ISTANBUL TECHNICAL UNIVERSITY ★ INSTITUTE OF SCIENCE AND TECHNOLOGY

Bacillus subtilis' DE SPORLANMA SÜRESİNCE *yvgW'* NUN FONKSİYONEL ROLÜ

M.Sc. Thesis by
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FUNCTIONAL ROLE OF *yvgW* GENE DURING SPORULATION IN *Bacillus subtilis*

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ABBREVIATIONS

ATP	: Adenosine triphospahe
bp	: Base pair
dH_2O	: Distilled water
DNA	: Deoxyribonucleic acid
DSM	: Difco's Sporulation Medium
EDTA	: Ethylenediaminetetraacetic acid
EtBr	: Ethidium bromide
IPTG	: Isopropyl-b D- thiogalactopyranoside
kb	: Kilobase
lacZ	: structural gene for β-galactosidase
LB- broth	: Luria Bertani broth
OD	: Optical density
PCR	: Polymerase chain reaction
SM	: Sterlini and Mandelstam Medium
TAE	: Tris acetate EDTA
Tris	: Hydroxymethyl aminomethane
cat	: Cm^R gene
erm	: erythromycin resistance gene
kan	: kanamycin resistance gene;
spc	: spectinomycin resistance gene
neo	: neomycin resistance gene
nm	: nanometer
SASP	: Small acid soluble proteins
DPA	: Dipicolinic acid

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FUNCTIONAL ROLE OF *yvgW* GENE DURING SPORULATION IN *BACILLUS SUBTILIS*

SUMMARY

Bacterial spores are now considered as the cells that show the greatest resistance abilities. Those though structures, they are formed when exposed to starvation and they may stay still against heat, UV, chemical treatments like lysozyme and chloroform and time. Moreover, dormant spores may return to their vegetative state, after cessation of starvation, through germination exposing a key component: spore coat. There are also other elements, found in bacterial spores that play important parts for the resistance characteristics and germination properties. Those are small, acid-soluble proteins, SASP, which are the predominant spore core proteins with a dual function during sporulation and germination and DPA (dipicolinic acid) which has an important role in spore core dehydration and provides the spore with a good stability during germination, leading to spontaneous germination.

Recently, under the light of the fact that, the timing of *yvgW* expression corresponds to the late sporulation stage, the dependence of *yvgW* expression on forespore-specific sigma factor σ^G was previously examined through construction of a new mutant strain containing transcriptional *yvgW::lacZ* fusion and an additional deletion on *spoIIIG* locus that is known to encode σ^G. The present research includes the studies to achieve the molecular characterization and elucidation of the functional role of *yvgW* gene in the sporulation process in *B. subtilis*. Effects of the deletion of *spoIIAC, spoIVCB, spoIVCB* and *sigB* genes, encoding for $\sigma^{F,}$ σ^E, σ^K and σ^B, respectively, on the expression of *yvgW-lacZ* were investigated. These subsequent analyzes of the effects of the sporulation-specific sigma factors on expression of *yvgW* gene strongly suggested that *yvgW* is transcribed in both mother cell and forespore under the control of mother-cell specific sigma factor σ^E and forespore-specific sigma factor σ^G, respectively. Moreover, through the construction of *yvgW*Δ537-1351*::spc, yvgW* mutant cells were investigated for their spore properties, such as their resistance profiles against heat chloroform and lysozyme, pointing out that spores of the mutant cells showed high sensitivity to heat and chloroform, but resistance to lysozyme. The level of dipicolinic acid was also investigated in *yvgW* spores and compared to wild type spores, revealing a significant reduction in *yvgW* spores as compared to wild type spores. Furthermore, the analyses of the nutrition specific germination and outgrowth characteristics of null mutant and wild type cells were performed in order to gain more insights about the functional role of *yvgW* gene in the sporulation process and those experiments showed that there was no defect in the initiation of *yvgW* spore germination but null mutant spores returned to vegetative state more slowly than the wild-type spores that .

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ÖZET

Günümüzde bakteri sporları, en çok dayanıklılık yetisi gösteren yapılar olarak bilinirler. Bu yapılar, açlıkla karşılaştıklarında oluşurlar ve sıcaklığa, mor ötesi ışınlara, kloroform ve lizozim gibi kimyasal uygulamalara ve zamana karşı da varlıklarını sürdürürler. Ayrıca, açlığın kalkması sonucunda önemli bir spor elemanı olan spor ceketi parçalanır ve çimlenme süreci ile "uyku" halindeki sporlar üreme hücresi durumlarına da dönebilirler. Bakteri sporlarında direnç karakterlerinde ve çimlenme özelliklerinde önemli yer tutan farklı etmenler vardır. Sporlanma ve çimlenme olmak üzere, ikili görevi olan, spor gömleği proteinlerin en önemlilerinden küçük, asitte çözünür proteinler ve DPA (dipikolinik asit) bunlardandır. DPA'nın, sporun iç bölge dehidrsayonunda önemli bir rolü vardır ve çimlenme boyunca sağladığı kararlı yapı ile spontane çimlenmeye yol açar.

yvgW ekspresyonun sporlanmanın geç evresinde gerçekleşmesi ışığı altında, *yvgW* geninin forspora-spesifik σ^G sigma faktörüne ekspresyon için bağımlılığı transkripsiyonel *yvgW::lacZ* füzyon ve fazladan *spoIIIG* lokusunda delesyon olan, σ^G'yi kodladığı bilinen yeni bir mutant suşun yaratılması ile incelenmiştir. Yapılmış olan bu çalışma, *B. subtilis*'te *yvgW* geninin sporlanma sürecinde işlevsel rolünün aydınlatılması ve moleküler karakterizasyonunun başarılması için yapılan deneysel araştırmaları içermektedir. Sırasıyla, $\sigma^{F,}$ σ^E, σ^K ve σ^B sigma faktörlerini kodlayan *spoIIAC, spoIVCB, spoIVCB* ve *sigB* genlerinin delesyonlarının, *yvgW-lacZ* geninin ekspresyonuna olan etkisi araştırılmıştır. Bu sporulasyona özgü sigma faktörlerin *yvgW* üzerindeki etkilerinin incelendiği analizler sonucu, yvgW geninin anne hücreye özgü sigma faktörü, σ^E ve forspora özgü sigma faktörü, σ^G' nin kontrolü altında, hem anne hücre hem de forsporda ifade edildiği önerilmiştir. Ayrıca, *yvgW*Δ537-1351*::spc* oluşturulması ile, *yvgW* mutantının sıcaklık, kloroform ve lizozime karşı dirençliliği incelenmiştir ve bu incelemeler mutant suşun sporlarının sıcaklık ve kloroforma karşı hassasiyet ve lizozime karşı dirençlilik gösterdiğini ortaya çıkarmıştır. *yvgW* sporlarında DPA düzeyleri de incelenmiş, yaban birey ile karşılaştırılmıştır ve sonuçta mutant suşun oluşturduğu sporların belirgin bir miktarda daha az DPA oranı gösterdiği görülmüştür. Nul mutant ve yaban bireyin gösterdikleri beslenmeye bağlı özel çimlenme ve çimlenmeden sonra tekrar vejetatif büyümeye geçme (outgrowth) özellik analizleri, *yvgW* geninin sporlanma sürecindeki işlevsel rolünü anlamada daha fazla bilgi sahibi olunması için gerçekleştirilmiş ve bu deneyler hücrelerin çimlenme başlangıcında sorun yaşamadıklarını ancak null mutant sporların çimlenmeden sonra vejetatif hallerine daha geç döndüklerini göstermiştir.

1. INTRODUCTION

1.1. *Bacillus subtilis*

Members of genus *Bacillus* represent aerobic, endospore-forming, rod-shaped Gram-positive bacteria which also have an industrial importance coming from their capability of producing antibiotics, proteases, insecticides and so on (Harwood et al., 1990). When their endospore-forming ability come along with the ease of working genetics and physiology of this bacteria, representatives of this genus took their milestone role in the trials of analyzing Gram-positive bacteria (Sonenshein et al., 2002).

Detection of first endospores was realized in *Bacillus subtilis* and *Bacillus anthracis* in 1876 by Cohn and Kock. Eventually, *Bacillus subtilis* has become not only one of the most intensively studied bacteria but also one of the most clearly understood organism found in nature. In fact, the particular investigation of this organism is counted as the most immaculate after *Escherechia coli*, among prokaryotes (Sonenshein et al., 2002 and Harwood et al., 1990).

Additionally, *Bacillus subtilis* is a chemoorganotroph, so that it is able to maintain a suitable environment containing factors it demands for its growth by simply oxidizing organic compounds belonging to a broad range of family. Moreover, just like many other members of its genus, *Bacillus subtilis* is mesophilic and may undergo growth and production of normal-sized colonies within a day when placed at 37^{o}C. Another important characteristic of this organism is its being aerobe and therefore it requires sufficient aeration during growth (Harwood et al., 1990).

Proved as the best representative of Gram-positive bacteria, soil bacterium *Bacillus subtilis* has a genome of 4.2 Mbp long (Franguel et al., 1999). Its genome sequence was completed in 1997 by an international collaboration, started initially by Japanese government and European Economic Union (Kunst et al., 1997). It is now known that *Bacillus subtilis* uses 275 genes, 25 of which are unknown, in order to grow in rich medium at moderate temperatures and in aerated environment (Kobayashi et al.,

2002). Its genome also consists of 17 sigma factors and approximately 250 DNA binding transcriptional regulators. In addition to these, 4106 protein-coding, 86 tRNA, 30 rRNA and 3 small stable RNA genes were located on its genome (Ando, 2002; Kobayashi et al., 2002).

In 1947, Burkholder and Giles reported that they isolated many auxotrophic mutants of *Bacillus subtilis*, one of which is a tryptophan requiring strain called BGSC1A1, *Bacillus subtilis* 168. Subsequently, in 1958, transformable characteristic of this strain was reported and following this information, *Bacillus subtilis* 168 has became the most useful strain for genetic researches based on this organism (Spizen, 1958; Harwood et al., 1990). Therefore, *Bacillus subtilis* PY79 has found its place as the wild type strain of this project as a consequence of its being prototrophic derivative of *Bacillus subtilis* 168.

1.2. Sporulation in *Bacillus subtilis*

A wide range of bacteria go for specialized differentiated cell types when exposed to difficult environmental conditions and in order to handle starvation of many sources, like carbon, nitrogen or in some circumstance a phosphorous source (Errington, 2003; Piggot and Hilbert, 2004). For instance in *Bacillus subtilis*, a variety of responses are stimulated to allow the bacteria survive in the increasingly hostile environment (Grossman, 1995; Phillips and Strauch, 2002). Production of antibiotics and macromolecular hydrolases, development of motility, chemotaxis and competance are among these responses (Jong et al., 2003; Kunst et al., 1997). Another response that makes *Bacillus subtilis* to form specialized cells is sporulation which is the final resort for this bacterium (Jong et al., 2003). *Bacillus subtilis* undergoes symmetric cell division, generating two identical daughter cells during vegetative growth, while during sporulation cell division is asymmetric, followed by engulfement of the smaller forespore by the larger sibling, the mother cell (Errington, 2003; Levin and Grossman; 1998).

The process of sporulation, which represents a series of morphological and physiological events, that occur through the sequential activation and silencing or blocking of genes, starts at the end of the exponential growth and this process requires approximately 8 to 10 hours in order to be completed. Steps of that morphological event are divided into seven stages that are designated with Roman

numerals. Figure 1.1 gives a schematic view of these stages (Errington, 2003). Sporulation mechanism begins with a vegetative cell, called the stage 0 of the sporulation and it ends with the release of mature spore, defined as stage VII (Errington, 1993). Cells enter the sporulation pathway by the formation of an axial filament, through which chromosomes generated by DNA replication become aligned across the long axis of the cell (Stragier and Losick, 1996). Next, the specialized spore septum is formed at a polar position stage II, giving rise to an asymmetric division, that subsequently produce two cells differing in size (Errington, 1993; Stragier and Losick, 1996). Stage I is no longer recognized because it was defined as not being specific to sporulation (Piggot and Coote, 1976). Engulfement of forespore by the mother cell is completed at stage III. In this stage forespore cells don't show their rigid shape because it lacks a thin layer of peptidoglycan called as the germ cell wall, which will end up later in spore cortex covering the forespore. Therefore, this defined shape starts to develop with the development of cortex between the forespore membranes during stage IV and furthermore the forespore starts to produce large amounts of small acid-soluble proteins known as SASP. Furthermore, this spore cortex is thought to be involved in attaining or maintaining the dehydrated and heat-resistant state of the spore. During this stage, proteinaceous spore coat also begins to be deposited outside the surface of the spore, stage V. The final period of sporulation is referred as the maturation, stage VI, along which properties of resistance, dormancy and germinability appear in sequence (Errington, 1993; Errington, 1996, Webb et al., 1997). Final stage of sporulation is the stage VII and mature spore is released following the lysis of the mother cell, undergoing programmed cell death, whereas the immortal forespore becomes the mature spore and gives rise to subsequent progeny (Errington, 1993; Nicholson and Setlow, 1990, Stragier and Losick, 1996).

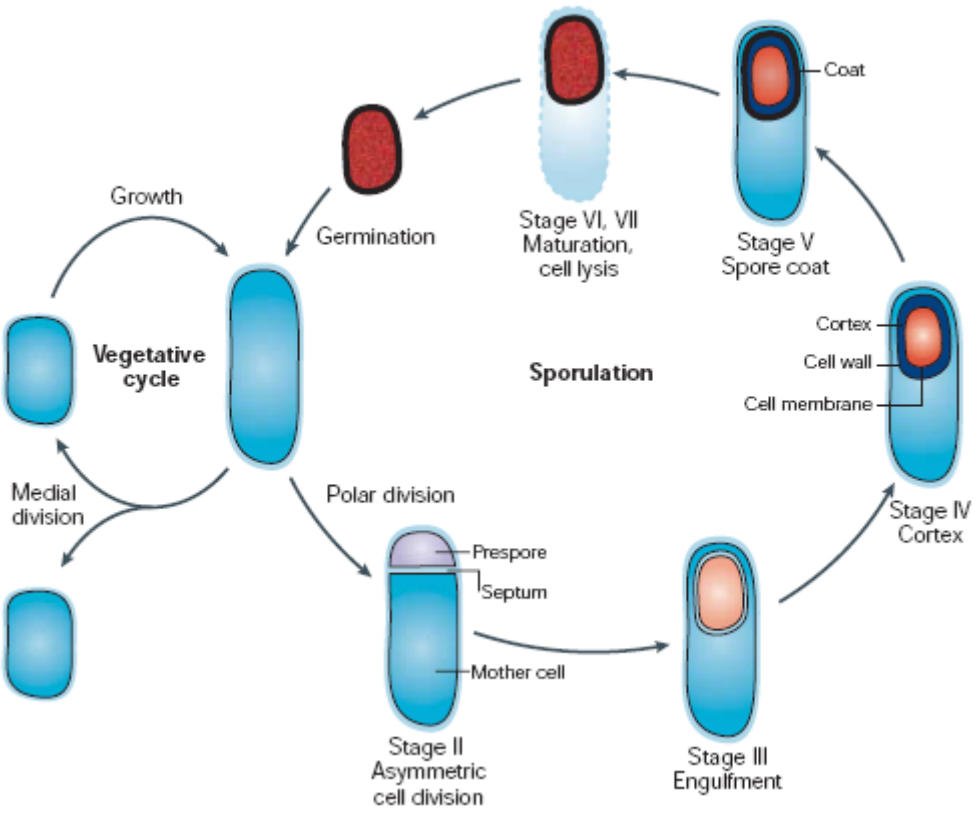


Figure 1.1: This simplified schematic shows only the key stages of the cycle designated by Roman numerals.

