ISTANBUL TECHNICAL UNIVERSITY ★ INSTITUTE OF SCIENCE AND TECHNOLOGY

CHARACTERIZATION OF A NOVEL SPORULATION GENE *yvgW*, IN *BACILLUS SUBTILIS*

M.Sc. Thesis by

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BACİLLUS SUBTİLİS' TE BULUNAN YENİ SPORULASYON GENİ, *yvgW'* NUN KARAKTERİZASYONU

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FEBRUARY 2005 Ayça Gülçin ÜLGEN

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ABBREVIATIONS

ATP	: Adenosine triphospahe
BLAST	: Basic Local Alignment Search Tool
bp	: Base pair
dH_2O	: Distilled water
DNA	: Deoxyribonucleic acid
DSM	: Difco's Sporulation Medium
EDTA	: Ethylenediaminetetraacetic acid
EtBr	: Ethidium bromide
GFP	: Green Fluorescent Protein
HMW	: High Molecular Weight
IPTG	: Isopropyl-b D- thiogalactopyranoside
kb	: Kilobase
lacZ	: ß-galactosidase
LB- broth	: Luria Bertani broth
LMP agarose	: Low melting point agarose
NCBI	: National Center for Biotechnology Information
OD	: Optical density
ORF	: Open Reading Frame
PCR	: Polymerase chain reaction
PBPs	: Penicilin Binding Proteins
SM	: Sterlini and Mandelstam Medium
TAE	: Tris acetate EDTA
Tris	: Hydroxymethyl aminomethane

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CHARACTERIZATION OF A NOVEL SPORULATION GENE *yvgW*, IN *BACILLUS SUBTILIS*

SUMMARY

Sporulation process is a developmental stage of differentiation in response to unfavourable conditions such as nutrient insufficiency. The process involves an extracellular or intracellular signal dependent pathway employing many different gene products and factors to create a response regulatory system.

The gram-positive bacterium *Bacillus subtilis* stands as the primary model organism for the research of sporulation process since its metabolism is well understood and it has a simple developmental system. Although this process is well defined in *Bacillus subtilis* up to now, following the genome sequencing studies, it has been found out that there are still some unrevealed genes with unknown functions affecting the sporulation processes.

The inactivation of *yvgW* gene in *Bacillus subtilis* has been reported to affect the sporulation frequency in this organism causing a dramatic decrease (Yazgan *et al.,* 2001). Dependently, as a part of studies comprising the elucidation of the functional role of *yvgW* gene in the sporulation process, we analyzed the expression of *yvgW* during growth and sporulation by generating a strain (AGU1), containing a transcriptional *yvgW::lacZ* fusion in *yvgW* locus.

In order to visualize the possible *yvgW* expression during vegetative growth, AGU1 and *B. subtilis* PY79 were grown in Luria Bertani medium and *yvgW*-directed β-galactosidase activities in these cells were measured. Thus, *yvgW* gene expression during the logarithmic phase was clarified and interpreted in a such way that although no significant *yvgW* gene expression was observed during exponential phase of growth, a significant one was observed at the end of the logarithmic phase and additionally, a low level activity was shown to be accumulated during the transition to the stationary phase.

Furthermore, AGU1 strain and *B. subtilis* PY79 standing as the control strain were induced to sporulation by the nutrient exhaustion and the resuspension methods, in order to analyse the *yvgW* expression during sporulation. Expression levels were analyzed in order to be able to decide sporulation-specificity of *yvgW*. These analyses revealed that *yvgW* expression is not only sporulation-specific but also can be commented to be a cell-compartment-specific-mother cell or forespor-.

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 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. Later, the expression of transcriptional *yvgW::lacZ* fusion in *spoIIIG* mutant background was analyzed. The finding that *yvgW* is expressed specifically in the forespore compartment of the sporulation cell and the transcription of *yvgW* is mainly controlled by σ^G.

BACİLLUS SUBTİLİS' TE BULUNAN YENİ SPORULASYON GENİ, *yvgW'* NUN KARAKTERİZASYONU

ÖZET

Sporulasyon, besin yetersizliği gibi yaşamsal tehlike taşıyan, çevre koşullarına yanıt olarak bakteri metabolizmasının farklılaşma sürecinde meydana gelen değişikler bütünüdür. Bu süreç, hücre dışı ve içi sinyallere bağlı olarak, farklı gen ürünlerini ve faktörlerini harekete geçirerek bir düzenleyici sistem oluşturur.

Gram pozitif bakteri olan *B. subtilis*, metabolizmasının iyi bilinmesi ve gelişimle ilgili olarak basit bir sisteme sahip olması nedeniyle, sporulasyon araştırmaları için örnek model organizma olarak seçilmiştir. *B. subtilis'*de sporulasyon günümüzde oldukça iyi açıklanmış olmasına rağmen, genom dizileme çalışmalarının ardından, sporulasyonu etkileyen ama fonksiyonu tanımlanmamış genlerin hala var olduğu tespit edilmiştir.

*B. subtilis'*te bulunan *yvgW* geninin inaktif edilmesi, organizmanın sporulasyon seviyesinin belirgin bir şekilde düşmesiyle sonuçlanmıştır (Yazgan ve ark. 2001). Bu bulguya dayanarak, *yvgW* geninin *B. Subtilis'* de endospor oluşumu üzerine olan fonksiyonel etkisini moleküler düzeyde karakterize edecek moleküler çalışmaların ilk basamağı olarak, büyüme ve sporlanma süresince *yvgW* gen aktivitesinin takip edilebilmesi amacıyla *yvgW* lokusunda transkripsiyonel *yvgW::lacZ* füzyonu içeren AGU1 suşu oluşturulmuştur.

Vejetatif büyüme sırasındaki *yvgW* ekspresyonun aydınlanması amacıyla, AGU1 ve geri plandaki β-galaktosidaz seviyesinin görüntülenmesi için bulunan kontrol suş PY79 Luria Broth besi yerinde büyütülerek, β-galaktosidaz seviyeleri takip edilmiştir. Logaritmik büyüme fazında belirgin bir gen aktivitesi gözlenmemesine rağmen, logaritmik fazın sonunda açığa çıktığı ve düşük seviyede bir aktivitenin durağan faza geçiş sırasında biriktiği tespit edilmiştir.

Ayrıca, AGU1 suşu, Difco sporulasyon ve süspansiyon besi yerlerinde, spor oluşturma düzeyine, sporülasyon sırasındaki *yvgW* ekspresyonunun analizi amacıyla indüklenmiş ve *yvgW* önderliğindeki gerçekleşen β-galaktozidaz aktiviteleri ölçülmüştür. Sonuçlar, *yvgW* ekspresyonunun sporulasyona özgülüğünü ve aynı zamanda, *yvgW* ekspresyonun ana hücre ya da öncül spora özgü hücre kompartmanlarında olduğu şeklinde yorumlanabileceğini ortaya çıkarmıştır.

yvgW geninin ifade zamanının geç sporulasyon evresinde olduğu gerçeğinin ışığı altında, *yvgW* geninin ifade edilişinin, öncül spora özgü sigma faktörü σ^{G}' ye olan bağımlılığının incelenmesi amacıyla, transkripsiyonel *yvgW::lacZ* füzyonu ve bunun yanında σ^{G}' yi kodladığı bilinen *spoIIIG* gen bölgesinde delesyon taşıyan yeni bir mutant suşu oluşturulmuştur. Daha sonra, transkripsiyonel *yvgW::lacZ* füzyonunun ifade edilişi *spoIIIG* mutant geri planında analiz edilmiştir. Sonuçlar *yvgW* expresyonunun öncül spora özgü hücre kompartmanlarında olabileceğini ve expresyonunun çoğunlukla sigma faktör σ^{G} bağımlı olduğunu göstermiştir.

