Exploring the evolutionary link between biofilms and spores formation in spore-formers

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A THESIS SUBMITTED FOR THE DEGREE OF MASTER IN MEDICAL MICROBIOLOGY

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Abstract

Bacteria are often thought as single cell organisms, however they can develop into morphologically complex multicellular communities composed of different subpopulations of specialized cell types. Biofilm is an example, in which bacteria organize for protection from harmful conditions in the host and to create nutrient-rich areas. In the last years biofilm have been show to comprise an important aspect of microbial persistence in the human gut. Endospore-formers, although thought not to be major constituents of the microbiota in the human intestine, cause several intestinal diseases, usually associated with antibiotic use. Whether these bacteria persist in the intestine in biofilms or as endospores is not totally elucidated since both, biofilms and endospores, are able to resist to antimicrobial agents. Most likely sporulation and biofilm formation are tightly linked processes. For some endosporeformers, spore differentiation is induced by a sub-population of cells within the biofilm.

In this work we tackled the link between bacterial biofilms and endosporulation in *Bacillus subtilis*. We showed that endospores produced in biofilms have higher resistance to UV radiation. We revealed that a gene, *remA*, conserved among endosporeformers and essential for biofilm formation is expressed during sporulation. *remA* is expressed in the forespore soon after asymmetric division and in the mother cell after engulfment completion. GerE represses *remA* expression in the mother cell at late stages of sporulation. Consequently, we found components of the biofilm matrix, TasA and BslA, on the coat of endospores produced in biofilms. We suggest that components of the biofilm matrix may be part of mature endospores. We hypothesize that some of the structural proteins that confer integrity to the matrix biofilm, as TasA, may have a role as a scaffold for the assembly of the endospore surface layers.

Resumo

A percepção instalada é a de que as bactérias são organismos unicelulares. No entanto, estes organismos são capazes de se organizarem em comunidades multicelulares complexas compostas de subpopulações de células diferenciadas. Os biofilmes são um exemplo deste tipo de organização. Os biofilmes conferem protecção contra as condições desfavoráveis encontradas no hospedeiro, ao mesmo tempo que criam nichos ricos em nutrientes facilitando a implantação da população. Nos últimos anos foi demonstrado que a persistência microbiana no trato gastrointestinal humano se deve em larga medida à formação de biofilmes. Algumas bactérias que podem ser encontradas no trato gastrointestinal humano são ainda capazes de diferenciar um tipo celular altamente resistente a insultos químicos e físicos, o esporo. Nestes casos, não é claro se são os biofilmes ou os endoesporos os principais responsáveis pela persistência destes organismos, já que ambos são resistentes aos antibióticos.

Neste trabalho exploramos a ligação genética entre a formação de biofilmes e a esporulação em *Bacillus subtilis*. Mostramos que os endoesporos produzidos em biofilmes exibem maior resistência aos UV. Mostramos que um gene, *remA*, conservado em bactérias formadoras de endoesporos e essencial para a formação de biofilmes é expresso durante a esporulação. *remA* é expresso no pré-esporo após a divisão assimétrica e na célula mãe após o envolvimento do pré-esporo. GerE reprime a expressão de *remA* na célula mãe em estádios tardios de desenvolvimento. Consequentemente, encontramos componentes da matriz do biofilme no manto de endoesporos maduros. Algumas das proteínas estruturais que conferem integridade à matriz do biofilme, como TasA, poderão servir como base para a montagem das camadas superficiais do esporo.

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Symbols and Abbreviations

%	Percentage
::	Insertion
~	Approximately
µg	Microgram
µl	Microliter
µm	Micrometer
µM	Micromolar
A	Adenine
B&W	Bott and Wilson
bp	Base pair
C	Cytosine
cm	Centimeter
cm	Chloramphenicol resistance cassette
Cm	Chloramphenicol
Cm^r	Chloramphenicol resistance
ddH_2O	Double distilled H_2O
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DSM	Difco Sporulation Medium
g	Gravitational force
G	Guanine
H_2O	Water
H_2O_2	Hydrogen Peroxide
IgG	Immunoglobulin G
kb	Kilobase
kDa	KiloDalton
km	Kanamycin resistance cassette
kV	Kilovolt
LA	Luria agar
LB	Luria Broth
lb/in^2	Pounds per square inch
M	Molar
mg	Milligram
min	Minute
mJ	Microjoule
ml	Millilitre
mM	Millimolar
mm	Millimiter
Neo	Neomycin
nm	Nanometer
nM	Nanomolar
ºC	Degrees Celsius
OD	Optical density
OD_{570}	Optical density at 570 nm
OD_{580}	Optical density at 580 nm
OD_{600}	Optical density at 600 nm

PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
P_{remA}	*remA* promoter region
P_{tapA}	*tapA* promoter region
P_{yloC}	*yloC* promoter region
RNA	Ribonucleic acid
rpm	Revolution per minute
RT	Room temperature
SDS-PAGE	Sodium dodecyl sulphate - Polyacrylamide gel electrophoresis
SEM	Scanning electron microscopy
SOE	Splicing by overlaying extension
sp	Spectinomycin resistance cassette
T	Thymine
UV	Ultraviolet
V	Volt
v/v	Volume/volume
w/v	Weight/volume
WT	Wild Type
Δ	Deletion
σ	Sigma factor

1. Introduction

1.1 The model organism *Bacillus subtilis*

The genus *Bacillus* has played an important role in the history of Microbiology and Medicine. In the XIX century, heat-attenuated *Bacillus anthracis* was used by Pasteur as the first antibacterial vaccine, and Koch used anthrax as the test case for the development of postulates relating microorganisms to specific diseases (*1*). In our days *Bacillus* is not only known for its role in human and animal infections, but it has also valued as a producer of important antibiotics, insecticides, proteases and other products (*2-4*).

Bacillus subtilis, a member of the genus *Bacillus*, is a Gram-positive, non-pathogenic organism, soil-dwelling bacteria commonly found throughout the environment (*5, 6*). The existence of sophisticated genetic manipulation tools, the sequencing of the complete genome of its laboratory strain 168 in 1997 and its ability to form endospores (called spores for simplicity) and biofilms allowed to established *B. subtilis* as a model organism for prokaryotic cell development and differentiation studies (*7, 8*).

This thesis focuses on the biofilm formation and endosporulation (sporulation for simplicity) of *B. subtilis* and the link between these two processes.

1.2 Biofilm formation by *B. subtilis*

1.2.1 Biofilm structure and function definition

Currently, biofilms are considered the most common bacterial life style in natural environments, negatively affecting human activity, ranging from industrial production (i.e. pipelines) to health (i.e. infection of medical devices and malformed valves, dental plaque) and food production (*9-11*). Life in a biofilm is very advantageous for bacteria. Biofilm structure and composition provides its inhabitants protection against antimicrobial agents, protozoa and host defences while enabling a division of labor between cells, which will benefit the whole community (*12*). By definition, biofilms are structured communities of microbial cells living adherent to a surface and encased in an extracellular self-produced polymeric matrix (*12-14*).

Undomesticated strains of *B. subtilis* proved to be a remarkable model system to study biofilms, and have led to the recognition of distinct subpopulations of differentiated cells (Fig. 1A)(*15*). During the first stage of biofilm development most of the cells are motile. As time progresses, the encasing cells start to differentiate phenotypically through the changes in their patterns of gene expression, into the following categories: motile, matrix producers, cannibals, spores, miners, competents and surfactin producers (*6, 10, 12*):

- Motile: responsible for expanding the biofilm and establishing new colonies in a more enriched environment.
- Matrix producers: responsible for production of the major components of the extracellular matrix that protects the whole colony.
- Cannibals: cells able to produce and secret two toxins (Skf e Sdp) responsible for killing their sensitive siblings. These deaths provide them with a new source of nutrients for the community delaying the need to enter in sporulation. Cannibalism is often performed by the same cells responsible for matrix production or sporulation.
- Miners: secret exoproteases that promote biopolymers degradation into smaller molecules providing a new source of nutrients to the colony. They also produce BlsA protein, which makes the colony water resistant.
- Competents: small population of cells with the ability to absorb and incorporate external DNA into their genome.
- Surfactin producers: small population of cells that produce surfactin.
- Spores: population of resistant cells present mostly in the aerial structures of the biofilm for easier dispersion.

Cell differentiation depends of spatiotemporal conditions causing certain types of cells to origin in certain zones of the biofilm at a certain point in time, for instance cells in outer layers of the biofilm tend to differentiate into motile cells or spores, while in the interior of the biofilm cells differentiate into matrix producers (Fig. 1A) (*10*).

Biofilms expand by swarming motility with the help of surfactant molecules, which also induce the production of flagella (*10, 13, 16*). Besides phenotypically differentiation, there is a morphologic evolution of the biofilm: as the biofilm grows, it develops wrinkles that expand the area of contact with the atmosphere enhancing the absorption of oxygen.

Wrinkles also create a network of liquid channels that facilitates nutrient transport throughout the biofilm (Fig. 1C) (*10*).

Finally, the biofilm ages and formation of fruiting bodies occurs at the surface. These fruiting bodies are the site of formation of spores at the edge of the biofilm facilitates their spreading (Fig. 1A, C and D).

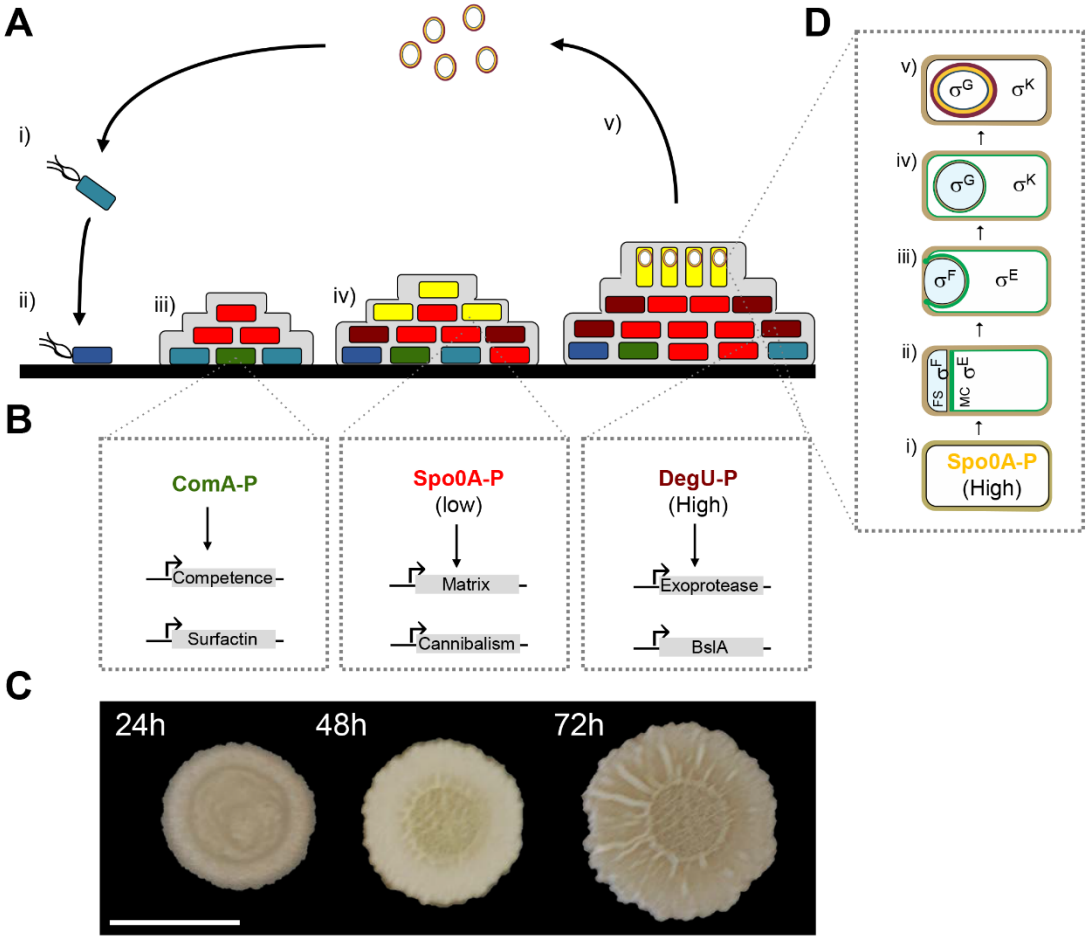

Figure 1 – *Bacillus subtilis* as a multicellular organism. (A) Schematic representation of biofilm development in *B. subtilis*: i) planktonic cell; ii) attachment; iii) formation of a microcolony; iv) formation of the biofilm; v) dispersion of the spores. In the biofilm there are distinct subpopulations of cell types that exhibit different spatiotemporal distributions: cells in blue are motile; competent cells and surfactin producers are show in green; cells shown in red produce matrix (in grey) and secrete peptide toxins to cannibalize their siblings; cells shown in brown are exoproteases producers and secrete BslA, a water repellent protein localized on the surface of the biofilm; sporulating cells are shown in yellow. **(B)** The different genetic programs related to cell differentiation (adapted from (*10*)). **(C)** Evolution of the NCIB 3610 colony morphology through time. The scale bar represents 1 cm **(D)** Representation of the main stages of sporulation: i) pre-divisional cell with high levels of Spo0A~P, the four cell type-specific factors that control spore development and their time of activity are shown; ii) cell that has completed asymmetric division forming a forespore (FS) and a mother cell compartment (MC); iii) and iv) a cell during and after engulfment completion of the forespore by the mother cell; v) assembly of the spore protective layers, the cortex peptidoglycan and the protein coat. At the end of the process, the mature spore is released upon lysis of the mother cell.

1.2.2 Analysis of biofilm development

As described above a biofilm is a bacterial community attached to an surface. Formation of these communities are usually studied in two type of assays either by examining attachment to a solid media or to each other in a liquid/air interface. *B. subtilis* forms robust biofilms that are manifested by highly structured floating pellicles that grow on the surface of liquid cultures and the architecture colonies that grow on agar plates (*17*). When attached to a solid media, bacteria normally form a colony with complex architecture, which seems to be directly linked to the ability to produce extracellular matrix, and the phenotypic variation in the morphology is indicative of its genotype (Fig. 1C). These colonies also allow the study of the different populations, within the biofilm, and their evolution throughout biofilm maturation (*15*). Liquid/air interface assays allows to study the formation of a pellicle in the top of the liquid media. The comparison between several strains in the thickness of the pellicle and its formation velocity allows for a fast screen of mutants. (*10, 18, 19*)

1.2.3 Composition of the biofilm matrix

The matrix plays a major role in holding cells together within the biofilm and at the same time in protecting them from external insults. The matrix produced during biofilm formation by *B. subtilis* contains manly exopolysaccharides (EPS) and proteins.

The genes responsible for the production of EPS are part of the *epsA-O* operon (herein *eps* operon). *eps*-defective mutants develop flat colonies and fragile pellicles (*17, 20*). Two secreted proteins give the structure integrity of the matrix: TasA and TapA. Both proteins are encoded by the operon *tapA-sipW-tasA* (herein *tapA*), and are secreted into the extracellular space by the signal peptidase SipW, encoded by the second gene of the operon. TasA forms amyloid fibres that bind cells together in the biofilm (*21*). TapA serves both to anchor the fibres to the cell wall and to help to assemble TasA into fibres (*22*). A *tasA* mutant does not form biofilm. TasA was also identified as a spore-associated protein with a role in the spore coat assembly (*23, 24*). A third protein is present in the biofilm matrix: BslA. BslA is secreted during biofilm maturation and self-assembles into a hydrophobic layer on the top of the biofilm where it serves as a water-repellent barrier (*25*).

