ISTANBUL TECHNICAL UNIVERSITY ★ INSTITUTE OF SCIENCE AND TECHNOLOGY

CHARACTERIZATION OF THE *yvfI* MUTANT SPORES

M.Sc. Thesis by
Ezgi ARSLAN

Department : Advanced Technologies

Programme: Mol. Bio. Genetics & Biotechnology

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(521071037)

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Supervisor (Chairman) : Assoc. Prof. Dr. Ayten Yazgan Karataş (ITU)
Members of Examining Committee: Assoc. Prof. Dr. Gamze KOSE (YEDITEPE UNI.)
Assis. Prof. Dr. Fatma Neşe KOK (ITU)

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yvfI MUTANT SPORLARIN KARAKTERİZASYONU

YÜKSEK LİSANS TEZİ
Ezgi ARSLAN
(521071037)

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Tez Danışmanı : Doç. Dr. Ayten Yazgan KARATAŞ (İTÜ)

Diğer Jüri Üyeleri : Doç. Dr. Gamze KOSE
(YEDİTEPE ÜNİ.)
Yrd. Doç. Dr. Fatma Neşe KOK (İTÜ)

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FOREWORD

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July, 2010

Ezgi ARSLAN

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ABBREVIATIONS

AGFK **:** AsparagineGlucose Fructose KCl
DPA **:** Dipicolinic Acid
DSM **:** Difco Sporulation Medium
Erm **:** Eritromycin
GPR **:** Germination Protease
LB **:** Luria-Bertani Liquid Medium
SASP **:** Small, Acid-Soluble Protein
SDS-PAGE **:** Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SMM **:** Spizizen's Minimal Medium
Spc **:** Spectinomycin
UV **:** Ultraviolet

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SUMMARY

CHARACTERIZATION OF THE *yvfI* MUTANT SPORES

In *Bacillus subtilis*, sporulation process is a developmental stage of differentiation in response to unfavourable conditions such as nutrient insufficiency. This process involves an extracellular or intracellular signal dependent pathway employing many different gene products and factors to create a response regulatory system. Upon completion of sporulation, if the conditions return to favourable for growth, fully maturated spore germinates and turn to vegetative stage. Moreover, spore's structure and chemical composition displays the important role in spore resistance and germination profiles. There are three crucial molecules in the spore that play major roles in providing resistance to spores. Those molecules are small acid-soluble proteins (SASPs), spore coat proteins and dipicolinic acid (DPA). SASPs are not only significant elements in spore resistance against heat, many chemicals, UV radiation but also they provide amino acids for protein synthesis during germination. Moreover, the coat provides resistance against some chemicals, exogenous lytic enzymes that can degrade spore cortex and predation by protozoa. Furthermore, the accumulation of DPA in the spore core is responsible for the reduction in core water content during sporulation and additionally DPA plays a significant role in the UV photochemistry of spore DNA.

There are so many reports regarding the involvement of the peptide antibiotics in the sporulation process. Consistently, previous studies have implied that bacilysin, which is a small dipeptide antibiotic of 125 kDa, might be a control element in the sporulation process. Recently, *yvfI* gene was identified as a noval gene essential for bacilysin biosynthesis in *Bacillus subtilis*. As a further step, based on the relationship between YvfI and bacilysin biosynhesis, the present project aims to elucidate a possible effect of the *yvfI* gene product on the sporulation process. Under this respect, spore properties of wild type PY79 and its YvfI defective derivative strain TEK1 (*yvfI::Tn10::spc*) strains were compared. In order to investigate resistance profiles, both mutant type and wild type spores were treated with lysozyme, heat, hydrogen peroxide and chloroform. Subsequently, both small acid soluble proteins (SASPs) and spore coat proteins were extracted from spores of both wild type PY79 and *yvfI* disrupted strains. In case of SASP analysis, both PY79 and TEK1 spores were observed to express α, β and γ type SASPs as visualized like three main bands on a low pH PAGE. On the other hand, PY79 spores were examined to differ from TEK1 spores by a small number of spore coat proteins. Germination and outgrowth characteristics of TEK1 and PY79 spores were performed in order to examine the functional role of *yvfI* during the sporulation process, in detail. Both strains have displayed germination and outgrowth properties not only in rich medium 2x YT and but also in SMM medium. Level of DPA of above mentioned strains were also compared. Finally, the morphology of colonies of strains PY79 and TEK1 formed on the agar plate containing DS medium were also examined in order to

fulfill the overall aim of exploring more details about the functional role of *yvfI* gene product on the sporulation process in *Bacillus subtilis*.

Keywords: *yvfI* gene, *Bacillus subtilis*, sporulation, spore properties

ÖZET

yvfI MUTANT SPORLARI KARAKTERİZASYONU

Bacillus subtilis de sporulasyon, besin yetersizliği gibi olumsuz koşullara yanıt olarak, bakteri metabolizmasının gelişim aşamasında meydana gelen farklılaşmadır. Bu süreç, hücre dışı ve hücre içi sinyallere bağlı olarak, farklı gen ürünlerini ve faktörlerini harekete geçirecek bir düzenleyici sistem içerir. Ayrıca, bu sıradışı olan gelişimsel aşama farklılaşmada rol oyanayan bir çok gen arasındaki ardışık ve karmaşık etkileşimlerden oluşur. Sporulasyonun tamamlanması üzerine eğer koşullar büyüme için tekrar elverişli hale gelirse, olgunlaşmış spor çimlenir ve çimlenme sonrası büyüme evresi olan outgrowth aşamasına geçer. Ayrıca, sporun yapısı ve kimyasal bileşimi spor direnci ve çimlenme profilinde önemli bir rol oynamaktadır. Spora dirençliliğini sağlamada büyük role sahip üç önemli molekül vardır. Bu moleküller küçük asitte çözülebilen proteinler (SASPs), spor ceket proteinleri ve dipiklonik asittir (DPA). SASP' ler sadece ısıya, çeşitli kimyasallara UV radyasyonuna karşı spora dirençlilik sağlamada önemli unsur olmayıp aynı zamanda çimlenme sırasında protein sentezi için amino asit sağlarlar. Dahası, spor ceketi bazı kimyasallara, spor korteksinin yıkımını sağlayan egzojen litik enzimlere ve saldırgan protozoalara karşı dirençlilik sağlar. Bunlara ek olarak, spor çekirdeğindeki DPA birikimi sporulasyonda spor çekirdeğindeki suyun azalmazından sorumlu olup aynı zamanda spor DNA'sının UV fotokimyasında önemli bir rol oynamaktadır.

Sporlanmaya spesifik genlerin çeşitli antibiyotiklerle etkileşime girdiğine ilişkin raporlar sonucunda antibiyotiklerin sporlanmayla ilgili fonksiyonlarının olduğu hipotezi güçlenmiştir. Önceki çalışmalarda 125 kDa ağırlığında küçük bir dipeptid antibiyotik olan basilisinin sporlanmada kontrol elementi olabileceği gösterilmiştir. Ayrıca, son zamanlarda *Bacillus subtilis* de *yvfI* geninin basilisin biyosentezinde gerekli olduğu iddia edilmektedir. Yukarıda belirtilen sporulasyon ve *yvfI* geni arasındaki olası ilişkiyi ileri süren bulguların ışığı altında, mevcut projenin amacı *yvfI* geni ürününün olası etki bölegerinin sporulasyon süreci sırasında incelemektir. Bu amaçla, bu çalışmada yabani tip PY79 ve onun basilisin defektif türevi TEK1 (*yvfI::Tn10::spc*) sporlarının temel spor özellikleri karşılaştırıldı. Dirençlilik profillerini karşılaştırmak amacıyla, hem mutant suş hem yabanil suş sporları lizozim, ısı, hidrojen peroksit ve kloroforma maruz bırakıldı. Bunu takiben, SASP ve spor ceketi proteinleri, hem yabanil suş PY79'dan hem basilisin defektif suş TEK1'den izole edilmiştir. SASPs analizinde, üç ana bant olarak görüntülenen α, β, γ-tipi SASP' lerin, düşük pH' lı poliakrilamit jel elektroforezi kullanılarak her iki suşta da eksprese edildikleri gözlemlenmiştir. Diğer yandan PY79 sporları, TEK1 sporlarından az sayıda spor ceketi proteiniyle ayrılır. TEK1 ve PY79 sporlarının çimlenme ve outgrowth karakteristikleri incelenmiştir ve bu sayede *yvfI* geninin sporlanmada fonksiyonu detaylı gözlemlenmiştir. Sonuçlar göstermiştir ki, TEK1 ve PY79 sporları yalnızca zengin besiyeri olan 2x YT' de değil aynı zamanda SMM fakir besiyerinde de çimlenme ve outgrowth profili göstermiştir. DPA seviyeleri

yukarıda belirtilen suşlarda karşılaştırılmış. Son olarak, *Bacillus subtilis* 'de sporulasyon sürecinde *yvfI* gen ürününün fonksiyonel rolünü incelemek amacıyla PY79 ve TEK1 sporlarının DSM agarda koloni morfolojisi incelenmiştir.

Anahtar Kelime : *yvfI* geni, *Bacillus subtilis*, sporlanma, spor özellikleri

1. INTRODUCTION

1.1. *Bacillus subtilis* lifecycle

Bacillus subtilis is a spore-forming Gram positive bacterium which follows a process of sporulation to form remarkably resistance endospore, in response to starvation (Setlow, 2003). Early in sporulation, a division septum is placed asymmetrically generating a larger compartment termed the mother cell, and a smaller compartment termed the forespore. After a series of highly regulated events, the mother cell lyses, releasing the mature spore into the environment. Although being metabolically dormant, the spore retains its ability to sense nutrients and when they become available, the spore will move through the processes of germination and outgrowth until it has been converted back into a growing cell (Errington, 1993).

1.2. Sporulation in *Bacillus subtilis*

Under starvation condition, the Gram positive soil bacterium *Bacillus subtilis* initiates a range of responses that allow it to survive in the difficult environmental conditions (De Jong et al., 2004). Included these responses are the production of antibiotics, the development of motility and competence, and as a final resort, the formation of dormant, environmentally resistant spore. Pursuing vegetative growth or entering sporulation includes radical changes in the genetic program and morphology [Figure 1.1] (Piggot and Hilbert, 2004; Errington, 1993; De Jong et al., 2004). During vegetative growth, the cell divides symmetrically and generates two identical cells, while during sporulation cell division is asymmetric and results in two different cell types: the forespore and the larger mother cell (Levin and Grossman, 1998; Michael, 2001).

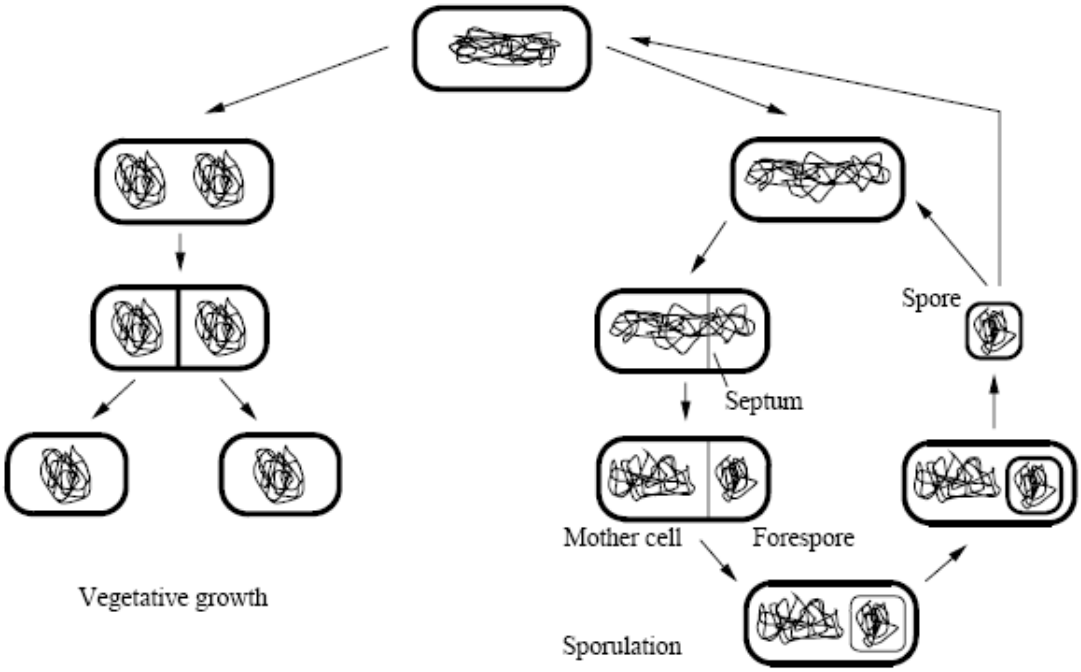


Figure 1.1 Life cycle of *B.subtilis* : decision between vegetative growth and sporulation.

1.2.1 Morphological stages of sporulation

During sporulation, the bacterial cell divides to produce two nonidentical cells of very different size and this process is divided into seven stages (Errington, 1993; Hilbert and Piggot, 2004).

Vegetative cells grow and divide to generate two identical cell, normally in stage 0. A division septum, composed the ring of the essential tubulin homolog FtsZ at midcell, generates two identical daughter cell. Under condition where the population density increases and nutrient are depleted, sporulation begins and cells enter stage I [Figure 1.2] (Piggot and Hilbert, 2004; Errington, 1993). In this stage, an axial filament of chromatin comprises and extends from one pole of the cell to the opposite site. RacA has been identified as a protein produced in sporulation which binds the chromosome and the polar division protein DivIVA, acting as a bridge, thereby anchoring the filament to the poles by RacA (Errington, 1993; Ben-Yehuda et al., 2003).

Following FtsZ divison septum relocalizes, to sites near the cell pole, the asymmetric division begins to form at one of those sites. The two unequal compartments will have significantly different developmental fates and size. The larger compartment,

the mother cell, will lyse at the ending of sporulation, while the smaller compartment, the forespore, will form into the mature spore and be released into the environment (Levin and Grossman, 1998; Chastanet and Losick, 2007). Completion of the spore septum is defined as stage II. After division, only the origin-proximal one-third of a choromosome is located in the forespore. The remainder of the choromosome is transported through the division septum into the forespore by the DNA translocase SpoIIIE, thereby mother cell and forespore have the identical genomes [Figure 1.2] (Piggot and Hilbert, 2004; Hilbert and Piggot, 2004).

Early compartmentalized gene expression results in modification of the asymmetric septum, so that its peptidoglycan is removed and it begins to migrate around the forespore which integrates to release the forespore within the mother cell, this process is known as a engulfment [Figure 1.2] (stage III). It has been shown that three critical engulfment proteins, SpoIID, SpoIIM, SpoIIP, are all produced in the mother cell and localize to the sporulation septum. These membrane bound proteins hydrolyze peptidoglycan so that membrane migration occurs around the forespore. These proteins are also needed to prevent a second asymmetric division in the mother cell. When they fail in functioning, a three-chambered 'abortivly disporic' organism is generated (Chastanet and Losick, 2007; Piggot and Hilbert, 2004).

Subsequently, the process of maturation begins [Figure 1.2] (stage IV). In stage IV, forespore becomes appearent as a layer and resistance to several environmental conditions. The first layers to be synthesized are the germ cell wall and the cortex. These layers are comprised of the peptidoglycan and are synthesized between the two membranes of the forespore. Then, the proteinaceouse spore coat begins to be deposited on the outsite surface of the spore (stage V). This step is followed by the stage VI (maturation), where forespore gains resistance against heat and most chemicals. The final step in sporulation (stage VI) includes lysis of the mother cell and the release of the mature spore into the environment. The spore is metabolically dormant and retains this state until nutrients become available. Eventually, the process of germination and outgrowth become initiated and spores return to their vegetatively growing state [Figure 1.2] (Errington, 1993; Nicholson et al., 2000; Eichenberger et al., 2001).

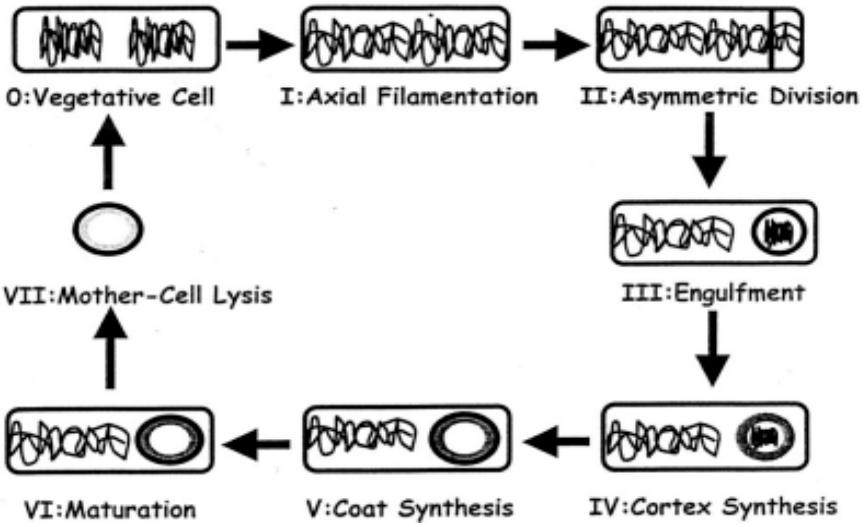


Figure 1.2 Morphological states of *B.subtilis* sporulation (Hilbert and Piggot, 2004). Stage 0 is defined as vegetative growth. Following, during stage I, an axial filament of chromatin condenses and forms along the long axis of the cell. Stage II is determined by the formation of asymmetric division septum, creating two different types of cells, the mother cell and the forespore. In stage III, forespore is engulfed by the mother cell. When engulfment has been completed, cortex and germ cell wall are deposited between membranes surrounding the forespore in stage IV. The proteinaceous spore coat is synthesized on the outside surface of the spore during stage V. Maturation is defined as stage VI, when the spore gains the characteristic properties of resistance. In stage VII, the mature spore is released through the lysis of the mother cell.

