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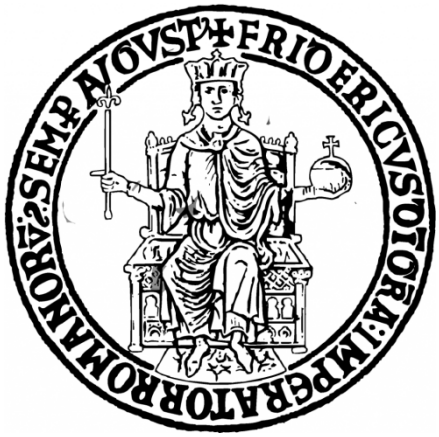

Doctorate in Applied Biology

XXV$^{th}$ Cycle

# The *Bacillus subtilis* spore: assembly of surface proteins and biotechnological applications

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***Outline of the Thesis***

**Outline**

As summarized in this Introduction (**Chapter 1**), *Bacillus subtilis* is a model organism to study a variety of biological phenomena, ranging from gene expression to microbial differentiation, protein-protein interactions and formation of subcellular structures. In addition, this organism is also widely studied for biotechnological reasons, from enzyme and antibiotic production to surface display and vaccine delivery.

I am particularly interested in both aspects and, therefore, I decided to focus my attention firstly, on the post-translational regulation of two crucial morphogenetic proteins, CotE and CotH, and secondly, on gaining a better understanding of the non-recombinant approach for surface display on bacterial spores. Those two aspects are clearly interconnected since understanding the details of how major morphogenetic factors direct spore coat assembly and therefore affect spore surface properties certainly have relevant consequences on the definition of the most appropriate strategy to display heterologous proteins on the spore surface.

Chapters 2 and 3 of this Thesis are focused on spore coat formation while Chapters 4 and 5 report experiments on the spore display of a model enzyme and an antigen. In particular, in **Chapter 2** I focused my attention on the timing of expression of CotH. This regulatory protein controls the assembly of at least 9 other coat components but is present in the sporangium for a very short time. It appears 4 hours after the start of sporulation, peaks one hour later and then rapidly disappear. In order to investigate the role of CotH I constructed a mutant strain in which CotH is over-produced and persist within the sporangium for a much longer time. The phenotypic analysis of this mutant showed that over-production of CotH leads to a partial by-pass of the requirement of the major morphogenetic factor CotE, suggesting a partial functional redundancy. A manuscript is in preparation with the experiments described in this part of the Thesis (**Sirec, T.**, Isticato, R., Giglio, R., Baccigalupi, L., Ricca, E., 2013. Over-production of CotH partially bypasses *cotE*-

associated phenotypes in *Bacillus subtilis* spores, *in preparation*). In **Chapter 3** I put together experiments aimed at understanding the machanism of action of the morphogenetic proteins CotE and CotH. The two proteins are shown to depend on each other for assembly within the spore coat and to directly interact. A collection of *cotE* deletion mutants has been used to verify the region of CotE involved in the interaction with CotH and, based on the information obtained, new mutants have been constructed to define in more details this protein-protein interaction. This part of my Thesis will be the object of a publication that is still in preparation. In **Chapter 4** I used a non-recombinant approach to display on the surface of *B. subtilis* spores the model enzyme beta-Galactosidase of the thermoacidophile bacterium *Alicyclobacillus acidocaldaricus.* My experiments clarified that the enzyme can be efficiently adsorbed to the spore surface and that once adsorbed it maintains its specific activity. Spore adsorption provides protection to the enzyme that appear protected from unphysiological conditions (acidic pH and high temperatures). This part of my work has been published in 2012 (**Sirec, T.,** Strazzulli, A., Isticato, R., De Felice, M., Moracci, M., Ricca, E. 2012. Adsorption of beta-galactosidase of *Alicyclobacillus acidocaldarius* on wild type and mutants spores of *Bacillus subtilis*. Microbial Cell Factories 11:100). In **Chapter 5** I expanded the use of the non-recombinant display system to expose on the spore surface the B subunit of the heat-labile toxin of *Escherichia coli*. Like the enzyme, also this antigen was efficiently presented on the spore surface and spore-displayed antigen was shown to induce a LTB-specific immune response in mice nasally immunized. A manuscript is in preparation with the experiments described in this part of the Thesis (Non-recombinant spores of *Bacillus subtilis* displaying LT-B induce strong humoral and cellular immune responses in mucosally immunized mice. Treppiccione, L., Isticato, R., **Sirec, T.**, Maurano, F., Rossi, M., Ricca, E., *in preparation*).

# *Chapter 1*

*Introduction*

## Sporulation and germination in *Bacillus subtilis*

### Bacterial spore

The bacterial spore is a dormant, highly resistant cell type, which allows spore-forming organisms to survive adverse conditions, such as starvation, high temperatures, ionizing radiations, mechanical abrasion, chemical solvents, detergents, hydrolytic enzymes, desiccation, pH extremes and antibiotics (Nicholson, el al., 2000). Spore formers mainly belong to the *Bacillus* and *Clostridium* genera. The most studied species of these two genera is *Bacillus subtilis*, a model system for gram-positive bacteria. Spore formation involves the cooperation of two sister cells in a series of developmental changes culminating in the packaging of one cell into a tough resistant coat. The other cell contributes most of its resources to the process and then lyses. Unlike most adaptive responses in bacteria, sporulation takes many hours and includes major changes in cellular morphology as well as in biochemistry and physiology. Because it also involves the differentiation of two cells that start out with identical genomes, it incorporates two of the main characteristics of developmental processes in all organisms, i.e., temporal change and cellular differentiation (Errington, J., 1993).

Morphological and metabolic diversity between two genetically identical cells is possible because they follow two different gene expression programmes of gene expression. In spore-forming bacteria the mechanisms responsible for the establishment of cell-specific gene expression and the temporal control of gene expression is due, in large part, to sequential appearance of four transcription factors, called sigma factors, alternate to $\sigma^A$ factor active during vegetative life ($\sigma^F$, $\sigma^E$, $\sigma^G$, $\sigma^K$), which bind to core of RNA polymerase and direct it to transcribe only from promoters of sporulation genes, called *spo* (Losick and Stragier, 1992). The process of spore formation is presented in Figure 1.

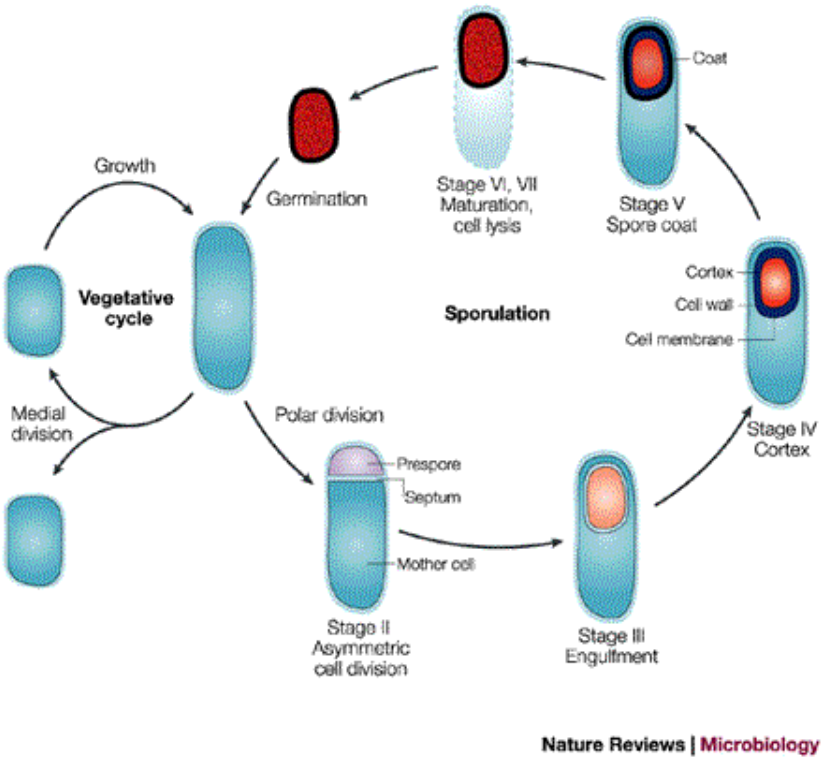


**Figure 1**: schematic representation of sporulation and germination in *Bacillus subtilis.*

The spore can stay in the environment as quiescent cell for a long time, but it must be able to return to active growth when nutrients are available through a process called germination (Figure 1). Dormant spores continuously monitor the environment for the renewed presence of nutrients.

**Morphological stages**

The morphological process of sporulation is driven by a temporally and spatially controlled program of gene expression. Commencement of this program requires the cell reaching a certain stage in the cell cycle (Grossman, 1995; Errington et al, 1996), it should have the tricarboxylic acid cycle is intact (Sonenshein, 1995), at least one extracellular pheromone present in the appropriate amount, and an unknown environmental stimulus that has activated a complex phosphorylation cascade (Hoch, 1993). Under these conditions (during starvation) sporulation ensues and the pattern of vegetative gene expression is largely replaced with the

specialized program of sporulation gene expression. An early morphological event is the formation of an asymmetrically placed division septum, which divides the cell into the forespore and mother cell compartments (Figure 1). The smaller compartment (the forespore) will go on to become the spore and the larger compartment (the mother cell) will serve to nurture the spore until its development completes. After the sporulation septum is laid down, the sporulation gene expression program splits and two distinct programmes activate, one in each of the resulting cellular compartments. As a result of these two divergent programmes of gene expression, the spore will be built from the outside (as a result of protein synthesis in the mother cell) and from the inside (as a consequence of the proteins produced in the forespore). After that, the edge of the septum migrates in the direction of the forespore pole of the cell, pinching the forespore compartment off to become a protoplast which sits free in the mother cell cytoplasm and is surrounded by a double layer of membranes.

Following the completion of the engulfment, two types of peptidoglycans are layed between the inner and outer membranes surrounding the forespore. The surface of the inner spore membrane is the site of assembly of a thin layer of peptidoglycan called the primordial germ cell wall, similar in composition to the vegetative cell wall that serves as the primordial wall of the newly formed vegetative cell following spore germination. The outer forespore membrane is the site of assembly of a second, thicker, and chemically distinct layer of peptidoglycan called the spore cortex which is essential for the attainment and maintenance of the dehydrated state of the spore core, for spore mineralization and for dormancy (Henriques and Moran, 2007). Concomitantly with cortex formation, the proteinaceus coat is deposited around the outer surface of the outer membrane. The coat is evident by about the fifth hour of sporulation. Two major coat layers can be observed by electron microscope analysis: a darkly stained outer coat and a more lightly stained lamellar inner coat. The final step is the lysis of the mother cell and the release of the fully formed spore.

When enviromental conditions are suitable, the spore can germinate and thereby convert back into a growing cell. When this occurs, first the spore core rehydrates and swells and then cortex and coat crack, releasing the nascent cell (Figure. 1).

**Genetic control of sporulation**

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

forespore, probably thanks to the presence of a recently identified channel, which connects the two compartments. This channel is formed by the protein products (AA-AH) of an eight-cistron *spoIIIA* operon, which is transcribed in the mother cell under the $\sigma^E$ control, and by a forespore protein Q (Camp and Losick, 2009). The gene encoding $\sigma^G$ is under the control of $\sigma^F$ and is expressed just after the appearance of the sporulation septum. However, the gene product remains inactive until forespore engulfment is complete (Stragier, 1992). The cytoplasm of the forespore dehydrates soon after $\sigma^G$ becomes active. $\sigma^K$ is activated by a proteolysis event that appears to occur on the outer forespore membrane, which is in contact with the mother cell cytoplasm. $\sigma^K$ is required for a variety of events that occur late in sporulation, including the synthesis of the coat. The last known phase of mother cell gene expression is directed by $\sigma^K$ along with the small DNA-binding protein GerE (Zheng et al., 1990). GerE controls coat protein genes and genes which may be involved in the glycosylation of the coat. The mechanism of regulation of gene expression in the two cell compartments which was briefly summarized above, was proposed in 1992 (Stragier and Losick), and called criss-cross regulation. Since then it has been confirmed by various experimental evidences and is schematically reported in the Figure 2.

**Germination and outgrowth**

When the environmental conditions are favorable for the vegetative life, the spore that is continuously monitoring the environment, returns to active growth through a process called germination. It is an irreversible process (Parker et al., 1996) that involves a series of rapid degradative reactions, leading to dismantlement of the unique spore structure and loss of spore dormancy and resistance. The subsequent

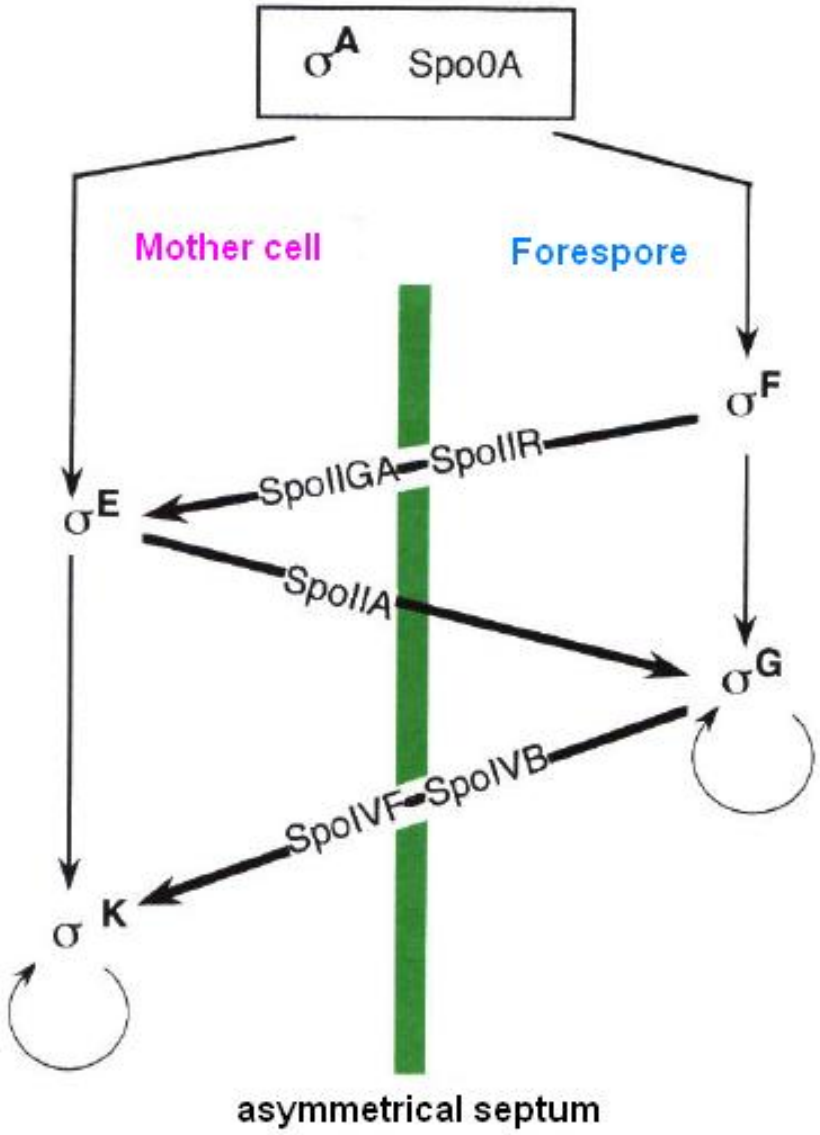


Figure 2: C*riss-cross regulation*. The thin arrows indicate the transcriptional control in the two different cells, while the thick arrows indicate the three checkpoints that coordinate the gene expression of one cell with the morphological changes of the other.

steps that lead to cell enlargement and cell-division are termed *outgrowth*, and are considered a separate process, distinct from germination. Outgrowth takes more than one hour to occur while germination takes just few minutes. The amino-acids necessary in this step for the protein synthesis during 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) degradations. The dormant spore is equipped with sensors to choose the right moment for germination. These

sensors are specific proteins called nutrient receptors, and are located at the inner membrane (Hudson et al., 2001; Paidhungat et al., 2001). They recognize specific molecules as a signal of nutrient-rich conditions. Effective germinants are amino-acids, sugars and robosides (Hornstra et al., 2005). In laboratory conditions, germination is efficiently induced by L-alanine and a mixture of molecules known as AGFK (asparagines, glucose, fructose, KCl). The correct assembly of the coat is also important for a proper germination, in fact null mutant for *cotT* and *cotD*, show defect in germination, also because the coat is responsible to suppress spontaneous, unwanted germination. Moreover, different classes of mutants are identified, that are not able or less able to germinate in presence of L-alanine and AGFK. These differences are probably a consequence of presence of different receptors nutrient-specific (Moir and Smith, 1990).

## Spore structure and spore coat

### Spore structure

The bacterial spore consist in various layers: a core, surrounded by the inner membrane, the cortex, the outer membrane, the coat and 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. The physical state of the core cytoplasm, however, is far from regular in comparison to vegetative cell cytoplasm, having a water content of only 30 – 50 % instead of the 70 – 88 % of the vegetative cytoplasm (Potts, 1994; Setlow, 1994; 2000).

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

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

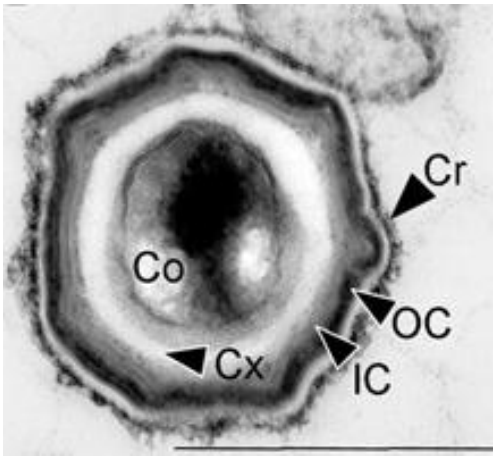


**Figure 3**: *Bacillu subtilis* spore ultrastructure. The figure shows the spore core (Co), the cortex peptidoglycan layer (Cx), the inner coat (IC) and the outer coat (OC) and the crust (Cr) (McKenney et al, 2010).

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 consist of two main layers (Figure 3): the inner layer (thick 20 - 40 nm) is formed by the juxtaposition of three to six lamellae aligned along the periphery of the spore, and the outer layer

(thick 40 - 90 nm) consisting of four to five electron-dense striations that also align parallel to the spore surface (Henriques and Moran, 2007) and a recently characterized outermost layer, spore crust (McKenney et al., 2010). Herein the use of ruthenium red stain, which stains acid polysaccharides, followed by electron microscopy revealed a glycoprotein layer that may represent a rudimentary exosporium at the periphery of *B. subtilis* spores (Figure 3). 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 this layer (Imamura et al., 2012).

Analysis of localization of various coat protein 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 protein; and at *the post-translational level*, with various kinds of modifications and with the assembly of coat components (Driks et al., 1994; Ricca et al., 1997).

**Role of gene expression in coat assembly**

Coat assembly is mainly a function of the mother cell and covers a period of about 6 hours, beginning with asymmetric division of the sporangial cell. Thus, expression of genes coding for coat components (*cot* genes) is under control of the two transcription factors that regulate mother cell gene expression: $\sigma^E$ and $\sigma^K$; and three DNA binding proteins called: SpoIIID, GerR and GerE. Due to the action of those transcriptional factors it is possible to identify four classes of *cot* genes (Figure 4).

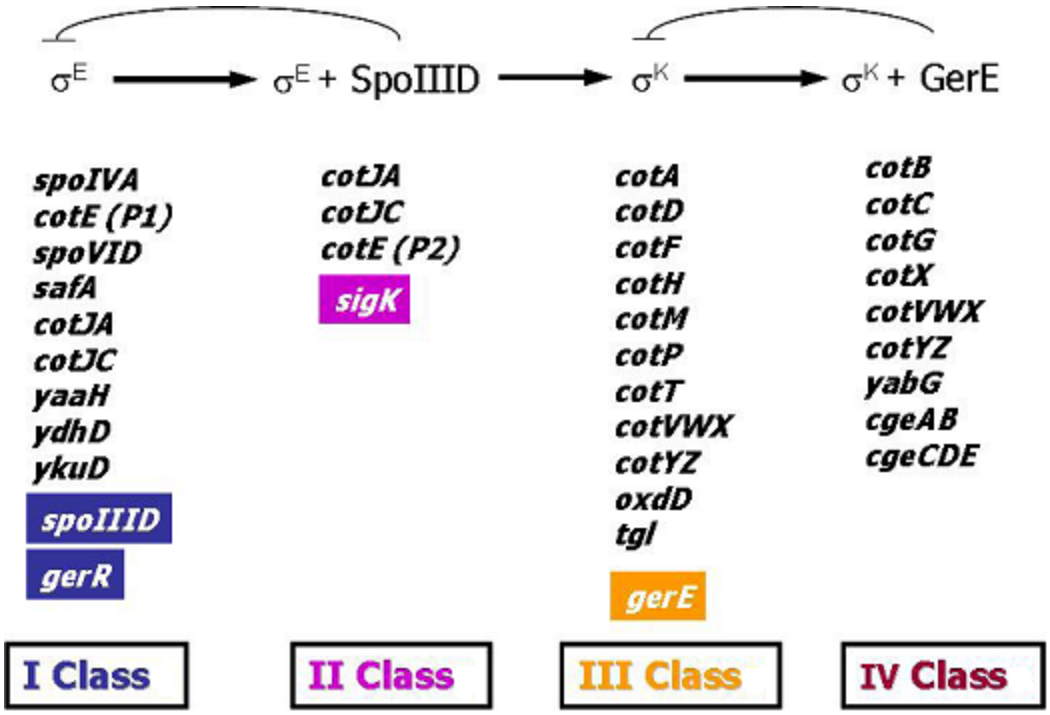


**Figure 4**: Program of mother cell gene expression.

The gene expression programme in the mother cell compartment involves the activation of 383 genes, that represent about 9% of *Bacillus subitlis* genome, and it is started with activation of $\sigma^E$. It directs the expression of at least two coat genes, *cotE* and *cotJ*. $\sigma^E$ is also responsible for the expression of the genes coding for the coat morphogenetic proteins SpoIVA and SpoVID, from pro-SigK and for genes coding for the transcriptional factors SpoIIID and GerR. SpoIIID can act as a repressor or as an activator while GerR, seems to work only as a repressor of genes activated by $\sigma^E$ (Eichenberger et al., 2004).

After engulfment, $\sigma^K$ directs the expression of a large group of coat protein genes. The first $\sigma^K$ –controlled regulon is composed of *cotA*, *cotD*, *cotF*, *cotH*, *cotM*, *cotT*, *cotV*, *cotW*, *cotY*, and *cotZ*. The transcription factor gene *gerE* is also part of this regulon. GerE works in conjunction with $\sigma^K$ to activate a final regulon, encompassing *cotB*, *cotC*, *cotG*, *cotS*, *cotV*, *cotW*, *cotX*, *cotY*, and *cotZ*. GerE can also modulate the expression of genes in the $\sigma^K$ regulon. It down regulates *cotA* and *cotM* and activates *cotD*, *cotV*, *cotW*, *cotX*, *cotY*, and *cotZ*. A further level of complexity in the control of mother cell gene expression comes from a feedback-like regulation in

which late regulatory events modulate ones that were initiated earlier. For example, $\sigma^K$ down regulates transcription of the gene encoding $\sigma^E$, thereby helping to terminate expression of $\sigma^E$–directed genes. GerE is also able to down regulate the activity of $\sigma^K$. The production of the spore coat proteins in the correct cellular compartment and at the proper time is critical to the formation of the coat. Mutants in which the time of $\sigma^K$ activation is altered, and the coat components of class III and IV, are synthesized one hour earlier than in wild type cells, produced spores impaired in their germination efficiency (Cutting et al., 1991; Ricca et al., 1992).

**Post-translational regulation during coat formation**

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

**SpoIVA** is produced in the mother cell early in sporulation, under the control of $\sigma^E$ and is essential for the assembly of both the spore cortex and the coat layers. SpoIVA localizes at or near the mother cell surface of the septum and at later stages, following engulfment of the forespore by the mother cell, the protein forms a shell that surrounds the forespore. Recent studies (Ramamurthi and Losick, 2008) have demonstrated that SpoIVA is an ATPase and that it allows its self assembly. It has been proposed that two interaction sites are involved in assembling of SpoIVA: one is used for ATP-dependent polymerization, and the second for lateral interactions between the polymers. Localization of SpoIVA marks the forespore outer membrane for its use both in the synthesis of the cortex and as the site of coat attachment. Mutants carrying a *spoIVA* null allele, show a coat correctly formed with inner and outer part but dispersed in the mother cell cytoplasm and not assembled around the forespore (Roels et al., 1992; Stevens et al., 1992). So its role is

probably to attach the undercoat to the forespore surface from the mother cell side (Driks et al., 1994). SpoIVA is also required for the localization of second key coat morphogenetic protein, SpoVID (Beall et al., 1993), to the surface of the developing spore. SpoVID governs a morphogenetic transition, called spore encasement, using a N-terminal, that seems to be essential to form multimeric structures (Wang et al., 2009). *spoVID* mutants like *spoIVA* mutants, have a spore coat dispersed in the cytoplasm. The localization of SpoIVA to the forespore outer membrane is a prerequisite also for the localization of another morphogenetic protein, CotE (Zheng et al., 1990).

**CotE** is a 24 kDa protein protein found in several *Bacillus* species and also in a *Geobacillus* and an *Oceanobacillus* (Henriques and Moran, 2007), produced in mother cell compartment. Expression of *cotE* relies on two promoters, designed P1 and P2. Transcription from P1 initiates soon after asymmetric division and is shut off by the repressive action of SpoIIID. Transcription from P2 appears to be under the joint control of $\sigma^E$ and SpoIIID, but remains on after the activation of $\sigma^K$, to be repressed in the final stages of sporulation by the GerE (Costa, et al., 2006).

CotE localizes about 75 nm from the forespore outer membrane and later encircles the engulfed forespore. Electron microscope analysis have shown that *cotE* mutants totally lack the outer coat layer (Zheng et al., 1990). The region delimited by the SpoIVA and the CotE ring 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 electrondense outer layer (Figure 7). Assembly of the outercoat involves the cooperation of CotE with the action of the additional morphogenetic protein CotH (Nacleiro 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 a 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 spore coat components (Kim et al., 2006, Figure 6) and is essential for formation of CotC-CotU hetero-oligomers (Isticato et al., 2010).

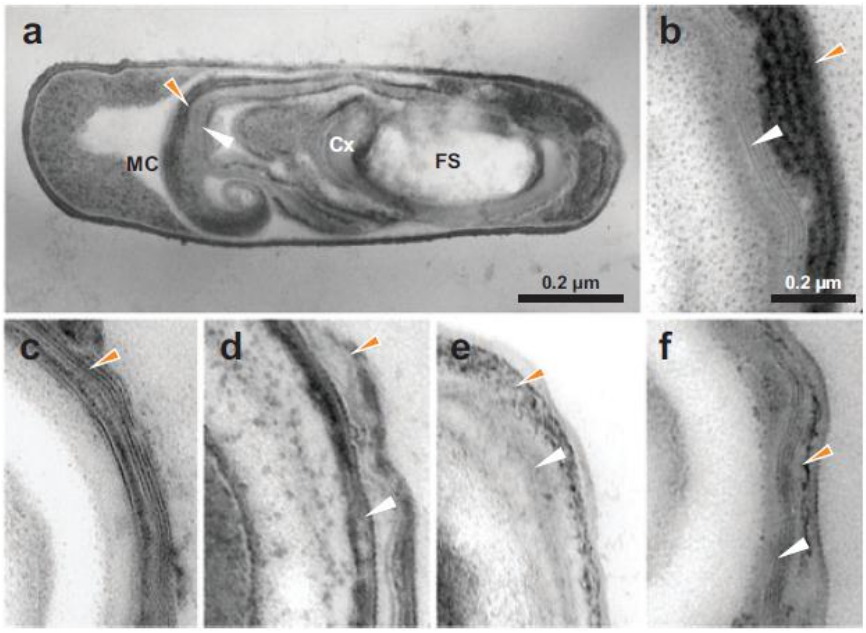


**Figure 5**. Phenotypes caused by mutations in the genes for various coat morphogenetic factors. (*a*) An electron micrograph of a *spoVID* sporangium showing swirls of coat material throughout the mother cell cytoplasm and an exposed cortex (Cx) around the developing spore. Also shown are electron micrographs of (*b*) wild-type spores or spores of the following mutants: (*c*) *cotE*, (*d* ) *safA*, (*e*) *cotH*, and ( *f* ) *cotG*. The white and orange arrowheads indicate the inner and outer coat regions, respectively. MC, mother cell; FS, forespore (Henriques and Moran, 2007).

**CotH** is a 42.8-kDa protein found in several *Bacillus* species 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 lysozyme resistance 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 *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 5). The structural gene coding for CotH is clustered together with *cotG* gene on the *Bacillus subtilis* chromosome but is divergently transcribed (Naclerio et al., 1996).

**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 factor $\sigma^K$ and of transcriptional regulator GerE. Gene *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 9 repeats of 13 amino acids (Sacco et al., 1995). More recently it has been suggested that CotG has a more complex organization with 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-containg *Bacilli* CotG has a modular structure although the number and the length of the repeats differs 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).

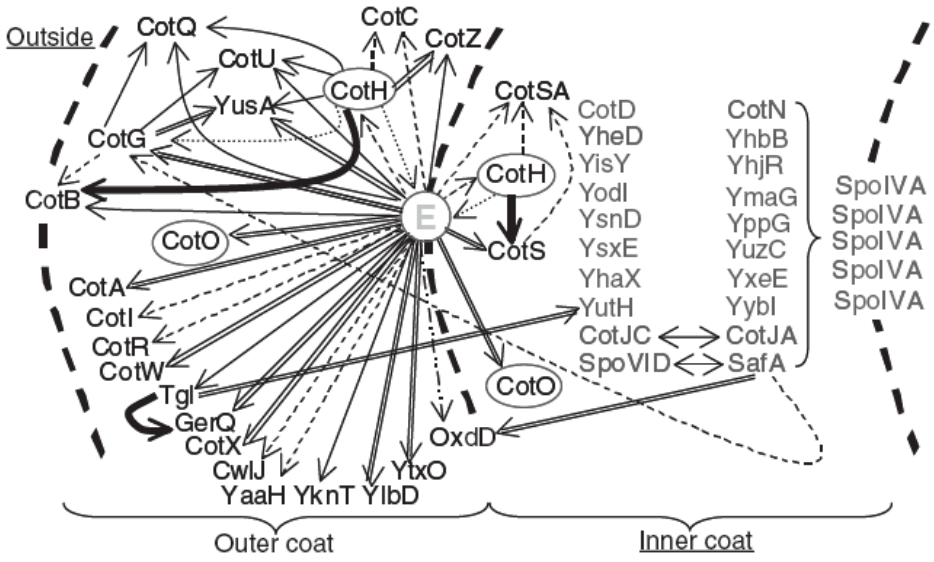


**Figure 6**: Model of the coat protein interaction network. An arc of the spore coat is indicated; E is CotE. The interior of the spore is to the right of the diagram. The inner and outer coat layers are indicated, as well as the outside of the spore (on the left). Directions of the arrows indicate 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).

**Post- translational modifications**

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

coat is reinforced by the preponderance of residues of tyrosine in the sequences of coat proteins. A trasglutaminase has been identified in the coat layers and the its structural gene, *tgl,* identified (Kobayashi et al., 1996). This enzyme generate a ε-(γ-glutamyl)lysine cross-link among the R groups of different amino-acids, belonging at the same chain or also at different chains. It has been also demonstrated that the assembly of GerQ within the is dependent on Tgl activity (Zilhao et al., 2005). In addition to modification by cross-linking, there is evidence for glycosylation of coat proteins late in development. A cluster of genes, *cge*, activated under the control of GerE, was identified (Roels et al., 1992). One of these, *cgeD*, is similar to glycosyl transferases that participate in polysaccharide biosynthesis. Mutations that deleted genes in this cluster resulted in spores that appeared normal by the standard assays and by electron microscopy. However, these spores tended to aggregate and had abnormal adsorption properties, suggesting a surface alteration. It is possible that the *cge* locus encodes one or more coat proteins, but it is perhaps more likely that it is responsible for a terminal step in coat formation that involves the glycosylation of the coat. Another cluster of 10 genes which also show homology to polysaccharide biosynthetic genes, and whose expression is repressed by GerE, was identified (Hullo et al.). One of these, *spsA*, is very similar to *cgeD*. The deletion of the *sps* locus does not result in a gross morphological defect in spore structure, but the spores are very hydrophobic. So, the glycosilation of the spore surface could determine the hydrophobicity of the spore and therefore its adherence properties (Wiencek, et al., 1990). An additional post-translational modification that has been shown to occur during coat formation is the proteolytic processing of certain coat proteins, such as CotF and CotT. The enzymes responsible for these cleavage events have not been identified, but a GerE-dependent coat associated protease activity has been found.

Recently a new family of spore coat proteins was defined, named bacterial spore kinases. They are widely distributed in spore-forming *Bacillus* and *Clostridium* species, and have a dynamic evolutionary history. Sequence and structure analyses

indicate that the bacterial spore kinases are CAKs (CDK-activating kinases), a prevalent group of small molecule kinases in bacteria that is distantly related to the eukaryotic protein kinases. Evolutionary constraint analysis of the protein surfaces indicates that members of the bacterial spore kinase family have distinct clade-conserved patterns in the substrate binding region, and probably bind and phosphorylate distinct targets. Several classes of bacterial spore kinases have apparently independently lost catalytic activity to become pseudokinases, indicating that the family also has a major noncatalytic function. Four bacterial spore kinases are found *in B. subtilis* and many other *Bacillaceae*: YutH and YsxE (pseudokinases) are present in almost all spore-forming species, whereas CotI and CotS (kinases),are more restricted. All four are experimentally implicated in sporulation (Scheef et al., 2010).

From the results discussed above, it is possible to propose a tentative model to explain how the layered structure of the coat is established and how coat formation is directed to occur at the location of the spore surface (Figure 7). In this view, there are four major steps in coat assembly: binding of SpoIVA to the forespore surface, formation of the precoat (both of these steps occur under the control of $\sigma^E$), a $\sigma^K$ dependent phase of inner and outer coat layer assembly, and a $\sigma^K$ plus- GerE-dependent phase of inner and outer coat layer assembly that includes post-assembly modification of the coat.