1.2.1 Control of initiation of sporulation

In bacteria, main elements of cellular regulatory pathways are two-component signal transduction systems and bacterial cells use these systems in order to sense environmental change and develop a response on the basis of gene expression (Hoch and Silhavy, 1995). First one of these two components is the signal ligand-responsive sensor histidine kinase and second one is the response regulator that is commonly a transcription factor. Receiving specific environmental signal sensor kinases bacteria cause an ATP-dependent autophosphorylation on a conserved histidine residue and this phosphorylated group is then transferred to a conserved aspartate residue in the regulatory domain of a specific response regulator-transcription factor, leading to the activation and/or repression properties of the output domain (Stephanson and Hoch, 2002). When these two-component system reveal a more complex signal transduction pathway, they turn into a phosporelay cascade during which signal integration circuits allow a higher level of control with more nodes and checkpoints for the input of both positive and negative signals. This system can be obviously

observed in Bacillus subtitlis sporulation phophorelay (Perego et al., 1994; Perego and Hoch, 1996; Stephenson and Hoch, 2002).

In *Bacillus subtitlis*, regulation of sporulation in its initial level contains *abrB* and *spo0H* genes and also seven response regulator proteins, Spo0A, Spo0B, Spo0E, Spo0F, Spo0J, Spo0K and Spo0L, with Spo0A being the most critical control factor (Frandsen et al., 1999, Fawcett et al., 2000). Sporulation initiation signals in *Bacillus subtitlis* result in the activation of the master regulatory protein, Spo0A, by phosporylation (Piggot and Hilbert; 2004). First of all, at least five sensor kinases , KinA, KinB, KinC, KinD and KinE feed their phosphate into the phosphorelay cascade by initially phophorylating the single domain response regulator Spo0F (Fig 1.2) (Fabret et al., 1999). Then this phosphate group is transferred to the relay protein Spo0B and finally to Spo0A (Stragier and Losick, 1996). Although the phosphate contribution of KinB may surpass that of KinA under certain environmental conditions, the two kinases may respond to different nutritional indicators (LeDeaux et al., 1995). The fact that KinA is cytoplasmic, whereas KinB is membrane-bound, may be used to explain these differences in sensing.

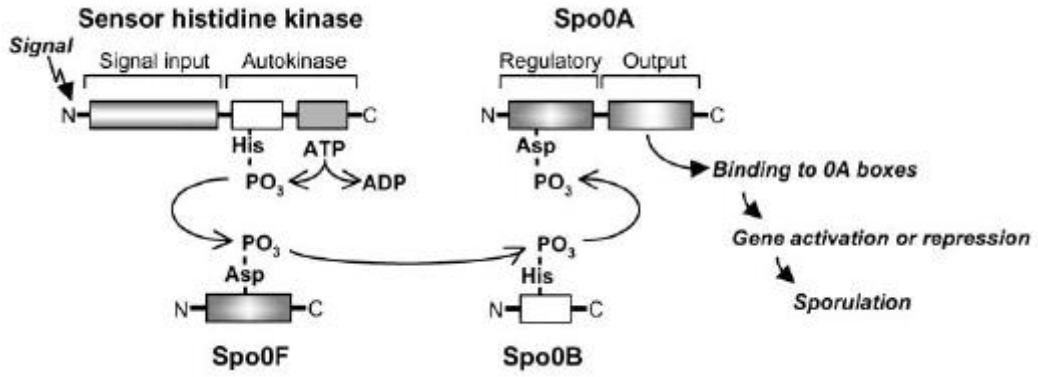


Figure1.2: The *B. subtilis* sporulation phosphorelay.

Phosphorylated Spo0A works by activating the transcription of several key sporulation-specific genes, and blocking expression of *abrB* gene that is a global negative regulator (Fujita and Losick, 2003). The product of *abrB* gene is a repressor that functions in the prevention of the expression of transition stage-specific genes during vegetative growth. *spo0E, spo0H, spoVG* genes, necessary for normal sporulation, stand among the genes regulated by *abrB*. Blockage of transcription of these genes affect sporulation, but among these genes, the crucial one is *spo0H*,

which encodes a nonessential sigma factor, σ^{H} that is involved in expression of vegetative and early stationary-phase genes (Stragier and Losick, 1996).

Additionally, the regulation of phosphate-flow through is largely reversible so that, dephosphorylating any of the components not only prevents new Spo0A~P from being generated, but also shifts the overall equilibrium away from Spo0A~P, resulting in removing what is already present, thereby blocking initiation (Stephens, 1998).

Activated spo0A shows mainly dual function; first one is the triggering the asymmetric division and the second function is the transcription of spo0IIA, spo0IIE and spo0IIG loci, which encode key furthermore developmental regulators (Piggot and Hilbert, 2004). Spo0A is known to regulate transcription of 121 genes, including several transcription factors and about 400 genes are indirectly controlled by spo0A (Stragier and Losick, 1996).

It is now that, different on different roles of spo0A are dependent on differing levels of phosphorylation (Piggot and Hilbert; 2004). Furthermore, within a population, Spo0A activity levels are variable and this variation causes deep impacts on development. For instance, cells that constitute of activated Spo0A produce on extracellular killing factor that kills cells that have not active Spo0A proteins. The ultimate purpose of this mechanism is enabling the procedures to feed on their non-producing siblings (Gonzalez-Pastor et al., 2003). Moreover, activated Spo0A (Spo0A~P) functions on switching from symmetric to asymmetric septation, such that a mutant, unable to produce a sufficient level of Spo0A~P, goes for a normal chromosomal segregation as it did during vegetative growth instead of forming on axial filament and a polar septum (Levin and Grossman, 1998). As shown by Fujita and Losick in 2003, Spo0A continues to function of polar septum, when it is present exclusively in mother cell, functioning as a mother-cell specific transcription factor.

1.2.2 Regulation of σ factor activity during sporulation in development

It has been 25 years that a set of sigma (σ) factors are known to control temporal gene expression during sporulation in *Bacillus subtilis* (Losick and Pero; 1981). *B. sutitlis* carries genes for 17 different σ factors, and least 6 of which direct RNA polymerase (RNAP) to transcribe sporulation-specific genes, σ^{A}, σ^{E}, σ^{F}, σ^{G}, σ^{K} and σ^{H} (Kroos and Yu, 2000) (Figure 1.3).

Moreover, σ^F and σ^E factors are achieved shortly after assymetric division during which σ^F directs gene expression in forespore and σ^E direct gene expression in mother cell. σ^F and σ^E factors direct the transcription of largely overlapping sets of genes and later in sporulation σ^F is replaced by σ^G and σ^E is replaced in the mother cell by σ^K (Mergolis et al., 1991; Driks and Losick, 1991, Li and Piggot, 2001; Losick and Stragier, 1992) (Figure 1.4).

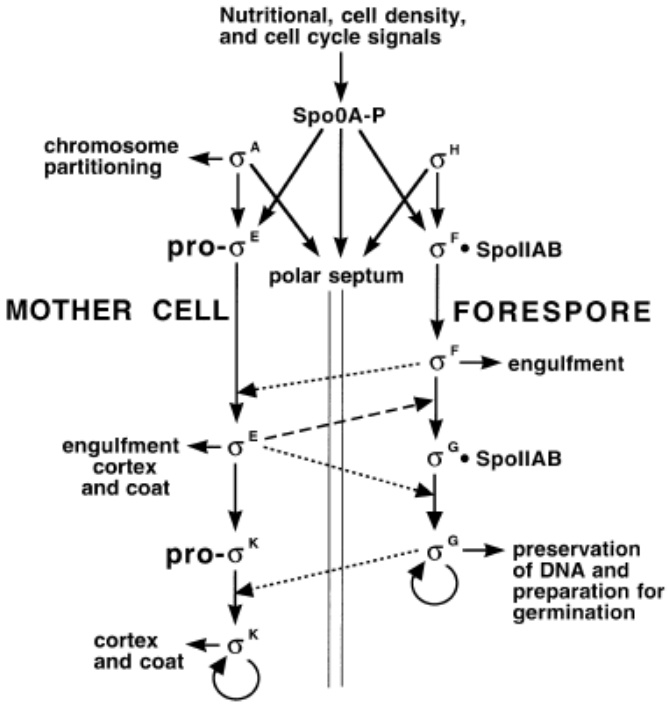


Figure 1.3: Regulatory network controlling sigma factor synthesis and activation. Solid arrows indicate dependence relationships for σ factors and the products of genes that bring out morphological change. The two vertical lines represent the membranes that separate the mother cell and forespore after polar septum formation. Dashed arrows show signaling interactions between the two cell types that govern σ factor activation (short dashes) or synthesis (long dashes) (Kroos *et. al.*, 1999).

1.2.2.1. Sigma A and Sigma H

Spo0A~P acts in cojunction with the housekeeping sigma factor σ^H in order to drive gene transcription at the onset of sporulation (Stragier and Losick 1996; Kroos and Yu, 2000). σ^H, when interacts with core RNAP, it directs this enzyme to initiate transcription from at least 49 promoters controlling 87 or more genes (Britton et al., 2002).

Additionally Spo0A~P binds to promoter regions and activates transcription by σ^A RNAP and σ^H RNAP probably by contacting homologous regions of σ factors with different surfaces of its carboxy terminal domain and by contacting RNAP also with

its amino-terminal domain (Buckner and Moran, 1998; Cervin and Spiegelman, 1999). Furthermore in response to starvation, σ^A which is the major factor present in growing cells and σ^H a minorly abundant σ factor, direct transcription of genes whose products redirect septum formation and partition of each copy of the replicated DNA to forespore or mother cell. Moreover early in sporulation, σ^A and σ^H complete with each other for binding to core RNAP. The level and activity of σ^H is controlled by transcriptional and post-transcriptional mechanisms. With the help of inhibition of AbrB production by Spo0A~P, transcription of the spo0H gene encoding σ^H increases (Kroos et al., 1999).

Furthermore, it is also reported by Fukushima et al. in 2003 a sigma A controlled veg gene was found to be expressed in the forespore, presumably functioning in further germination process suggesting that σ^A controls expression of some specific genes both before and after engulfment, due to its housekeeping characteristics

1.2.2.2. sigma F

In the time course of sporulation, the first activated sigma factor is σ^F, which becomes active in the forespore soon after asymmetric septation. σ^F is already present in the cell before sporulation begins, but it is kept inactive until asymmetric septation occurs. If σ^F is activated at the right time and in the right compartment, then the remaining sigma factors become correctly activated in their turn and sporulation progress to completion. Thus, the accurate regulation of σ^F is crucial for the success of sporulation (Kroos et. al., 1999).

Activity of σ^F is confined to the forespore cell and cell-specific activation of σ^F is governed by a pathway involving the proteins SpoIIAB, SpoIIAA and SpoIIE (Mergolis et al. 1991; Duncan and Losick, 1993)

In the predivisional sporangium and in the mother cell σ^F is held in an inactive complex with SpoIIAB, which is referred to as an antisigma factor (Duncan and Losick, 1993). σ^F is liberated from the SpoIIAB- σ^F complex in the forespore due to the action of SpoIIAA, an anti-antisigma factor that binds to and antagonizes the action of spoIIAB (Alper et al., 1994; Diedrich et al., 1994). Action of SpoIIAA is regulated by it inteconversion between a phosphorylated and a dephosphorylted state (Duncan et al, 1995). This interconversion is governed by opposing actions of a serine kinase and a phosphatase. Serine kinase is SpoIIAB, which is a dual function

protein that is capable of binding to and inhibiting σ^F and of phosphorylation and thereby inactivating SpoIIAA (Alper et al., 1994; Diedrich et al., 1994). This reverse action is catalysed by the third member of σ^F regulatory pathway, spoIIE (Duncan et al., 1995). SpoIIE is a phosphatase that is responsible for converting the inactive, phosphorylated form of SpoIIAA (SpoIIAA-P) to its active, dephosphorylated form (Seavers et al., 2001). Therefore, SpoIIE indirectly activates σ^F through the dephosphorylation of SpoIIAA-P. SpoIIAB and SpoIIAA is mutually antagonistic: ATP-containing SpoIIAB can inactivate spoIIAA by covalent modification can inhibit ADP-containing spoIIAB by sequestering it in an inactive spoIIAA-AB complex. The capacity of spoIIAB to form alternative complexes with spoIIA is known as partner switching (Alper et al., 94; Diedrich et al., 94).

1.2.2.3 Sigma E

Establishment of forespore specificity through activation of σ^F is soon followed by the induction of a large set of genes in the mother cell, under the control of the transcription factor, σ^E (Londono-Vallejo and Stragier, 1995). σ^E is initially synthesized as an inactive proprotein pro-σ^E and this synthesis starts prior to septation but the conversion of pro-σE to mature σ^E does not take place unit after asymmetric division when the active form of the transcription factor is found in the mother cell. Proteolytic processing of pro-σ^E is mediated by the proprotein processing enzyme spoIIGA which is activated by a secreted signaling protein spoIIR that is produced in the forespore under the control of σ^F (Londono-Vallejo and Stragier, 1995; Peters and Haldenwang, 1994; Hofmeister et al., 1995; Karow et al., 1995).

It was previously suggested by Helmann and Moran at 2002 that the largest regulon of four cell-specific σ factors, 70 genes are directly controlled by σ^E. However in 2003, Eichenberg and friends reported that σ^E directs the transcription of more than 250 genes during sporulation in *Bacillus subtilis*.

The first major function accomplished by genes under the control of σ^E is to promote the engulfment of the forespore by the mother cell. Three genes have been shown to be essential for this process (*spoIID*, *spoIIM* and *spoIIP*) (Fraudsen and Stragier, 1995). Their products are involved in degradation of the septal peptidoglycan and mutations in *spoIID*, *spoIIM* or *spoIIP* block sporulation at morphological stage II,

and prior to the stage of engulfment. Furthermore, it has been shown recently that these genes are also necessary for preventing septation at the other pole of the sporangium, creating a disporic formation (Poglieno et al. 1999; Eichenberg et al., 2001)

Second major function of σ^E controlled genes is the synthesis of the protective envelopes around the spore, the cortex and the different spore-coat layers. For instance SpoVB, SpoVD and SpoVE one σ^E controlled genes that function in cortex formation (Popham and Stragier, 1991; Daniel et al., 1994; Piggot et al., 1986)

The third major function, carried out by a large group of genes under the control of σ^E is to maintain a sufficient level of metabolic activity to enable the progression of the sporulation process under conditions of limiting nutrient availability (Eichenberg et al. 2003). Many genes in this group, like *ylbK*, *yvjE* and *ywnE* appear to be involved in lipid metabolism. This suggests a possible way of generating energy in the absence of nutrients by oxidation of fatty acids from the cytpolasmic membrane and also of way polyhydroxyalkanate other genes like catabolism *mlpA*, *pepE* and *yufN* also encodes putative peptidases and proteases for the protein degradation in order to generate nutrients (Eichenberg et al., 2003; Jendrossek and Handrich, 2002)

The forth major function of σ^E is to set the stage for the next and final steps of sporulation. Three major transcriptional regulators are controlled by σ^E, *spoIIID*, σ^G and σ^K (Stragier et al., 1989; Kroos et al., 1989; Halberg and Kroos, 1994; Sato et al., 1994). Meanwhile, both σ^E and σ^K activity are needed in the mother cell for synthesis of the cortex and coat layers that encase the forespore. The cortex, produced by σ^E-controlled genes, is a loosely cross-linked peptidoglycan formed between the membranes surrounding the forespore (Eichenberg et al., 2003).

1.2.2.4. Sigma G

Transcription of the *spoIIIG* gene, encoding the late forespore regulator σ^G, is driven by the σ^F related RNA polymerase. However, transcription of *spoIIIG* is delayed towards the end of the engulfment process, when compared to transcription of first class σ^F-dependent genes. Additionally, it requires both the activity of σ^E in the mother cell and expression of the σ^F-controlled gene *spoIIQ* (Sun et al., 2000).