1.2.2. Initiation of sporulation

In response to limited nutrient sources and cell density signals, *B.subtilis* can differentiate and form a highly resistant endospore. This process requires a high input of energy and its initiation must be tightly regulated. Initiation of sporulation is controlled by the master regulator Spo0A, which is activated by phosphorylation with a multicomponent phosphorelay involving the primary kinase KinA and two intermediate phosphotransferases (Veening et al., 2009). Once phosphorylated, Spo0A~P directly regulates more than 100 genes. Initial activation of Spo0A occurs at the end of exponential growth leading to the transition state where cells produce proteases, become competent for transformation, show biofilm formation and even enter in cannibalism (Piggot and Hilbert, 2004; Molle et al., 2003). As sporulation progresses, there is a increase in the amount of phosphorylated Spo0A (Spo0A~P) in the mother cell, and a decrease in the forespore. It has been shown that both of these changes are necessary for continued spore formation (Piggot and Hilbert, 2004;

Molle et al., 2003; Errington, 1993; Doan and Rudner, 2007; Ramamurti and Losick, 2005).

Phosphorelay system consists of five histidine autokinases (KinA, KinB, KinC, KinD, KinE) and two phosphorelay proteins (Spo0F and Spo0B). Each of these kinases can phosphorylate Spo0F, a protein which serves as an intermediate in the phoshorelay. Moreover, KinA is primary kinase and is necessary for phosphorylation of Spo0A [Figure 1.3] (Veening et al., 2005; Piggot and Hilbert, 2004). Spo0B is a phosphotransferase that moves phosphate from Spo0F~P to Spo0A [Figure 1.3]. The phosphorylation of Spo0A leads to the activation of a number of sporulation genes as well as the repression of *abrB* [Figure 1.3]. *abrB* encodes the protein which represses the expression of a number of genes involving *kinA* and *sigH.* Similar to Spo0A , SigH is needed for the initiation of sporulation as it transcribes many of the genes included in the phosphorelay involving *kinA, Spo0F* and *Spo0A* [Figure 1.3].

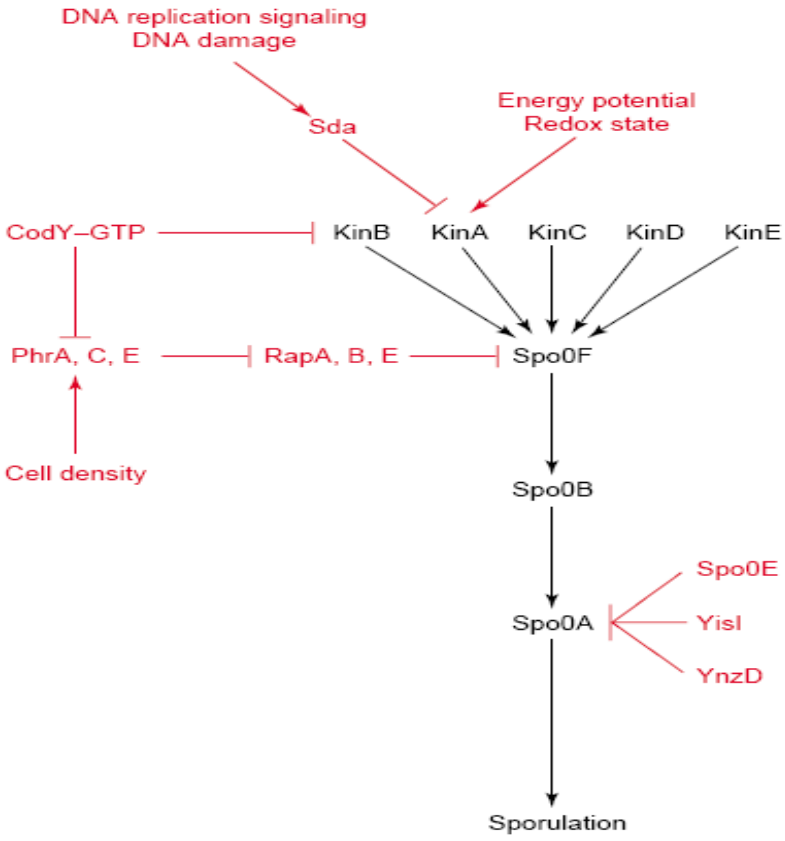


Figure 1.3 Initiation of sporulation. KinA-E, five histidine kinases phosphorylate Spo0F, that transfers the phosphate to Spo0B. Spo0B transfers it to Spo0A, activating transcription of crucial sporulation genes. Sda expression is activated by DNA damage, thereby preventing

sporulation. When cell density is sensed by Phr peptides, Rap proteins dephosphorylate Spo0F. Transcription of *kinB* and several *phr* genes are repressed by CodY and also Spo0A can be repressed by Spo0E, YisI, YnzD.

Sporulation is initiated in response to a number of external and internal signals. Initiation takes place only when there is a high population density, nutrients are depleted, DNA damage is at a minimum level and there is no block in DNA replication (Veening et al., 2005; Piggot and Hilbert, 2004). When cell density is low, the Rap proteins A, B and E which are aspartly-phosphatases, dephosphorylate Spo0F~P, inhibiting phosphorylation of Spo0A and therefore initiation of sporulation. Spo0A~P is dephosphorylated through the action of Spo0E and two homologs YisI and YnzD; expression of each protein is increased in non-sporulation conditions (Piggot and Hilbert, 2004). As the cell density increases, expression of phosphatase inhibitors, *phrA, phrC, phrE* cause stopping the activity of RapA-B-E proteins and this inhibition allows sporulation to begin [Figure 1.3] (Levin and Grossman, 1998; Veening et al., 2005; Piggot and Hilbert, 2004). Importantly, these genes are repressed by CodY which is the key sensor of guanine nucleotide levels. When nutrient levels are high, there is a quantity of available GTP in the cell. CodY is a protein that senses the level of available guanine nucleotides. When GTP levels are high, CodY represses expression of *phrA* and *kinB*, but when GTP levels are low, these genes are derepressed and sporulation is initiated. Finally, DNA damage and / or a block in DNA replication lead to the expression of Sda (suppressor of *dnaA*) , a protein that inhibits KinA autophosphorylation thereby inhibiting sporulation (Hilbert and Piggot, 2004; Veening et al., 2009; Piggot and Hilbert, 2004). Importantly, this internal cell cycle regulation allows only those cells that have intact, replicating choromosomes to enter sporulation (Micheal, 2001; Veening et al., 2005; Burkholder et al., 2001; Ozin et al., 2001; Stephenso and Hoch, 2002).

1.2.3. Compartmentalized gene expression of sporulation

Activation of Spo0A and σ^H play crucial roles during sporulation and are essential for initiation of this differentiated state as mentioned above. However, σ^H conducts the transcription of *spo0A* and *ftsZ*, an operon which is needed for asymmetric division (Levin and Grossman, 1998; Molle et al., 2003). When Spo0A~P and σ^H levels are high, immediately asymmetric division septum occurs and compartmentalized gene expression follows this septum formation [Figure 1.4].

Before septation, the transcriptional factor σ^F is kept inactive by the anti-σ factor SpoIIAB. Inhibition is reversed by the binding anti-anti-σ factor SpoIIAA to SpoIIAB- σ^F complex and this reverse activation is catalyzed by SpoIIE thereby releasing free σ^F in the prespore [Figure 1.4] (Hilbert and Piggot, 2004; Errington, 1993; Levin and Grossman, 1998).

Furthermore, mother cell specific sigma factor σ^E is synthesized in the mother cell, initially as the inactive membrane-bound precursor, pro-σ^E. Expression of *spoIIG*, encoding pro-σ^E, is under control of the master response regulator Spo0A. σ^F activates the expression of *spoIIR* in the forespore which creates a signal for the mother cell to process pro-σ^E to an active state. Once the level of pro-σ^E reaches a critical threshold in the prespore, this contributes to compartmentalized σ^E activity and more than 253 genes become directly controlled by sigma-E, including σ^F encoding gene [Figure 1.4] (Piggot and Hilbert, 2004; Chastanet and Losick, 2007; Blaylok et al., 2004).

Activation of the σ^E is followed by activation of the another forespore sigma factor, σ^G transcription of which is directed by σ^F [Figure1.4]. Following complete engulfment, the σ^E-dependent SpoIIIA proteins act as signals to activate σ^G in the forespore. This activation of σ^G into an active state, allows the transcription of *spoIVB* which encodes a protein leading to processing of σ^K from its precursor pro-σ^K. It should also be noted that both σ^G and σ^K can stimulate their own synthesis by a positive feedback mechanism (Doan et al., 2005; Wang et al., 2007; Piggot and Hilbert, 2004).

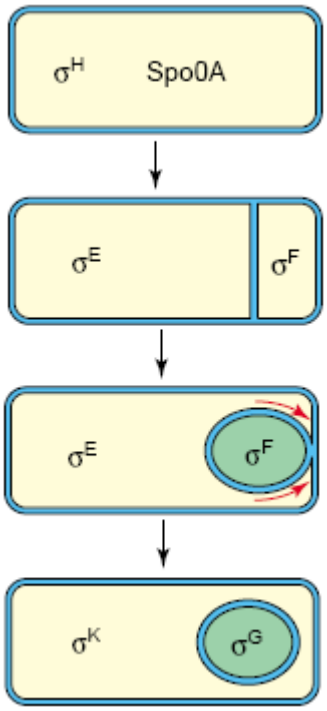


Figure 1.4 Gene regulation during spore formation (Piggot and Hilbert, 2004). High level of Spo0A~P and σ^H leads to asymmetric division. Compartmentalized gene expression starts with σ^F, becoming active in the forespore and σ^E, in the mother cell. σ^F transcribes σ^G in the forespore and sends a signal to mother cell to process pro-σ^E to σ^E. Activation of σ^E causes the transcription of σ^K in the mother cell as well as transcription of σ^G in the forespore following completion of engulfment. Eventually, the late mother cell transcription factor σ^K is formed from pro-σ^K.

1.2.4. Resistance Properties and Structure of Spores

Spores have a number of mechanisms that are responsible for spore resistance against enzymes, chemicals, heat and radiation. Complex spore structures not only promote the survival of the spores but also give the spore extended properties in case of spore resistance profiles (Setlow, 2005). Additionally, the level of spore core mineral ions and stability of total spore proteins also play crucial roles in providing spore with resistance. α-β type small-acid soluble proteins (SASPs) and dipicolinic acid (DPA) which are deposited in spore core, are the structural elements that function in protecting DNA against vital damages. Moreover, chemical agents are reacted by spore coat proteins and the relative impermeability of spore's inner membrane restricts access of chemicals through the spore core (Paidhungat et al., 2000; Cabrera-Hernandez et al., 1999; Setlow, 2005).

As mentioned, spore structure and composition are important in spore resistance. Starting from the inside and moving to the outside, the spore layers include a central core, inner membrane, germ cell wall, cortex, outer membrane, coats and the exosporium but not in case of *B. subtilis* which does not possess an exosporium [Figure 1.5] (Henriques et al., 2000; Driks, 1999). The spore core includes most of the spore enzymes, DNA, ribosomes, tRNAs. Furthermore, there are also three small molecules that act as keys in spore resistance: the first one is water. In the growing cell, water contains about 75-80 % of the wet weight, on the contrary, in the spore, water comprises only 27-55 % of wet weight. Due to the low water content in the spore core, macromolecular movement is found to be minimal. This is likely the major factor in the spore's enzymatic dormancy, and plays a major role in the spore's resistance to wet heat (Setlow, 2005; Magge et al., 2008). The spore core is dehydrated until nutrients become available and germination processes starts. In the first minutes of germination, the spore core restores its water content and consequently, macromolecular motion and enzymes activity are regained (Setlow, 2005).

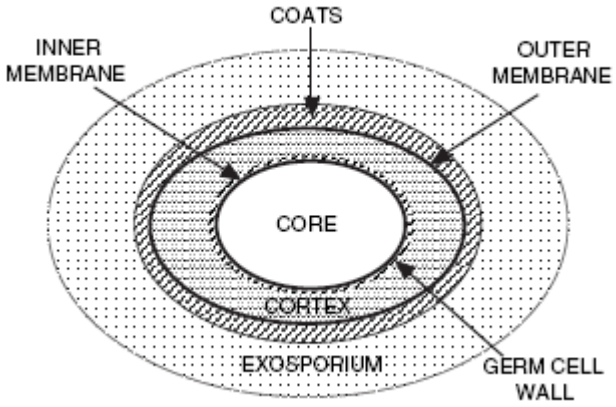


Figure 1.5 Spore structure (Setlow, 2005). The inner core layer is surrounded by an inner membrane. This is followed by the germ cell wall and the layer of peptidoglycan called the cortex. An outer membrane surrounds the cortex and proteinaceous coat is located just under the exosporium but not in case of *B. subtilis* which does not possess an exosporium.

The second important small molecule is pyridine-2,6-dicarboxylic acid (dipicolinic acid, DPA) [Figure 1.6]. DPA is synthesized in the mother cell and taken up into the forespore during stages V and VI of sporulation (Paidhungat et al., 2000). It comprises 5-15 % of dry weight of spores and is located only in the core. Besides, it

is likely chelated with divalent cations, especially Ca^{2+} in the spore core, but the mechanism of DPA deposition in the spore is still unknown (Magge et al., 2008). Yet, it is known that the level of the DPA in the spore core is above its solubility, and it has been suggested that the core is in a glass-like state, but this has not been proven. DPA also plays a significant role in spore resistance to UV radiation and wet heat (Setlow, 2005). By replacing water in the core during sporulation, DPA protects the spore core from wet heat. However, it is well established that dehydrated spores are resistant to wet heat, the mechanism for this resistance is still unknown (Magge et al., 2008; Ragkouski et al., 2003; White et al., 2004).

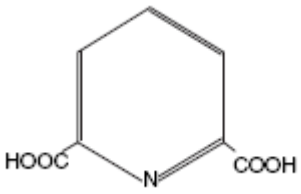


Figure 1.6 Structure of dipicolinic acid. DPA is synthesized in mother cell and taken up by the forespore.

In addition to its role in spore resistance, DPA is an effective germinant for spores of *Bacillus subtilis*. DPA synthesis is catalyzed by DPA synthase, consisted of the SpoVFA and SpoVFB protein which are encoded by the sporulation specific operon *spoVA* , but the mechanism of the DPA uptake is not known (Magge et al.,2008; Eriington, 1993). While the Ca-DPA pool in the spore core is highly stable, DPA and its associated cations are released rapidly when spore germination is begins. Recent research has suggested that SpoVA proteins are involved in the release of Ca-DPA. During spore germination, the release of Ca-DPA also directs the signal transduction such that while this release is considered as stage I, it also functions in signaling the initiation of stage II of germination, and moreover it also leads to the crucial event of the spore's peptitoglycan cortex hydrolysis by either of the redundant cortex-lytic enzymes, CwlJ or SleB (Setlow, 2003; Magge et al., 2008).

The third type of small molecule in the spore core is a group of small, acid-soluble proteins (SASP). The major type of SASP found in the core are of the α/β- type. These proteins refer to 3-6% of total spore proteins and are synthesized in the forespore during stage IV of sporulation under transcriptional control (Setlow, 2005). The SASPs have two main functions. First, they bind to the spore's DNA and protect it from physical damage. Interestingly, these proteins do not contain any known

DNA binding motifs, suggesting that their interaction with DNA is by a novel mechanism. By binding to the spore's DNA, these proteins prevent the formation of cyclobutane-type pyrimidine dimmers induced by UV radiation. Instead of this typical DNA damage, a novel spore photoproduct is created, but only between adjacent thymine residues. This photoproduct can be easily and accurately repaired during germination and outgrowth. The SASPs are also important in spore resistance to heat and many chemicals. The second major role of these small proteins occurs during germination and outgrowth. Towards the end of germination SASPs are readily degraded by an aspartic acid protease, termed germination protease (GPR). The products of this proteolysis serve as sources of amino acids for the outgrowing spores (Nicholson et al., 2000; Lee et al., 2007; Coshon et al., 1999; Mohr et al., 1991; Moeller et al., 2009).