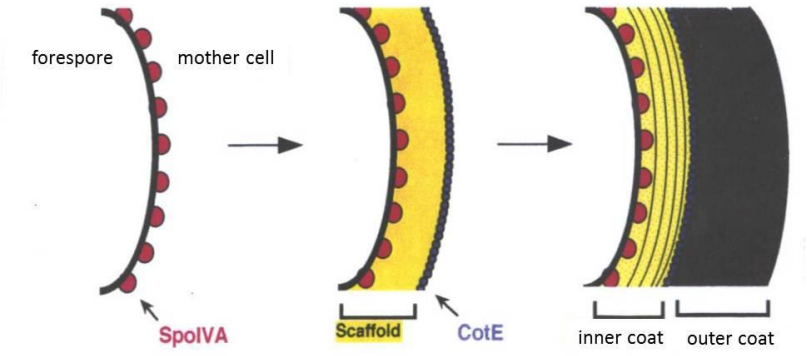


**Figure 7**: Spore coat assembly model.

**New applications of spores as display system**

A variety of bioactive peptides and proteins have been successfully displayed on the surface of recombinant spores of *Bacillus subtilis* and other spore formers. In most cases spore display has been achieved by stably anchoring the foreign molecules to endogenous surface proteins. Recombinant spores have been then proposed for a large number of potential applications, ranging from oral vaccine vehicles to bioremediation tools, and including biocatalysts, probiotics for animal or human use as well as the generation and screening of mutagenesis libraries. In addition, a non-recombinant approach has been recently developed and proposed to adsorb antigens and enzymes on the spore surface. This non-recombinant approach appears particularly well suited for applications involving the delivery of active molecules to human or animal mucosal surfaces. Both the recombinant and non-recombinant spore display systems have a number of advantages over cell- or phage-based systems. The stability, safety and amenability to laboratory manipulations of spores of several bacterial species, together with the lack of some constrains limiting the use of other systems, make the spore a highly efficient platform to display heterologous proteins.

**Surface display on *B. subtilis* spores on recombinant approach**

*Bacillus* spores are encased into the coat, a protein structure protecting the spore from toxic chemicals, lytic enzymes (Henriques and Moran, 2007) and phagocytic predation by protozoans (Klobutcher et al., 2006). The rigidity and compactness of the spore coat immediately suggest the possibility of using its structural components as anchoring motifs for the expression of heterologous polypeptides on the spore surface. A genetic system to engineer the coat of *B. subtilis* spores has been developed and a model passenger efficiently displayed (Isticato et al., 2001). The spore-based approach provides several advantages over other display systems, such as a high stability even after a prolonged storage, the possibility to display large multimeric proteins and the safety for a human use.

Attempts to expose heterologous proteins on the spore surface were focused mainly on CotB protein selected for the surface location (Isticato et al., 2001) and CotC selected for the high relative abundance in spore coat ( Mauriello et al., 2004). Also other fusion partners as CotG, In the first attempt of using CotG as an anchoring motif the biotin-binding protein Streptavidin was fused to the C-terminal end of CotG (Naclerio et al., 1996) The β-Galactosidase of *E. coli* (Kwon et al., 2007), the phytase of *B. subtilis* (Potot et al., 2010) and the urease A of *H. acinonychis* (Hinc et al., 2010) were also fused to the C terminus of CotG but those chimeras were encoded by recombinant DNA integrated into the *B. subtilis* chromosome.

The last attempt to expose heterologous proteins was made with OxdD, which is a 43 kDa inner coat component, not exposed on the spore surface (Ozin et al., 2001) with oxalate decarboxylase activity. Because of its internal location OxdD has been proposed as a carrier able to provide a high degree of protection to the passenger protein, that is not surface exposed but rather covered by the most external layers of the coat (Potot et al., 2010). OxdD has been successfully tested as carrier by fusing to its C terminus a phytase (Phy) and a b-glucuronidase (b-Glu) (Potot et al., 2010). Phytases are monomeric enzymes widely used in animal nutrition since they catalyze the hydrolysis of indigestible, organic form of phosporous found in grains and release a usable form of inorganic phosphorus. In all examples reported, the efficiency of exposure was ~ $10^3$ heterologous protein molecules per spore.

**Surface display on non-recombinant spores**

Spore-based display systems summarized above rely on the genetic engineering of the host. This is a major drawback when the application of the display system involves the release into nature of the recombinant host (field applications) and, in particular, when the display system is thought for human or animal use (delivery of antigens or enzymes to mucosal surfaces). Serious concerns over the use of live genetically modified microorganisms, their release into nature and their clearance from the host following oral delivery have been raised (Detmer and Glenting, 2006).

To overcome this obstacle, non-recombinant approaches would be highly desirable. In this context, various non-recombinant approaches to display heterologous proteins on the spore surface have been recently proposed. In the first study suggesting that heterologous proteins can be adsorbed on the spore surface, the gene encoding the NADPH-cytochrome P450 reductase (CPR), a diflavin-containing enzyme, was over-expressed in *B. subtilis* cells by using the IPTG-inducible vegetative promoters (P*groE* and P*tac*) (Yim et al., 2009). The expressed CPR was released into the culture medium after sporulation by autolysis of the mother cell and was found associated to the spore surfaces. Purified spores showed CPR activity and the enzyme was accessible to anti-CPR antibodies in FACS experiments (Yim et al., 2009). Therefore, these results provided strong evidence that CPR was spontaneously targeted to the outer spore surface, resulting in *in vivo* immobilization of the enzyme on the spore. It is noteworthy that the enzyme used in this study contains two flavin cofactors (FMN and FAD) and, for this reason, cannot be produced-secreted by standard expression systems (Yim et al., 2009). Although this first study did not address the mechanisms involved in the spontaneous adhesion of the enzyme to the spore surface and did not investigate if other enzymes had a similar behaviour, it clearly suggested a new approach for spore display. A similar observation was obtained using spores of *B. thuringiensis.* The endo-β-*N*-acetylglucosaminidase (Mbg) of *B. thuringiensis* is a putative peptidoglycan hydrolase containing two LysM domains at its N terminus, and had been used to display of GFP on the surface of *B. thuringiensis* cells (Shao et al., 2009). However, by fusion to Mbg, GFP and a bacterial laccase (WlacD) were also found associated to spores, inducing the authors to propose Mbg as a carrier also for spore surface display Jiang et al., 2011). Since Mbg is expected to bind peptidoglycan through its LysM domains, it is unlikely to bind the spore coat and the interaction with the spore is then most likely due to a spontaneous adhesion as in the case of CPR. A different approach for spore adsorption was followed by Huang et al. (2010). Previously purified proteins were mixed with purified spores of *B. subtilis* and adsorption

conditions developed. A collections of antigens (the TTFC of *C. tetani*, PA of *B. anthracis*, Cpa of *C. perfringens* described above and glutathione S transferase (Sj26GST) from *Shistosomas japonica* (Walker et al., 1993)) were expressed in *E. coli* and purified by affinity chromtography. Adsorbed spores were shown able to induce specific and protective immune responses in mice immunised mucosally (Huang et al., 2010). Spore adsorption resulted more efficient when the pH of the binding buffer was acidic (pH 4) and less efficient or totally inhibited at pH values of 7 or 10 (Huang et al., 2010). A combination of electrostatic and hydrophobic interactions between spores and antigen were suggested to drive the adsorption that was shown to be not dependent on specific spore coat components but rather due to the negatively charged and hydrophobic surface of the spore (Huang et al., 2010). In addition, the same study showed that killed or inactivated spores were equally effective as live spores in adsorbing the various antigens (Huang et al., 2010). A similar approach was also used to adsorb enzymes, the phytase of *E. coli* to spores of a probiotic strain of *B. polyfermenticus* (Cho et al., 2011). In the case of the phytase, immobilization on the spore surface stabilized the enzyme by increasing its half-life at temperatures ranging between 60 to 90°C, but also caused a loss of activity of about 30% (Cho et al., 2011).

**Physicochemical propreties of spores important for protein adhesion**

The physicochemical properties of the spore surface have been addressed in different studies with different approaches. A first study showed that spores of *B. subtilis* are negatively charged by time-resolved micropotentiometry (Kazakov et al., 2008). In an aqueous environment the spore behaves like an almost infinite ionic reservoir and is able of accumulating billions of protons (approximately $2 \times 10^{10}$ per spore) (Kazakov et al., 2008). The carboxyl groups were identified as the major ionizable groups in the spore and on the basis of the diffusion time analysis, it was found that proton diffusion is much lower in the spore core than within the coats and cortex (Kazakov et al., 2008). This, then, suggested that the inner membrane,

separating core from cortex and coats in a dormant spore, is probably a major permeability barrier for protons (Kazakov et al., 2008). The role of electrostatic forces in spore adhesion to a planar surface has been also addressed studying spores of *B. thuringiensis* (Chung et al., 2010). The surface potentials of a spore and a mica surface were experimentally obtained using a combined atomic force microscopy (AFM)-scanning surface potential microscopy technique (Chung et al., 2010). By these techniques, the surface charge density of the spores was estimated at 0.03 $\mu C/cm^2$ at 20% relative humidity and decreased with increasing humidity. This work showed that the electrostatic force can be an important component in the adhesion between the spore and a planar surface (Chung et al., 2010). Adhesion to abiotic surfaces has been also studied using *B. subtilis* and *B. anthracis* spores and attributed to attractive interactions between the spores and the porous media (Chen et al., 2010). Surface thermodynamic properties of wild type and mutant spores of both *Bacillus* species were estimated based on contact angle measurements and related to their transport in silica sand. This study showed that spores of *B. subtilis* and *B. anthracis* were monopolar and negatively charged, in spite of their different surface structure (*B. anthracis* spores have an exosporium surrounding the coat). In both species, the spore surface thermodynamic properties changed in a similar ways when the spore surface was altered due to mutations and, in general, adhesion increased in the mutants (Chen et al., 2010).

**Advantages of the spore surface display systems**

Spore-based surface display systems provide several advantages with respect to other display approaches using microbial cells or bacteriophages. A first advantage comes from the well documented robustness of the *Bacillus* spore which grants high stability to the display system even after a prolonged storage. This aspect has been tested with spores displaying CotB$\Delta_{105}$-TTFC. Aliquots of purified recombinant spores were stored at -80°C, -20°C, +4°C and at room temperatures and assayed for the amount of heterologous protein present on the spore after different storage

times. A dot-blot analysis with anti-TTFC antibody showed identical amounts of displayed TTFC at all time points (Isticato et al., 2004). The stability of the display system is an extremely useful property for a variety of biotechnological applications. Heat-stability is, for example, a stringent requirement in the development of new mucosal vaccine delivery systems, mainly for those intended for use in developing countries, where poor distribution and storage conditions are main limitations. High stability of the display system is an extremely useful property also for industrial and environmental applications, in which cell-based systems have found so far limited use in the field because of the inability of bacterial cells to survive long term, especially under extreme conditions (Knecht et al., 2011). The safety record of several *Bacillus* species (Cutting, 2011) is another important advantage of spores over other systems. Several *Bacillus* species, including *B. subtilis*, are widely used as probiotics and have been on the market for human or animal use for decades in many countries (Cutting, 2011). Although most of the study so far performed have been carried out with laboratory strains of *B. subtilis*, in some cases intestinal isolates and strains with probiotic properties have been also used to display heterologous proteins (Cutting et al., 2009). The safety of the live host is obviously an essential requirement if the display system is intended for human or animal use, such as delivery of vaccines or therapeutic molecules to mucosal surfaces. A limitation of cell- and phage-based display systems is the size of the heterologous protein to be exposed, since it may affect the structure of a cell membrane-anchoring protein or of a viral capsid. In addition, cell-based systems need a membrane translocation step in order to externally expose a protein produced in their cytoplasm (Knecht et al., 2011). Spore-based systems do not have such limitations. As described in sections above large proteins (for example, TTFC of 51.8 kDa) and multimeric proteins (for example, the tetrameric Streptavidin) have been successfully displayed on the spore surface. A first reason for the successful display of those large proteins is that the coat components used as carriers are dispensable for the formation of an apparently normal spore as well as for its

germination (Henriques and Moran, 2007). Therefore, if the coat component-carrier does not behave as a wild type protein as a consequence of the presence of the heterologous part fused to it, this is unlikely to affect coat structure and function. Moreover, all known coat proteins are expressed in the mother cell compartment of the sporulating cell and, as a consequence, coat-based chimera do not need to undergo a cell wall translocation step, thus eliminating a severe limitation often encountered with cell-based display systems. Identical arguments apply for display systems based on spore adsorption. Independently purified proteins or proteins synthesized in the mother cell during sporulation can be attached to spores and the immobilization has been shown to provide increased stability to the protein without interfering with the spore structure or the need to translocate across a cell membrane.

**References**

1. **Baccigalupi, L., Castaldo, G., Cangiano, G., Isticato, R., Marasco, M., De Felice, M., and Ricca, E. 2004**. GerE independent expression CotH leads to CotC accumulation in the mother cell compartment during *Bacillus subtilis* sporulation. Microbiology 150: 3441-3449.
2. **Beall, B., Driks, R., and C. P. Moran. Jr. 1993**. Cloning and characterization of a gene required for assembly of the *Bacillus subtilis* spore coat. J. Bacteriol. 175:1705-1716.
3. **Camp, A. H., Losick, R. 2009**. A feeding tube model for activation of a cell-specific transcription factor during sporulation in *Bacillus subtilis*. Gene & Develop. 23:1014-1024.
4. **Cangiano, G., Mazzone, A., Baccigalupi, L., Isticato, R., Eichenberger, .P, De Felice, M., Ricca, E. 2010.** Direct and indirect control of late sporulation genes by GerR of *Bacillus subtilis*. J. Bacteriol. 192:3406-3413.
5. **Ceragioli, M., Cangiano, G., Esin, S., Ghelardi, E., Ricca, E., Senesi, S. 2009.** Phagocytosis, germination and killing of *Bacillus subtilis* spores presenting heterologous antigens in human macrophages. Microbiol. 155:338-46.
6. **Chada, V. G., E. A. Sanstad, Wang, R., Driks, A. 2003**. Morphogenesis of bacillus spore surfaces. J. of Bacteriol. 185: 6255-61.
7. **Chen, G., Driks, A., Tawfiq, K., Mallozzi, M., Patil, S. 2010.** *Bacillus anthracis* and *Bacillus subtilis* spores surface properties and transport. Colloid and Surfaces B: Biointerfaces 76:512-518.
8. **Cho, E. A., Kim, E. J., Pan, J. G. 2011**. Adsorption immobilization of *Escherichia coli* phytase on probiotic *Bacillus polyfermenticus* spores. Enzyme Microb Technol. 49:66-71.
9. **Chung, E. A., Iacoumi, S., Lee, I., Tsouris, C. 2010.** The role of the electrostatic force in spore adhesion. Environ. Sci. Technol. 44:6209–6214.
10. **Costa, T., M., Serrano, L., Steil, U., Völker, C. P., Moran, Jr., Henriques, A. O. 2006.** The timing of *cotE* expression affects *Bacillus subtilis* spore coat morphology but not lysozyme resistance. J. of Bacteriol. 189 (6):2401-2410.
11. **Cutting, S. M., 2011**. *Bacillus* probiotics. *Food Microbiology* 28:214-220.
12. **Cutting, S., Driks, A., Shimdt, R., Kunkel, B., and Losick, R. 1991.** Forespore specific transcription of a gene in the signal transduction pathway that governs pro-$\sigma^K$ processing in *Bacillus subtilis*. Genes & Dev. 5:456-466.
13. **Cutting, S. M., Hong, H. A., Baccigalupi, L., Ricca, E. 2009.** Oral vaccine delivery by recombinant spore probiotics. Intern. Rev. Immunol. 28:487-505.
14. **Detmer, A., Glenting, J. 2006**. Live bacterial vaccines—a review and identification of potential hazards. Microb Cell Fact 5:23.
15. **Driks, A. 1999**. *Bacillus subtilis* spore coat. Microbiol Mol Biol Rev; 63:1–20.
16. **Driks, A., Roels, S., Beall, B., Moran, C. P., Jr, Losick, R. 1994**. Subcellular localization of proteins involved in the assembly of the spore coat of *Bacillus subtilis*. Genes Dev. 8 (2): 234-44.
17. **Duc le H. Hong, A. H., Fairweather N., Ricca, E., Cutting S. M. 2003**. Bacterial spores as vaccine vehicles., Infect. Immun., 71(5):2810-2818.
18. **Eichenberger, P., Fujita, M., Jensen, S. T., Conlon, E. M., Rudner, D. Z., Wang S. T., Ferguson, C., Haga, K., Sato, T., Liu, J. S., Losick, R. 2004.** The program of gene transcription for a single differentiating cell type during sporulation in *Bacillus subtilis*. PLOS Biology 2(10):1664-1683**.**
19. **Errington, J. 1993**. *Bacillus subtilis* Sporulation: regulation of gene expression and control of morphogenesis. Microbiol. Rev. 57:1-33.
20. **Fischetti V.A., Medaglini, D., Pozzi, D. 1996**. Gram positive commensal bacteria for mucosal vaccine delivery. *Curr. Opin. Biotechnol.* 7:659–666.

21. **Giglio R. , Fani R., Isticato R., De Felice, M., Ricca E., Baccigalupi L. 2011.** Organization and Evolution of the *cotG* and *cotH* Genes of *Bacillus subtilis.* Journal of Bacteriol., 193(23):6664–6673.
22. **Green, D., Wakeley, P., Page, A., Barnes, A., Baccigalupi, L., Ricca, E., Cutting, S. 1999**. Characterization of two *Bacillus* probiotic species. Appl Environ Microbiol. 65:4288–4291.
23. **Grossman, A. D. 1995**. Genetic networks controlling the initiation of sporulation and the development of genetic competence in *Bacillus subtilis*. Annu. Rev. Genet. 29:477-508.
24. **Halberg, R., Oke, V., Kroos, L. 1995.** Effects of *Bacillus subtilis* sporulation regulatory protein SpoIIID on transcription by sigma K RNA polymerase in vivo and in vitro. J. Bacteriol.177:1888-1891.
25. **Henriques, A. O., Moran, C. P. Jr. 2000**. Structure and assembly of the bacterial endospore coat. Methods 20:95-110.
26. **Henriques, A. O., Moran, C. P. Jr. 2007**. Structure, assembly and function of the spore surface layers. Annu. Rev. Microbiol. 61:555-88.
27. **Henriques A. O., Melsen L. R., Moran C. P. 1998**. Involvement of superoxide dismutase in spore coat assembly in *Bacillus subtilis*. J. Bacteriol. 180:2285-2291.
28. **Hinc, K., Isticato, R., Dembek, M., Karczewska, J., Iwanicki, A., Peszyńska-Sularz, G., De Felice, M., Obukowski, M., Ricca, E. 2010.** Expression and display of UreA of *Helicobacter acinonychis* on the surface of *Bacillus subtilis* spores". Microbial Cell Factories 9:2.
29. **Hoa, N.-T., Baccigalupi, L., Huxham, A., Smertenko, A., Van, P. H., Ammendola, S., Ricca, E., Cutting, S. 2000.** Characterization of *Bacillus* species used for oral bacteriotherapy and bacterioprophylaxis of gastrointestinal disorders. Appl Environ Microbiol. 66:5241–5247.
30. **Hoch, J. A. 1993**. Regulation of phosphorelay and the initiation of sporulation in *Bacillus subtilis*. Annu. Rev. Microbiol. 47:441-465.
31.**Hofmeister, A. E. 1998**. Activation of the proprotein transcription factor pro-∂E is associated with its progression through three patterns of subcellular localization during sporulation in *Bacillus subtilis*. J. Bacteriol. 1980:2426-2433.
32. **Huang, J.M., Hong, H.A., Van Tong, H., Hoang, T.H., Brisson, A., Cutting, S.M. 2010.** Mucosal delivery of antigens using adsorption to bacterial spores. Vaccine 28:1021-1030.
33. **Ireton, K., S. Jin, A. D., Grossman, Sonenshein, A. L. 1995**. Krebs cycle function is required for activation of Spo0A transcription factor in *Bacillus subtilis*. Proc. Natl. acad. Sci. USA 92:2845-2849.
34. **Isticato, R., Cangiano, G., Tran, H. T., Ciabattini, A., Medaglini, D., Oggioni, R. M., De Felice, M., Pozzi, G., Ricca, E. 2001.** Surface display of recombinant proteins on *Bacillus subtilis* Spores, J. Bacteriol., 183(21):6294 - 6301.
35. **Isticato, R., Cangiano, G., De Felice, M., Ricca, E. 2004.** Display of molecules on the spore surface. p 193-200. In: E. Ricca, AO Henriques and SM Cutting (ed), Bacterial Spore Formers: Probiotics and Emerging Applications. Horizon Biosciences, Norfolk, UK.
36. **Isticato, R., Esposito, G., Zilhao, R., Nolasco, S., Cangiano, G., De Felice, M., Henriques, A. o., and Ricca, E. 2004**. Assembly of multiple CotC forms into the *Bacillus subtilis* spore coat. J. Bacteriol.186(4):1129-1135.
37. **Isticato, R., Pelosi, A., De Felice, M., Ricca, E. 2010.** CotE binds to CotC and CotU and mediates their interaction during spore coat formation in *Bacillus subtilis.* Journal of Bacteriol.,192(4):949–954.
38. **Jiang, M., Shao, X., Ni, H., Yu, Z., Li, L. 2011**. In vivo and in vitro surface display of heterologous proteins on Bacillus thuringiensis vegetative cells and spores. Process Biochem. 46:1861-1866.
39. **Ju, J., T., Luo, W., Haldenwang. 1997**. *Bacillus subtilis* pro-sigma E fusion protein localizes to the forespore septum and fails to be processed when synthesized in the forespore. J. Bacterial. 179:4888-4893.

40. **Kazakov, S., Bonvouloir, E., Gazaryan, I. 2008.** Physicochemical characterization of natural ionic microreservoirs: *Bacillus subtilis* dormant spores. J. Phys. Chem. 112:223 3-2244.
41. **Kim, H., Hahn, M., Grabowski, P., McPherson, D.C., Otte, M.M., Wang, R., Ferguson, C.C., Eichenberger, P. and Driks, A. 2006.** The *Bacillus subtilis* spore coat protein interaction network. Mol. Microbiol. 59:487-502.
42. **Kim, H. J., Lee, S. C. Kim, G. B.** Spore-displayed streptavidin: a live diagnostic tool in biotechnology, Biochem. Biophys. Res. Commun.,331(1):210 - P214.
43. **Klobutcher, L.A,, Ragkousi, K., Setlow, P. 2006**. The *Bacillus subtilis* spore coat provides ‘‘eat resistance’’ during phagocytic predation by the protozoan *Tetrahymena thermophila*. Proc Natl. Acad. Sci USA 103:165–170.
44. **Knecht, L. D., Pasini, P., Daunert, S. 2011.** Bacterial spores as platforms for bioanalytical and biomedical applications. Anal. Bioanal. Chem. 400:977-989.
45. **Kobayashi, K., Kumazawa, Y., Miwa, K. and Yamanaka, S. 1996.** ε-(γ-glutamyl)lysine cross-links of spore coat proteins and transglutaminase activity in *Bacillus subtilis*. FEMS Microbiol. Lett. 144:157-160.
46. **Krajcíkova´, D., Luka´cova´, M., Mu¨llerova´, D., Cutting, S. M., and Bara´k, I. 2009**. Searching for protein-protein interactions within the *Bacillus subtilis* spore coat. J. Bacteriol. 191:3212–3219
47. **Kroos, L., Kunkel, B. and Losick, R. 1989**. Switch protein alters specificity of RNA polymerase containing a compartment-specific sigma factor. Science 243:526-529.
48. **Kunkel, B., Kroos, L., Poth, H., Youngam, P. and Losick, R. 1989.** Temporal and spatial control of the mother-cell regulatory gene *spoIIID* of *Bacillus subtilis*. Genes Dev.3:1735-1744.
49. **Kwon, S.J., Jung, H.C., Pan, J.G. 2007.** Transgalactosylation in a water-solvent biphasic reaction system with β-Galactosidase displayed on the surfaces of *Bacillus subtilis* spores. *Appl. Environ. Microbiol.* 73:2251-2256.
50. **Little, S., Driks, A. 2001**. Functional analysis of the *B. subtilis* morphogenetic spore coat proteins. Molecular Microbiology.42(4): 1107-1120.
51. **Losick, R., and P. Stragier. 1992**. Crisscross regulation of cell-type-specific gene expression during development in *Bacillus subtilis*. Nature 355:601-604.
52. **Mauriello, M. F. E., Duc, H. L., Isticato, R., Cangiano, G., Hong, A. H., De Felice, M., Ricca, E., Cutting, M. S. 2004.** Display of heterologous antigens on the *Bacillus subtilis* spore coat using CotC as a fusion partner, Vaccine, 22(9-10):1177-1187.
53**. McKenney, P. T., Driks, A., Eskandarian, H. A., Grabowski, P., Guberman, J., Wang, K. H., Gitai, Z., Eichenberger, P. 2010.** A Distance-Weighted Interaction Map Reveals a Previously Uncharacterized Layer of the *Bacillus subtilis* Spore Coat. Current Biology 20: 934–938
54. **Moir, A. and Smith, D. A. 1990**. The genetics of bacterial spore germination. Annu. Rev. Microbiol. 44:531-553.
55. **Nacleiro, G., Baccigalupi, L., Zilhao, R., De Felice, M. and Ricca, E. 1996**. *Bacillus subtilis* spore coat assembly require *cotH* gene expression. J. Bacteriol. 178: 4375-4380.
56. **Nicholson, W. L., Munakata, N., Horneck, G., Melosh, H. J., and Setlow, P. 2000.** Resistance of *Bacillus* endospores to extreme terrestrial and extraterrestrial environments. Microbiol. Mol. Biol. Rev. 64: 548-572.
57. **Ozin, A.J., Samford, C.S., Henriques, A.O., Moran, C.P. 2001.** SpoVID guides SafA to the spore coat in *Bacillus subtilis*. J. Bacteriol. 183:3041–3049.
58. **Parker, G. F., Daniel, R. A., and Errington, J. 1996**. Timing and genetic regulation of commitment to sporulation in *Bacillus subtilis*. Microbiology 142:3445-3452.
59. **Pogliano, K., Hofmeister, A. E. M. and Losick, R. 1997**. Disappearance of the σE transcription factor from the forespore and the SpoIIE phosphatase from the mother cell contributes to establishment of cell-specific gene expression during sporulation in *Bacillus subtilis*. J. Bacteriol. 179:3331-3341.

60. **Potot, S., Serra, C.R., Henriques, A.O., Schyns, G. 2010**. Display of recombinant proteins on *Bacillus subtilis* spores using a coat-associated enzyme as the carrier. Appl. Environ. Microbiol. 76: 5926-5933.
61. **Ramamuthi, K.S. and Losick, R. 2008**. ATP-driven self assembly of morphogenetic protein in *Bacillus subtilis*. Mol. Cell. 31(3): 406-414.
62. **Ricca, E., Baccigalupi, L., Nacleiro, G. and Cutting, S. 1997**. Spore coat differentiation. Res. Microbiol. 148: 5-9.
63. **Ricca, E., Cutting, S., and Losick, R. 1992**. Characterization of *bofA*, a gene involved in intercompartimental regulation of pro-∂K processing during sporulation in *Bacillus subtilis*. J. Bacteriol. 174:3177-31.
64. **Roels, S., Driks, A. and Losick, R. 1992**. Characterization of *spoIVA*, a sporulation gene involved in coat morphogenesis in *Bacillus subtilis*. J. Bacteriol. 174: 575-585.
65. **Sacco, M., Ricca, E., Losick, R. and Cutting, S. 1995.** An additional GerE controlled gene encoding an abundant spore coat protein from *Bacillus subtilis*. J. Bacteriol. 177**:**372–377.
66. **Scheeff, E. D., Axelrod, H. L., Miller, M. D., Chiu, H. J., Deacon, A. M., Wilson, I. A., Manning, G.** Genomics, evolution, and crystal structure of a new family of bacterial spore kinases. Proteins 2010; 78:1470–1482.
67**. Shao, X., Jiang, M., Yu, Z., Cai, H., Li, L. 2009.** Surface display of heterologous proteins in *Bacillus thuringiensis* using a peptidoglycan hydrolase anchor. Microbial Cell Factories 8:48.
68. **Silvaggi, J.M., Popham, D.L., Driks, A., Eichenberger, P., Losick, R. 2004**. Unmasking novel sporulation genes in *Bacillus subtilis*. *J Bacteriol* 186(23):8089-95.
69. **Stevens, C. M., Daniel, R., Illing, N. and Errington, J. 1992**. Characterization of a sporulation gene *spoIVA* involved in spore coat morphogenesis in *Bacillus subtilis*. J. Bacteriol. 174: 586-594.
70. **Stragier, P. 1992.** Establishment of forespore-specific gene expression during sporulation of *Bacillus subtilis*, p. 297-310. In J. A. Cole, F. Mohan, and C. Dow (ed.), Procaryotic structure and function. Society for General Microbiology, London, United Kingdom.
71. **Sun, D., Stragier, P. and Setlow, P. 1989**. Identification of a new σ-factor involved in compartmentalized gene expression during sporulation of *Bacillu subtilis*. Genes Dev. 3:141-149.
72. **Walker, J., Crowley, P., Moreman, A. D., Barrett, J. 1993**. Biochemical properties of cloned glutathione S-transferases from *Schistosoma mansoni* and *Schistosoma japonicum*. Mol Biochem Parasitol 61:255–264.
73.**Wang Katherine, H., Isidro, A. L., Domingues, L., Eskandarian, H. A., McKenney, P. T., Drew, K., Grabowski, P., Chua, M. H., Barry, S. N., Guan, M., Bonneau, R., Henriques, A. O. Eichenberger P. 2009.** The coat morphogenetic protein SpoVID is necessary forespore encasement in *Bacillus subtilis*. Molecular Microbiol. 74: 634-649.
74. **Wiencek, K.M., Klapes, N. A., Kubler, O. 1990**. Hydrophobicity of *Bacillus* and *Clostridium* spores. Appl. Environ. Microbiol. 56:2600-2605.
75. **Zhang, B., Daniel, R. A., Errington, J., Kroos L. 1997**. *Bacillus subtilis* SpoIIID protein binds to two sites in the *spoVD* promoter and represses transcription by sigma E RNA polymerase. J. Bacteriol. 179:972-975.
76. **Zheng, L., and Losick, R. 1990**. Cascade regulation of spore coat gene expression in *Bacillus subtilis*. J. Mol. Biol. 212:645- 660.
77. **Zihlao, R., Isticato, R., Martins, I. O., Steil, I., Volker, U., Ricca, E., Moran, C. P. Jr., and Henriques, A., O. 2005**. Assembly and function of a spore Coat-associated transglutaminase of *Bacillus subtilis*. J. Bacteriol. 7753-7764.
78. **Yim, S.K., Jung, H.C., Yun, C.H., Pan, J.G. 2009.** Functional expression in *Bacillus subtilis* of mammalian NADPH-cytochrome P450 oxidoreductase and its spore-display. Protein Expression and Purification 63:5–11.

79. **Zilhao, R., Serrano, M., Isticato, R., Ricca, E., Moran, P. M., Henriques, A. O. 2004.** Interactions among CotB, CotG and CotH during assembly of the *Bacillus subtilis* spore coat. J. Bacteriol. 186(4):1110-1119.

## *Chapter 2*

# Over-production of CotH partially bypasses *cotE*-associated phenotypes in *Bacillus subtilis* spores.

## Introduction

In response to nutrient limitation, the gram-positive soil bacterium *Bacillus subtilis* forms an alternative cell type, called the spore, during a process known as sporulation. A multilayered proteinaceous shell, called the coat, surrounds the spore and provides an extraordinarily high degree of resistance to a variety of environmental assaults, including mechanical stress, and degradative proteins, such as lysozyme (1). These features are due to its particular structure, a heavily dehydrated core, a cortex composed mainly of peptidoglycan, a thick spore coat which can be divided in inner and outer layer and the spore crust. There were more than 70 protein species found in the spore coat. Most of them have a strictly structural role, some of them possess even enzymatic activity, and only a relatively small number of proteins have a disproportionately large impact on morphogenesis of the spore. These so-called morphogenetic proteins have the job of co-ordinating the assembly of the major structural proteins in a macromolecular complex. Studying how these proteins are able to direct the assembly of so many other proteins is an important issue which should be addressed in details.

CotE is one of the most important morphogenetic factors involved in late stages of sporulation, as far as deletion of *cotE* gene entirely prevents the assembly of the outer coat layer and of some inner coat proteins (7; 8; 11). In particular, it affects the assembly of at least 24 components of inner and outer coat (3). The effect of CotE on coat formation appears not to be at the level of expression of coat protein genes but, rather, on protein assembly (7). CotE is therefore a morphogenetic protein that nucleates coat protein assembly (2). Additionally, CotE directs the assembly of CotH, another morphogenetic protein, which controls the assembly of at least 9 spore coat components (3) and is required for the proper assembly of both coat layers (12). We refer to those proteins whose assembly depends on CotE and/or on CotH as CotE- and/or CotH-controlled proteins.

CotH and CotE cooperate in regulation of coat assembly. CotH directs deposition of a subset of the CotE-dependent coat proteins and is, itself, at least partially CotE dependent (3; 12; 13). The set of proteins known to be absent or reduced in amount in a in a *cotH* null mutant spore is currently CotB, CotC, CotG, CotS, CotSA, CotQ, CotU, CotZ and YusA (3). Moreover, CotE and CotH are important in conferring some resistance propreties. A previous report (13) showed that spores of a strain carrying a *cotH* null mutation maintain its full resistance to lysozyme, while spores of the double *cotE cotH* null mutant have an increased sensitivity to lysozyme compared with the single *cotE* null mutant.

To explore further the roles of CotE and CotH in spore coat assembly, we constructed a strain, which over-products CotH and we observed, that CotH partially bypasses *cotE*-associated phenotypes in *Bacillus subtilis* spores.

**Materials and methods**

**Bacterial strains and transformation.** *B. subtilis* strains used in this study are listed in Table 1. Plasmid amplification for subcloning experiments, nucleotide sequence analysis, and transformation of *Escherichia coli* competent cells were performed with *E. coli* strain DH5 (19). Bacterial strains were transformed by previously described procedures: $CaCl_2$-mediated transformation of *E. coli* competent cells (19) and two-step transformation of *B. subtilis* (4).

**Genetic and molecular procedures.** Isolation of plasmids, restriction digestion, and ligation of DNA were carried out by standard methods (19). Chromosomal DNA from *B. subtilis* was isolated as described elsewhere (4). RG24 strain was obtained by fusing *cotA* promoter to a DNA fragment containing the 5' region of the coding part of *cotH* (Fig. 3). This fusion was obtained by using the gene splicing by overlap extension (SOEing) technique (17). Briefly, two PCR products were obtained with oligonucleotide pairs PCotAs and CotA3anti (to amplify the *cotA* promoter region of

577 bp) and H32s/H13 (amplifying the 850 bp fragment starting from the ATG to the unique EcoRI site internal of *cotH* coding sequence) (table 2). The obtained products were used as templates to prime a third PCR with the external primers CotApS and H13 (table 2). The modified version of *cotH* was cloned into the vector pER19 (32), and the correct gene fusion was verified by sequencing reactions. The resulting plasmid, pRG24, was introduced by single reciprocal (Campbell-like) recombination at the *cotH* locus of the *B. subtilis* chromosome. Several chloramphenicol-resistant clones were analyzed by PCR to select the clone containing the modified *cotH* promoter sequence upstream the entire *cotH* gene, yielding the strain RG24.

A RG24 lacking *cotE* strain was obtained by transforming a competent cell of strain RH211 (*cotE*::spc) (table 1) with chromosomal DNA extracted from strain RG24, generating strain AZ555.