The late forespore-specific σ factor, σ^G is regulated at least three levels. First, its gene sigG or spoIIIG is transcribed from a promoter recognized by the first

forespore-specific σ factor, σ^F and later by σ^G itself thus restricting its location to the porespore compartment (Sun et al., 1991). Secondly, unlike other σ^F-dependent genes, *sigG* is not transcribed in the presence of mutations in the SpoIIG gene, which encodes first mother-cell specific sigma factor σ^E. Therefore, sigG transcription is also dependent on as yet unidentified signal transduction pathway of which at least one component is expressed in mother cell. Thirdly, *sigG* begins to be transcribed appoximately 120 minute after the initiation sporulation. However σ^G-dependent genes expression does not begin until 30 minute later (Patridge and Errington, 1993). Mutations in spoIIB, spoIID, spoIIIA and spoIIIJ, prevents transcription of σ^G-depenendent genes without effecting σ^G synthesis, impliying that their products play a role in σ^G activation (Perez et al., 2000; Partridge and Errington, 1993). *spoIIB*, *spoIID* and *spoIIM* required for forespore engulfment, suggesting a link bw activation of σ^G and the completion of engulfment (Perez et al. 2000; Smith et al., 1993)

Evans et al. 2003 suggested that σ^G and anti sigma factor SpoIIAB form a nucleotide-dependent complex although this interaction is much whether than that of σ^F and spoIIAB. Furthermore anti-anti σ factor spoIIAA efficiently disrupts SpoIIAB:σ^G complex, thereby relasing σ^G. So that they report that spoIIAB interacts with σ^G in the same way as with σ^G that may be another mechanism to keep σ^G inactive while σ^F is active.

The function of the σ^G is activating transcription of a large set of genes in the engulfed forespore, including the *ssp* genes encoding members of the SASP family. Among the genes activated by σ^G, the *spoVT* gene appears to play a specific regulatory role by encoding a protein required for expression of a subset of σ^G-controlled genes in the forespore, such as *spoVA*. The SpoVT protein is related to the AbrB protein, a well-studied *B. subtilis* DNA-binding protein, and it is believed that, when the time needed for its concentration to reach a critical threshold expires, the SpoVT protein binds to some regulatory DNA sequences and allows expression of the latest class of forespore-specific genes. Moreover, via inhibiting *spoIIIG* transcription, SpoVT may also contribute to a progressive shut-off of the forespore-specific transcription (Serrano et al., 2003).

1.2.2.5. Sigma K

The late-appearing, mother-cell-specific transcription factor σ^K is synthesized as an inactive precursor protein, known as pro-σ^K, under the direction of σ^E acting in conjunction with the DNA-binding protein SpoIIID. Following this, σ^K collaborates with SpoIIID and directs transcription of its structural gene. Later in sporulation, transcription of *sigK* is repressed by the product of *gerE* that is controlled by σ^K (Kroos et al., 2002).

The first level of regulation involves the creation of the *sigK*, interrupted by a large DNA element known as *skin*. The rearrangement of the intact σ^K coding sequence is accomplished by SpoIVCA, which is a recombinase that catalyzes the excision of *skin*. Furthermore, SpoIVCA is transcribed by the action of σ^E that works in conjunction with SpoIIID. As a consequence, the two truncated coding elements become joined in-frame. Because σ^E and SpoIIID are produced exclusively in the mother cell, *skin* is not excised from the germ line chromosome of the forespore and hence is passed to the subsequent progeny (Stragier and Losick, 1996).

The second level of regulation is the transcription of *sigK* that initially requires the concerted action of σ^E and SpoIIID. Once again, this helps to ensure that σ^K is produced only in the mother cell (Kroos et al., 1999).

Finally, the third level of regulation involves proteolytic removal of pro amino acid sequence of σ^K, like its early counterpart σ^E, achieved through initially signaling from the forespore. The signaling pathway involves SpoIVB, a serine peptidase produced in the forespore, which is believed to cross the innermost membrane of the forespore and activates a complex of proteins, including BofA, SpoIVFA, and SpoIVFB, located in the outermost membrane surrounding the forespore. Activation of the complex allows proteolytic processing of pro-σ^K, and the resulting σ^K RNA polymerase transcribes genes in the mother cell (Wakeley et al., 2000).

σ^K directs the expression of the final regulon of sporulation genes whose products are involved in maturation of the spore coat and cortical layers (Kroos et al., 1999). Thus, premature expression of σ^K-controlled genes, leads to the production of a reduced number of spores with a disorganized coat structure, leading to defective germination (Nicholson and Setlow et al., 1990). In addition to this function, σ^K is

thought to be directing the transcription of genes involved in lysis of the mother cell and release of the mature spore (Errington, 2003).

1.3. Spore Germination in *Bacillus subtilis*

Bacterial endospores have a unique structure that determines their extreme resistance properties. The structure of *Bacillus subtilis* endospores includes three distinct features, i.e. spore coat, cortex and core. The spore cortex, which is required for spore dormancy and heat resistance, consists of a thick layer of peptidoglycan of a spore-specific structure. Despite their resistance and dormancy, the dormant spore monitors its environment, and when conditions are again favorable for growth, the spore germinates and goes through outgrowth, ultimately being converted back into a growing cell (Chirakkal et al., 2002). Resuscitation of spores is associated with a loss of phase brightness caused by hydration of all internal structural compartments which results in a phase dark, germinated spore when viewed under phase contrast optics of a light microscope. Germination of a spore population is a diverse, heterogeneous process and it is necessary to correlate the characteristic changes in a population of germinating spores with the behavior of individual spores in the same population (Vary and Halvorson, 1965).

1.3.1. Earliest events in spore germination

Earliest events in spore germination involve receptor proteins encoded by homologues of the GerAA, AB and AC proteins (Paidhungat and Setlow, 1999; Moir et al., 2002), which are located in the inner membrane (Hudson et al., 2001; Paidhungat and Setlow, 2001). Receptor±germinant interaction results in the triggering of a cascade of biochemical events, including loss of heat resistance and ion movements from the core (Thackray et al., 2001; Southworth et al., 2001).

1.3.1.1. Germinant receptors

The action of nutrient germinants is initiated by binding to receptors located in the spore's inner membrane. In *B. subtilis* these receptors are encoded by the homologous tricistronic *gerA*, *gerB* and *gerK* operons (termed *gerA* operon homologs) expressed in the forespore late in sporulation. Similar GerA-type proteins are present in spores of other *Bacillus* and *Clostridium* species. In *B. subtilis* spores,

the GerA receptor recognizes L-alanine, whereas the GerB and GerK receptors are required together for germination with AGFK (Paidhungat and Setlow, 2002; Moir et al., 2002).

In addition to the gerA operon homologs, there are several other genes whose products play roles in *B. subtilis* spore germination (Paidhungat and Setlow, 2001). These include: *gerF*, whose product adds diacylglycerol to membrane proteins, probably including the proteins encoded by the B cistrons of the *gerA* operon homologs; *gerC*, which encodes an enzyme of quinone biosynthesis; and *gerD*, whose product is needed in some unknown fashion for nutrient germination (Setlow, 2003).

1.3.1.2. Nutrient Germinants

Spores in nature germinate probably only in response to nutrients, termed germinants. These germinants are generally single amino acids, sugars or purine nucleosides, but there are also combinations of nutrients that trigger spore germination, one being a mixture of asparagine, glucose, fructose and K^+ (AGFK) that triggers *B. subtilis* spore germination. Within seconds of mixing spores and germinants, the spore becomes committed to germinate, and germination will proceed even after removal of the germinant (Paidhungat and Setlow, 2001).

1.3.2. Subsequent steps in germination

A common activation treatment for spore germination is a sub-lethal heat shock, but the mechanism of spore activation is not well understood, Following the germination initiation during which receptor±germinant interaction take place, subsequent steps of germination are triggered as seen figure 1.4 (Paidhungat and Setlow, 2001). First, spore H^+, monovalent cations and Zn^{2+} are released probably from the spore core and the release of H^+ elevates the core pH from 6.5 to 7.7 which is a change essential for spore metabolism once spore core hydration levels are high enough for enzyme action (Jedrzejas and Setlow, 2001). Second, the spore core's large depot (10% of spore dry wt) of pyridine-2, 6-dicarboxylic acid (dipicolinic acid [DPA]) and its associated divalent cations, predominantly Ca^{2+} is released. Third, replacement of DPA by water takes place, resulting in an increase in core hydration and causing some decrease in spore wet-heat resistance, although this initial increase in core hydration is not sufficient for protein mobility or enzyme action in the spore core

(Setlow et al., 2001; Cowan et al., 2003). Fourth, spore's peptidoglycan spore cortex is hydrolysed and fifth, swelling of the spore core through further water uptake and expansion of the germ cell wall take place (Setlow et al., 2001). Only after this further increase in core hydration does protein mobility in the core return, thus allowing enzyme action (Setlow et al., 2001; Cowan et al., 2003). These events take place without detectable energy metabolism, and comprise the process of germination (Paidhungat and Setlow, 2001). This process is divided into two stages; stage I comprise the first three steps of the process, and stage II comprises the fourth and fifth steps. The two stages can be separated experimentally by either chemical treatments or mutations (Paidhungat and Setlow, 2001; Setlow et al., 2001; Setlow et al., 2002). The initiation of enzyme action in the spore core after completion of stage II allows initiation of spore metabolism, followed by the macromolecular synthesis that converts the germinated spore into a growing cell (Paidhungat and Setlow, 2001). This period is termed spore outgrowth.

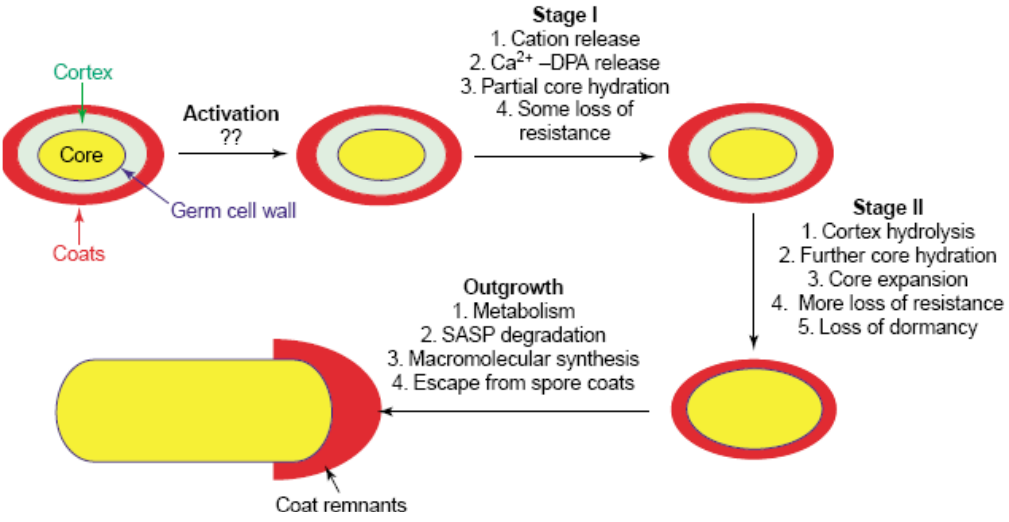


Figure 1.4: Events in spore germination.

Spore germination is divided into two stages, as cortex hydrolysis is not required for stage I. SASP degradation denotes the hydrolysis of the large depot of small, acid-soluble spore proteins (SASP) that make up 10–20% of the protein in the spore core. One type of SASP, the α/β-type, saturates spore DNA and prevents many types of DNA damage (Setlow, 2000). Whereas metabolism and SASP degradation (which require enzyme action in the spore core) are shown as taking place only after stage II is complete, these events may begin partway through stage II when the core water

content has risen sufficiently for enzyme action. Also shown in figure 1.4 is that the spore's germ cell wall must expand significantly to complete stage II of germination (Paidhungat and Setlow, 2001). The events in stage I may take only seconds for an individual spore, although there may be a lag of several minutes after addition of a germinant before these events begin, and spore cortex degradation may take several minutes for an individual spore. However, because of significant variation between individual spores, particularly in the times for the initiation of the first events in stage I after addition of a germinant, these events may take many minutes for a spore population (Setlow, 2003).

1.3.3. Crucial roles of SASP and DPA in resistance characteristics and germination properties of *Bacillus subtilis*

Since DPA is found only in dormant spores of *Bacillus* and *Clostridium* species and since these spores differ in a number of properties from vegetative cells, in particular in their dormancy and heat resistance, it is not surprising that DPA and divalent cations have been suggested to be involved in some of the spore's unique properties. There is some evidence in support of this suggestion, since mutants whose spores do not accumulate DPA have been isolated in several *Bacillus* species, and often these DPA-less spores are heat sensitive (Balassa et al., 1979; Coote, 1972; Piggot et al., 1980; Wise et al., 1967; Zytkovicz and Halvorson., 1972).

DPA is synthesized from an intermediate in the lysine pathway, and the enzyme that catalyzes DPA synthesis is termed DPA synthetase (Daniel and Errington, 1993). In *B. subtilis* this enzyme is encoded by the two cistrons of the *spoVF* operon, which is expressed only in the mother cell compartment of the sporulating cell, the site of DPA synthesis. Mutants of *B. subtilis* likely to be in or known to be in *spoVF* result in lack of DPA synthesis during sporulation, and the spores produced never attain the wet heat resistance of wild-type spores (Balassa et al., 1979; Coote, 1972; Piggot et al., 1986; Daniel and Errington, 1993). Unfortunately, it has been impossible to isolate and purify free spores from these *spoVF* mutants of *B. subtilis*, since the spores are extremely unstable and germinate and lyse during purification. This observation suggests that, at least in *B. subtilis*, DPA is needed in some fashion to maintain spore dormancy (Errington, 1993; Lewis, 1969), although the specific mechanism whereby this is achieved is not clear. In addition to its possible roles in

spore dormancy and resistance, DPA complexed with a divalent cation, usually Ca^{2+}, is an effective germinant of spores of almost all *Bacillus* and *Clostridium* species. These and other data have led to the suggestion that DPA may activate, possibly allosterically, some enzyme involved in spore germination (Lewis, 1969). To date, this spore enzyme involved in spore germination has not been identified. However, DPA does allosterically modulate the activity of the germination protease (GPR) that initiates the degradation of the spore's depot of small, acid-soluble spore proteins (SASPs) during spore germination. GPR is synthesized as an inactive zymogen (termed P46) during sporulation, and P46 autoprocesses to a smaller active form (termed P41) approximately 2h later in sporulation. This conversion of P46 to P41 is stimulated allosterically by DPA, and only the physiological DPA isomer is effective. The activation of this zymogen is also stimulated by the acidification and dehydration of the spore core, and together these conditions ensure that P41 is generated only late in sporulation, when the conditions in the spore core preclude enzyme action. As a result, GPR's SASP substrates, which are synthesized in parallel with P46, are stable in the developing and dormant spore (Illades-Aguiar and Setlow, 1994; Setlow and Setlow; 1993).

This is important for spore survival, as some major SASP have two important functions; one exerted during dormancy and other one during germination (Slieman and Nicholson, 2001). SASP α/β are known to be DNA-binding proteins which form a complex with DNA in the spore core, causing a conformational change in DNA and eventually leading to the accumulation of spore photoproduct in dormant UV-irradiated spores (Setlow, 1995; Setlow, 1999; Dirks and Setlow, 1999; Mason and Setlow, 1988). Furthermore, SASP γ are degraded to amino acids in order to supply germinating spores with those amino acids also during outgrowth (Setlow, 1995; Setlow 1999; Dirks and Setlow, 1999; Hackett and Setlow, 1988; Sanchez-Salas et al., 1992).

1.3.4. Ion/DPA channels

The release of cations (the question of parallel anion release is not resolved), followed by release of DPA and associated divalent cations early in spore germination suggests that one or more channels for these ions must be opened in the inner spore membrane upon binding of a germinant to its receptor. Similarly, during

sporulation, there must be a mechanism for the uptake of DPA into the forespore from its site of synthesis in the mother cell compartment. DPA uptake into the forespore probably requires energy, given that extremely high concentrations of DPA are accumulated, but there is no obvious need for energy in the release of DPA and cations in germination. Unfortunately, the proteins involved in these ion movements are not known (Setlow, 2003).

Another small molecule that exhibits significant movement in spore germination, but in the opposite direction of ions and DPA, is water. The *B. subtilis* spore core volume increases 2- to 2.5-fold through stage II of germination, and does so by water uptake. However, the mechanism for this water uptake is not known, and *B. subtilis* has no homologs of the aquaporins found in other organisms (Paidhungat and Setlow, 2002).