These 59-75 residue proteins are encoded by multiple genes, encoded by *sspA, sspB,* and comprise of a protein family with highly conserved intraspecies amino acid sequences. Furthermore, those are nonspecific DNA-binding proteins with apparent binding constants for random sequence DNA of 15-100 mM (Lee et al., 2007; Loshon et al., 1997; Moeller et al., 2009; Price and Kosick., 1999; Setlow et al., 1998). High levels of α/β-type SASPs in spores are sufficient to saturate the spore DNA and this nucleoprotein complex is protected from DNA damage caused by heat, many genotoxic chemicals, enzymes and UV radiation. In addition, the third major SASP in *B. subtilis* spores, SASP-γ, encoded by *sspE*, play no role in spore resistance (Loshon et al., 1997). The only known fuction of SASP-γ is providing amino acid for protein synthesis during developmental period. In addition to this three major SASPs, *B.subtilis* spores also contain a large number of minor SASPs, encoded by *sspC, sspD, sspF, sspH, sspI, sspJ, sspK, sspL, sspM, sspN, sspO, sspP*. Since these minor SASPs are very small proteins and their exact number was not identified as ORFs in the initial analysis of complete *B. subtilis* genome (Cabrera-Hernandez and Setlow, 2000; Cabrera-Hernandez et al., 1999; Cucchi and Rivas., 1998; Connors et al., 1986; Yamamoto et al., 1999)

Located just outside of the core is the inner membrane [Figure 1.5] (Setlow, 2005). During sporulation this membrane is derived from the plasma membrane that surrounds the forespore compartment. The inner membrane functions as a strong permeability barrier for the spore and plays an important role in spore resistance to

many chemicals. In the dormant spore, the lipids within this membrane are immobile, however, they become fluid upon completion of germination (Setlow, 2005; Wuytack and Michiels, 2001). During the first minutes of germination, the cortex is degraded and the volume encompassed by the inner membrane expands approximately two-fold as the spore rapidly rehydrates (Setlow, 2005).

Surrounding the inner membrane, two layers of peptidoglycan are located; the germ wall and the cortex [Figure 1.5]. The germ wall is located just beneath the cortex and has a structure identical to the peptidoglycan layer of a vegetative cell. In this layer the peptidoglycan is made of alternating β-1-4-linked N-acetly glucosamine and N-acetly muramic acid (Atrih and Foster, 1999). This layer is highly cross-linked and is not degraded during germination and outgrowth. Instead, the germ wall becomes the cell wall of the outgrowing spore.

In contrast, the cortex is consisted of peptidoglycan that is different from that of a vegetative cell. The cortex peptidoglycan is made of muramic acid,~50% of which is muramic acid δ lactam, which acts as a recognition determinant for enzymes that degrade the cortex during germination and outgrowth (Popham et al., 1996). Degradation of the cortex is essential for the expansion of the spore core as well as for outgrowth. Moreover, the cortex is much less cross-linked than the germ wall suggesting that the spore peptidoglycan is more flexible and thus perhaps more easily degraded than the bacterial cell wall (Setlow, 2005; Moriyama et al., 1996; Boland et al., 2000).

The outer spore membrane is located outside of the cortex [Figure 1.5]. This membrane is essential during spore formation, however, its exact role in the dormant spore is not known. Unlike the inner spore membrane, the outer membrane is not a strong permeability barrier for the spore. Researches have show that when the coat and the outer membrane are removed, there is no noticeable effect on spore resistance against heat, radiation, and at least some chemicals (Setlow, 2005; Popham et al., 1996).

The outmost layer in spores of *Bacillus subtilis* is the spore coat [Figure 1.5]. This layer is a complex structure made of more than 50 proteins (Setlow, 2005). In case of spore resistance profiles, coat proteins are important against chemicals, exogenous lytic enzymes, predation by protozoa, but have no definite role against heat, radiation and some other chemicals. Electron microscopy has revealed that the coat of

B.subtilis spore has two major layers: a thicker and layered outer coat and an inner coat (Driks, 2002; Henriques and Moran, 2000). The coat proteins are synthesized in the mother cell during sporulation and then layered onto the developing forespore in a series of steps that are coupled to developmental events that drive spore formation. Any loss of at least five coat morphogenetic proteins, SpoIVA, SpoVID, SafA, CotE, and CotH has significant and in same cases dramatic effects on coat assembly and final coat structure (Driks, 1999; Henriques and Moran, 2000; Driks, 2002; Beall et al., 1993; Aronson and Fitz-James., 1976).

Under the control of σ^E, CotE and CotJ are expressed in the mother cell as well as the coat morphogenetic genes *spoIVA* and *spoVID* [Figure 1.7.]. Proper localization of SpoIVA to the outer forespore surface needs at least one other protein, SpoVM, whose expression is also under control of σ^E. In the absence of SpoIVA, a sporulating cell will begin normal development, but will not form a cortex and thus will not complete sporulation (Serrano et al., 1999; Driks 1999; Munoz et al., 1978; Pandey and Aronson, 1979). Two additional proteins, SpoVID and SafA also play significant roles in coat morphogenesis. Both proteins are under the control of σ^E, have been shown to interact during the early stage of coat assembly (Lai et al., 2003).

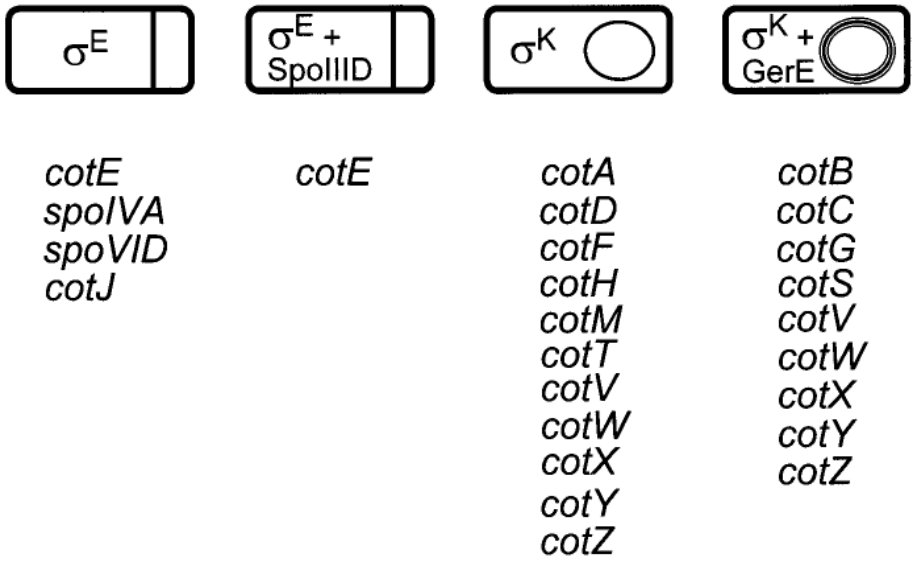


Figure 1.7. Gene expression in Coat assembly. The morphological stages of sporulation are represented with mother cell cascade of gene expression.

Level of CotE expression is also under control of σ^E and SpoIIID that can either repress or activate gene expression. Once SpoIVA is localized on the outer surface of the forespore, a ring of CotE, SpoIVA-dependent morphogenetic protein is

assembled 75 nm from SpoIVA (Little and Driks, 2001). The space between SpoIV and CotE is called the matrix and is of unknown composition, subsequently this structure is called precoat. After engulfment, σ^K becomes active and directs the expression of a large group of coat protein genes and the cortex appears. The σ^K – dependent regulon is consisted of *cotA, cotD, cotF, cotH, cotM, cotT, cotV, cotW, cotY* and *cotZ* genes [Figure1.7.]. When σ^K becomes active, the matrix is converted into the inner spore coat, while the outer spore coat forms around the ring of CotE (Little and Driks, 2002; Driks,1999; Ichikawaet et al., 2000; Drink et al., 1993). CotD, CotH, CotS and CotT assemble into the inner coat, CotA and CotM are formed into the outer coat. CotH is proposed to be localized near CotE at the interface between the inner and the outer coat layers. The final transcriptional factor gene is *gerE,* encoding a DNA-binding protein that acts in conjuction with σ^K to regulate transcription of several coat protein genes, encompassing *cotB, cotC, cotG, cotS, cotV, cotW, cotX, cotY* and *cotZ* [Figure 1.7.]. In a final stage of coat assembly, under the control of GerE, CotB and CotG are synthesized and incorporated into the outer coat. Further modifications, consisting of glycosylation, proteolysis and cross-linking, bring the coat to its final form (Henriques et al., 1997; Isticato et al., 2004; Naclerio et al., 1996; Ragkousi and Setlow, 2004; Zilhão et al., 2004).

1.3. Germination and Outgrowth of *Bacillus subtilis*

Dormant spores of the *Bacillus subtilis* continuously monitor their environment and when nutrient become available, they begin the processes of germination and outgrowth (Setlow, 2003). In nature, spores will germinate in response to specific nutrients including single amino acids, sugar or purine nucleosides. However, in the laboratory it is a also possible to germinate spores with different combinations of nutrients such as a mixture of asparagine, glucose, fructose and potassium (AGFK), as well as other agents including lysozyme, salts, high pressure, Ca^{2+}-DPA and dodecylamine. A spore will become committed to germinate within seconds of sensing one of these germinants, but the mechanism of commitment is still unknown (Chirakkal et al., 2002; Vepachedu et al., 2007; Aertsen et al., 2005; Bounne et al., 1991). The mechanism by which a spore becomes activated in nature prior to germination is also unknown, but it is understood that for many species, activation can increase synchrony of germination in a spore population [Figure1.8,] (Setlow,

2003). In the laboratory a sub-lethal heat shock is often used to activate a population of *B. subtilis* spores before germination (Aertsen et al., 2005; Keijser at al., 2007).

Once the spore is activated, Stage I of germination begins when a germinant permeates the spore coat and the cortex layers, and comes into contact with a germinant receptor located in the inner spore membrane (Masayama et al., 2007). This reception triggers the release of monovalent and divalent cations, as well as the larger depot of Ca^{2+}-DPA from the spore coat [Figure1.8]. Consequently, germinating spores take up some water and rehydrate the core, although the amount of water that enters the core is not sufficient for enzyme activity or protein mobility. The increase in core water content in Stage I does, however, cause some loss of resistance to wet heat (Setlow, 2003; Horsburgh et al., 2001; Hundson et al., 2001; Pelczar et al., 2007)

Stage II of germination in *B.subtilis* spores begins with hydrolysis of the peptidoglycan cortex by the cortex lytic enzymes SleB and CwIJ [Figure 1.8]. In the laboratory, lysozyme can also be used to degrade the cortex, However, this chemical degradation first requires removal of the spore coat to allow access to the cortex. Spores lacking both *sleB* and *cwIJ* are unable to outgrow or form colonies on agar media unless the cortex is hydrolyzed by treatment with lyzosyme. Both SelB and CwIJ need muramic acid-δ-lactam peptidoglycan for their action. This ensures that only the cortex peptidoglycan is hydrolyzed during germination and germ wall is kept intact (Paidhungat and Setlow, 1999; Hornstra et al., 2005; Ducros et al., 2001).

SleB is synthesized in the forespore and localized both in the inner membrane and the outer layers of the spore. For proper localization in the spore, SleB requires an additional protein, YpeB, which also gets localized in the inner membrane and outer layers of the spore. In *B.subtilis,* SleB functions as a lytic-transglycosylase type muramidase, but the mechanism by which it is activated is still unknown (Igarashi et al., 2004).

In contrast to SleB, CwlJ is synthesized in the mother cell during sporulation and is then localized on the outer layers of the spore. However, like SleB, in *B. subtilis* CwlJ also requires an additional spore coat protein, GerQ, for proper localization into the spore. Even though it is known that CwlJ is necessary for germination, its enzymatic function is not yet understood. Potentially, CwlJ is activated when the

spore's large depot of Ca^{2+}-DPA is released from the core during the first stage of germination (Vepachedu et al., 2007; Vepachedu et al., 2004; Setlow, 2003).

Both SleB and CwlJ are synthesized suggesting that there is a mechanism that keeps these enzymes inactive in the dormant spore. The mechanism that keeps SleB in an inactive state in the dormant spore is not clear. However, it is thought that CwlJ may remain inactive until after Satge I of germination because it needs Ca^{2+}-DPA for activaton (Setlow, 2003; Aertsen et al., 2005; Keijser et al., 2007; Ragkousi et al., 2000).

After cortex hydrolysis, the next step in germination includes swelling of the spore core due to additional water uptake, and expansion of the germ cell wall [Figure1.8]. This level of core hydration is sufficient for protein mobility and enzyme activity in the core, and also leads to the loss of spore resistance as well as dormancy (Atrih and Foster, 2001; Atluri et al., 2006). Once the cortex has been hydrolyzed and the core has been rehydrated, the spore begins the process of outgrowth. The enzymatic activity that began in Stage II of germination is continued during outgrowth along with metabolism, degradation of the SASPs, and macromolecular synthesis [Figure1.8] (Setlow,2003; Bagyan and Setlow, 2002). Upon activation, GPR acts on SASP for degradation in order to set spore DNA free for transcription following spore germination. The oligopeptides, that are the end result of SASP degradation, are further degraded to amino acids by peptidases and these amino acids are used for protein synthesis and energy metabolism during spore outgrowth. The final stage of outgrowth involves cracking of the spore coat and release of the new vegetative cell into the environment [Figure1.8] (Setlow, 2003; Black et al., 2005).

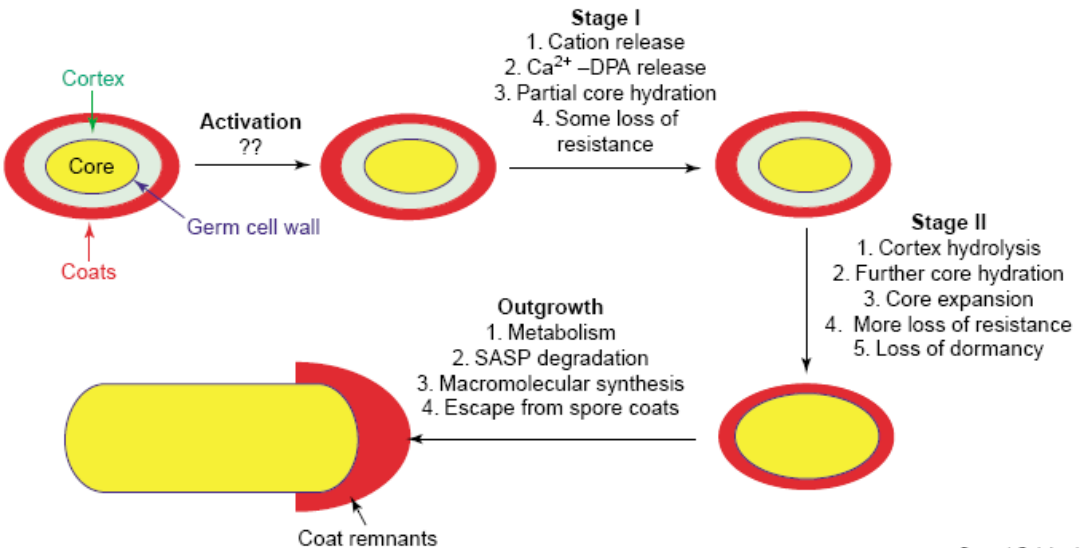


Figure1.8. Events of Spore Germination and Outgrowth. The events occurring during the activation stage of germination are not well understood. Spores are heat shocked at a sub-lethal treatment to induce activation.

1.3.1 Germination Receptors

Germination of *B. subtilis* spores begins with the binding of specific nutrient germinats, either L-alanine or AGFK, to specific receptors that are located in the spore inner membrane (Setlow, 2003; Black et al., 2006). Three functional nutrient receptors are found in *B. subtilis* spores which are encoded by homologous tricistronic *gerA*, *gerB*, and *gerK* operons. These operons are expressed late during sporulation and under control of σ^G (Setlow, 2003; Igarashi and Setlow, 2006). The GerA nutrient receptor responds to L-alanine while the GerB and GerK nutrient receptors are required together to respond to AGFK (Setlow, 2003). When all three germinant receptors are deleted, spores fail to germinate with nutrients but they do maintain a slow spontaneous rate of germination, the mechanism of which is not fully known (Paidhungat et al., 2001). The necessity for the presence of both the GerB and GerK receptors to initiate germination with AGFK suggests that these two receptors may interact in some manner. Other analysis demonstrates that the germinant receptors are capable of interacting with SpoVAE and SpoVAD proteins which may be involved in Ca^{2+}-DPA release during germination (Igarashi and Setlow, 2005; Vepachedu and Setlow, 2007). It should be noted that germination receptors themselves and protein subunit within receptors may interact with each

other, moreover, these receptors may have the ability to interact with other proteins (Vepachedu and Setlow, 2007).

1.3.2 Non-nutrient Germination

In addition to nutrients, there are also alternative pathways, in case followed, germination of *B. subtilis* spores may be achieved without involving germination receptors. Some agents which lead to this type of germination are lysozyme, Ca^{2+}-DPA, high pressure and salts, although the direct mechanism of action for many of these agents is not fully understood (Paidhungat et al., 2001; Black et al., 2006; Black et al., 2005). Lysozyme not only degrades the spore cortex but also degrades the germ wall allowing spores lysis. However, if germination is induced in a hypertonic medium, spores retain their structure, the cortex is degraded and DPA is released allowing these spores to germinate and give rise to colonies (Moir, 2005; Tovar-Rojo et al., 2002).