1. INTRODUCTION

1.1. *Bacillus subtilis*

Bacillus subtilis is one of the most intensively studied, well characterized gram-positive, soil bacterium which has served as a model in biochemical, genetic and molecular biological studies mostly due to the availability of their entire genome sequence that has been published in 1997 (Sonenshein *et. al.,* 2002). This organism has been placed in the family *Bacillaceae* because of the distinguishable feature of this family about the production of endospores. However, the bacteria belonging to genus *Bacillus* differ from the other endospore-forming bacteria on the basis of being rod-shaped and strict or facultative aerobes. Additionally, they also show a great diversity in their physiology. Among members of that genus, *Bacillus subtilis* is a chemoorganotroph that has the ability to survive when exposed to very simple growth conditions such as simple salt medium containing glucose or other sugars as carbon and energy source, inorganic nitrogen and adequate supply of oxygen (Nicholson and Setlow, 1990). Furthermore, characteristic feature of the physiology of this organism is the tendency to a "fast and feast" existence, which is controlled by a bunch of strategies that can be observed as a complex network in post-exponential growth. Post-exponential growth corresponds to a range of responses including carbon and nitrogen repression, stringency, chemotaxis associated motility, and the production of antibiotics. Besides, *Bacillus subtilis* have presumably evolved various hydrolytic enzymes to increase its fitness by limiting competition with other microorganisms through enlargement of the scale of carbon/nitrogen sources they use (Nicholson and Setlow, 1990).

1.2. Genetic View of *Bacillus*

The complete genome of *Bacillus subtilis* that is placed in a 4215 kb long, single chromosome 4215 kb long, includes 4106 putative protein-coding sequences, for about 2 300 genes with determined possible functions (Kobayashi and Ogasawara, 2002). Among 4106 protein-coding genes, 86 tRNA genes, 30 rRNA genes and three

small stable RNA genes are annotated. When the sequence was published, possible functions was assigned to about 58% (2379 genes) of the protein-coding genes, however the number of the genes with assigned function has increased to 63% (2562 genes) in the current database (Kobayashi and Ogasawara, 2002). Beside these, only 4% of essential genes display unknown functions (Kobayashi *et. al.*, 2003).

In order to determine complete genome sequence, 2810 knockout and 135 IPTG-dependent mutants have been constructed. This approach is based on gene inactivation via insertion of a nonreplicating plasmid into the target gene by a single crossover recombination. Under the aim of expression of genes from the same operon, IPTG regulated promoter presented on the inserted plasmid was used. As a result, 275 genes, including 25 genes of unknown function, were found to be essential for *B. subtilis* growth in rich medium at a moderate temperature in aerobic conditions. *B. subtilis* genome codes for 17 sigma factors and about 250 DNA binding transcriptional regulators (Kobayashi and Ogasawara, 2002). In addition to this, 1443 genes, some of which are conserved in a variety of organisms, have not reveled any functional information (Kobayashi *et. al.*, 2003).

1.3. Sporulation in *Bacillus subtilis*

Exposed to nutritional starvation, *B. subtilis* stops growing and initiates responses to restore growth by increasing the metabolic diversity. These responses include the induction of motility and chemotaxis, and the production of macromolecular hydrolases (proteases and carbohydrases) and antibiotics. If these responses fail to re-establish growth, the cells are mediated to form endospores which are small, metabolically dormant cells, showing remarkable resistance to heat, desiccation, radiation and chemical influence (Kunst *e.t al.,* 1997)

Although, the main stimulus for sporulation is starvation, the sporulation developmental program is not initiated immediately when growth slows due to nutrient limitation. In addition, highest population density is important beyond nutrient limitation, and no single nutritional effect acts as the trigger. Rather, the cell has an extremely complex and sophisticated decision-making apparatus, which monitors a huge range of internal and external signals such as chromosome integrity, the state of chromosomal replication, and the functioning of the Krebs cycle (Appleby *et. al.,* 1996, Errington, 2003). Before deciding to sporulate, a variety of

alternative responses occur, including the activation of flagellar motility to seek new food sources by chemotaxis, the production of antibiotics to destroy competing soil microbes, the secretion of hydrolytic enzymes to scavenge extracellular proteins and polysaccharides. Another alternative response arises in the induction of competence for uptake of exogenous DNA for consumption, with a possible side effect of stable integration of new genetic information. In other words, sporulation is the last-ditch response to starvation and is suppressed until alternative responses prove that conditions turned favorable (Grossman and Losick, 1997).

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 (Errington, 2003). 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. The initial stage, named as stage 0, represents the stage during which cells cease exponential growth in a sporulation medium without facing any obvious morphological change. The most characteristic feature of the sporulation is the formation of the polar septum following chromosomal DNA replication and the alignment of this DNA in the axial filament. As a consequence of this formation, stage II ends up with mother cell and forespore each receiving one of the replicated chromosomes. Between stage 0 and stage II, there is another step called stage I, during which cells synthesize a number of different enzymes such as α-amylases and proteases. In the next stage of development, stage III, the forespore becomes engulfed by the mother cell, resulting in a manner that the forespore is completely pinched off as a free structure within the mother cell, becoming a cell-within-a-cell (Stragier and Losick, 1996). Next, the forespore starts to produce large amounts of variety of small acid-soluble proteins known as SASP at stage IV. Meanwhile, in the intermembrane space between the forespore and mother cell, a thin layer of peptidoglycan called as the germ cell wall, which will end up later in spore cortex covering the forespore, is produced. Furthermore, this spore cortex is thought to be involved in attaining or maintaining the dehydrated and heat-resistant state of the spore. This step is followed by the deposition of layers of coat proteins around the outer membrane that envelops the cortex and by the generation of spore coat at stage V. Later, development of spore

resistance such as heat, organic solvents, and radiation occurs fully during stage VI (Errington, 1996, Webb *et. al.,* 1997). Eventually, when maturation is completed, the fully ripened spore is liberated by the lysis of the mother cell. Therefore, the mother cell undergoes programmed cell death, whereas the immortal forespore becomes the mature spore and gives rise to subsequent progeny (Nicholson and Setlow, 1990, Stragier and Losick, 1996).

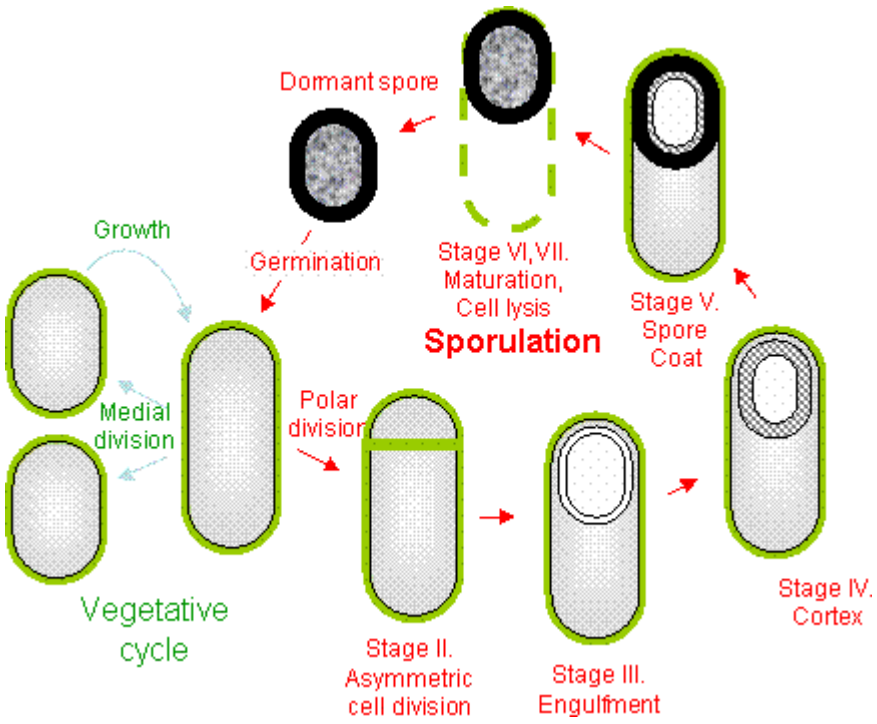


Figure1.1: The morphological stages of sporulation that are designated by Roman numerals (http://users.path.ox.ac.uk/~erring/index.htm).

1.3.1. The Regulatory Network in Initiation of Sporulation

The initiation of sporulation in *Bacillus subtilis* is regulated by a phosphorylation-mediated signal transduction pathway, which was termed as phosphorelay (Burbulys et al., 1991). The 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). The activity of master response regulator protein Spo0A depends on phosphorylation that is a typical 'response-

regulator' component of the 'two-component' signaling systems, controlling the entry into the developmental pathway for endospore formation (Stephens, 1998).

Two-component system and phosphorelay are both believed to function as environmental sensors that program expression of the genes as an answer to environmental stimuli. In bacterial cells, two-component signal transduction systems are central elements of cellular regulatory pathways and are commonly used to sense environmental change and to influence gene expression (Hoch and Silhavy, 1995). These systems depend on histidine kinases that serve both to perceive a particular stimulus and to respond to it. Therefore, binding of the signal ligands to the kinases induces ATP hydrolyses and autophosphorylation of a histidine residue. This phosphoryl group is subsequently transferred to a conserved aspartate residue in the regulatory domain of a specific response regulator–transcription factor. After regulatory domain is phosphorylated, it activates the transcriptional activation and/or repression properties of the output domain (Perego, 1997).