**Overexpression of CotH.** Strain VS13 overexpressing his-tagged CotH (Table 1) was obtained by cloning *cotH* coding region in pBAD and was available in the lab (Baccigalupi personal communication). Strain VS13 was collected after autoinduction (50 mL culture) by centrifugation and resuspended in 5 ml of lysis buffer (50 mM NaH2PO4, 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 (20-min pulses at 20 Hz with a Sonicator Ultrasonic liquid processor; Heat System Ultrasonic Inc., NY). The suspension was clarified by centrifugation at 13,000 g at 4°C for 20 min, and protein concentration was determined by a Bio-Rad assay. The protein extract was diluted 1:3 in binding buffer (20 mM sodium phosphate, 500 mM NaCl, 20 mM imidazole [pH 7.4]). The diluted sample was applied to a HisTrap HP column (GE Healthcare Europe GmbH, Milan, Italy), which was previously equilibrated with 10 ml of binding buffer. After the loading the *E. coli* protein extract, the column was washed with 10 ml of binding buffer. Then proteins were eluted with the same

buffer, supplemented with increasing concentrations of imidazole (50 mM, 100 mM and 250 mM). Purified protein was desalted using the PD10 column (GE Healthcare Europe GmbH, Milan, Italy) to remove high NaCl and imidazole concentrations.

**Transcriptional analysis.** RNA were extracted from cells in sporulation at 5, 7 and 9 hours after the onset of sporulation (T5, T7 and T9). For reverse transcription-PCR (RT-PCR) analysis a sample containing 2 µg of DNase-treated RNA was incubated with oligonucleotide H6 at 65°C for 5 min and slowly cooled to room temperature to allow the primer annealing. RNAs were then rettrotrascribed with oligo H6, which is annealing in the coding region of *cotH*. We obtained cDNA by incubating the mixture at 50°C for 1 h in the presence of 1 µl AffinityScript multiple-temperature reverse transcriptase (Stratagene), 4 mM dNTPs, reaction buffer Stratagene), and 10 mM dithiothreitol (DTT). The enzyme was then inactivated at 70°C for 15 min. We obtained cDNA, which was amplified by PCR, firstly, using primers CotApS and H to analyze expression from *cotA* promoter, and secondly, primers H12anti and H to analyze the expression starting from *cotH* promoter. As a control, PCRs were carried out with RNA non-retrotranscribed to exclude the possibility that the amplification products could derive from contaminating genomic DNA.

**Sporulating cells lysates and immunoblot analysis.** Sporulation of all *B. subtilis* strains was induced by the exhaustion method (4). Sporulating cells were harvested during the sporulation and 18 hours from the onset of sporulation, and mother cells and forespore fractions were isolated as described before (9). Whole-cell lysates of sporulating cells were prepared by sonication (9) followed by detergent treatment (62.5 mM Tris-HCl [pH 6.8], 4% SDS, 5% glycerol, 2% β-mercaptoethanol, 0.003% bromophenol blue) at 100°C for 7 min. Fifty micrograms (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 was performed with treatment at 65 °C in SDS-DTT extraction buffer or at 4 °C in 0.1M NaOH (9). Western blot analysis were performed by standard procedures. For electrotransfer was used nitrocellulose membrane and the proteins were then hybridated with either anti-cotH, anti-CotB, anti-cotG, anti-CotC or anti-CotA antibodies as described previously (9).

**Physiological assays.** Lysozyme resistance (4). Purified spores were resuspended in Tris 10 mM at pH 7.2 and $OD_{600}$ of spore suspensions was measured before and after adding 50 µg/mL of lysozyme. $OD_{600}$ was measured at 1 minute intervals for first 10 minutes and after 30 minutes of incubation of spores with lysozyme at 25 °C. Germination assays. Spore germination was assayed as described previously (21).

**Table 1**. List of strains.

| **Strain** | **Genotype** | **Source** |
|---|---|---|
| *B. subtilis* | | |
| PY79 | wild type | [22] |
| RH211 | *cotE::spc* | [9] |
| DZ213 | *cotE::cm* | [25] |
| ER220 | *cotH::spc* | [23] |
| RG24 | *pAH::cm* | This study |
| AZ555 | *cotE::spc pAH::cm* | This study |
| KS450 | *gerΔ36* | [20] |
| *E. coli* | | |
| VS13 | *cotH-his* | Lab stock |

**Table 2.** List of primers.

| Primer | Sequence[a] |
|---|---|
| H32s | AATAAAAGCCTAAAATTTGTAAGGAGGATTATGG |
| H | CGCGGATCCGCGCCGGAATTCAGCGATATCAATATCCAG |
| H13 | TCCTTGAATTCTTACATAATCG |
| H12anti | GTATAAGTGTTCCGCTGAGATCTT |
| H6 | CTCTGATATGTGATCCCC |
| CotApS | CGTCTATCTTGTCATCGCC |
| CotAp3a | CAAATTTTAGGCTTTTATTTACTATAGTTAATGACAATAAGG |

[a] Capital and underlined letters indicate nucleotides complementary to corresponding gene DNA and unpaired flanking sequences carrying a restriction site, respectively.

**Results and Discussion**

**CotH is present in the sporulating cell for a short time during sporulation.** CotH structural gene is transcribed under the control of the sporulation-specific sigma factor $\sigma^K$ and is negatively controlled by the transcriptional regulator GerE (6). As a consequence, transcription of the gene is induced 4 hours after the beginning of sporulation, peaks one hour later and then rapidly decreases (6). The CotH protein follows the same pattern. A western blotting analysis with anti-CotH antibody of proteins extracted from forespores at various times during sporulation showed that CotH appeared about five hours after the onset the sporulation, reached its maximal amount one hour later and then rapidly decreased (Fig. 1A). The reduction of the amount of CotH present around the forming spore when the *cotH* gene is no longer active (after T6) is unusual and could be due to several different reasons: i) CotH could fail to assemble around the spore and accumulate in the mother cell compartment of the sporangium, ii) CotH could become non-extractable because of putative post-translational modifications occurring in the sporangium after T6, or iii) CotH could be degraded either because of an intrinsic instability or because of a specific protease. To distinguish between those possibilities we first analysed by western blot with anti-CotH antibody the two cell compartments during sporulation. As shown in Fig. 1B, CotH was never detected in the mother cell of a wild type strain at all time points analyzed. In a *cotE* mutant, in which CotH assembly is impaired (12), CotH accumulated in the mother cell indicating that CotH can be detected in that cell compartment (Fig. 1B). To verify whether CotH becomes non-extractable at late times during sporulation we extracted the coat proteins with a procedure developed to extract the spore coat proteins from inner and outer spore coat layers using a decoating extraction buffer, but were unable to detect CotH (Fig. 1C). Those results, therefore suggest that CotH does not accumulate in the mother cell and does not become non-extractable.

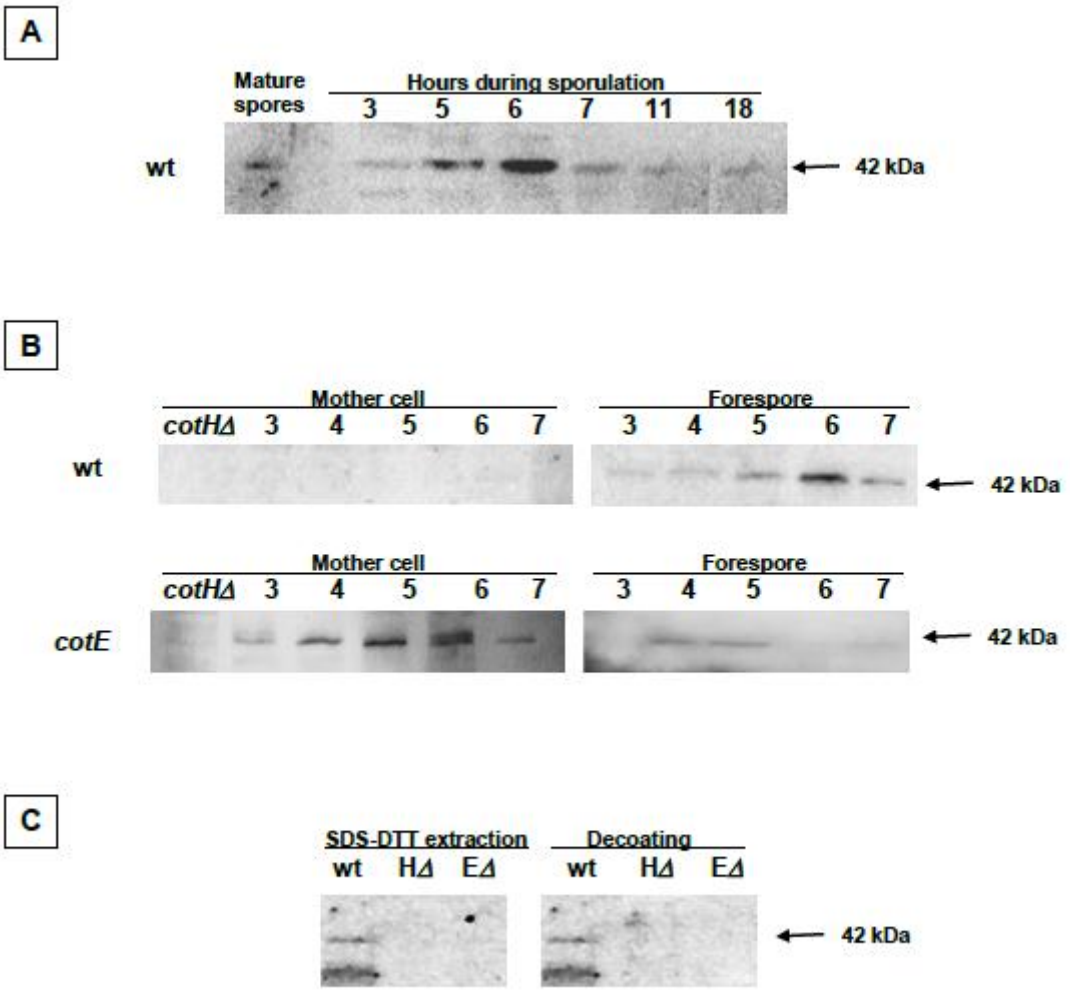


**Figure 1.** Western blotting analysis with anti-CotH antibody with anti-CotH antibody. In panel A we analysed protein extract form mature spores and at various times after the onset of sporulation starting at $T_3$ to $T_{18}$. In panel B, both cellular compartments were analysed of wild type and *cotE* null mutant strain. *CotH* null mutant (ER220) was used as negative control and proteins were analysed at 3, 4, 5, 6 and 7 hours after the onset of sporulation. In Fig. C, the proteins were extracted from mature spores of wild type (AZ54), *cotH* mutant (ER220) and *cotE* mutant (DZ213) spores with classical SDS-DTT extraction buffer and with decoating buffer (SDS 2 %, DTT 100 mM, NaOH 100 mM, NaCl 100 mM).

To verify whether CotH is particularly unstable we overexpressed CotH in *E. coli* and incubated the purified protein overnight at 25 or 37°C. The protein samples were then compared to non treated CotH (stored at -20°C) and analysed by western and dot blot to monitor the amount of CotH still present after the incubation at 25 and at 37°C. As shown in Fig. 2, a reduced amount of CotH was found after the incubation at 37°C than at 25°C, suggesting that the protein is particularly unstable.

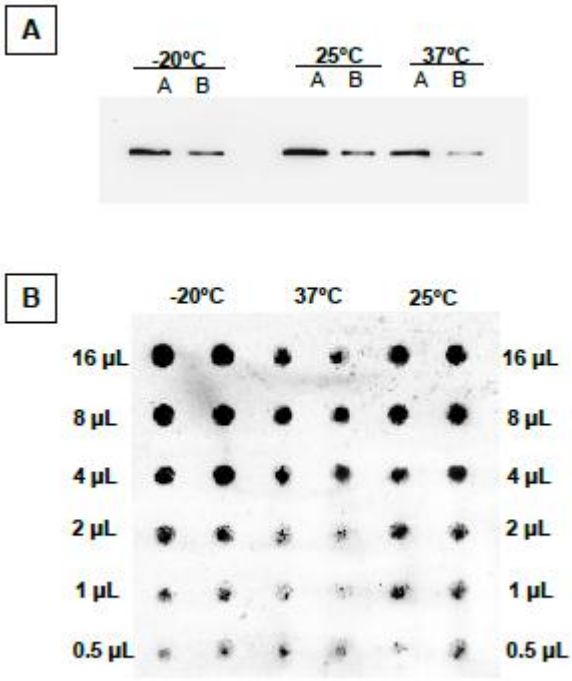


**Figure 2.** Western (A) and dot (B) blot of purified CotH protein. CotH was purified from VS13 strain as described in materials and methods and incubated overnight (16 hours) at 25 and at 37°C. The samples were then compared to non treated protein (stored at -20°C). In panel A, 1 µg (A) and 0.5 µg (B) of purified CotH were loaded. In panel B, the same amounts of CotH were loaded onto the nitrocellulose membrane. Anti-CotH antibody was used to detect the protein.

**Expression of *cotH* under the control of the *cotA* promoter.** To elucidate the role of CotH in spore coat morphogenesis we decided construct a mutant in which CotH was more abundant and/or present for a longer time during sporulation than in the wild type strain. To this aim we decided to clone the *cotH* coding part under the transcriptional and translational signals of the *cotA* gene. *cotA* is transcribed by $\sigma^k$ but its promoter is more efficient than the *cotH* promoter in initiating transcription (16). The *cotA* promoter was fused to a DNA fragment containing the 5' region of the coding part of *cotH* (Fig. 3). The gene fusion, carried by the integrative plasmid pRG24, was used to transform competent cells of a wild type strain of *B. subtilis,* PY79. By a single (Campbell-like) cross-over recombination event between homologous DNA regions present on the plasmid and on the chromosome the *cotA* promoter was inserted upstream of an entire *cotH* coding part yielding strain RG24 (Fig. 3).

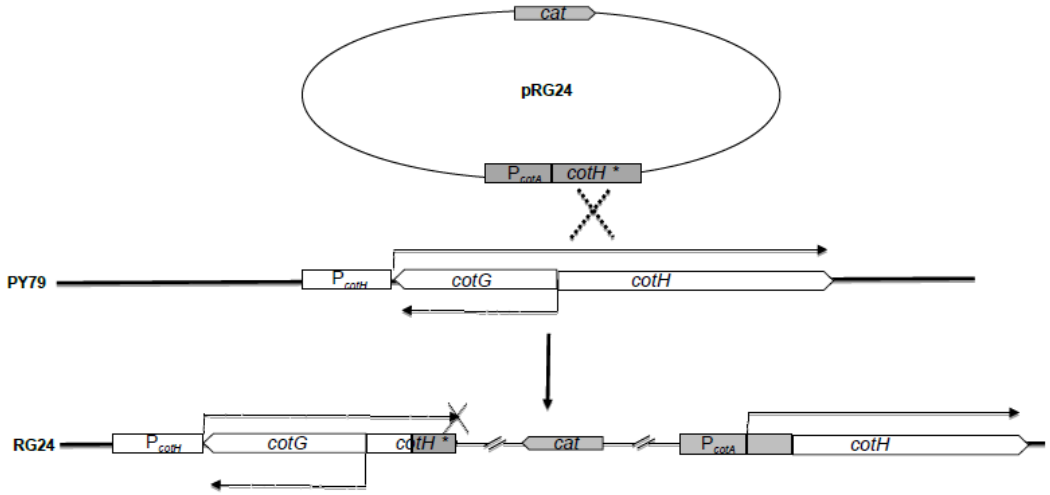


**Figure 3**. Schematic representation of RG24 strain construction. Promoter of *cotA* was fused to the coding region of *cotH* using "gene soeing" technique as described in materials and methods. The fragment obtained was cloned into the integration vector pER19, yielding plasmid pRG24, then used to transform competent cells of a wild type strain of *B. subtilis.* The resulting strain, RG24, a *cotH* promoter followed by an interrupted *cotH* gene and a *cotA* promoter followed by an entire *cotH* coding part.

Strain RG24 was used to monitor by RT-PCR the expression driven by the *cotA* and *cotH* promoter during sporulation. Total RNA was extracted from sporulating cells and used as a template to produce cDNA using a primer annealing in the *cotH* coding part. The same primer was also used for the susquent PCR paired with a primer annelaing just downstream of the *cotA* promoter (transcript drived by *cotA* promoter) or within cotG (transcript drived by *cotH* promoter).

As shown in Fig. 4A, a *cotA*-driven transcript was more abundant than that driven by the *cotH*-promoter at all time points analyzed. In addition, the *cotA*-driven transcript was also present at late time of sporulation (T9). In agreement with the transcriptional analysis a western blot experiment (Fig. 4B) showed that in strain RG24, CotH is present at late times during sporulation.

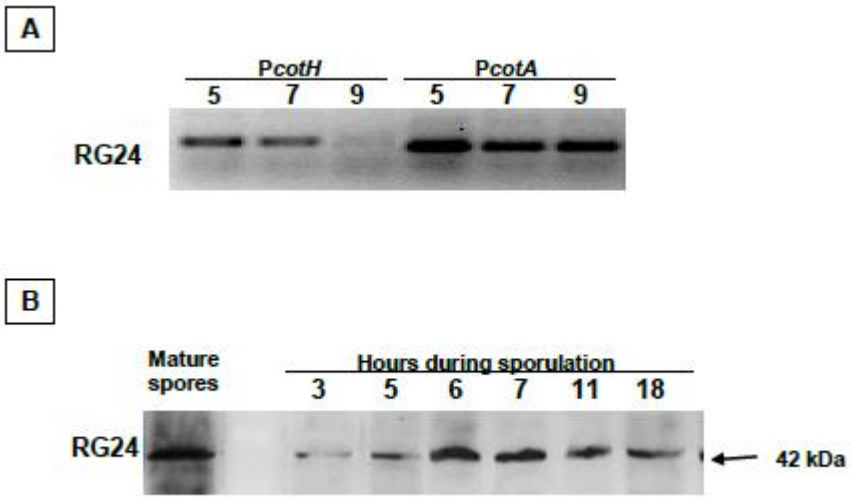


**Figure 4.** (A) Expression of *cotH* in RG24 strain was monitored by RT-PCR. Total RNA was extracted from sporulating cells 5, 7 and 9 hours after the onset of sporulation. cDNA synthesis was primed with oligonucleotide H6 (Table 2). In the amplification reactions, cDNA obtained was used as a template and the PCR reactions were primed with oligonucleotide pairs H12anti/H to analyze expression from *cotH* promoter and CotApS/H to analyze expression from *cotA* promoter (Table 2). (B) Proteins extracted from RG24 strain were extracted from mature spores and from forespore compartment at various times during the sporulation (at 3, 5, 6, 7, 11 and 18 hours after the onset of sporulation). Proteins were analysed by western blot and with specific anti-CotH antibody.

**Over-production of CotH partially bypasses *cotE*-associated phenotypes.** To investigate the effects of CotH over-expression we analyzed the efficiency of sporulation, the resistance of spores to chemicals and lytic enzymes and the efficiency of spore germination of strain RG24. Spores of strain RG24 were identical to isogenic wild type spores for all phenotypes analyzed (not shown), indicating that CotH over-expression does not affect spore structure and function in an otherwise wild type strain. However, when CotH was over-expressed in a mutant strain lacking CotE, we observed a partial suppression of the typical *cotE* phenotypes, the sensitivity to lysozyme (Fig. 5A) and the reduced efficiency of germination (Fig. 5B).

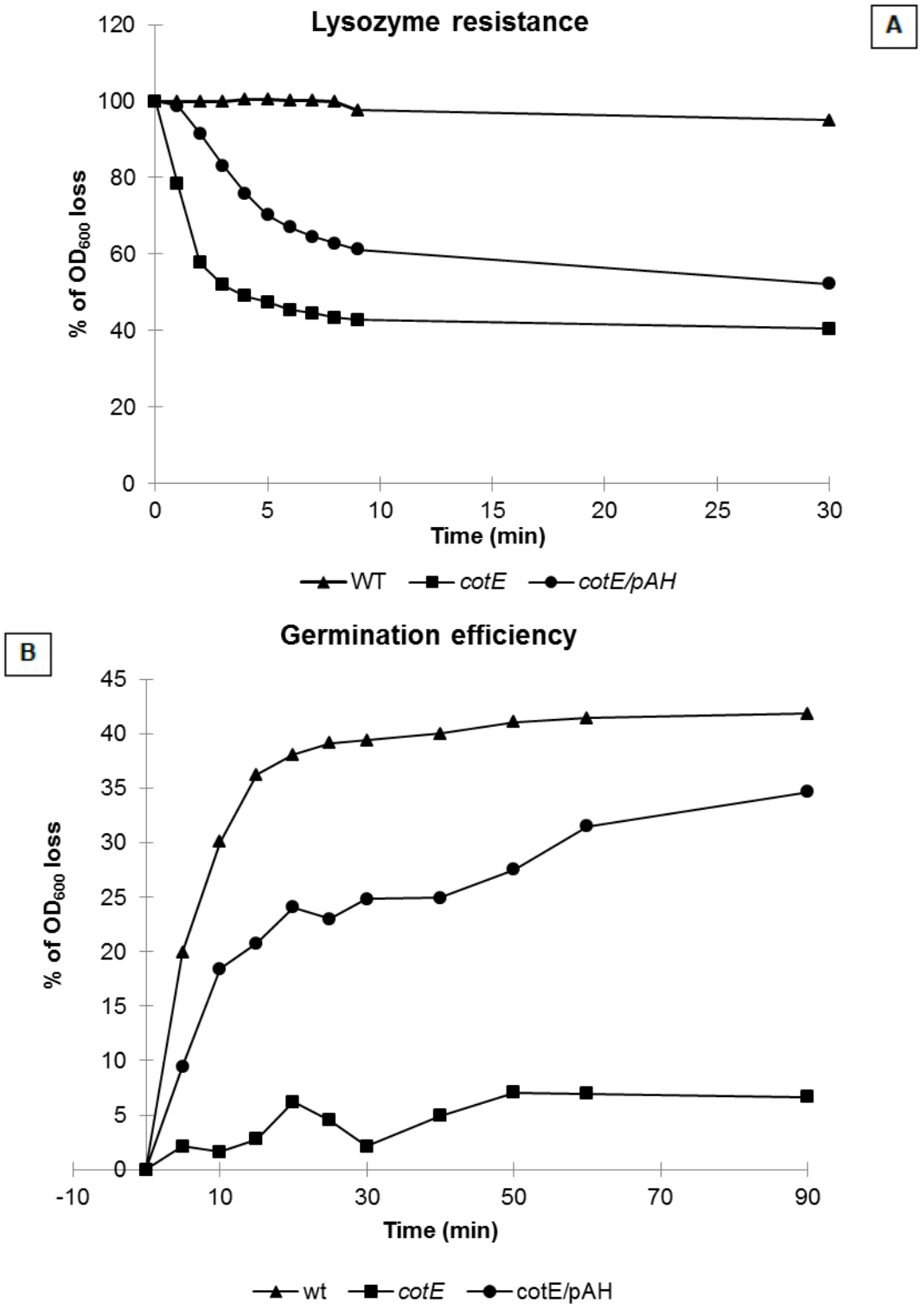


**Figure 5.** Wild type spores (▲), *cotE* mutant (■) and *cotE*/pAH (●) were assayed for their resistance to lysozyme (panel A) and efficiency in germination (panel B).

A previous report (13) showed that spores of a strain carrying a *cotH* null mutation do not show any difference with respect to the wild type, while spores of the double *cotE cotH* null mutant have an increased sensitivity to lysozyme compared with the single *cotE* null mutant. Therefore, the previous report suggest that CotH contributes with CotE in determining the resistance to lysozyme and the efficience of germination. Now, we observe that the increased/prolonged presence of CotH partially restores the resistance to lysozyme and the germination efficiency lost because of the *cotE* null mutation. Our results then suggest that CotH can partially replace CotE in driving the formation of the outer coat.

**Over-production of CotH bypasses the CotE requirement for the assembly of CotH-dependent outer coat components.** It is well established that in a *cotE* mutant the outer coat is not assembled (7). To explain how the over-production of CotH can partially by-pass the requirement of CotE for lysozyme-resistance and germination efficiency we analyzed the coat assembly of various CotE-dependent coat components in a *cotE* mutant and in the isogenic mutant lacking CotE but over-expressing CotH. As a control we also analysed strain RG24 over-expressing CotH in a otherwise wild type strain. In all cases analysed, over-production of CotH in a wild type strain did not affect the assembly of other coat components. However, over-expression of CotH in the absence of CotE allowed the assembly of some CotE-dependent outer coat components (Fig. 6). In particular, over-production of CotH affected the assembly of the CotE-/CotH-dependent CotB, CotG, CotC and CotU proteins but not of the assembly of CotA or TgI that are CotE-dependent and CotH-independent (Fig. 6).

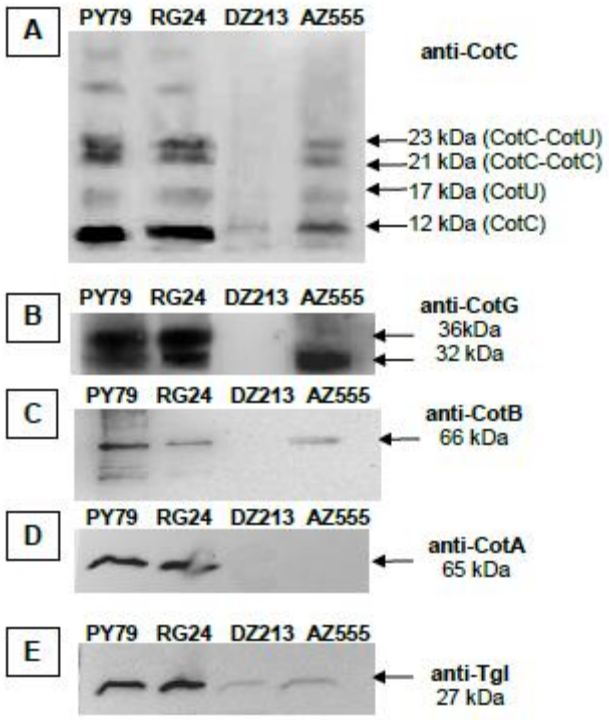


**Figure 6.** Western blot of proteins extracted from wild type spores (PY79), RG24**,** DZ213 (*cotE* null mutant ) and RG24 lacking *cotE* strain (AZ555). The proteins were extracted from spores with SDS-DTT extraction buffer or with 0.1 M NaOH (only in panel C). Membranes were hybridated with anti-CotC antibody (panel A), anti-CotG (panel B), anti-CotB (panel C), anti-CotA (panel D) and anti-TgI (panel E).

As shown in Fig. 6 all CotE/CotH-dependent proteins analyzed were assembled in a a *cotE* mutant strain over-producing CotH. CotC is an abundant spore coat protein known to assemble in the outer coat in various forms: a monomer of 12 kDa, a homodimer CotC-CotC of 21 kDa and two less abundant forms of 12.5 and 30 kDa, probably due to posttranslational modifications of CotC (9). CotU is a structural homolog of CotC and it assembles on spore as a monomer of 17 kDa and as a CotC-CotU heterodimer of 23 kDa (10). The two proteins, which share an almost identical N terminus and high identity in amminoacid sequence, are both recognized by anti-CotC and anti CotU specific antibodies. CotC and CotU are also dependent on CotH (9), as far as *cotH*-null mutation prevents CotC and CotU assembly in the

coat as well as their accumulation in the mother cell cytoplasm (10). In contrast, a mutation causing *cotH* overexpression allows CotC and CotU accumulation in the mother cell cytoplasm (6), and it has been proposed that CotH acts by stabilizing CotC and CotU (1, 10). The formation of homo- and hetero-dimers instead is driven by different factors, in particular the formation of CotC-CotC homodimer seems to be a spontaneous process as far as it occurs also in a heterologous host (*E. coli*) (9). In contrast, it was clearly shown that formation of heterodimer CotC-CotU requires CotE protein, which is essential to mediate heterodimerization (9). In our immunoblot analysis we detected all four of CotC-CotU associated forms (Fig. 6A) in wild type, RG24 and *AZ555* strain, while only in *cotE* null mutant none of these proteins were found on spore surface, showing that overexpression of CotH can bypass the requirement for CotE for assembly and dimerization of CotC-CotU related forms.

CotG is an outer coat protein with calculated molecular weight of 24 kDa but specific anti-CotG antibodies recognise in wild type spores two specific bands with molecular weight of 32 kDa and 36 kDa. This abnormal migration could be due to CotG dimerization or to eventual post-translational modifications, but this aspect was not addressed yet in details. It is CotE and CotH dependent protein (5) and nonetheless in *cotE* mutant, CotH is present in low amounts on prespore and in higher amounts in mother cell compartment, it results non sufficient for CotG assembly. In strain AZ555 lacking *cotE* and overexpressing *cotH*, assembly of a 32 kDa form of CotG protein occurs (Fig. 6B). Moreover, CotG protein plays an essential role together with CotH in the conversion of CotB from an immature 46-kDa form into a mature 66-kDa form (14). In a *cotE* null mutant CotB does not form a 46-kDa form and does not even assemble on the spore (14). In strain AZ555 instead we detected a mature form of CotB in spore protein extract (Fig. 6, panel C), suggesting, that a 32 kDa form of CotG detected in this strain is sufficient for maturation and assembly of mature CotB, obeying a hierarchical control CotH-CotG-

CotB as proposed by Zilhao *et al.* in 2004. This data confirm our observations based on resistance studies (Fig. 5A and 5B) which suggest, that overexpression of *cotH,* which results in higher quantities of CotH protein and its prolonged presence during late stages of sporulation, is able to partially replace CotE in directing outer spore coat assembly and partially restoring resistance to lytic enzymes and germination efficiency.

Fig. 6 also shows that CotE-dependent, CotH-independent proteins are not affected by the over-production of CotH. CotA is a 65 kDa protein with a laccase activity (15) and is strictly CotE dependent (5) and its assembly on spores was not affected by CotH overproduction (Fig. 6D). Tgl is another spore coat component, which possess transglutaminasic activity (22) and its assembly on spore is CotH independent and partially CotE-dependent (5). Neither the assembly of Tgl was affected by CotH overproduction (Fig. 6E).

**Conclusions**

In conclusion, our data indicate that over-production of CotH allows the bypass of CotE requirement for development of full resistance to lysozyme and full efficiency of germination. Such bypass is most likely accomplished by allowing the assembly of some coat components under the dual control of CotE and CotH.

**References**

1**. Aronson, A. I., and P. Fitz-James. 1976.** Structure and morphogenesis of the bacterial spore coat. Bacteriol. Rev. 40:360–402.
2**. Bauer, T., Little, S., Sto¨ver, A.G., and Driks, A**. **1999.** Functional regions of the *B. subtilis* spore coat morphogenetic protein CotE. J Bacteriol 181: 7043–7051.
3**. Little, S., and Driks, A. 2001.** Functional analysis of the *Bacillus subtilis* morphogenetic spore coat protein CotE. Mol Microbiol 42: 1107–1120.
4. **Cutting, S., and P. B. Vander Horn**. **1990.** Genetic analysis, p. 27–74. In C. Harwood and S. Cutting (ed.), Molecular biological methods for Bacillus. John Wiley & Sons, Chichester, United Kingdom.
5. **Kim, H., Hahn, M., Grabowski, P., McPherson, D.C., Otte, M.M., Wang, R., Ferguson, C.C., Eichenberger, P. and Driks, A. 2006.** The *Bacillus subtilis* spore coat protein interaction network. Mol. Microbiol. 59:487-502.
6. **Baccigalupi, L., G. Castaldo, G. Cangiano, R. Isticato, R. Marasco, M. De Felice, and E. Ricca. 2004.** GerE-independent expression of *cotH* leads to CotC accumulation in the mother cell compartment during *Bacillus subtilis* sporulation. Microbiology 150:3441–3449.
7. **Zheng, L., W. P. Donovan, P. C. Fitz-James, and R. Losick. 1988.** Gene encoding a morphogenic protein required in the assembly of the outer coat of the *Bacillus subtilis* endospore. Genes Dev. 2:1047–1054.
8. **Abe, A., H. Koide, T. Kohno, and K. Watabe. 1995.** A *Bacillus subtilis* spore coat polypeptide gene, *cotS*. Microbiology 141:1433–1442.
9**. Isticato, R., G. Esposito, R. Zilhao, S. Nolasco, G. Cangiano, M. De Felice, A. O. Henriques, and E. Ricca. 2004.** Assembly of multiple CotC forms into the *Bacillus subtilis* spore coat. J. Bacteriol. 186:1129–1135.
10. **Isticato, R., A. Pelosi, R. Zilhao, L. Baccigalupi, A. O. Henriques, M. De Felice, and E. Ricca. 2008.** CotC-CotU heterodimerization during assembly of the *Bacillus subtilis* spore coat. J. Bacteriol. 190:1267–1275.
11. **Takamatsu, H., Y. Chikahiro, T. Kodama, H. Koide, S. Kozuka, K. Tochikubo, and K. Watabe**. **1998.** A spore coat protein, CotS, of *Bacillus subtilis* is synthesized under the regulation of sK and GerE during development and is located in the inner coat layer of spores. J. Bacteriol. 180:2968–2974.
12. **Zilhao, R., Naclerio, G., Henriques, A.O., Baccigalupi, L., Moran, C.P., Jr, and Ricca, E. 1999**. Assembly requirements and role of CotH during spore coat formation in *Bacillus subtilis.* J Bacteriol 181: 2631–2633.
13. **Naclerio, G., Baccigalupi, L., Zilhao, R., De Felice, M., and Ricca, E. 1996.** *Bacillus subtilis* spore coat assembly requires *cotH* gene expression. J Bacteriol 178: 4375–4380.
14. **Zilhao, R., Serrano, M., Isticato, R., Ricca, E., Moran, C. P., Henriques A. O. 2004.** Interactions among CotB, CotG, and CotH during assembly of the *Bacillus subtilis* spore coat. J. Bacteriol. 186:1110–1119.
15. **Martins, L. O., C. M. Soares, M. M. Pereira, M. Teixeira, T. Costa, G. H. Jones, and A. O. Henriques. 2002.** Molecular and biochemical characterization of a highly stable bacterial laccase that occurs as a structural component of the *Bacillus subtilis* endospore coat. J. Biol. Chem. 277:18849–18859.
16**. Eichenberger, P., Fujita, M., Jensen, S. T., Conlon, E. M., Rudner, D. Z., Wang S. T., Ferguson, C., Haga, K., Sato, T., Liu, J. S., Losick, R. 2004.** The program of gene transcription for a single differentiating cell type during sporulation in *Bacillus subtilis*. PLOS Biology 2(10):1664-1683.
17. **Horton, R. M., H. D. Hunt, S. N. Ho. Pullen, and J. K. R. Pease. 1989.** Engineering hybrid genes without the use of restriction enzymes: gene splicing by overlap extension. Gene 77:61–68. 1

18**. Ricca, E., S. Cutting, and R. Losick. 1992.** Characterization of *bofA*, a gene involved in inter-compartmental regulation of pro-sK processing during sporulation in *Bacillus subtilis*. J. Bacteriol. 174:3177–3184.
19. **Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989.** Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
20**. Zheng, L. B. & Losick, R. 1990.** Cascade regulation of spore coat gene expression in *Bacillus subtilis*. J Mol Biol 212:645–660.
21. **Paidhungat, M., and P. Setlow. 1999.** Isolation and characterization of mutations in *Bacillus subtilis* that allow spore germination in the novel germinant D-alanine. J. Bacteriol. 181:3341–3350.
22. **Zilhao, R., Isticato, R., Martins L. O., Steil, L., Volker, U., Ricca, E., Moran, C. P. Jr., and Henriques, A. O. 2005.** Assembly and Function of a Spore Coat-Associated Transglutaminase of *Bacillus subtilis*. Journal of Bacteriology, 187(22): 7753–7764.
23. **Naclerio G, Baccigalupi L, Zilhao R, De Felice M, Ricca E**. **1996.** *Bacillus subtilis* spore coat assembly requires *cotH* gene expression. J Bacteriol,178:4375–4380.