Ca^{2+}-DPA has been suggested to function in germination through direct or indirect activation of CwlJ, however the mechanism of this activation is still not known (Paidhungat et al., 2001). Spores lacking CwlJ do not germinate with addition of exogenous Ca^{2+}-DPA, while spores lacking SleB do germinate with exogenous Ca^{2+}-DPA, supporting the hypothesis that CwlJ is crucial for Ca^{2+}-DPA induced germination (Paidhungat et al., 2001).

Cationic surfactants such as dodecylamine also germinate spores, and have been suggested to open the Ca^{2+}-DPA channels of the spore (Setlow, 2003; Tovar-Rojo et al., 2002). Germination with dodecylamine does not need any germinant receptors, whereas either CwlJ or SelB are required for this process (Setlow, 2003; Tovar-Rojo et al., 2002; Vepachedu and Setlow, 2007).

1.4. Bacilysin and *yvfI* gene

Bacillus species produce a wide variety of secondary metabolites with antimetabolic and pharmacological activities. Most of these metabolites are small peptides that have unusual components and chemical bonds (Yazgan et al., 2003). Certain strains of *Bacillus subtilis* produce and secrete extracellularly a simple dipeptide antibiotic; bacilysin (Hilton et al., 1988). Bacilysin is 125 kDa dipeptide antibiotic which is composed of a N-terminal L-alanine and C-terminal L-anticapsin (Köroğlu et al.,

2008). Antibiotic moiety of bacilysin comes from its L- anticapsin residue when its released from alanine residue following the uptake into the cell and hydrolysis by peptidases in the cytosol. Glucosamine syntetase activity that determines bacterial peptidoglycan biosynthesis is inhibited by free anticapsin molecules (Kenig et al., 1975). Using Tn10 mutagenesis technique, previous study showed that the biosynthesis of bacilysin antibiotic is under control of quorum sensing global regulation involving the action of ComQ/ComX, PhrC (CSF), ComP/ComA and in a Spo0K (Opp)-dependent manner in *B. subtilis* (Yazgan et al., 2003). According to the data given by Yazgan et al. In 2003, *srfA* operon is possibly not only responsible for the surfactin biosynthesis but also necessary for the bacilysin production. In addition, *spo0A* blocked-mutants showing bacilysin-negative phenotype and *abrB* disrupted-mutants indicating an increase in production of bacilysin and the suppression of bacilysin-negative phenotype by an *abrB* mutation in *spo0A* blocked-mutants revealed that the transcription of some genes involved in bacilysin production is under the control of *abrB* gene and Spo0A protein (Yazgan et al., 2003). Biosynthetic core function on bacilysin production depends on *ywfBCDEFGH* cluster and this operon was renamed as *bacABCDE*. *bacD (ywfE)* is recently announced as a novel gene synthesizing L-amino acid ligase belonging to ATP-dependent carboxylate-amine/thiol ligase superfamily and this ligase is functional in the amino acid ligation of anticapsin to alanine (Stein, 2005). Besides, *yvfI* was recently identified as a novel gene required for bacilysin production by transposon mutagenesis. The *yvfI* gene is already known to encode an unknown protein that resembles to the GntR family of transcriptional regulator (Kunst et al., 1997). Regulators from this family are consisted of a conserved N-terminal DNA binding domain followed by C-terminal domain involved in the effector binding and/or oligomerization (Köroğlu et al., 2008). Very recent study has shown that the *lutABC* operon is under the control of *yvfI,* consequently renamed as *lutR* (Chai et al., 2009).

1.5. The Aim of the Present Project

In *Bacillus subtilis*, bacilysin production is regulated via global regulation system and this system is also responsible for sporulation (Köroğlu et al., 2008). Previous work published by Özcengiz and Alaeddinoğlu in 1991 suggested that bacilysin-negative mutant (bac^-) NG79, which was obtained by NTG mutagenesis, was less

resistant to heat, chloroform, lysozyme treatments and these spores contained less dipicolinate. Moreover, the transfer of the *bac* locus to the bacilysin-negative mutant NG79 reassembled its sporulation ability. Furthermore, external addition of bacilysin to the cultures of the bacilysin-negative mutant NG79 to investigate if the bacilysin is directly responsible for the sporulation defect, demonstrated that NG79 has restored its sensitive profile (Özcengiz and Alaeddinoğlu, 1991). All of these findings suggested that bacilysin can be a control element in the sporulation process in *Bacillus subtilis*. Consistently, the fact that *yvfI* gene product is required for bacilysin production rise the possibility that YvfI has a functional role in sporulation process. Therefore, objective of this study was to examine the possible action sites of *yvfI* gene product in sporulation process. Under this respect, in this study, *B.subtilis* TEK1 (*yvfI*::*Tn10*::*spc*) strain that was previously constructed by using Transposon *Tn10* Mutagenesis, and that manifests bacilysin negative phenotype was used for the comparison studies on spore properties. Initially, both wild type PY79 spores and *yvfI* mutant TEK1 spores were treated with heat, chloroform, lysozyme and hydrogen peroxide to investigate resistance profiles of *yvfI* spores. Furthermore, the level of accumulation or production of dipicolinic acid were also determined in TEK1 spores and compared to wild type spores. Moreover, in order to examine the effect of spore coat proteins on spore resistance profiles and germination process, spore coat proteins on the outer face of outer forespore membrane were extracted from purified spores of *yvfI::Tn10::spc* mutant (TEK1) and wild type (PY79) strains. Besides, possible differences in small acid-soluble protein (SASPs) profiles between both strains were also exploited. Subsequently, nutrition specific germination and outgrowth characteristics of TEK1 and PY79 spores, in rich and minimal media, were characterized in germination assays. Finally, colony morphology of the strains PY79 and TEK1 on the agar plate containing Difco Sporulation medium were investigated in order to fulfill the overall aim of exploring more details about the functional role of *yvfI* gene product on the sporulation process in *Bacillus subtilis*.

2. MATERIALS and METHODS

2.1. Materials

2.1.1. Bacterial Strains used in this study

Bacillus subtilis PY79, which is a prototrophic derivative of standard strain *Bacillus subtilis* 168 was used as the wild type strain. Previously TEK1 (*yvfI::Tn10::spc*) was constructed by Türkan Ebru Köroğlu. Strains and their genotypes that are used in this project are listed in Table 2.1.

Table 2.1: Bacterial strains and their genotypes used in this project

Strain	Genotype	Source
Bacillus subtilis PY79	Wild type, BSP cured prototrophic derivative of *B.subtilis* 168	P.Youngman
Bacillus subtilis TEK1	*yvfI::Tn10::spc*	T.E.Köroğlu

2.1.2. Bacterial Culture Media

The compositions and preparation of bacterial culture media are given in Appendix A.

2.1.3. Buffers and Solutions

The compositions and preparation of buffers and solutions are given in Appendix B.

2.1.4. Chemicals and Enzymes

The chemicals and enzymes used and their suppliers are given in Appendix C together with their suppliers.

2.1.5. Laboratory Equipment

The laboratory equipment used during the project is listed in Appendix F.

2.1.6. Maintanence of Bacterial Strains

B.subtilis PY79 and *B.subtilis* TEK1 (*yvfI*::*Tn10*::*spc*) strains were grown in Luria-Bertani (LB) liquid medium and kept on Luria-Bertani (LB) agar plates. Difco's Sporulation Medium (DSM) agar was used for the maintenance of *B.subtilis* PY79. 2xYT medium and Tryptophan and Glucose containing Spipizen's Minimal Medium (SMM) was used for the germination of *B. subtilis* PY79 and *B. subtilis* TEK1 strains. Difco's Sporulation Medium (DSM) were used for the induction of sporulation.

2.2. Methods

2.2.1. Construction of *yvfI* deletion in *B. Subtilis*

B.subtilis TEK1 (*yvfI*::*Tn10*::*spc*) strain was previously constructed by Türkan Ebru Köroğlu using Transposon *Tn10* Mutagenesis as a part of her master thesis project.

2.2.2. Mature spore isolation

Cells were grown in DSM and harvested at the end of 48th hour of culturing by centrifugation (10 000 g, 10 min, 4^{o}C). Spores were purified through washes in ice cold deionized water two times and then through a further lysozyme treatment (0.1 mg/ml, 37^{o}C, 10 min). Following this step, spores were collected once again and washed multiple times by centrifugation (10 000 g, 10 min, 4^{o}C) with ice cold deionized water. For the storage, purified spores were suspended in ice cold deionized water and kept in dark at -20^{o}C for a short-term preservation.

2.2.3. SASP protein extraction from spores

Prufied spores were lyophilized and spores (25 mg dry weight; strain PY79 and TEK1) were pulverized with 100 mg of glass beads at room temperature in a dental amalgamator (Wig-L-Bug). Ten 1-min periods of shaking were sufficient to disrupt > 90% of all mature spores. The 1-min periods of shaking were separated by 1-min periods with the machine at rest. The dry powder was removed from the Wig-L-Bug capsule and suspended with 2.5 ml of ice cold 0.3 M HCl in 15 ml centrifuge tube and then incubated 30 min on ice. After 30 min on ice with intermittent vortex mixing, the suspension was centrifuged (10000 g, 10 min, 4^{o}C). The supernatant was saved and the pellet was re-suspended with an additional 1.5 ml of 0.3 M HCl. After

centrifugation, both supernatant fluids was pooled and re-centrifuged if some particulate matter remains. The pooled supernatant fluid was ultrafiltrated, using low cut-off ultrafiltration tubes with 1% acetic acid, multiple times by centrifugation (3500 g, 20 min, 4^{o}C). Then the ultrafiltrated materials were lyophilized and the dry residue was suspended in 100 µl of 8M urea for analysis by polyacrylamide gel electrophoresis at low pH as described in Appendix B. For analysis by polyacrylamide gel electrophoresis, 10 µl of sample diluent was added to each sample (20 µl) and gel was run with the electrode polarity set appropriately for the fact that SASPs are positively charged and will run towards the cathode at 20 mA and 4^{o}C.

2.2.4. Coat protein extraction from spores

Approximately 5x10^{9} spores/ml were suspended in 1 ml of ST solution (1% (w/v) SDS, and 50mM dithiothretiol) and incubated the suspension in ST solution at 70 ^{o}C for 30 min. Spores were centrifuged (10000 g, 10 min) and the supernatant fluids were dialyzed overnight at 4^{o}C against 0.5 M sodium acetate/acetic acid buffer (pH 5.0), and then against several changes of distilled water at 4^{o}C using of low molecular weight cut-off dialysis tubing. The dialyzed materials were lyophilized and then dry residue was suspended in 40 µl of TMS buffer (130 mM Tris.Cl (pH 6.8), 2% SDS, 5% (v/v) β-mercaptoethanol, and 10% (v/v) glycerol) for analysis by SDS-PAGE as described in Appendix B. To run the gel, 10 µl of 0.002% bromphenol blue was added to each samples (20 µ) and boiled for 5 min before SDS-PAGE. Gel should be run at 25 mA until the dye is 0.5-1.0 cm from the bottom.

2.2.4.1. Preparation of Dialysis Tubing

The tubes were boiled on stir plate in a 4L volume of 2% (w/v) Sodium bicarbonate and 1 mM EDTA pH 8.0 and were rised in distilled water. Then they were boiled again in 1 mM EDTA (pH 8.0) throughout 10 min and rised in distilled water. Finally, they were cooled then stored them in 50% Ethanol at 4^{o}C. Before using the tubes, they were washed with distilled water.

2.2.5. Germination and outgrowth of *Bacillus subtilis* spores

Purified spores in water were heat activated at 70 ^{o}C for 30 min, cooled down and suspended in 2xYT and SMM. Spores were diluted to an OD_{600} of 0.4 in 2xYT and

to an OD_{600} of 0.8 in SMM. After 15 min of adaptation at 37°C, germination agents of either L-alanine (10 mM) or AGFK (3.3 mM L, asparagine, 5.6 mM D-glucose, 5.6 mM D-fructose, 10 mM KCl) was added to the medium in order to provide germination initiation. Furthermore, germination was monitored by measurement of loss in optical density at 600 nm at 37°C for 90 min with 10 minute intervals.

2.2.6. Spore resistance

Cells were grown in DSM at 37°C for 18 h following the end of exponential growth, which stands for approximately 24 hours, and a sample was removed from the cultures. Spores were purified through washes in ice cold deionized water five times and then were diluted to OD_{600} of 5.0 in cold deionized water. Purified spores were diluted serially 10-fold in 0,85% saline solution and 0.1 ml aliquots of dilutions were plated on LB agar plates for total viable cell count. Afterwards these dilutions were heated at 80°C for 30 min and plated once again for total viable cell count. For the chloroform and lysozyme treatments, samples from the cultures were taken, diluted serially 10-fold in 0,85% saline solution and plated. Then, new samples were taken from the cultures and treated with lysozyme (final concentration, 0.25 mg/ml) at 37°C for 10 min or with 10% v/v chloroform at room temperature for 10 min. Following the treatments, lysozyme or chloroform-treated cultures were serially diluted and plated on LB agar medium containing plates. For the H_2O_2 treatments, prufied spores were diluted to OD_{600} of 2.0 in 1x PBS and were diluted serially 10-fold in 1x PBS solution and 0.1 ml aliquots of dilutions were plated as above mentioned. Afterwards 8.58 ml of the spore suspension was placed in schott tube and 1.42 ml of 30% H_2O_2 was added to the spore suspension. The final H_2O_2 concentration was set to be 5%. The suspension was incubated at room temperature with continuous gentle mixing for 40 min, and 1.165 ml samples were removed and immediately diluted 1:10 with a solution of bovine catalase (100 µg/ml in PBS) that previously had been filter sterilized with a 0.45-µm-pore size filter. Serial 1:10 dilutions of the catalase treated spore suspension were then plated on LB agar plates in order to determine the number of viable colonies. All the plates were incubated overnight at 37°C.

2.2.7. Assay for dipicolinic acid (DPA)

Purified spores were diluted to OD_{600} of 2,5 in cold deionized water and 2 ml from these suspensions were harvested by 1 min. of centrifugation at 13.000 rpm and pellets were suspended in 1 ml of deionized water. Following this step, tubes were held in boiling water for 25 min. and then cooled down on ice. Subsequently, samples were centrifuged for 2 min. and their supernatants were saved. Assay reagent, composed of 25 mg L-cystein, 170 mg iron sulfate, 80 mg ammonium sulfate in 25 ml of 50 mM sodium acetate (pH 4.6 with glacial acetic acid) was prepared and 0,2 ml of this reagent was mixed with 0.4 ml of the supernatants taken and 0.4 ml of dH2O. These mixtures were centrifuged again for 2 min. and their optical densities at 440 nm were measured against a blank prepared from 0.8 ml of dH2O and 0.2 ml of assay reagent. Aiming the determination of exact DPA concentration (µg/ml), a calibration curve was prepared and treated similarly with pure DPA using standards of 0, 10, 20, 40, 70 and 100 µg DPA/ml.

2.7.8 Colony Morphology Analysis

For assay of colony morphology on DSM agar (Difco's Sporulation Medium), cells were first grown in LB medium. A total of 3 µl of cells was spotted to DSM agar. The plates were incubated at 37 ^{o}C. Images of colonies were taken using GS-800 Calibrated Densitometer.

3. RESULTS AND DISCUSSION

3.1. Construction of TEK1, *yvfI::Tn10::spc*, strain

TEK1 was previously constructed by Türkan Ebru Köroğlu as a part of her master thesis project at Istanbul Technical University. In order to construct the transposon library, transposon Tn10 mutagenesis was carried out in PY79 strain using temperature sensitive mini-*Tn*10 (*ori-spc*) containing vector pIC333. pIC333 vector was introduced into competent PY79 cells and transformants were selected on erythromycin (Erm) and spectinomycin (Spc). Spc^R and Erm^R mini-*Tn10* insertion mutants from the transposon library were screened for the loss of activity aganist *S. aureus* ATCC 9144 and bacilysin-negative mutants were determined by the paper disc-agar diffusion assay and the verified mutant was named as TEK1 (Köroğlu et al., 2008). Following this analysis, to identify the gene affecting bacilysin biosynthesis in PY79, inserted mini-*Tn10* (*spc*) transposons and their flanking DNA segments were cloned and sequenced. Nucleotide sequence analysis revealed that the insertion in TEK1 took place in the *yvfI* gene as based on the sequence determined by the *B. subtilis* genome consortium (Kunst et al.; 1997).

3.2. Response of *yvfI* spores to heat, chloroform, lysozyme, H_2O_2

In this study, in order to understand if the distruption of *yvfI* gene would show an effect on sporulation phenotype, as a first step, resistance profiles of TEK1 and wild type PY79 spores against chemical and physical stresses such as heat, chloroform, lysozyme and H_2O_2 were analyzed (Table 3.2).

Table 3.2: Heat, chloroform, lysozyme and H_2O_2 resistance of cells grown for sporulation in DSM.