1.2.4 The genetic regulation of biofilm formation

In biofilms, differentiation programs are spatiotemporally regulated (*6*). The main differentiation programs (mobility, matrix production, cannibalism and sporulation) are regulated by Spo0A, where the activation of a chosen program depends of Spo0A~P cellular levels (Fig. 1B and D). There are two other master regulators: DegU (which controls the differentiation of miners and BslA production) and ComA (which controls competence and surfactin production) (Fig. 1B) (*6, 10*).

Spo0A is activated through a phosphorylation cascade. First, the kinases KinA-D autophosphorylates in response to different stimuli: KinA and B to respiration, KinC to surfactin and KinD to glycerol, manganese and L-malic acid. Then, they transfer their phosphate group to Spo0B; Spo0B~P transfers the phosphate group to Spo0F, which finally transfers it to Spo0A (*10, 26-28*). ComA and DegU are both phosphorylated by their respective kinases, ComP and DegS. ComP reacts to a quorum-sensing signal, the accumulation of ComX in the media resulting of a high cell density. DegS responds to flagellar arrest which occurs in the adherent cells (*29-31*).

Master regulators are inhibited by a family of Rap phosphatases that dephosphorylate either the regulator itself as in ComA~P and DegU~P, or the phosphotransferases as Spo0B~P and Spo0F~P. Each group of phosphatases has its target: RapC, K, F, G, and H target ComA~P; RapA, B and E target Spo0B~P and Spo0F~P and RapG and RapH target DegU~P. DegU~P is also degraded by ClpCP proteases (*28, 31-34*).

As described before, the activation of the biofilm's regulators are controlled by a number of external signs like nutrients or oxygen shortages, surface adherence, quorum-sensing and secondary metabolites. These signs cause planktonic cells to localize at the base of the future biofilm and switch into a sessile state were they start to form long chains of cells held together and protected by a self-made extracellular matrix (*10, 12, 35*). In *B. subtilis* this switch occurs due to simultaneous repression of flagella genes (*fla-che* operon) and autolysins genes (*lytA, lytD and lytF*) and up regulation of matrix production genes (*epsA-O* and *tapA-sipW-tasA*) (*6, 10*). Increasing levels of Spo0A~P repress the *fla-che* operon (mobility) and activate SinI, a matrix production regulatory protein (*36-38*). Flagella synthesis is expensive to the cells, therefore, instead of destroying them, this cells synthetize a protein EpsE (part of the *eps* operon) which when expressed, interacts

with flagellum protein FliG, acting like a clutch in the flagella motor, preventing cell mobility. This mechanism ensures that the cell is not going to spend energy trying to move while it is producing matrix. Also this provides a chance for the individual cells to survive, if the environment conditions change drastically since cells do not have to synthesise the whole flagella all over again, they simply have to reactivate the flagella motor to escape (*6, 10, 39, 40*).

SinI is an antagonist of SinR, the repressor of the matrix production operons *eps* and *tapA*. As SinI binds and inactivates SinR, its repression is gradually releived, allowing for transcription of the matrix production operons (*40-42*). At the same time Spo0A~P inhibits the expression of AbrB, which is a second repressor of the *eps* and *tapA* operons, leading to a fine-tune of the expression of the matrix-related genes (*43*).

Downstream from SinR are three biofilm matrix transcriptional regulators: SlrR, RemA and RemB (formerly YlzA and YaaB, respectively) (*40, 44*). SlrR is a secondary antagonist of SinR that is necessary for full expression of the *tapA* operon (*10, 40*). RemA and RemB are necessary for expression of the *tapA* operon and also regulates expression of the *eps* operon and the *bslA* gene (*40, 45*).

Disruption or deletion of *remA* results in a severe defect in biofilm formation due to complete loss of expression of the matrix synthesis genes. *remA* appears to be expressed from two regulation region. The region upstream of the *yloC* gene and the region just upstream of the *remA* gene are both required for biofilm formation. The region just upstream of *remA*, includes a vegetative promoter and a second promoter under the control of a sporulation-specific sigma factor (σ^F). The role of this promoter is not yet clear (see below) (*40, 46*). RemA is a 89 aminoacid DNA-binding protein that binds to multiple sites upstream of the *tapA* and *eps* operons, and is necessary and sufficient for their transcription. SinR acts on these operons by competing with RemA for binding (*45*). Transcriptional profiling indicates that RemA is primary a regulator of the extracellular matrix genes, but it also activates genes involved in osmoregulation, such as the *opuA* operon (*45*).

As previously mentioned DegU~P controls the differentiation of miners (exoproteases producers) and it also activates expression of *bslA* gene, whose protein BslA (formerly YuaB) is responsible for the formation of a water repellent layer that helps to protect the

colony. Activation of DegU is achieved only in cells where flagellar rotation is inhibited and high levels of DegU~P help maintain this repression. This mechanism ensures that activation of DegU only occurs in attached cells, so they can benefit from the action of exoproteases. (*47-51*)

ComA activates competence, which allows the cells to assimilate and incorporate external DNA in order to acquire new genes that might help adaptation to new environmental conditions or help repair errors in the DNA, and surfactin production, which is important to the community for two reasons: surfactin assists in the expansion of the colony by motile cells and triggers the differentiation of matrix producers (*10, 13, 52*).

In some of these cells, Spo0A~P levels continue to rise due to shortness of nutrients, and these cells become cannibals. Slightly higher levels of Spo0A~P activate the expression of two other operons: *skf* (sporulation killing factor) and *sdp* (sporulation-delaying protein). These operons code for two bacteriocins-like products, Skf and Sdp, and their secretion systems. These bacteriocins, when secreted to the media, kill the cannibal's sensitive siblings (Spo0A-OFF), providing a new source of nutrients and delaying the need to enter in sporulation. Some authors also believe that these toxin secretion systems provide cannibal cells with an increased protection against antibiotics, which creates a population of persister cells (*12, 38, 53*). If nutrient depletion continues, Spo0A~P levels rise and the cells enter in sporulation. This differentiation path ensures that, in case of total absence of nutrients, there is a population of resistant cells able to restart the biofilm when a food source becomes available. This population normally localizes in aerial structures along the wrinkles, termed fruiting bodies due to their similarity with the fungi structures, probably to facilitate their dispersion (*6, 10, 17*).

1.3 Sporulation by *B. subtilis*

1.3.1 An overview of sporulation

Since the late 1800s, it is known that some species have the ability to form a specialized type of dormant cells kwon as spores (*5, 28, 54-56*). Several species with low G/C content from the Firmicutes phylum are spore-producers, including important pathogens of the

Clostridium and *Bacillus* genera. The sporulation process has been studied for decades in *B. subtilis*. Sporulation proceeds through a series of well-defined morphological stages that culminates about 8 hours after the onset of the process in the production of a dormant spore (*28, 57-59*) (Fig. 1D). Sporulation begins with a vegetative cell that contains two copies of the chromosome (stage 0). The nucleoid is then condensed to form a single filament that stretches across the long axis of the cell (stage I). A hallmark of sporulation is the formation of an asymmetric septum that divides the developing cell into a small forespore and a larger mother cell (stage II). At this stage the two cells lie side by side. Asymmetric division traps about 30% of one chromosome in the forespore, while the remaining of the chromosome is pumped into the forespore following division (*60-62*). Later, the mother cell engulfs the forespore to produce a free protoplast independent of the external medium (stage III). After stage III the engulfed forespore is surrounded by two peptidoglycan layers (named germ cell wall and cortex; stage IV) and by a proteinacious layer (named coat; stage V). At this point, the spore develops full resistance to physical and chemical agents (stage VI). At the end of the differentiation process the mother cell lyses releasing a fully mature spore into the environment (stage VII).

1.3.2 The genetic regulation of sporulation

Following asymmetric division the mother cell and the forespore follow different but interdependent programs of gene expression that are coupled to the course of morphogenesis (*28*). Gene expression is controlled by a cascade of compartment-specific RNA polymerase sigma (σ) subunits. The sporulation-specific RNA polymerase sigma factors are σ^F, σ^E, σ^G and σ^K, whose activity is under temporal and spatial regulation (Fig. 1D). The σ^F and σ^G regulators control gene expression during the early and late stages of forespore development, respectively, whereas σ^E controls the expression of early mother cell genes, and is replaced at later stages by σ^K.

During sporulation further control of gene expression is imposed by auxiliary transcription factors that influence the activity of RNA polymerase and help driving the cascade towards late genetic and morphological events. Five auxiliary transcriptional regulators are known, four of which can act as both activators or repressors and one only as a repressor: RsfA (which works with σ^F), SpoIIID and GerR (which works with σ^E;

GerR acts only as a repressor), SpoVT (which works with σ^G) and GerE (which works with σ^K) (*55, 63*).

Sporulation is a time and energy consuming process, which is irreversible following asymmetric division (*28, 64*). In order to confirm the necessity to enter in sporulation, the cells rely on regulatory loops and regulator's thresholds (*28, 64*). As described above, sporulation is initiated by the rise of the Spo0A~P levels. Together with σ^A and σ^H, Spo0A directs the transcription of early sporulation genes, like the *spoIIA* and *spoIIG* operons, which encode the first compartment-specific transcription factors, σ^F and σ^E (*28*). Therefore σ^F and σ^E are synthesized prior to septation in the pre-divisional cell but are held inactive until the formation of the asymmetric septum. Only after the septum is complete does σ^F becomes active in the forespore compartment. An intercompartment signal transduction pathway emanating from the forespore then causes the activation of σ^E in the mother cell (*28*). σ^F and σ^E then direct the transcription of the genes that code for the late forespore (σ^G) and mother cell-specific factors (σ^K), respectively. σ^G is activated in the forespore in a way that requires σ^E-dependent gene expression (*65*). σ^K is activated in the mother cell as a result of a signal generated in the forespore under σ^G control (*66*). Cells express pools of the sporulation sigma factors ahead of time, maintaining them in an inactive form until certain morphological events occur. Interestingly, the mother cell and forespore have developed different strategies for regulating the compartment specific σ factors activity. In the mother cell σ^E and σ^K are synthesized in an inactive pro-protein form. In the forespore σ^F and probably σ^G are kept inactive by an anti-sigma factor (*67, 68*)

1.3.3 Spore morphology and proprieties

Spores are able to resist to several extreme environmental conditions such as heat, solvents, lytic enzymes, UV radiation, oxidizing agents and also to predators. This resistance is conferred by the structure and assembly of the spore layers (*5, 54, 55*).

Although there is a large diversity of spore-formers, the spore morphology seems to be conserved (Fig. 2A) (*55*). The spore is composed by its core where the genome is housed and a set of protective layers: the cortex peptidoglycan and a multi-protein coat (Fig. 2B). The spore cortex is required for the maintenance of the dehydrated state of the

core, spore mineralization, dormancy and also for protection against organic solvents and heat (*24, 56*). The spore coat is arranged into several layers: the basement layer, the inner coat, the outer coat and the crust (Fig. 2B). The coat prevents access of lytic enzymes to the cortex (i.e. lysozyme) and confers resistance to chemicals, UV radiation and predation (*69*). In *B. subtilis* the coat is the outermost layer of the spore mediating the interaction with the surrounding environment.

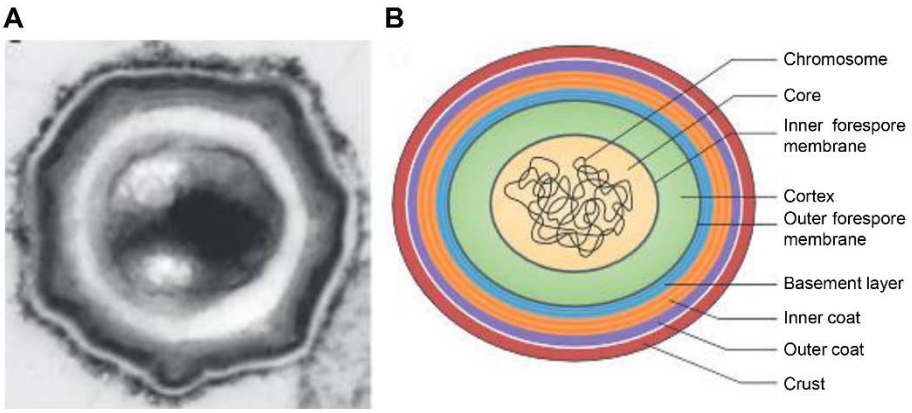

Figure 2 – *B. subtilis* spore morphology. (A) Thin sectioning transmission electron micrograph of a *B. subtilis* spore stained with ruthenium red. **(B)** A schematic representation of the main spore structurs and layers. Adapted from (*56*).

Even with these shields, spores are able to sense the environment conditions around them and germinate if favourable conditions are restored, becoming once again metabolic active cells (*54*).

1.3.4 A genomic signature for sporulation

Recently our laboratory defined a genomic signature for sporulation, as the genes which are found in 90% of the endospore forming bacteria, and present in no more than 10% of the non-endosporulators (Fig. 3A) (*46*). This signature includes 48 genes and is dominated by genes with an established function in sporulation. Among these are for example the products of the genes coding for the RNA polymerase sigma factors that control wild type-specific gene expression during sporulation (*28, 70, 71*). This list also includes genes that code for global transcriptional regulators, such as *spo0A*, *sigH* or *abrB*, which have a crucial role in both sporulation and biofilm formation (*46*). Our analysis of the signature genes produced two striking observations. First, approximately 20% of the genes have no known function in sporulation and code for products with no similarity to known proteins. Second, at least for one of these genes, *remA*, a role in

biofilm development, but not in sporulation, was described (see above) (Fig. 3B). Previous results obtained in the laboratory reveal that *remA* is also expressed during sporulation, mainly in the forespore, from a σ^F-dependent promoter (*46*) (Fig. 3D). Interestingly, the product encoded by one of the genes expressed under the control of RemA, TasA, was previously shown to accumulate in mature spores produced from planktonic cultures (*23*, *24*).