1.3.5. Cortex-lytic enzymes

In *B. subtilis* two enzymes, CwlJ and SleB, play redundant roles in the degradation of the spore's peptidoglycan cortex during germination (Paidhungat and Setlow, 2002; Setlow et al., 2001; Boland et al. 2000; Atrich and Foster, 2001). Germination of cwlJ and sleB spores is relatively normal and even *cwlJ sleB* spores go through stage I of germination relatively normally. However, the double mutant spores cannot degrade their cortex and do not progress beyond stage I of germination. CwlJ and SleB require muramic-dlactam in peptidoglycan for their action, with SleB being a lytic transglycosylase while the specificity of CwlJ is unknown (Atrich and Foster, 2001). The muramic-d-lactam requirement for peptidoglycan cleavage by CwlJ or SleB ensures that the spore's germ cell wall, which lacks this modification, is not degraded during germination and becomes the cell wall of the outgrowing spore (Popham, 2002). Note that the germ cell wall must expand considerably to encompass the increased volume of the stage II germinated spore core. This expansion can be blocked by some treatments of spores, but its mechanism is not understood. CwlJ and SleB are synthesized only in sporulation, CwlJ in the mother cell and SleB in the forespore; neither is synthesized in zymogen form, although SleB is synthesized with a signal peptide that is rapidly removed (Melly et al., 2002; Paidhungat and Setlow, 2002). CwlJ is located in the spore coat fraction and is readily removed by decoating procedures (Bagyan and Setlow, 2002). CwlJ is also

absent from cotE spores, which have a severe coat defect. SleB is found in the spore integuments (coats, outer membrane and cortex). Since SleB has a potential peptidoglycan-binding domain, this protein may be located in the cortex or the coat/cortex boundary. However, a significant amount of SleB is also associated with the inner spore membrane. Both CwlJ and SleB require specific proteins for assembly and/or stability in spores. For SleB the protein required is YpeB. The ypeB gene is co-transcribed with sleB, and YpeB is in the same location in spores as SleB. Similarly, in most Bacillus species, *cwlJ* is co-transcribed with gerQ (originally called ywdL). Although this is not the case in *B. subtilis*, even in this organism, *gerQ* and *cwlJ* are transcribed in the mother cell at the same time in sporulation. GerQ is essential for the presence of CwlJ in spores and GerQ is, like CwlJ, a coat protein. The reason for the absence of CwlJ in *gerQ* spores is not clear, but gerQ spores have no gross coat defects and GerQ is not needed for *cwlJ* transcription (Setlow, 2003).

Because both SleB and CwlJ are synthesized in a mature form, there must be a mechanism (or mechanisms) keeping these enzymes in an inactive state in dormant spores. For SleB, this mechanism is not clear, although it may be that SleB only works on a cortex in which the peptidoglycan has a level of stress much higher than that in dormant spores (Foster and Johnstone, 1987, Makino et al., 1994). However, the inactivity of CwlJ in dormant spores and its activation after stage I of germination is probably because CwlJ requires Ca^{2+}– DPA for its action (Setlow, 2003). Whereas CwlJ and SleB are the only enzymes needed for cortex degradation during *B. subtilis* spore germination, other enzymes may be involved in this process, yet not be essential. One such enzyme is the yaaH gene product that encodes a cortex-lytic enzyme (termed SleL in *B. cereus*). The situation in spores of *Clostridium perfringens* may be somewhat different from that in Bacillus spores, as *C. perfringens* spores have a cortex-lytic enzyme (termed SleC) that is activated by proteolysis in the first minutes of spore germination (Chen et al., 2000).

1.3.6. Non-nutrient germinants

In addition to nutrients, spores are germinated by a variety of non-nutrients (Gould, 1969), including lysozyme, Ca^{2+}– DPA, cationic surfactants, high pressures and salts. These various non-nutrients can bypass individual components of the nutrient germination pathway. Because lysozyme can degrade the cortex of most spores, this

is a potential pathway for spore germination, although lysozyme will also degrade the spore's germ cell wall. In order for spores to be germinated by lysozyme, the spore coats must first be removed. With this pretreatment, spores are readily germinated by lysozyme, undergo DPA release and, if the lysozyme treatment is in a hypertonic medium, give rise to colonies. Exogenous Ca^{2+}–DPA also is a good spore germinant. These findings strongly suggest that germination by exogenous Ca^{2+}–DPA is via some direct or indirect activation of CwlJ. Spore germination by cationic surfactants like dodecylamine, was recognized over 40 years ago (Rode and Foster, 1961). Complete spore germination induced by dodecylamine requires either CwlJ or SleB, but does not require the spore's germinant receptors. Because dodecylamine causes rapid Ca^{2+}–DPA release from spores that cannot degrade their cortex, yet causes no release of other small molecules from these spores, this agent may open the spore's channels for Ca^{2+}–DPA. Spores of many species can be germinated at very high pressures (100–600 megaPascals [MPa]) (Setlow, 2003). At lower pressures (100–200 MPa), germination is caused by the activation of the germinant receptors (Vuytagk et al. 2000). However, at higher pressures (500–600 MPa), spores that lack nutrien receptors trigger germination rapidly, suggesting that these pressures somehow open the spore's Ca^{2+}–DPA channels (Paidhungat et al., 2002).

1.4. The Aim of the Present Project

yvgW gene was first reported as a cadmium resistant gene in *Bacillus subtilis* that can be induced with the existence of Cd^{+2} ions (Solovieva and Entian, 2002). After this, the other publication was that it is a CPx-type ATPase which is selectively induced by Zn (II) and Co (II) as well as Cd(II) ions in *B. subtilis* (Gaballa and Helmann, 2003). On the other hand, Yazgan et. al. (2001) mentioned that inactivation of *yvgW* caused reduction in sporulation efficiency in *Bacillus subtilis.*

Under the light of the fact that, the timing of *yvgW* expression corresponds to the late sporulation stage, the dependence of *yvgW* expression on forespore-specific sigma factor σ^G was previously examined through construction of a new mutant strain containing transcriptional *yvgW::lacZ* fusion and an additional deletion on *spoIIIG* locus that is known to encode σ^G. The present research includes the studies to achieve the molecular characterization and elucidation of the functional role of *yvgW* gene in the sporulation process in *B. subtilis.* Effects of the deletion of *spoIIAC,*

spoIVCB, *spoIVCB* and *sigB* genes, encoding for $\sigma^{F,}$ σ^{E}, σ^{K} and σ^{B}, respectively, on the expression of *yvgW-lacZ* were investigated. Moreover, through the construction of *yvgWΔ*537-1351*::spc*, *yvgW* mutant cells were investigated for their spore properties, such as their resistance profiles against heat chloroform and lysozyme. The level of dipicolinic acid was also investigated in *yvgW* spores and compared to wild type spores. Furthermore, the nutrition specific germination and outgrowth characteristics of null mutant and wild type cells were analyzed in order to gain more insights about the functional role of *yvgW* gene in the sporulation process.

2. MATERIALS and METHODS

2.1. Materials

2.1.1. Bacterial Strains

Strains used in this project are listed in Table 2.1. The mini-Tn10 delivery vector pIC333 (Figure 2.1) was supplied by Prof. Tarek Msadek (Pasteur Institute, France) and pDrive vector. pDrive vectorfor cloning of PCR products (Figure 2.2) was obtained from Qiagen

Table 2.1: Bacterial strains used in the project

Strain or plasmid	Relevant Genotype, phenotype, and/or characteristics	Construction, source or reference
B. subtilis PY79	wild type, BSP cured prototrophic derivative of *B.subtilis* 168	P.Youngman
AGU1	*yvgW::yvgW-lacZ erm*	Ayça Gülçin Ülgen
AGU2	*yvgW::yvgW-lacZ erm ΔspoIIIG::cat*	Ayça Gülçin Ülgen
OY1	*yvgW::yvgW-lacZ erm ΔspoIIGB::kan*	This work
OY2	*yvgW::yvgW-lacZ erm ΔspoIIAC::kan*	This work
OY3	*yvgW::yvgW-lacZ erm ΔspoIVCB::neo*	This work
OY4	*yvgW::yvgW-lacZ erm ΔspoIVCB::neo*	This work
RL560	*ΔspoIIIG::cat*	Richard Losick
ASK202	*ΔspoIIAC::kan*	Kazuhito Watabe
ASK203	*ΔspoIIGB::kan*	Kazuhito Watabe Kei Asai
SR276	*ΔspoIVCB::neo*	Lee Kroos
ML6	*ΔsigB::cat*	Michael Hecker
OY5	*ΔyvgW::spc*	This work
E.coli Top 10 F’	[*lac*Iq Tn10(Tet^r)], *mcrA* Δ(*mrr-hsdRMS-mcrBC*), *f80lacZΔM15 ΔlacX74, deoR, recA1, araD139 Δ(ara-leu)7697, galU, galK,rpsL (Strr), endA1, nupG*	M.A.Marahiel

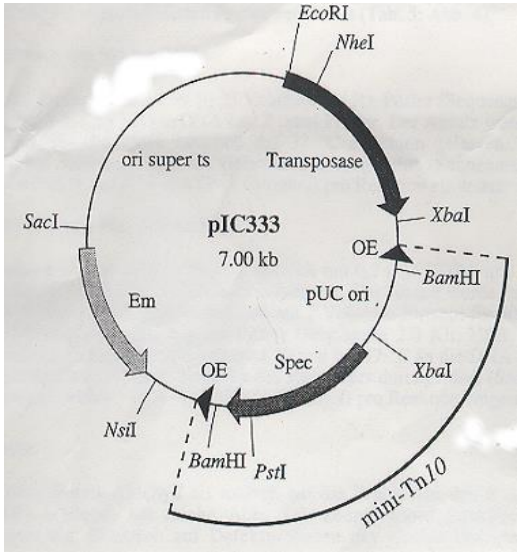


Figure 2.1: Shematic presentation of the 7 kb pIC333 vector.

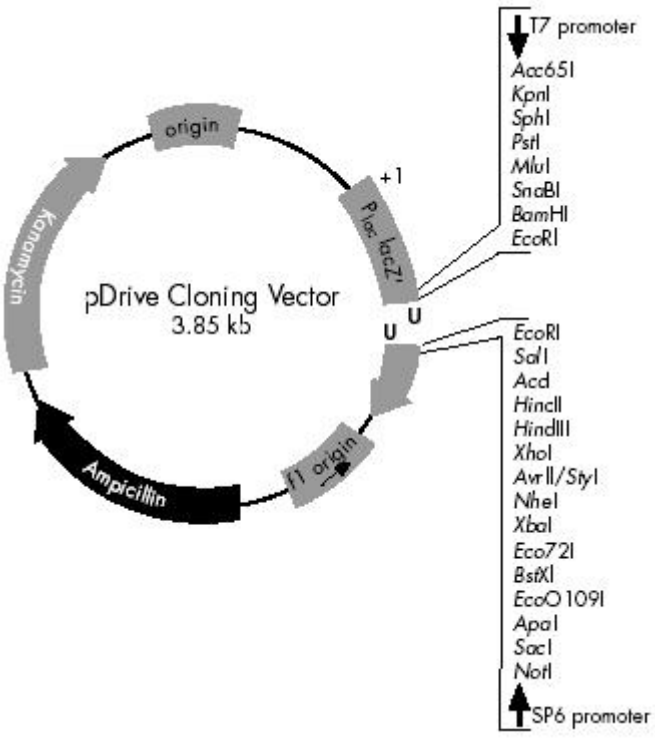


Figure 2.2: Genomic map of pDrive cloning vector including the functional genes in the structure as well as the restriction map (http://www1.qiagen.com/HB/PCRCloning).

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. Maintenance of Bacterial Strains

The bacterial strains and plasmids used in this study are described in Table 2.1. *B. subtilis* PY79, OY1, OY2, OY3, OY4 and OY5 strains were grown in Luria-Bertani (LB) broth medium and kept on Luria-Bertani (LB) agar plates. DSM agar was used for the maintenance of *B. subtilis* PY79 strain, DSM and SM (Sterlini, J.M., Mandelstam, J., 1969) medium were used for the induction of sporulation. *E. coli* Top 10 F' was grown in LB and in 2xYT liquid mediums and kept on LB and 2xYT agar plates. 2xYT was used for the transformation of the *E. coli* Top 10 F' strain and HS and LS mediums were used for the transformation of *B. subtilis* strains. All cultures were stored at +4^{o}C. 10% glycerol stocks of each strain were prepared and kept at -80^{o}C. Erythromycin (Erm) (1μg/ml), spectinomycin (Spc) (100 μg/ml), lincomycin (Ln) (25μg/ml), kanamycin (Kan) (10 μg/ml), ampicilin (Amp) (100 μg/ml), neomycin (Neo) (5μg/ml) and chloramphenicol (Cm) (5μg/ml) were used for *B. subtilis* strains. Amp (100 μg/ml) and Tetracycline (Tet) (20 μg/ml) were used for *E.coli* Top 10 F' strain as the selective antibiotics. Germination assays were performed in 2xYT or Spizizen's minimal medium (SMM) (Spizizen 1958).

2.2. DNA techniques and Manipulations

2.2.1. Polymerase Chain Reaction – PCR

The primers which are used during this study are shown in the Table 2.2. In PCR, all cycles lasted for 1 minute. The denaturation temperature was 94ºC and the extention temperature was 72ºC.The annealing temperature for the first 5 cycles was 55ºC and 60ºC for the next 25 cycles. The concentration of chromosomal DNA was 0.01 to 0.001 ng/µl. Primers were used at 1-10 pM (equimolar) and deoxyribonucleoside 5'triphosphates (dNTPs) were used at a final concentration of 2 mM.

Table 2.2: The oligonucleotide primers used in this study

Primers	Oligonucleotide Sequence	Target Sequence	Location
*yvgW Sac*I R	5'-CGG GAG CTC TTC ACT AGG CAA AAG CTT-3'	408 bp long	from 1351 bp to 1757 bp of *yvgW* gene
*yvgW Xba*I F	5'-GCC TCT AGA GGG AAA ATG CTT TCC TCC-3'		
*yvgW Pst*I F	5'-GGC CTG CAG CAA TGG GTC ACG AAT AAA-3'	377 bp long	from 160 bp to 537 bp of *yvgW* gene
*yvgW Bam*HI R	5'-CGG GGA TCC AAG CTC ACC GAT TTG ATA-3'		
spc screen F	5' -TTG CCA GAA CTA ATT GAG GGG- 3'	1100 bp long	700 bp *spc* cassette - from 50bp to 750 bp of *yvgW* 5' end
yvgW screen R	5'-CAG CGG CAA TAC GAC AAA AT-3'		
spc cassette R	5' -CTA ATT GAG AGA AGT TTC TAT- 3'	800 bp long	from 98 bp to 898 bp of *spc* cassette
spc cassette F	5' -CTC TAG AGG ATC GAT CTG TAT- 3'		

2.2.2. Restriction Enzyme Digestion

Digestion reactions were carried out as the instruction manual, in a way that the amount of 10X digestion buffer was 1/10 of the total reaction mix. The reaction mix was incubated for 1-4 hours at 37°C, and then enzyme was denaturated at 65°C for 20 min.

2.2.3. Agarose Gel Electrophoresis

Depending on the purpose of the electrophoresis, different concentrations of agarose gel were used as indicated below:

2.2.3.1. DNA Molecular Weight Markers

Marker 1: Φx174 DNA / *Bsu*RI (*Hae*III) (Appendix D)

Marker 3: Lambda DNA / *Eco*RI + *Hind*III (Appendix D)

GeneRuler: 1kb DNA Ladder (Appendix D)

Agarose gel with the necessary concentration was prepared and boiled to accomplish the agarose network. Then, the gel was cooled down to a degree that allows the addition of 1.5 µl EtBr. Gel loading dye (6X) was added to the samples depending on the sample amount. Gel was poured into tray and the comb was placed. Gel was left for solidification. Markers and samples were loaded and electrophoresis was carried out at 80V.