Strain	Treatment[a]	Cell titer before treatment[b] (cfu/ml)	Cell titer after treatment (cfu/ml)	Survival frequency[c]
PY79	Heat	509×10^7	460×10^7	0.90
TEK1	Heat	458×10^7	385×10^7	0.84
PY79	Chloroform	441×10^7	207×10^7	0.46
TEK1	Chloroform	511×10^7	124×10^7	0.24
PY79	Lysozyme	372.5×10^7	315×10^7	0.84
TEK1	Lysozyme	459×10^7	458×10^7	0.99
PY79	H_2O_2	321×10^7	69×10^7	0.21
TEK1	H_2O_2	206.5×10^7	14×10^7	0.06

a. heat treatment: incubation of the sample at 80°C for 30 min, chloroform treatment: with 10% v/v chloroform at room temperature for 10 min, lysozyme treatment: with lysozyme (final concentration, 0.25 mg/ml) at 37°C for 10 min, H_2O_2 treatment: with 5% H_2O_2 at 37°C for 40 min.
b. cfu/ml of culture following growth for 24 h at 37°C in DSM.
c. Survival is calculated by dividing cfu/ml after treatment to cfu/ml before treatment

In order to determine whether there was a difference in resistance of TEK1 and PY79 spores, spores were exposed to heat, chloroform, lysozyme and hydrogen peroxide (H_2O_2) treatments following which viable colonies and therefore survival frequencies were counted. For this purpose, TEK1 and PY79 were treated with 10% (v/v) chloroform at room temperature for 10 min (Table 3.2). 46% of PY79 spores stayed viable against chloroform treatment, whereas the rate of survival of TEK1 spores was only 24%. Furthermore, TEK1 and PY79 spores were treated with lysozyme (final concentration, 0.25 mg/ml) at 37°C for 10 min. TEK1 and wild type spores were found to be similarly resistant against lysozyme. Moreover, TEK1 and PY79 spores were treated with 5% H_2O_2 at room temperature for 40 min. Levels of H_2O_2 resistance of wild type PY79 and its *yvfI* distrupted derivative TEK1 spores were examined and the survival rate of TEK1 spores was determined to be 6%, while the viability rate of PY79 spores reached up to 21%. Furthermore, survival frequencies of PY79 and TEK1 spores were compared following the incubation at 80°C for 20 minutes and consequently TEK1 spores were observed to be heat-resistant as well as PY79 spores. According to these results, TEK1, *yvfI::Tn10::spc* and wild type spores

were explored to be equally resistant to lysozyme and heat, whereas spores produced by TEK1 strain were relatively chloroform and H_2O_2–sensitive.

Due to the these explored differences, revealing out that wild type PY79 spores differ from its *yvfI* distrupted derivative TEK1 spores with its chloroform and H_2O_2 resistance properties, TEK1 spores and PY79 spores were further compared according to their coat proteins profiles (Figure 3.3).

3.3 Analysis of spore coat proteins of *yvfI* spores

One of the final events in sporulation is the formation of the proteinaceous structure surrounding the spores known as the coat (Drinks, 1999). Electron microscopy has revealed out that the coat of *B. subtilis* spores is composed of two major layers: a thicker and layered outer coat, and an inner coat, which is comprised of highly cross-linked proteins which play a role together in spore resistance and spore germination (Drinks, 2002). In this study, coat proteins of *B. subtilis*, which constitute one of the major known spore proteins, were resolved by SDS-PAGE [Figure 3.3]. The predicted characteristics of 85 proteins are described in the *B. subtilis* Genome Database (http:// bacillus.genome.ad.jp/). 41 of these proteins have been previously reported as sporulation-specific proteins, and another 44 proteins have been previously reported to be synthesized in vegetatively growing cell or sporangia (Kuwana et al., 2002).

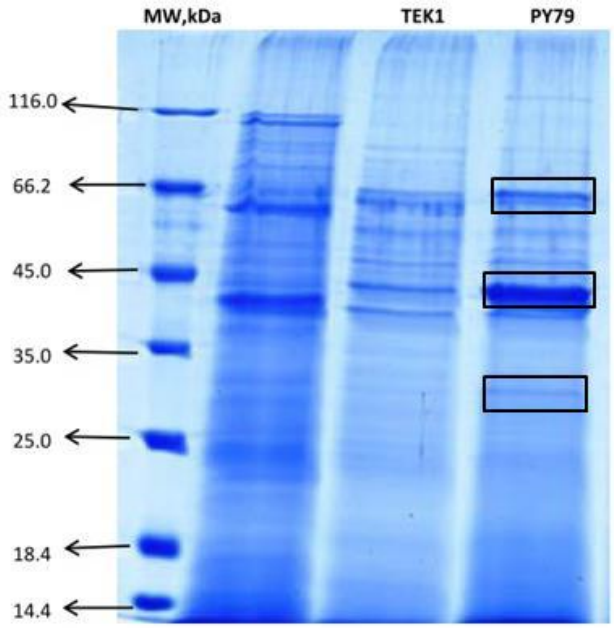


Figure 3.3. Coat proteins of *yvfI::Tn10::spc* mutant (TEK1) and wild type (PY79). Proteins were extracted from spores as described in Methods. The protein preparation was resolved by SDS-PAGE (12%, w/v, polyacrylamid gel) and the proteins were stained with Coomassie brilliant blue.

In this experiment, the molecular weights of corresponding spore coat protein bands were analyzed. According to SDS-PAGE analysis of total spore coat protein, *yvfI* gene functions directly or indirectly in the production of some coat proteins. Our results suggest that PY79 spores and TEK1 spores differ from each other with protein expression levels at the bands approximately between 35 kDa-25 kDa, 45 kDa-35 kDa, and at approximately 66.2 kDa [Figure 3.3]. Although protein bands were seen in extracts of both wild type and TEK1 spore coat proteins, the expression level of coat protein of TEK1 spores was lower than that of wild type coat protein [Figure 3.4.1]. One of these bands stands between 45 kDa and 35 kDa. Molecular weight of CotS (Takamatsu et al., 1999), CotH (Zilhao et al., 1999), CotSA (Takamatsu et al., 1999), CotB (Kakeshita et al., 2001), YtaA (unknown protein),YtcC (unknown protein) were depicted as 42 kDa. The *cotS* operon consists of *cotSA* (*YtxN*), *cotS*, *ytaA*, *ytcC* genes and has a promoter in the upstream of *cotSA*; the expression of this operon is regulated by both σ^K and GerE. *CotSA* is 1131 nucleotides long and potentially encodes a polypeptide of 377 amino acids with a predicted molecular mass of 42 kDa (Takamatsu et al., 1999). A previous study by Watabe in 2002, amino acid sequences data showed that YtaA is a paralogue of

CotS, and YtcC is a paralogue of CotSA. In case of both YtaA and YtcC, their functions are still unknown. Therefore, functions of CotS and YtaA and those of CotSA and YtcC were predicted to be similar to each other (Kuwana et al., 2002). Additionally, *cotH* locus encodes a polypeptide of 42.8 kDa (CotH) and CotH was known to be under the control of CotE and GerE proteins (Zilhao et al., 1999). Electron microscopy results from previous studies suggested that CotH function was needed for the correct formation of both inner and outer coat structures (Zilhao et al., 1999). CotB was encoded downstream of *cotH* and its location was depicted as 42.9 kDa (Bauer et al., 1999; Henriques et al., 1997). CotB is outer coat protein and relies on CotG, another coat protein, for its assembly into coat (Bauer et al., 1999). Kuwana et al. have pointed out that some spore proteins are subjected to processing or cross-linking in which CotB and CotS are involved.

The second band stands between 35 kDa and 25 kDa. YrbA protein was found to be involved in the proper formation of the coat and/or cortex. Takamatsu et al have pointed out that molecular mass of YrbA (SafA) protein was initially 45 kDa and was found to be reduced to 30 kDa late in sporulation. In the case of this report, processing of YrbA occurred early in sporulation in *B. subtilis* but not in *yabG* mutant because of YabG (sporulation specific protease) was involved in the proteolysis of several coat proteins directly or indirectly (Takamatsu et al., 2000). Many identified coat protein were indicated in the *B. subtilis* database and their molecular mass were observed to be smaller than 66.2 kDa. However, the possible presence of several unidentified coat proteins in *B. subtilis* was proposed to be the explanation of the third differing band which stands at approximately 66.2 kDa. In addition, *B. subtilis* spores contain several unidentified proteins. Some of these proteins were supposed to contribute directly or indirectly to the characteristics of spores, such as a high degree of resistance and cell morphology. The above results indicated that *yvfI* gene may function in the synthesis of a number of coat proteins, directly or indirectly. Henriques and Moran have pointed out that protein-protein crosslinking and their interactions leading to coat formation confer a high degree of chemical and mechanical resistance to the modified structure (Henriques and Moran, 2000; Kim et al., 2006; Riesenman and Nicholson, 2000). Although *yvfI* spores are lysozyme and heat resistant, it presumably became sensitive to chloroform and H_2O_2. Despite the fact that coat layer is fundamental to spore resistance, absence of any

coat protein in *B. subtilis* composed only a subtle effect on the essential properties of the coat (Drinks, 2002). Therefore, based on these obtained data, it was suggested that disruption of *yvfI* gene in *B. subtilis* might cause alterations on more than one coat protein or on their protein-protein interactions. In addition to this, *yvfI* gene was presumably proposed to function in synthesis of unidentified proteins that are required for resistance.

3.4 DPA contents of TEK1 and PY79 spores

Furthermore, TEK1 spores were compared with PY79 spores according to their DPA contents. On the basis of dipicolinic acid assay, PY79 spores were found to synthesize about 14.86 ± 2.18 μg/OD_{600} DPA, while *yvfI* mutant spores synthesized about 13.76 ± 2,82 μg/ OD_{600} DPA.

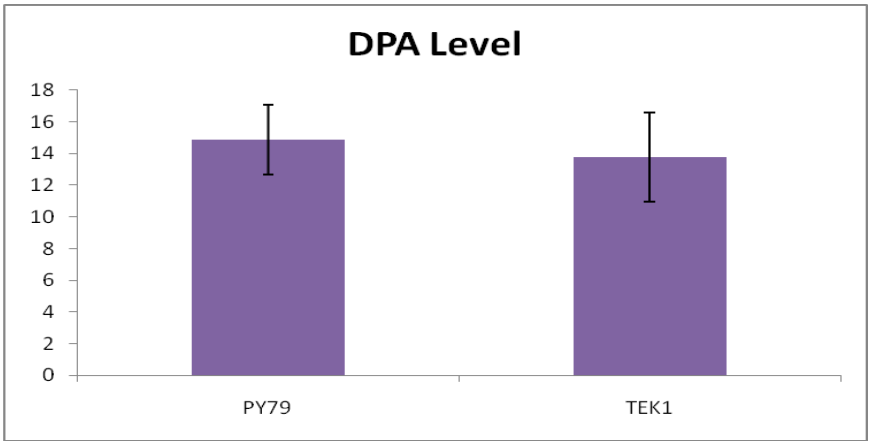


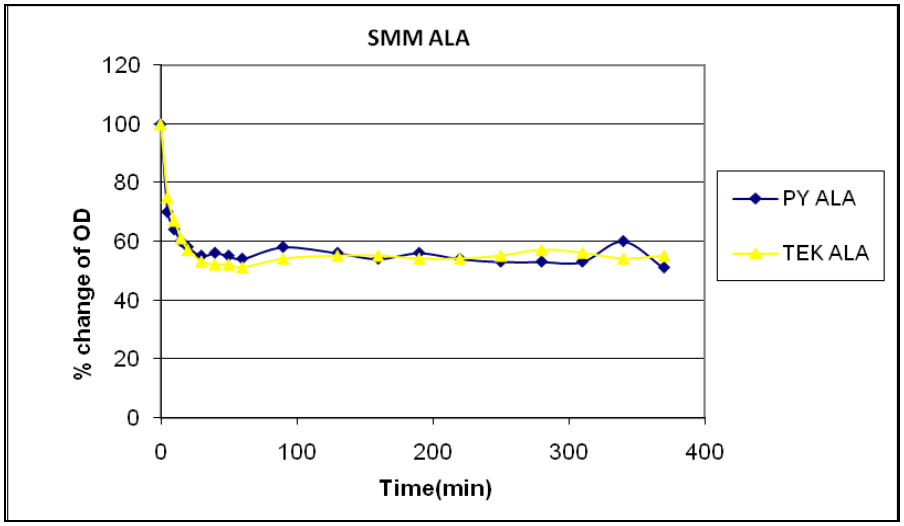
Figure 3.4 DPA contents of TEK1 and PY79 spores

yvfI spores were found to accumulate or synthesize same level of DPA when compared to the wild type spores. In *B. subtilis*, the high core DPA level stabilizes the spore's dormant state, an important element in reducing the spore's core water content and thereby increasing its heat resistance (Tovar-Rojo et al., 2002) and DPA also plays a more direct role in protecting spore DNA against several types of damage (Magge et al., 2008). Similarity of heat resistance of PY79 and TEK1 was proposed to be the consequence of the same levels of DPA synthesis and/or accumulation in the spores (Figure 3.4).

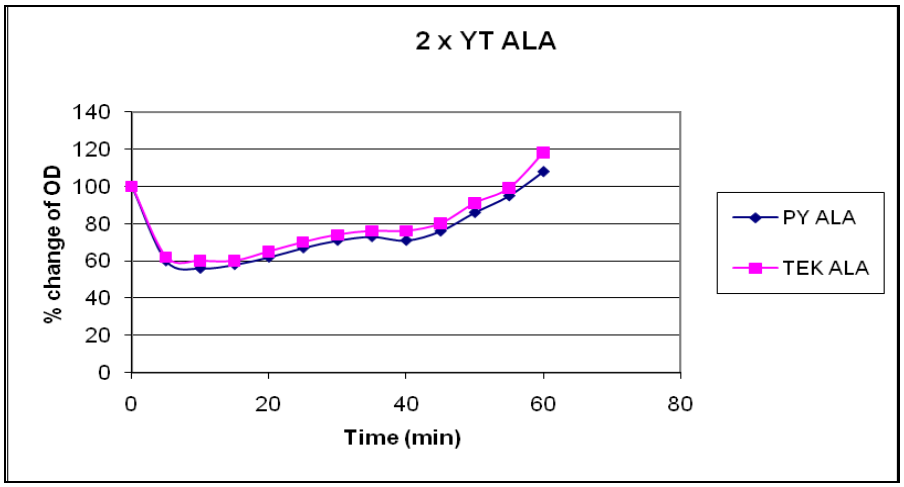
3.5 Response of *yvfI* spores to germination

For the germination properties, pure spores from TEK1 and PY79 were treated with different germination agents at different media. Germination of *yvfI* spores and PY79 spores with L-alanine or AGFK was observed through monitoring of spore germination by measuring the OD_{585} of cultures (Figure3.5). Firstly, spores were grown in rich medium 2x YT and germination was observed to develop in a similar pattern even though spores were treated with different germination agents (Figure3.5-B, 3.5-D).

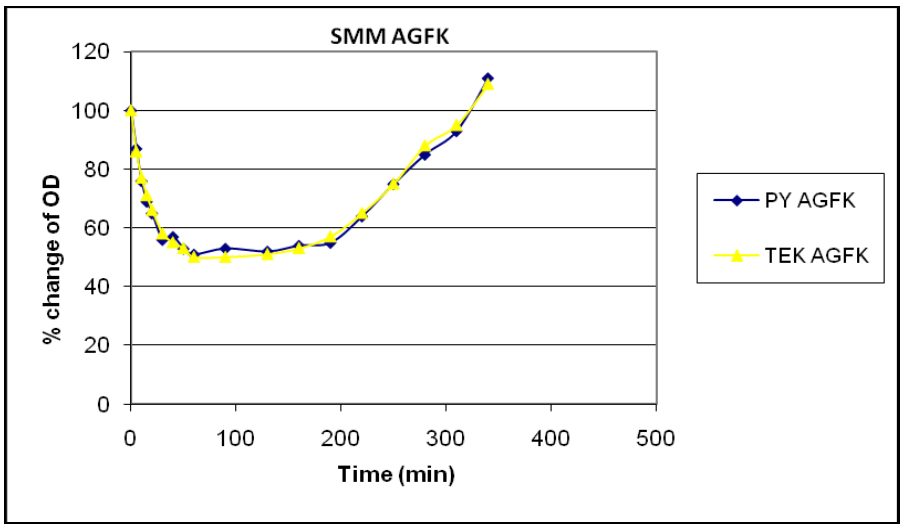
(A)



(B)



(C)



(D)

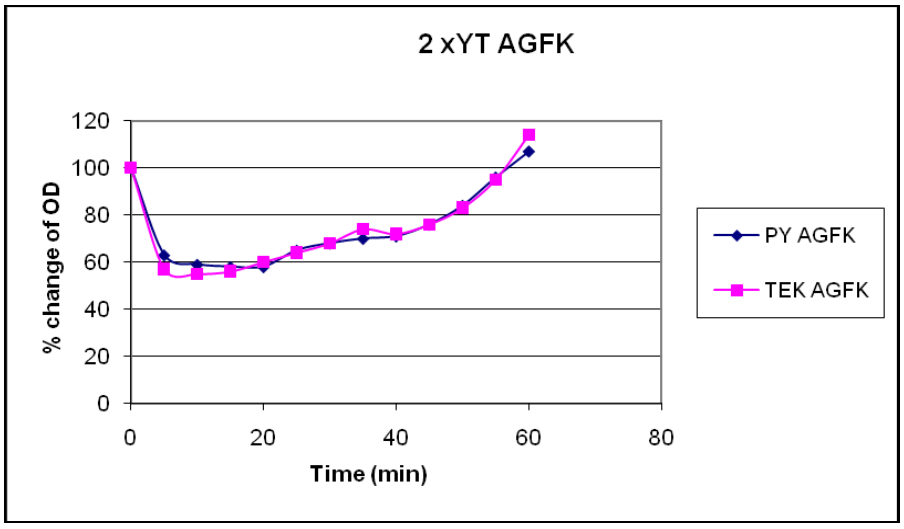


Figure 3.5 Effects of different germination agents L-alanine and AGFK on TEK1 and PY79 spores, when grown in either SMM or 2xYT. Spores were prepared, induced by heat and germinated at 37 ^{o}C with 4mM L-alanine in SMM medium (A) and in 2xYT (B) and with AGFK (3.3 mM L-asparagine, 5.6 mM D-glucose, 5.6 mM D-fructose, 10 mM KCl) in SMM (C) and in 2xYT (D). Germination and outgrowth profile of TEK1 and PY79 spore cells were drawn through measurement of loss in optical density at 585 nm at 37^{o}C for 90 min at 10 min intervals and further measurements continued until outgrowth is observed at 30 min intervals.