# Chapter 3

CotE and CotH interact and control each other assembly within the spore coat of *Bacillus subtilis.*

## Introduction

*Bacillus subtilis* is a gram-positive ubiquitous bacterium, which is able to survive harsh environmental conditions triggering the expression of specific program, which leads to formation of extremely resistant structure, called spore. *Bacillus subtilis* cells activate this survival mechanism at the onset of nutrient limitation and sporulate and give rise to spores that are both dormant and remarkably resistant to many stress factors (2). Detailed studies of sporulation have given us much insight into the spatial and temporal regulation of gene expression that occurs during this process (3, 16). Early in sporulation, the division septum is placed asymmetrically in the dividing cell instead of in the middle, as seen during vegetative cell growth. The small compartment generated after the asymmetric division develops into the spore, and the large compartment serves as the mother cell. Different sets of genes are switched on, and different proteins are produced at different times during spore development, which is determined largely by the timing and compartmentation of the synthesis and activation of four RNA polymerase sigma factors (two active in the mother cell and two active in the developing spore). One of the late events in sporulation is the formation of a complex, multilayer protein structure that surrounds the spore, known as the “coat.” In *B. subtilis*, there are two major coat layers, as shown by electron microscopy: the lamellar inner coat and the thicker outer coat (17, 18, 19). Coat protein assembly is a good system to learn how multiprotein complexes are formed, since the spore coat is composed of many different proteins. Regulation of coat formation is firstly, at the level of gene expression, and secondly, at post-translational level, involving various coat proteins with morphogenetic activity, i.e. proteins that do not affect the synthesis of other coat proteins, but affect their assembly within the coat layers. The most important morphogenetic proteins are: SpoIVA, SpoVID, CotE, CotH and are all produced in the mother cell compartment (20, 23).

SpoIVA is produced in the mother cell on early stages of sporulation, and is essential for the assembly of both the spore cortex and the coat layers. It localizes at or near the mother cell surface of the septum. At later stages, following engulfment of the forespore by the mother cell, SpoIVA forms a shell that surrounds the forespore and is also required for the localization of second key coat morphogenetic protein, SpoVID (21) and another morphogenetic protein, CotE (25), to the surface of the developing spore. SpoVID governs a morphogenetic transition, called spore encasement, (26) and CotE is involved in the assembly of outer spore coat.

CotE is a well-conserved protein found in several *Bacillus* species and also in a *Geobacillus* and an *Oceanobacillus* (27), produced in mother cell compartment. CotE localizes about 75 nm from the forespore outer membrane and later encircles the engulfed forespore. Electron microscope analysis have shown that *cotE* mutants totally lack the outer coat layer (25). The region delimited by the SpoIVA and the CotE ring 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 (20). Instead, the outer coat proteins assemble simultaneously around the CotE layer to form the electrondense outer layer. Assembly of the outercoat involves the cooperation of CotE with the action of additional morphogenetic proteins, including CotH (31). 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 a N-terminal domain that, together with the internal domain, directs the formation of CotE homo-multimers (13, 15). Moreover, it has shown that CotE physically interacts with many spore coat components (5) and is essential for formation of CotC-CotU hetero-oligomers (8).

In this work we examined the interaction between CotE and CotH. These two proteins are mutually dependent for assembly within the coat and directly interact. A collection of deletion mutants of *cotE* were used to show that the C terminus of CotE is involved in the interaction with CotH and new mutants constructed to precisely define the amino acids of CotE needed to contact CotH.

## Materials and methods

**Bacterial strains and transformation.** *B. subtilis* strains used in this study are listed in Table 1. Plasmid amplification for subcloning experiments, nucleotide sequence analysis, and transformation of *Escherichia coli* competent cells were performed with *E. coli* strain DH5 (24). Bacterial strains were transformed by previously described procedures: $CaCl_2$-mediated transformation of *E. coli* competent cells (24) and two-step transformation of *B. subtilis* (4).

**Genetic and molecular procedures.** Isolation of plasmids, restriction digestion, and ligation of DNA were carried out by standard methods (24). Chromosomal DNA from *B. subtilis* was isolated as described elsewhere (4). CotEΔPEE, CotEΔFLE, CotEΔFLD and CotEΔFLK proteins were expressed in *B. subtilis*. In particular, purified 920 bp fragments for every CotEΔ form were obtained by PCR amplification of wild type *B. subtilis* strain chromosomal DNA using a CotE-P forward primer with PEE, FLD, FLE and FLK reverse primers (primers reported in Table 2). These purified fragments were then digested with BamHI and EcoRI and cloned in a previously BamHI-EcoRI-digested plasmid pDG364 (32). All four pDG364-CotEΔ constructs were checked for nucleotide sequence and then transformed in a *cotE* null mutant (RH211), obtaining AZ559, AZ560, AZ561 and AZ562 mutant strains.

**Overproduction of six-His-tagged CotEΔ, six-His-tagged CotH and un-tagged CotE.** The *cotE* coding region was amplified by PCR from *B. subtilis* chromosomal DNA with primers E-NdeI-F and PEE, FLD, FLE and FLK to form CotE proteins with terminal amminoacids corresponding to the primer name in capital letters, respectively (Table 2). The 540-bp PCR product of all four CotE forms was cleaved with EcoRI and HindIII and ligated into EcoRI-HindIII-digested expression vector pRSETB (Invitrogen). Recombinant plasmids carrying an in-frame fusion of the 5' end of the *cotE* coding region to six histidine codons under the transcriptional control of a T7 promoter was used to transform competent cells of *E. coli* BL21(DE3) (Invitrogen), yielding strains TS1, TS2, TS3 and TS4. Plasmids used for BL21 transformation were previously checked for nucleotide sequence. Untagged CotE and his-tagged CotH proteins were obtained by cloning *cotE* and *cotH* coding region in pBAD and were available in the lab (Baccigalupi personal communication).

His tagged proteins CotEΔPEE, CotEΔFLE, CotEΔFLD, CotEΔFLK, CotE and CotH and un-tagged CotE were produced by autoinduction by growing cells at 37°C for 18 h with orbital shaking (150 rpm) and by using the Overnight Express autoinduction system 1 according to the manufacturer's instructions (Novagen).

**Pull-down experiments.** Strains VS13 (CotH-his) and RH136 (CotE) (Table 1) were collected after autoinduction (15 mL culture) by centrifugation and resuspended in 1.5 ml of lysis buffer (50 mM NaH2PO4, 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 (20-min pulses at 20 Hz with a Sonicator Ultrasonic liquid processor; Heat System Ultrasonic Inc., NY). The suspension was clarified by centrifugation at 13,000 g at 4°C for 20 min, and protein concentration was determined by a Bio-Rad assay. Three hundred micrograms of extract from strain VS13 was applied to Ni-NTA magnetic agarose beads (Qiagen), separately. After 1 h of incubation at room temperature with shaking, the beads were washed with 2.5

ml of wash buffer (50 mM NaH2PO4, 300 mM NaCl, 10 mM imidazole), and 300 µg of extract from strain RH136 was added to the sample and incubated for 1 h at room temperature with shaking. Unbound proteins were removed by washing with wash buffer at three different concentrations of imidazole (40 mM, 100 mM, and 250 mM [indicated asWin Fig. 2]). Bound proteins were eluted using the wash buffer at increasing concentrations of imidazole (500 mM and 1 M [indicated as E1 and E2 in Fig. 2, respectively]). Eluted proteins were resolved on SDS-12.5% PAGE gels and subjected to immunoblot analysis.

**Sporulating cells lysates and immunoblot analysis.** Sporulation of all *B. subtilis* strains was induced by the exhaustion method (4). Sporulating cells were harvested during the sporulation and 18 hours from the onset of sporulation, and mother cells and forespore fractions were isolated as described before (10). Whole-cell lysates of sporulating cells were prepared by sonication (10) followed by detergent treatment (62.5 mM Tris-HCl [pH 6.8], 4% SDS, 5% glycerol, 2% β-mercaptoethanol, 0.003% bromophenol blue) at 100°C for 7 min. Fifty micrograms (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 was performed with treatment at 100 °C in sample buffer (1 mM DTT, 10% β-mercaptoetanol, 4% SDS, 0.05% bromophenol blue, 125 mM Tris pH 6.8, 10% glycerol) or at 4 °C in 0.1M NaOH (10). Western blot analysis were performed by standard procedures. For electrotransfer was used nitrocellulose membrane and the proteins were then hybridated with either anti-CotH, anti-CotB, anti-CotG, anti-CotC or anti-CotA antibodies as described previously (10).

**Table 1.** List of strains used in this study.

| Strain | Genotype | Source |
|---|---|---|
| *B. subtilis* | | |
| PY79 | wild type | [22] |
| RH211 | *cotE::spc* | [9] |
| DZ213 | *cotE::cm* | [25] |
| ER220 | *cotH::spc* | [31] |
| RG24 | *pAH::cm* | Chapter 2 |
| NC5 | *cotH-cotG-mut1 cotGΔ::erm* | [11] |
| TB70 | *cotE::cat::spc amyE::cotEΔ159-181* | [12] |
| TB51 | *cotE::cat::spc amyE::cotEΔ147-160* | [12] |
| TB71 | *cotE::cat::spc amyE::cotEΔ80-102* | [12] |
| TB95 | *cotE::cat::spc amyE::cotEΔ58-75* | [12] |
| TB83 | *cotE::cat::spc amyE::cotEΔ30-55* | [12] |
| TB126 | *cotE::cat::spc amyE::cotEΔ179–181* | [13] |
| SL483 | *cotE::cat::spc amyE::cotEΔ176–181* | [13] |
| SL507 | *cotE::cat::spc amyE::cotEΔ170–181* | [13] |
| SL484 | *cotE::cat::spc amyE::cotEΔ162–181* | [13] |
| AZ559 | *cotE::cat::spc* amyE::*cotEΔ176–181PEE* | This study |
| AZ560 | *cotE::cat::spc* amyE::*cotEΔ176–181FLE* | This study |
| AZ561 | *cotE::cat::spc* amyE::*cotEΔ176–181FLD* | This study |
| AZ562 | *cotE::cat::spc* amyE::*cotEΔ176–181FLK* | This study |
| *E. coli* [a] | | |
| TS1 | *cotEΔPEE::6his* | This study |
| TS2 | *cotEΔFLE::6his* | This study |
| TS3 | *cotEΔFLD::6his* | This study |
| TS4 | *cotEΔFLK::6his* | This study |
| VS13 | *cotH::6his* | Lab stock |
| AP19 | *cotE::6his* | [8] |
| RH136 | *cotE* | [8] |

[a] 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.

**Table 2.** List of primers

| Oligonucleotide | Sequence[a, b] | Restriction site | Position of annealing[c] |
|---|---|---|---|
| CotE-P | CGggatccCGAGCTCGTTGCACACACC | BamHI | -370/-388 |
| PEE | aagcttATTCCTCCGGGTTGATGC | HindIII | +505/+519 |
| FLD | aagcttATTCTAAAAACTCCGGGTTG | HindIII | +510/+526 |
| FLE | aagcttAGTCTAAAAACTCCGGGTTG | HindIII | +510/+526 |
| FLK | aagcttATTCTAAAAACTCCGGGTTG | HindIII | +510/+526 |
| E-NdeI-F | TAGgaattcCATATGTCTGAATACAGGGAAATT | EcoRI | +1/+21 |

[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.

## Results and Discussion

**CotE is required for CotH assembly.** CotE is notable among the coat proteins in that deletion of its gene entirely prevents the assembly of the outer coat layer and of some inner coat components (25, 29, 30). The effect of CotE on coat formation appears not to be at the level of expression of coat protein genes but, rather, on protein assembly (25). CotE is therefore a morphogenetic protein that nucleates coat protein assembly (17). CotE directs the assembly also of CotH, which is required for the proper assembly of both coat layers (28). In a wild type strain (Figure 1A), CotH appears around the forming spore three hours after the onset of the sporulation. In agreement with a previous study on *cotH* expression (11), we detected CotH only around the prespore and up to 6 hours after the onset of sporulation (Fig. 1A). In a *cotE* mutant strain (Figure 1B), CotH is hardly able to assemble on the spore surface as far as a very weak 42 kDa band was detected with specific anti-CotH antibody. In the absence of CotE CotH was unable to assemble around the forming spore and accumulated in the mother cell compartment.

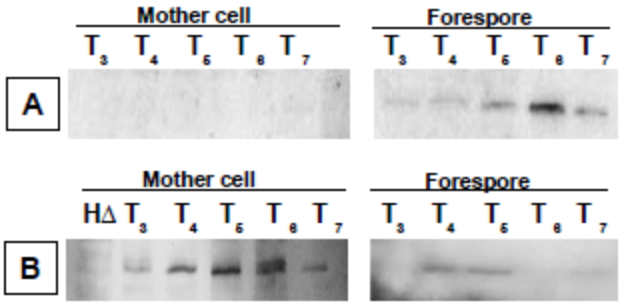


**Figure 1.** Western blots of proteins, extracted from mother cell and forespore compartment at various times during sporulation. Proteins were fractionated on 12.5 % polyacrylamide gels, electro transferred to membranes, and reacted with anti-CotH antibody. We analyzed wild type strain (PY79) in panel A and *cotE* null mutant (DZ213) in panel B.

**CotH is required for CotE assembly.** CotH is present in the sporangium in low quantity and for a restricted time. Nonetheless it is required for the assembly of at least 9 outer coat components (5). In addition we report here that CotH is also required for CotE assembly. As shown in Fig. 2, the amount of CotE extracted is higher from purified spores of a wild type strain (lane 2) than in a *cotH* mutant (lane 3). Consistently, spores of a mutant over-expressing *cotH*, NC5 (11), contain more CotE (lane 1) than the isogenic wild type (lane 2).

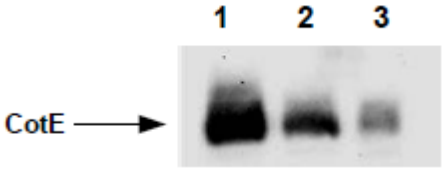


**Figure 2.** Western blot of mature spore protein extracts of NC5 strain (lane 1), wild type (PY79) (lane 2) and *cotH* null mutant (ER220) (lane 3). The proteins were blotted on nitrocellulose membrane and hybridated with anti-CotE specific antibodies.

A similar result was obtained analyzing the amount of CotE present in the mother cell and around the forming spore during sporulation. Fig. 3 shows a western blot analysis with anti-CotE antibody a various time during sporulation of a wild type (panel A), a *cotH* (panel B) and a mutant over-expressing *cotH* (11) (panel C).

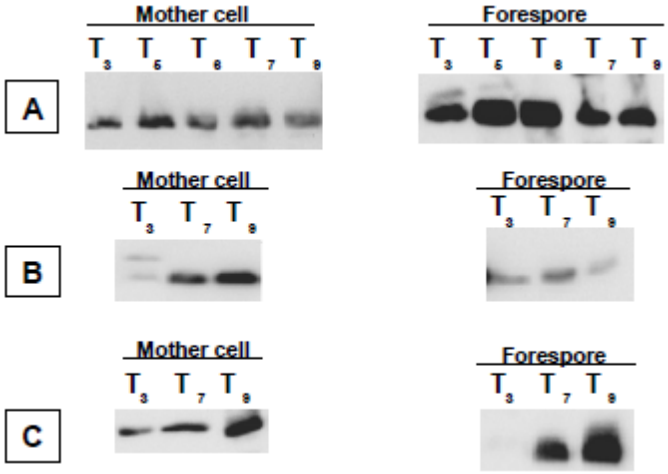


**Figure 3.** Western blot with anti-CotE antibody of protein extracted from mother cell and forespore compartments of wild type (PY79) (A), *cotE* null mutant (DZ213) (B) and NC5 strain (C). Proteins were extracted at various times during the sporulation, separated on 12.5% poliacrialammide gel and then electro-transferred on nitrocellulose membrane.

Based on the results of Figs. 2 and 3, we can conclude that the amount of CotH present in the sporangium seems to directly affect the amount of CotE assembled in the spore coat. Our results are in agreement with a previous report (5), showing that the fluorescence due to a CotE-GFP fusion protein was totally found around the forming spore in a wild type strain and reduced and partially diffused in the mother cell in a *cotH* mutant. The presence of at least some CotE on the forespore of a *cotH* mutant is also consistent with findings indicating that *cotH* mutant spores possess more outer coat material than *cotE* spores (31, 28).

Results of Figs. 2 and 3, together with previous fluorescence microscopy data (5), point to a partial dependence of CotE assembly on CotH. Since CotH depends on CotE (Fig. 1), we can conclude that the two proteins are mutually dependent.

**CotE and CotH directly interact *in vitro*.** To verify whether CotE and CotH directly interacts, we over-expressed in *E. coli* a His-tagged version of CotH (CotH-His), and an untagged version of CotE and performed *in vitro* pull-down assay. After autoinduction, *E. coli* cells were lysed by sonication, and Ni-NTA magnetic beads were incubated with extracts of cells expressing CotH-His. Beads were then washed and incubated with the extract of cells expressing untagged CotE. After additional washes, proteins were eluted and used for Western blot experiments with anti-CotH and anti-CotE antibodies. Fig. 4 shows the same fractions analyzed with anti-CotH panel A) and anti-CotE (panel B) and indicates that CotE is retained by beads pre-adsorbed with CotH-His. When CotE was adsorbed to beads without CotH-His (panel C) all CotE was washed away with the first wash. This the demonstrates that CotE is retained on Ni-NTA magnetic beads only when CotH-His had been previously bound to the beads, and therefore that CotE directly binds to CotH.

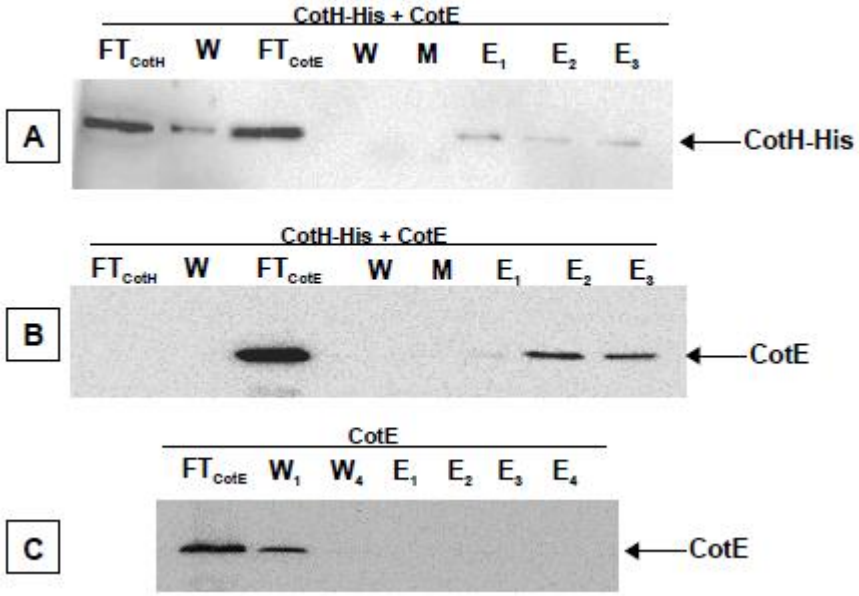


**Figure 4.** Pull-down experiment. Flowthrough (FT), washed (W), and eluted (E1, E2 and E3) fractions were fractionated on 12.5% polyacrylamide gels, electrotransferred to membranes, and reacted with anti-CotH (A) and anti-CotE antibodies (BC).

**The C-terminus of CotE is required for CotH assembly.** The analysis by western blot of collection of *cotE* deletion mutants (provided by Adam Driks, Loyola University, US) was used to show that the C-terminal part of CotE is involved in the interaction with CotH. Fig. 5 shows mutant forms of CotE, lacking the C-terminal 3, 6, 12 or 20 amino acid residues, were still able to assemble within the coat although with a reduced efficiency compared with the wild type. Proteins extracted from the same mutants were then use in western blot with anti-CotH antibody. Fig. 6 shows that CotH was normally assembled in mutants lacking the last 3 or the last 12 amino acids while was not assembled in mutants lacking the C-terminal 6 or 20 amino acids.

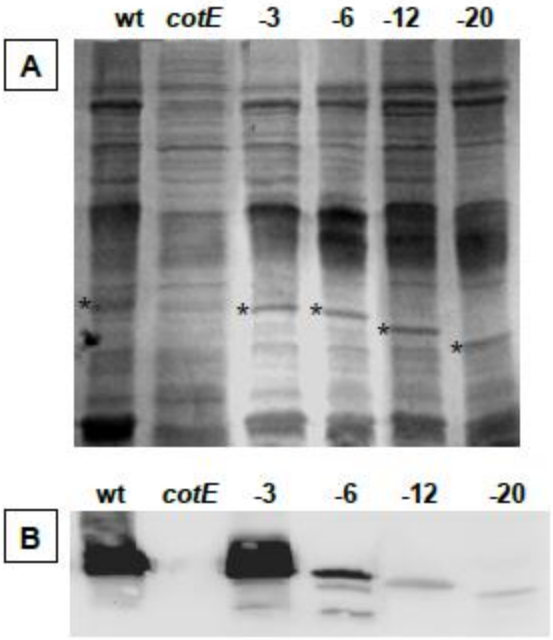


**Figure 5.** SDS-PAGE (A) and western blot (with anti-CotE specific antibody) (B) of wild type strain (PY79), *cotE* mutant (DZ213), mutants lacking -3 (TB126), -6 (SL483), -12 (SL507) and -20 (SL484) amino acids at C-terminal end of CotE. In panel A, stars indicate the band corresponding to CotE.

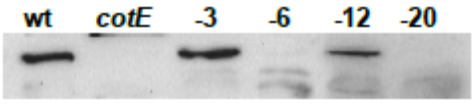


**Figure 6.** Western blot with anti-CotH specific antibody of wild type (PY79), *cotE* (DZ213), mutants lacking -3 (TB126), -6 (SL483), -12 (SL507) and -20 (SL484) amino acids at C-terminal end of CotE.

The analysis of the amino acidic sequence of the C terminus of CotE (Fig. 7A) showed an unusual pattern with the positively charged amino acids glutamate and aspartate present several times (21 Asp (E) or Glu (D) in the C-terminal 38 amino acids, i.e. 55%). In addition we noticed that all mutants but the one lacking 6 amino acids ended with one or two positively charged residues. The mutant lacking 6 residues and not ending with a positively charged residue is one of the two mutants

that fail to assemble CotH (Fig. 6). The other one is the -20 mutant lacking the last 20 residues (Fig. 6). Based on this we hypothesize that the interaction with CotH requires a positively charged domain ending at the C terminus with al least one positively charged amino acid and that the -20 mutant fails to assemble CotH because CotE is not functioning properly, as suggested by the weak assembly of the -20 version of CotE (Fig. 5). To test this hypothesis we designed specific primers and we constructed 3 more *cotE* deletion mutants starting from mutant -6 (that does not assemble CotH): i) adding a single Asp residue (strain FLE); ii) adding a single Glu residue (strain FLD); iii) replacing the two final residues (Phe, Leu) with one Glu residue (strain PEE) (Fig. 7B). As a negative control we also constructed a mutant carrying as C terminus a negatively charged lys residue (strain FLK).

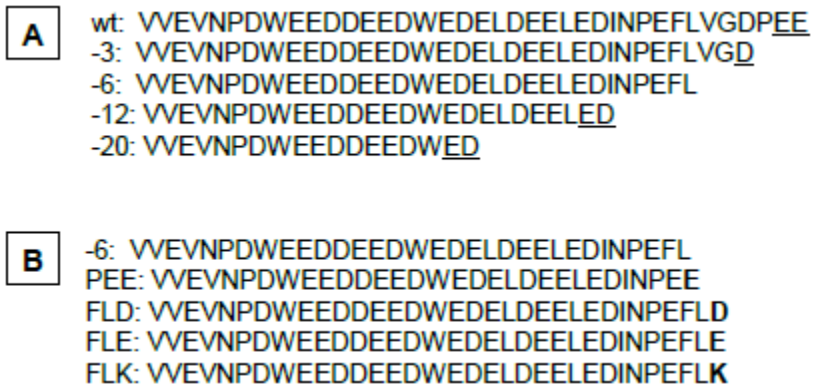


**Figure 7.** (A) Primary structure of C-terminal end of CotE (wild type) and mutants lacking -3, -6, -12 and -20 amino acids. (B) Amino acid sequence of mutant strains constructed starting from mutant -6.

The mutants indicated in Fig. 7B have been constructed in *E. coli.* The experiments to transfer them in *B. subtilis* and to analyze their ability to drive the assembly of CotH are still in progress. Moreover, all four mutants indicated in Fig. 7B were also efficiently overexpressed and purified from *E. coli*. The experiments on interaction *in vitro* between CotE, four CotE mutants (Fig. 7B) and CotH are in progress.

**Preliminary conclusions**

Regulation of protein assembly in *Bacillus subtilis* spores is an important issue for understanding the unique process of sporulation which leads to formation of a spore, an extremely resistant structure, which can support exposure to various range of physicochemical insults. Spore coat protects these dormant cells against the attack of lytic enzymes and toxic chemicals and is able to detect favorable conditions for microbial growth as the presence of nutrients, by triggering the spore germination. Nonetheless intensive research and many excellent publications on spore coat structure and assembly were made in last years, it is still not clear how spore coat proteins are organized and packed in this extremely resistant structure, which can support a series of extreme environmental insults. More insights into the molecular details of spore surface morphogenesis will provide avenues for exploitation of the spore surface layers in applications for biotechnology and medicine.

In this work we report that CotE and CotH, two important morphogenetic factors of spore coat formation depend on each other for coat assembly. The mutual dependence is due the direct interaction that occurs between the two proteins. The C terminus of CotE is essential for the interaction with CotH and apparently a positively charged amino acid residue has to be the C terminus of CotE to allow such interaction. Further experiments are still in progress to evaluate the need of a positively charged amino acid at the C terminus of CotE to drive the interaction with CotH.

**References**


1. **Aronson, A. I., and P. Fitz-James. 1976.** Structure and morphogenesis of the bacterial spore coat. Bacteriol. Rev. 40:360–402.
2. **Setlow, P. 1994.** Mechanisms which contribute to the long-term survival of spores of *Bacillus* species. J. Appl. Bacteriol. Symp. Suppl. 76:49S–60S.
3. **Errington, J. 1993.** *Bacillus subtilis* sporulation: regulation of gene expression and control of morphogenesis. Microbiol. Rev. 57:1–33.
4. **Cutting, S., and P. B. Vander Horn. 1990.** Genetic analysis, p. 27–74. In C. Harwood and S. Cutting (ed.), Molecular biological methods for Bacillus. John Wiley & Sons, Chichester, United Kingdom.
5. **Kim, H., Hahn, M., Grabowski, P., McPherson, D.C., Otte, M.M., Wang, R., Ferguson, C.C., Eichenberger, P. and Driks, A. 2006.** The Bacillus subtilis spore coat protein interaction network. Mol. Microbiol. 59:487-502.
6. **Giglio R. , Fani R., Isticato R., De Felice, M., Ricca E., Baccigalupi L. 2011.** Organization and Evolution of the *cotG* and *cotH* Genes of *Bacillus subtilis*. Journal of Bacteriol., p. 6664–6673.
7. **Henriques A. O., Melsen L. R., Moran C. P. 1998.** Involvement of superoxide dismutase in spore coat assembly in *Bacillus subtilis*. J. Bacteriol. 180:2285-2291.
8. **Isticato, R., Pelosi, A., De Felice, M., Ricca, E. 2010.** CotE binds to CotC and CotU and mediates their interaction during spore coat formation in *Bacillus subtilis*. Journal of Bacteriol., p. 949–954.
9. **Isticato, R., G. Esposito, R. Zilha˜o, S. Nolasco, G. Cangiano, M. De Felice, A. O. Henriques, and E. Ricca. 2004.** Assembly of multiple CotC forms into the *Bacillus subtilis* spore coat. J. Bacteriol. 186:1129–1135.
10. **Isticato, R., A. Pelosi, R. Zilhao, L. Baccigalupi, A. O. Henriques, M. De Felice, and E. Ricca. 2008.** CotC-CotU heterodimerization during assembly of the *Bacillus subtilis* spore coat. J. Bacteriol. 190:1267–1275.
11. **Baccigalupi, L., G. Castaldo, G. Cangiano, R. Isticato, R. Marasco, M. De Felice, and E. Ricca. 2004.** GerE-independent expression of cotH leads to CotC accumulation in the mother cell compartment during *Bacillus subtilis* sporulation. Microbiology 150:3441–3449.
12. **Bauer, T., Little, S., Sto¨ver, A.G., and Driks, A. (1999)** Functional regions of the *B. subtilis* spore coat morphogenetic protein CotE. J Bacteriol 181: 7043–7051.
13. **Little, S., and Driks, A. (2001)** Functional analysis of the *Bacillus subtilis* morphogenetic spore coat protein CotE. Mol Microbiol 42: 1107–1120.
14. **Zilhao, R., Serrano, M., Isticato, R., Ricca, E., Moran, C. P., Henriques A. O. 2004.** Interactions among CotB, CotG, and CotH during assembly of the *Bacillus subtilis* spore coat. J. Bacteriol. 186:1110–1119.
15. **Krajcíkova´, D., Luka´cova´, M., Mu¨llerova´, D., Cutting, S. M., and Bara´k, I. 2009.** Searching for protein-protein interactions within the *Bacillus subtilis* spore coat. J. Bacteriol. 191:3212–3219
16. **Piggot, P. J., and R. Losick. 2002.** Sporulation genes and intercompartmental regulation, p. 483–515. In A. L. Sonenshein, J. A. Hoch, and R. Losick (ed.), *Bacillus subtilis* and its closest relatives: from genes to cells. American Society for Microbiology, Washington, D.C.
17. **Driks, A. 1999.** *Bacillus subtilis* spore coat. Microbiol. Mol. Biol. Rev. 63:–20.
18. **Driks, A. 2002.** Proteins of the spore core and coat, p. 527–535. In A. L. Sonenshein, J. A. Hoch, and R. Losick (ed.), *Bacillus subtilis* and its closest relatives: from genes to cells. American Society for Microbiology, Washington, D.C.
19. **Henriques, A. O., and C. P. Moran, Jr. 2000**. Structure and assembly of the bacterial endospore coat. Methods 20:95–110.
20. **Driks, A., Roels, S., Beall, B., Moran, C. P., Jr, Losick, R. 1994**. Subcellular localization of proteins involved in the assembly of the spore coat of *Bacillus subtilis*. Genes Dev. 8 (2): 234-44.

21. **Beall, B., Driks, R., and C. P. Moran. Jr. 1993.** Cloning and characterization of a gene required for assembly of the *Bacillus subtilis* spore coat. J. Bacteriol. 175:1705-1716.
22. **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.
23. **Ricca, E., Baccigalupi, L., Nacleiro, G. and Cutting, S. 1997.** Spore coat differentiation. Res. Microbiol. 148: 5-9.
24. **Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989**. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
25. **Zheng L, Donovan WP, Fitz-James PC, Losick R.** 1988. Gene encoding a morphogenic protein required in the assembly of the outer coat of the *Bacillus subtilis* endospore. Genes Dev, 2:1047–1054.
26. **Wang Katherine, H., Isidro, A. L., Domingues, L., Eskandarian, H. A., McKenney, P. T., Drew, K., Grabowski, P., Chua, M. H., Barry, S. N., Guan, M., Bonneau, R., Henriques, A. O. Eichenberger P. 2009.** The coat morphogenetic protein SpoVID is necessary forespore encasement in *Bacillus subtilis*. Molecular Microbiol. 74: 634-649.
27. **Henriques, A. O., Moran, C. P. Jr. 2007.** Structure, assembly and function of the spore surface layers. Annu. Rev. Microbiol. 61:555-88
28. **Zilhao, R., Naclerio, G., Henriques, A.O., Baccigalupi, L., Moran, C.P., Jr, and Ricca, E. (1999)** Assembly requirements and role of CotH during spore coat formation in *Bacillus subtilis*. J Bacteriol 181: 2631–2633.
29. **Abe, A., Ogawa, S., Kohno, T., and Watabe, K. (1993)** Purification of *Bacillus subtilis* spore coat protein by electrophoretic elution procedure and determination of NH2-terminal amino acid sequences. Microbiol Immunol 37: 809–812.
30. **Takamatsu, H., Chikahiro, Y., Kodama, T., Koide, H., Kozuka, S., Tochikubo, K., and Watabe, K. (1998a)** A spore coat protein, CotS, of *Bacillus subtilis* is synthesized under the regulation of sigmaK and GerE during development and is located in the inner coat layer of spores. J Bacteriol 180: 2968–2974.
31. **Naclerio G, Baccigalupi L, Zilhao R, De Felice M, Ricca E:** *Bacillus subtilis* spore coat assembly requires *cotH* gene expression. J Bacteriol 1996, 178:4375–4380.
32. **Karmazyn-Campelli C, Fluss L, Leighton T, Stragier P.** The *spoIIN279*(ts) mutation affects the FtsA protein of *Bacillus subtilis*. Biochimie. 1992 Jul-Aug;74(7-8): 689-94 (PMID: 1391048)

**Chapter 4**

# “Adsorption of β-galactosidase of *Alicyclobacillus acidocaldarius* on wild type and mutants spores of *Bacillus subtilis*”

*Published in August 2012 on Microbial Cell Factories*

**Abstract**

**Background:** The *Bacillus subtilis* spore has long been used as a surface display system with potential applications in a variety of fields ranging from mucosal vaccine delivery, bioremediation and biocatalyst development. More recently, a non-recombinant approach of spore display has been proposed and heterologous proteins adsorbed on the spore surface. We used the well characterized β-galactosidase from the thermoacidophilic bacterium *Alicyclobacillus acidocaldarius* as a model to study enzyme adsorption, to analyze whether and how spore adsorption affects the properties of the enzyme and to improve the efficiency of the process.

**Results:** We report that purified β-galactosidase molecules were adsorbed to purified spores of a wild type strain of *B. subtilis* retaining ca. 50% of their enzymatic activity. Optimal pH and temperature of the enzyme were not altered by the presence of the spore, that protected the adsorbed β-galactosidase from exposure to acidic pH conditions. A collection of mutant strains of *B. subtilis* lacking a single or several spore coat proteins was compared to the isogenic parental strain for the adsorption efficiency. Mutants with an altered outermost spore layer (crust) were able to adsorb 60-80% of the enzyme, while mutants with a severely altered or totally lacking outer coat adsorbed 100% of the β-galactosidase molecules present in the adsorption reaction.