In the case of phosphorelay, signal ligands activate sensor histidine kinases as in a basic two-component system, but with a difference such that the phosphoryl group is transferred to an aspartate residue of a single-domain response regulator that lacks a DNA-binding output domain (Stephenson and Hoch, 2002, Sonenshein *et. al.,* 2002). Phosphorelays are signal integration circuits that allow greater levels of control than basic two-component systems by providing more nodes and checkpoints for the input of both positive and negative signals. This complex structure is nowhere more evident than in the *Bacillus subtilis* sporulation phosphorelay, which responds to multiple signal inputs through sensor kinases and phosphatases (Perego *et. al*., 1994, Perego and Hoch, 1996).

In this signal dependent phosphorelay system of sporulation, at least five sensor kinases, KinA, KinB, KinC, KinD and KinE, feed phosphate group to system, probably as a response to a different stimulus (Figure 1.2). However, phosphate is not directly transferred to Spo0A, but instead is transferred by two intermediates, Spo0F and Spo0B, which gives rise to the concept of a phosphorelay system (Burbulys *et. al.*, 1991). The process continues with Spo0A, receiving its phosphate from the Spo0B protein. Spo0B is simply an intermediary protein, taking its phosphate from the Spo0F protein, which is in turn phosphorylated by one of two related histidine kinases, KinA and KinB. KinA is probably responsible for most of

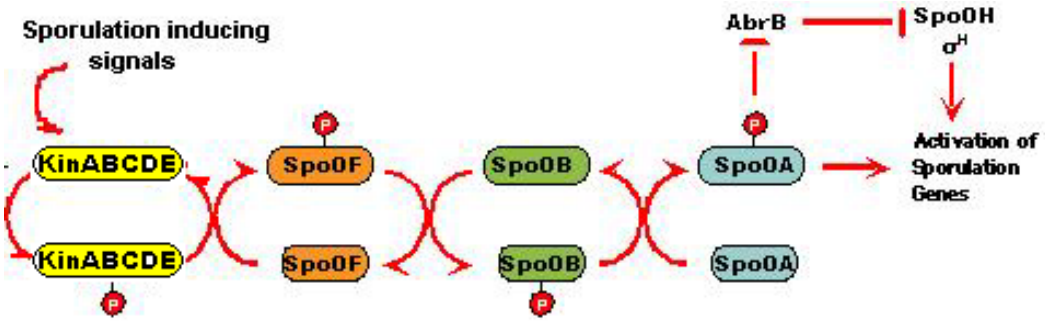


Figure 1.2: The *Bacillus subtilis* signal dependent phosphorelay sporulation system (http://www.mun.ca/biochem/courses/4103/figures/Spo0A.GIF).

the phosphate entering the phosphorelay (Trach and Hoch, 1993). 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. At the end of this process, Spo0A turns to its phosphorylated form and it 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).

1.3.2 Sporulation Specific Sigma Factors

Gene expression during spore formation in *Bacillus subtilis* is controlled by the temporal and spatial action of five developmental sigma factors; σ^{H}, σ^{F}, σ^{E}, σ^{G} and σ^{K} which become active at different times, in diverse cell types (Figure 1.3)

Moreover, each of them direct RNA polymerase to transcribe a different set of genes. The activity and sequential activation of each σ factor in the cascade is carefully regulated by multiple mechanisms (Errington, 1993, Kroos *el al*., 1999).

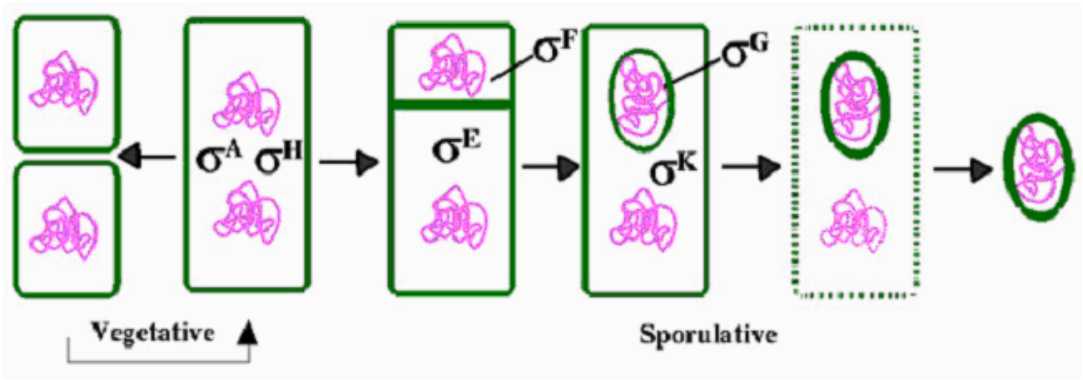


Figure 1.3: Schematic representation of endospore formation in *Bacillus subtilis* indicating the stages at and the compartments in which sigma factors are expressed (Errington, 1993).

Manufacturing and initiation of each σ factor, at the proper time and location, are ensured by a complex regulatory network (Losick and Stragier, 1992). The phosphorylated Spo0A resides at the top of the regulatory hierarchy. An elevated level of Spo0A-P triggers entry into sporulation by activating transcription by σ^{A}-RNAP and σ^{H}–RNAP that are already present in the cell (Figure 1.4). Besides, the level of σ^{H} rises following the transcription of *sigH* gene and is depressed by the action of Spo0A on the expression of *abrB* gene, and subsequently, both the synthesis and the stability of σ^{H} are enhanced by post-transcriptional mechanisms (Haldenwang, 1995). σ^{H}-RNAP and σ^{A}-RNAP transcribe operons that include the genes for σ^{F} and σ^{E} that are encoded by s*poIIAC* and *spoIIGB* respectively (Duncan and Losick, 1993).

1.3.2.1 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 (Kross *et. al.,* 1999).

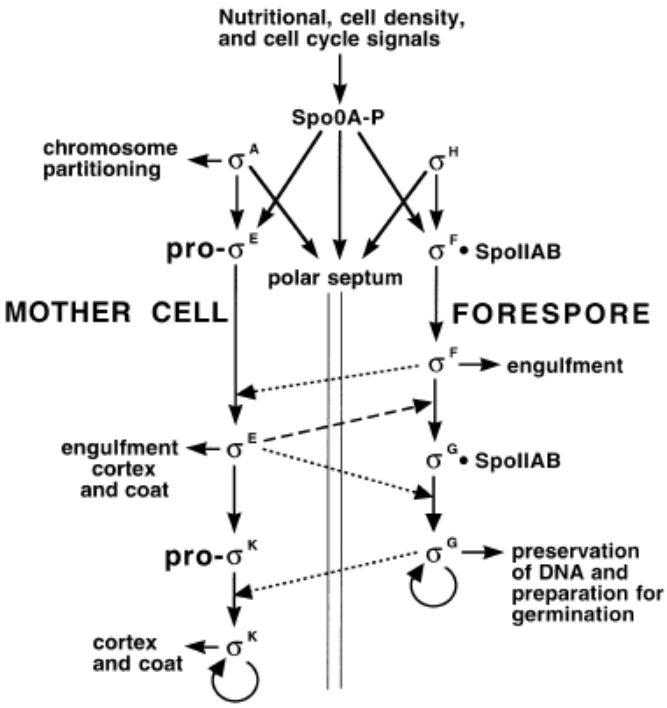


Figure 1.4: Regulatory network controlling sigma factor synthesis and activation. Solid arrows indicate dependence relationships for σ factors and the products of genes that bring out morphological change. The two vertical lines represent the membranes that separate the mother cell and forespore after 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).

Compartment specific activation of σ^F is governed by a pathway involving regulatory proteins SpoIIAB, SpoIIAA and SpoIIE. The first one, SpoIIAB is a dual-function protein that acts as an anti-sigma factor by binding and thus inhibiting σ^F and, as a serine protein kinase by phosphorylating and thereby inactivating SpoIIAA. The other regulatory protein SpoIIAA is an anti-anti-sigma factor that induces the release of σ^F from the SpoIIAB– σ^F complex (Garsin *et al.,* 1998). Finally, SpoIIE is a phosphatase that is responsible for converting the inactive, phosphorylated form of SpoIIAA (SpoIIAA-P) to its active, dephosphorylated form. Therefore, SpoIIE indirectly activates σ^F through the dephosphorylation of SpoIIAA-P. All three proteins are encoded within a three-cistron operon known as *spoIIA*, whereas SpoIIE is the product of the *spoIIE* gene (Seavers *et al.,* 2001).