Following similar germination patterns in 2xYT, the germination of *yvfI* spores was examined in a minimal medium, SMM. As a result, PY79 and TEK1 spores were

found to germinate in a similar pattern regardless of the medium type or different germination agents (Figure 3.5-A, 3.5-C). Spore germination is a process that can be divided into three stages: activation, which is followed by germination and finally outgrowth. Outgrowth is the process during which anabolic reactions starts to take place of catabolic ones, so that spores regain their vegetative forms. Consequently, outgrowth is validated as the initial optical densities of purified spores are restored at the end of the second stage of germination. Therefore, when the outgrowth of TEK1 and PY79 spore cells was visualized, it was noticed that returning of TEK1 cells to their vegetative state was similar to that of wild-type germinating spores.

According to the germination data that was obtained in this study, TEK1 and PY79 spores were reported not to differ from each other.

3.6 Analysis of small acid soluble proteins (SASPs) of *yvfI* spores

The core contains many important molecules that play a role in spore resistance and spore germination including α, β, and γ-type SASP. These α/β-type and γ-type SASPs are produced only in the forespore (Setlow et al., 2000), and their degradation is initiated during the first minutes of germination (Ross and Setlow, 2000). In order to determine the effect of *yvfI* gene product on the small, acid-soluble proteins, SASPs were extracted from both TEK1 and PY79 spores (Figure 3.6).

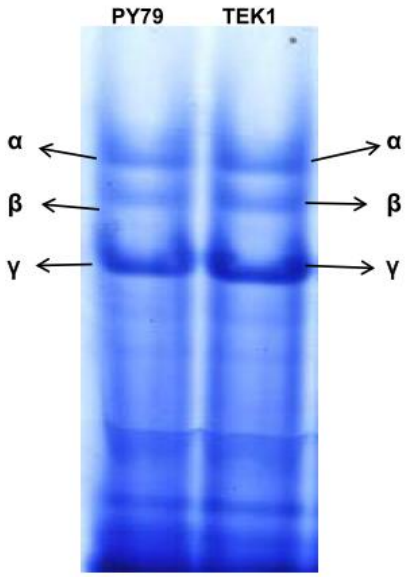


Figure 3.6 Polyacrylamid gel analysis at low pH of extracts from wild type (PY79) and *yvfI::Tn10::spc* mutant (TEK1) spores. Proteins were extracted from spores as described Methods. The three major SASP (α, β, γ) were labeled by arrows.

This three major SASP is important for spore survival, as two major SASP (the α/β-type) are essential for the protection of spore DNA from variety types of damage and α^- β^- spores are much more sensitive than wild-type spores to killing DNA damage from agents such as UV radiation, heat and some chemicals. According to data observed in this study, equal level of SASP-α and –β in TEK1 and PY79 spores have backed up similar resistance to heat and estimated UV resistance profiles. While degradation of both the α/β-type SASP, the other major SASP (γ) provides amino acids for protein synthesis early in spore germination and SASP-γ^- spores are retarded in spore outgrowth (Roos and Setlow, 2000; Setlow et al., 2000). Therefore, germination and outgrowth profiles reported in this study were also completed by equal levels of SASP-γ of PY79 and TEK1 spores.

Germination protease (GPR) initiates SASP degradation by cleaving these proteins during spore germination. GPR is a sequence-specific endoprotease that is synthesized as an inactive zymogen (P_{46}) during sporulation at about the same time as its SASP substrate. P_{46} autoprocesses to a smaller form (P_{41}) and this autoprocessing is triggered by pyridine-2,6-dicarboxylic acid (dipicolinic acid [DPA]) and decreases in pH and hydration level (Carroll and Setlow, 2005). The activation of this zymogen is also stimulated by the acidification and dehydration of the spore core, and together these conditions ensure that P_{41} is generated only late in sporulation, when the conditions in the spore core preclude enzyme action (Paidhungat et al., 2000). According to results, the level of DPA in *yvfI* spores that found in wild-type suggested that heat-resistance spores should be generated as mentioned before resistance data and the SASP specific protease, GPR, might be efficiently processed in these spores, since both DPA accumulation and core dehydration stimulate conversion of P_{46} to P_{41}. P_{41} action would most likely be on SASP-γ since this protein is not protected from proteolysis by binding to some macromolecule, unlike SASP-α and –β which are bound to spores DNA (Paidhungat et al., 2000).

3.7. Determination of Colony Morphology of PY79 and TEK1 Spores

To compare the colony morphology of wild type PY79 and its *yvfI* distrupted derivative TEK1 (*yvfI::Tn10::spc*) strains were first cultivated overnight in LB medium. A total of 3 µl of cells was then applied to DSM agar and plates were incubated at 37^{o}C for 22 days. There was not a considerable difference between the morphology of colonies of PY79 and TEK1, but the dark brown pigment accumulation was only observed in the colony of PY79 (Fig. 3.7). It is already known that the *cotA* gene encodes 65 kDa protein CotA, located in the outer spore coat, which is responsible for brownish pigmentation of the colonies (Hullo et al., 2001). According to this results, it was suggested that *yvfI* gene migth function in synthesis and regulation of *cotA* gene that involved in dark brown pigmentation in *B. Subtilis* during sporulation.

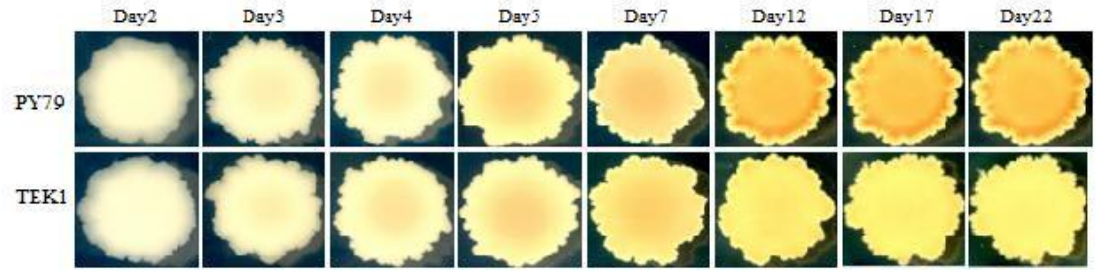


Figure 3.7 Colonies architecture of PY79 and TEK1 spores.

4. CONCLUSION

Present study was focused on the characterization of *yvfI* mutant spores. In order to understand the effect of the disruption of *yvfI* gene on the spore properties, TEK1 and PY79 were treated with 10% (v/v) chloroform at room temperature for 10 min. Furthermore, spores of both strains were treated with lysozyme (final concentration, 0.25 mg/ml) at 37^{o}C for 10 min and with 5% H_2O_2 at room temperature for 40 min. Lastly, survival frequencies of both *yvfI* mutant and wild type spores were compared following the incubation at 80^{o}C for 20 min. As a result, TEK1 spores are found to be more sensitive to chloroform and H_2O_2 than PY79 spores. On the other hand, similar survival frequencies were observed when spores were subjected to heat and lysozyme. Under the aim of backing up these explored resistance properties, spore coat proteins which grant the spore with resistance to treatment with chloroform or challenge by lysozyme, were extracted from both PY79 and TEK1 spores and compared. Results obtained in this part of the study suggested that PY79 spores and TEK1 spores differ from each other with protein expression levels at the bands approximately between 35 kDa-45 kDa, 25 kDa-35 kDa and 66.2 kDa. Although protein bands were seen in extracts of both wild type and TEK1 spore coat protein, the expression level of coat protein of TEK1 spores was lower than that of wild type coat protein. The band that stands at approximately 42 kDa was proposed to be CotS, CotH, CotSA, CotB, YtaA (unknown protein),YtcC (unknown protein) and that the other band that resides between 35 kDa-25 kDa put forward to be YrbA (unknown protein). The band at approximately 66.2 kDa was considered to be one of unidentified coat proteins. The results presented here showed that the distruption of *yvfI* gene resulted in changes in spore coat proteins profiles which can be the reason for the sensitivity of the YvfI spores to chloroform and hydrogen peroxide. Furthermore, similarity of heat resistance of PY79 and TEK1 spores was suggested to be a consequence of the same levels of DPA synthesis and/or accumulation in the

spores which was found to be 14.86 ± 2.18 μg/OD_{600} DPA in PY79 spores and about 13.76 ± 2,82 μg/ OD_{600} DPA in case of *yvfI* mutant spores.

Moreover, small-acid soluble proteins (SASPs) of TEK1 and PY79 spores were compared. Both PY79 and TEK1 spores showed three main bands and these bands would correspond to the α, β and γ SASP. Thus, this data suggested that *yvfI* gene did not function in production or functioning of SASPs. Equal level of SASP-α and –β in TEK1 and PY79 spores has also backed up similar resistance to heat and estimated UV resistance profiles. Only known function of SASP-γ is to provide amino acid for protein synthesis during period of development. The analysis of germination and outgrowth characteristic of TEK1 spores and wild type spores, which displayed no differences in both SMM and 2x YT medium, also confirmed equal levels of SASP-γ of PY79 and TEK1 that was reported in this study. Finally, the result of colony morphology analysis revealed that *yvfI* gene migth function in synthesis and regulation of *cotA* gene that is involved in brownish pigmentation of the colonies.

Consequently, in this study it was demostrated that *yvfI* gene plays a regulatory role on the expression of some coat proteins and thereby, it is also important for gaining of resistance of spores to chloroform and H_2O_2.

REFERENCES

Aertsen, A., Opstal, I.V., Vanmuysen, S.C., Wuytack, E.Y., Michiels, C.W., 2005. Screening for *Bacillus subtilis* mutants deficient in pressure induced spore germination: identification of *ykvU* as a novel germination gene, *Fems Microbiolgy Letters*, **243**, 385-391

Aronson, A.I., Fitz-James, P., 1976. Structure and Morphogenesis of the Bacterial Spore Coat, *American Society for Microbiology*, Vol.**40**, No.2

Atluri, S., Ragkousi, K., Cotezzo, D.E., Setlow, P., 2006. Cooperativity Between Different Nutrient Receptors in Germination of Spores of *Bacillus subtilis* and Reduction of This Cooperativity by Alterations in the GerB Receptor, *Journal of Bacteriolgy*, Vol. **188**, No. 1

Atrih, A., Foster, S.J., 2001. In vivo roles of the germination-specific lytic enzymes of *Bacillus subtilis* 168, *Microbiology*, **147**, 2925-2932

Atrih, A., Foster, S.J., 1999. The role of peptidoglycan structure and structural dynamics during endospore dormancy and germination, *Antonie van Leeuwenhoek* **75**, 299–307

Bagyan, I., Setlow, P., 2002. Localization of the Cortex Lytic Enzyme CwlJ in Spores of *Bacillus subtilis, Journal of Bacteriology*, Vol. **184**, No. 4

Bauer, T., Little, S., Stover, A.G., Drinks. A., 1999. Functional Regions of the *Bacillus subtilis* Spore Coat Morphogenetic Protein CotE, *Journal of Bacteriology*, Vol. **181**, No. 22

Beall, B., Drinks, A., Losick, R., Moran, C.P., 1993. Cloning and Characterization of a Gene Required for Assembly of the *Bacillus subtilis* Spore Coat, *Journal of Bacteriology*, Vol.**175**, No. 6

Ben-Yehuda, S., Rudner, D.Z., Losick, R., 2003. RacA, a Bacterial Protein That Anchors Chromosomes to the Cell Poles, *Science*, Vol **299**

Black, E.P., Koziol-Dube, K., Guan, D., Wei, J., Setlow, B., Cotezzo, D.E., Hoover, D.G., Setlow, P., 2005. Factors Influencing Germination of *Bacillus subtilis* Spores via Activation of Nutrient Receptors by High Pressure, *Applied and Environmental Microbiolgy*, Vol. **71**, No. 10

Black, E.P., Wei, J., Atluri, S., Cortezzo, D.E., Koziol-Duce, K., Hoover, D.G., Setlow, P., 2006. Analysis of factors influencing the rate of germination of spores of *Bacillus subtilis* by very high pressure, *Journal of Applied Microbiology*, 1364-5072

Blaylock, B., Jiang, X., Rubio, A., 2004. Zipper-like interaction between proteins in adjacent daughter cells mediates protein localization, *Genes Dev.*, **18**, 2916-2928

Burkholder, W.F., Kurtser, I., Grossman, A.D., 2001. Replication Initiation Proteins Regulate a Developmental Checkpoint in *Bacillus subtilis*, *Cell*, Vol. **104**, 269-279

Boland, F.M., Atrih, A., Chirakkal, H., Foster, S.J., Moir, A., 2000. Complete spore-cortex hydrolysis during germination of *Bacillus subtilis* 168 requires SleB and YpeB, *Microbiology*, **146**, 57-64

Bourne, H., Fitzjames, P.C., Aronson, A.I., 1991. Structural and Germination Defects of *Bacillus subtilis* Spores with Altered Contents of a Spore Coat Protein, *Journal of Bacteriology*, Vol. **173**, No. 20

Cabrera-Hernandez, A., Sanchez-Salas, J.L., Paidhungat, M., Setlow, P., 1999. Regulation of four genes encoding small, acid-soluble spore proteins in *Bacillus subtilis*, *Gene*, **232**

Cabrera-Hernandez, A., Setlow, P., 2000. Analysis of the regulation and function of five genes encoding small, acid-soluble spore proteins of *Bacillus subtilis*, *Gene*, **248**, 160-181

Carroll, T.M., Setlow, P., 2005. Site-Directed Mutagenesis and Structural Studies Suggest that the Germination Protease, GPR, in Spores of *Bacillu*s Species Is an Atypical Aspartic Acid Protease, *Journal of Bacteriology*, Vol. **187**, No. 20

Chai, Y., Kolter, R., Losick, R., 2009. A Widely Conserved Gene Cluster Required for Lactate Utilization in *Bacillus subtilis* and Its Involvement in Biofilm Formation, *Journal of Bacteriology*, Vol. **191**, No. 8

Chastanet, A., Losick, R., 2007. Engulfment during sporulation in *Bacillus subtilis* is governed by a multi-protein complex containing tandemly acting autolysins, *Molecular Microbiology*, **64**, 139-152

Chrikkal, H., O'Rourke, M., Atrih, A., Foster, S.J., Moir, A., 2002. Analysis of spore cortex lytic enzymes and related proteins in *Bacillus subtilis* endospore germination, *Microbiology*, **148**, 2383-2392

Connors, M.J., Mason, J.M., Setlow, P., 1986. Cloning and Nucleotide Sequencing of Genes for Three Small, Acid- Soluble Proteins from *Bacillus subtilis* Spores, *Journal of Bacteriology*, Vol. **166**, No. 2

Cucchi, A., De Rivas, C.S., 1998. SASP (Small, Acid-Soluble Spore Proteins) and Spore Properties in *Bacillus thuringiensis israelensis* and *Bacillus sphaericus*, *Current Microbiology*, Vol. **36,** pp. 220-225

De Jong, H., Geiselmann, J., Batt, G., Hernandez, C., Page, M., 2004. Qualitative Simulation of the Initiation of Sporulation in *Bacillus subtilis*, *Bulletin of Mathematical Biology*, **66**, 261–299

Doan, T., Marquis, K.A., Rudner, D.Z., 2005. Subcellular localization of a sporulation membrane protein is achieved through a network of interactions along and across the septum, *Molecular Microbiology*, 1365-2958

Doan, T., Rudner, D.Z., 2007. Perturbations to engulfment trigger a degradative response that prevents cell–cell signalling during sporulation in *Bacillus subtilis*, *Molecular Microbiology*, 64, 500-511

Drinks, A., 1999. *Bacillus subtilis* Spore Coat, *Microbiology and Molecular Biology Reviews,* Vol. **63**, No. 1

Drinks, A., 2002. Maximum shields: the assembly and function of the bacterial spore coat, *Trends in Microbiology*, Vol.**10**, No.6