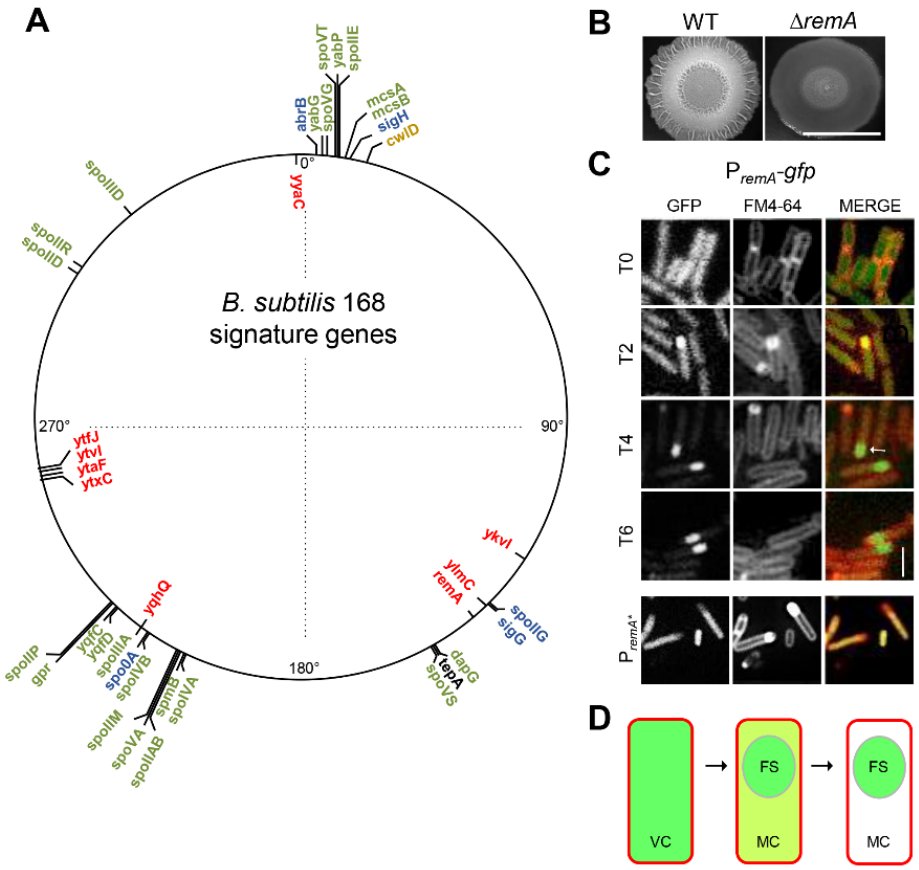

Figure 3 – Functional analysis and pattern of expression of *remA*. (A) Genomic signature for sporulation defined as those genes present in 90% of endosporulating bacteria and in no more than 10% of the remaining bacterial species. Genes with an established function in sporulation are shown in green in the outside part of the circle; genes coding for the RNA polymerase sigma factors that control gene expression during sporulation and genes encoding for global transcriptional regulators are represented in blue; a gene, *tepA*, with a predicted function in protein secretion but which has not yet been implicated in sporulation is represented in black. Genes with no assigned function are shown in red, in the inner part of the circle. The position of the genes is indicated in the chromosome of the *B. subtilis* 168. **(B)** Top view of biofilm development in NCIB 3610 and a congenic *remA* deletion mutant. The scale bar represents 1 cm. **(C)** Expression of a transcriptional fusion of *remA* promoter to *gfp*, inserted at the nonessential *amyE* locus of a wild-type strain during sporulation (T0). The cells were stained with the membrane dye FM4-64. The scale bar represents 2 µm. **(D)** Schematic representation of the expression pattern found for *remA*; pale green denotes weak expression, and the darker green indicates stronger accumulation of GFP.

1.4 Aims of this thesis

For a long time sporulation has been studied in the laboratory in liquid cultures. Although this traditional way of culturing bacteria in liquid medium has been tremendously important in the study of sporulation, planktonic growth is rarely how bacteria exist in nature. Rather, it is the biofilm mode of growth that has been proposed to be the bacterial major life style in nature. Moreover, genes like *remA* seem to indicate a strong interconnection between these two processes: *remA* is essential for the expression of the matrix production genes, which are required for biofilm formation and at the same time are part of the mature spore. Based in these observations, the major goal of this thesis was to dissect the function of the *remA* gene in sporulation during biofilm formation. The specific goals of this work were:

1. Characterization of the resistance proprieties of spores produced in a biofilm;
2. Characterization of spores produced by the *remA* mutant;
3. Studies on *remA* gene expression during sporulation;
4. Studies on *tapA* gene expression operon during sporulation.
5. Localization of TasA during sporulation.

2. Material and methods

2.1 Microbiological techniques

2.1.1 Strains, growth conditions and general techniques

All *B. subtilis* strains used in this study and their relevant properties are listed in table 1. In this study was used *B. subtilis* strains congenic derives of the undomesticated strain NCIB3610 (*Bacillus* Genetic Stock Center, BGSC). The *Escherichia coli (E. coli)* strain DH5α (Bethesda Research laboratories) was used for molecular cloning. Strains were routinely propagated and maintained in Luria Broth media (LB) or Luria Agar (LA) and antibiotics were added (Table 2) when needed. All growth media and solutions used in this work are described in table 3 and 4 respectively. All strains were stored at -80ºC in 15% glycerol.

2.1.2 Biofilm formation and quantification

Biofilm growth was done in liquid or solid MSgg (*17*) inoculated from a pre-inoculum grown in LB for 6 hours at 37ºC and 180 rpm. For pellicle assays, 10 ml of MSgg in a 6-well plate were inoculated with 10 µl of pre-inoculum and grown for 3 days at 30ºC, without agitation. For colony morphology analysis, a 3 µl drop of pre-inoculum was carefully placed in MSgg plate and allowed to dry out before moving the plate. The plates were grown for 3 days at 30ºC.

Biofilm and planktonic cells were quantified as described previously (*72*). In 96 well plates 100 µl of MSgg were inoculated with 1 µl of an LB culture grown for 8 hours, diluted to an optical density of 600 nm (OD_{600}) of about 0.3, and incubated at 30ºC for 24 hours in order to form a pellicle. After the 24 hours the liquid fractions containing the planktonic cells were carefully transferred to a new plate and OD_{600} was measured. To the pellicle was added 150 µl of 1% (w/v) filtered crystal violet and incubated for 25 min at RT. The crystal violet was removed and the wells were washed with H_2O two times to remove the excess of dye. The plate were allowed to dry at RT and 150 µl of DMSO was added to each well. The solubilized dye was transferred to a new plate and OD_{570} was measured in a SpectraMax Plus 384 and the software SpectraPro (both from Molecular Devices).

2.1.3 Spores production and purification

B. subtilis spores were purified from colonies and liquid cultures. In liquid cultures sporulation was induced by growth at 37°C and depletion of nutrients in the Difco Sporulation Media (DSM) medium (*73*). From a pre-inoculum grown in LB for 6 hours at 37ºC and 180 rpm, 100 ml of DSM supplemented with 1 mM $Ca(NO_3)_2$; 0.01 mM $MnCl_2$; 1 µM $FeSO_4$ were inoculated to a OD_{600}~0.05, and grown at 37ºC and 180 rpm, for 24 hours. The cultures were then centrifuged at 7 000 *g* for 10 min and 4ºC, and the pellets were resuspended in cold ddH_2O and left in cold for at least 24 hours.

Spores formed in biofilm were purified from colonies growing in MSgg for 5 days at 30ºC (see above). The colonies were recovered from the MSgg to tubes containing 2 ml of cool ddH_2O and then the samples were sonicated for 15X one second high intensity pulse (Bioruptor® Plus Sonication System, from Diagenode). The cell suspension were then centrifuged at 7 000 g for 10 min at 4ºC and the pellets were resuspended in cold ddH_2O for at least 24 hours.

The cell suspension in water was centrifuged at 7 000 *g* for 10 min at 4ºC. The spore pellet was resuspended in 1 ml of a 20% solution of Gastografin (Shering). This suspension was layered on top of 20 ml of a 50% solution of Gastografin and centrifuged at 12 000 *g* for 30 min. The spore pellet was washed 5 times with cold ddH_2O, resuspended in ddH_2O, and stored at -20ºC. The spore suspension was quantified spectrophotometry by measuring the OD_{580}.

2.1.4 Sporulation assays

Sporulation assays were preformed from colonies and liquid cultures. Strains were grown as described above for spore production. Liquid cultures were grown in DSM for 24 hours, then serial dilutions in B&W salts were performed and 100 µl of 10^{-5}, 10^{-6} dilutions were plated to determine the viable cell title. A selection of dilutions was then incubated at 80ºC for 20 min and 100 µl were plated to determine the heat resistant spore title. Plates were incubated overnight at 37ºC and counted in the morning

For sporulation assays from colonies, one plate of MSgg agar per assay was inoculated, as described above, and incubated at 30ºC. The colonies were recovered and placed in tubes with 1 ml of B&W salts. Then the samples were sonicated 12 times with 1 second

pulses (Sonicator Branson 450D, microtip 32 mm, 10 % power). The cell suspensions were diluted to an OD_{600}~1, then serial dilutions in B&W salts were performed and 100 µl of 10^{-5}, 10^{-6} were plated to determine the viable cell title. A selection of dilutions was then incubated at 80ºC for 20 min and 100 µl were plated to determine the heat resistant spore title. Plates were incubated overnight at 37ºC and counted after overnight growth.

For UV resistance assays, purified spores were resuspended in 5 ml of ddH_2O to an OD_{580}~0.005, and a 100 µl was collected, then serial dilutions in B&W salts were performed and 100 µl of 10^{-5}, 10^{-6} were plated to determine the viable cell title. The suspension was then placed in a sterile glass petri dish sealed with cling film and exposed to UV in 30 seconds periods (120 000 mJ) where 500 µl samples were collected to assess UV resistance.

For chloroform resistance assays, purified spores were resuspended in 550 µl of ddH_2O to an OD_{580}~0.005, and a 100 µl was collected, then serial dilutions in B&W salts were performed and 100 µl of 10^{-5}, 10^{-6} were plated to determine the viable cell title. To the remaining of the suspension 50 µl of chloroform was added and agitated in a vortex at high speed for 15 min to determine chloroform resistance.

For H_2O_2 resistance assays, purified spores were resuspended in 933 µl of PBS to an OD_{580}~0.005, and a 100 µl was collected, then serial dilutions in B&W salts were performed and 100 µl of 10^{-5}, 10^{-6} were plated to determine the viable cell title. To the remaining suspension 30% (v/v) H_2O_2 was added to the final concentration of 5%, and the mixture was agitated in a vortex at low speed for 15 min and diluted 1:10 in a solution of filtered bovine catalase.

From a selection of dilutions from the UV samples, chloroform sample and catalase-spores suspension, 100 µl were plated to determine the resistant spore title. Plates were incubated overnight at 37ºC and counted in the morning.

For germination assays, purified spores were resuspended in 100 µl of H_2O to an OD_{580}~1, and were activated by heat, at 70ºC for 20 min. The spores were then washed with H_2O, resuspended in 100 µl of GFK buffer (Table 4), and dispensed to 4 micro-wells, 45 µl of suspension each. After an incubation at 37ºC for 15 min, was added to one

of the wells 5 µl of H_2O and to the others 5 µl of 100 mM L-Alanine, and while the plate was incubated at 37ºC the OD_{580} was measured every 5 min.

For lysozyme resistance assays, purified spores were resuspended in 100 µl of 10 mM Tris-HCl pH 7.0, and dispensed to 4 micro-wells, 45 µl of suspension each. After an incubation at 37ºC for 15 min, was added to one of the wells 5 µl of 10 mM Tris-HCl pH 7.0 and to the others 5 µl of 10 mg/ml of lysozyme and while the plate was incubated at 37ºC the OD_{580} was measured every minute during 10 min.

For both germination and lysozyme resistance assays the % of OD loss in time *t* was determined using the following formula: $(OD_t x100)/OD_{t0}$.

2.2 Biochemical techniques

2.2.1 Spore coat extraction and spore fractioning

To analyse the profile of spore coat proteins, *B. subtilis* spores were resuspended in SDS-PAGE loading buffer and boiled for 8 min (SDS-DTT extraction). The alkaline extraction was performed by adding NaOH solution (0.1 M) to the cell pellets of purified spores (centrifuged 2 min, 13 000 *g* at RT) and left on ice, 15 min (shaking the tubes regularly). After further centrifugation (2 min, 13 000 *g* at RT) the supernatant was neutralized with HCl (0.1 M) and loading buffer was added to a final 1X concentration. The amount of purified spores applied in well of the acrylamide gel was determined by measure of OD_{580} spore suspension previously diluted 1:200 in ddH_2O, and using the following formula: µl/well= $3600/OD_{580}$ x 200 (where µl represents the volume of the spore suspension to apply to a well of an SDS-PAGE gel). Proteins extracted were resolved by 15% SDS-PAGE (*74*).

For spore fractioning, spores corresponding to an OD_{580}~2 were boiled for 5 min in extraction buffer without bromophenol blue and centrifuged for 2 min at 16 200 *g*. The supernatant, consisting in the soluble coat fraction, was removed and stored in a new tube, where bromophenol blue was added (for final concentration of 0.05%) before analysis in SDS-PAGE 15%. The sediment, containing the decoated spores, was washed twice with PBS-Tween and divided into two equal volume samples. These samples were incubated at 37°C for 2 hours in 50 mM Tris buffer (pH 8.0), with or without lysozyme (2 mg/ml). Then, they were boiled for 5 min in protein loading buffer and proteins were resolved in

SDS-PAGE 15%, along with half of the volume of the coat fractions (above).

The molecular size marker used was the Precision Plus All blue (BioRad) and electrophoresis was conducted using the Mini-Protean Cell system (Bio-Rad). After electrophoretic separation, depending on the purpose, the gels were stained or used for immunologic detection of proteins (see below). The staining of the gels was performed in a solution of 50% (v/v) ethanol, 10% (v/v) acetic acid and 0.1% Coomassie Blue R-250 for 30 min. The gels were then destained with a solution of 30% (v/v) Ethanol 10% (v/v) acetic acid, and finally scanned.

For protein identification, protein bands were excised and digested with trypsin, before analysis by matrix-assisted laser desorption ionization (MALDI), performed by the Mass Spectrometry Laboratory Services at Instituto de Tecnologia Química e Biológica António Xavier, Universidade Nova de Lisboa.

2.2.2 Immunoblot analysis

For immunoblot analysis the proteins resolved by SDS-PAGE were transferred to a 0.2 μm nitrocellulose membrane, in the presence of transfer buffer for 90 min at a constant potential difference of 100 V. After transfer, the membrane was incubated for 1 hour with orbital shaking in 20 ml of blocking solution, and washed with 10 ml wash solution. Then, the membranes were incubated overnight at 4ºC in the anti-TasA (1:1000) primary antibody solution. In the next day, after three washes in 10 ml of wash solution, the membranes were incubated for 30 min with orbital stirring at RT, with secondary antibody solution, rabbit's anti-IgG conjugated to horseradish peroxidase (HRP, Sigma) , at a dilution of 1:10000. After three further washes of 10 min each, the detection of protein antigens was performed according to the manufacturer's instructions, by incubation in 2 ml of detection solution (SuperSignal West Pico Chemiluminescent Substrate, Pierce), followed by exposure of the membranes to an autoradiography film (Hyperfilm, GE Healthcare). Exposure times varied between 5 seconds and 20 min. The films were then developed and fixed (Kodak).

2.3 Cell biology techniques

2.3.1 Photography, fluorescence microscopy and image analysis

Colonies growing in MSgg plates were photographed with a Leica DC 200 camera and WILD MZ8 stereomicroscope (both form Leica microsystems) and the images were processed with *Adobe Photoshop CS6* version 13.0 (Adobe Systems Incorporated).

For fluorescence microscopy the strains were induced to sporulate by resuspension medium (*73*). Supplemented Luria Broth (SLB) were inoculated from overnight strains in LA, and then grown overnight at 37ºC and 80 rpm. 30 ml of SLB were inoculated with the pre-inoculum to an OD_{600}~0.03, and grown at 37ºC and 180 rpm until OD_{600}~0.6. The cultures were then centrifuged at 3 500 *g* for 5 min at room temperature, resuspended in 30 ml of Resuspension Medium (T0) and grown at 37ºC and 180 rpm for the remaining assay's length. In the case of strain containing *gfp* fusions, 1 ml samples were collected at the indicated times and centrifuged for 2 min at 3 500 *g*. The sediment was resuspended in 100 µl of PBS buffer 1X and FM4-64 (membrane dye from Molecular Probes) was added to a concentration of 10 µg/ml. In the case of strain containing SNAP-tag fusions 0.2 ml samples were taken and incubated with SNAP-tag substrate added at a final concentration of 250 nM (505-STAR, New England biolabs). Samples were incubated in the dark for 30 min at 37ºC and then centrifuged for 2 min at 3 500 *g*. The sediment was resuspended in 1 ml of PBS buffer 1X and FM4-64 was added to a concentration of 10 µg/ml. The solutions were then centrifuged for 2 min at 3 500 *g* and resuspended in 10 µl. 3 µl of each sample were then applied in a agarose (1.7%) slide and observed in bright phase contrast and fluorescence at 1600X with a Leica SM6000B microscope. Images were taken with a cooled camera iXon-em+885 (Andor Technology) and analysed with *Methamorph* (Molecular devices) and *Adobe Photoshop CS6* version 13.0 (Adobe Systems Incorporated). Standard filters for GFP (green) and FM4-64 (red) were used for collecting the fluorescence images. For quantification of the *gfp* signal resulting from transcriptional fusions, 6x6 pixel regions were defined in the desired cell and the average pixel intensity was calculated, and corrected by subtracting the average pixel intensity of the background.