An important aspect of the regulation of σ^F is that its activation depends on the formation of the polar septum. Asymmetric division can therefore be thought as a developmental checkpoint so that σ^F is activated only when and if sporulation polar septum is formed (Kroos *et. al.,* 2002).

1.3.2.2 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, that is synthesized as an inactive pre-protein in the predivisional sporangium, but is not converted to the mature form until after septation, when σ^E enters to the mother cell. Removal of pro-sequence requires proteolytic processing achieved by SpoIIGA that has a putative serine protease activity (Londono-Vallejo and Stragier, 1995). SpoIIGA requires the action of a forespore-specific protein, SpoIIR, which is a secreted protein that can exit the forespore and, in principle, can contact the membrane-bound SpoIIGA protease on the outer face of the opposing septal membrane of the mother cell. The main checkpoint here is that; *spoIIR* is the only σ^F controlled gene needed for activation of σ^E. Thus, pro-σ^E processing is tied to septation because σ^F-directed transcription depends on polar division (Karow *et. al.,* 1995).

Mutations that prevent the activation of either σ^F or σ^E result in a phenotype in which the mother-cell compartment undergoes another asymmetric division, which forms a second forespore-like compartment at the opposite pole, indicating that the sporulating cell is initially prepared for division near either of its cell poles and that the σ^F/σ^E activation cascade culminates with one or more genes that fix the fate of the mother cell by blocking the second potential polar division (Lewis *et. al.,* 1994). This blockage requires the concerted action of three different σ^E-dependent genes; *spoIID*, *spoIIM* and *spoIIP* that presumably encode cell-wall lytic enzymes that degrade the material in the developing septum (Eichenberger *et al.,* 2001).

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 (Eichenberger *et al.,* 2003).

1.3.2.3 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).

Following synthesis, σ^G does not become active, until engulfment of the forespore by the mother cell is complete (Piggot and Losick, 2001). Once active, σ^G directs expression of its own gene, allowing a rapid increase in its cellular level. On the other hand, mutations in several different genes, including *spoIIB*, *spoIID*, *spoIIM*, *spoIIIA*, and *spoIIIJ*, prevent transcription of σ^G -dependent genes without affecting σ^G synthesis, implying that their products play a role in σ^G activation. Three of the proteins, SpoIIB, SpoIID, and SpoIIM, are required for forespore engulfment, suggesting a link between activation of σ^G and the completion of engulfment. Besides, less is known about how σ^G is held inactive prior to engulfment, but there is some evidence that SpoIIAB which regulate σ^F activation also regulates σ^G activity (Abanes-De Mello *et. al.,* 2002).

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).

1.3.2.4 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 aminoacid 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).

1.4. Some Novel Sporulation Genes Found in *Bacillus subtilis*

At the time when *Bacillus subtilis* genome sequencing was completed, the research activities have been centered on functional genomics of *Bacillus subtilis* and essentially focused on the identification of the novel genes related with growth, motility, metabolism and sporulation. Identification was performed by using several insertional vector systems to disrupt gene function. By this way, structural and

functional roles of the identified genes related with such processes could have been immediately characterized. Below are given some of these recent studies that have subjected mainly on sporulation, based on the fact that *yvgW* gene was predicted to be effective in *Bacillus subtilis* endospore formation.

1.4.1 *yabQ* Gene, Essential for Spore Cortex Formation

The research group of Asai (2001) reported the function of the *yabQ* gene, which was located in *yabP–yabQ–divIC–yabR* operon and expressed during sporulation following a σ^E dependent manner. This function was involved in formation of the spore cortex. Additionally, the operon was identified through screening of transcripts with probes that were specific to the genes in a 108 kb region from *rrnO* to *spo0H* of the *Bacillus subtilis* chromosome and furthermore, the expression of these transcripts was initiated at the second hour of sporulation and in a σ^E -dependent manner (Asai *et. al.,* 2001).

During its function analysis, *yabQ* gene was deleted by an in-frame deletion, achieved via two-allele replacement, resulted in a large decrease in numbers of chloroform, lysozyme and heat-resistant spores, when compared to numbers found within the wild-type strain. Electron microscopy revealed that development of the spore cortex was blocked in the *yabQ* mutant. In addition to this observation, it was found that the inner coat layer of the mutant was partially detached from the outer coat, although the spore coat was visible. The localization of YabQ protein was determined by an in-frame fusion of the green fluorescent protein gene to *yabQ*, and subsequently it was found out that it resides around the forespore. This localization did not depend on SpoIVA or on CotE functions, both of which determine proper localization of coat proteins and cortex formation. The *yabQ* deletion did not affect expression of genes involved in cortex synthesis. According to these datas, it was suggested that the YabQ protein reaches its final location in the membrane of the forespore and plays an important role in synthesis of spore cortex and coat (Asai *et. al.,* 2001).

1.4.2 A Polysaccharide Deacetylase Gene (*pdaA*): *yfjS*

yfjS was reported and renamed as polysaccharide deacetylase gene (*pdaA*) by the research group of Fukushima (2002). The predicted amino acid sequence of *Bacillus subtilis yfjS* exhibited high similarity to those of several polysaccharide deacetylases.

A *pdaA-lacZ* gene fusion was constructed through the use of an integrative pMUTIN2 vector in order to determine expression time of *yfjS* by performing *β*-galactosidase assay. The observation was that *β*-galactosidase activity was low but significant at t4 and maximal at t6 with a 3-miller unit specific activity. Moreover, RNAs from four sigma factor-deficient (null) strains, $SigF^-$, $SigE^-$, $SigG^-$, $SigK^-$, were analyzed by Northern blotting, using a RNA probe containing the internal region of the *pdaA* gene and the obtained result has shown that *pdaA* was transcribed by σ^G RNA polymerase (Fukushima *et. al.,* 2002).

In addition to these datas, the result of *pdaA* disruption was characterized by a *pdaA*-deficient mutant. Cells showed normal growth, cell separation, and motility and produced bright refractile spores. Exposed to the heat activation, cells were forced to undergo sporulation and then suspended to germinate. During the incubation, a dramatic difference in the manner of hexosamine release was revealed between mutants and wild type. A complete lack of hexosamine release has been reported for a mutant of *cwlD*, which is one of the homologous genes for cell wall-lytic N-acetylmuramoyl-Lalanine amidase. Since the *pdaA*- and *cwlD*-deficient spores have similar phenotypes and the *cwlD*-deficient spores completely lack muramic acid δ-lactam, RP-HPLC was performed for the determination of the spore cortex muropeptides. The same peaks were revealed from the single mutants of *pdaA* and *cwlD.* Localization of *pdaA* by *pdaA*-GFP fusion has pointed out sporangia and spores. These results have indicated that the PdaA protein was localized in spores (Fukushima *et. al.,* 2002).

According to the overall scheme, *pdaA* is required for germination and for production of muramic δ-lactam residues in the spore cortex of *Bacillus subtilis.* The δ-lactam was recently shown to serve as a substrate recognition signal for the different GSLEs involved in cortex hydrolysis during germination. Spores, lacking this cortex-specific moiety, neither hydrolyze peptidoglycan nor outgrow. This data has correlated with the phenotype of the *pdaA* mutant spores and confirmed the role of *pdaA* (Fukushima *et. al.,* 2002).

1.4.3 A Putative High-Molecular-Weight Class A Penicillin-Binding Protein: *ywhE*

Pederson *et. al.* (2000) has characterized *ywhE* gene in *Bacillus subtilis* which was identified as the gene that potentially encodes a putative high-molecular-weight A class penicillin-binding protein, defined in the *Bacillus subtilis* genome sequencing project (Kunst *et al.,* 1997). Analysis of the expression of a translational *ywhE–lacZ* fusion, which was constructed with pJF751 that is a vector for construction of translational *lacZ* fusions, showed that *ywhE* expression is sporulation-specific, and is controlled predominantly by the forespore-specific sigma factor σ^F, and to a lesser extent by σ^G. Additionally, primer extension analysis identified two transcription start sites upstream of the *ywhE* translational initiation codon. Sequences located in the -10 and -35 regions relative to the transcription start sites showed good homology to the consensus sequences for promoter elements of σ^F -dependent genes (Pederson *et.al.,* 2000).