**Conclusion:** Our results indicate that the spore surface structures, the crust and the outer coat layer, have an negative effect on the adhesion of the β-galactosidase. Electrostatic forces, previously suggested as main determinants of spore adsorption, do not seem to play an essential role in the spore-β-galactosidase interaction. The analysis of mutants with altered spore surface has shown that the process of spore adsorption can be improved and has suggested that such improvement has to be based on a better understanding of the spore surface structure. Although the molecular details of spore adsorption have not been fully elucidated, the efficiency of

the process and the pH-stability of the adsorbed molecules, together with the well documented robustness and safety of spores of *B. subtilis*, propose the spore as a novel, non-recombinant system for enzyme display.

**Background**

Display systems to present biologically active molecules on the surface of microorganisms have become an increasingly used strategy to address biotechnological issues [1, 2]. For biomedical applications surface display systems have been mostly used for the identification of neutralizing epitopes, the development of whole cell diagnostic tools, or vaccine delivery [3, 4]. More recent

is a strategy to engineer bacterial endospores (spores) to display heterologous proteins on their surface [5]. Endospore-forming bacteria are Gram-positive microorganisms belonging to different genera and including more than 1,000 species [6]. The common feature of these organisms is the ability to form a quiescent cellular type (the spore) in response to harsh environments. The spore can survive in this dormant state for long periods, resisting to a vast range of stresses such as high temperature, dehydration, absence of nutrients, presence of toxic chemicals. When the environmental conditions ameliorate, the spore germinates originating a vegetative cell able to grow and sporulate [6]. The ability of the spore to survive non-physiological conditions is, in part, due to the presence of the spore coat, a proteinaceous structure surrounding the spore. At least seventy different proteins (Cot proteins) form the multilayered coat structure, composed of an inner part, an outer part [7] and the crust, the latter being a recently discovered outermost layer of the spore [8, 9].

Spore-based display systems provide several advantages with respect to systems based on the use of phages and bacterial cells [10]. The remarkable and well documented resistance of spores to various environmental and toxic effects [7]

ensures high stability of the display system. Proteins to be displayed on the spore are produced in the mother cell compartment of the sporangium and are assembled around the forming spore without the need to be translocated across a membrane, thus eliminating the size constrains of cell-based display systems [5, 10]. The safety record of several endospore-forming species [11], makes spores of those species ideal candidates as vehicles to deliver molecules to mucosal surfaces [6].

The strategy to obtain the spore surface display of heterologous proteins is based on the construction of gene fusions between the gene coding for a selected spore surface protein (carrier) and the heterologous DNA coding for the protein to be displayed [5]. By this approach a variety of heterologous proteins have been displayed and recombinant spores proposed as vaccine vehicles (see ref. 6 for a review), as biocatalysts (see ref. 58 10 for a review), or as a bioremediation tool [12]. To optimize and rationalize this display strategy an inner (OxdD [13]) and various outer (CotB [5], CotC [14, 15], CotG [16]) coat components have been tested as carriers.

The spore-based display system, like other cell- or phage-based systems, relies on the genetic engineering of the host to display immunogenic peptides or proteins and obtain a recombinant organism to be used as a live biotechnological tool [5, 6, 10]. This is a major drawback since it causes the release of live recombinant organisms into nature, raising concerns over the use and clearance of genetically modified microorganisms [17]. To overcome this obstacle, a nonrecombinant approach to use spores as a display system has been recently proposed and model proteins efficiently exposed. In the first study suggesting that heterologous proteins can be adsorbed on the spore surface, the mammalian NADPH-cytochrome P450 reductase (CPR), a diflavin-containing enzyme, was over-expressed in sporulating *B. subtilis* cells and released into the culture medium after sporulation by autolysis of the mother cell. However, part of the CPR activity was found associated to spores and the displayed enzyme shown to be accessible to anti-CPR antibodies [18]. In a

different study a collections of purified antigens (TTFC of *Clostridium tetani*, PA of *Bacillus anthracis*, Cpa of *Clostridium perfringens* and glutathione S-transferase of *Shistosomas japonica*) were adsorbed to *B. subtilis* spores and shown to be able to induce specific and protective immune responses in mucosally immunised mice [19]. Spore adsorption resulted to be more efficient when the pH of the binding buffer was acidic (pH 4) and less efficient or totally inhibited at pH values of 7 or 10 [19]. A combination of electrostatic and hydrophobic interactions between spores and antigens were suggested to drive the adsorption, that was shown to be not dependent on specific spore coat components but rather on the negatively charged and hydrophobic surface of the spore [19]. Hydrophobic and electrostatic interactions were suggested as the main forces involved also in the interaction between the *E. coli* phytase and spores of *B. polyfermenticus* [20].

We used a well-characterized and biotechnologically important enzyme, a β-galactosidase of the thermoacidophilic bacterium *Alicyclobacillus acidocaldarius* [21], as a model to study enzyme adsorption on *B. subtilis spores.* This enzyme belongs to the glycoside hydrolase family 42 (GH42) and is characterized by an optimal activity and stability at 65° C [21]. By using this system we tested whether adsorbed β-galactosidase molecules retained their activity and whether and how spore-adsorption affected the properties of the enzyme. With the dual aim of identifying spore surface structures involved in β-galactosidase adsorption and to improve the efficiency of the process we also screened for enzyme binding a collection of mutant strains of *B. subtilis* lacking a single or several spore coat proteins. A better understanding of the spore surface structure is likely to lead to a rationalization of the adsorption system, such that wild type or mutant spores will be utilized, depending upon the specific application or the heterologous enzyme to display.

**Results**

β-Galactosidase of *A. acidocaldarius* adsorbs to *B. subtilis* spores and retains its enzymatic activity. In an initial experiment 0.5 μg of β-Galactosidase (β-Gal) of *A. acidocaldarius*, over-expressed in *E. coli* and purified by affinity chromatography with GST columns (Methods), were incubated with $1.0x10^{10}$ spores of *B. subtilis* strain PY79 [22], purified by renographin gradient, as previously described [23]. The adsorption reaction was performed in citrate buffer at pH 4.0, as previously described for various antigens [19]. After adsorption, the mixture was either assayed for β-Gal activity ("Total Units" in Fig. 1) or fractionated by centrifugation. The pellet ("Spore- Bound Units" in Fig. 1) and supernatant ("Unbound Units" in Fig. 1) fractions were then assayed independently. Fig. 1 schematizes the experiment and reports that ca. 50% of the β-Gal units were found in the pellet fraction and, therefore, were most likely associated to the spores.

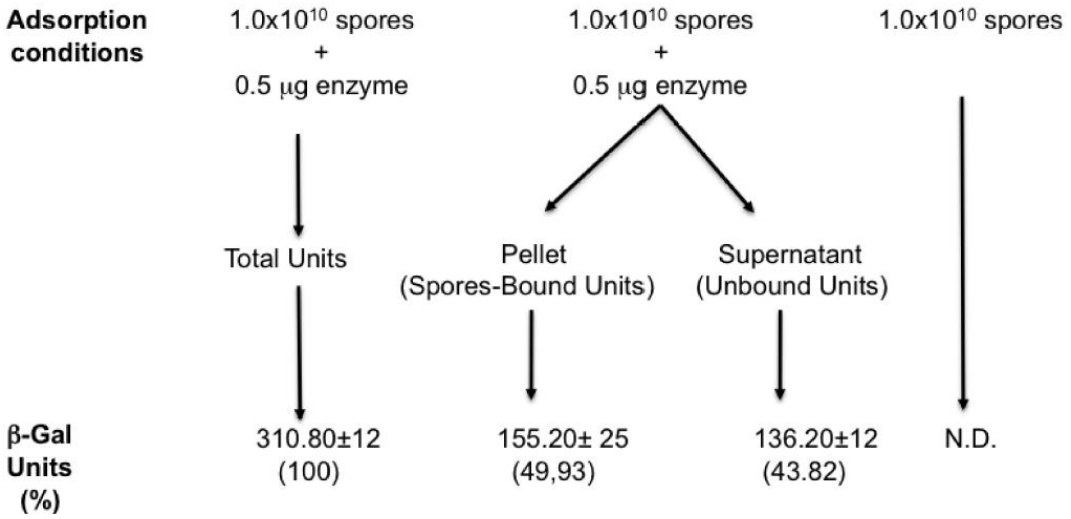


**Fig. 1.** Schematic representation of a typical adsorption experiment. Purified spores were mixed to the purified enzyme in PBS buffer (pH 4.0) and incubated one hour at 25°C. The reaction was then stopped in ice and sample either directly assayed (Total Units) or fractionated by centrifugation and fractions assayed independently.

The spore-associated β-Gal activity was not due to an endogenous enzyme, since purified spores alone did not show any activity (Fig. 1). Only a limited amount (less than 10%) of β-Gal units was lost during the procedure (Fig. 1) and the adhesion appeared to be stable, since 95% of the units was still associated to the pellet fraction after two washes with phosphate buffer pH 4.0 or 5.5 (not shown).

In a previous report Huang et al. [19] have shown that spore adsorption of four different antigens is more efficient at pH 4.0 than at pH 7.0 or 10.0. In agreement with those observations we found that adsorption of β-Gal to $1.0x10^{10}$ spores was strictly dependent on the pH of the adsorption buffer, with a similar amount of units adsorbed at pH values of 3.5 and 4.0 and a strongly decreased amount of units adsorbed at pH 4.5 (Table 1). β-Gal has a deduced isoelectric point of 5.77, therefore at all pH tested in the experiments of Table 1, it is expected to have a net positive charge.

**TABLE 1.** pH-dependent adsorption efficiency with $1x10^{10}$ spores.

| | pH values of the adsorption reaction | | | |
|---|---|---|---|---|
| | **3.0** | **3.5** | **4.0** | **4.5** |
| **Total units** | 0 | 262.55±5.15 | 308.92±11.13 | 353.64±23.80 |
| **(%)** | | (100) | (100) | (100) |
| **Spore-bound units** | 0 | 139.64±5.65 | 158.98±2.41 | 26.89±4.68 |
| **(%)** | | (53.18) | (51.46) | (7.60) |
| **Unbound-units** | 0 | 92.46±8.24 | 127.77±12.38 | 306.72±26.96 |
| **(%)** | | (35.21) | (41.36) | (86.73) |

[1] β-galactosidase unit is defined as an amount of β-Gal which is able to hydrolyze 1 μmol of substrate in 1 min at standard conditions [19].

At pH values lower than 3.5 no activity was found in any of the fractions as well as in the unfractionated sample (Total Units), indicating that exposure at pH 3.0 totally inactivated the enzyme (Table 1). At both pH values of 3.5 and 4.0 the amount of β-Gal units lost in the experimental procedure was about 10% (Table 1). To check that

our experimental conditions were not limiting for the number of spores, we measured the β-Gal activity associated to each spore using different amounts of enzyme and spores in the adsorption reaction. As shown in Fig. 2, with both $2.0x10^9$ (open symbols) and $1.0x10^{10}$ (closed symbols), increasing the amount of enzyme used in the adsorption reaction up to 4 μg, the β-Gal units bound to each spore increased, indicating that the spores present in the reaction buffer were not saturated by the enzyme and therefore were not a limiting factor for the reaction.

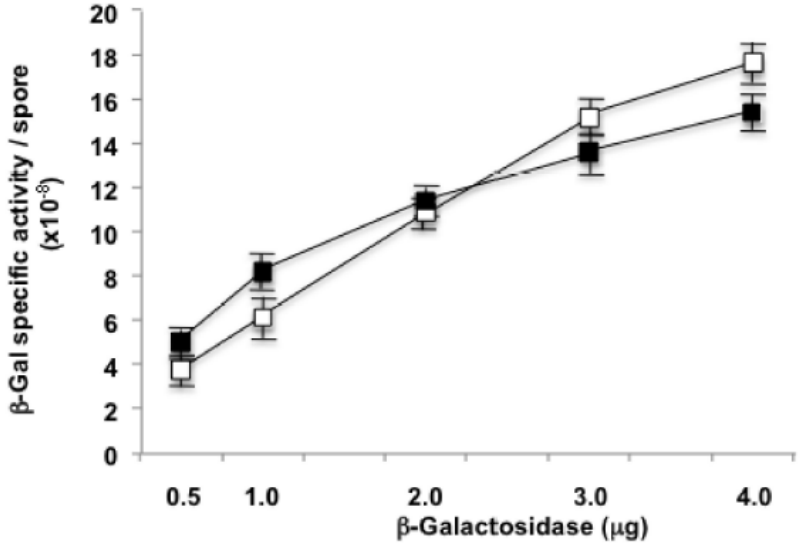


**Fig. 2** β-galactosidase units per spore obtained with increasing amounts of enzyme. The enzyme was adsorbed to $2.0x10^9$ (open symbols) or $1.0x10^{10}$ (closed symbols) spores.

**Adsorption to spores stabilizes β-Gal**

It has been previously reported that the β-Gal of *A. acidocaldarius* has an optimal activity and stability at 65° C and at pH 6.5 [21]. To verify whether spore adsorption affected these properties, we compared the β-Gal activity of the free and the spore-adsorbed enzyme, after exposing both to the adsorption conditions (1 hour at pH 4.0). As shown in Fig. 3, the optimum temperature (panel A) and pH (panel B) were identical with (open symbols) and without (closed symbols) spores, indicating that the spore-bound and the free enzyme have identical properties.

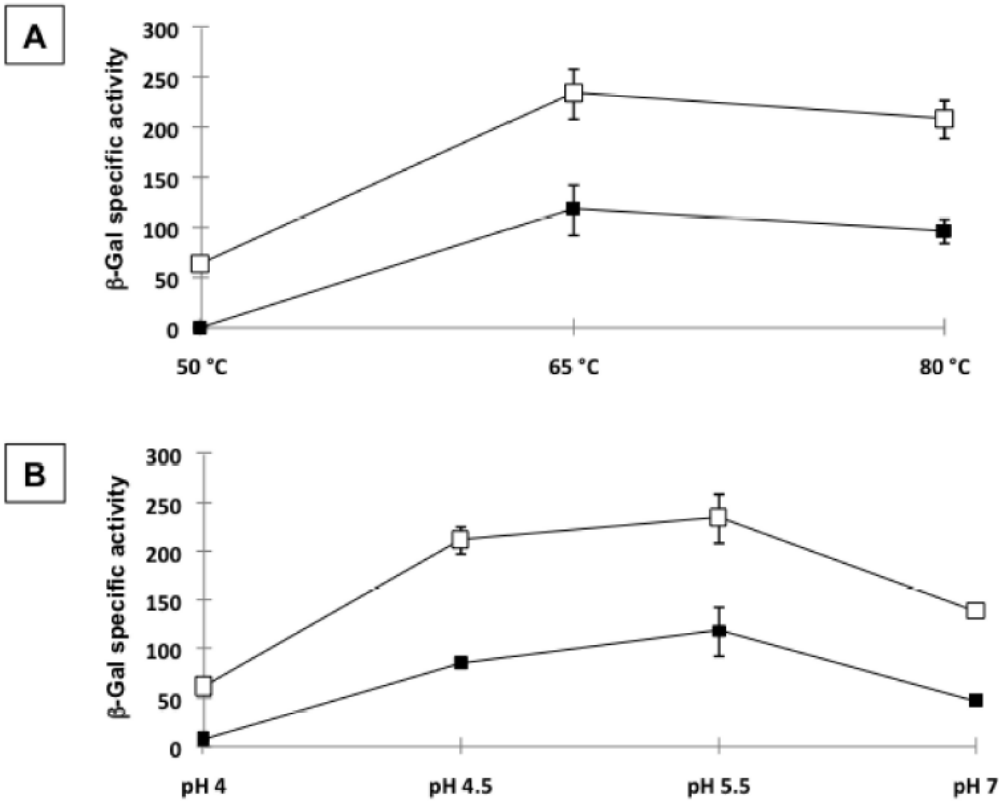


**Fig. 3.** Spore-associated β-galactosidase units obtained assaying the samples at pH 5.5 and various temperatures (A) or at 65°C and various pH values (B).

Results of Fig. 3 indicate that at all tested temperatures and pH values the spore-bound enzyme is more active than the free enzyme. A stabilization effect of spores on an enzyme has been previously suggested for a different enzyme and for spores of a different bacterial species [19]. To address this point in more detail we compared the activity of spore-bound and free β-Gal after exposure at low pH or high temperatures. Although the enzyme is from a thermoacidophilic bacterium, the activity of the free enzyme decreased overtime after exposure at both low pH or high temperature and was protected by denaturation when adsorbed to spores. After exposure at pH 4.0 the activity of the free β-Gal was completely lost after 24 hours while the spore-bound enzyme was still fully active (Fig. 4A). After 2 hours of incubation at 75°C the activity of the free β-Gal was completely lost while the spore-

bound enzyme retained about 80% of its original activity (black and white squares in Fig. 4B, respectively). At 80°C the activity of the free enzyme was completely lost after 1 hour while the spore-bound enzyme retained over 30% of its activity even after two hours of incubation (black and white circles in Fig. 4B, respectively).

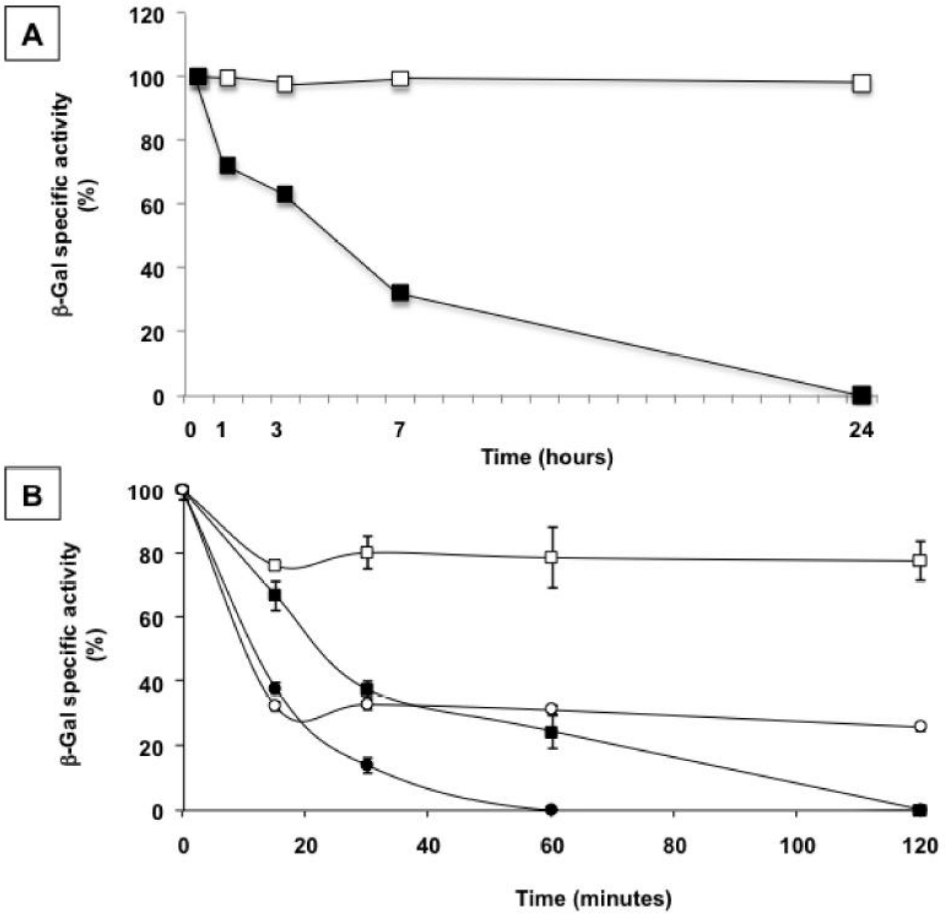


**Fig. 4.** (A) Percentage of β-galactosidase units observed after incubation at pH 4.0 for various times of free (closed squares) and spore-bound (open squares) enzyme. (B) Percentage of β-galactosidase units observed after incubation at 75°C (squares) and 80°C (circles) for various times of free (closed symbols) and spore-bound (open symbols) enzyme. The data shown are representative of three independent experiments, each with three biological replicates.

**Spores with altered surface show increased efficiency of adsorption.** In a previous report it has been shown that adsorption of various antigens on the surface of *B. subtilis* spores does not depend on a direct interaction with any of the major outer coat components (CotA, CotB, CotC, CotG and CotF) [19]. In agreement with

the previous results we showed that β-Gal adsorption is similar in wild type spores (PY79) and in isogenic null mutants lacking *cotB, cotC, cotG* or *cotS* (Fig. 5A). However, it has been recently shown that the *B. subtilis* spore is surrounded by an outermost layer, the crust, not identified before [8]. The crust is composed of proteins and the products of the *cgeA* gene and of the *cotVWXYZ* cluster are involved in crust formation [9]. One of those proteins, CotZ, has been proposed to be essential for the spore crust to surround the spore but not for the formation of the other coat layers [9]. Based on this, we examined whether the presence of the crust had an effect on the adsorption of β-Gal by comparing the efficiency of adsorption of wild type strain (PY79) with that of isogenic null mutants lacking *cotZ*, *cgeA cotX* or *cotXYZ*. As shown in Fig. 5B, the efficiency of β-Gal adsorption was increased in all four mutants. Only a slight increase was observed with mutant spores lacking *cotZ, cgeA* or *cotX* (from ca. 50% of activity associated to wild type spores to ca. 60% associated to the mutant spores), while a stronger effect was observed with spores lacking *cotXYZ* (ca. 80% of spore-associated activity). Since the mutant lacking *cotXYZ* showed a higher increase of β-Gal adhesion than mutants lacking only *cotZ* or *cotX,* we hypothesize that either CotY is an important factor in mediating the adhesion of β-Gal or that CotZ, CotX and CotY have an additive action in modifying the spore surface and, as a consequence, reducing adhesion of the enzyme (Fig. 5B). Although further experiments will be needed to fully address this point, 1 results of Fig. 5B indicate that a normally formed crust has an inhibiting role on β-Gal adhesion.

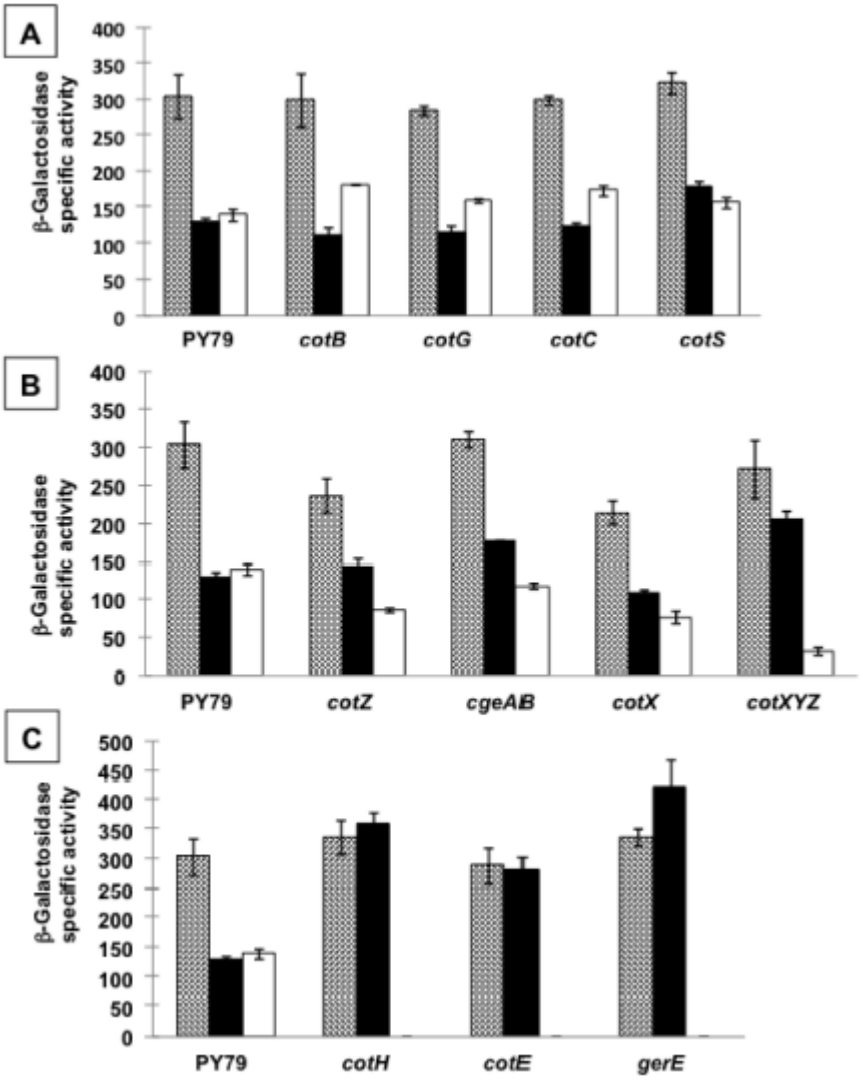


**Fig. 5.** Total Units (grey bars), Spore-Bound Units (black bars) and Unbound Units (white bars) of β-galactosidase obtained using wild type (PY79) or isogenic mutant spores. Panel A includes mutants lacking a single outer coat component; panel B includes mutants totally or partially lacking the crust; and panel C includes mutants with a severely defective (*cotH* and *gerE*) or totally absent (*cotE*) outer coat.

Next, we decided to test the adsorption efficiency of mutants lacking also other coat layers. In particular, we used spores of strains lacking *cotH, gerE* or *cotE. cotH* spores lack at least 9 outer coat components [24], *gerE* codes for a transcriptional regulator and *gerE* mutant spores lack the latest class of coat components [7], and *cotE* spores totally lack the outer coat [25]. As shown in Fig. 5C, with spores of all three mutants 100% of the β-Gal activity was adsorbed, indicating that the spores lacking the crust and at least part of the outer coat are extremely efficient in adsorbing β-Gal.

**Adsorption to mutant spores lacking *cotE* or *cotH***

Wild type spores of *B. subtilis* are negatively charged [26] and able to accumulate a large number of protons in an aqueous solution [27]. The presence of a large number of spores is then likely to alter the pH of an aqueous solution. Because of the pH-dependence of β-Gal adsorption (Table 1), we hypothesized that wild type and mutant spores had a different effect on the pH of the binding solution and, as a consequence, on the efficiency of β-Gal adsorption. To verify this hypothesis we measured the effects of spores on the pH of pure water and of the adsorption buffer (citrate buffer pH 4.0). We tested *cotE* or *cotH* mutant spores lacking either the entire outer coat or 9 major outer coat components [24], while we did not consider *gerE* mutant spores, although they also showed 100% of β-Gal adhesion (Fig. 5C), since the outer coat structure of *gerE* spores has not been studied in details and not all genes controlled by the transcriptional regulator GerE have been identified and functionally characterized. As shown in Table 2, both $2.0x10^9$ and $1.0x10^{10}$ wild type spores were able to reduce the pH of pure water of over one pH unit, while the same numbers of *cotE* or *cotH* spores had a minor, if any, effect. In all cases no effects on the pH were observed at the adsorption conditions, that buffered the effect of spores (not shown). Those results seem to suggest that the different efficiency of adsorption between wild type and mutant spores was not caused by a spore-dependent alteration of the pH in the adsorption reaction and that wild type spores have a net negative charge higher than that of both *cotE* or *cotH* mutant spores.

Wild type and mutant spores were then compared for their efficiency in adsorbing and stabilizing β-Gal molecules. *cotE* and *cotH* mutant spores showed identical adsorption efficiencies that increased with increasing amounts of enzyme in the adsorption reaction (Fig. 6A). These results confirm and expand the results of Fig. 2, indicating that the spores present in the reaction buffer ($2.0x10^9$ ) were not saturated by 20 µg of enzyme and therefore were not a limiting factor for the reaction. β-Gal has a molecular mass of 77,737 daltons, therefore the maximal amount of enzyme

used in the experiment of Fig. 6A (20 µg) contains approximately $1.55x10^{14}$ molecules. Since in the adsorbing reaction with $1.0x10^{10}$ spores about 50% of the enzyme is bound to wild type spores and 100% to mutant spores, we can calculate that about $7.75x10^{3}$ and $1.55x10^{4}$ molecules of β-Gal were adsorbed to each wild type and mutant spores, respectively.

**Table 2.** Effect of wild type and mutant spores on pH

| | n° of spores[a] | pH[b] |
|---|---|---|
| Water | 0 | 5.98±0.08 |
| Water + WT spores | $2.0x10^{9}$ | 7.23±0.07 |
| | $1.0x10^{10}$ | 7.14±0.24 |
| Water + *cotE* spores | $2.0x10^{9}$ | 6.06±0.07 |
| | $1.0x10^{10}$ | 6.18±0.04 |
| Water + *cotH* spores | $2.0x10^{9}$ | 5.88±0.07 |
| | $1.0x10^{10}$ | 5.85±0.07 |

[a] The number of purified spores was measured by direct counting with a Burker chamber under an optical microscope (Olympus BH-2 with 40xlenses); [b] pH was measured using a pH-meter with a combined glass microelectrode (LLBiotrode, Metrohm) probe in a final volume of 200 µL.

We then decided to check whether also *cotE* and *cotH* spores, like their isogenic parental strain, were able to stabilize the enzyme at an acidic condition or at high temperatures able to fully denaturate the free enzyme (Fig. 4). The enzyme bound to either *cotE* or *cotH* spores retained over 70% of its initial activity after 24 hours at pH 4.0, whereas the free enzyme was totally inactive (Fig. 6B). The enzyme bound to mutant spores lacking either *cotE* (Fig. 7A) or *cotH* (Fig. 7B) retained over 50% of their original activity after 2 hours of incubation at 75°C while the free enzyme was totally denatured. At 80°C only a minor protective effect was shown by both *cotE* or *cotH* mutant spores (Fig. 7). While β-Gal bound to wild type spores was fully active after 24 hours at pH 4.0 and retained about 80 and 40% of its activity after 2 hours at 75°C or 80°C, respectively (Fig. 4), adsorption to *cotE* or *cotH* spores resulted in reduced stabilization effect to both acidic pH (Fig. 6B) and high temperatures (Fig.

7). However, considering that mutant spores adsorb a higher amount of β-Gal activity than wild type spores (Fig. 5C and 6A), even with a loss of activity during incubation at denaturing conditions, the use of *cotE* or *cotH* spores can be considered advantageous for specific applications that involve exposure of the enzyme at low pH conditions or high temperatures.

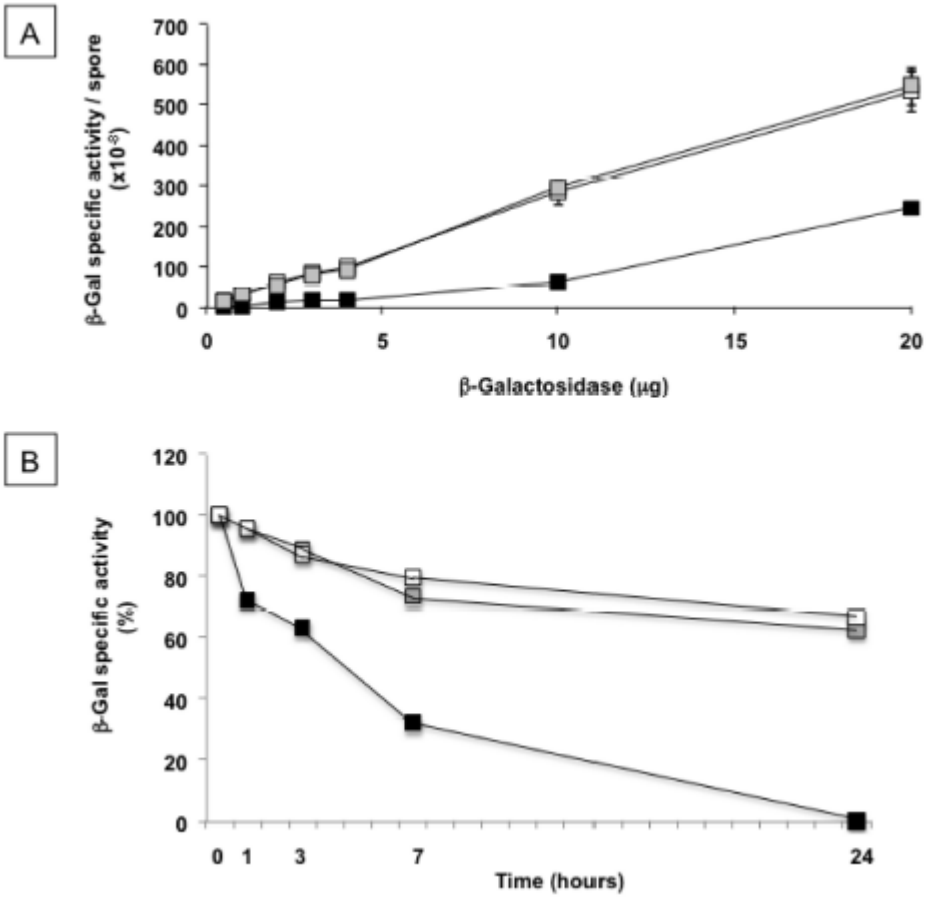


**Fig. 6.** (A) β-galactosidase units per spore obtained with increasing amounts of enzyme. The enzyme was adsorbed to wild type spores (black symbols) or mutant spores lacking *cotE* (grey symbols) or *cotH* (white symbols). (B) Percentage of β-galactosidase units observed after incubation at pH 4.0 for various times of the free enzyme (black symbols) and of the enzyme bound to *cotE* (grey symbols) or *cotH* (white symbols) spores. The data shown are representative of three independent experiments, each with three biological replicates.

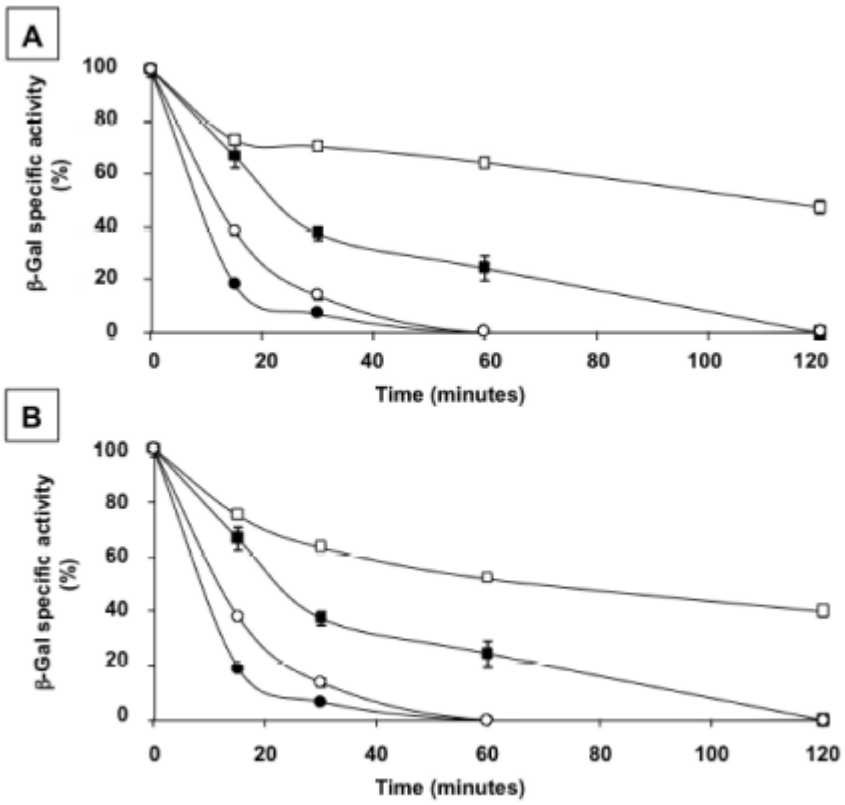


**Fig. 7.** Percentage of β-galactosidase units observed after incubation at 75°C (squares) and 80°C (circles) for various times of free enzyme (closed symbols) and of the enzyme bound to *cotE* (A) or *cotH* (B) spores (open symbols). The data shown are representative of three independent experiments, each with three biological replicates.