For scanning electron microscopy (SEM) purified spores were fixed with 2.5% glutaraldehyde, 1% formaldehyde, 0.1 M phosphate buffer during 30 min at RT. Samples were washed 3 times with 0.2 M $CaCl_2$ and 0.1% Tween 20, dehydrated at RT with 10% increasing concentrations of ethanol starting from 50% to 100%, and finally dehydrated in 100% acetone during 5 min. Samples were mounted on gold cover glass slides, and then sputtered with 10 nm layer of gold using a Cressington sputter model 108. Scanning electron microscopy was performed on a Hitachi TM 3000 electron microscope operated at 15 kV.

2.3.2 SNAP-Tag fusions detected by In-gel fluorescence

The SNAP-tag fusions were detected by in-gel fluorescence as described in (*46*) with some adaptations. Strains containing SNAP-tag translational fusions were induced to sporulate by resuspension medium (see above). At the desired times following resuspension, samples of 5 ml of the sporulation cultures were collected and the SNAP-tag substrate added at a final concentration of 250 nM (505-STAR, New England biolabs). Samples were incubated in the dark for 30 min at 37ºC. Cells were collected by centrifugation at 4 000 g for 5 min at 4ºC and wash twice with the same volume of PBS as the culture. The pellet was resuspended in 1 ml French Press buffer and the cells lysed using a French pressure cell at 18000 lb/in^2. The protein present in each lysate was quantified by the biuret method (BCA Protein Assay Kit Pierce, Thermo Scientific) and loaded in a 15% SDS-PAGE. After electrophoresis, the gel was scanned on a FLA-5100 imaging system (FujiFilm). For detection of proteins labeled with 505-STAR was used the 532 nm laser and for molecular maker detection was used the 635 nm laser.

2.4 Genetic and molecular biology general techniques

2.4.1 Plasmid construction

Inserts for cloning were obtained by Polymerase Chain Reaction (PCR). The thermostable enzyme used was Phusion High- Fidelity DNA Polymerase (Finnzymes) and the reaction conditions were setted based on the oligonucleotides melting temperature, amplified product size and the manufacturer's indications for the DNA polymerase. All cloning experiments were according to established methodologies (*75*). All plasmids were sequenced to verify that no mutations were generated during PCR or

cloning. Plasmids used in this study and the oligonucleotide primers used for PCR, or sequencing are listed, respectively, in table 5 and 6.

2.4.2 Plasmid DNA extraction

To identify transformants carrying the plasmid of interest, plasmid DNA was extracted on a small scale (minipreps) and subjected to cleavage with appropriated restriction endonucleases . Single colonies of *E. coli* DH5α were grown overnight in LB medium with appropriate antibiotics, and the next day the culture was centrifuged (16 200 *g*, 5 min). Cell lysis was performed enzymatically by resuspending the pellet in a solution of 360 µl STET buffer, 0.6 mg/ml lysozyme and 0.25 mg/ml of RNAse A, followed by incubation at 37ºC for 30 min. Then the sample was boiled for 1 min, which enables the denaturation of chromosomal DNA. After centrifugation (16 200 *g*, 10 min) the pellet was removed, isopropanol was added to a final concentration of 70% (v/v) and the sample was centrifuged at 16 200 *g* for 45 min at 4ºC, in order to precipitate the plasmid DNA. The supernatant was discarded and the pellet was allowed to dry out before be resuspended in 20 µl of ddH_2O and stored a -20ºC.

For isolation of clean plasmid DNA from *E. coli* was used the NZYMiniprep kit (nzytech) according to the protocol provided by the manufacturer. The method is based on the alkaline lysis of the cells and DNA adsorption by the silica matrix, in the presence of high salt concentration. The purity, quality and concentration of material are ideal for genetic transformation experiments, cloning and DNA sequencing.

2.4.3 Transformation of competent cells of *E. coli*

In this study we used chemically competent cells of *E. coli* DH5α prepared by the $CaCl_2$ method (*75*). An isolated colony of *E. coli* was inoculated into 50 ml of LB and incubated at 37ºC and 120 rpm on an orbital shaker to an OD_{600}~0.6. The culture was then centrifuged for 2 min at 7 000 g and 4ºC. The supernatant was discarded and the pellet was resuspended with 25 ml of a 0.1 M $CaCl_2$ cold solution. The cells were incubated on ice for about 1 hour and centrifuged again. The cell pellet was resuspended in 3.3 ml CaCl2 and 15% glycerol, and then distributed in aliquots of 150 µl were stored at -80ºC until use.

For transformation, 150 µl of the suspension of competent cells were transformed with 1 µl of plasmid DNA (about 1 µg) or 10 µl of a ligation mixture and incubated for 1 hour on ice. Following a 90 seconds heat-shock at 42ºC, were incubated on ice for 2 min, 1 ml of pre-equilibrated medium LB was added to the cells, incubated at 37ºC for 2 hours with agitation and plated onto LB agar supplemented with the appropriate antibiotic.

2.4.4 *B. subtilis* genomic DNA extraction

A single colony of *B. subtilis* was inoculated into 5 ml of LB and incubated at 37ºC and 120 rpm on an orbital shaker overnight. The cell culture was pelleted (10 min at 5 800 *g* and 4ºC), resuspended in 1 ml of SET buffer, 0.5 mg/ml of lysozyme and 0.05 mg/ml de RNase A and incubated at 37ºC for 30-60 min. Then, 25 µl of proteinase K (10 mg/ml) and 25 µl of Sarkosyl 10% were added and the solution was incubated again at 37ºC for 30-60 min. An equal volume of phenol:chloroform:isoamilic acid (25:24:1) was added to the lysate and mixed by inversion for 10 min and then centrifuge for another 10 min at 16 200 *g*. The aqueous phase was transferred into a new tube and this extraction was repeated. To the final aqueous phase an equal volume of chloroform was added and mixed by inversion for 10 min and then centrifuge for another 10 min at 16 200 *g*. The aqueous phase was retrieved, dialyzed for 30-60 min against water and stored at 4ºC.

2.4.5 Preparation and transformation of competent cells of *B. subtilis*

A single colony of *B. subtilis* was inoculated into 5 ml of Growth Media 1 (GM1) and incubated at 37ºC and 200 rpm. Growth was followed by measuring OD_{600} and T0 was determined as the 1st time point after exponential phase. 90 min after T0 1 ml of GM1 culture was inoculated in 4 ml of Growth Media 2 (GM2) and grown for 2 hours. 500 µl of competent cells were transformed with 5 µl of genomic DNA or 20 µl of plasmid DNA, and incubated 1 hour at 37ºC and 200 rpm. 250 µl of transforming mixture were plated in LA supplemented with the appropriated antibiotics.

If the transformation was intended to insert DNA constructs into the *amyE* locus another test has to be performed. As *amyE* is a non-essential locus is a prime location for inserting, by double cross-over, promoter fusions to reporter genes or genes for complementation of deletion mutants. *B. subtilis* strains with an intact *amyE* locus, the gene coding for α-amylase, are able to degrade starch, the insertion interrupts the *amyE* locus, and therefor α-amylase production, making this strains unable to degrade starch.

Iodine when combined with starch forms a dark blue complex. In order to confirm the interruption of the *amyE* locus the colonies resultant from the transformation of *B. subtilis* were streak into LA plates complemented with the appropriate antibiotics and a replica LA + 1% starch plate. After an overnight incubation at 37ºC, to the plate that contain starch was added a solution of 1% I_2 + 10% KI. The colonies were considered positives for insertion in *amyE* locus when the orange halo around the colony, resultant from the starch degradation by α-amylase, was absent (*76*).

2.2.6 DNA electrophoresis

The DNA samples were subjected to electrophoresis in 1% agarose gels in TAE 1X buffer. Gels were run in the presence of ethidium bromide, at a concentration of 0.001% (v/v). Before application to the gel, was added buffer Orange G to each sample. Gels were run at constant voltage of 110 V, the DNA visualized under UV radiation of low wavelength and the gels were documented by digital photography. The molecular weight marker used to infer the molecular weight of the DNA fragments was the 1 kb plus DNA ladder (Gibco, BRL).

3. Results

3.1 Analysis of the proprieties of the spores produced in the biofilm

For years, spores of *B. subtilis* produced from planktonic cultures (DSM) have been extensively study and their resistance proprieties characterized. However biofilm communities are now widely accepted as the main life style in natural environments, and so spores produced in a planktonic culture may not be the best representatives of the spores produced in nature. Therefore this study started with the characterization of spores produced within biofilms with respect to germination, lysozyme, UV and hydrogen peroxide (H_2O_2) resistance, as well as coat composition.

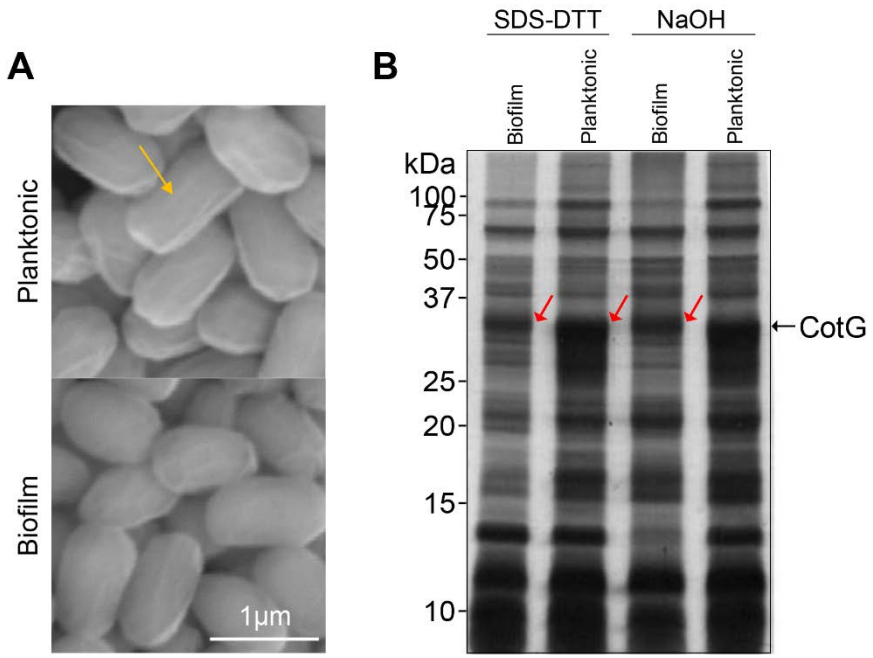

Figure 4 – SEM images of WT spores and its coat protein profiles. (A) Spores from MSgg and DSM cultures were purified and imaged by SEM; the yellow arrow indicates the characteristic ridges along the longer axis of the spore. **(B)** Profile of coat protein extracted by SDS-DTT or NaOH methods from WT spores purified from MSgg and DSM cultures.

Strain NCIB 3610 (WT) was induced to sporulate in DSM or to produce biofilm in MSgg and spores from these cultures were purified. Density gradient purified spores were first examined by scanning electron microscopy (SEM) (Fig. 4A). As described previously for other *Bacillus* species (*77*), *B. subtilis* spores are oval, with surface ridges

extending along the long axis of the spore (Fig. 4A, yellow arrow). We found that both planktonic or biofilm spores morphologies were indistinguishable by SEM. We then used SDS-DTT or alkaline treatment (see Materials and Methods) to extract proteins from the surfaces of purified spores. The extracted proteins were then resolved by SDS-PAGE. The pattern of released proteins from spores produced in MSgg is slightly different from that of spores produced in DSM (Fig. 4B). In particular, a band of about 32 kDa appears more diffuse in spores from DSM then in spores from MSgg. This band migrates in the same position of CotG, a previously characterized coat protein (*78*).

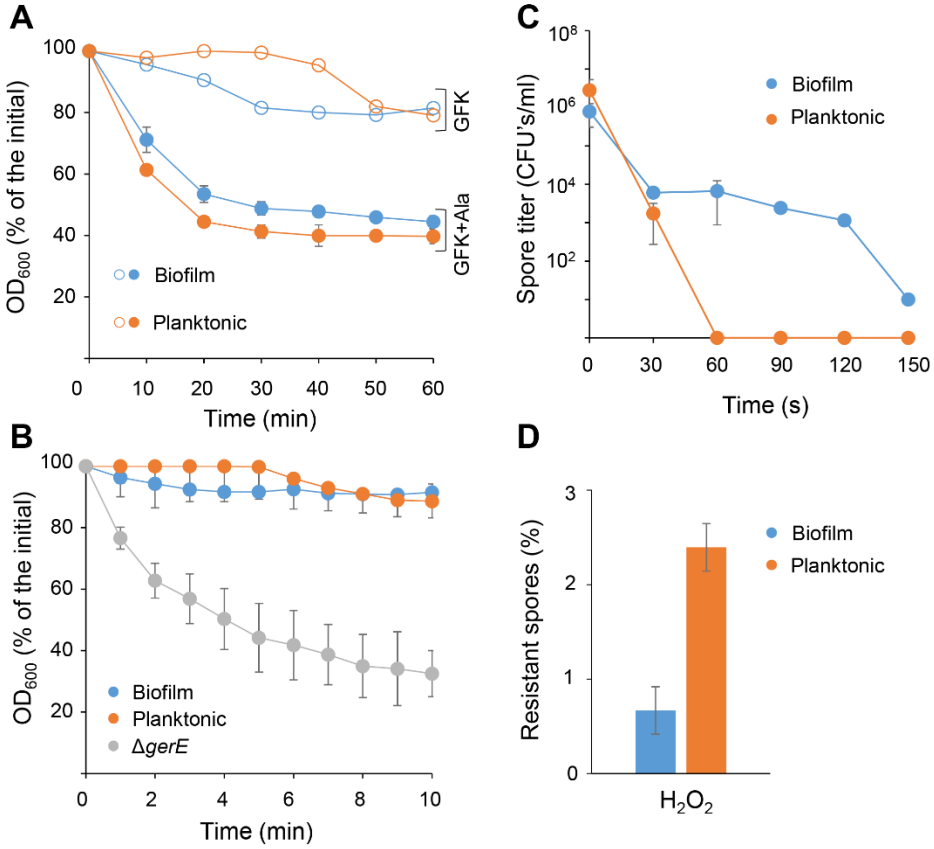

Figure 5 – Spore germination and resistance assays. (A) Germination of WT spores produced in MSgg and DSM media triggered by alanine. The germination buffer without addition of alanine was used as negative control. **(B, C and D)** Resistance of WT spores produced in MSgg and DSM to lysozyme **(B)**, UV radiation **(C)** and H_2O_2 **(D)**, show that biofilm spores have a higher resistance to UV radiation than spores produced during planktonic gwroth. Δ*gerE* strain was used as control for the lysozyme resistance assay.