2.2.4. Gel Extraction

"QIAquick Gel Extraction Kit" was used for the gel extraction of the PCR products. The fragment was excised from the gel and 3 volumes of buffer QG were added depending on the weight of the fragment. If the color of the solution was not yellow, 10 µl of 3M sodium acetate (pH 5.0) was added. Following, the solution was incubated for 10 min at 50°C by shortly vortexing every 2-3 min, until the gel was dissolved completely. After addition of 1 volume of isopropanol, the sample was applied to the QIAquick column and centrifuged at 13000 rpm for 1 minute. Then the flow through was discarded and the QIAquick column was placed back into the same collection tube. Later, 0.5 ml of buffer QG was added to the column and centrifuged at 13000 rpm for 1 minute. Subsequently, the flow through was discarded and 0.75 ml of buffer PE was added to wash. The column was standed for 2-5 min and then centrifuged at 13000 rpm for 1 minute, which was followed with an additional 1 minute at 13000 rpm. Eventually, the column was placed into a clean 1.5 ml microfuge tube and 30 µl from EB buffer was dropped to the center of the QIAquick membrane within the column and it was let to stand for 1 minute and then

centrifuged for 1 minute. The resulting solution within the 1.5 microfuge containing the plasmid DNA was stored at –20 °C.

2.2.5. Ligation of the PCR Product into pDRIVE Cloning Vector

Purified PCR fragments were inserted into pDrive (Qiagen) vector by using the componenets of Qiagen Cloning Kit, the ligation reactions were set up as follows:

Table 2.3: Volume of components used in ligation of the PCR product into pDRIVE cloning vector.

Component	Volume/reaction
pDrive Cloning Vector (50 ng/µl)	1 µl
PCR product	2 µl
Distilled water	2 µl
Ligation Master Mix, 2x	5 µl
Total volume	10 µl

The ligation mix was then incubated at 16 ºC for 1.5 hour and then ligase was denatured at 70°C for 10 min.

2.2.6. Ligation with T4 Ligase

Firstly, 9.5µl of PCR products as insert fragments and 0.5µl of vector were mixed in an eppendorf tube and incubated for 5 min at 65°C. Then, the tube was cooled on ice and spanned down to collect the whole mixture. Following, 2µl of ligation 10xbuffer, 2µl of Polyethylene glycol (50% PEG 8000), 2µl of T4 DNA ligase, 4µl of dH_2O were added into the same eppendorf tube. Following centrifugation, the ligation mix was incubated at 16°C for 16 hours.

2.2.7. Preparation and Transformation of Electrocompetent *E.coli* Top 10 Cells

Overnight *E. coli* Top 10 F' cells were inoculated into 400 ml 2xYT broth containing 20 µg/ml Tet with a 1/100 dilution and incubated at 37°C until OD_{600} reached 0.6. Then cells were incubated on ice for 30 min and centrifuged at 5000 rpm for 15 min. Later, pelleted cells were resuspended in 40 ml of cold sterile dH_2O and centrifuged at 5000 rpm for 15 min. The pellet was gained by removing supernatant and it was resuspended in 20 ml of cold sterile dH_2O and centrifuged at 5000 rpm for 15 min. Cells were resuspended in 1 ml of 10% glycerol (cold) and 40 µl volumes were

aliquoted in the 1.5 ml eppendorf tubes. Subsequently, the samples were quick freezed in the liquid nitrogen and stored at -80°C.

For transformation of *E.coli*, firstly, electrocompotent *E. coli* Top 10 F' cells were thawed on ice and centrifuged for a quick spin. Then, 20 µl of ligation sample was added and all together were transferred into an electroporation tube. Following, the electroporation tube was placed into electroporation machine and the process was carried out at 1800V. After addition of 1 ml 2xYT broth, the mixture was transferred to a 1.5 ml tube. Later the mixture was incubated for 1 hour at 37°C and then centrifuged at 5000 rpm for 15 min in order to harvest cells. Then the supernatant was discarded and the pellet was resuspended in 100 µl of 85% NaCl. At last, 100 µl of culture was spread out for each 2xYT (Amp100 µg/ml) plate and incubated overnight at 37°C.

2.2.8. Plasmid DNA isolation

Plasmid DNA isolation was applied through using the buffers and solutions of the "QIAquick Plasmid DNA Isolation Kit". For Plasmid DNA isolation, 1.5 ml of bacterial cells were harvested by centrifugation at 13000 rpm for 5 min. The supernatant was removed and the pellet was resuspended in 300 µl P1 buffer. After addition of 300 µl of P2 buffer, the solution was incubated at room temperature for 5 min. Then, 300 µl of P3 buffer was added and mixed through inverting until the lysate is no longer viscous. Following, the sample was incubated for 15 min on ice and centrifuged at 13000 rpm for 15 min. Supernatant was next transferred to a new 1.5 ml eppendorf tubes and 0.7 volume of isopropanol was added depending on the volume of the supernatant and the solution was centrifuged at 13000 rpm for 30 min. The pellet was washed with 1ml of 70% ethanol by centrifugation at 13000 rpm for 5 min. After the supernatant was removed, ethanol was dried out at 37°C for 15. At last, 15 µl of EB buffer was added and the tubes were incubated at 37°C for 15 min at 350 rpm.

2.2.9. Preparation of *B. subtilis* Competent Cells and Transformation

Preparation of *B. subtilis* competent cells and transformation were performed as described by Klein et al, 1992. HS and LS media were used for the preparation of *B. subtilis* competent cells. At first, 3 ml of overnight culture was prepared in HS medium by incubating at 37°C and shaking at 250 rpm. Then 0.5 ml of this overnight

inoculum was transformed into 20 ml of freshly prepared LS medium and incubated at 30°C with shaking at 100 rpm. until OD_{600} of cultures reached 0.55. Following, 1 ml of competent cells was transferred into 2 ml eppendorf tube and 2 µl of DNA was added. Cells were incubated at 37°C for 2 hours with shaking at 250 rpm and then they were harvested via centrifugation at 5000 rpm for 15 min. Finally, cells were resuspended in 100 µl of sterile %85 NaCl and plated onto selective LB agar plates and incubated at 37°C for 16 hours.

2.2.10. Chromosomal DNA isolation

1,5 ml of overnight culture was centrifuged at 13000 rpm for 5 min. The pelleted cells were resuspended in 567 µl of TE by repeated vortexing. Then, 10 µl of proteinase K (20mg/ml), 6 µl of RNase (10 mg/ml), 24 µl of lysozyme (100mg/ml) and 30 µl of 10% SDS were added and the mixture was incubated for 1 hour at 37°C water bath. After addition of 100 µl of 5M NaCl solution, the sample was mixed without vortexing until the mucosal white substance become visible. Following, 80 µl of CTAB / NaCl (prewarmed at 65°C) solution was added and the mixture was incubated for 10 min in 65°C water bath. The sample was then extracted with the same volume of freshly prepared phenol/chloroform/isoamyl alcohol (25:24:1) solution and centrifuged at 13000 rpm for 10 min. At later stage, the upper phase was transferred to a new 1.5 ml microfuge tube and 0.7 volume isopropanol was added. After mixing shortly, the sample was centrifuged at 13000 rpm for 15 min. The pellet was washed with 1ml of 70% ethanol centrifuged at 13000 rpm for 5 min. Subsequently, the pellet was dried at 37°C for 1 hour and dissolved in 10 µl of TE buffer via incubation at 37°C for 30 min. Finally, the isolated DNA was made run on 0.6% agarose gel and absorbance at 260 nm and 280 nm were measured to determine the concentration and purity of the isolated DNA.

2.3. Induction of Sporulation by Resuspension Method

Inoculums for resuspension medium (Appendix A) was prepared just like in DSM, then they were used to inoculate 35ml of freshly prepared growth medium (Appendix A) to an initial optical density at 595 nm (OD_{595}) of about 0.17. Later, the cultures were incubated at 37°C (250 rpm) until OD_{595} was 0.5-0.8 and centrifuged at 8000g for 5 min. Hereafter, cultures were resuspended in an equal volume of freshly prepared warm resuspension medium in the same flask and returned back to

incubation at 37°C. Starting from t_0 that was defined as the point at which cells are resuspended, 1 ml of culture was taken as duplicates at each hour for the later execution of β-galactosidase assay. For the measurement of growth at OD_{595}, the necessary amount of cultures were taken as dilutions. The following procedure was applied to the 1 ml of cultures.

2.4. β-galactosidase Assay with ONPG

After each sampling, the culture was centrifuged at 13000 rpm for 5 min and the supernatant was discarded. Then, the pellet was washed with 500 µl of ice-cold 25 mM Tris-Cl (pH 7.4) by centrifugation. Following, the removal of the supernatant, the pellet was washed in 0.5 ml of ice old 25 mM TrisHCl (pH 7.4) for 5 min at 13000 rpm in a microfuge. Then supernatant were discarded and cells were put on ice.

Following the removal of the supernatant, the pellet was resuspended in 640 µl of Z-buffer via vortexing and 160 µl of lysozyme was added. Later, the solution was vortexed for a second and incubated at 37°C for 5 min. Subsequently, the samples were taken on ice and 8 µl of 10% Triton-X100 was added. After vortexing for a while, the extracts were stored on ice.

β-galactosidase assay was continued by prewarming the extracts in 30°C water bath for 5 min. Subsequent to this, 200 µl of ONPG solution was added and the solution was controlly watched for a yellowish color formation. Following the complete settlement of yellowish color, the reaction was stopped by adding 0.4 ml of 1 M Na_2CO_3 and reaction time was recorded. Reaction time refers to a period that was started by adding ONPG and continued until the settlement of the yellow color. At last, the samples were centrifuged at 13000 rpm for 5 min and the supernatant was taken to measure A_{420} and A_{550} of it. Calculations for *β*-galactosidase activity were carried out according to the formulation below (Miller, 1972) and graphs for β-galactosidase activities were drawn.

$$\text{Miller units} = \frac{A_{420} - (1.75 \times A_{550})}{\text{Reaction time (min)} \times OD_{595}} \times 1000$$

2.5. 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.

2.6. Germination and outgrowth of *Bacillus subtilis* spores

Purified spores in water were heat activated at 65 ^{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^{o}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^{o}C for 90 min with 10 minute intervals.

2.7. Spore resistance

Cells were grown in DSM at 37^{o}C for 18 h following the end of exponential growth, which stands for approximately 24 hours, and a sample was removed from the cultures, 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^{o}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^{o}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. All the plates were incubated overnight at 37^{o}C.

2.8. 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.

3. RESULTS AND DISCUSSION

3.1. Expression of *yvgW* in *B. subtilis* during sporulation

Mutant strain AGU1 (*yvgW::lacZ::erm*) that was previously constructed through the use of *Bacillus subtilis* specific vector pMutinT3 involving *lacZ* reporter gene leading to a gene fusion into *yvgW* gene locus was previously assayed for the measurement of yvgW expression during sporulation, suggesting that the expression of *yvgW* in SM was showed to be induced at about 5^{th} hour of its sporulation with a maximum *β*-galactosidase activity (approximately 16 Miller units) detected at 7th hour of sporulation. These results have already been reported to verify that expression of *yvgW* gene is sporulation specific and may be cell-compartment-specific (mother cell or forespore) since genes expressed well after t_2 of sporulation may be expressed in only one cell compartment and *yvgW* expression appeared after fourth hour in sporulation, reaching maximum levels at the seventh hour in sporulating cells (Gülçin Ülgen, M.Sc thesis in 2005). This time period corresponds to the late sporulation events during which accumulation of peptidoglycan in the cell wall, formation of cortex, and accumulation of spore coat proteins occur (Stragier and Losick, 1996).

3.2. Sigma factor dependence of *yvgW* expression

Following determination of the expression time of *yvgW* during sporulation process, which sigma factor is responsible for transcription of *yvgW* gene was aimed to be demonstrated.

3.2.1. σ^K and σ^G dependences of *yvgW* expression

Among four different sigma subunits of RNA polymerase, sigma K (σ^K) and sigma G (σ^G) function at late sporulation phase in mother cell and forespore respectively (Leichert et al., 2003). Since expression time of *yvgW* corresponds to late sporulation process, firstly dependence of *yvgW* expression on sporulation specific σ^G factor was

previously examined by constructing a mutant strain, AGU2, containing transcriptional *yvgW::lacZ* fusion and deletion on *spoIIIG* locus that encodes sigma G marked by *cat* resistance gene by using chromosomal DNA of RL560 and using it to transform AGU1. Furthermore, expression of transcriptional *yvgW::lacZ* fusion in *spoIVCB* mutant background was analyzed and a mutant named OY3 was constructed following this aim. Firstly, chromosomal DNA of SR276 (*B. subtilis* PY79 *ΔspoIVCB::neo*) was used to transform AGU1 and possible transformants were selected against Neo^R and Erm^R. During transformation, deletion of *spoIVCB* gene in AGU1 was revealed by double cross over in a way that, *spoIIIG* was replaced by the neo-cassette belonging to SR276. Transformant colonies were picked up and screened on DSM- agar plate, incubated for two days at 37^0C, to check sporulation negative mutant phenotype generated with deletion of *spoIVCB*. All of them were *spo*$^-$, therefore one of them was chosen randomly and called as OY3 that contains transcriptional *yvgW::lacZ* fusion and deletion on *spoIVCB* gene with *neo* gene. In order to observe the effect of deletion of *spoIIIG* and *spoIVCB* genes on expression of *yvgW* gene, AGU1, AGU2 and OY3 strains were induced to sporulation in SM medium and *β*-galactosidase activity was measured during sporulation process. All the strains exhibited similar growth pattern, indicating that they were in the same stage of sporulation. Therefore, the *β*-galactosidase activity of AGU1, AGU2 and OY3 were comparable to each other giving idea about the sigma G and sigma K activities on the expression of *yvgW.* As seen on figure 3.1, deletion of the *spoIIIG* coding for σ^G, caused a drastic reduction of the level of *yvgW* driven *lacZ* expression of about 50- 60 % relative to AGU1. However, Deletion of *spoIVCB* gene coding for σ^K had no significant effect in the timing and level of the *yvgW-lacZ* expression.

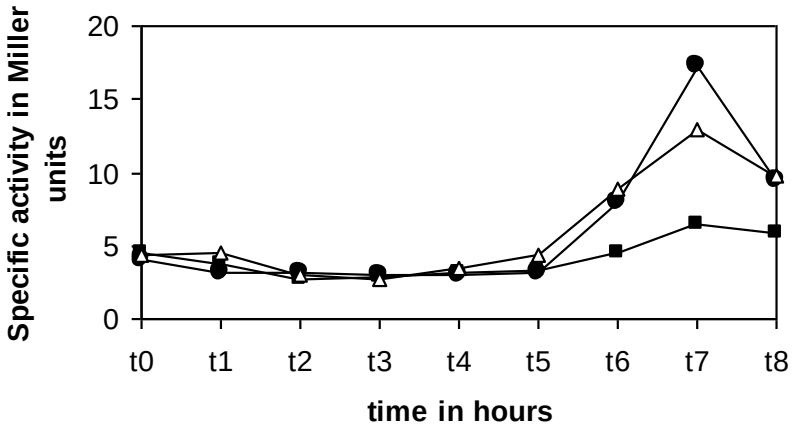


Figure 3.1: Effects of deletion of genes encoding sporulation-specific sigma factors, σG and σK on expression of *yvgW-lacZ.* Time zero is the time of resuspension of the culture to initiate sporulation. The symbols used for the various strains are; (●) AGU1 (*yvgW::yvgW-lacZ er*), (Δ) OY3 (*yvgW::yvgW-lacZ er ΔspoIVCB::nm),* (■) AGU2 (*yvgW::yvgW-lacZ er ΔspoIIIG::cat.*

3.2.2. σ^F and σ^E dependences of *yvgW* expression

These data in section 3.2.1 suggested that *yvgW* is expressed specifically in the forespore compartment of the sporulation cell and *yvgW* transcription is controlled by mainly by σ^G. However to certain the role of σ^G in controlling *yvgW* expression, the expression of transcriptional *yvgW::lacZ* fusion not only in early forespore specific sigma factor σ^F mutant but also in early and late mother-cell specific sigma factors σ^E mutants' background was analyzed. Therefore, chromosomal DNA of strain ASK202, carrying a deletion on *spoIIAC* that encodes sigma F marked by *kan* resistance gene and chromosomal DNA of strain ASK203, carrying a deletion on *spoIIGB* that encodes sigma E marked by *kan* resistance gene were used to transform AGU1 and possible transformants were selected against Kan^R and Erm^R. During transformation, all the deletions in AGU1 were created by double cross over in a way that, *spoIIIAC* and *spoIIGB* were replaced by the kan-cassettes. Transformant colonies were picked up and screened on DSM- agar plate, incubated for two days at 37^0C, to check sporulation negative mutant phenotype generated with deletions. All of them were *spo*$^-$, therefore one of them was chosen randomly and called as OY1 and OY2 that contains transcriptional *yvgW::lacZ* fusion and deletion on *spoIIIAC* and *spoIIGB* genes with *kan* gene, respectively.