## Discussion

The adsorption of antigens and enzymes to bacterial spores has been reported previously and the involvement of a combination of physicochemical forces has been suggested [18, 19, 20]. In this frame, our work was aimed at gaining a better understanding of the spore-enzyme interaction. We used spores of a laboratory strain of *B. subtilis*, for which a collection of isogenic mutants altered in spore surface proteins was available, and as a model enzyme the well characterized β-Gal of the thermoacidophilic bacterium *Alicyclobacillus acidocaldarius* [21].

Our results indicate that spores bind β-Gal without affecting its properties but, instead, stabilizing it at acidic pH and high temperatures. Spore adsorption is a very

efficient process with each wild type spore able to adsorb about $7.75x10^3$ molecules of β-Gal. Gene fusion-based display of heterologous antigens was slightly less efficient than adsorption, with $1.5x10^3$ molecules of TTFC (5) and $4.8x10^3$ molecules of LTB (15) exposed on each spore. Spores lacking the outermost structures, the crust and the outer coat layer, have an adsorption efficiency even higher than wild type spores, with $1.55x10^4$ molecules of β-Gal adsorbed on each spore. This indicates that those structures, mainly formed by proteins and glycoproteins [7], have an inhibitory effect on the adhesion of the enzyme. While the efficiency of adhesion is improved, the stabilization at low pH is reduced in the mutants after 24 hours of incubation at pH 4.0. In the case of β-Gal, the loss of activity (30%) at low pH is compensated by the high amount of enzyme adsorbed. Although other enzymes have not been tested yet, our data suggest that the use of wild type or mutants spores can be planned according to the specific application and the heterologous enzyme to be displayed.

The carboxyl groups were identified as the major ionizable groups in the spore and proton diffusion was found much lower in the spore core than within the coats and cortex, suggesting the inner membrane, separating core from the external layers of the spore, as a major permeability barrier for protons [27]. Then, the carboxyl groups in the coat and in the cortex have been suggested as responsible of the negative charge of spores [27]. The different effects of wild type and mutant spores on the pH of an aqueous solution (Table 2), indicate that wild type spores attract more protons than *cotE* or *cotH* mutant spores and therefore, suggest that they have a net negative charge higher than that of the mutant spores. Since spores of those mutants lack either the entire outer coat (*cotE*) or a large part of it (*cotH*), they have a reduced number of proteins, and as a consequence of carboxyl groups, in their cortex and coat. Based on this we hypothesize that the reduced number of carboxyl groups present in the regions of the spore more permeable to proton diffusion [27] is responsible of the reduced number of protons attracted by mutant spores. However,

the different efficiency of β-Gal adsorption observed between wild type and mutant spores is not due to a different effect on the pH, since the effect of spores on the pH is buffered at the adsorption conditions (citrate buffer pH 4.0).

Our results, indicating that spores with altered surface structures have altered adsorption efficiency, point to the physicochemical properties of the spore surface as responsible of the interaction with the model enzyme. β-Gal, having a deduced isoelectric point of 5.77, at the adsorption conditions (pH 4.0) is expected to have a net positive charge and to be attracted by negatively charged spores. In spite of this, β-Gal binds more efficiently to mutant (lower negative charge) than wild type (higher negative charge) spores. We conclude that, at least in the case of β-Gal, the electrostatic force does not seem to be the predominant force involved in the interaction with the spore.

The hypothesis that the different negative charge of wild type and mutant spores is somehow responsible of the different stabilization effect observed at pH 4.0 is an intriguing possibility that needs to be addressed. Answering the questions of what is the basis of spore adsorption and of enzyme stabilization are challenging future goals that will necessarily require further experiments and the use of sophisticated physicochemical tools.

Although the molecular details of adsorption and enzyme stabilization have not been totally elucidated, spores have shown clear potentials as a novel, surface display system that, being non-recombinant, able to protect the heterologous enzyme from acidic pH and based on a host with a remarkable safety record [10, 11], appear particularly well suited for the delivery of biotherapeutic molecules to animal and human mucosal surfaces.

## Methods

### Bacterial strains and transformation

*B. subtilis* strains used in this study are listed in Table 3. *B. subtilis* PY79 [21] was used as recipient strain in transformation procedures. Plasmid amplification for DNA sequencing, subcloning experiments, and transformation of E. coli competent cells were performed with strain DH5α [28]. Bacterial strains were transformed by previously described procedures, i.e., $CaCl_2$-mediated transformation of *E. coli* competent cells [28] and two-step transformation of *B. subtilis* [23].

**TABLE 3.** *B. subtilis* strains

| Strain | Genotype | Source |
|---|---|---|
| PY79 | Wild type | 22 |
| RH201 | *cotB::spc* | 5 |
| RH101 | *cotC::spc* | 34 |
| ER203 | *cotG::Δerm* | 35 |
| AZ541 | *cotS::cm* | This study |
| GC347 | *cgeA::spc* | This study |
| AZ542 | *cotZ::neo* | This study |
| AZ543 | *cotX::neo* | This study |
| AZ544 | *cotXYZ::neo* | This study |
| DZ213 | *cotE::cm* | 25 |
| ER220 | *cotH::spc* | 36 |
| KS450 | *gerE36* | 35 |

### Genetic and molecular procedures

Isolation of plasmids, restriction digestion, and ligation of DNA, were carried out by standard methods [28]. Chromosomal DNA from *B. subtilis* was isolated as described elsewhere [29].

For the construction of insertional null mutations DNA fragments internal to genes *cotS, cotZ* and *cgeA* were amplified by PCR using the *B. subtilis* chromosome as a substrate and synthetic oligonucleotides listed in Table 4 to prime the reactions. The PCR products were visualized on ethidium bromide-stained agarose gels and gel purified by the QIAquick gel extraction kit (Qiagen) as specified by the manufacturer. Amplified fragments were ligated into plasmid3 pBEST501 [30] (*cotZ* mutant) or pER19 [31] (*cotS* mutant), carrying an antibiotic resistance cassette selectable in *B. subtilis*. The mutant in *cgeAB* was obtained by cloning amplified internal fragment of

*cgeA* gene in a pGEM-T (Promega) vector in which was previously cloned a spectinomycin cassette (from pAH256 [32]) in a *Pst*I restriction site. Those recombinant plasmids were used to transform competent cells of strain PY79. Transformants were the result of a single (Campbell-like) recombination event between homologous DNA present on the plasmid and on the chromosome. Transformants were selected by antibiotic resistance and confirmed by PCR analysis of chromosomal DNA. Mutants in the *cotX* and *cotXYZ* genes were already available but carried in a *B. subtilis* strain with a different genetic background (strain MB24) [33]. To obtain *cotX* and *cotXYZ* mutants isogenic with the wild type and other mutants used in this study, chromosomal DNA of the existing strains was extracted and used to transform competent cells of PY79 competent cells.

**TABLE 4.** Synthetic oligonucleotides

| Oligonucleotide | Sequence (5'- 3')[a] | Restriction site | Position of annealing[b] |
|---|---|---|---|
| cotS3 | ggatccATCGACCATGTGGCGCTG | *Bam*HI | +64/+82 |
| cotS4 | aagcttAACCATCACTTTATTCAG | *Hind*III | +771/+758 |
| cotZ1 | gcatgcGCTGTTGAAGAAGACTGC | *Sph*I | +64/81 |
| cotZ2 | gtcgacAACTTCAATACAGTAGTTCG | *Sal*I | +378/+359 |
| cgeA1 | gcatgcCAACTGCAACAGAAGGAG | *Sph*I | +64/+81 |
| cgeA2 | gtcgacGTGAACCTGATCGAAAGC | *Sal*I | +297/+279 |

[a] Capital and lowercase letters indicate nucleotides complementary to corresponding gene DNA and unpaired flanking sequences carrying a restriction site, respectively. [b] Referred to *cotS*, *cotZ* or *cgeA* sequences, taking the first nucleotide of the initiation codon as +1.

**Purification of spores and β-galactosidase**

Sporulation of wild type and recombinant strains was induced by the exhaustion method. After 30 h of growth in Difco Sporulation medium (DSM) at 37 °C with vigorous shaking [29], spores were collected, washed three times with distilled water and purified by gastrografin gradient as described before [29]. Spore counts were determined by serial dilution and plate-counting. A recombinant plasmid containing the *lacB* gene of *Alyciclobacillus acidocaldarius* into the expression vector pET29a has been previously described [20]. Expression of *lacB* was induced by 0.1 mM isopropyl-β D-thiogalactoside (IPTG) in *E. coli* BL21RB791 cells and β-Gal purified

using the GST-tag and the thrombin cleavage on the matrix as described by the manufacturer (Amersham Biotech).

**Binding assay and enzyme detection**

Purified β-Gal was added to a suspension of $1x10^{10}$ spores in sodium citrate 50 mM at pH 4.0 at 25°C in a final volume of 200 µl. After 1 hour of incubation, an aliquot (70 µl) of the binding mixture was stored at 4°C while the remaining part of the binding mixture was centrifuged (10 min at 13,000 rpm) to fractionate pellet and supernatant. All fractions were then used for β-Gal assays: 20 µl of each fraction were added to the reaction buffer (50 mM sodium citrate buffer at pH 5.5, 2NP-β-D-Gal 10 mM) and mixtures incubated at 65 °C for 5 minutes; the reaction was then blocked by addition of 800 µl of 1M $Na_2CO_3$. When the assay was performed on samples containing spores, the samples were centrifuged prior to measurement of optical density at 420 nm. We expressed results of enzymatic assays in total units, where 1 unit is defined as an amount of β-Gal able to hydrolyze 1 µmol of substrate in 1 min at standard conditions [20].

**Competing interests**

The authors declare that they have no competing interests.

Authors' contribution

TS - performed most of the experiments; AS - contributed to enzyme purification and enzymatic assays; RI - contributed to the construction of mutants and to develop the adsorption conditions; MDF - contributed to experiment design and manuscript writing; MM - contributed to experiment design and manuscript writing; ER - contributed discussions and suggestions during the work and wrote most of the manuscript. All authors read and approved the final manuscript.

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**References**

1. **Wu CH, Mulchandani A, Chen W.** Versatile microbial surface-display for environmental remediation and biofuels production. *Trends Microbiol.* 2008, 16:181-188.

2. **Lee, S.Y., J.H. Choi, Z. Xu.** Microbial cell-surface display. *Trends Biotechnol.* 2003, 21:45-52.

3. **Wells J.** Mucosal vaccination and therapy with genetically modified lactic acid bacteria. *Annu Rev Food Sci Technol.* 2011, 2:423-45.

4. **Villaverde A.** Nanotechnology, biotechnology and microbial cell factories. *Microb. Cell Factories* 2010. 9:53.

5**. Isticato, R., Cangiano, G., Tran, H.,T., Ciabattini, A., Medaglini, D., Oggioni, M.,R., De Felice, M., Pozzi, G., Ricca, E.** Surface display of recombinant proteins on *Bacillus subtilis* spores. *J. Bacteriol.* 2001, 183: 6294-6301.

6. **Cutting, S.M., Hong, H.A., Baccigalupi, L., Ricca, E.** Oral Vaccine Delivery by Recombinant Spore Probiotics. *Intern. Rev. Immunol.* 2009, 28:487-505.

7. **Henriques, A.O., C.P. Moran Jr.** Structure, assembly and function of the spore surface layers. *Ann. Rev. Microbiol.* 2007, 61:555-588.

8. **McKenney PT, Driks A, Eskandarian HA, Grabowski P, Guberman J, Wang KH, Gitai Z, Eichenberger P.** A distance-weighted interaction map reveals a previously uncharacterized layer of the *Bacillus subtilis* spore coat. *Current Biology* 2010, 20:934–938.

9. **Imamura D, Kuwana R, Takamatsu H, Watabe K.** Proteins involved in formation of the outermost layer of *Bacillus subtilis* spores. *J. Bacteriol.* 2011, 193:4075-4080.

10. **Knecht, L.D., Pasini, P., Daunert, S.** Bacterial spores as platforms for bioanalytical and biomedical applications. *Anal Bioanal Chem* 2011, 400:977-989.

11. **Cutting, SM.** *Bacillus* probiotics. *Food Microbiol.* 2011, 28:214-220.

12. **Hinc K, Ghandili S, Karbalaee G, Shali A, Noghabi K, Ricca E, Ahmadian G.** Efficient binding of nickel ions to recombinant *Bacillus subtilis* spores. *Res. Microbiol.* 2010, 161:757-764.

13. **Potot S, Serra CR, Henriques AO, Schyns G.** Display of recombinant proteins on *Bacillus subtilis* spores, using a coat-associated enzyme as the carrier. *Appl Environ*
*Microbiol.* 2010, 76:5926-5933.

14. **Mauriello EMF, Duc LH, Isticato R, Cangiano G, Hong HA, De Felice M, Ricca E, Cutting SM.** Display of heterologous antigens on the *Bacillus subtilis* spore coat using CotC as a fusion partner. *Vaccine* 2004, 22:1177-1187.

15**. Isticato R, Scotto Di Mase D, Mauriello EMF, De Felice M, Ricca E.** Amino terminal fusion of heterologous proteins to CotC increases display efficiencies in the *Bacillus subtilis* spore system. *BioTechniques* 2007, 42:151-156.

16. **Hinc K, Isticato R, Dembek M, Karczewska J, Iwanicki A, Peszyńska-Sularz G, De Felice M, Obukowski M, Ricca E.** Expression and display of UreA of *Helicobacter acinonychis* on the surface of *Bacillus subtilis* spores. *Microbial Cell Factories* 2010, 9:2.

17. **Detmer, A., Glenting, J.** Live bacterial vaccines—a review and identification of potential hazards. *Microb Cell Fact* 2006, 5:23.

18. **Yim S-K, Jung H-C, Yun C-H, Pan J-G.** Functional expression in *Bacillus subtilis* of mammalian NADPH-cytochrome P450 oxidoreductase and its spore-display. *Protein Expression and Purification.* 2009. 63:5–11.

19. **Huang, J.M., Hong, H.A., Van Tong, H., Hoang, T.H., Brisson, A., Cutting, S.M.** Mucosal delivery of antigens using adsorption to bacterial spores. *Vaccine* 2010, 28:1021-1030.

20. **Cho EA, Kim EJ, Pan JG.** Adsorption immobilization of *Escherichia coli* phytase on probiotic *Bacillus polyfermenticus* spores. *Enzyme Microb Technol.* 2011, 49:66-71.

21. **Di Lauro B, Strazzulli A, Perugino G, La Cara F, Bedini E, Corsaro MM, Rossi M, Moracci M.** Isolation and characterization of a new family 42 beta-galactosidase from the thermoacidophilic bacterium *Alicyclobacillus acidocaldarius*: identification of the active site residues. *Biochim Biophys Acta*. 2008, 1784:292-301.
22. **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.
23. **Cutting S, Vander Horn PB.** Genetic analysis *In*: C. Harwood and S. Cutting (Eds.), Molecular Biological Methods for *Bacillus.* John Wiley and Sons, Chichester, UK. 1990, pp. 27-74.
24. **Kim H, Hahn M, Grabowski P, McPherson D, Otte MM, Wang R, Ferguson CC, Eichenberger P, Driks A.** The *Bacillus subtilis* spore coat protein interaction network. *Mol. Microbiol.* 2006, 59:487-502.
25. **Zheng L, Donovan WP, Fitz-James PC, Losick R.** Gene encoding a morphogenic protein required in the assembly of the outer coat of the *Bacillus subtilis* endospore. *Genes Develop.* 1988, 2:1047-1054.
26. **Chen G, Driks A, Tawfiq K, Mallozzi M, Patil S.** *Bacillus anthracis* and *Bacillus subtilis* spores surface properties and transport. *Colloid and Surfaces B: Biointerfaces.* 2010. 76:512-518.
27. **Kazakov S, Bonvouloir E, Gazaryan I.** Physicochemical characterization of natural ionic microreservoirs: *Bacillus subtilis* dormant spores. 2008. *J. Phys. Chem.* 112:2233- 2244.
28. **Sambrook J, Fritsch EF, and Maniatis T.** Molecular cloning, laboratory manual, 2nd ed. 1989. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, USA.
29. **Nicholson WL and Setlow P.** Sporulation, germination and out-growth, p. 391–450.InC. Harwood and S. Cutting (ed.), Molecular biological methods for *Bacillus*. John Wiley and Sons, Chichester, United Kingdom.
30. **Itaya M, Kondo K, Tanaka T.** A neomycin resistance cassette selectable in a single copy state in the *Bacillus subtilis* chromosome. *NucleicAcid Res*. 1989. 17:4410.
31**. Ricca E, Cutting S, and Losick R.** Characterization of *bofA*, a gene involved in intercompartmental regulation of pro-K processing during sporulation in *Bacillus subtilis. J. Bacteriol.* 1992. 174:3177–3184.
32. **Henriques A, Beall BW, Moran CP**. CotM of *Bacillus subtilis*, a member of the alphacrystallin family of stress proteins, is induced during development and participates in spore outer coat formation. *J. Bacteriol.* 1997. 179:1887-1897.
33. **Zilhao R, Isticato R, Martins LO, Steil L, Völker U, Ricca E, Moran CP, Henriques AO.** Assembly and function of spore-coat associated transglutaminase of *Bacillus subtilis. J. Bacteriol.* 2005. 187:7753-7764.
34. **Isticato R, Esposito G, Zilhão R, Nolasco S, Cangiano G, De Felice M, Henriques AO, Ricca E.** Assembly of Multiple CotC Forms into the *Bacillus subtilis* Spore Coat. *J. Bacteriol.* 2004. 186:1129-1135.
35. **Sacco M, Ricca E, Losick R, Cutting S.** An additional GerE-controlled gene encoding an abundant spore coat protein from *Bacillus subtilis*. *J. Bacteriol.* 1995. 177:372-377.
36. **Naclerio G, Baccigalupi L, Zilhao R, De Felice M, Ricca E.** *Bacillus subtilis* spore coat assembly requires *cotH* gene expression. *J. Bacteriol.* 1996. 178:4375-4380.

# *Appendix*

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MICROBIAL CELL FACTORIES

RESEARCH Open Access

# Adsorption of β-galactosidase of *Alicyclobacillus acidocaldarius* on wild type and mutants spores of *Bacillus subtilis*

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## Abstract

**Background:** The *Bacillus subtilis* spore has long been used as a surface display system with potential applications in a variety of fields ranging from mucosal vaccine delivery, bioremediation and biocatalyst development. More recently, a non-recombinant approach of spore display has been proposed and heterologous proteins adsorbed on the spore surface. We used the well-characterized β-galactosidase from the thermoacidophilic bacterium *Alicyclobacillus acidocaldarius* as a model to study enzyme adsorption, to analyze whether and how spore-adsorption affects the properties of the enzyme and to improve the efficiency of the process.

**Results:** We report that purified β-galactosidase molecules were adsorbed to purified spores of a wild type strain of *B. subtilis* retaining ca. 50% of their enzymatic activity. Optimal pH and temperature of the enzyme were not altered by the presence of the spore, that protected the adsorbed β-galactosidase from exposure to acidic pH conditions. A collection of mutant strains of *B. subtilis* lacking a single or several spore coat proteins was compared to the isogenic parental strain for the adsorption efficiency. Mutants with an altered outermost spore layer (crust) were able to adsorb 60-80% of the enzyme, while mutants with a severely altered or totally lacking outer coat adsorbed 100% of the β-galactosidase molecules present in the adsorption reaction.

**Conclusion:** Our results indicate that the spore surface structures, the crust and the outer coat layer, have an negative effect on the adhesion of the β-galactosidase. Electrostatic forces, previously suggested as main determinants of spore adsorption, do not seem to play an essential role in the spore-β-galactosidase interaction. The analysis of mutants with altered spore surface has shown that the process of spore adsorption can be improved and has suggested that such improvement has to be based on a better understanding of the spore surface structure. Although the molecular details of spore adsorption have not been fully elucidated, the efficiency of the process and the pH-stability of the adsorbed molecules, together with the well documented robustness and safety of spores of *B. subtilis*, propose the spore as a novel, non-recombinant system for enzyme display.

## Background

Display systems to present biologically active molecules on the surface of microorganisms have become an increasingly used strategy to address biotechnological issues [1,2]. For biomedical applications surface display systems have been mostly used for the identification of neutralizing epitopes, the development of whole cell diagnostic tools, or vaccine delivery [3,4]. More recent is a strategy to engineer bacterial endospores (spores) to display heterologous proteins on their surface [5]. Endospore-forming bacteria are Gram-positive microorganisms belonging to different genera and including more than 1,000 species [6]. The common feature of these organisms is the ability to form a quiescent cellular type (the spore) in response to harsh environments. The spore can survive in this dormant state for long periods, resisting to a vast range of stresses such as high temperature, dehydration, absence of nutrients, presence of toxic chemicals. When the environmental conditions ameliorate, the spore germinates originating a vegetative cell able to grow and sporulate [6]. The ability of the spore to survive non-physiological conditions is, in part,

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due to the presence of the spore coat, a proteinaceous structure surrounding the spore. At least seventy different proteins (Cot proteins) form the multilayered coat structure, composed of an inner part, an outer part [7] and the crust, the latter being a recently discovered outermost layer of the spore [8,9].

Spore-based display systems provide several advantages with respect to systems based on the use of phages and bacterial cells [10]. The remarkable and well documented resistance of spores to various environmental and toxic effects [7] ensures high stability of the display system. Proteins to be displayed on the spore are produced in the mother cell compartment of the sporangium and are assembled around the forming spore without the need to be translocated across a membrane, thus eliminating the size constrains of cell-based display systems [5,10]. The safety record of several endospore-forming species [11], makes spores of those species ideal candidates as vehicles to deliver molecules to mucosal surfaces [6].

The strategy to obtain the spore surface display of heterologous proteins is based on the construction of gene fusions between the gene coding for a selected spore surface protein (carrier) and the heterologous DNA coding for the protein to be displayed [5]. By this approach a variety of heterologous proteins have been displayed and recombinant spores proposed as vaccine vehicles (see ref. 6 for a review), as biocatalysts (see ref. 10 for a review), or as a bioremediation tool [12]. To optimize and rationalize this display strategy an inner (OxdD [13]) and various outer (CotB [5], CotC [14,15], CotG [16]) coat components have been tested as carriers.

The spore-based display system, like other cell- or phage-based systems, relies on the genetic engineering of the host to display immunogenic peptides or proteins and obtain a recombinant organism to be used as a live biotechnological tool [5,6,10]. This is a major drawback since it causes the release of live recombinant organisms into nature, raising concerns over the use and clearance of genetically modified microorganisms [17]. To overcome this obstacle, a non-recombinant approach to use spores as a display system has been recently proposed and model proteins efficiently exposed. In the first study suggesting that heterologous proteins can be adsorbed on the spore surface, the mammalian NADPH-cytochrome P450 reductase (CPR), a diflavin-containing enzyme, was over-expressed in sporulating *B. subtilis* cells and released into the culture medium after sporulation by autolysis of the mother cell. However, part of the CPR activity was found associated to spores and the displayed enzyme shown to be accessible to anti-CPR antibodies [18]. In a different study a collections of purified antigens (TTFC of *Clostridium tetani*, PA of *Bacillus anthracis*, Cpa of *Clostridium perfringens* and glutathione S transferase of *Shistosomas japonica*) were adsorbed to *B. subtilis* spores and shown to be able to induce specific and protective immune responses in mucosally immunised mice [19]. Spore adsorption resulted to be more efficient when the pH of the binding buffer was acidic (pH 4) and less efficient or totally inhibited at pH values of 7 or 10 [19]. A combination of electrostatic and hydrophobic interactions between spores and antigens were suggested to drive the adsorption, that was shown to be not dependent on specific spore coat components but rather on the negatively charged and hydrophobic surface of the spore [19]. Hydrophobic and electrostatic interactions were suggested as the main forces involved also in the interaction between the *E. coli* phytase and spores of *B. polyfermenticus* [20].

We used a well-characterized and biotechnologically important enzyme, a β-galactosidase of the thermoacidophilic bacterium *Alicyclobacillus acidocaldarius* [21], as a model to study enzyme adsorption on *B. subtilis* spores. This enzyme belongs to the glycoside hydrolase family 42 (GH42) and is characterized by an optimal activity and stability at 65°C [21]. By using this system we tested whether adsorbed β-galactosidase molecules retained their activity and whether and how spore-adsorption affected the properties of the enzyme. With the dual aim of identifying spore surface structures involved in β-galactosidase adsorption and to improve the efficiency of the process we also screened for enzyme binding a collection of mutant strains of *B. subtilis* lacking a single or several spore coat proteins. A better understanding of the spore surface structure is likely to lead to a rationalization of the adsorption system, such that wild type or mutant spores will be utilized, depending upon the specific application or the heterologous enzyme to display.

## Results

### β-Galactosidase of *A. acidocaldarius* adsorbs to *B. subtilis* spores and retains its enzymatic activity

In an initial experiment 0.5 μg of β-Galactosidase (β-Gal) of *A. acidocaldarius*, over-expressed in *E. coli* and purified by affinity chromatography with GST columns (Methods), were incubated with $1.0\times10^{10}$ spores of *B. subtilis* strain PY79 [22], purified by renographin gradient, as previously described [23]. The adsorption reaction was performed in citrate buffer at pH 4.0, as previously described for various antigens [19]. After adsorption, the mixture was either assayed for β-Gal activity ("Total Units" in Figure 1) or fractionated by centrifugation. The pellet ("Spore-Bound Units" in Figure 1) and supernatant ("Unbound Units" in Figure 1) fractions were then assayed independently. Figure 1 schematizes the experiment and reports that ca. 50% of the β-Gal

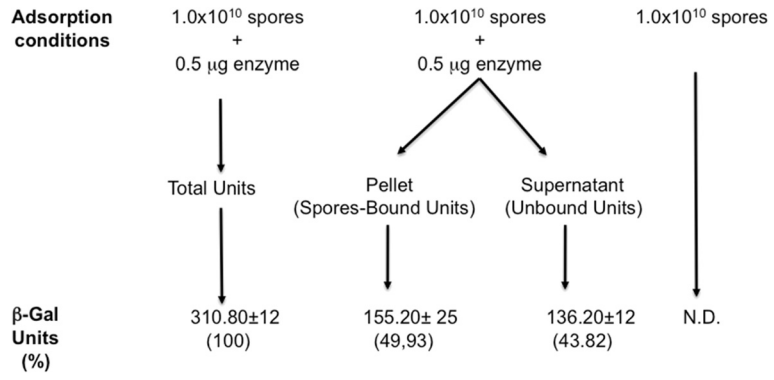


**Figure 1 Schematic representation of a typical adsorption experiment.** Purified spores were mixed to the purified enzyme in PBS buffer (pH 4.0) and incubated one hour at 25°C. The reaction was then stopped in ice and sample either directly assayed (Total Units) or fractionated by centrifugation and fractions assayed independently.

units were found in the pellet fraction and, therefore, were most likely associated to the spores. The spore-associated β-Gal activity was not due to an endogenous enzyme, since purified spores alone did not show any activity (Figure 1). Only a limited amount (less than 10%) of β-Gal units was lost during the procedure (Figure 1) and the adhesion appeared to be stable, since 95% of the units was still associated to the pellet fraction after two washes with phosphate buffer pH 4.0 or 5.5 (not shown).

In a previous report Huang et al. [19] have shown that spore adsorption of four different antigens is more efficient at pH 4.0 than at pH 7.0 or 10.0. In agreement with those observations we found that adsorption of β-Gal to $1.0\times10^{10}$ spores was strictly dependent on the pH of the adsorption buffer, with a similar amount of units adsorbed at pH values of 3.5 and 4.0 and a strongly decreased amount of units adsorbed at pH 4.5 (Table 1). β-Gal has a deduced isoelectric point of 5.77, therefore at all pH tested in the experiments of Table 1, it is expected to have a net positive charge. At pH values lower than 3.5 no activity was found in any of the fractions as well as in the unfractionated sample (Total Units), indicating that exposure at pH 3.0 totally inactivated the enzyme (Table 1). At both pH values of 3.5 and 4.0 the amount of β-Gal units lost in the experimental procedure was about 10% (Table 1).

To check that our experimental conditions were not limiting for the number of spores, we measured the β-Gal activity associated to each spore using different amounts of enzyme and spores in the adsorption reaction. As shown in Figure 2, with both $2.0\times10^{9}$ (open symbols) and $1.0\times10^{10}$ (closed symbols), increasing the amount of enzyme used in the adsorption reaction up to 4 μg, the β-Gal units bound to each spore increased, indicating that the spores present in the reaction buffer were not saturated by the enzyme and therefore were not a limiting factor for the reaction.

### Adsorption to spores stabilizes β-Gal

It has been previously reported that the β-Gal of *A. acidocaldarius* has an optimal activity and stability at 65°C and at pH 6.5 [21]. To verify whether spore adsorption affected these properties, we compared the β-Gal activity of the free and the spore-adsorbed enzyme, after exposing both to the adsorption conditions (1 hour at pH 4.0). As shown in Figure 3, the optimum temperature (panel A) and pH (panel B) were identical with (open symbols) and without (closed symbols) spores, indicating that the spore-bound and the free enzyme have identical properties.

Results of Figure 3 indicate that at all tested temperatures and pH values the spore-bound enzyme is more active than the free enzyme. A stabilization effect of spores on an enzyme has been previously suggested for a different enzyme and for spores of a different bacterial species [20]. To address this point in more detail we

**Table 1 pH-dependent adsorption efficiency with $1\times10^{10}$ spores**

| pH values of the adsorption reaction | | | | |
|---|---|---|---|---|
| | **3.0** | **3.5** | **4.0** | **4.5** |
| **Total units (%)** | 0 | 262.55 ± 5.15 (100) | 308.92 ± 11.13 (100) | 353.64 ± 23.80 (100) |
| **Spore-bound units (%)** | 0 | 139.64 ± 5.65 (53.18) | 158.98 ± 2.41 (51.46) | 26.89 ± 4.68 (7.60) |
| **Unbound-units (%)** | 0 | 92.46 ± 8.24 (35.21) | 127.77 ± 12.38 (41.36) | 306.72 ± 26.96 (86.73) |

[1]β-galactosidase unit is defined as an amount of β-Gal which is able to hydrolyze 1 μmol of substrate in 1 min at standard conditions [19].

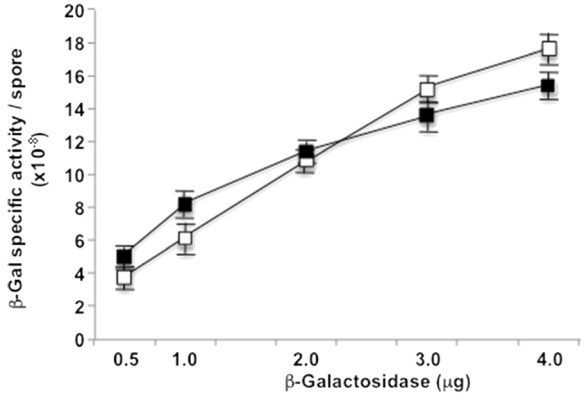


**Figure 2 β-galactosidase units per spore obtained with increasing amounts of enzyme.** The enzyme was adsorbed to $2.0\times10^9$ (open symbols) or $1.0\times10^{10}$ (closed symbols) spores.

compared the activity of spore-bound and free β-Gal after exposure at low pH or high temperatures. Although the enzyme is from a thermoacidophilic bacterium, the activity of the free enzyme decreased overtime after exposure at both low pH or high temperature and was protected by denaturation when adsorbed to spores. After exposure at pH 4.0 the activity of the free β-Gal was completely lost after 24 hours while the spore-bound enzyme was still fully active (Figure 4A). After 2 hours of incubation at 75°C the activity of the free β-Gal was completely lost while the spore-bound enzyme retained about 80% of its original activity (black and white squares in Figure 4B, respectively). At 80°C the activity of the free enzyme was completely lost after 1 hour while the spore-bound enzyme retained over 30% of its activity even after two hours of incubation (black and white circles in Figure 4B, respectively).