Spores were then used to measure lysozyme, UV and hydrogen peroxide resistance and the efficiency of germination (Fig. 5). These are spore properties known to be determined in part by the coat layers (*69*, *79*). We also measured heat resistance of the spores, a property that is determined by the status of the cortex layer (*80*). Spores

produced in the biofilm did not have altered heat resistance properties, 70.59% of heat resistant spores (MSgg) vs. 81.77% (DSM). The spores were also indistinguishable in their resistance to lysozyme and hydrogen peroxide (Fig. 5B and D). We also found that both types of spores have similar kinetics of germination in response to L-alanine (Fig. 5A) on GFK. In contrast, spores produced in MSgg were more resistant to UV radiation then spores produced in DSM (Fig. 5C). Interestingly spores purified from biofilms show a dark brown pigment in contrast with the light grey spores produced in DSM. A protective role of a brown pigment in the spore resistance to environmental UV radiation has been previously reported (*79*). Formation of this pigment was dependent on spore coat protein A (*81*).

In conclusion, these results show that spores produced in the biofilm have a slightly altered profile of surface proteins that may have an impact on the increased UV resistance.

3.2 Role of *remA* sporulation-specific expression on biofilm formation

With the recent definition of an endosporulation genomic signature (*46*), a particular set of genes raised an interesting question: why do genes, with no known role on sporulation, are conserved in sporulating bacteria? One gene in particular, *remA*, intrigued us due to its known function in biofilm formation (*40*). Previous work in the laboratory shown that, even though *remA* is a signature gene for endo-sporulating organisms, expressed during sporulation, it is not required for sporulation (*46*). However *remA* has been confirmed to have an important role in biofilm development, as a DNA binding protein required for the transcription of the matrix encoding genes (*45*). As described above, *remA* is transcribed from a promoter region upstream of the *yloC* gene (P_{yloC}) and from a promoter region located just upstream of the *remA* gene, which includes a σ^F-dependent promoter (P_{remA}; Fig. 6A). Both promoter regions are required for full transcription of *remA* and biofilm formation. The σ^F promoter has been shown to be sufficient for *remA* expression in the forespore (*46*), however the role of this promoter on biofilm and spore formation during biofilm development had not been investigated.

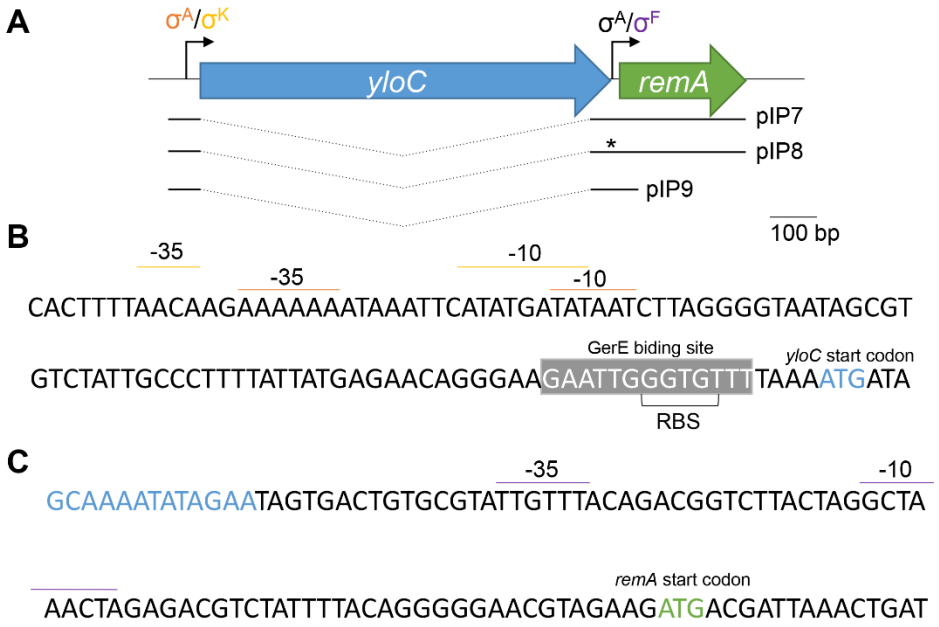

Figure 6 – Regulatory region of the *remA* gene. (A) Schematic representation of *yloC* and *remA* genomic region with its putative promoters (broken lines). The lines bellow represent the DNA fragments cloned into the indicated plasmids. **(B)** Regulatory region of the *yloC* gene (P_{yloC}), with the -35 and -10 elements of the putative σ^A- (orange) and σ^K-dependent (yellow) promoters. The putative GerE biding site (grey) and start codon are also represented. **(C)** Regulatory region of the *remA* gene (P_{remA}), with the -35 and -10 elements of the putative σ^F- dependent (purple) promoter. The start codon is also represented.

In order to study the role of *remA* in spore and biofilm development, complementation strains were constructed by fusing the *yloC* promoter to the *remA* promoter and coding region. The *yloC* promoter region (567 bp) and the *remA* gene (500 bp) were amplified from WT chromosomal DNA using primers PyloC362D/PyloC-remA and remA842D/remA2194R (Table 6). The two fragments were joined by splicing by overlay extension (SOE) PCR using primers PyloC362D and remA2194R (Table 6) and cloned between the BamHI and EcoRI sites of pDG364 (*46*), to produce pIP7 (Fig. 6A). We used pIP7 and specific primers (remASigFD and remASigFR, Table 6) to mutate the σ^F-specify promoter present in the *remA* promoter region (the *remA* gene with the altered promoter is herein designated as *remA**), to produce pIP8 (Fig. 6A). pIP 7 and 8 were then used to transform a Δ*remA* strain (Table 1). In these strains a copy of the *remA* gene is inserted at the *amyE*, a non-essential locus of *B. subtilis*

Strains Δ*remA*, Δ*remA* $remA^{wt}$ ($remA^{wt}$ for simplicity) and Δ*remA* $remA^*$ ($remA^*$ for simplicity) were induced to form biofilms in MSgg agar, for colony architecture comparison, and liquid MSgg for biofilm quantification. As described before, the Δ*remA* mutant is not able to form an elaborated colony in the solid MSgg, forming instead a flat

and moist colony, easily disrupted (Fig. 7A); biofilm's crystal violet quantification also shows that this mutant is completely defective in pellicle formation (Fig. 7B). We were able to fully complement the *remA* mutation by insertion of the *remA*^{wt} and *remA** alleles at *amyE*. Both strains form elaborated colonies in solid MSgg and pellicle in liquid MSgg (Fig. 7A and B). These results show that the σ^F-specific promoter of *remA* is not required to proper biofilm formation. We also studied the rate of sporulation during biofilm development, which revealed that Δ*remA* strain is defective in spore formation, with spores recovered only 5 days after the beginning of biofilm formation. This is characteristic of mutants defective on biofilme formation (*82*). In contrast, for the *remA*^{wt} and *remA** strains spores could be recovered since day one (Fig. 7C).

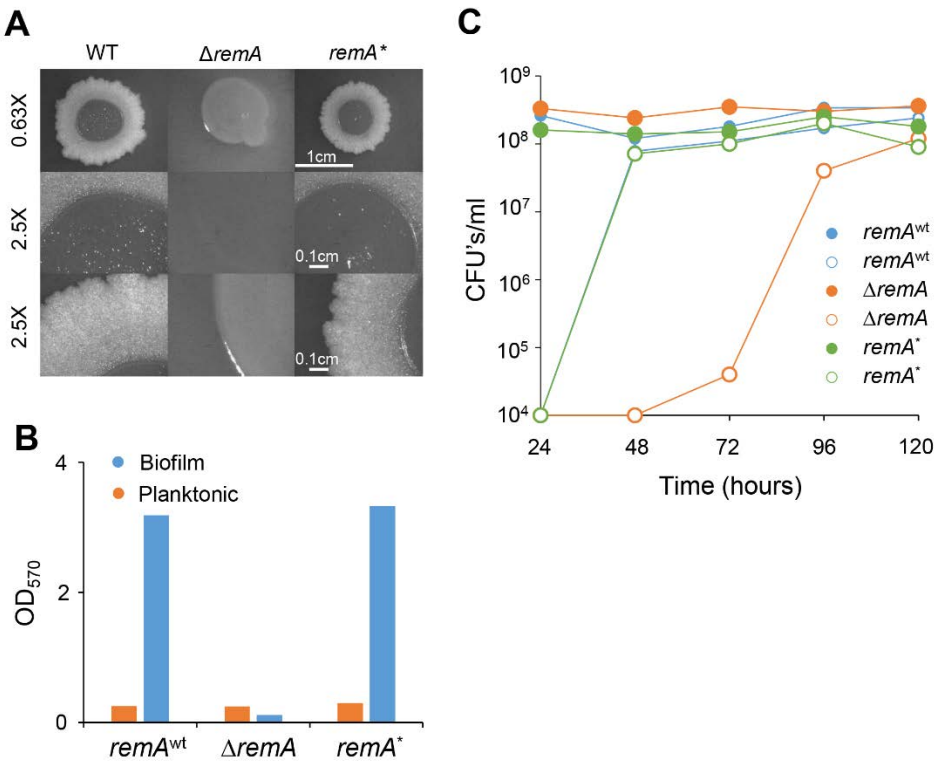

Figure 7 – Functional analysis of *remA*. (A) Top view of biofilm development by a WT strain, a Δ*remA* mutant and the *remA** complementation strain. **(B)** Quantification of biofilm formation using the crystal violet assay. **(C)** Sporulation efficiency of the same strains as in **A** and **B**, during biofilm formation. Viable cells are shown in full symbols, heat resistance cells are sown in empty symbols.

3.3 Spore coat profile of the *remA* mutant

As mentioned previously *remA* is a transcription factor required for the expression of the matrix encoding genes (*45*). One of these genes is *tasA*, the third gene of a tricistronic operon, which includes *tapA* and *sipW* (*23, 41*). In addition, TasA was first characterized as a protein from the spore coat, in fact as the predominant product solubilized by NaOH treatment from spores of a *gerE* mutant (*23, 24*). The *gerE* mutant is pleiotropically impaired in the transcription of several of the *cot* genes (*83*) and forms spores with altered coat layers which lack several of the proteins that can be extracted from wild-type spores (*84*). We therefore decided to study the impact of *remA* in the association of TasA with the mature spores. For that, pIP 7 and 8 (see above) were used to transform a Δ*gerE* Δ*remA* strain (Table 1). Spores from the WT, *remA*^{wt}, Δ*remA, remA**, Δ*gerE*, Δ*gerE remA*^{wt}, Δ*gerE* Δ*remA,* Δ*gerE remA**, Δ*tasA* were purified from biofilms grown in MSgg agar and the coat proteins extracted by SDS-DTT or alkaline treatment. The extracted proteins were then resolved by SDS-PAGE. Figure 8A and B shown that the *remA* mutant have a pattern of coat proteins similar to the WT. In contrast, when the *remA* mutation is inserted in a *gerE* mutant there are at least two polypeptides (about 30 kDa and 15 kDa) present in the *gerE* mutant, that are absent from the protein profile of the double mutant (Fig. 8A and B). Mass spectrometry confirmed the ~30 kDa protein as TasA, and identified the ~15 kDa protein as BslA. BslA is also a matrix protein from the biofilm with a role in biofilm hydro protection (*25, 85*). Furthermore, transcription of the *bslA* gene, as that of the *tasA* gene, also requires RemA (*45*). These data suggest that other components of the biofilm matrix under the control of RemA may also be part of mature spores.

We further investigated the presence or absence of TasA by immunoblot analysis using an anti-TasA antibody. In contrast to what was observed in WT spores produced in DSM (*23*), TasA is extracted from WT spores produced in biofilms. TasA is absent in the *remA* mutants, and is more extractible from the *gerE* mutants as described previously (*23*). Surprisingly, the strains carrying the *remA* allele with mutations on the σ^F promoter (*remA**) show an increased level of TasA. To determine whether TasA was loosely associated with the spores produced in biofilm, possibly as a contaminant from the matrix, the coat proteins were extracted by SDS/DTT treatment before or after three washes of the spores with a 1 M KCl solution (*86*). The results from these experiments are shown

in figure 8C. Treatment with KCl did not significantly change the amount of TasA released from the coats of WT or *gerE* spores. These results suggest that TasA is a component of the spore coat, and its association with the mature spore is regulated by two transcriptional factors, *remA* and *gerE*.

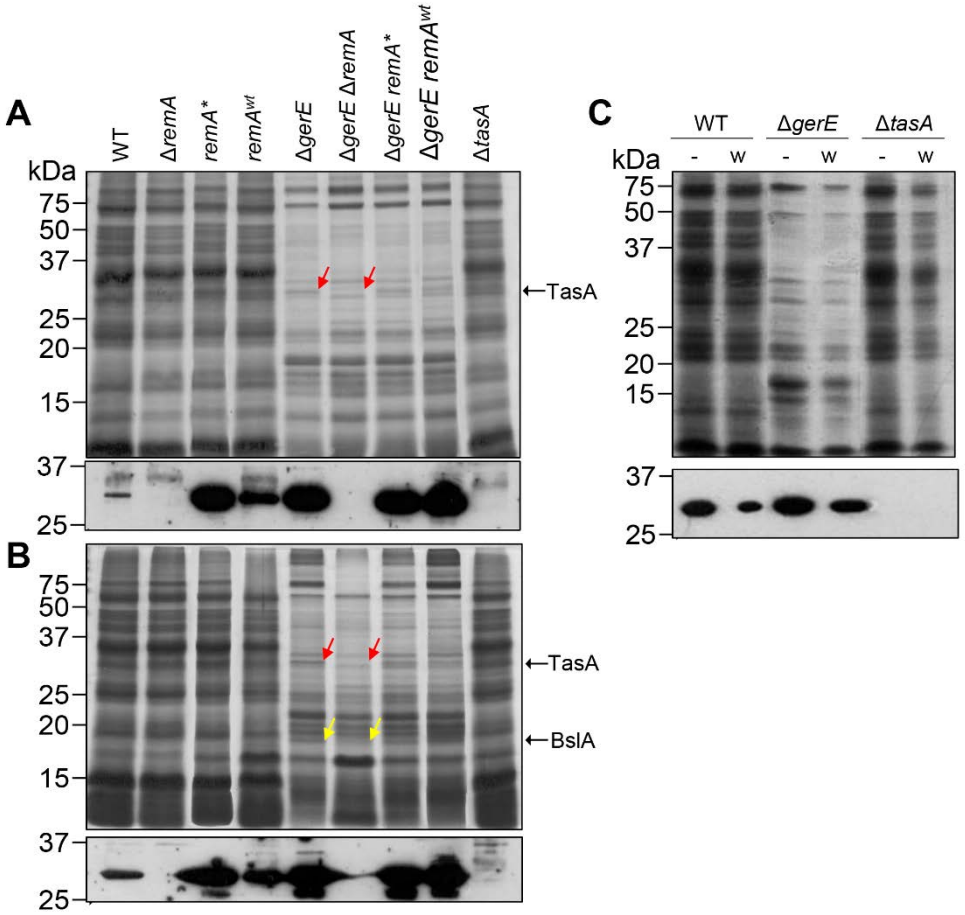

Figure 8 – Role of *remA* in spore coat assembly. Coat proteins were extracted from WT, Δ*remA*, *remA**, *remA*^{WT}, Δ*gerE*, Δ*gerE* Δ*remA*, Δ*gerE remA**, Δ*gerE remA*^{WT} and Δ*tasA* spores by SDS-DTT **(A)** and NaOH **(B)** methods, resolved in 15% SDS-PAGE gels which were stained with Coomassie. TasA was detected by immunoblotting with anti-TasA (bottom panels). The position of TasA and BslA is indicated by red and yellow arrows, respectively. **(C)** Spore coat proteins from unwashed and washed (1 M KCl) spores from the WT, Δ*gerE* and Δ*tasA* strains, extracted by the SDS-DTT method and immunobloted with anti-TasA antibodies (bottom panel).