Drinks, A., Roels, S., Beall, B., Moran, C.P., Losick, R., 1993. Subcellular localization of proteins involved in the assembly of the spore coat of *Bacillus subtilis, Genes & Development***, 8**, 234-244

Ducros, V.M.A., Lewis, R.J., Verma, C.S., Dodson, E.J., Leonard, G., Turkenburg, J.P., Murshudov, G.N., Wilkinson, A.J., Brannigan, J.A., 2001. Crystal Structure of GerE, the Ultimate Transcriptional Regulator of Spore Formation in *Bacillus subtilis, J. Mol. Biol.*, **306**, 759-771

Eichenberger, P., Fawcett, P., Losick, R., 2001. A three-protein inhibitor of polar septation during sporulation in *Bacillus subtilis, Molecular Microbiology*, **42**, 1147-1162

Errington, J., 1993. *Bacillus subtilis* Sporulation: Regulation of Gene Expression and Control of Morphogenesis, *Microbiological Reviews*, Vol. **57**, No.1

Henriques, A.O., Beall, B.W., Moran, C.P., 1997. CotM of *Bacillus subtilis*, a Member of the a-Crystallin Family of Stress Proteins, Is Induced during Development and Participates in Spore Outer Coat Formation, *Jornal of Bacteriology*, Vol.**179**, No.6

Henriques, A.O., Moran, C.P., 2000. Structure and Assembly of the Bacterial Endospore Coat, *Methods*, **20**, 95-100

Hilbert, D.W., Piggot, P.J., 2004. Compartmentalization of Gene Expression during *Bacillus subtilis* Spore Formation, *Microbiology and Molecular Biology Reviews*, Vol**. 68**, No. 2

Hilton, M.D., Alaeddinoglu, N.G., Demain, A.L., 1988. *Bacillus subtilis* Mutant Deficient in the Ability To Produce the Dipeptide Antibiotic Bacilysin: Isolation and Mapping of the Mutation, *Journal of Bacteriology*, Vol. **170**, No. 2

Hudson, K.D., Corfe, B.M., Kemp, E.H., Feavers, I.A., Coote, P.J., Moir, A., 2001. Localization of GerAA and GerAC Germination Proteins in the *Bacillus subtilis Spore, Journal of Bacteriology*, Vol. **183,** No. 14

Hullo, M.F., Moszer, I., Danchin, A., 2001. CotA of *Bacillus subtilis* Is a Copper-Dependent Laccase, *Journal of Bacteriology*, 183 (18): 5426-5430

Hornstra, L.M., Vries, Y.P., De Vos, W.M., Abee, T., Wells-Bennik, M.H.J., 2005. *gerR*, a Novel ger Operon Involved in L-Alanine- and Inosine-Initiated Germination of *Bacillus cereus* ATCC 14579, *Applied and Environmental Microbiology*, Vol. **71**, No. 2

Horsburg, M.J., Thackray, P.D., Moir. A., 2001. Transcriptional responses during outgrowth of *Bacillus subtilis* endospores, *Microbiology*, **147**, 2933-2941

Ichikawa, H., Kroos, L., 2000. Combined Action of Two Transcription Factors Regulates Genes Encoding Spore Coat Proteins of *Bacillus subtilis*, *The Journal of Biological Chemistry*, Vol. **275**, No. 18

Igarashi, T., Setlow, B., Paidhungat, M., Setlow, P., 2004. Effects of a *gerF* (*lgt*) Mutation on the Germination of Spores of *Bacillus subtilis*, *Journal of Bacteriology*, Vol. **186**, No. 10

Igarashi, T., Setlow, P., 2005. Interaction between Individual Protein Components of the GerA and GerB Nutrient Receptors That Trigger Germination of *Bacillus subtilis Spores, Journal of Bacteriology*, Vol. **187**, No. 7

Igarashi, T., Setlow P., 2006. Transcription of the *Bacillus subtilis gerK* Operon, Which Encodes a Spore Germinant Receptor, and Comparison with That of Operons Encoding Other Germinant Receptors, *Journal of Bacteriology*, Vol. **188**, No. 11

Isticato, R., Esposito, G., Zilhão, R., Nolasco, S., Cangiano, G., Felice, M.D., Henriques, A.O., Ricca, E., 2004. Assembly of Multiple CotC Forms into the *Bacillus subtilis* Spore Coat, *Journal of Bacteriology*, Vol.**186**, no.4

Kakeshita, H., Takatmatsu, H., Amikura, R., Nakamura, K., Watabe, K., Yamane, K., 2001. Effect of depletion of FtsY on spore morphology and the protein composition of the spore coat layer in *Bacillus subtilis*, *Fems Microbiology Letters*, **195**, 41-46

Keijser, B.J.F., Beek, A.T., Rauwerda, H., Schuren, F., Montij, R., Spek, H., Brul, S., 2007. Analysis of Temporal Gene Expression during *Bacillus subtilis* Spore Germination and Outgrowth, *Journal of Bacteriology*, Vol. **189**, No. 9

Kenig, M., Vandamme, E., Abraham, P., 1975. The Mode of Action of Bacilysin and Anticapsin and Biochemical Properties of Bacilysin-resistant Mutants, *Journal of General Microbiology*, **94**, 46-54

Kim, H., Hanh, M., Grabowski, P., McPherson, D.C., Otte, M.M., Wang, R., Ferguson, C.C., Eichenberger, P., Drinks.A., 2006. The *Bacillus subtilis* spore coat protein interaction network, *Molecular Microbiolgy*, **59**(2), 487-502

Koroğlu, T.E., Kurt-Gür, G., Ünlü, E.C., Yazgan-Karataş, A., 2008. The novel gene *yvfI* in *Bacillus subtilis* is essential for bacilysin biosynthesis, *Antonie van Leeuwenhoek*, **DOI 10.1007/s10482-008-9265-8**

Kunst, F., Ogasawara, N, Moszer, I., Albertini, A.M., Alloni, G., Azevedo, V., Bertero, M.G., Bessiéres, P., Bolotin, A., Borchert, S., Borriss, R., Boursier, L., Brans, A., Braun, M., Brignell, S.C., Bron, S, Brouillet, S., Bruschi, C.V., Caldwell, B., Capuano, V., Carter, N.M., Choi, S.-K., Codani, J.-J., Connerton, I.F., Cummings, N.J., Daniel, R.A., Denizot, F., Devine, K.M., Düsterhöft, A., Ehrlich, S.D., Emmerson, P.T., Entian, K.D., Errington, J., Fabret, C., Ferrari, E., Foulger, D., Fritz, C., Fujita, M., Fujita, Y., Fuma, S., Galizzi, A., Galleron, A., Ghim, S.-Y., Glaser, P., Goffeau, A., Golightly,E.J., Grandi, G., Guiseppi, G., Guy, B.J., Haga, K., Haiech, J., Harwood, C.R., Hénaut, A., Hilbert, H., Holsappe, S.,

Hosono, S., Hullo, M.-F., Itaya, M., Jones, L., Joris, B., Karamata, D., Kasahara, Y., Klaerr-Blanchard, M., Klein, C., Kobayashi, Y., Koetter, B., Koningstein, G., Krogh, S., Kumano, M., Kurita, K., Lapidus, A., Lardinois, S., Lauber, J., Lazarevic, V., Lee, S.-M., Levine, A., Liu, H., Masuda, S., Mauel, C., Médigue, C., Medina, N., Mellado, R.P., Mizuno, M., Moest, D., Nakai, S., Noback, M., Noone, D., O'Reilly, M., Ogawa, K., Ogiwara, A., Oudega, B., Park, S.-H., Parro, V., Pohl, T.M., Portetelle, D., Porwollik, S., Prescott, A.M., Presecan, E., Pujic, P., Purnelle, B., Rapoport, G., Rey, M., Reynolds, S., Rieger, M., Rivolta, C., Rocha, E., Roche, B., Rose, M., Sadaie, Y., Sato, T., Scanlan, E., Schleich, S., Schroeter, R., Scoffone, F., Sekiguchi, J., Sekowska, A., Seror, S.J., Serror, P., Shin, B.-S., Soldo, B., Sorokin, A., Tacconi, E., Takagi, T., Takahashi, H., Takemaru, K., Takeuchi, M., Tamakoshi, A.,Tanaka, T., Terpstra, P., Tognoni, A., Tosato, V., Uchiyama, S., Vandenbol, M., Vannier, F., Vassarotti, A., Viari, A., Wambutt, R., Wedler, E., Wedler, H., Weitzenegger, T., Winters, P., Wipat, A., Yamamoto, H., Yamane, K., Yasumoto, K., Yata, K., Yoshida, K., Yoshikawa, H.-F., Zumstein, E., Yoshikawa, H., and Danchin, A. (1997). The complete genome sequence of the Gram-positive bacterium *Bacillus subtilis. Nature* **390** : 249-256.

Kuwana, R., Kasahara, Y., Fujibayashi, M., Takamatsu, H., Ogasawara, N., Watabe, K., 2002. Proteomics characterization of novel spore proteins of *Bacillus subtilis, Microbiology*, **148**, 3971-3982

Lai, E., Pdahke, N.D., Kachman, M.T., Giorno, R., Vazquez, S., Vazques, J.A., Maddock, J.R., Drinks, A., 2003. Proteomic Analysis of the Spore Coats of *Bacillus subtilis* and *Bacillus anthracis*, *Journal of Bacteriology*, Vol. 185, No. 4

Lee, K.S., Bumbaca, D., Kosman, J., Setlow, P., Jedrzejas, M.J., 2007. Structure of a protein–DNA complex essential for DNA protection in spores of *Bacillus* species, *PNAS*, Vol. **105**. No. 8

Levin, P.A., Grossman, A.D., 1998. Cell cycle and sporulation in *Bacillus subtilis, Current Opinion in Microbiology*, **1**, 630-635

Little, S., Drinks,A., 2001. Functional analysis of the *Bacillus subtilis* morphogenetic spore coat protein CotE, Molecular Microbiology, **42**(4), 1107-1120

Loshon, C.A., Genest, P.C., Setlow, B., Setlow, P., 1999. Formaldehyde kills spores of *Bacillus subtilis* by DNA damage and small, acid-soluble spore proteins of the α/β-type protect spores against this DNA damage, *Journal of Applied Microbiology*, **87**, 8-14

Loshon, C.A., Kraus, P., Setlow, B., Setlow, P., 1997. Effects of Inactivation or Overexpression of the *sspF* Gene on Properties of *Bacillus subtilis* Spores, *Journal of Bacteriology*, Vol**. 179**, No. 1

Magge, A., Granger, A.C., Wahome, P.G., Setlow, B., Vepachedu, V.R., LoshonC.A., Peng, L., Chen, D., Li, Y., Setlow, P., 2008. Role of

Dipicolinic Acid in the Germination, Stability, and Viability of Spores of *Bacillus subtilis*, *Journal of Bacteriology*, Vol.**190**,No.14

Masayama, A., Kuwana, R., Takamatsu, H., Hemmi, H., Yoshimura, T., Watabe, K., Moriyama, R., 2007. A Novel Lipolytic Enzyme, YcsK (LipC), Located in the Spore Coat of *Bacillus subtilis*, Is Involved in Spore Germination, *Journal of Bacteriology*, Vol. **189,** No. 6

Michael, W.M., 2001. Cell cycle: Connecting DNA replication to sporulation in *Bacillus*, *Current Biology*, **11**, 443-445

Moeller, R., Setlow, P., Horneck, G., Berger, T., Reitz, G., Rettberg, P., Doherty, A.J., Okayasu, R., Nicholson, W.L., 2008. Roles of the Major, Small, Acid-Soluble Spore Proteins and Spore-Specific and Universal DNA Repair Mechanisms in Resistance of *Bacillus subtilis* Spores to Ionizing Radiation from X Rays and High-Energy Charged-Particle Bombardment, *Journal of Bacteriology*, Vol. **190**, No. 3

Moeller, R., Setlow, P., Reitz, G., Nicholson, W.L., 2009. Roles of Small, Acid-Soluble Spore Proteins and Core Water Content in Survival of *Bacillus subtilis* Spores Exposed to Environmental Solar UV Radiation, *Applied and Environmental Microbiology*, Vol. **75**, No. 16

Mohr, S.C., Sokolov, N.H.A., He, C., Setlow, P., 1991. Binding of small acid-soluble proteins from *Bacillus subtilis* changes the conformation of DNA from B to A, *Proc. Natl. Acad. Sci. USA*, Vol. **88**, pp. 77-81

Moir, A., 2005. How do spores germinate?, *Journal of Applied Microbiology*, 1364-5072

Molle, V., Fujita, M., Jensen, S.T., Eichenberger, P., Gonzalez-Pastor, J.E., Liu, J.S., Losick, R., 2003. The Spo0A regulon of *Bacillus subtilis*, *Molecular Microbiology*, **50**, 1683-1701

Moriyama, R., Kudoh, S., Miyata, S., Nonobe, S., Hattori, A., Makino, S., 1996. A Germination-Specific Spore Cortex-Lytic Enzyme from *Bacillus cereus* Spores: Cloning and Sequencing of the Gene and Molecular Characterization of the Enzyme, *Journal of Bacteriology*, Vol.**178**, No. 17

Munoz, L., Sadai, Y., Doi, R.H., 1978. Spore Coat Protein of *Bacillus subtilis*, *The Journal of Biological Chemistry*, Vol. **253**, No. 19

Naclerio, G., Baccigalupi, L., Zilhao,R., De Felice, M., Ricca, E., 1996. *Bacillus subtilis* Spore Coat Assembly Requires *cotH* Gene Expression, *Journal of Bacteriology*, Vol.**178**, No.15

Nicholson, W.L., Munakata, N., Horneck, G., Melosh, H.J., Setlow, P., 2000. Resistance of Bacillus Endospores to Extreme Terrestrial and Extraterrestrial Environments, *Microbiology and Molecular Biology Reviews*, Vol. **64**, No. 3

Ozcengiz, G., Alaeddinoglu, N.G., 1991. Bacilysin Production and Sporulation in *Bacillus subtilis*, *Current Microbiology*, Vol. **23,** pp. 61-64

Ozin, A.J., Samford, C.S., Henriques, A.O., Moran, C.P., 2001. SpoVID Guides SafA to the Spore Coat in *Bacillus subtilis*, *Journal of Bacteriology*, Vol.**183**, No.10

Paidhungat, M., Ragkousi, K., Setlow, P., 2001. Genetic Requirements for Induction of Germination of Spores of *Bacillus subtilis* by Ca^{2+} - Dipicolinate, *Journal of Bacteriology*, Vol. **183**, No. 16

Paidhungat, M., Setlow, B., Drinks, A., Setlow, P., 2000. Characterization of Spores of *Bacillus subtilis* Which Lack Dipicolinic Acid, *Journal of Bacteriology*, Vol. **182**, No. 19

Paidhungat, M., Setlow, P., 1999. Isolation and Characterization of Mutations in *Bacillus subtilis* That Allow Spore Germination in the Novel Germinant D-Alanine, *Journal of Bacteriology*, Vol. **181**, No. 11

Pandey, N.K., Aronson, A.I., 1979. Properties of the *Bacillus subtilis* Spore Coat, *Journal of Bacteriology*, Vol. **137**, No. 3

Pelczar, P.L., Igarashi,T., Setlow, B., Setlow, P., 2007. Role of GerD in Germination of *Bacillus subtilis* Spores, *Journal of Bacteriology*, Vol. **189**, No. 3

Piggot, P.J., Hilbert, D.W., 2004. Sporulation of *Bacillus subtilis*, *Current Opinion in Microbiology*, **7**, 579-586

Popham, D.L., Helin, J., Costello, C.E., Setlow, P., 1996. Muramic lactam in peptidoglycan of *Bacillus subtilis* spores is required for spore outgrowth but not for spore dehydration or heat resistance, *Proc. Natl. Acad. Sci.*, Vol.**93**, pp. 15404-15410

Price, K.D., Losick, R., 1999. A Four-Dimensional View of Assembly of a Morphogenetic Protein during Sporulation in *Bacillus subtilis*, *Journal of Bacteriology*, Vol. **181**, No. 3

Ragkousi, K., Cowan, A.E., Ross, M.A., Setlow, P., 2000. Analysis of Nucleoid Morphology during Germination and Outgrowth of Spores of *Bacillus* Species, *Journal of Bacteriology*, Vol. **182**, No. 19

Ragkousi, K., Eichenberger, Ooji, C.V., Setlow, P., 2003. Identification of a New Gene Essential for Germination of *Bacillus subtilis* Spores with Ca^{2+}- Dipicolinate, *Journal of Bacteriology*, Vol. **185**, No. 7

Ragkousi, K., Setlow, P., 2004. Transglutaminase-Mediated Cross-Linking of GerQ in the Coats of *Bacillus subtilis* Spores, *Journal of Bacteriology*, Vol.**186**, No.17

Ramamurthi, K.S., Losick, R.M, 2005. Protein Localization: Reach out and Touch the Forespore, *Current Biology*, Vol. **15**, No. 5

Riesenman, P.J., Nicholson, W.I., 2000. Role of the Spore Coat Layers in *Bacillus subtilis* Spore Resistance to Hydrogen Peroxide, Artificial UV-C, UV-B, and Solar UV Radiation, *Applied and Environmental Microbiology*, Vol**. 66**, No. 2