In order to observe effects of *ywhE* gene disruption on growth and sporulation, *ywhE* was insertionally inactivated. However, an insertional mutation in *ywhE* had no significant effect on growth, morphology, and sporulation, and *ywhE* spores had normal heat-resistance, cortex structure, and germination and outgrowth properties. Beside these, overexpression of *ywhE* in *Escherichia coli* has resulted in cell lysis. In agreement with these datas, the possible role of *ywhE* during sporulation becomes obvious only in the absence of one or several of the other three class HMW PBPs. (Pederson *et.al.,* 2000).

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 this knowledge, the studies on *yvgW* gene were to be furthered to cover the molecular characterization and the elucidation of the functional role of *yvgW* gene in the sporulation process. As a part of these molecular studies, the

present research aimed at (i) the analysis of expression time of *yvgW* gene through growth and sporulation, (ii) specifying sigma factor dependence of *yvgW* during sporulation process.

2. MATERIALS AND METHODS

2.1. Materials

2.1.1. Bacterial Strains

Bacillus subtilis PY79 that is a prototrophic derivative of standard strain *Bacillus subtilis* 168 was used as the wild type strain for the structural and the functional characterization of *yvgW* gene. *Esherischia coli* Top 10F'[*lac*Iq Tn10 (Tetr)], *mcrA* Δ(*mrr-hsdRMS-mcrBC*), *f80lacZΔM15 ΔlacX74, deoR, recA1, araD139 Δara-leu) 7697, galU, galK, rpsL (strr), endA1, nupG*) was used for cloning *B. subtilis* DNA. Integrative vector pMUTINT3 was used for the construction of *yvgW-lacZ* transcriptional fusion. AGU1 (*yvgW*::*lacZ*::*erm*) and AGU2 (*ΔspoIIIG::cat yvgW::lacZ::erm*) mutant strains which were constructed in this work were used for characterization of *yvgW* gene.

Table 2.1: Bacterial strains and their genotype used through the project.

Strain	Genotype	Source
Bacillus subtilis PY79	Wild type, BSP cured prototrophic derivative of *B.subtilis* 168	P.Youngman
AGU1	*yvgW::lacZ::erm*	This project
AGU2	*ΔspoIIIG::cat yvgW::lacZ::erm*	This project
E.coli Top10F'	[*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

2.1.2. Culture and Media

Composition and preparation of culture medias are given in the 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. Maintenance of Bacterial Strains

B. subtilis PY79, AGU1 and AGU2 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) broth mediums were used for the induction of sporulation. *E. coli* Top 10 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* Top10F' strain and HS and LS mediums were used for the transformation of *B. subtilis* strains. All cultures were stored at $+4^0$C and subcultured every week. 10% glycerol stocks of each strain were prepared and kept at -80^0C. Erythromycin (Erm) (1μg/ml), Lincomycin (Ln) (25μg/ml) and Chloramphenicol (Cm) (5μg/ml) were used for *B. subtilis* strains, Ampisilin (Amp) (100 μg/ml) and Tetracycline (Tet 20 μg/ml) were used for *E.coli* Top 10 strain as the selective antibiotics.

2.1.6. pMUTINT3: Insertional Gene Inactivation Vector for *Bacillus subtilis*

Insertional mutagenesis is a leading method to study the characterization of unknown open reading frames belonging to *Bacillus subtilis* chromosomal DNA. Therefore, several vectors were constructed to apply systematic gene inactivation through insertional mutagenesis and to observe the changes in the phenotype or life cycle of the bacterium (Vagner *et al*., 1998). For this purpose a special integrational plasmid, called pMUTINT3, was constructed which is a member of the pMUTIN set of plasmids. The main properties of these plasmids are that: (1) they are unable to replicate in *B.subtilis* to perform insertional mutagenesis, (2) in order to be able to measure the target gene expression they carry a reporter *lacZ* gene, (3) in order to

control the expression of the genes found downstream of the target gene, they include an inducible promoter. The necessity for an inducible promoter comes from the observation that most of the *B.subtilis* genes are present in multicistronic units, which leads to separation of the downstream genes from their promoter, when an insertion is applied to an operon. During the characterization of a specific gene function, this kind of polar effects are not desired so an inducible promoter is added to the plasmid structure, which is also used to regulate the expression of the target gene creating conditional mutants.

8834 bp long pMUTINT3 plasmid contains Pspac promoter developed by Yansura and Henner (1984) that can be induced via IPTG while it is normally repressed by plasmid encoding LacI protein (Figure 2.1). The Pspac inducible promoter includes one of the three-lac operators, known as "O1" and RNA polymerase recognition sequences of the SPO1 phage.

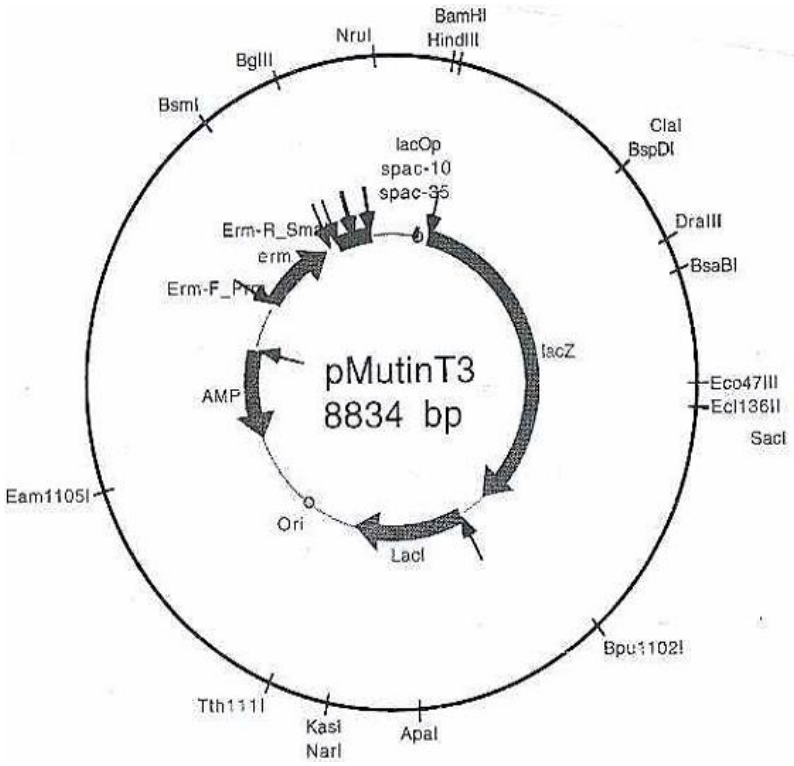


Figure 2.1: Genomic map of pMUTINT3 vector including the functional genes in the structure as well as the restriction map (Vagner *et al*., 1998).

The other properties of the plasmid are that, it contains specific selection markers; Ampicillin resistance gene expressed in *E.coli,* while Erythromycin resistance gene is expressed in *B.subtilis*. The Ori region from ColE1 replication sequences in the plasmid structure enables the plasmid to carry on replication in *E.coli*. The modified

lacZ reporter gene enables to carry out transcriptional fusions and measurement of gene expression in *B.subtilis*. (Vagner *et al*., 1998)

2.1.7. pDrive Cloning Vector

pDrive Cloning Vector is supplied in a linear form with a U overhang at each end and was used to direct-cloning of PCR products that were generated by non-proofreading DNA polymerases just like *Taq* Polymerase. Since, proofreading DNA polymerases generate predominantly blunt-ended PCR products, they won' t be efficiently ligated into the pDrive Cloning Vector. Additionally, this vector has *amp* and *kan* resistance genes, beside these, blue/white colony screening is available while using this vector for cloning purposes. pDrive also contains several unique restriction endonuclease recognition sites around the cloning site that allows easy restriction analysis of recombinant plasmids. In order to carry out transcription of cloned PCR products, as well as sequence analysis, it contains a T7 and SP6 promoter on either sides of the cloning site. Moreover, the pDrive Cloning Vector has a phage f1 origin to allow preparation of single-stranded DNA (http://www1.qiagen.com/HB/PCRCloning).