3.4 *remA* expression is regulated by GerE during sporulation

We next investigated *remA* expression during sporulation. TasA was described as being easily extractible from *gerE* spores (*23*). However because there are other matrix components which are RemA-dependent for transcription, such as BslA, that were also

identified in the *gerE* mutant, we decided to investigate the effect of a *gerE* mutation on the expression of *remA*.

As described above *remA* is expressed from two promoter regions, one upstream of the *remA* and that includes the σ^F-dependent promoter (P_{remA}) and one upstream of the *yloC* gene (P_{yloC}). Earlier studies using a transcriptional fusion of P_{remA} to *gfp* (green fluorescent protein gene) have shown that *remA* expression is detected during vegetative growth and in the mother cell following asymmetric division at the onset of sporulation (Fig. 3C and D) (*46*). In addition, *remA* is expressed in the forespore soon after asymmetric division in a σ^F-dependent manner (*46*). Studies using both promoters fused to *gfp* were not performed. In order to study the expression of *remA*, strains carrying both promoters fused to *gfp* were constructed. The P*yloC* region (567 bp) was amplified using the primers PyloC362D and PyloC-remA (Table 6). The P*remA* fragment (175 bp) was generated using the primers remA842D and remA1005R (Table 6). The two fragments were joined by SOE PCR using primers PyloC362D and remA1005R (Table 6) and cloned between the BamHI and SmaI sites of pIP6 (Table 5), to produce pIP9 (Fig. 6A). pIP 9 and pMS406 (carrying the fusion P*remA-gfp*, (*46*)) were used to transform WT and Δ*gerE* strains (Table 1). The transcriptional fusions were inserted at the nonessential *amyE* locus, and the time and compartment of expression during sporulation were examined by fluorescence microscopy. As described previously, fluorescence from the P_{remA}-*gfp* was detected during vegetative growth, in the mother cell following asymmetric division, and accumulated strongly in the forespore (Fig. 9A and D, (*46*)). Late during sporulation, fluorescence is not detected in the mother cell (Fig. 9A). The *gerE* mutation does not affect the pattern of expression of the P_{remA}-*gfp* fusion (Fig. 9B and C). Fluorescence from $P_{yloC}P_{remA}$-gfp was detected in vegetative cells, and soon after asymmetric division in the forespore (Fig. 9D). In addition, fluorescence is also detected in the mother cell until late in sporulation (Fig. 9D). In a *gerE* mutant the GFP intensity increases in the mother cell following engulfment completion (Fig. 9E and F).

Inspection of the region upstream of the *yloC* gene revealed possible −35 (AACA) and −10 (CATATGATA) elements (located between −100 and −66 relative to the start codon of *yloC*) recognized by σ^K, the late mother cell-specific σ factor (−35, --AC-; −10, -CATA---T-; hyphens indicate spaces) (Fig. 6B). We have also identified a possible GerE

binding site (RWWTRGGYNNYY; R, A or G; Y, C or T; W, A or T; N, A, C, G, or T) located between positions –16 and – 4 relative to the start codon ((*87*), Fig. 6B).

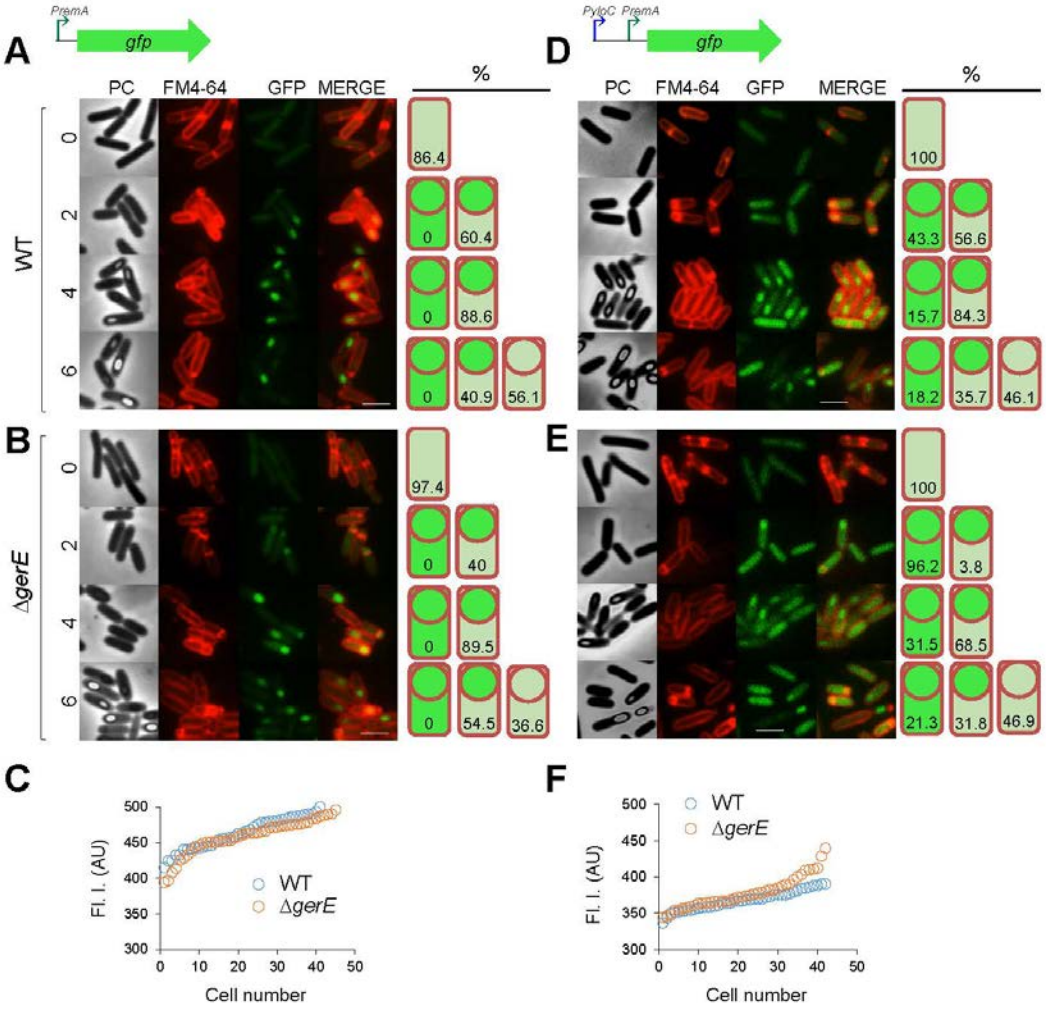

Figure 9 – Expression of P_{remA} and $P_{yloC}P_{remA}$ during sporulation. Expression of the transcriptional fusion of P_{remA} **(A and B)** or $P_{yloC}P_{remA}$ **(D and E)** to *gfp* in the WT **(A and D)** or *gerE* mutant **(B and E)**, at the indicated times (in hours, number on the left side of the panels) after the onset of sporulation (0). Membranes were stained with FM4-64, the scale bars represent 1 µm. Spatial and temporal expression patterns are represented, pale green denotes weak expression, and darker green indicates strong accumulation of GFP. **(C and F)** Quantitative analysis of the fluorescence intensity (Fl. I.) in arbitrary units (AU) per each cell. The data shown are from a representative experiment of tree independent experiments.

Together these observations are in line with the idea that *remA* is expressed during vegetative growth and during sporulation, in the forespore soon after asymmetric division and in the mother cell after engulfment completion. GerE represses *remA* expression in the mother cell at late stages of sporulation. In a *gerE* mutant, increased *remA* expression may lead to transcriptional activation of the matrix-encoded genes at late stages of sporulation.

3.5 *tasA* expression during sporulation

We next investigated whether the increased expression of *remA* in the *gerE* mutant has an impact on *tasA* expression. *tasA* is expressed from the promoter upstream of *tapA*, the first gene of the operon (Fig. 10A) (*41*). Thus, we constructed a P_{tapA}-*gfp* fusion. The promoter region (252 bp) was amplified from WT chromosomal DNA, using primers PtapA774 and PtapA1017 (Table 6). The PCR fragment was digested with SalI and HindIII and inserted into plasmid pMS157 (*88*), creating pIP2 (Table 5). pIP3 was created by the replacement of the neomycin resistance cassette by a chloramphenicol resistance gene (Fig. 10A).

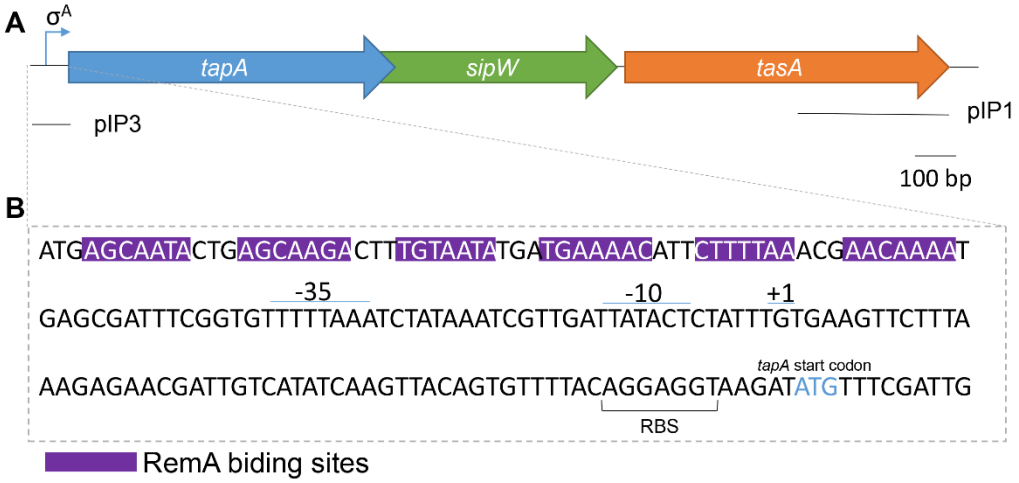

Figure 10 –The *tapA* operon. (A) Schematic representation of *tapA* genomic region with its putative promoter (broken lines). The lines represent the DNA fragments cloned into the indicated plasmids. **(B)** Regulatory region of the *tapA* operon (P_{tapA}), with the -35 and -10 elements of the putative σ^A-dependent (blue) promoter. The putative RemA biding sites (purple), ribosomal biding site (RBS) and start codon (light blue) are also represented.

pIP3 contains, in addition to the P*tapA* insert and the gene coding for GFP, two homology regions with the *amyE* gene allowing integration of DNA placed between them at the *amyE* locus by double cross-over recombination (Table 5). pIP3 was transferred to the *amyE* locus of the WT, Δ*remA*, Δ*gerE* and Δ*gerE* Δ*remA* strains, and the localization of GFP was visualized in sporulation cultures (Table 1).

Figure 11 shows that P*tapA* is expressed in vegetative cells and after asymmetric division in the mother cell. In the *gerE* mutant there is an increase in the fraction of sporulating cells showing GFP signal in the mother cell and an increase in the fluorescence intensity per cell, compared with the wild type background (Fig. 11E). *gfp*

expression was not detected in the *remA* mutant nor in the double *gerE remA* mutant (Fig. 11C and D).

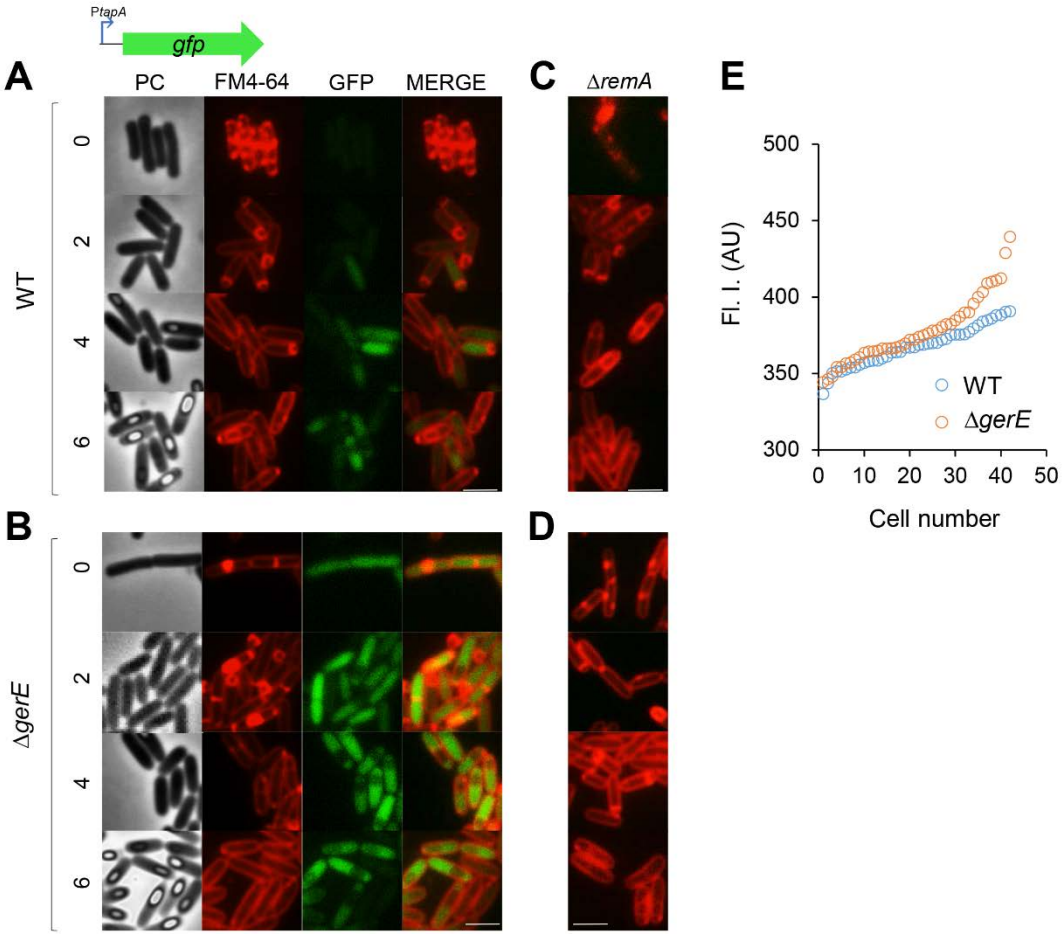

Figure 11 – Expression of P_{tapA} during sporulation. (A, B, D and E) Expression of the transcriptional fusion of P_{tapA} to the *gfp* inserted in the WT**(A)**, *gerE* mutant **(B)**, *remA* mutant **(C)** and *remA gerE* double mutant during sporulation at the indicated times after the onset of sporulation (0). Membranes were stained with FM4-64, the scale bars represent 1 µm. **(D)** Quantitative analysis of the fluorescence intensity (FI) in each cell. The data shown are from one experiment representative of three similar, independent experiments.

Moreover, although *remA* is expressed in the forespore ((*46*); Fig. 3C), we did not detected expression from the *tapA* promoter in the forespore (Fig. 11A and B). This may occur because other factors required for *tapA* expression are not present in the forespore. Alternatively *tapA* expression can be specifically repressed in the forespore. These results show that *remA* is essential for *tapA* expression not only during vegetative growth but also during sporulation in the mother cell, and that the GerE phenotype concerning TasA accumulation may be explained by repression of *remA* expression by GerE.