### Spores with altered surface show increased efficiency of adsorption

In a previous report it has been shown that adsorption of various antigens on the surface of *B. subtilis* spores does not depend on a direct interaction with any of the major outer coat components (CotA, CotB, CotC, CotG and CotF) [19]. In agreement with the previous results we showed that β-Gal adsorption is similar in wild type spores (PY79) and in isogenic null mutants lacking *cotB, cotC, cotG* or *cotS* (Figure 5A). However, it has been recently shown that the *B. subtilis* spore is surrounded by an outermost layer, the crust, not identified before [8]. The crust is composed of proteins and the products of the *cgeA* gene and of the *cotVWXYZ* cluster are involved in crust formation [9]. One of those proteins, CotZ, has been proposed to be essential for the spore crust to surround the spore but not for the formation of the other coat layers [9]. Based on this, we examined whether the presence of the crust had an effect on the adsorption of β-Gal by comparing the efficiency of adsorption of a wild type strain (PY79) with that of isogenic null mutants lacking *cotZ*, *cgeA cotX* or *cotXYZ*. As shown in Figure 5B, the efficiency of β-Gal

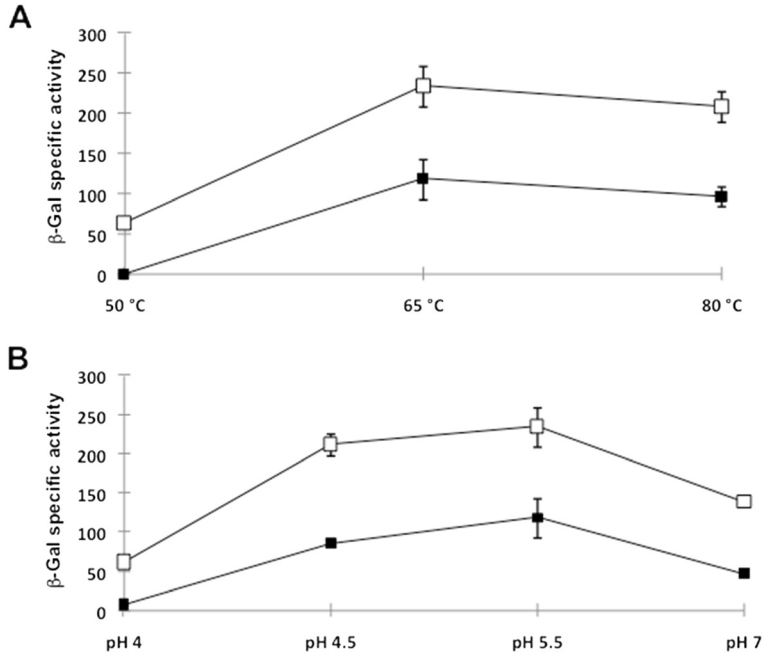


**Figure 3 Spore-associated β-galactosidase units obtained assaying the samples at pH 5.5 and various temperatures (A) or at 65°C and various pH values (B).**

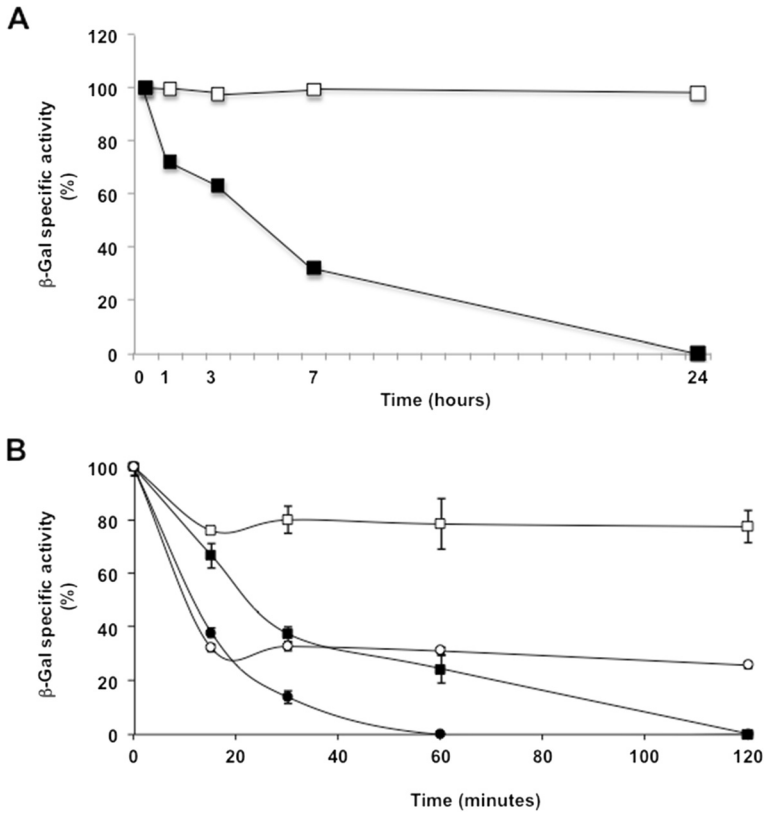


**Figure 4 (A) Percentage of β-galactosidase units observed after incubation at pH 4.0 for various times of free (closed squares) and spore-bound (open squares) enzyme.** (**B**) Percentage of β-galactosidase units observed after incubation at 75°C (squares) and 80°C (circles) for various times of free (closed symbols) and spore-bound (open symbols) enzyme. The data shown are representative of three independent experiments, each with three biological replicates.

adsorption was increased in all four mutants. Only a slight increase was observed with mutant spores lacking *cotZ, cgeA* or *cotX* (from ca. 50% of activity associated to wild type spores to ca. 60% associated to the mutant spores), while a stronger effect was observed with spores lacking *cotXYZ* (ca. 80% of spore-associated activity). Since the mutant lacking *cotXYZ* showed a higher increase of β-Gal adhesion than mutants lacking only *cotZ* or *cotX,* we hypothesize that either CotY is an important factor in mediating the adhesion of β-Gal or that CotZ, CotX and CotY have an additive action in modifying the spore surface and, as a consequence, reducing adhesion of the enzyme (Figure 5B). Although further experiments will be needed to fully address this point, the results of Figure 5B indicate that a normally formed crust has an inhibiting role on β-Gal adhesion.

Next, we decided to test the adsorption efficiency of mutants lacking also other coat layers. In particular, we used spores of strains lacking *cotH, gerE* or *cotE. cotH* spores lack at least 9 outer coat components [24], *gerE* codes for a transcriptional regulator and *gerE* mutant spores lack the latest class of coat components [7], and *cotE* spores totally lack the outer coat [25]. As shown in Figure 5C, with spores of all three mutants 100% of the β-Gal activity was adsorbed, indicating that the spores lacking the crust and at least part of the outer coat are extremely efficient in adsorbing β-Gal.

### Adsorption to mutant spores lacking *cotE* or *cotH*

Wild type spores of *B. subtilis* are negatively charged [26] and able to accumulate a large number of protons in an aqueous solution [27]. The presence of a large number of spores is then likely to alter the pH of an aqueous solution. Because of the pH-dependence of β-Gal adsorption (Table 1), we hypothesized that wild type and mutant spores had a different effect on the pH of the binding solution and, as a consequence, on the efficiency of β-Gal adsorption. To verify this hypothesis we measured the effects of spores on the pH of pure water and of the adsorption buffer (citrate buffer pH 4.0). We tested *cotE* or *cotH* mutant spores lacking either the entire outer coat or 9 major outer coat components [24], while we did not consider *gerE* mutant spores, although they also showed 100% of β-Gal adhesion (Figure 5C), since the outer coat structure of *gerE* spores has not been studied in details and not all genes

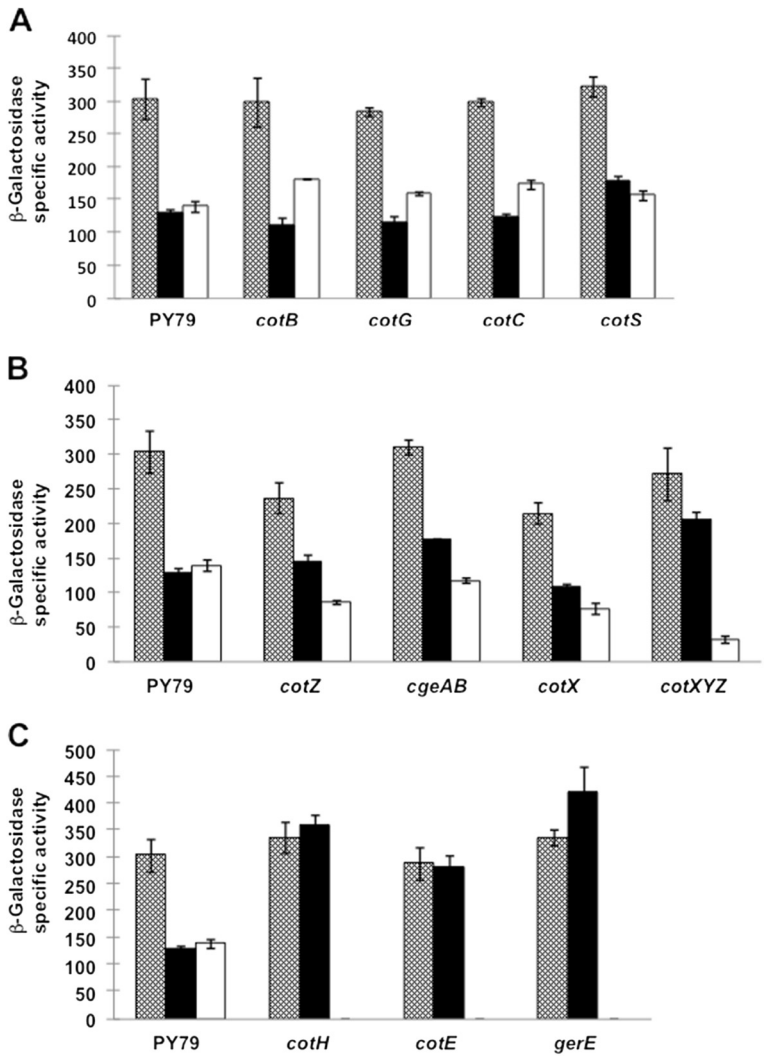


**Figure 5 Total Units (grey bars), Spore-Bound Units (black bars) and Unbound Units (white bars) of β-galactosidase obtained using wild type (PY79) or isogenic mutant spores.** Panel **A** includes mutants lacking a single outer coat component; panel **B** includes mutants totally or partially lacking the crust; and panel **C** includes mutants with a severely defective (*cotH* and *gerE*) or totally absent (*cotE*) outer coat.

controlled by the transcriptional regulator GerE have been identified and functionally characterized. As shown in Table 2, both $2.0\times10^9$ and $1.0\times10^{10}$ wild type spores were able to reduce the pH of pure water of over one pH unit, while the same numbers of *cotE* or *cotH* spores had a minor, if any, effect. In all cases no effects on the pH were observed at the adsorption conditions, that buffered the effect of spores (not shown). Those results seem to suggest that the different efficiency of adsorption between wild type and mutant spores was not caused by a spore-dependent alteration of the pH in the adsorption reaction and that wild type spores have a net negative charge higher than that of both *cotE* or *cotH* mutant spores.

Wild type and mutant spores were then compared for their efficiency in adsorbing and stabilizing β-Gal molecules. *cotE* and *cotH* mutant spores showed identical adsorption efficiencies that increased with increasing amounts of enzyme in the adsorption reaction (Figure 6A). These results confirm and expand the results of Figure 2, indicating that the spores present in the reaction buffer ($2.0\times10^9$) were not saturated by 20 μg of enzyme and therefore were not a limiting factor for the reaction. β-Gal has a molecular mass of 77,737 daltons, therefore the maximal amount of enzyme used in the experiment of Figure 6A (20 μg) contains approximately $1.55\times10^{14}$ molecules. Since in the adsorbing reaction with $1.0\times10^{10}$ spores about 50% of the enzyme is bound to wild type spores and 100% to mutant spores, we can calculate that about $7.75\times10^3$ and $1.55\times10^4$ molecules of β-Gal were adsorbed to each wild type and mutant spores, respectively.

**Table 2 Effect of wild type and mutant spores on pH**

| | n° of spores[a] | pH[b] |
|---|---|---|
| Water | 0 | 5.98 ± 0.08 |
| Water + WT spores | $2.0 \times 10^9$ | 7.23 ± 0.07 |
| | $1.0 \times 10^{10}$ | 7.14 ± 0.24 |
| Water + *cotE* spores | $2.0 \times 10^9$ | 6.06 ± 0.07 |
| | $1.0 \times 10^{10}$ | 6.18 ± 0.04 |
| Water + *cotH* spores | $2.0 \times 10^9$ | 5.88 ± 0.07 |
| | $1.0 \times 10^{10}$ | 5.85 ± 0.07 |

[a] The number of purified spores was measured by direct counting with a Burker chamber under an optical microscope (Olympus BH-2 with 40xlenses); [b] pH was measured using a pH-meter with a combined glass microelectrode (LLBiotrode, Metrohm) probe in a final volume of 200 μL.

We then decided to check whether also *cotE* and *cotH* spores, like their isogenic parental strain, were able to stabilize the enzyme at an acidic condition or at high temperatures able to fully denaturate the free enzyme (Figure 4). The enzyme bound to either *cotE* or *cotH* spores retained over 70% of its initial activity after 24 hours at pH 4.0, whereas the free enzyme was totally inactive (Figure 6B). The enzyme bound to mutant spores lacking either *cotE* (Figure 7A) or *cotH* (Figure 7B) retained over 50% of their original activity after 2 hours of incubation at 75°C while the free enzyme was totally denatured. At 80°C only a minor protective effect was shown by both *cotE* or *cotH* mutant spores (Figure 7). While β-Gal bound to wild type spores was fully active after 24 hours at pH 4.0 and retained about 80 and 40% of its activity after 2 hours at 75°C or 80°C, respectively (Figure 4), adsorption to *cotE* or *cotH* spores resulted in reduced stabilization effect to both acidic pH (Figure 6B) and high temperatures (Figure 7). However, considering that mutant spores adsorb a higher amount of β-Gal activity than wild type spores (Figures 5C and 6A), even with a loss of activity during incubation at denaturing conditions, the use of *cotE* or *cotH* spores can be considered advantageous for specific applications that involve exposure of the enzyme at low pH conditions or high temperatures.

## Discussion

The adsorption of antigens and enzymes to bacterial spores has been reported previously and the involvement of a combination of physicochemical forces has been suggested [18-20]. In this frame, our work was aimed at

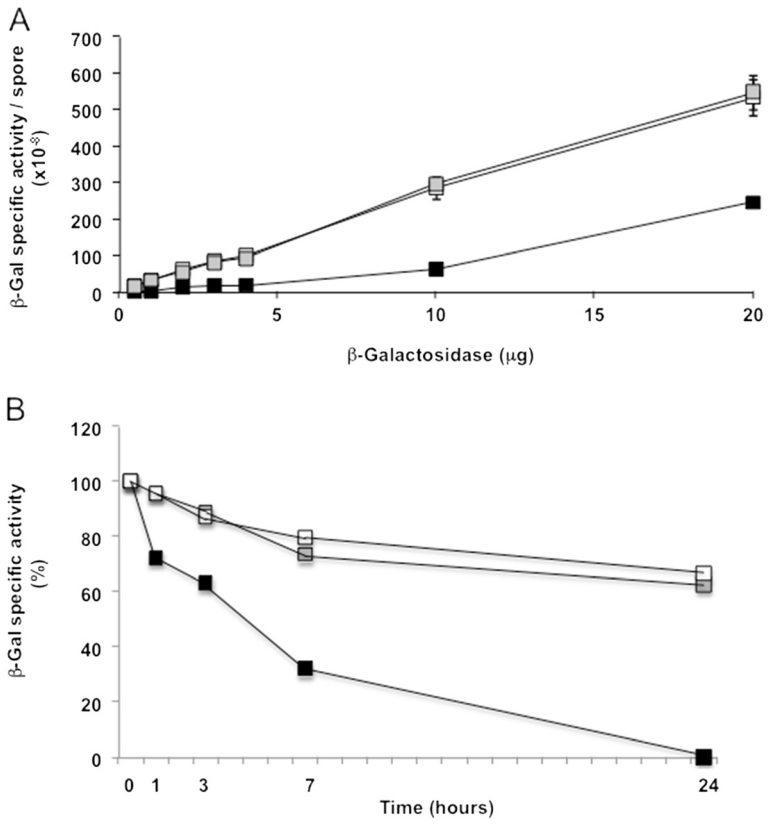


**Figure 6 (A) β-galactosidase units per spore obtained with increasing amounts of enzyme.** The enzyme was adsorbed to wild type spores (black symbols) or mutant spores lacking *cotE* (grey symbols) or *cotH* (white symbols). (**B**) Percentage of β-galactosidase units observed after incubation at pH 4.0 for various times of the free enzyme (black symbols) and of the enzyme bound to *cotE* (grey symbols) or *cotH* (white symbols) spores. The data shown are representative of three independent experiments, each with three biological replicates.

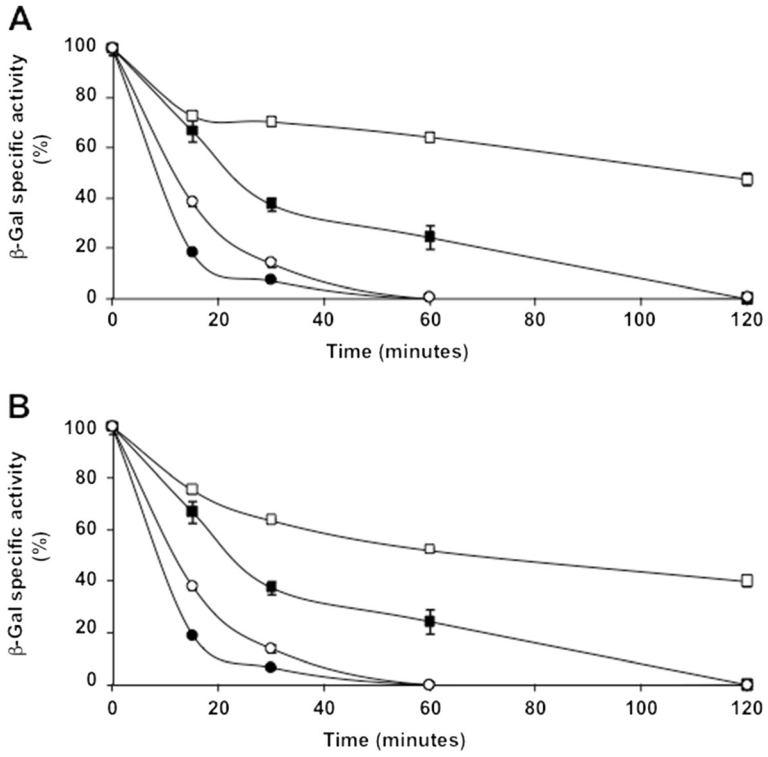


**Figure 7 Percentage of β-galactosidase units observed after incubation at 75°C (squares) and 80°C (circles) for various times of free enzyme (closed symbols) and of the enzyme bound to *cotE* (A) or *cotH* (B) spores (open symbols).** The data shown are representative of three independent experiments, each with three biological replicates.

gaining a better understanding of the spore-enzyme interaction. We used spores of a laboratory strain of *B. subtilis*, for which a collection of isogenic mutants altered in spore surface proteins was available, and as a model enzyme the well characterized β-Gal of the thermoacidophilic bacterium *Alicyclobacillus acidocaldarius* [21].

Our results indicate that spores bind β-Gal without affecting its properties but, instead, stabilizing it at acidic pH and high temperatures. Spore adsorption is a very efficient process with each wild type spore able to adsorb about $7.75\times10^3$ molecules of β-Gal. Gene fusion-based display of heterologous antigens was slightly less efficient than adsorption, with $1.5\times10^3$ molecules of TTFC (5) and $4.8\times10^3$ molecules of LTB (15) exposed on each spore. Spores lacking the outermost structures, the crust and the outer coat layer, have an adsorption efficiency even higher than wild type spores, with $1.55\times10^4$ molecules of β-Gal adsorbed on each spore. This indicates that those structures, mainly formed by proteins and glycoproteins [7], have an inhibitory effect on the adhesion of the enzyme. While the efficiency of adhesion is improved, the stabilization at low pH is reduced in the mutants after 24 hours of incubation at pH 4.0. In the case of β-Gal, the loss of activity (30%) at low pH is compensated by the high amount of enzyme adsorbed. Although other enzymes have not been tested yet, our data suggest that the use of wild type or mutants spores can be planned according to the specific application and the heterologous enzyme to be displayed.

The carboxyl groups were identified as the major ionizable groups in the spore and proton diffusion was found much lower in the spore core than within the coats and cortex, suggesting the inner membrane, separating core from the external layers of the spore, as a major permeability barrier for protons [27]. Then, the carboxyl groups in the coat and in the cortex have been suggested as responsible of the negative charge of spores [27]. The different effects of wild type and mutant spores on the pH of an aqueous solution (Table 2), indicate that wild type spores attract more protons than *cotE* or *cotH* mutant spores and therefore, suggest that thay have a net negative charge higher than that of the mutant spores. Since spores of those mutants lack either the entire outer coat (*cotE*) or a large part of it (*cotH*), they have a reduced number of proteins, and as a consequence of carboxyl groups, in their cortex and coat. Based on this we hypothesize that the reduced number of carboxyl groups present in the regions of the spore more permeable to proton diffusion [27] is responsible of the reduced number of protons attracted by mutant

spores. However, the different efficiency of β-Gal adsorption observed between wild type and mutant spores is not due to a different effect on the pH, since the effect of spores on the pH is buffered at the adsorption conditions (citrate buffer pH 4.0).

Our results, indicating that spores with altered surface structures have altered adsorption efficiency, point to the physicochemical properties of the spore surface as responsible of the interaction with the model enzyme. β-Gal, having a deduced isoelectric point of 5.77, at the adsorption conditions (pH 4.0) is expected to have a net positive charge and to be attracted by negatively charged spores. In spite of this, β-Gal binds more efficiently to mutant (lower negative charge) than wild type (higher negative charge) spores. We conclude that, at least in the case of β-Gal, the electrostatic force does not seem to be the predominant force involved in the interaction with the spore.

The hypothesis that the different negative charge of wild type and mutant spores is somehow responsible of the different stabilization effect observed at pH 4.0 is an intriguing possibility that needs to be addressed. Answering the questions of what is the basis of spore adsorption and of enzyme stabilization are challenging future goals that will necessarily require further experiments and the use of sophisticated physicochemical tools.

Although the molecular details of adsorption and enzyme stabilization have not been totally elucidated, spores have shown clear potentials as a novel, surface display system that, being non-recombinant, able to protect the heterologous enzyme from acidic pH and based on a host with a remarkable safety record [10,11], appear particularly well suited for the delivery of biotherapeutic molecules to animal and human mucosal surfaces.

## Methods

### Bacterial strains and transformation

*B. subtilis* strains used in this study are listed in Table 3. *B. subtilis* PY79 [21] was used as recipient strain in transformation procedures. Plasmid amplification for DNA sequencing, subcloning experiments, and transformation of *E. coli* competent cells were performed with strain DH5α [28]. Bacterial strains were transformed by previously described procedures, i.e., $CaCl_2$-mediated transformation of *E. coli* competent cells [28] and two-step transformation of *B. subtilis* [23] (Table 4).

### Genetic and molecular procedures

Isolation of plasmids, restriction digestion, and ligation of DNA, were carried out by standard methods [28]. Chromosomal DNA from *B. subtilis* was isolated as described elsewhere [29].

For the construction of insertional *null* mutations DNA fragments internal to genes *cotS, cotZ* and *cgeA* were amplified by PCR using the *B. subtilis* chromosome as a substrate and synthetic oligonucleotides listed in Table 2 to prime the reactions. The PCR products were visualized on ethidium bromide-stained agarose gels and gel purified by the QIAquick gel extraction kit (Qiagen) as specified by the manufacturer. Amplified fragments were ligated into plasmids pBEST501 [30] (*cotZ* mutant) or pER19 [31] (*cotS* mutant), carrying an antibiotic resistance cassette selectable in *B. subtilis*. The mutant in *cgeAB* was obtained by cloning amplified internal fragment of *cgeA* gene in a pGEM-T (Promega) vector in which was previously cloned a spectinomycin cassette (from pAH256 [32]) in a *Pst*I restriction site. Those recombinant plasmids were used to transform competent cells of strain PY79. Transformants were the result of a single (Campbell-like) recombination event between homologous DNA present on the plasmid and on the chromosome. Transformants were selected by antibiotic resistance and confirmed by PCR analysis of chromosomal DNA. Mutants in the *cotX* and *cotXYZ* genes were already available but carried in a *B. subtilis* strain with a different genetic background (strain MB24) [33]. To obtain *cotX* and *cotXYZ* mutants isogenic with the wild type and other mutants used in this study, chromosomal DNA of the existing strains was extracted and used to transform competent cells of PY79 competent cells.

**Table 3 *B. subtilis* strains**

| Strain | Genotype | Source |
|---|---|---|
| PY79 | Wild type | [22] |
| RH201 | *cotB::spc* | [5] |
| RH101 | *cotC::spc* | [34] |
| ER203 | *cotG::*Δ*erm* | [35] |
| AZ541 | *cotS::cm* | This study |
| GC347 | *cgeA::spc* | This study |
| AZ542 | *cotZ::neo* | This study |
| AZ543 | *cotX::neo* | This study |
| AZ544 | *cotXYZ::neo* | This study |
| DZ213 | *cotE::cm* | [25] |
| ER220 | *cotH::spc* | [36] |
| KS450 | *gerE36* | [35] |

### Purification of spores and β-galactosidase

Sporulation of wild type and recombinant strains was induced by the exhaustion method. After 30 h of growth in Difco Sporulation medium (DSM) at 37°C with vigorous shaking [29], spores were collected, washed three times with distilled water and purified by gastrografin gradient as described before [29]. Spore counts were determined by serial dilution and plate-counting.

A recombinant plasmid containing the *lacB* gene of *Alyciclobacillus acidocaldarius* into the expression

**Table 4 Synthetic oligonucleotides**

| Oligonucleotide | Sequence (5'- 3')[a] | Restriction site | Position of annealing[b] |
|---|---|---|---|
| cotS3 | ggatccATCGACCATGTGGCGCTG | *Bam*HI | +64/+82 |
| cotS4 | aagcttAACCATCACTTTATTCAG | *Hind*III | +771/+758 |
| cotZ1 | gcatgcGCTGTTGAAGAAGACTGC | *Sph*I | +64/81 |
| cotZ2 | gtcgacAACTTCAATACAGTAGTTCG | *Sal*I | +378/+359 |
| cgeA1 | gcatgcCAACTGCAACAGAAGGAG | *Sph*I | +64/+81 |
| cgeA2 | gtcgacGTGAACCTGATCGAAAGC | *Sal*I | +297/+279 |

[a] Capital and lowercase letters indicate nucleotides complementary to corresponding gene DNA and unpaired flanking sequences carrying a restriction site, respectively. [b] Referred to *cotS*, *cotZ* or *cgeA* sequences, taking the first nucleotide of the initiation codon as +1.

vector pET29a has been previously described [20]. Expression of *lacB* was induced by 0.1 mM isopropyl-β D-thiogalactoside (IPTG) in *E. coli* BL21RB791 cells and β-Gal purified using the GST-tag and the thrombin cleavage on the matrix as described by the manufacturer (Amersham Biotech).

### Binding assay and enzyme detection

Purified β-Gal was added to a suspension of $1\times10^{10}$ spores in sodium citrate 50 mM at pH 4.0 at 25°C in a final volume of 200 μl. After 1 hour of incubation, an aliquot (70 μl) of the binding mixture was stored at 4°C while the remaining part of the binding mixture was centrifuged (10 min at 13,000 rpm) to fractionate pellet and supernatant. All fractions were then used for β-Gal assays: 20 μl of each fraction were added to the reaction buffer (50 mM sodium citrate buffer at pH 5.5, 2NP-β-D-Gal 10 mM) and mixtures incubated at 65°C for 5 minutes; the reaction was then blocked by addition of 800 μl of 1 M $Na_2CO_3$. When the assay was performed on samples containing spores, the samples were centrifuged prior to measurement of optical density at 420 nm. We expressed results of enzymatic assays in total units, where 1 unit is defined as an amount of β-Gal able to hydrolyze 1 μmol of substrate in 1 min at standard conditions [20].

#### Competing interests

The authors declare that they have no competing interests.

#### Authors' contributions

TS performed most of the experiments. AS contributed to enzyme purification and enzymatic assays. RI contributed to the construction of mutants and to develop the adsorption conditions. MDF contributed to experiment design and manuscript writing. MM contributed to experiment design and manuscript writing. ER contributed discussions and suggestions during the work and wrote most of the manuscript. All authors read and approved the final manuscript.

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#### References

1. Wu CH, Mulchandani A, Chen W: **Versatile microbial surface-display for enviromental remediation and biofuels production.** *Trends Microbiol* 2008, **16:**181–188.
2. Lee SY, Choi JH, Xu Z: **Microbial cell-surface display.** *Trends Biotechnol* 2003, **21:**45–52.
3. Wells J: **Mucosal vaccination and therapy with genetically modified lactic acid bacteria.** *Annu Rev Food Sci Technol* 2011, **2:**423–445.
4. Villaverde A: **Nanotechnology, biotechnology and microbial cell factories.** *Microb Cell Fact* 2010, **9:**53.
5. Isticato R, Cangiano G, Tran HT, Ciabattini A, Medaglini D, Oggioni MR, De Felice M, Pozzi G, Ricca E: **Surface display of recombinant proteins on *Bacillus subtilis* spores.** *J Bacteriol* 2001, **183:**6294–6301.
6. Cutting SM, Hong HA, Baccigalupi L, Ricca E: **Oral Vaccine Delivery by Recombinant Spore Probiotics.** *Int Rev Immunol* 2009, **28:**487–505.
7. Henriques AO, Moran CP Jr: **Structure, assembly and function of the spore surface layers.** *Ann Rev Microbiol* 2007, **61:**555–588.
8. McKenney PT, Driks A, Eskandarian HA, Grabowski P, Guberman J, Wang KH, Gitai Z, Eichenberger P: **A distance-weighted interaction map reveals a previously uncharacterized layer of the *Bacillus subtilis* spore coat.** *Curr Biol* 2010, **20:**934–938.
9. Imamura D, Kuwana R, Takamatsu H, Watabe K: **Proteins involved in formation of the outermost layer of *Bacillus subtilis* spores.** *J Bacteriol* 2011, **193:**4075–4080.
10. Knecht LD, Pasini P, Daunert S: **Bacterial spores as platforms for bioanalytical and biomedical applications.** *Anal Bioanal Chem* 2011, **400:**977–989.
11. Cutting SM: ***Bacillus* probiotics.** *Food Microbiol* 2011, **28:**214–220.
12. Hinc K, Ghandili S, Karbalaee G, Shali A, Noghabi K, Ricca E, Ahmadian G: **Efficient binding of nickel ions to recombinant *Bacillus subtilis* spores.** *Res Microbiol* 2010, **161:**757–764.
13. Potot S, Serra CR, Henriques AO, Schyns G: **Display of recombinant proteins on *Bacillus subtilis* spores, using a coat-associated enzyme as the carrier.** *Appl Environ Microbiol* 2010, **76:**5926–5933.
14. Mauriello EMF, Duc LH, Isticato R, Cangiano G, Hong HA, De Felice M, Ricca E, Cutting SM: **Display of heterologous antigens on the *Bacillus subtilis* spore coat using CotC as a fusion partner.** *Vaccine* 2004, **22:**1177–1187.
15. Isticato R, Scotto Di Mase D, Mauriello EMF, De Felice M, Ricca E: **Amino terminal fusion of heterologous proteins to CotC increases display efficiencies in the *Bacillus subtilis* spore system.** *Biotechniques* 2007, **42:**151–156.
16. Hinc K, Isticato R, Dembek M, Karczewska J, Iwanicki A, Peszyńska-Sularz G, De Felice M, Obukowski M, Ricca E: **Expression and display of UreA of *Helicobacter acinonychis* on the surface of *Bacillus subtilis* spores.** *Microb Cell Fact* 2010, **9:**2.
17. Detmer A, Glenting J: **Live bacterial vaccines—a review and identification of potential hazards.** *Microb Cell Fact* 2006, **5:**23.

18. Yim S-K, Jung H-C, Yun C-H, Pan J-G: **Functional expression in *Bacillus subtilis* of mammalian NADPH-cytochrome P450 oxidoreductase and its spore-display.** *Protein Expr Purif* 2009, **63:**5–11.
19. Huang JM, Hong HA, Van Tong H, Hoang TH, Brisson A, Cutting SM: **Mucosal delivery of antigens using adsorption to bacterial spores.** *Vaccine* 2010, **28:**1021–1030.
20. Cho EA, Kim EJ, Pan JG: **Adsorption immobilization of *Escherichia coli* phytase on probiotic *Bacillus polyfermenticus* spores.** *Enzyme Microb Technol* 2011, **49:**66–71.
21. Di Lauro B, Strazzulli A, Perugino G, La Cara F, Bedini E, Corsaro MM, Rossi M, Moracci M: **Isolation and characterization of a new family 42 beta-galactosidase from the thermoacidophilic bacterium *Alicyclobacillus acidocaldarius*: identification of the active site residues.** *Biochim Biophys Acta* 2008, **1784:**292–301.
22. 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.
23. Cutting S, Vander Horn PB: **Genetic analysis**. In *Molecular Biological Methods for Bacillus*. Edited by Harwood C, Cutting S. John Wiley and Sons: Chichester, UK; 1990:27–74.
24. Kim H, Hahn M, Grabowski P, McPherson D, Otte MM, Wang R, Ferguson CC, Eichenberger P, Driks A: **The *Bacillus subtilis* spore coat protein interaction network.** *Mol Microbiol* 2006, **59:**487–502.
25. Zheng L, Donovan WP, Fitz-James PC, Losick R: **Gene encoding a morphogenic protein required in the assembly of the outer coat of the *Bacillus subtilis* endospore.** *Genes Dev* 1988, **2:**1047–1054.
26. Chen G, Driks A, Tawfiq K, Mallozzi M, Patil S: **Bacillus anthracis and Bacillus subtilis spores surface properties and transport.** *Colloids Surf B Biointerfaces* 2010, **76:**512–518.
27. Kazakov S, Bonvouloir E, Gazaryan I: **Physicochemical characterization of natural ionic microreservoirs: Bacillus subtilis dormant spores.** *J Phys Chem* 2008, **112:**2233–2244.
28. Sambrook J, Fritsch EF, Maniatis T: *Molecular cloning, laboratory manual*. 2nd edition. Cold Spring Harbor, NY, USA: Cold Spring Harbor Laboratory Press; 1989.
29. Nicholson WL, Setlow P: **Sporulation, germination and out-growth**. In *Molecular biological methods for Bacillus*. Edited by Harwood C, Cutting S. Chichester, United Kingdom: John Wiley and Sons; 391–450.
30. Itaya M, Kondo K, Tanaka T: **A neomycin resistance cassette selectable in a single copy state in the Bacillus subtilis chromosome.** *Nucleic Acid Res* 1989, **17:**4410.
31. Ricca E, Cutting S, Losick R: **Characterization of *bofA*, a gene involved in inter-compartmental regulation of pro-K processing during sporulation in *Bacillus subtilis*.** *J Bacteriol* 1992, **174:**3177–3184.
32. Henriques A, Beall BW, Moran CP: **CotM of *Bacillus subtilis*, a member of the alpha-crystallin family of stress proteins, is induced during development and participates in spore outer coat formation.** *J Bacteriol* 1997, **179:**1887–1897.
33. Zilhao R, Isticato R, Martins LO, Steil L, Völker U, Ricca E, Moran CP, Henriques AO: **Assembly and function of spore-coat associated transglutaminase of *Bacillus subtilis*.** *J Bacteriol* 2005, **187:**7753–7764.
34. Isticato R, Esposito G, Zilhão R, Nolasco S, Cangiano G, De Felice M, Henriques AO, Ricca E: **Assembly of Multiple CotC Forms into the Bacillus subtilis Spore Coat.** *J Bacteriol* 2004, **186:**1129–1135.
35. Sacco M, Ricca E, Losick R, Cutting S: **An additional GerE-controlled gene encoding an abundant spore coat protein from Bacillus subtilis.** *J Bacteriol* 1995, **177:**372–377.
36. Naclerio G, Baccigalupi L, Zilhao R, De Felice M, Ricca E: **Bacillus subtilis spore coat assembly requires cotH gene expression.** *J Bacteriol* 1996, **178:**4375–4380.

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# Chapter 5

Non-recombinant spores of *Bacillus subtilis* displaying LT-B induce strong humoral and cellular immune responses in mucosally immunized mice

**Abstract**

**Background:** Mucosal infections are a major global health problem and it is generally accepted that mucosal vaccination strategies, able to block infection at their entry site, would be preferable with respect to other prevention approaches. However, there are still relatively few mucosal vaccines available, mainly because of the lack of efficient delivery systems and of mucosal adjuvants. Recombinant bacterial spores displaying a heterologous antigen have already been shown to induce protective immune responses and, therefore, proposed as a mucosal delivery system. A non-recombinant approach has been recently developed and tested to display antigens and enzymes.