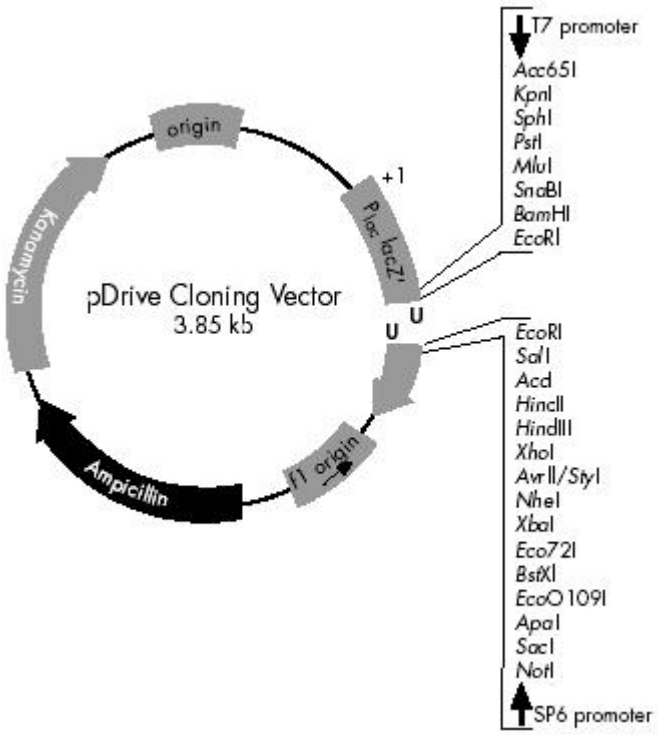


Figure 2.2: Genomic map of pDrive cloning vector including the functional genes in the structure as well as the restriction map (http://www1.qiagen.com/HB/PCRCloning).

2.2. DNA techniques and Manipulations

2.2.1. Polymerase Chain Reaction – PCR

The oligonucleotide primers were purchased from IONTEK. *yvgW* F_1 and *yvgW* R_1 primers are the forward and the reverse primers for the amplification of *yvgW* fragment to be cloned, respectively (Table 2.2). Underlined sequences represent recognition sites for *Hind*III and *Bam*HI restriction enzymes on forward and reverse primers respectively. Except *yvgW* F1 and *yvgW* R1 primers, rest of them was used for screening purposes.

Table 2.2: Sequences and Locations of Oligonucleotide Primers

Primer	Oligonucleotide Sequence	Target Sequence	Location
yvgW F_1	5'-CTT AAG CTT AGC ACC TTA ACT GTC TCA-3'	375 bp long	From 124 bp to 498 bp ORF of of *yvgW* gene
yvgW R_1	5'-GGA TCC CTC AGG GTA TTG CTG AAT CAG-3'		
pMUTINT3 F	5'-TAA GAC GGT TCG TGT TCG TGC- 3'	703 bp long	*erm* gene on pMUTINT3
pMUTINT3 R	5'-GCT GGC AGC TTA AGC AAT TGC- 3'		
yvgW F_2	5' -GGA TCC GTG AGA CTA GTG AAA CAG GAA- 3'	987 bp long	987 bp 5`-end of *yvgW* gene
yvgW R_2	5' –GGC AAG CTT CAT TCG ATT ACG ATA GCC- 3'		
yvgW F_3	5' -GGA TCC GTG AGA CTA GTG AAA CAG GAA- 3'	2106 bp long	Entire *yvgW* gene
yvgW R_3	5' -GGC TCG AGT AGT TTG TTT TTG AGA CGC AT- 3'		

PCR conditions were shown in table 2.3: (a). A master mix composed of the materials that can be seen on the table 2.3: (b) was prepared according to the number of samples. Then, the master mix was divided into separate PCR tubes and 1 µl of chromosomal DNA of *Bacillus subtilis* was added into each tube as template DNA. Finally, 0.5 µl of Taq polymerase was added into each tube separately.

Table 2.3: a) PCR conditions, **b)** The volume of components in PCR.

a)

94 °C	3 min	Initial Denaturation	
94 °C	1 min	Denaturation	5 cycle
55 °C	1 min	Annealing	
72 °C	1 min	Extension	
94 °C	1 min	Denaturation	25 cycle
60 °C	1 min	Annealing	
72 °C	1 min	Extension	
72 °C	10 min	Final extension	

b)

	1X
F Primer	4 µl
R Primer	4 µl
dNTP	3 µl
10XBuffer	3 µl
$MgCl_2$	3 µl
deH_2O	13 µl
Total	30 µl

2.2.2. Enzymatic 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 minutes.

2.2.3. Agarose Gel Electrophoresis

Depending on the purpose of the electrophoresis, different concentrations of agarose gel were used, which were given in Table 2.4.

Table 2.4: Agarose gel concentrations for different samples

Sample	Concentration
Chromosomal DNA	0.6 %
Plasmid DNA	0.8 %
Digestion products of plasmid	1 %
PCR products with pMut primers	1.5 %
PCR products with *yvgW* primers	2 %

2.2.3.1. DNA Molecular Weight Markers

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

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

Low melting 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 (4X) was added to the samples depending on the sample amount. Gel was poured into tray and the comb was placed and 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 minutes at 50°C by shortly vortexing every 2-3 minutes, 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. Subsequent, the flow through was discarded and 0.75 ml of buffer PE was added to wash. The column was standed for 2-5 minutes 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 Clonning Vector

All supplied components are mixed in an eppendorf tube according to volumes that was given at table 2.5. After adding all components, the tube was incubated at 16 ^{o}C for 1.5 hour and then ligase was denatured at 70 °C for 10 minutes.

Table 2.5: 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

2.2.6. Ligation of the PCR Product Into pMUTINT3 Vector

Firstly, 9.5µl of *yvgW* PCR products as insert fragments and 0.5µl of pMUTINT3 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. Finally, the mixture was again centrifuged for a quick spin and incubated at 16°C for 16 hours.

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

Initially, for electrocompotent *E.coli* top 10 cell preparation, the overnight *E.coli* Top10F' cells were inoculated into 400 ml 2xYT broth (Tet 20 µg/ml) with a 1/100 dilution rate and incubated at 37°C until OD_{600} reached 0.6. Then cells were incubated on ice for 30 minutes and centrifuged at 5000 rpm for 15 minutes. Later, supernatant was removed and pellet was resuspended in 40 ml of cold sterile dH_2O and centrifuged at 5000 rpm for 15 minutes. 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 minutes. Again supernatant was discarded and cells were resuspended in 1 ml of 10% glycerol (cold) and 40 µl volumes were aliquoted in the 1.5 ml eppendorf tubes. Consequently, 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 cells were thawed on ice and centrifuged for a quick spin. Then, 20 µl (for ligation reaction which pDRIVE vector was used, 10 µl of the mixture was transformed) of ligation sample was added and all together were transferred into electroporation apparatus. Following, the electroporation apparatus 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 minutes in order to harvest cells. Then the supernatant was discarded and the pellet was resuspended in 100 µl of 85% $NaCl_2$. 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" but with a different procedure of isolation than the one given below.

The bacterial cells were harvested by centrifugation at 13000 rpm for 5 minutes. The supernatant was removed and the pellet was resuspended in 300 µl P1 buffer. After addition of 300 µl P2 buffer, the solution was incubated at room temperature for 5 minutes. Then, 300 µl P3 buffer was added and mixed through inverting until the lysate is no longer viscous. Following, the sample was incubated for 15 minutes on ice and centrifuged at 13000 rpm for 15 minutes. Next, supernatant was transferred to a new 1.5 ml eppendorf tubes and depending on the volume of the supernatant 0.7 volume isopropanol was added and the solution was centrifuged at 13000 rpm for 30 minutes. The pellet was washed with 1ml of 70% ethanol by centrifugation at 13000 rpm for 5 minutes. 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 minutes 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 (Appendix A) mediums 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. At this time, 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 minutes. Finally, cells were resuspended in 100 µl of sterile %85 $NaCl_2$ and plated onto selective LB – agar plates and incubated at 37°C for 16 hours.

2.2.9.1. Induction of MLS Resistance Gene

After the transformation was carried out as explained above and before plating transformed cells onto LB agar plates containing selective antibiotics; the following procedure was used to obtain transformed cells. First of all, 100 µl of 10 µg /ml erythromycin solution was added to a small test tube containing LB overlay agar that contains % 0.7 agar in it and was kept molten at 45^{O} C. Then, transformed cells were added to this tube and were poured onto an antibiotic-free LB agar plate. After incubation at 37°C for 2 hours, a second overlay of LB soft agar containing100 µl of a 400 µg /ml/10 mg/ml erythromycin/lincomycin mixture was poured onto the first overlay. Colonies were grown out through the surface of the agar plate, after incubation at 37°C for 2 days.