Following construction, AGU1, OY1 and OY2 were induced to sporulation by resuspension of a growing culture in a poor medium which is Sterlini-Mandelson

sporulation medium (SM) (Sterlini and Mandelstam, 1969) and samples were started to be taken at 1 hr intervals during the mid-log phase of their growth and continued until eight hr of sporulation (t_8) with a final sample being taken at about t_{24} to assay for β –galactosidase using ONPG as substrate. In this method, time zero, reflecting the beginning of sporulation, was determined after incubating cultures until OD_{595} at 0.5-0.8 in growth medium and resuspending them in resuspension medium. All the strains exhibited similar growth pattern, indicating that they were in the same stage of sporulation so that their β –galactosidase activities were compared and as seen in Fig.3.2, β –galactosidase assay revealed out that the deletion of *spoIIAC* and *spoIIGB* genes, coding for σ^F and σ^E, completely abolished *yvgW-lacZ* expression.

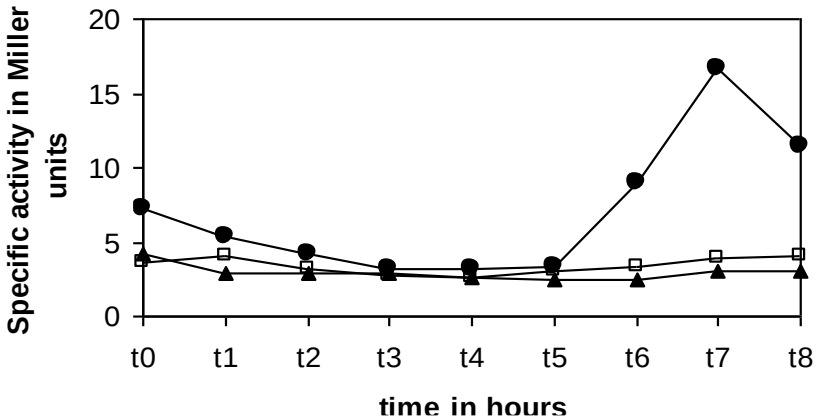


Figure 3.2: Effects of deletion of genes encoding sporulation-specific sigma factors, σF and σE on expression of *yvgW-lacZ.* Time zero is the time of resuspension of the culture to initiate sporulation. The symbols used for the various strains are; (●) AGU1 (*yvgW::yvgW-lacZ erm*), (□)OY1 (*yvgW::yvgW-lacZ erm ΔspoIIAC::kan*) and (▲) OY2 (*yvgW::yvgW-lacZ erm ΔspoIIGB::kan*)

To sum up, in this study, although the deletion of *spoIIAC* and *spoIIGB* genes, coding for σ^F and σ^E, respectively, resulted in the complete elimination of *yvgW-lacZ* expression, the deletion of the *spoIIIG* coding for σ^G caused a 50 to 60% reduction, compared to wild type strain in the level of *yvgW-lacZ* expression. In contrast, mutation in *spoIVCB*, encoding σ^K, had no significant effect on the expression of *yvgW-lacZ*. Since, σ^F drives not only the synthesis of σ^G but also the activation of σ^E, and σ^E is required for the activation of σ^G (Haldenwag, 1995), these results strongly suggest that *yvgW* is transcribed in both mother cell and forespore under the control of mother-cell specific sigma factor σ^E and forespore-specific sigma factor σ^G,

respectively, as in the case of the muramic-δ-lactam biosynthetic gene, *cwlD* (Sekiguchi *et al*., 1995).

3.2.3. σ^B dependences of *yvgW* expression

Additionally, the presence of σ^A and σ^B recognition sequences at the upstream of *yvgW* coding region (Gaballa and Helmann, 2003) and some recent reports about σ^B and σ^A directed sporulation genes such as *obg* (Scott and Haldenwang, 1999), *spoVC* (Price et al., 2001), *kat X* (Bagyan et al., 1998), and *veg* gene (Fukushima et al., 2003) suggested another possibility that *yvgW* might be transcribed primarily by RNA polymerase containing σ^A or σ^B at the late stage of sporulation but somehow, pre and/or post transcriptional regulation of compartment-specific *yvgW* expression can be exclusively under the control of σ^E and σ^G. To check this hypothesis, the *yvgW-lacZ* transcription in σ^B mutant background was analyzed in this study by constructing a new mutant, using chromosomal DNA of ML6, carrying a deletion on *sigB* gene, encoding sigma factor σ^B, and a *cat* gene on this deleted area to transform AGU1. Possible transformants were selected against Cm^R and Erm^R and during transformation, deletion of *sigB* gene in AGU1 was revealed by double cross over in a way that, *sigB* was replaced by the *cat*-cassette belonging to ML6. One of the transformant colonies was picked up and named as OY4 and in order to observe the effect of deletion of *sigB* on expression of *yvgW* gene, AGU1 and OY4 strains were induced to sporulation in SM medium and *β*-galactosidase activity was measured during sporulation process. All the strains exhibited similar growth pattern, indicating that they were in the same stage of sporulation. Therefore, the *β*-galactosidase activity of AGU1 and OY3 were comparable to each other giving idea about the sigma B activities on the expression of *yvgW*. As seen on figure 3.3, mutation in *sigB* had no significant effect on the level and or timing of sporulation specific *yvgW-lacZ* expression.

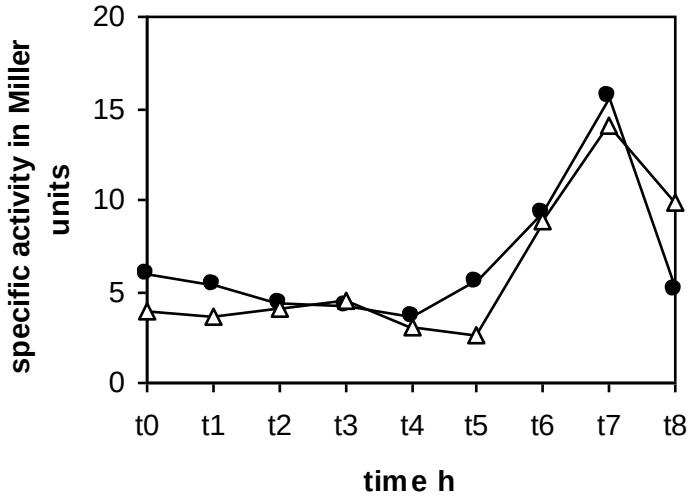


Figure 3.3: Effect of deletion of gene encoding σ^B on expression of *yvgW-lacZ*. Time zero is the time of resuspension of the culture to initiate sporulation. The symbols used for the various strains are; (●) AGU1 (*yvgW::yvgW-lacZ erm*) and (Δ)OY4 (*yvgW::yvgW-lacZ erm ΔsigB::cat*)

3.3. Construction of *yvgW* deletion vector

3.3.1. Obtaining *spc* cassette

7.0 kb long pIC333 vector was digested with *BamH*I for the interest of obtaining 2.4 kb long mini-*Tn10* fragment (Figure 3.4). Afterwards, mini-*Tn10* fragment was double digested with *Xba*I and *BamH*I and 900 bp long *spc* cassette was obtained which had been isolated from the agarose gel.

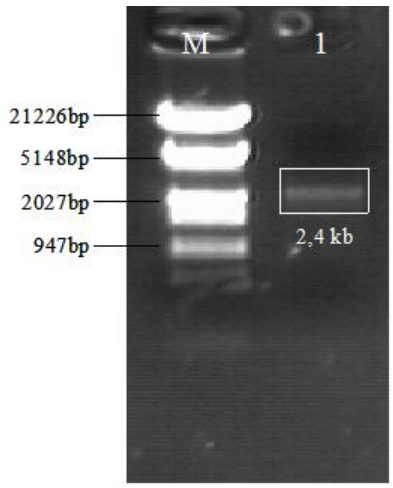


Figure 3.4: 2.4 kb long mini-*Tn10* fragment obtained from pIC333 vector (lane 1). M: Marker 3: Lambda DNA / *Eco*RI + *Hind*III

Under the aim of cloning it into pDrive Cloning Vector (Qiagen), *spc* cassette was digested with *Xba*I and *Bam*HI restriction enzymes and ligated into similarly digested pDrive Cloning Vector (Qiagen). This ligation mixture was used to transform electrocompetent cells of *E.coli* Top10 and transformants were selected on LB agar plates containing 100µg Ampicillin ml^{-1}.

Resulting transformants were picked up and 10 of them were used for plasmid DNA isolation for the verification of the cloning of 900 bp *spc cassette* fragment (Figure 3.5).

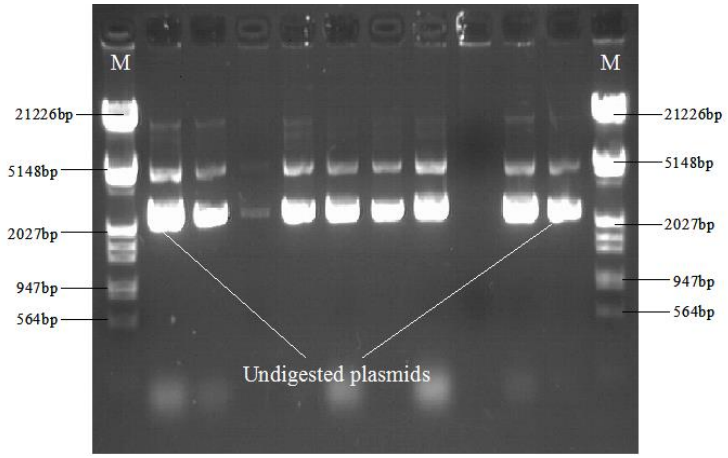


Figure 3.5: Plasmid DNA' s isolated from *E.coli* Top10 Amp^R transformants and M: Lambda / *Eco*RI+*Hind*III Marker DNA fragments.

Plasmid DNA's isolated from transformants were double digested with *Bam*HI and *Xba*I restriction enzymes for further confirmation (Figure 3.6). The vector pDrive itself was 3850bp long while the insert was 900 bp long. Thus, molecular weight of the resulting recombinant plasmid was expected to be about 4750 bp long.

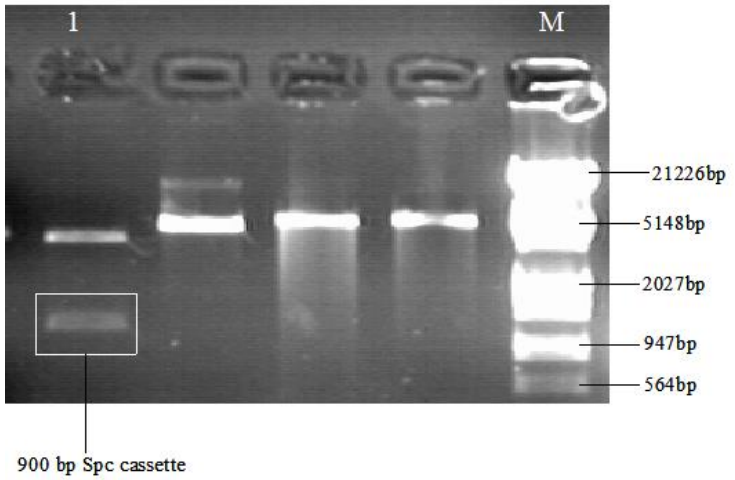


Figure 3.6: pDrive cloning vector and spc cassette, after *Xba*I and *BamH*I double digestion (1). M : Marker 3: Lambda DNA / *Eco*RI+*Hind*III

As a consequence of plasmid double digestion (Figure 3.6 lane 1), a 900 bp DNA fragment, which is equal to the cloned *spc cassette* from pIC333 (Figure 3.6 lane 1) and a 3850 bp DNA fragment which represents linear pDrive Cloning Vector DNA (Figure 3.6 lane 1) were observed on the agarose gel. Therefore, plasmid DNA's isolated from this transformant was selected as the desired construct.

3.3.2. Insertion of the PCR Fragments into pDrive Cloning Vector Containing *spc* Cassette

An internal fragment of the *B. subtilis* PY79 *yvgW* gene, which stands between 1351 bp to 1757 bp downstream of translational start codon was amplified by PCR using the chromosomal DNA of wild type strain *Bacillus subtilis* PY79 as template (Figure 3.7). The reverse sequence was representing recognition site for *Sac*I and the forward sequence was representing recognition site for the *Xba*I restriction endonucleases.

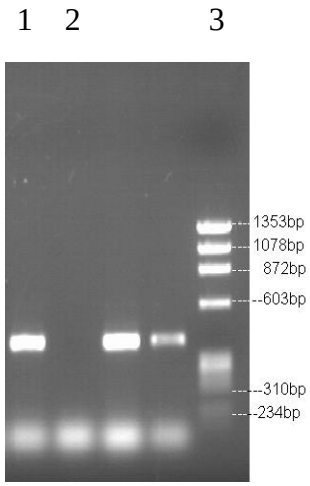


Figure 3.7: 406 bp *yvgW* fragment amplified with PCR (lane 1), control PCR (lane 2) and Marker 1: PhiX174 DNA / *Bsu*RI (*Hae*III) (lane 3).

In order to clone into the costructed plasmid, the resulting PCR fragment was digested with *Sac*I and *Xba*I restriction enzymes and ligated into similarly digested pDrive containing *Spc* cassette vector (Figure 3.8). The ligation mixture was used to transform electrocompetent cells of *E.coli* Top10 and transformants were selected on LB agar plates containing 100µg Ampicillin ml^{-1}.

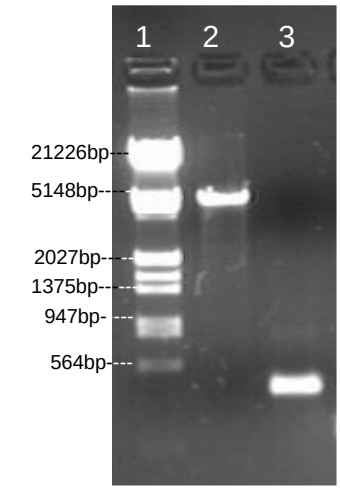


Figure 3.8: Gel extraction result of 4750 bp *spc* cassette containing pDrive cloning vector (lane 2) and 406 bp long PCR fragment double digestions with *Xba*I and *BamH*I after gel extraction (lane 3). Marker 3: Lambda DNA / *Eco*RI+*Hind*III (lane 1).

Following that, second internal fragment of *B. subtilis* PY79 *yvgW* gene, which stand between 160 bp to 537 bp downstream of translational start codon of the *B. subtilis* PY79 *yvgW* gene were amplified by PCR (Figure 3.9). The reverse sequence were representing recognition site for *Bam*HI and the forward sequence were representing recognition site for the *Pst*I restriction endonucleases.