Ross, M.A., Setlow, P., 2000. The *Bacillus subtilis* HBsu Protein Modifes the Effects of α/β -Type, Small Acid-Soluble Spore Proteins on DNA, *Journal of Bacteriology*, Vol. 182, No. 7

Serrano, M., Zilhao, R., Ricca, E., Ozin, A.J., Moran, C.P., Henriques, A.O., 1999. A *Bacillus subtilis* Secreted Protein with a Role in Endospore

Coat Assembly and Function, *Journal of Bacteriology*, Vol. 181, No. 12

Setlow, B., McGinnis, K.A., Ragkousi, K., Setlow, P., 2000. Effects of Major Spore-Specific DNA Binding Proteins on *Bacillus subtilis* Sporulation and Spore Properties, *Journal of Bacteriology*, Vol. **182**, No. 24

Setlow, B., Tautvydas, K.J., Setlow, P., 1998. Small, Acid-Soluble Spore Proteins of the α/β Type Do Not Protect the DNA in *Bacillus subtilis* Spores against Base Alkylation, *Applied and Environmental Microbiology*, Vol. 64, No. 5

Setlow, P., 2003. Spore Germination, *Current Opinion in Microbiology*, **6**, 550-556

Setlow, P., 2005. Spores of *Bacillus subtilis*: their resistance to and killing by radiation, heat and chemicals, *Journal of Applied Microbiology*, 1364-5072

Stein, T., 2005. *Bacillus subtilis* antibiotics: structures, syntheses and specific functions, *Molecular Microbiology*, **56**, 845-857

Stephenson, K., Hoch, J.S., 2002. Evolution of signalling in the sporulation phosphorelay, *Molecular Microbiology*, **2,** 297-304

Takamatsu, H., Hiraoka, T., Kodama, T., Koide, H., Kozuka, S., Tochikubo, K., Watabe, K., 1998. Cloning of a novel gene *yrbB*, encoding a protein located in the spore integument of *Bacillus subtilis, Fems Microbiology Letters*, **166**, 361-367

Takamatsu, H., Imamura, A., Kodama, T., Asai, K., Ogasawara, N., Watabe, K., 2000. The *yabG* gene of *Bacillus subtilis* encodes a sporulation specific protease which is involved in the processing of several spore coat proteins, *Fems Microbiolgy Letters*, **192**, 33-38

Takatmatsu, H., Kodama, T., Watabe, K., 1999. Assembly of the CotSA coat protein into spores requires CotS in *Bacillus subtilis, Fems Microbiology Letters*, **174**, 201-206

Tovar-Rojo, F., Chander, M., Setlow, B., Setlow, P., 2002. The Products of the *spoVA* Operon Are Involved in Dipicolinic Acid Uptake into Developing Spores of *Bacillus subtilis, Journal of Bacteriology*, Vol. **184**, No. 2

Veening, J.W., Hamoen, L.W., Kuipers, O.P., 2005. Phosphatases modulate the bistable sporulation gene expression pattern in *Bacillus subtilis, Molecular Microbiology*, **56**, 1481-1494

Veening, J.W., Murray, H., Errigton, J., 2009. A mechanism for cell cycle regulation of sporulation initiation in *Bacillus subtilis, Genes Dev*., **23**, 1959-1970

Vepachedu, V.R., Hirneisen, K., Hoover, D.G., Setlow, P., 2007. Studies of the release of small molecules during pressure germination of spores of *Bacillus subtilis, Letters in Applied Microbiology*, 0266-8254

Vepachedu, V.R., Setlow, P., 2004. Analysis of the germination of spores of *Bacillus subtilis* with temperature sensitive spo mutations in the *spoVA* operon, *Fems Microbiology Letters*, **239**, 71-77

Vepachedu, V.R., Setlow, P., 2007. Role of SpoVA Proteins in Release of Dipicolinic Acid during Germination of *Bacillus subtilis* Spores Triggered by Dodecylamine or Lysozyme, *Journal of Bacteriology*, Vol. **189,** No. 5

Wang, L., Perpich, J., Drinks, A., Kroos, L., 2007. One Perturbation of the Mother Cell Gene Regulatory Network Suppresses the Effects of Another during Sporulation of *Bacillus subtilis, Jornal of Bacteriology*, Vol. **189**. No. 23

White, B.J., Legako, A., Harmon, H.J., 2004. Interaction of dipicolinic acid with water-soluble and immobilized porphyrins, *Sensor and Actuators B*, **97**, 277-283

Wuytack. E.Y., Michiels, C.W., 2001. A study on the effects of high pressure and heat on *Bacillus subtilis* spores at low pH, *International Journal of Food Microbiology*, **64**, 333-341

Yamamoto, H., Mori, M., Sekiguchi, J., 1999. Transcription of genes near the *sspE* locus of the *Bacillus subtilis* genome, *Microbiology*, **145**, 2171-2180

Yazgan-Karataş, A., Çetin, S., Özcengiz, G., 2003. The effects of insertional mutations in *comQ, comP, srfA, spo0H, spo0A* and *abrB* genes on bacilysin biosynthesis in *Bacillus subtilis*, *Biochimica et Biophysica Acta* , **1626**, 51– 56

Zilhao, R., Naclerio, G., Henriques, A.O., Baccigalupi, L., Moran, C.P., Ricca, E., 1999. Assembly Requirements and Role of CotH during Spore Coat Formation in *Bacillus subtilis, Journal of Bacteriology*, Vol. **181**, No. 8

Zilhao, R., Serrano, M., Isticato. R., Ricca, E., Moran, C.P., Henriques, A.O., 2004. Interactions among CotB, CotG, and CotH during Assembly of the *Bacillus subtilis* Spore Coat, *Journal of Bacteriology*, Vol. **186**, No. 4

(http://genolist.pasteur.fr/SubtiList/)

(http:/bacillus-genome.ad.jp/)

APPENDIX A

Compositions and Preparation of Culture Media

Luria Bertani (LB) Medium (1000ml)

Tryptone 10 g/L

Yeast Extract 5 g/L

NaCl 5 g/L

Distilled H_2O was added up to 1000 ml and then autoclaved for 15 min.

Luria Bertani (LB) Agar Medium (1000 ml)

Tryptone 10 g/L

Yeast Extract 5 g/L

$NaCl_2$ 5 g/L

Agar 15 g/L

Distilled H_2O was added up to 1000 ml and then autoclaved for 15 min.

SMM (1000 ml)

$(NH_4)_2SO_4$ 2 g

K_2HPO_4 14 g

KH_2PO_4 6 g

Na_3.citrate.$2H_2O$ 1 g

$MgSO_4.7H_2O$ 0,2 g

Distilled H_2O was added up to 1000 ml and then autoclaved for 15 min. After cooling down, followings were added into the medium.

50% glucose 10 ml

L-tryptohan(3mg/ml) 10 ml

2xYT Medium (1000 ml)

Tryptone 16 g

Yeast Extract 10 g

NaCl 5 g

Distilled H_2O was added up to 1000 ml and then autoclaved for 15 min.

Agar 15 g (Add before autoclaving for solid 2xYT medium)

DSM (Difco's sporulation medium / agar) (1000 ml)

Nutrient Broth 8 g

KCl (10% w/v) 10 ml

$MgSO_4.7H_2O$ (1.2%) 10 ml

NaOH (1M) 0.5 ml

Autoclaved for 30 min and cooled down to 50°C.

$Ca(NO_3)_4$ (1M) 1 ml

$MnCl_2$ (0.01M) 1 ml

$FeSO_4$ (1mM) 1 ml (resuspend before use)

% 1.5 Agar was added if necessary before autoclaving.

For antibiotic resistance LB liquid and solid media:

Final concentration:

Spc: 100 μg/ml

Added to the liquid media after autoclaving and cooling down

Added to the liquid agar media after cooling down to 50°C but before pouring into petri dishes

APPENDIX B

Compositions of Buffers and Solutions

Reagents and solutions for gel electrophoresis of SASPs at low pH.

Solution needed:

A.	1M KOH	24.0 ml
	glacial acetic acid	11.2 ml
	tetramethylenediamine (TEMED)	2.3 ml
	H_2O	62.5 ml
B.	1M KOH	24.0 ml
	glacial acetic acid	1.44 ml
	tetramethylenediamine (TEMED)	0.23 ml
	H_2O	74.33 ml
C.	acrylamide	28 g
	bis-acrylamide	0.74 g
	H_2O	to 100 ml
	(filter solution C through Whatman #1 paper)	
D.	acrylamide	10.0 g
	bis-acrylamide	2.5 g
	H_2O	to 100 ml
	(filter solution D through Whatman #1 paper)	
E.	riboflavin	0.4 g
	H_2O	to 100 ml

F.	ammonium persulfate	0.14 g
	H_2O	to 100 ml

Running Buffer (8X)

β-alanine	31.2 g
Glacial acetic acid	8.0 ml
H_2O	to 1 litre
(dilute 1/8 before use)	

Sample Diluent

Glycerol	1.0 ml
Solution B	2.0 ml
0.25% methyl green	0.25 ml

Gel Stain

Methanol	50 ml
Glacial acetic acid	10 ml
H_2O	50 ml
Coomassie Brilliant Blue R	0.275 g

Destaining Solution

Methanol	75 ml
Glacial acetic acid	50 ml
H_2O	875 ml

Preparation of gels

	Solution	Relative amount
Lower resolving gel	A	1
	C	2
	F	4
	H_2O	1
Upper stacking gel	B	1
	D	2
	E	4
	H_2O	1

The total volume used fort he upper gel should be 1/5 that used fort he lower gel. The upper gel is polymerized for 30-60 min in strong sunlight; a long-wavelength transilluminator can be used to promote polymerization. To run samples on a 15x17x0.15 cm slab gel, mix two parts dissolved SASP extract with one part diluent and run 20 µl of the extract. Gel are stained for 45 min with gentle agitation, and destained overnight.

Reagents and solutions for SDS-PAGE analysis of spore coat proteins.

Solutions

A.	acrylamide	30.0 g
	bis-acrylamide	0.5 g
	H_2O	to 100 ml
	(filter solution A through Whatman #1 paper)	
B.	acrylamide	10.0 g
	bis-acrylamide	0.76 g
	H_2O	to 100 ml
	(filter solution B through Whatman #1 paper)	
C.	1.5 M Tris.Cl, pH 6.8	100 ml
	SDS	0.4 g

D.	0.5 M Tris.Cl, pH 6.8	100 ml
	SDS	0.4 g
E.	ammonium persulfate	100 mg
	dH_2O	1 ml
F.	tetramethylenediamine (TEMED)	

Running Buffer (8X)

Tris base	24.0 g
Glycine	115.2 g
H_2O	to 1 litre
	(pH 8.3)

dilute running buffer to 1X and add SDS to 0.1% final concentration just prior to use

Gel Stain

2-propanol	25 ml
Glacial acetic acid	10 ml
H_2O	56 ml
Coomassie Brilliant Blue R	0.2 g

Gel destain

2-propanol	100 ml
methanol	50 ml
glacial acetic acid	100 ml
dH_2O	750 ml

Gel storage

Ethanol	5 ml
Glacial acetic acid	10 ml
H_2O	85 ml

Gel formulation

	Solution	**volume(for 32 ml gel)**
Lower resolving gel	A	16 ml
	C	4 ml
	E	160 µl
	F	16 µl
	H_2O	11.82 ml
Upper stacking gel	B	5 ml
	D	2.5. ml
	E	100 µl
	F	10 µl
	H_2O	2.4 ml

The total volume used for the upper gel should be 1/5 that used for the lower gel.

APPENDIX C

ENZYMES AND CHEMICALS

Enzyme	**Supplier**
Lysozyme	Sigma

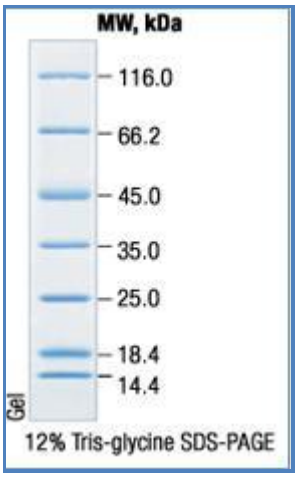
Chemical	**Supplier**
Acrylamide	Merck
Agar	Sigma
Alanine	AppliCHEM
Ammonium persulfate	Merck
Asparagine	Sigma
Bis-acrylamide	Merck
Choloroform	Merck
Ca $(NO_3)_4$	Merck
Coomassie Brilliant Blue R	Sigma
D(+)-Glucose	Merck
Ethanol	Riedel-de Haën
Fructose	Merck
Glacial Acetic Acid	Riedel-de Haën
Glycerol	Merck
Glycine	Merck
HCl	Merck
H_2O_2	Merck
Iron(III) sulfate – 7 – hydrate ($FeSO_4.7H_2O$)	Riedel-de Haën
KH_2PO_4	Merck

K_2HPO_4	Merck
KOH	Sigma
L-Cystein	Aldaich
L-Tryptophan	Merck
Methanol	Riedel-de Haën
$MnCl_2.4H_2O$	Merck
Magnesium sulphate ($MgSO_4.7H_2O$)	Riedel-de Haën
$Na_2SO_4.10H_2O$	Merck
Na_3citrate.$2H_2O$	Merck
N-acetlyglucoseamin	Sigma
Natrum hydroxid (NaOH)	Riedel-de Haën
Nutrient broth	Merck
Potassium chloride (KCl)	Riedel-de Haën
2-propanol	Riedel-de Haën
Riboflavin	Merck
SDS	Merck
Sodium chloride (NaCl)	Riedel-de Haën
Sodium hydrogen phosphate($Na_2HPO_4.7H_2O$)	Merck
TEMED	Carlo Ederba
Tris (hydrocymethyl) aminomethane	Merck
Tryptone	Sigma
Yeast Extract	Sigma

APPENDIX D

MARKERS……Fermentas

Unstained Protein Molecular Weight Marker



APPENDİX E

yvfI DNA Sequence

```
  1 atgaaacagg gagaaggcac gtatctgaag gaatttgagc tcaatcaaat ttctcagccg
 61 ctctcagccg cccttctgat gaaaaaagag gacgtcaagc agctgctcga ggtcagaaaa
121 ctgcttgaaa tcggcgtggc ttcactagcg gctgaaaaaa ggacagaagc agatctcgaa
181 agaattcagg atgcactaaa ggaaatgggc agcattgaag cggacgggga gctgggagag
241 aaagcagact ttgcatttca tcttgcgctt gcggacgctt ctcaaaatga acttcttaaa
301 cacttgatga atcacgtgtc atcattgctg ctggaaacaa tgagggaaac gaggaaaatc
361 tggctgtttt ccaagaagac ctccgttcag cggctgtatg aggagcacga acggatttac
421 aatgctgtgg ctgccgggaa cggtgcacag gcggaagccg ccatgctggc gcatttgacg
481 aatgtggaag atgtgctttc gggatatttc gaggaaaatg tgcaataa
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APPENDİX F

LABORATORY EQUIPMENT

Autoclave: Tuttnauer Systec Autoclave (2540 ml)

Balances: Precisa 620C SCS

Precisa 125 A SCS

Centrifuge: Beckman Coulter, Microfuge 18

Centrifuge rotor: F241.5P

Deep freezes and refrigerators: -80°C Heto Ultrafreeze 4410

-20°C Arçelik 209lt

+4°C Arçelik

Electrophoresis equipments: E – C mini cell primo EC320

Gel documentation system: UVI PHotoMW Version 99.05 for Windows

Incubators: Nüve EN400

Nüve EN500

Orbital shaker incubators: Sertomat S – 2

Thermo 430

Pipettes: Gilson pipetteman 10 µl, 20 µl, 200 µl, 1000 µl

Volumate Mettler Toledo 10 µl, 20 µl, 200 µl, 1000 µl

Eppendorf research 10 µl, 20 µl, 200 µl, 1000 µl

pH meter: Mettler Toledo MP220

Spectrophotometer: PerkinElmer Lambda25 UV/VIS Spectrometer

Thermomixer: Eppendorf thermomixer comfort (1.5 ml)

Transillumunator: Biorad UV transilluminator 2000

Vortexing machine: Heidolph Raax top

Waterbaths: Memmert wb-22

Amalgamator: YDM

Ultrafiltration tube: VIVASPIN

Lyophilizator : ALPHA 1-2 LD plus

Power supply: Bio-Rad

Dialysis Tubing Cellulose Membrane: Sigma-Aldrich

SDS-PAGE Apparatus: Bio-Rad

Polyacrylamide Gel Electrophoresis Apparatus: Bio-Rad

GS-800 CALIBRATED DENSITOMETER: Bio-Rad

CV

Ezgi Arslan was born in Istanbul in 1985. After getting her high school diploma from Halide Edip Adıvar (Yabancı Dil Ağırlıklı) Lisesi, in 2003, she started to study in Istanbul University, Science and Literature Faculty, Department of Biology 2003. She was graduated in 2007 and at the same year, she was accepted to Advanced Technologies in Molecular Biology, Genetics and Biotechnology's program in Istanbul Technical University, Department of Molecular Biology and Genetics.