2.2.10. Chromosomal DNA isolation

At the beginning, 1,5 ml of overnight culture was centrifuged at 13000 rpm for 5 minutes. The pellet was handled by discarding the supernatant and 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 / $NaCl_2$ (prewarmed at 65°C) solution was added and the mixture was incubated for 10 minutes 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 minutes. 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 minutes. Later the supernatant was removed and the pellet was washed with 1ml 70% ethanol centrifuged at 13000 rpm for 5 minutes. 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 minutes at thermomixer. Finally, the isolated DNA was made run on 0.6% agarose gel and the absorbance values at 260nm and 280nm were read to determine the concentration and purity of the DNA.

2.3. Induction of *yvgW::lacZ* Fusion by Cd^{+2} and Zn^{+2}

AGU1 colonies picked from LB agar plates were inoculated into lincomycin (25μg/ml) and erythromycin (1μg/ml) containing 10 ml LB broth and were made grow overnight at 37°C (250 rpm). For the negative control, PY79 colonies were inoculated into 10 ml LB broth and were made grow overnight at 37°C (250 rpm). Then, 100 μl of each culture were used to inoculate fresh LB broth mediums. Following this inoculation, cultures were left at 37°C (250 rpm) for incubation. Treatment was performed when the OD_{595} of the cultures reached 0,55. Before the induction 1 ml from each culture were taken in order to measure later the *β*-galactosidase activity of untreated AGU1 and PY79 cells at that OD_{595}. At this point 1 μl from 1M $ZnSO_4$ stock solution for 0,1 mM final concentration and 5 μl from $CdCl_2$. $6H_2O$ stock solution for 5 μM final concentration, were added individually to AGU1 and PY79 cultures. One of the AGU1 cultures was left untreated as a control. Then cultures were placed again at 37°C (250 rpm) for an hour incubation. At the end of this hour, optical densities of the cultures were read at 595 nm. and 1 ml from each culture were once again taken in order to measure their *β*-galactosidase activity. After sampling has been completed, *β*-galactosidase assay was performed.

2.4. Induction of Sporulation in DSM

Initially, all strains were grown overnight at 37°C on solid DSM. The cells were harvested by washing the plate with 3 ml of DSM, then, they were used to inoculate 35ml of DSM broth (Appendix A) to an initial optical density at 595nm of about 0.17. Later, the cultures were incubated at 37°C (250 rpm) until OD_{595} was 0.5 and starting from this point, 1 ml culture was withdrawn as duplicates at hourly intervals for the measurement of β-galactosidase activity. Meanwhile, OD_{595} was measured to observe t0 that is the initiation of sporulation represented by entry into stationary phase. In stationary phase, the value of OD_{595} is stable and doesn't increase dramatically. When t0 was defined, starting from this point sampling continued for eight hours and β-galactosidase procedure that is explained below was performed.

2.5. 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 595nm 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 t0 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.

2.6. β-galactosidase Assay with ONPG

After each sampling, the culture was centrifuged at 13000 rpm for 5 minutes 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 resuspended in 640 µl Z-buffer by vortex 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 until performing β-galactosidase assay.

β-galactosidase assay was executed in the first place 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 watched for the progression of the yellow color. Following, the reaction was stopped by the addition 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 formation of the yellow color, and so this period was recorded. 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 (Miller, 1972) below and a graph for both the β-galactosidase activity and log OD_{595} was drawn.

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

3. RESULTS AND DISCUSSION

3.1. Construction of *yvgW* Insertional Plasmid T1

In order to achieve the integration of pMUTINT3 vector into *B. subtilis* PY79 *yvgW* gene, firstly a 375 bp long fragment which stands between 124 to 498 bp downstream of translational start codon, belonging to 2106 bp *yvgW* ORF spanning region was amplified by PCR using the chromosomal DNA of wild type strain *Bacillus subtilis* PY79 as template (Figure 3.1) and cloned into pDrive Cloning Vector (Qiagen) for sequence analysis. The deduced nucleotide sequence data was compared to *B. subtilis* genome sequence, which can be found in National Center for Biotechnology Information (NCBI) database, using Blast search. As a consequence of this comparison, amplification of *yvgW* PCR fragment was confirmed. To clone into integrative plasmid pMUTINT3, the resulting PCR fragment was digested with *Hind*III and *Bam*HI restriction enzymes and ligated into similarly digested pMUTINT3 integrative 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}.

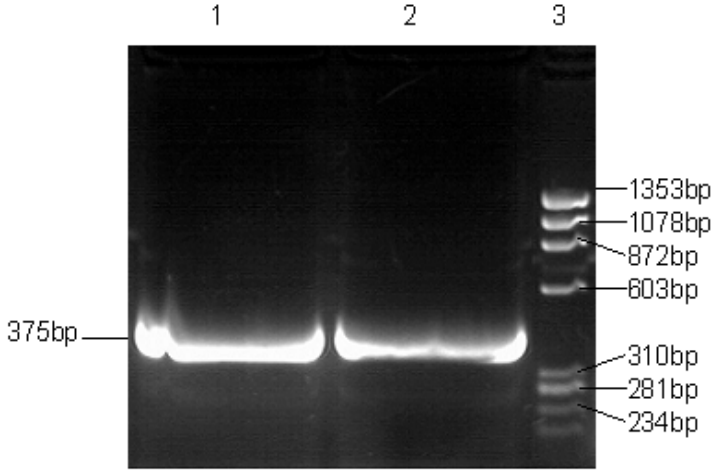


Figure 3.1: Amplification of 375 bp *yvgW* fragment with PCR.

The resulting 50 transformants were picked up and 10 of them were used for plasmid DNA isolation to verify the cloning of 375 bp *yvgW* PCR fragments (Figure 3.2).