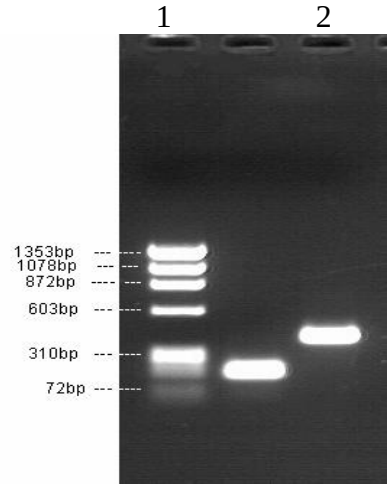


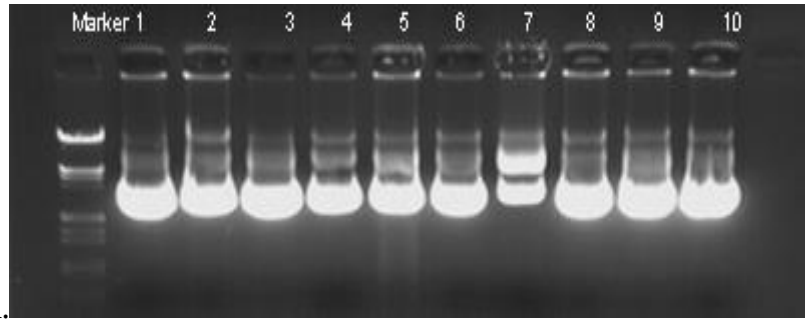
Figure 3.9: 377 bp *Bam*HI-*Pae*I PCR fragment after gel extraction (lane 2). Marker 1: PhiX174 DNA / *Bsu*RI (*Hae*III) (lane1).

To clone this second PCR product into the constructed vector containing both *Spc cassette* and *Xba*I-*Sac*I fragment, and second PCR product was double digested with *Bam*HI-*Pae*I and ligated into similarly digested constructed vector. The ligation

mixture was used to transform electrocompetent cells of *E.coli* Top10 and transformants were selected on LB agar plates containing 100µg Ampicillin ml^{-1}.

The resulting transformants were picked up and all of them were used for plasmid DNA isolation (Figure 3.10.A) to verify the cloning of the inserted fragments. Following this, restriction digestion analysis was carried out and the rescued plasmids were linearized with *Sac*I (Figure 3.10.B) As a consequence of these digestions, one plasmid was identified as having the expected molecular weight and containing the cloned fragment (Figure 3.10.B).

A.



B.

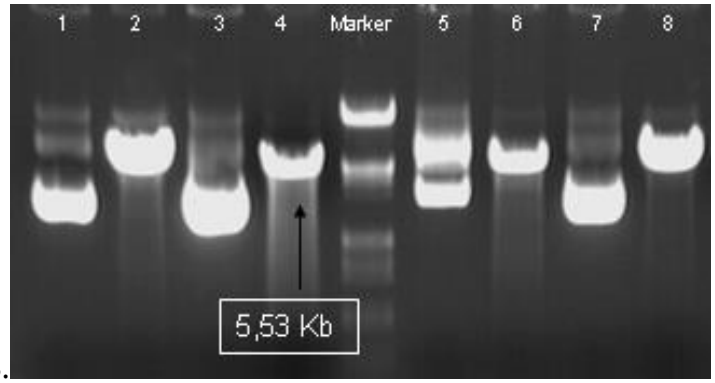


Figure 3.10: A. Undigested plasmids, **B.** Uncut plasmids 1,4,7,8 (lanes 1,3,5,7), Linearization of the obtained plasmids 1,4,7 and 8 with *Sac*I (lane 2,4,6,8) M: Marker 3: Lambda DNA / *Eco*RI+*Hind*III.

The vector pDrive itself was 3,85 kb long while the inserts were totally 1,68 kb long. The molecular weight of the resulting recombinant plasmid was expected to be about 5,53 kb long. Therefore, a band app. 5,53 kb in size was obtained from the plasmid digestion with *Sac*I (Figure 3.13.B).

With the objective of size determination, this chosen plasmid was amplified in *E. coli* Top10, and the recombinant plasmid was further screened with the sequence analysis, using the specific primers to the ends of *yvgW* gene; *Pst*I forward and *Sac*I reverse and M13 forward(-40) specific primer, respectively. It was shown that this

constructed vector contains both the ends of the *yvgW* gene and also the *spc* cassette (Figure 3.11).

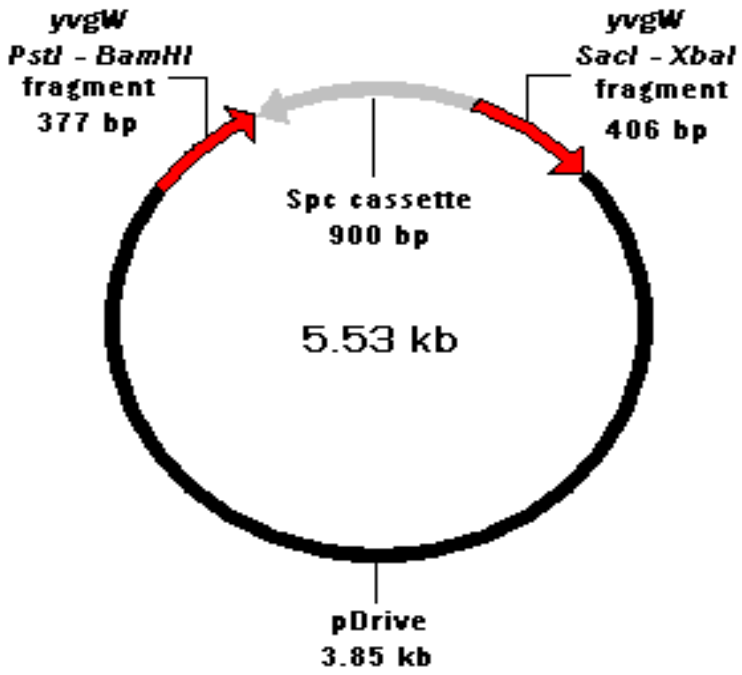


Figure 3.11: Constructed Deletion Vector

3.3.3. Construction of *yvgW* deletion in *B. Subtilis*

In order to provide construction of *yvgW* deletion mutant, constructed deletion vector DNA was used to transform competent cells of *B. subtilis* PY79 to spectinomycin resistance. Recombinant plasmid was driven into the chromosomal DNA of *B. subtilis* PY79 by a double cross over event. Chromosomal DNA's of resultant Spc^R transformants were isolated and the deletion on their *yvgW* locus was analyzed through performing PCR reaction, using two different sets of primers (Figure 3.12). First set contained the primers for the amplification of a fragment belonging to spc-cassette. Second set contained the primers for the amplification of a DNA fragment containing both a part of the *spc*-cassette and a part of the *yvgW* fragment that was standing at the 3' end of the spc-cassette. The verified mutant was named as OY5.

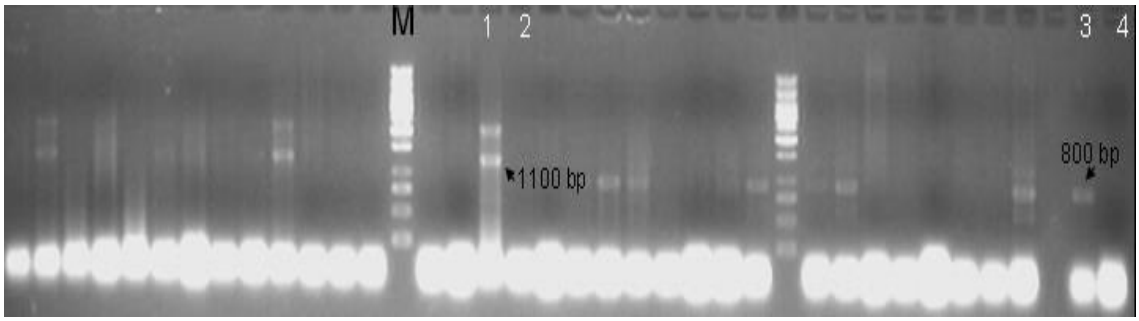


Figure 3.12: *yvgW* deletion mutant verification through PCR. A 800 bp fragment belonging to spc cassette was amplified by using primers specific to spc-cassette (lane 3). Control PCR with PY79 chromosomal DNA using primers specific to spc-cassette (lane 4). A 1100 bp fragment amplified from the same mutant which can be seen in the lane 3, containing both a part of the spc-cassette and a part of the *yvgW* fragment that was standing at the 3' end of the spc-cassette (lane 1). Control PCR with PY79 chromosomal DNA using primers specific to a part of the spc-cassette and a part of the *yvgW* fragment (lane 2). M: Gene Ruler 1 kb Marker.

3.4 Properties of *yvgW* spores

In this study, deletion of *yvgW* showed a clear effect on sporulation phenotype, it produced heat-sensitive and chloroform-sensitive spores. When sporulation frequencies of wild type, PY79 strain and *ΔyvgW::spc*, OY5 strain are compared (Table 2), it revealed an drastic decrease in the spore formation at OY5, when spores produced by this strain are incubated at 80^{o}C for 30 min.

Furthermore, survival frequencies of PY79 and OY5 spores, when treated with 10% (v/v) chloroform at room temperature for 10 min, differed dramatically, giving a 6-fold greater frequency for PY79 spores (Table 2). On the other hand, it is shown in this study that spores produced by OY5 strain were lysozyme-resistant just like PY79 spore cells (Table 2). Vegetative growth of the *yvgW*-deleted cells in DSM medium was also less efficient giving a 30-50% less total cell titer when compared to vegetative growth of PY79 cells in DSM medium (Table 2).

Table 3.1: Heat, chloroform and lysozyme 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	43.7×10^7	41.25×10^7	0.94
OY1	heat	33×10^7	2.55×10^7	0.077
PY79	chloroform	59×10^7	41.7×10^7	0.71
OY1	chloroform	30×10^7	3.8×10^7	0.13
PY79	lysozyme	53×10^7	47×10^7	0.89
OY1	lysozyme	31×10^7	26.5×10^7	0.85

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.
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

On the basis of dipicolinic acid assay, PY79 spores were found to synthesize about 32.75 ± 3,06 μg/OD_{600} DPA, while *yvgW* mutant spores had only 63 % of this level, synthesizing about 20.75 ± 2,49 μg/ OD_{600} DPA.

For the germination properties, pure spores from OY5 and PY79 were treated with different germination agents at different media. When made grow in minimal medium, PY79 and OY5 spores were found to germinate in a similar pattern the germination agent was L-alanine or AGFK. (Figure 3.13-A, 3.13-C). Additionally, in case of rich 2xYT medium, germination and outgrowth patterns did not differ from each other; even if spores were treated with different germination agents (Figure 3.13-B, 3.13-D). 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 OY5 and PY79 spore cells was visualized, it was noticed that OY5 cells return to their vegetative state in a more delayed pattern, compared to wild-type germinating spores. This defect was obviously observed in SMM minimal medium (Figure 3.13-A; 3.13-C), while no

differences in outgrowth properties between OY5 and PY79 spores were captured in 2xYT medium (Figure 3.13-B; 3.13-C).

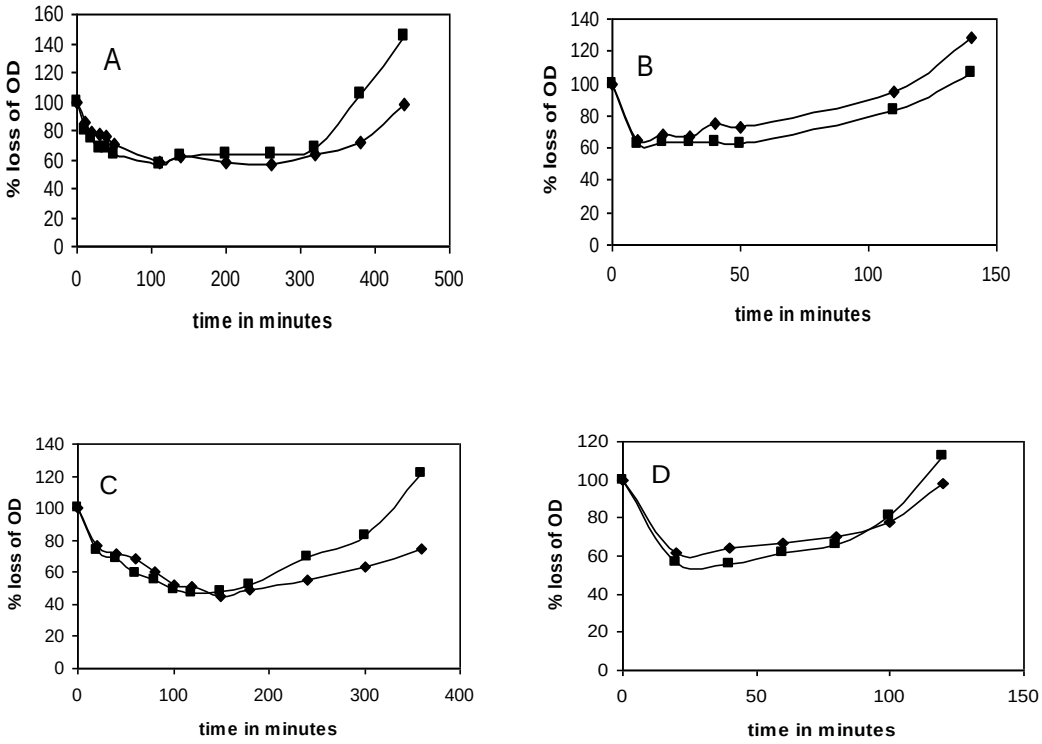


Figure 3.13: Effects of different germination agents L-alanine and AGFK on OY5 and PY79 spores, when grown in either SMM or 2xYT. Spores were prepared, induced by heat and germinated at 37 ^{o}C with 4mM L-alanine in SMM medium (A) and in 2xYT (B) and with AGFK (3.3 mM L-asparagine, 5.6 mM D-glucose, 5.6 mM D-fructose, 10 mM KCl) in SMM (C) and in 2xYT (D). Germination and outgrowth profile of OY5 and PY79 spore cells were drawn through measurement of loss in optical density at 600 nm at 37^{o}C for 90 min at 10 min intervals and further measurements continued until outgrowth is observed at 60 min intervals. The symbols used for the strains are; (♦) OY5 (*ΔyvgW::spc*) and (■) PY79 (wild type).

All of these available data strongly suggest that deficiency of *yvgW* effects a late step in spore synthesis; spore coat/cortex synthesis, dehydration of spores, SASP synthesis, DPA synthesis and/or accumulaton in the spores. Since *yvgW* spores are lysozyme resistant and also have no defect in the initiation of spore germination, indicated that there is no major defect in the coats of *yvgW* spores but it presumably became more permeable to chloroform because of the pleiotropic effect of *yvgW* on the spore coat protein profile. The level of DPA in *yvgW* spores was significantly lower than in wild type spores, which could be the reason for heat sensitive phenotypes, as well as the slow outgrowth phenotype of *yvgW* spores. In *B. subtilis*,

the specifically blockage of DPA synthesis results in DPA-less spores with decreased resistance to wet heat by the increased core hydration (Balassa et al.; 1979, Coote, 1972) which is the major determinant of spore heat resistance (Todd et al., 1986; Popham et al., 1995). In the case of recent report, spores of *B. subtilis* mutants lacking DPA because of null mutations in *spoVF* have a lower core wet density and are sensitive to wet heat (Paidhungat et al., 2000).

The great majority of SASP degradation during spore germination is initiated by a single protease, GPR. The major phenotype of the *gpr* mutants is a slow return to vegetative growth after spore germination in rich medium. Because GPR is synthesized as a 46-kDA precursor (termed P_{46}) during sporulation, and P_{46} autoprocesses into a smaller active form (termed P41) ca. 2 hr later in sporulation. This conversion of P_{46} to P_{41} is stimulated allosterically by DPA only late in spore core maturations, when the core dehydrated and acidified. This dehydration and acidification also stimulates conversion of P_{46} to P_{41}, and this condition in the spore core preclude enzyme action thus keeping SASPs as stable in developing and dormant spores (Sanchez-Salas et al., 1993). It was recently reported that very little P_{46} is processed to P_{41} in the more hydrated and DPA-less *Δger3 spoVF* spores which accompanies the degradation of SASP-γ during sporulation (Paidhungat et al., 2000). SASP-γ is an amino acid storage protein and its degradation helps support protein synthesis early in spore germination and outgrowth and SASP-γ^- spores are retarded in spore outgrowth (Hackett and Setlow, 1988). Consistently, slow outgrowth phenotype of heat-sensitive *yvgW* spores can be the consequence of the low level of DPA in *yvgW* spores due to the less efficient conversion of GPR P_{46} to P_{41} and thus presumably resulted in slow SASP degradation during early germination and/or the condition in *yvgW* spores core may not enough to preclude protease activity entirely and resulting in the loss of some SASP proteins especially SASP-γ during sporulation or spore incubation periods due to the residual protease activity in spores as suggested by Paidhungat et al., 2000. Even the fact that slow outgrowth phenotypes of *yvgW* spores suppressed in the presence of rich medium (2xYT) containing large amount of free amino acids get stronger the possibility of later suggestion, a further detailed work on the level of dehydration of *yvgW* spore core, the level of GPR and SASPs in *yvgW* spores is needed.