3.6 TasA localizes around the spore

We then wanted to investigate the localization of TasA during sporulation. Since TasA is a secreted protein that has a classical signal peptide recognized by the Sec system (*89*), and GFP is not able to fold and fluoresce after secretion through this system, we fused the SNAP-tag (New England Biolabs, Inc.) to the C-terminal of TasA. The SNAP-tag is a 20 kDa engineered form of the human repair protein O^6-alkylguanine-DNA alkyltransferase (hAGT) that covalently reacts with O^6-benzylguanine (BG) derivatives, in an irreversible manner (*90*).

The 3' region of *tasA* (592 bp) was amplified from WT chromosomal DNA, using the primers tasA2606 and tasA(linkerSNAP)R, and the *SNAP* gene (556 bp) was amplified from plasmid pFT58 (*91*), using primers SNAP D and SNAP^c R. The two fragments were fused by SOE PCR using the primers tasA2606 and SNAP^c R. The 1126 bp fragment was digested with EcoRI and XhoI and inserted into the plasmid pMS38 (*92*), creating the plasmid pIP1 (Fig. 10A; Table 5). pIP1 was used to transform WT were it integrated into the native *tasA* gene by a single reciprocal cross-over event (Table 1).

Since *tasA* is essential for biofilm formation (*21*), we tested the functionality of the TasA-SNAP fusion growing the WT, the *tasA* mutant and the strain containing the *tasA-SNAP* fusion in MSgg plates. Figure 12A show that the strain containing the translational fusion as an architecture similar to the WT, confirming that the activity of TasA is not grossly inhibited or altered by its fusion to the SNAP-tag.

The strain containing the TasA-SNAP fusion was grown in resuspension medium, and at the indicated times samples were taken and the SNAP-tag was labelled with the 505-Star substrate. Figure 12B shows that during sporulation TasA-SNAP localizes in the asymmetric septum and around the forespore in some cells. However a large number of cells (73.89%) show a fluorescence signal spread in the mother cell, which is not expected for a secreted protein. The reaction of the substrates with the SNAP -tag is irreversible and thus suited for the detection of labelled protein via in-gel fluorescence scanning following SDS-PAGE. Therefore cells extract of the culture at hour 2 was labelled and run in a SDS-PAGE gel. Cell extracts of a WT culture (without the fusion protein) were used as a control. As seen in figure 12C the TasA-SNAP-tag protein accumulates at very

low levels, and a putative product of proteolysis of around 25 kDa is present. This product may correspond to the SNAP-tag alone and may be responsible for the fluorescence detected in the mother cell cytoplasm. Since the fusion TasA-SNAP protein is proteolysed we cannot be confident about its localization.

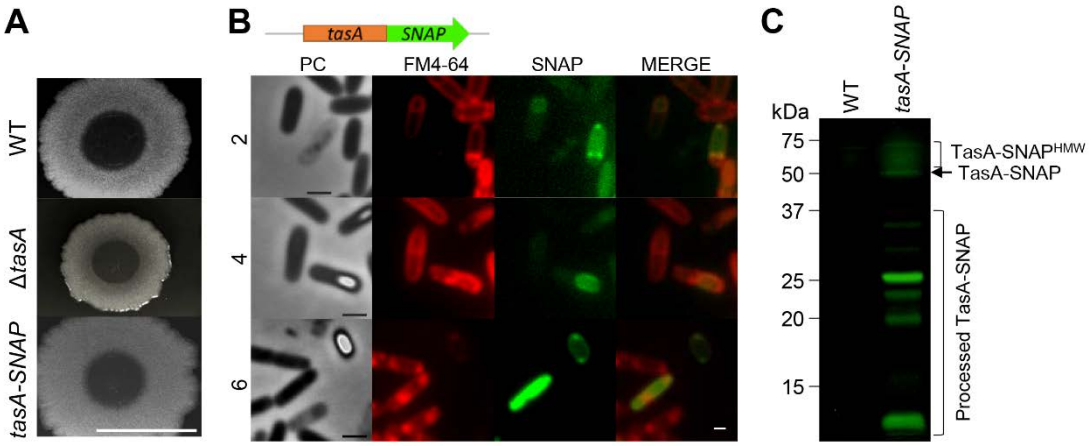

Figure 12 – Localization of TasA-SNAP fusion during sporulation. (A) Top view of biofilm development in the WT, *tasA* mutant and in the strain carrying the *tasA*-SNAP fusion. The scale bar represents 1 cm. **(B)** Fluorescence microscopy of the strain carrying the *tasA-SNAP* during sporulation at the indicated times after the onset of sporulation. Cells were labelled with FM4-64 (membranes) and 505-Star (SNAP). The scale bar represents 1 µm. **(C)** Total cell extracts from the WT and the strain producing TasA-SNAP were prepared from cultures 2 hours after the onset of sporulation. Proteins (30 µg) were resolved by SDS-PAGE and the gel scanned using a fluorimager. The position of the SNAP fusion protein and possible degradation products is indicated. High molecular weight species of TasA-SNAP (HMW) are also indicated.

Since *tasA* is expressed in the mother cell we infer that TasA is translocated across the outer forespore membrane to the space between the two forespore membranes, where cortex synthesis takes place. Therefore we tried to elucidate if TasA was underneath the coat, in the cortex region, or at an interior location within the coat. For that, density gradient purified spores from the WT, Δ*tasA*, Δ*gerE* and Δ*gerE*Δ*tasA* strains were produced. Then, coat proteins were extracted (coat fraction), and the decoated spores were submitted to a lysozyme treatment that digests the exposed cortex and releases the cortex-associated proteins (cortex fraction). Proteins within these spore fractions were resolved by SDS-PAGE, stained with coomassie or immunobloted for the detection of TasA. In spores from the WT or Δ*gerE* strains TasA was detected exclusively in the coat fraction (Fig. 13). This result indicates that TasA is a coat protein. However, observation of the SDS-PAGE stained with coomassie show that an increase number of proteins is released

from the cortex of the spores in the absence of TasA (Fig. 13). Thus, TasA seems to have an impact on the proteins extracted in the cortex fraction.

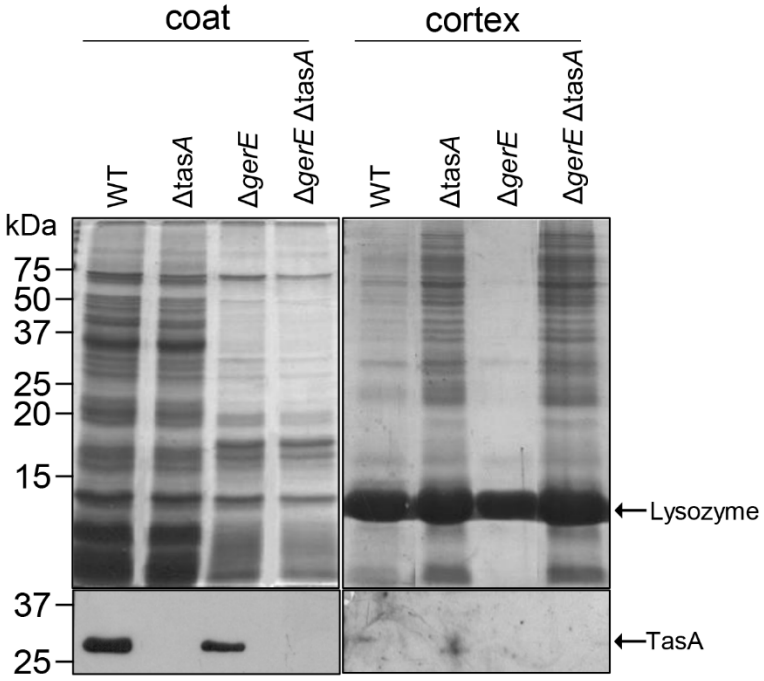

Figure 13 – TasA is a spore coat component. Spores from the WT, Δ*tasA*, Δ*gerE* and Δ*tasA* Δ*gerE* strains produced in MSgg were gradient purified and decoated. The exposed cortex was then digested with lysozyme and the proteins within the different spore fractions were resolved by SDS-PAGE, stained with coomassie and immunobloted with anti-TasA antibodies (bottom panels). The position of molecular weight markers (kDa) is shown on the left side of the panels. The arrows on the right side show the position of the lysozyme and TasA.

3. Discussion and conclusions

Bacterial biofilms cause many complications in several aspects of human life. They are responsible for clogging industrial pipelines, food contamination, persistence in the gastro-intestinal tract, and are one of the most common post-operatory infections, especially in procedures involving prosthetic implants, being estimated by the National Institute of Health (United States of America) that more than 60% of all human infections are caused by biofilms (*93-100*). Although in nature, biofilms are usually multispecies, the study of single-specie, isogenic biofilms is able to provide important insight on how a biofilm is formed and developed. We focused on biofilms produced by the Wild Type strain – NCIB 3610 – of *Bacillus subtilis* (*101*) because this is a reference strain for biofilm study. NCIB 3610 is very consistent throughout biofilm development, which allows for comparison between different phenotypes. Although *B. subtilis* is not a pathogenic organism, the study of its developmental processes can quickly reveal multiple leads to deal with the biofilms formed by pathogens.

The work performed for this thesis started with the characterization of spores produced within biofilms. We found that this spores have a higher resistance to UV radiation than the spores produced in planktonic cultures (Fig. 5C). Higher resistance to UV had already been shown in bacterial and fungal spores from high altitudes, associated with the accumulation of a darker pigmentation in the spores (*79*). Spores purified from biofilms also show a dark brown pigmentation; this pigmentation may be the same pigment that is produced in some species of *Bacilli* and accumulates during sporulation in a manner that depends on the spore-associated CotA lacase (*81, 102*). This CotA-dependent pigment behaves like a melanin, but its identity has not yet been confirmed (*81, 102*). It will be interesting to determine, in future work, the role of CotA in the production of the pigment and in the UV resistance of spores produced in biofilm. The higher UV resistance of the spores might work as a biofilm mechanism of defense, as the spores are usually localized at the top of the biofilm (Fig. 1A) and in this way they can shield the cells of the biofilm from UV radiation (*17*).

Previous work from our laboratory showed that *remA*, an essential gene for biofilm formation, is also expressed during sporulation under the control of the forespore-specific sigma factor, σ^F (*45, 46*). This observation strongly suggests that *remA* may also have a

role during sporulation or that the expression during sporulation may be important for biofilm formation. In any case, this observation indicates a link between sporulation and biofilm formation.

Our results show that *remA* forespore-specific expression is not required for biofilm formation (Fig. 7). However characterization of the coat proteins of spores produce within the biofilm reveal that TasA is part of the coat of the WT spores, which as described previously is an abundant component of the coat of a *gerE* mutant and its presence is completely dependent on *remA* (Fig.8;(*23*)). *remA* forespore-specific expression is not required for the accumulation of TasA in the spore. In fact, the strain that do not expressed *remA* in the forespore (*remA**) accumulate more TasA than the WT. The cause of this increase is not known at the moment. Previous studies have shown that *remA* is necessary for biofilm formation because it is the only transcription activator of biofilm matrix operons *eps* and *tapA* and the gene *bslA* (*45*). Interestingly, we were able to identify BslA in the coat of the *gerE* mutant (Fig.8B). Our data suggest that other components of the biofilm matrix may also be part of mature spores. We hypothesize that some of the structural proteins that confer integrity to the biofilm matrix, as TasA, may have a role as a scaffold for assemble of the spore surface layers. Remarkably, previous studies have shown that deletion of *tasA* results in the accumulation of misassembled material, in an inner coat not attached to the cortex layer and an altered outer coat (*23*). BslA confers hydroprotection by coating the biofilm and helps stabilize the interface formed by the biofilm and air or liquid. BslA is classified as a hydrophobin, because it shares the function with this class of fungal proteins (*103*). Hydrophobins are known for their ability to form a hydrophobic coat on the surface of cells. These proteins are the main component of the hydrophobic sheath covering the surface of many fungal spores (*104*). The presence of BslA in the biofilm spores could mean that these have a higher hydrophobicity than planktonic spores. It will be interesting to characterize the hydrophobicity of the spores produced in biofilm and the role of BslA in the development of this propriety.

Both BslA and TasA are secreted proteins and our mass spectrometry results show that the form that is present in the coat is the processed one, without signal peptide. This means that these proteins are translocate through the forespore outer membrane and may accumulated in the space between the two membranes that surround the forespore (Fig. 14). Given this it would be likely that these proteins localize to the spore cortex. However

our fractionation results show that TasA is a coat protein (Fig. 13). This may be explained if the outer membrane loses its integrity during cortex formation, releasing this protein to the coat layer. In any case TasA seems also to have an effect on the cortex, since the amount of cortex proteins released in the *tasA* mutant is larger than in the WT (Fig. 13). In the biofilm, TasA fibers are anchored to the cells and form a robust protein scaffold that holds cells together (*22*). By analogy, in the spore, TasA fibers may have a role in anchoring proteins to the cortex and to the coat.

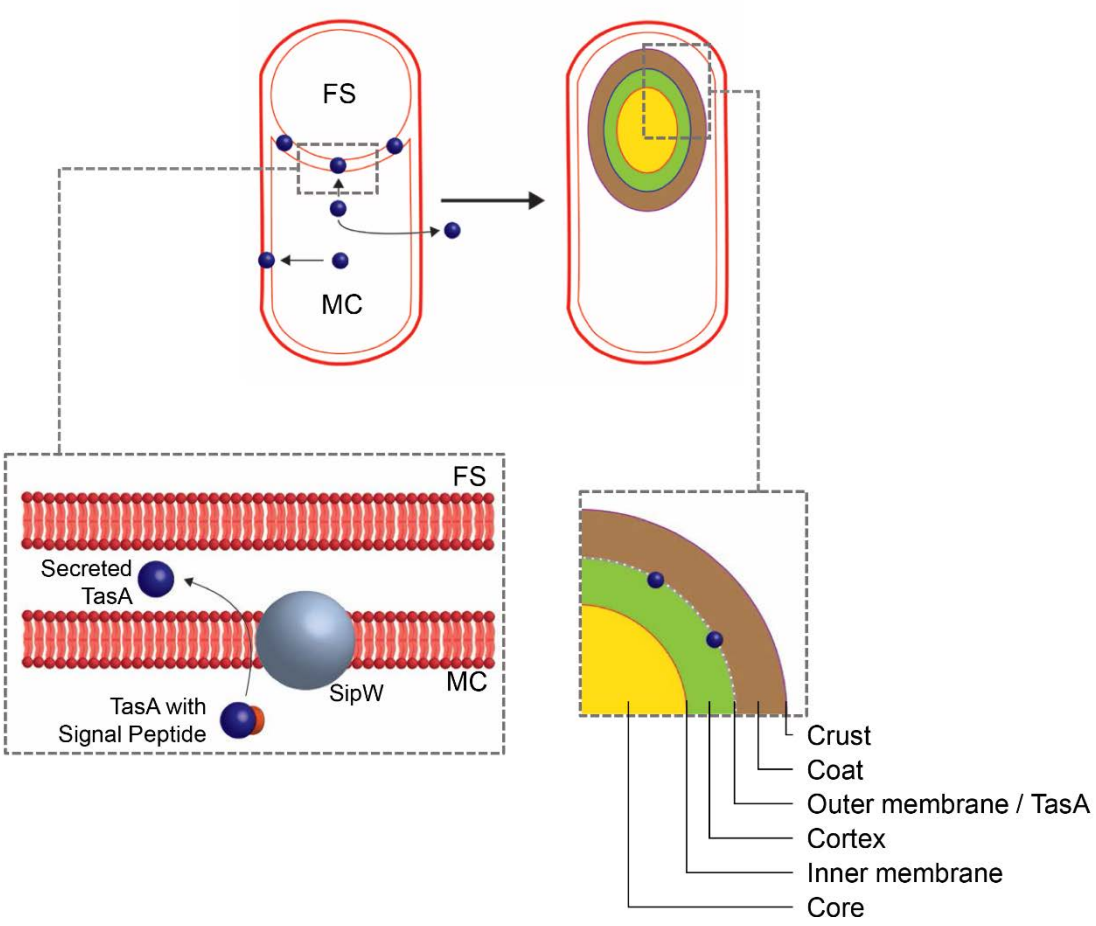

Figure 14 – TasA localization in sporulating cells. TasA is produced only in the mother cell compartment, and could be exported to the cell membrane, sporulation septum and extracellular media. SipW cleaves the TasA signal peptide. TasA around the forespore follows the migration of the spore membranes, indicating that protein migrates, and is translocated from the mother cell to this membrane, and not from the extracellular media.