**Results:** We report the use of the *Bacillus subtilis* spore as a non-recombinant system for the mucosal delivery of LT-B, the binding subunit of the heat-labile toxin of *Escherichia coli.* LT-B adsorption occurred in a pH-dependent way and mutant spores, altered in their surface structure, were found to be more efficient than wild type spores in displaying the antigen. Dot blotting and ELISA experiments indicated, respectively, that the non-recombinant approach was more efficient than the recombinat system in displaying LT-B and that it was able to allow the formation of LT-B pentamers on the spore surface. Both wild type and mutant spores displaying LT-B were used for nasal immunization of groups of mice and shown to induce strong LT-B-specific humoral and cellular immune responses. Analysis of the IgG subclasses of the cytokines produced by the immunized mice indicated a Th2-biased immune response.

**Conclusion:** Our results indicate that recombinant and non-recombinant spores displaying LT-B induce a different immune response, probably because of the formation of LT-B pentamers only with the non-recombinant approach. Efficiency of display, ability to display the native form of the antigen and to induce both humoral and cellular immune responses propose this non-recombinant delivery system as a powerful mucosal vaccination strategy.

**Background**

Several vaccination strategies based on the development of microbial and viral systems to deliver molecules with antigenic properties have been proposed and summarized in recent reviews [1, 2]. Bacterial endospores (spores) have also been proposed and several antigens exposed and tested in murine models [3, 4]. Spores are mainly formed by Gram-positive microorganisms belonging to different genera and including more than 1,000 species [5]. The common feature of these organisms is the ability to form a quiescent cellular type (the spore) in response to harsh environments. The spore can survive in this dormant state for long periods, resisting to a vast range of stresses such as high temperature, dehydration, absence of nutrients, presence of toxic chemicals. When the environmental conditions ameliorate, the spore germinates originating a vegetative cell able to grow and sporulate [5]. The ability of the spore to survive non-physiological conditions is, in part, due to the presence of the spore coat, a proteinaceous structure surrounding the spore. At least seventy different proteins (Cot proteins) form the multilayered coat structure [5].

The use of spores as a vaccination vehicle has several advantages over other microbial, viral or synthetic delivery systems. A first advantage comes from the well documented robustness of the *Bacillus* spore which grants high stability to the display system even after a prolonged storage [6]. The stability and, in particular, heat-stability is a stringent requirement in the development of new vaccine delivery systems, mainly for those intended for use in developing countries, where poor distribution and storage conditions are main limitations [6]. Another advantage of spores is the safety record of several *Bacillus* species [7], obviously an essential requirement for a delivery system for human or animal use. Several *Bacillus* species, including *B. subtilis*, are widely used as probiotics and have been on the market for human or animal use for decades in many countries [7]. In addition, spore-display systems do not require a membrane translocation step to display

heterologous proteins on the spore surface and, therefore, efficiently expose large antigens [4], overcoming limitations often encountered with cell- and phage-based display systems [1, 2]. Finally, spores carrying antigens have been shown to induce strong and protective immune responses when administered to mice by the mucosal (nasal and oral) route [4]. This is an extremely relevant issue since mucosal vaccines are still not commonly used because of the lack of appropriate delivery systems althought they are potentially considered as the best vaccination strategy for many infectious diseases.

More recent is the development of a non-recombinant approach to display heterologous proteins on bacterial spores [8, 9, 10]. A non-recombinant approach to deliver antigens is highly desirable since it does not cause the release of recombinant microrganisms into the environment [11]. The non-recombinant approach is based on the ability of spores to spontaneously adsorb proteins on their surface [12]. A collections of purified antigens (TTFC of *Clostridium tetani*, PA of *Bacillus anthracis*, Cpa of *Clostridium perfringens* and glutathione S transferase of *Shistosomas japonica*) have been adsorbed to *B. subtilis* spores and shown to be able to induce specific and protective immune responses in mucosally immunised mice [8]. Spore adsorption resulted to be more efficient when the pH of the binding buffer was acidic (pH 4) and less efficient or totally inhibited at pH values of 7 or 10 [8, 9]. A combination of electrostatic and hydrophobic interactions between spores and antigens were suggested to drive the adsorption, that was shown to be not dependent on specific spore coat components but rather on the negatively charged and hydrophobic surface of the spore [8]. However, at least in the case of the enzyme beta-galactosidase of *Alicyclobacillus acodocaldaricus*, the electrostatic force were shown to be not involved in spore adsorption [9].

Here we report the use of the non-recombinant approach to display on the surface of *B. subtilis* spores the B subunit of the heat-labile toxin (LT) of *Escherichia coli*. LT is similar to the cholera toxin and is formed by an enzymatically active subunit (LT-

A) and a pentameric non-toxic B subunit (LT-B), responsible to bind a receptor on the host cell membrane. Orally administered LT-B pentamers are known to potentiate the immune response acting by mucosal adjuvants [13]. LT-B has been previously displayed on the spore surface by the recombinant approach [14]. In that case, DNA coding for LT-B was genetically fused to the 3' of the *cotC* gene, coding for a small but very abundant component of the *B. subtilis* spore coat, CotC [14]. Recombinant spores were used to orally immunize mice and shown to induce the production of LT-B-specific fecal sIgA, while the production of LT-B-specific serum IgG was modest, if any [14].

## Results and Discussion

### *B. subtilis* spores adsorb LT-B of *E. coli* in a pH dependent way

LT-B was over-expressed in *E. coli* and purified by affinity chromatography as described in the Methods section. Purified LT-B (2 μg) were incubated with $2.0x10^9$ spores of *B. subtilis* strain PY79 [15], purified by renographin gradient, as previously described [20]. The adsorption reaction was performed in PBS at pH 4.0, 7.0 or 10.0, as previously described for various antigens and enzymes [8, 9]. After adsorption reaction, the mixture was fractionated by centrifugation and the pellet, containing all spores, was used to extract spore coat proteins, as previously described [17]. Extracted proteins were then analysed by western blotting with anti-LT-B antibody. As shown in Fig. 1, spore incubated with LT-B at pH 4.0 released a higher amount of LT-B than spores incubated at pH 7.0 while those incubated at pH 10.0 only released a minimal amount of LT-B.

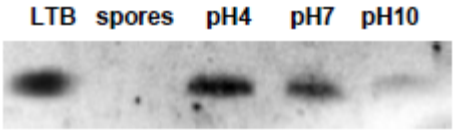


**Figure 1**. Western blot analysis with anti-LT-B antibody. Purified LT-B and spores were analysed independently and after incubation at different pH values, as described in Methods.

Spore not incubated with LT-B did not shown any protein recognized by anti-LT-B antibody (Fig. 1). Results of Fig. 1, then, clearly indicate that LT-B is adsorbed on the spore surface and that this process is more efficient at acidic pH values, as previously reported for other heterologous proteins [8, 9]. Based on this all therein adsorption experiments were performed in citrate buffer at pH. 4.0.

**Spores with strongly altered surface show increased efficiency of adsorption**

It has been previously reported that spores of mutant strains lacking a single or few structural coat components can adsorb heterologous proteins with an efficiency comparable to that of spores of the wild type strain [8, 9]. However, when an enzyme was tested for spore-adsorption, spores strongly altered in their surface structure were shown to adsorb better than wild type [9]. We first analysed the efficiency of LT-B adsorption with a collection of isogenic mutants lacking either a single or few coat components (Fig. 2).

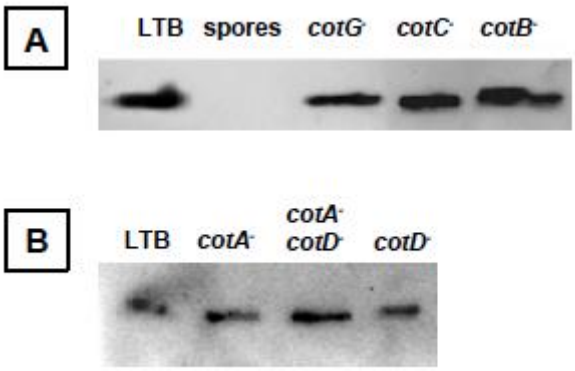


**Figure 2**. Western blot analysis with anti-LT-B antibody. Purified LT-B was adsorbed to spores of a collection of mutants.

Spores of mutants lacking a single (*cotC* or *cotB* in panel A and *cotA* or *cotD* in panel B) or few coat proteins (*cotG* mutant spores lack CotG and CotB [18] in panel A or the double mutant *cotA cotD* in panel B) all showed comparable efficiency of adsorption. This analysis, then, confirmed previous results obtained with other antigens [8] or an enzyme [9]. Next, we analysed mutants strongly altered in their spore surface structure. In particular, we tested spores of strains lacking *cotH* or *cotE. cotH* spores lack at least 9 outer coat components [19] while *cotE* spores totally lack the outer coat [20]. As shown in Fig. 3A, with spores of the two mutants behaved differently. While *cotH* spores released a similar amount of LT-B than wild

type spores (see Fig. 1 for a comparison), *cotE* mutant spores did not release any LT-B, suggesting that they adsorbed the same amount of antigen than the wild type and no antigen at all, respectively. Those results were quite surprising since both mutant spores had been previously shown to adsorb the beta-galactosidase of *A. acidocaldaricus* with efficiency much higher than the efficiency of wild type spores [9].

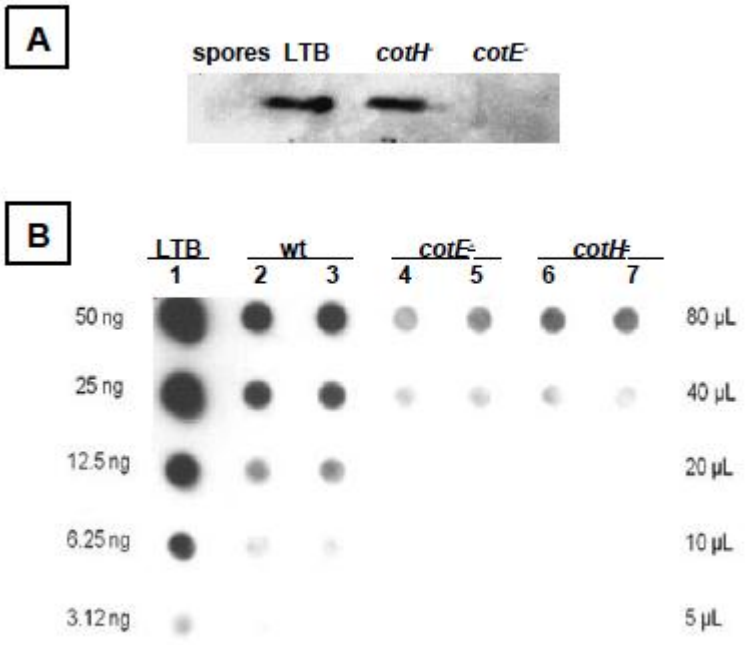


**Figure 3.** Western (A) and dot (B) blot analysis with anti-LT-B antibody. Purified LT-B was adsorbed to spores of *cotH* or *cotE* mutants.

To clarify this point we decided to follow a different experimental approach. Instead of measuring by western blot the amount of LT-B adsorbed and then released by the purified spores, we measured by dot blot the amount of LT-B not adsorbed by the spores and left in solution. After the adsorption reaction, spores were collected by centrifugation and the spore-free supernatant analyzed by dot blot. As shown in Fig. 3B the amount of LT-B not adsorbed to wild type spores was much higher than that not adsorbed by *cotE* or *cotH* mutant spores. In addition, the amount of not

adsorbed LT-B was similar for the two mutant spores. Based on the results of Fig. 3 and on the adsorption efficiency of mutant spores measured with the beta-galactosidase of *A. acidocaldaricus* [9] we hypothesize that: i) both *cotE* and *cotH* mutant spores are more efficient that wild type spores in adsorbing LT-B, since almost no LT-B is left unadsorbed (Fig. 3B); ii) *cotH* spores release part of the adsorbed antigen upon coat protein extraction while *cotE* mutant spores retain LT-B attached to their surface, since no anti-LT-B signals were detected by western blot with *cotE* spores (Fig. 3A). Our data do not allow us to rule out the possibility that *cotE* mutant spores totally degrade LT-B, maybe because of a protease activity exposed in the absence of the outer coat layer. However, we believe this is an unlikely possibility since the beta-galactosidase of *A. acidocaldaricus* was not degraded but rather efficiently adsorbed and stabilized by *cotE* spores [9]. Additional experiments will be needed to visualize LT-B on the surface of *cotE* spores and thus clarify this point.

**Recombinant and non-recombinant spores have a similar efficiency of LT-B display**

Each recombinant spore of a *B. subtilis* strain carrying the *cotC::ltb* gene fusion was previously shown to display $2.7x10^3$ LT-B molecules. We then decided to compare the efficiency of LT-B display by the recombinant and non-recombinant approach. Recombinant spores displaying LT-B and non-recombinant spores after LT-B adsorption were treated with SDS-DTT to extract coat proteins, as previously reported [17]. Protein concentrations were determined and identical amounts of proteins used in a dot blot experiment with anti-LT-B antibody (Fig. 4). Purified LT-B was used as a standard. As shown in Fig. 4, the same amount of extract of recombinant (CotC::LT-B) and non-recombinant (spores+LT-B) spores contained almost identical amounts of LT-B, suggesting that by either method similar amounts of antigen can displayed and presented to the immune system.

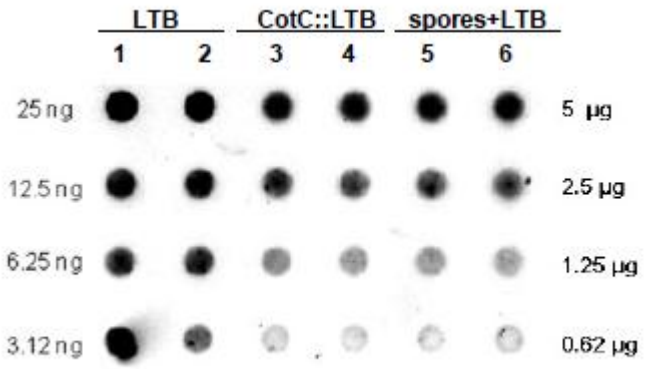


**Figure 4**. Dot blot experiment with anti-LT-B antibody. Nanograms on the left refer to the amount of purified LT-B while micrograms on the right to the amount of spore coat extract.

**Non-recombinant spores displaying LT-B induce a Th2-type immune response**

The oral immunization of groups of mice with recombinant spores displaying LT-B induced the production of specific fecal sIgA at a low levels, however, significantly higher than control groups [14]. Production of specific serum IgG was very low not allowing the analysis of the IgG subclasses [14]. To analyse the immunogenicity of LT-B adsorbed on the surface of non-recombinant spores we immunized groups of mice by the nasal route with purified LT-B (2 µg/dose/mouse), wild type spores ($2x10^9$/dose/mouse), or the same number of wild type spores adsorbed with 2 µg of LT-B. Each animal was dosed once a week for 8 weeks and all animals were sacrificed for analysis one week after the last dosage. Fecal sIgA were measured once a week from week 4 to week 8 and showed levels of LT-B-specific sIgA significantly higher in mice dosed with spore-adsorbed LT-B than in mice dosed with purified LT-B or spores alone (Fig. 5A). Therefore, results of Fig. 5A confirm previous results obtained with recombinant spores and indicate the production of fecal sIgA in the animals immunized with spores adsorbed with LT-B.

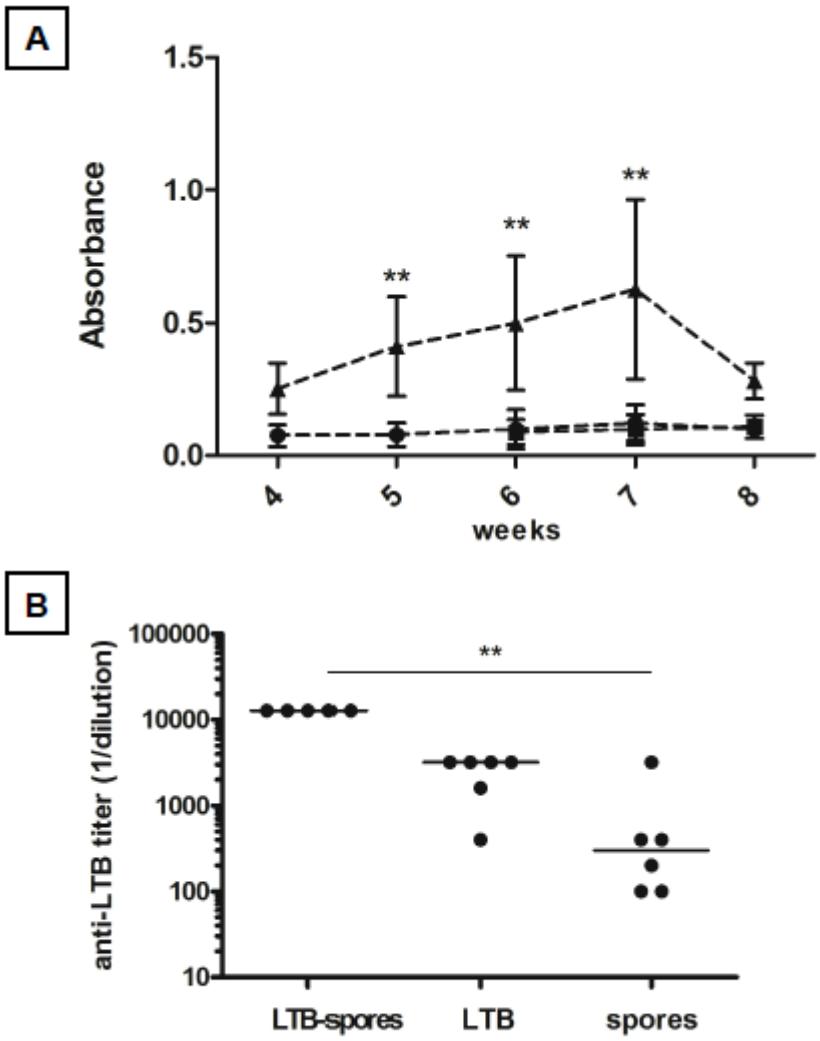


**Figure 5.** (A) Levels of LT-B-specific fecal sIgA produced by animals immunized with spores adsorbed with LT-B (triangles), purified LT-B (circles) or spores alone (squares). (B) Levels of LT-B-specific serum IgG produced by animals immunized with spores adsorbed with LT-B (LT-B-spores), purified LT-B (LT-B) or spores alone (spores).

Sacrificed animals were then analysed for the levels of LT-B-specific serum IgG. As shown in Fig. 5B, animals immunized with spore-adsorbed LT-B produced higher levels of IgG than animals immunized with purified LT-B or spores not displaying LT-B. Production of serum IgG was not observed when animals were immunized with

recombinant spores displaying LT-B [14], suggesting that recombinant and non-recombinant spores displaying the same antigen induce a different immune response. The high levels of IgG produced in the experiment of Fig. 5 allowed the analysis of the IgG subclasses produced by the immunized animals.

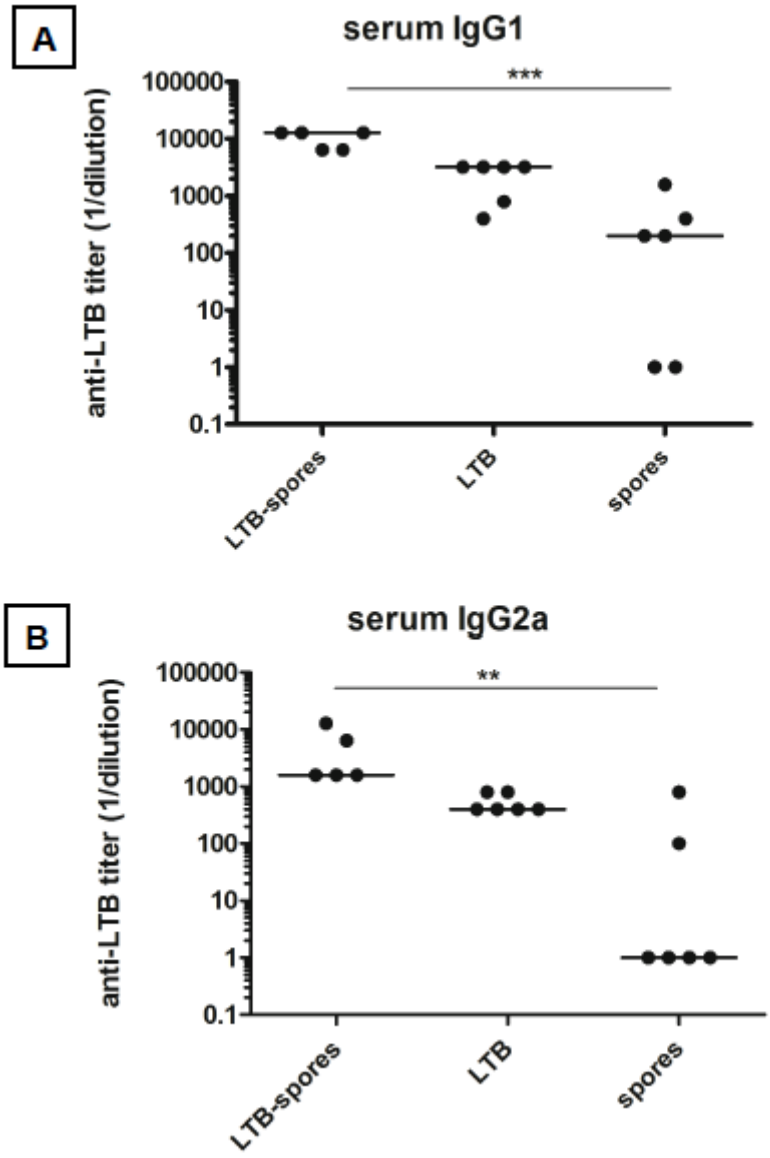


**Figure 6.** LT-B-specific serum IgG subclasses produced by mice immunized with spore-adsorbed LT-B (LT-B-spores), purified LT-B (LT-B) and spores alone (spores).

As shown in Fig. 6 mice immunized with spore-adsorbed LT-B produced more serum IgG1 than serum IgG2a, which is indicative of a Th2 (B cell) type of immune response. While this result has to be confirmed by the analysis of the pattern of cytokines produced by the immunized animals (in progress) it is interesting to note that recombinant and non-recombinant spores seem to induce different immune responses, with only fecal sIgA production induced by the recombinant spores and fecal sIgA and serum IgG production induced by non-recombinant spores. Although we cannot definitely exclude that the display of LT-B fused to a spore coat component (CotC) may alter the spore surface and somehow affect the type of immune response induced in mice, we believe that it is highly unlikely. Recombinant spores expressing a variety of heterologous proteins, including LT-B and other of larger size, have been shown identical to wild type spores in terms of stability, heat-resistance, lysozyme-resistance and ability to germinate [9, 21, 22, 23]. Therefore, we hypothesize that the different immune response observed with recombinant and non-recombinant spores is depending on the antigen, exposed in a different form on the surface of the two types of spores. In particular, we hypothesize that recombinant spores expose LT-B as a monomer, since it is unlikely that the CotC-LT-B chimera was able to form pentamers, as LT-B alone does. On the contrary, non-recombinant spores could display LT-B pentamers, since pentamers spontaneously form when purified LT-B is in solution. Therefore, we believe that part of the purified LT-B pentamerizes in solution before incubation with spores and adheres to spores as monomers and pentamers. If this is true, then, the different immune response observed in mice is not surprising since it is known that LT-B pentamers and not monomers can be recognized by the immune system as an immunoadjuvant [13]. Experiments to address this point by using a GM1-based ELISA are in progress.

## Materials and methods

### Bacterial strains and transformation

*B. subtilis* strains used in this study are listed in Table 1. Plasmid amplification for DNA sequencing, subcloning experiments, and transformation of *E. coli* competent cells were performed with strain DH5α [27]. Bacterial strains were transformed by previously described procedures, i.e., $CaCl_2$-mediated transformation of *E. coli* competent cells [27] and two-step transformation of *B. subtilis* [16].

**Table 1.** List of strains

| Strain | Genotype | Source |
|---|---|---|
| *B. subtilis* | | |
| PY79 | *Wild type* | [15] |
| DZ213 | *cotE::cm* | [16] |
| ER220 | *cotH::spc* | [24] |
| ER203 | *cotG::erm* | [18] |
| RH101 | *cotC::spc* | [25] |
| RH201 | *cotB::spc* | [21] |
| AZ67 | *cotA::cm* | Lab stock |
| AZ70 | *cotD::spc* | Lab stock |
| AZ558 | *cotA::sm cotD::spc* | Lab stock |
| RH114 | *cotC::LT-B* | [14] |
| *E. coli* | | |
| RH153 | *LT-B-6his* | Lab stock |

### Genetic and molecular procedures

Isolation of plasmids, restriction digestion, and ligation of DNA, were carried out by standard methods [27]. Chromosomal DNA from *B. subtilis* was isolated as described elsewhere [17].

### Overexpression of LT-B

The LT-B coding region was amplified by PCR from *E. coli* chromosomal DNA and it was cloned in expression vector pRSET-B vector (R. Isticato personal communication). Recombinant plasmid carrying an in-frame fusion of the 5’ end of

the LT-B coding region to six histidine codons under the transcriptional control of a T7 promoter was used to transform competent cells of *E. coli* BL21(DE3) (Invitrogen), yielding strain RH153. LT-B was produced by autoinduction by growing cells at 37 °C for 18 h with orbital shaking (150 rpm) and by using the Overnight Express autoinduction system 1 according to the manufacturer's instructions (Novagen). Strain RH153 was collected after autoinduction (50 mL culture) by centrifugation and resuspended in 5 ml of lysis buffer (50 mM $NaH_2PO_4$, 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 (20-min pulses at 20 Hz with a Sonicator Ultrasonic liquid processor; Heat System Ultrasonic Inc., NY). The suspension was clarified by centrifugation at 13,000 g at 4 °C for 20 min, and protein concentration was determined by a Bio-Rad assay. The protein extract was diluted 1:3 in binding buffer (20 mM sodium phosphate, 500 mM NaCl, 20 mM imidazole [pH 7.4]). The diluted sample was applied to a HisTrap HP column (GE Healthcare Europe GmbH, Milan, Italy), which was previously equilibrated with 10 ml of binding buffer. After the loading the *E. coli* protein extract, the column was washed with 10 ml of binding buffer. Then proteins were eluted with the same buffer, supplemented with increasing concentrations of imidazole (50 mM, 100 mM and 250 mM). Purified protein was desalted using the PD10 column (GE Healthcare Europe GmbH, Milan, Italy) to remove high NaCl and imidazole concentrations.

**Purification of spores**

Sporulation of wild type and recombinant strains was induced by the exhaustion method. After 30 h of growth in Difco Sporulation medium (DSM) at 37 °C with vigorous shaking [17], spores were collected, washed three times with distilled water and purified by gastrografin gradient as described before [17]. Spore counts were determined by serial dilution and plate-counting.

**Binding assay and LT-B detection**

Suspensions containing $2\times10^9$ purified spores were centrifuged and resuspended in 0.2 ml of PBS 0.15 M at pH 4, pH 7 or pH 10. 2 µg of purified LT-B was added to the spore suspension and the binding mixture incubated at room temperature for 1 hour. Spores were centrifuged and both spore pellet and supernatants were examined for LT-B. The spores were then resuspended in 100µl of spore coat extraction buffer [17], incubated at 68 ◦C for 1 h to solubilize spore coat proteins and one tenth loaded onto a 12% SDS-PAGE gel and blotted on nitrocellulose membrane. Specific anti-LT-B antibodies were used to detect LT-B in protein extracts examined. Supernatants post-binding were instead analysed by dot blot. Serial dilutions of supernatants were loaded on nitrocellulose membrane and later incubated with specific anti-LT-B antibody.

**Immunization**

Groups of six mice (female, Balb/C, 8 weeks old) were inoculated by nasal route with suspensions of spores expressing CotC-LTB (RH114) and LTB-adsorbed wild type spores (PY79). Groups of four mice with negative control $2\times10^9$ wild type spores (PY79) without an antigen attached. For positive control were used 4 mice with 2 µg of purified LTB. Nasal immunizations were performed with $2\times10^9$ spores in a volume of 0.2 ml administered once a week for 8 weeks total. Mice were sacrificed at week 9.

**Antigen specific response detection**

Fecal pellets and serum were collected to detect specific anti-LTB IgA and IgG immunoglobulins. Fecal pellets were collected after 4, 5, 6, 7 and 8 weeks after first somministration of spores for IgA titer and terminal bleeds were used for IgG analysis. Determination of antibody titers was performed by ELISA as described elsewhere (26).

**References**


1. **Lee, S.Y., J.H. Choi, Z. Xu. 2003.** Microbial cell-surface display. *Trends Biotechnol.* 21:45-52.
2. **Wells J. 2011.** Mucosal vaccination and therapy with genetically modified lactic acid bacteria. *Annu Rev Food Sci Technol.* **2**:423-45.
3. **Knecht, L.D., Pasini, P., Daunert, S. 2011.** Bacterial spores as platforms for bioanalytical and biomedical applications. *Anal Bioanal Chem* 2011, 400:977-989.
4. **Cutting, S.M., Hong, H.A., Baccigalupi, L., Ricca, E. 2009.** Oral Vaccine Delivery by Recombinant Spore Probiotics. *Intern. Rev. Immunol.* 28:487-505.
5. **McKenney, P.T., Driks, A., Eichenberger, P. 2013.** The *Bacillus subtilis* endospore: assembly and functions of the multilayered coat. *Nature Rev Microbiol.* 2013. 11:33-44.
6. **Isticato, R., Cangiano, G., De Felice, M., Ricca, E. 2004.** Display of molecules on the spore surface. p 193-200. *In:* E. Ricca, AO Henriques and SM Cutting (ed), *Bacterial Spore Formers: Probiotics and Emerging Applications.* Horizon Biosciences, Norfolk, UK.
7. **Cutting, S.M.** *Bacillus* probiotics. 2011. *Food Microbiology* **28**:214-220.
8. **Huang, J.M., Hong, H.A., Van Tong, H., Hoang, T.H., Brisson, A., Cutting, S.M. 2010.** Mucosal delivery of antigens using adsorption to bacterial spores. *Vaccine* 2010, 28:1021-1030.
9. **Sirec, T., Strazzulli, A., Isticato, R., De Felice, M., Moracci, M., Ricca, E. 2012.** Adsorption of beta-galactosidase of *Alicyclobacillus acidocaldarius* on wild type and mutants spores of *Bacillus subtilis*. *Microb Cell Fact* 11:100
10. **Song, M., Hong, H.A., Huang, J.M., Colenutt, C., Khang, D.D., Nguyen, T.V., Park, S.M., Shim, B.S., Song, H.H., Cheon, I.S., Jang, J.E., Choi, J.A., Choi, Y.K., Stadler, K., Cutting, S.M. 2012.** Killed *Bacillus subtilis* spores as a mucosal adjuvant for an H5N1 vaccine. *Vaccine* 30:3266-77.
11. **Detmer, A., Glenting, J. 2006.** Live bacterial vaccines—a review and identification of potential hazards. *Microb Cell Fact*, 5:23.
12. **Yim S-K, Jung H-C, Yun C-H, Pan J-G. 2009.** Functional expression in *Bacillus subtilis* of mammalian NADPH-cytochrome P450 oxidoreductase and its spore-display. *Protein Expression and Purification.* 63:5–11.
13. **Leach, S., Clements, J.D., Kaim, J., Lundgren, A. 2012.** The Adjuvant Double Mutant Escherichia coli Heat Labile Toxin Enhances IL-17A Production in Human T Cells Specific for Bacterial Vaccine Antigens. *PLOS ONE*. 7: e51718.
14. **Mauriello EMF, Duc LH, Isticato R, Cangiano G, Hong HA, De Felice M, Ricca E, Cutting SM. 2004.** Display of heterologous antigens on the *Bacillus subtilis* spore coat using CotC as a fusion partner. *Vaccine*, 22:1177-1187.
15. **Youngman P, Perkins JB, Losick R. 1984.** A novel method for the rapid cloning in *Escherichia coli* of *Bacillus subtilis* chromosomal DNA adjacent to Tn917 insertion. *Mol. Gen. Genet.*, 195:424-433.
16. **Cutting S, Vander Horn PB.** Genetic analysis *In*: C. Harwood and S. Cutting (Eds.), Molecular Biological Methods for *Bacillus.* John Wiley and Sons, Chichester, UK. 1990, pp. 27-74.
17. **Nicholson WL and Setlow P.** Sporulation, germination and out-growth, p. 391–450.InC. Harwood and S. Cutting (ed.), Molecular biological methods for *Bacillus*. John Wiley and Sons, Chichester, United Kingdom.
18. **Naclerio G, Baccigalupi L, Zilhao R, De Felice M, Ricca E. 1996.** *Bacillus subtilis* spore coat assembly requires *cotH* gene expression. *J. Bacteriol.* 178:4375-4380.
19. **Kim H, Hahn M, Grabowski P, McPherson D, Otte MM, Wang R, Ferguson CC, Eichenberger P, Driks A. 2006.** The *Bacillus subtilis* spore coat protein interaction network. *Mol. Microbiol.*, 59:487-502.
20. **Zheng L, Donovan WP, Fitz-James PC, Losick R. 1988.** Gene encoding a

morphogenic protein required in the assembly of the outer coat of the *Bacillus subtilis* endospore. *Genes Develop.*, 2:1047-1054.

21. **Isticato, R., Cangiano, G., Tran, H.,T., Ciabattini, A., Medaglini, D., Oggioni, M.,R., De Felice, M., Pozzi, G., Ricca, E. 2001.** Surface display of recombinant proteins on *Bacillus subtilis* spores. *J. Bacteriol.*, 183: 6294-6301.
22. **Isticato R, Scotto Di Mase D, Mauriello EMF, De Felice M, Ricca E. 2007.** Amino terminal fusion of heterologous proteins to CotC increases display efficiencies in the *Bacillus subtilis* spore system. *BioTechniques*, 42:151-156.
23. **Hinc K, Isticato R, Dembek M, Karczewska J, Iwanicki A, Peszyńska-Sularz G, De Felice M, Obukowski M, Ricca E. 2010**. Expression and display of UreA of *Helicobacter acinonychis* on the surface of *Bacillus subtilis* spores. *Microbial Cell Factories*, 9:2.
24. **Sacco, M., Ricca, E., Losick, R. and Cutting, S. 1995.** An additional GerE controlled gene encoding an abundant spore coat protein from *Bacillus subtilis*. J. Bacteriol. 177**:**372–377.
25. **Isticato, R., Esposito, G., Zilhao, R., Nolasco, S., Cangiano, G., De Felice, M., Henriques, A. o., and Ricca, E. 2004.** Assembly of multiple CotC forms into the *Bacillus subtilis* spore coat. J. Bacteriol.186(4):1129-1135.
26. **Mauriello, E., Cangiano, G., Maurano, F., Saggese V., De Felice, M., Rossi, M., Ricca, E. 2007.** Germination-independent induction of cellular immune response by *Bacillus subtilis* spores displaying the C fragment of the tetanus toxin. Vaccine, 25: 788–793.
27. **Sambrook J, Fritsch EF, and Maniatis T. 1989.** Molecular cloning, laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, USA