The ultimate purpose of this research is to achieve the molecular characterization and the elucidation of the functional role of *yvgW* gene in the sporulation process. Under these respects, *yvgW* promoter region used during sporulation and vegetatif growth will be identified by primer extension method and further detailed characterization of mutant spores will be performed.

4. CONCLUSION

Present study was focused on the functional characterization of *yvgW* in the sporulation process of *B. subtilis*. Previous analysis of *yvgW* expression showed that a significant compartment-specific *yvgW* expression take place during the late stage of sporulation (T_5-T_7) though a slight *yvgW* expression was induced in the vegetative cell upon with the cessation of logarithmic growth. Subsequently, the effect of sporulation specific sigma factors on expression of *yvgW* gene was further analyzed. The deletion of *spoIIAC* and *spoIIGB* genes, coding for σ^F and σ^E, respectively, resulted in the complete elimination of *yvgW-lacZ* expression. Although, the previous deletion of the *spoIIIG* coding for σ^G caused a remarkable reduction in the level of *yvgW-lacZ* expression, mutation in *spoIVCB* encoding σ^K had no significant effect on the expression of *yvgW-lacZ*. Moreover, through the construction of *ΔyvgW::spc*, *yvgW* mutant cells were investigated for their spore properties, giving that spores of mutant cells showed high sensitivity to heat and chloroform, and resistance to lysozyme. The level of dipicolinic acid was also significantly reduced (37 %) in *yvgW* spores compared to wild type spores. Furthermore, the analyses of the nutrition specific germination and outgrowth characteristics of null mutant and wild type cells showed that no defect in the initiation of *yvgW* spore germination but they return to vegetative state more slowly then wild-type spores.

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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

HS medium (30 ml)

10X-S-base	3 ml
Glucose (50%)	300 µl
Yeast Extract (10%)	300 µl
Casaminoacid (2%)	300 µl
Arg (8%) + His (0.4%)	3 ml
Tryptophan (0.5%)	300 µl
Phenylalanine (0.3%)	450 µl

Completed up to 30 ml with sterile distilled H_2O and stored at cold room (+4°C) up to one week at most.

LS Medium (20 ml)

10X-S-Base	2 ml
Glucose	200 µl
Tryptophan	200 µl
Phenylalanine	30 µl
Casaminoacid	100 µl
Beef Extract	200 µl
Spermine (50mM)	200 µl
$MgCl_2$ (1M)(filter steriled)	50 µl

Freshly prepared and completed up to 20 ml with sterile distilled H_2O.

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 (Schaeffer'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.

Sterlini – Mandelstan Medium (SM medium)

CH I + II

Casein hydrosylate (Oxoid L41)	10 g
L – Glutamic acid	3,68 g
L – Alanine	1,25 g
L – Aspargine	1,39 g
KH_2PO_4	1,36 g
NH_4Cl	1,34 g
Na_2SO_4	0,11 g
NH_4NO_3	0,10 g
$FeCl_3.6H_2O$	1 mg

Dissolve the glutamic acid in distilled water by adjusting the pH to 7,0 with 10 M NaOH. Then add the other ingredients, adjust the final volume to 940 ml, dispense into 94 ml aliquots and autoclave. Do not replace casein hydrosylate with casamino acids.

CH III

$MgSO_4.7H_2O$	1,98 g
10% (w/v) $CaCl_2$	4 ml

Add separately to distilled water and bring the volume to 1 L. Autoclave and dispense into appropriate aliquots (100 ml)

CH VI

Dissolve 1.1 g of $MnSO4.4H_2O$ in 100 ml distilled water, dispense into 19 ml aliquots and autoclave.

CH V

This is L-Tryptopan (2 mg/ml) dissolved in water and fitler sterilized.

Sporulation salts

d H_2O	989 ml
solution A	1 ml
solution B	10 ml

Solution A

Per 100 ml:

$FeCl_3.6\ H_2O$	0,089 g
$MgCl_2.6\ H_2O$	0,830 g
$MnCl_2.4\ H_2O$	1,979 g

Autoclave, aliquot and store at 4^0C

Solution B

NH_4Cl	53,5 g
Na2SO4	10,6 g
KH_2PO_4	6,8 g
NH_4NO_3	9,7 g

Dissolve in 800 ml d H_2O.

Adjust pH to 7,0 with 2 M NaOH and bring volume to 1 L. Store at 4^0C

Solution C

This is 5% L-Glutamate. (L-Glutamic acid; pH 7.0 with 10 M NaOH) Autoclave.

Solution D

This is 0.1 M $CaCl_2$. Autoclave.

Solution E

1 M MgSO4.7 H_2O. Autoclave

Growth Medium

Growth medium (100 ml) is prepared on the day of use from the following sterile components.

CH I + II	94 ml
CH III	5 ml
CH VI	0,2 ml
CH V	1 ml

Tryptophan (CH V) is incorporated into the growth medium because this amino acid is lost during acid hydrolysis of casein.

Resuspension Medium

Resuspension medium (100 ml) is prepared on the day of use from the following sterile components :

Sporulation salts	90 ml
Solution C	4 ml
Solution D	1 ml
Solution E	4 ml

The appropriate growth requirements must be added to this minimal medium.

For antibiotic resistance LB liquid and solid media:

Final concentration:

Amp:	100 μg/ml
Erm:	1 μg/ml
Ln:	25 μg/ml
Spc:	100 μg/ml
Neo:	5μg/ml
Kan:	10 μg/ml
Cm:	5μg/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

TAE Buffer (50X)

Tris base (2 moles)	242 g
Glacial acetic acid	57.1 ml
EDTA	100 ml (0.5 M, pH 8.0)

Distilled H_2O was added up to 1L and pH was adjusted to 8 by HCl

Low Melting Agarose Gel (1%)

Agarose	0.5 g
TAE buffer (1X)	50 ml

1.5µg/mL EtBr was added before pouring the gel into tray

Low Melting Agarose Gel (2%)

Agarose	1 g
TAE buffer (1X)	50 ml

Addb 1.5µl EtBr (final concentration: 0.5 µg/ml) was added before pouring the gel into tray.

Physiological Sodium Chloride Solution (0.85%) (1000 ml)

$NaCl_2$ 8.5 g

Dissolved in 1000 ml distilled water and autoclaved.

Z Buffer (500 ml, pH 7.0)

$Na_2HPO_4.7H_2O$	60mM, 5.33 g
Na_2HPO_4	40 mM, 3.12 g
KCl_2	10 mM, 0.373 g

$MgSO_4.7H_2O$ 1 mM, 0.123 g

All mixed and dissolved within 500 ml distilled water and the pH was adjusted to a value of 7.0.

β-mercaptoethanol final concentration: 270 µl / 100 ml (add to Z buffer on the day of the use)

Lysozyme final concentration: 2.5 mg/ml

ONPG final concentration: 4.0 mg/ml

Tris-Cl Solution (25mM, 1000 ml, pH 7.4)

Tris (hydromethyl)aminomethane 3.03 g

Dissolved in 1000 ml distilled water and pH was adjusted to 7.4 with HCl (1 M)

APPENDIX C

ENZYMES AND CHEMICALS

Enzyme	**Supplier**
*Bam*HI	Fermentas
*Hind*III	Fermentas
*Sac*I	Fermentas
*Pst*I	Fermentas
*Xba*I	Fermentas
Taq polymerase	Fermantas
T4 DNA Ligase	Fermentas

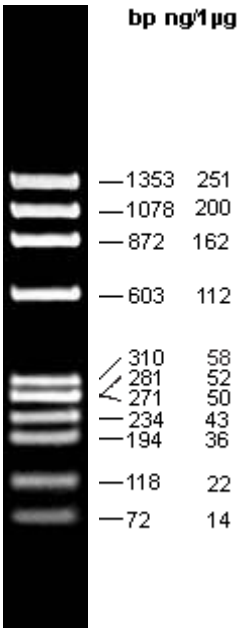
Chemical	**Supplier**
Agar	Sigma
Agarose	Sigma
β-mercapthoethanol	Merck
Calcium chlorid ($CaCl_2$)	Merck
D(+)-Glucose monohydrate	Merck
Iron(III) sulfate – 7 – hydrate ($FeSO_4.7H_2O$)	Riedel-de Haën
L- Argininemonohydrochlorid	Merck
L-Histidinmonohydrochlorid	Merck
L-Tryptophan	Merck
Lysozyme	Sigma
Magnesium sulphate ($MgSO_4.7H_2O$)	Riedel-de Haën
Natrum hydroxid (NaOH)	Riedel-de Haën
Natrium sulfate (Na_2SO_4)	Riedel-de Haën
Nutrient broth	Merck
ONPG	Sigma
Polyethyleneglycol ($HO(C_2H_4O)_nH$)	Merck

Potassium chloride (KCl)	Riedel-de Haën
Sodium carbonate (Na_2CO_3)	Riedel-de Haën
Sodium chloride (NaCl)	Riedel-de Haën
Sodium hydrogen phosphate($Na_2HPO_4.7H_2O$)	Merck
Tris (hydrocymethyl) aminomethane	Merck
Triton-X100	Sigma
Tryptone	Sigma
Yeast Extract	Sigma

APPENDIX D

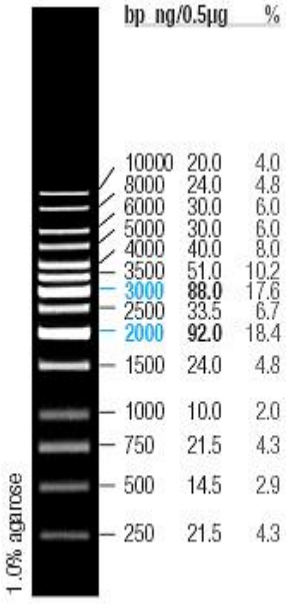
MARKERS……Fermentas

Marker 1 : PhiX174 DNA / *Bsu*RI (*Hae*III) Marker, 9



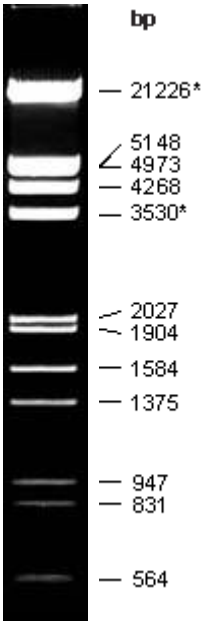
1.7% agarose
0.5µg/lane,
8cm length gel,
1X TBE, 12V/cm

GeneRuler 1kb DNA Ladder



0.5µg/lane,
8cm length gel,
1X TAE, 7V/cm, 45min

Marker 3: Lambda DNA / *Eco*RI+*Hind*III Marker, 3



1.0% agarose
0.5µg/lane,
8cm length gel,
1X TAE, 17V/cm

APPENDIX E

yvgW DNA Sequence

```
1    gtgagactag tgaaacagga atatgttctg gacggtttgg attgcagcaa ttgtgcccga
61   aaaatcgaaa atggagtcaa aggcataaaa ggcattaacg gatgcgcggt aaattttgcg
121  gcaagcacct taactgtctc agccgatggg aaagaagagc aatgggtcac gaataaagta
181  gagaaaaaag tgaaatcaat tgatccgcat gtaacggttc gccaaaagca tataaagaaa
241  tcagctgatg atggctatcg taatcgaatg gtcaatatgc tgatcagaat ggcggcggct
301  gtcattctcg gcgcagcggc atatttggtt cagtcgggaa ccattgaatt tttccttttc
361  ctcggtgctt atttgattat cggcggtgac attataatcc gcgcagtcaa aaacatcatc
421  cgcggtcagg tgtttgatga gcatttctta atggctcttg ccacaatcgg cgcttttctg
481  attcagcaat accctgaggg ggtcgccgtt atgctgtttt atcaaatcgg tgagcttttt
541  caaggagccg cggtcagccg ttccagaaaa tcaattagtg cgttaatgga catacggcct
601  gattacgcca atctcaaaac aaagaacggc atcgaacagg tttcgtcgga ggacgttcag
661  acaggagaca tcattgtggt taatccggga gaaagcatac ccctggacgg taaggtcgtg
721  caagggtcag cgatggttga tacatctgct ttaacaggtg agtccgttcc gagaaaagct
781  gcggaaggac aagatgtaat gtcaggcttt atcaatcaaa acggcgttct gcatattgaa
841  gtcacaaagg gctatcagga atcagcagta tcaaaaatat tagacttggt tcagaatgcg
901  agcagccgta aagcccggac agaaaacttt atcacgaagt ttgccaagta ttatacacct
961  gctgtggtga tcattgcggt attgctggcc tttgttccgc cgcttgttct ttctggcgcc
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1141 gtgcttgtaa agggaagcaa ttatttagaa gctttgaatc aagtcaaata cgcggtgttt
1201 gataaaacgg gaacactaac aaaaggcagc tttgaagtga cagaaatcaa acccgcggaa
1261 ggttttacaa aggatagatt acttgaagcg gcggcatacg ctgagcttca ttcacagcat
1321 ccgattgctg aatctgtgcg taaagcttac gggaaaatgc tttcctccga tgagattgag
1381 tcatacgaag aaatctccgg tcacggcatt tttgcaaagg taaatggaac tgagatcctt
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1561 gagataaagg aagacgcagc acaggcggtt gctgatctga agtcgcttgg gatcaaacag
1621 acagctatgc tgacgggtga ttcgaaacaa acgggagaag ccgtcggaaa acagcttggg
1681 attggtgagg tatatgcaga gctgctgccg caagataaag tcgcgcaggt cgaagcatta
1741 gaagctaagc ttttgcctag tgaaaaactc atttttgtcg gtgacggcat caatgatacg
1801 ccggtgcttg cacgcgcaga tatagggttt gcgatgggag gccttggctc agacgccgcg
1861 gtagaagccg cagacatcgt gctgatgaca gatcagccgt caaaaatagc agaagcgatt
1921 cggattgcga aacggacacg gcgaatcgtg tggcaaaaca tcggctttgc tttaggtgtg
1981 aaagcgatct ttctcattct tggtgcattt ggaattgcga ccatgtggga agcggttttc
2041 tcagatgtcg gcgttacact gcttgctgtg gcgaacgcca tgcgcgtcat gcgtctcaaa
2101 aacaaataaa ttgtcggaga gaattcattt tttcggggtt ttcttatcag cgcttgtctt
2161 gttaaaataa agaagtgggt caattgcgat ccgcttc
```

APPENDIX 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

Thermocycler: Techne FTGENE 5D

Thermomixer: Eppendorf thermomixer comfort (1.5 ml)

Transillumunator: Biorad UV transilluminator 2000

Vortexing machine: Heidolph Raax top

Waterbaths: Memmert wb-22

CV

Öykü İrigül was born in İSTİNYE-İSTANBUL in 1983. After getting her high school diploma from Galatasaray high school, in 2000, she started to study in Istanbul Technical University, Science and Literature Faculty, Department of Molecular Biology and Genetics in 2000. She was graduated in 2004 and at the same year, she was accepted to Advanced Technologies in Molecular Biology, Genetics and Biotechnology's program in Department of Molecular Biology and Genetics. She is still pursuing her studies in the same department. Microbial biotechnology, molecular microbiology, molecular genetics are among her professional interest topics. The titles of her publications are as follows:

ABSTRACTS

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