The *remA* gene appears to be expressed from two promoters. Complementation of the mutant *remA* biofilm phenotypes required that *remA* is expressed from the P_{yloC} promoter region, suggesting that *remA* may, under some circumstances, be cotranscribed with *yloC*, which codes for a 291-amino-acid protein (YloC) of unknown function (*40*). Nonetheless,

remA gene is expressed independently from sequences just upstream in the *yloC-remA* gene region. This region contains the σ^F -specific promoter. In this work we show that *remA* is expressed during sporulation, first in the forespore from the previously characterized σ^F promoter in the P_{remA} region, and after engulfment is cotranscribed with *yloC* in the mother cell from a putative σ^K promoter in the P_{yloC} region (Fig. 9A and D). The σ^K promoter is then subject to repression by GerE (Fig. 9D, E and F). In the future, the effect of a *sigK* (encoding for σ^K) mutation on *remA* expression will be addressed. The action of σ^K and GerE, with GerE repressing the gene activated by σ^K, will give rise to a pulse of *remA* expression in the mother cell (Fig. 15).

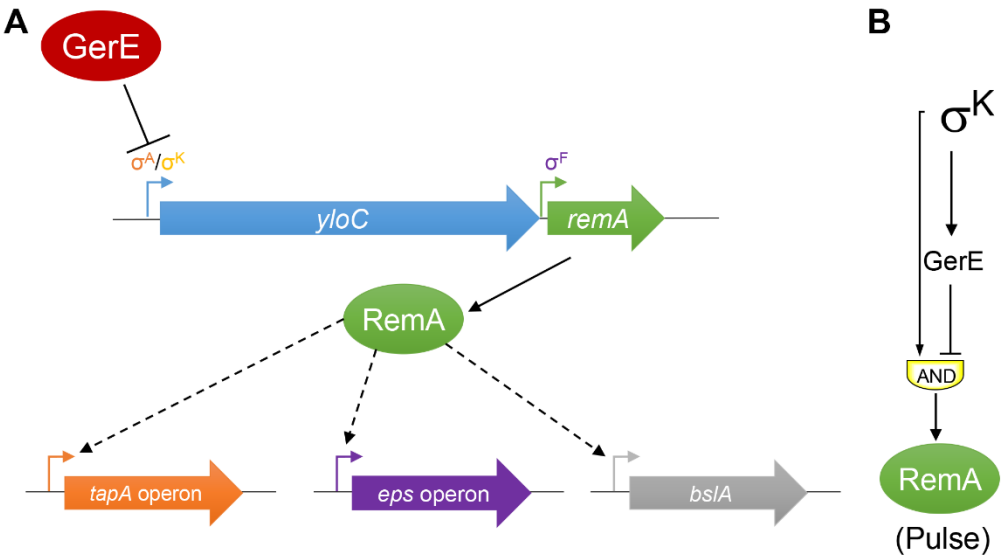

Figure 15 – Regulatory circuits governing *remA* expression. (A) Regulation of *yloC* promoter region by GerE, and the regulation of matrix production genes by RemA. **(B)** In the mother cell σ^K and GerE define a incoherent feed/forward loop with an AND gate logic that controls the expression of *remA*

In this work, we also show that the *tapA* promoter (which controls *tasA* expression) is also expressed in the mother cell during sporulation, in a *remA* dependent manner (Fig. 11). We also show that in the *gerE* mutant expression of *tapA* is increased (Fig. 11B and E), but is still dependent on *remA*. Our interpretation is that in the *gerE* mutant, *remA* expression is maintained in the mother cell until late in the development, leading to an increase in transcription from the *tapA* promoter. This would explain the increased accumulation of TasA in the coat of *gerE* mutant spores. Although we did not study *bslA* expression we speculate that the same should be observed for this gene, since BslA also accumulates more in the spore coat of the *gerE* mutant.

Still to explore is the role of RemA in the forespore. Although *remA* is expressed in the

forespore, at least the expression of the *tapA* operon is not activated in the forespore (Fig. 11). This may be explained by the absence of Spo0A from the forespore. Spo0A is required for the repression of the matrix-encoded genes repressors, SinR and AbrB, which may repress *tapA* in the forespore (*20, 43, 105*). RemA is a DNA binding protein that is involved in the activation of gene expression; hence we speculate that RemA may also work as a transcriptional regulator in the forespore. To gain insight into the role of RemA it will be interesting to use RNA sequencing for gene expression profiling the WT and the *remA** mutant early during sporulation.

In the case of sporeforming bacteria we are faced with an antibiotic- and immune system-resistant biofilm that is able to disperse in the form of highly resistant spores (Fig.1A). A link between spore differentiation and the formation of complex multicellular communities is also observed in bacteria such as *Streptomyces coelicolor* or the gram-negative *Myxococcus xanthus*, even though the spores formed by these organism are different from the endospores formed by the *Bacillus* and *Clostridia* genera (*106, 107*). This study makes a contribution in the functional analysis of a gene conserved among sporeformers essential for biofilm formation that may be a new target for inhibition of biofilm development by these organisms.

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6. Annex

Table 1 – *B. subtilis* strains used in this study

Strain	Relevant genotype	Source
NCIB 3610	Undomesticated strain	Laboratory stock
AH6864	Δ*remA*::sp	(*46*)
AH6865	Δ*remA::sp* Δ*amyE::remA*, Cm^r	(*46*)
MSB40	Δ*tasA::c*m	Laboratory stock
MSB54	Δ*gerE*::km	"
MSB55	Δ*remA::sp* Δ*gerE::km*	"
MSB56	Δ*remA::sp* Δ*gerE::km* Δ*amyE::remA**,Cm^r	This study
MSB57	Δ*tasA*::*tasA-SNAP*, Cm^r	"
MSB66	Δ*amyE::*P_{tapA}-*gfp*, Cm^r	"
MSB67	Δ*remA::sp* Δ*amyE::*P_{tapA}-*gfp*, Cm^r	"
MSB68	Δ*gerE::km* Δ*amyE::*P_{tapA}-*gfp*, Cm^r	"
MSB69	Δ*remA::sp* Δ*gerE::km* Δ*amyE::*P_{tapA}-*gfp*, Cm^r	"
MSB83	Δ*remA::sp* Δ*amyE::*P_{yloC} *remA**, Cm^r	"
MSB84	Δ*remA::sp* Δ*gerE::km* Δ*amyE::* P_{yloC} *remA**, Cm^r	"
MSB85	Δ*amyE::*P_{yloC} P_{remA}-*gfp*, Cm^r	"
MSB86	Δ*remA*::sp Δ*amyE::*P_{yloC} P_{remA}-*gfp*, Cm^r	"
MSB87	Δ*gerE*::km Δ*amyE::*P_{yloC} P_{remA}-*gfp*, Cm^r	"
MSB88	Δ*remA::sp* Δ*gerE::km* Δ*amyE::*P_{yloC} P_{remA}-*gfp*, Cm^r	"

Table 2 – Antibiotics

Antibiotic	Stock concentration	Solvent	Work concentration	
Spectinomycin	100 mg/ml	ddH_2O	100 µg/ml	
Chloramphenicol	30 mg/ml	95% etanol	5 µg/ml	
Ampicillin	100 mg/ml	ddH_2O	100 µg/ml	
Kanamycin	10 mg/ml	ddH_2O	1 µg/ml *B. subtilis*	3 µg/ml *E. coli*

Table 3 – Growth media

Media	Composition (for 1L of media)
Luria Broth (LB)	10 g tryptone; 5 g yeast extract; 5 g NaCl
Luria Agar (LA)	10 g tryptone; 5 g yeast extract; 5 g NaCl; 16 g agar
Difco Sporulation Media (DSM)	8 g nutrient broth; 1 g KCl, 0.25 g $MgSO_4.7H_2O$ or 0.12 g $MgSO_4$
Growth Media 1 (GM1)	960 ml B&W Salts; 10 ml 100 mM MgSO4; 10 ml 50% glucose; 10 ml 10% yeast extract; 10 ml B&W Aminoacids 5X
Growth Media 2 (GM2)	965 ml GM1; 5 ml 50 mM $CaCl_2$; 25 ml 1 M $MgCl_2$
MSgg	5 mM potassium phosphate pH 7; 100 mM morpholinepropanesulfonic acid pH 7; 2 mM $MgCl_2$; 700 µM $CaCl_2$; 50 µM $MnCl_2$; 50 µM $FeCl_3$; 1 µM $ZnCl_2$; 2 µM thiamine; 0.5% glycerol; 0.5% glutamate; 50 µg/ml tryptophan; 50 µg/ml phenylalanine and 50 µg/ml threonine; 15 g agar (solid version)
Supplemented Luria Broth (SLB)	250 ml LB; 50 ml CH III and 2 ml CH IV
Ressuspension Media	900 ml sporulation salts; 40 ml Solution C; 10 ml Solution D and 40 ml Solution E

Table 4 – Buffers and other solutions

Solution	Composition (for 100ml)
Bott &Wilson salts (B&W salts)	1.24 g K2HPO4; 0.76 g KH2PO4; 0.1 g tri-sodic citrate and 0.6 g $(NH_4)_2SO_4$
B&W aminoacids 5X	250 mg of Trp, Arg, Lys, Gly, Met, His, Val, Thr, Asp
1M potassium phosphate pH=7	32.37 g 1M K_2HPO_4 and 20.26 g 1M KH_2PO_4
CH III	1.98 g $MgSO_4.7H_2O$ and 4 ml $CaCl_2$
CH IV	1.1 g $MnSO_4.4H_2O$
GFK buffer	7 mM Tris-HCl pH 7; 1 mM D-Frutose; 1 mM D-Glucose; 10 mM KCl
Solution A	0.089 g $FeCl_3.6H_2O$; 0.83 g $MgCl_2.6H_2O$ and 1.979 g $MnCl_2.4H_2O$
Solution B	5.35 g NH_4Cl; 1.06 g Na_2SO_4; 0.68 g KH_2PO_4 and 0.97 g NH_4NO_3
Sporulation Salts	0.1 ml Solution A and 1 ml Solution B
Solution C	5 g L-glutamate
Solution D	0.1 M $CaCl_2$
Solution E	1 M $MgSO_4.7H_2O$

SET Buffer	20 g sucrose; 2 ml 1M Tris.HCl pH8 and 2 ml 0.5M EDTA pH8
STET Buffer	8% sucrose; 0.5% TritonX-100; 50 mM EDTA and 10 mM Tris-HCl pH 8
Resolution gel	15% Acrilamide (29:1); 0.375 M Tris.Cl; 0.1% SDS pH 8.8; 0.8% APS and 10% (v/v) TEMED
Staking gel	4.5% Acrilamide (29:1); 0.125 M Tris.Cl; 0.1% SDS, pH 6.8; 0,01% APS and 10% (v/v) TEMED
SDS-PAGE Loading Buffer	1X Upper Tris; 5% Glycerol; 2 % (w/v) SDS; 0.5 mM DTT; 5 % 2-mercaptoetanol and 0.025 % (w/v) bromophenol blue
Transfer Buffer	25 mM Tris, 192 mM Glicine and 10% Ethanol
PBS 10X	137 mM NaCl; 2.7 mM KCl; 4.3 mM Na2HPO4 and 1.4 mM KH2PO4
PBS-Tween	10 ml PBS 10X, 0.1 ml Tween 20
French Press Buffer	10 mM Tris, pH 8; 10 mM $MgCl_2$; 0.5 mM EDTA; 0.2 M NaCl; 10% grycerol and 0.1 mM DTT
Blocking solution	5% (w/v) powder skin milk diluted in PBS-Tween
Orange G	0.2% orange G; 0.37% EDTA and 50% glycerol
TAE 1X	40 mM Tris; 20 mM Acetic acid and 1 mM EDTA

Table 5 – Plasmids constructed during this study

Plasmid	Features	Selection mark in *B. subtilis*
pIP1	Plasmid derived from pMS38 carrying the *tasA-SNAP* translational fusion	Cm
pIP2	Plasmid derived from pMS157 carrying the P_{tapA} to create P_{tapA}-*gfp* transcriptional fusion	Neo
pIP3	Plasmid derived from pMS157 carrying the P_{tapA}-*gfp* fusion, where Neo resistance cassette was replaced by Cm resistance gene	Cm
pIP6	Plasmid derived from pMS157 where Neo resistance cassette was replaced by Cm resistance gene	Cm
pIP7	Plasmid derived from pGD364 carrying P_{yloC} *remA*	Cm
pIP8	Plasmid derived from pGD364 carrying P_{yloC} *remA**	Cm
pIP9	Plasmid derived from pIP6 carrying P_{yloC} P_{remA} to create P_{yloC} P_{remA} -*gfp* transcriptional fusion	Cm
pIP10	Plasmid derived from pIP6 carrying P_{yloC} P_{remA*} to create P_{yloC} P_{remA*} -*gfp* transcriptional fusion	Cm

Table 6 – Primers

Primer	Sequence (5’ to 3’)
PyloC362D	CAT GGA ATT CAG CCG GAA GGA GAC GTC
PyloC-remA	CGA CAA GCT TTG TGA TCT GAT GAT CTT TAA AAC ACC CAA TTC TTC CCT G
PtapA1017	GTG AAA GCT TAA TTA AAA CAT ATC TTA CCT CC
PtapA774	TAT GTC GAC TTT AAA TTC TCA CAT AAC AAT CCC
SNAP D	ATG GGA TAA AGA TTG TGA AAT GAA GAA GAA CC
SNAP^{c} R	CTC GAG TTA CCC AAG TCT GGT TTC CCC AAA CG
tasA(linkerSNAP)R	CAT TTC ACA ATC TTT ATC CAT AGC AGC TGC GGA TCC ACC ACC AAG ATT TTT ATC CTC GCT ATG CGC
tasA2606	CAA AGA ATT CTA ATG GCG CTT AAT TAT GGA G
remA1005R	CGG GAT CCT TAC GTC ATC TTC TAC GTT CCC CC
remA2194R	CGA ACG GAT CCT TTA CCA ACT CCT GAG GG
remA842D	GAT CAT CAG ATC ACA AAG CTT GTC G
remASigF D	CAG ACG GTC TTA CTA GGC TCA ACC AGA GAC GTC TAT TTT AC
remASigF R	GTA AAA TAG ACG TCT CTG GTT GAG CCT AGT AAG ACC GTC TG