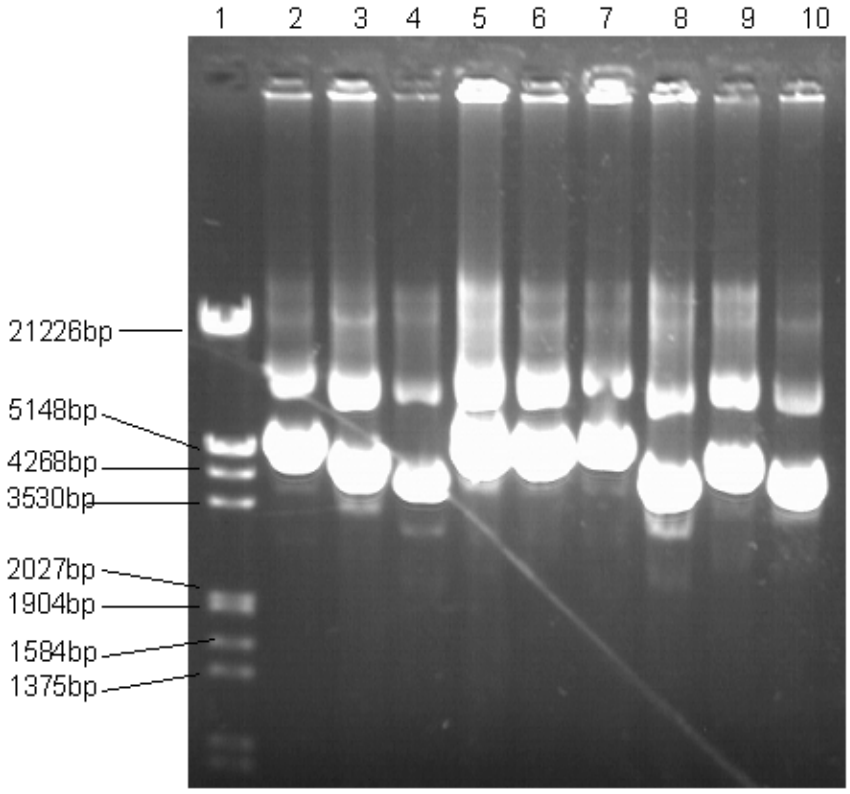
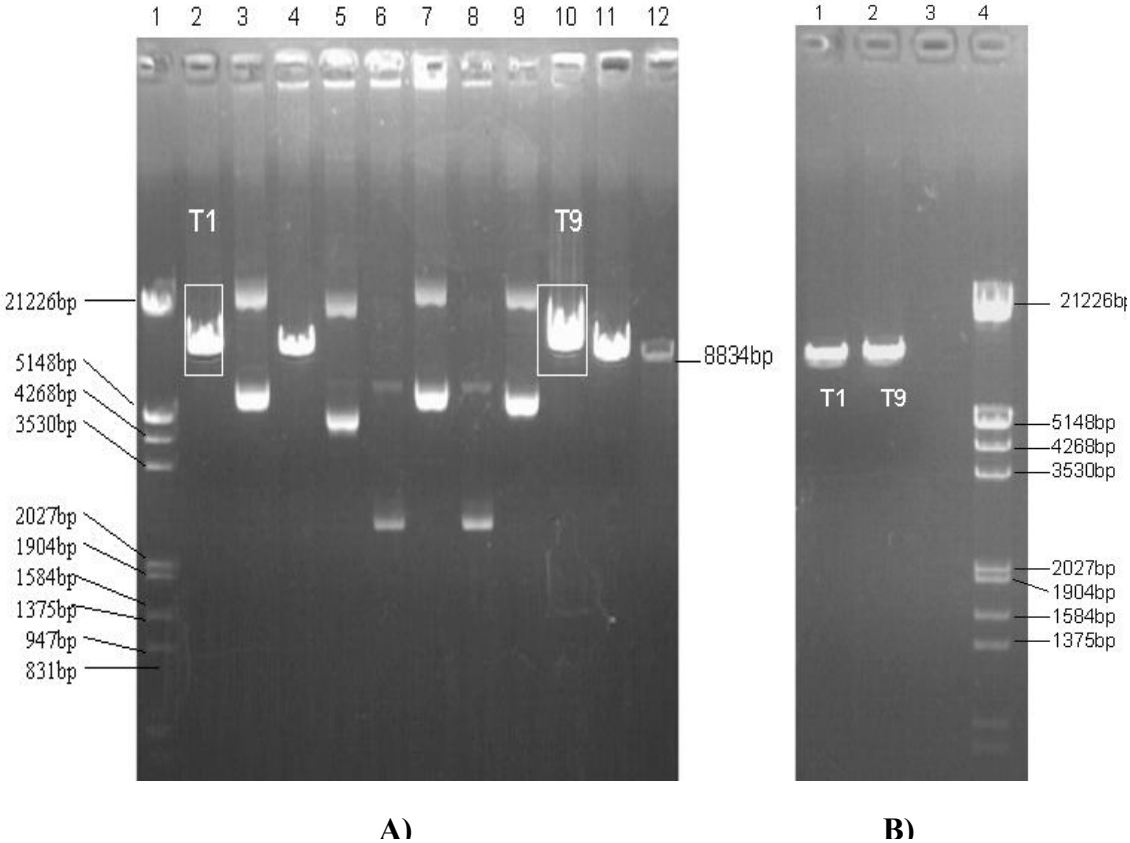


Figure 3.2: Plasmid DNAs isolated from *E.coli* Top10 Amp^R transformants (lane 2 to lane 10) and Lambda / *Eco*RI+*Hind*III Marker DNA fragments (lane1).

The plasmids DNAs isolated from transformants were linearized with *Bam*HI restriction enzyme for molecular weight confirmation (Figure 3.3(a)). The vector pMUTINT3 itself was 8834bp long while the insert was 375 bp long. The molecular weight of the resulting recombinant plasmid was expected to be about 9209 bp long. Therefore, plasmid DNAs isolated from transformant T1 and T9, which were expected size, were selected and linearized with *Cla*I restriction enzyme that has a unique restriction site on pMUTINT3 just like *Bam*HI (Figure 3.3(b), lane 1 and lane 2). The same result was obtained from *Cla*I restriction enzyme digestion. Thereby, T1 and T9 were selected as candidates for further analysis.



A) **B)**

Figure 3.3: A) Linearization of plasmid DNAs isolated from transformants with *Bam*HI restriction enzyme. *Bam*HI digested plasmid DNAs (lane 2 to lane 11) and pMUTINT3 (lane 12). Marker: Lambda DNA / *EcoR*I+*Hind*III (lane 1). **B)** Linearization of T1 (lane1) and T9 (lane 2) with *Cla*I. Marker: Lambda DNA / *Eco*RI+*Hind*III (lane 4).

With the objective of screening T1 and T9 for the size determination of pMUTINT3 vector and *yvgW* insert fragment, double restriction analysis with *Hind*III and *BamH*I was performed. As a consequence of T1 double digestion (Figure 3.4 lane 2), a 375 bp DNA fragment, which is equal to cloned *yvgW* PCR fragment (Figure 3.4 lane 5) and a 8834 bp DNA fragment which represents linear pMUTINT3 plasmid DNA (Figure 3.4 lane 3 and lane 6) was observed on the agarose gel. Meanwhile T9 double digestion (Figure 3.4 lane 4) was given an 8834 bp DNA fragment (Figure 3.4 lane 3 and lane 6) and a 603 bp DNA fragment. From these observations, we confirmed that recombinant plasmid T1 was the desired recombinant construct that consists of cloned 375 *yvgW* PCR fragment and pMUTINT3 vector.

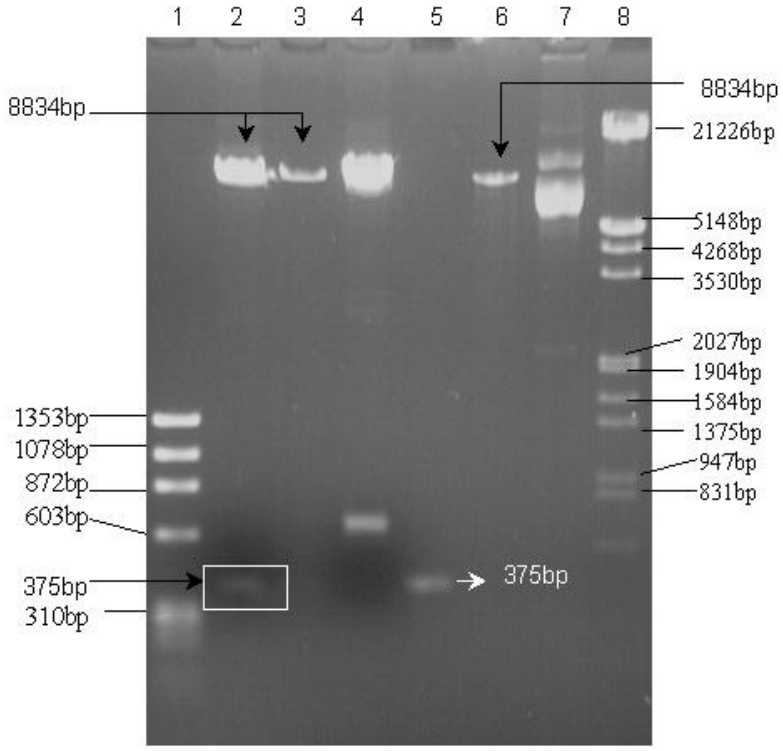


Figure 3.4:*Bam*HI and *Hind*III double digestion of plasmid DNA T1 (lane 2) and T9 (lane 4). Linearized pMUTINT3 (lane 3 and lane 6), 375 bp *yvgW* fragment amplified by PCR (lane 5) and undigested T1. Markers: PhiX174 DNA / *Bsu*RI (*Hae*III) DNA fragments (lane 1) and Lambda DNA / *Eco*RI+*Hind*III DNA fragments (lane 8).

After the amplification of plasmid T1 in *E coli* Top10, the recombinant T1 plasmid was further verified with PCR analysis by using the primers specific to *erm* gene present on pMUTINT3 and specific to cloned *yvgW* fragment was employed by using T1 plasmid DNA as template (Figure 3.5).

In figure 3.5, the band seen on lane 2 is the fragment of pMUTINT3 vector obtained as a result of the PCR reaction, which was performed with primers specific to *erm* gene on pMUTINT3 by using pMUTINT3 as template. The size of this fragment was 703 bp, and as expected, a 703 bp DNA fragment was obtained when; the PCR reaction was performed by using the same primers with plasmid T1 as template (Figure 3.5 lane 3). Furthermore, the other PCR reaction with primers specific to cloned *yvgW* fragment was executed by using the plasmid T1 as template to check whether the plasmid T1 still includes *yvgW* insert fragment or not (Figure 3.5 lane 4). The consequence of this PCR reaction, a 375 bp DNA fragment, which is equal to cloned *yvgW* PCR fragment (Figure 3.5 lane 7) was observed. Therefore, we concluded that plasmid T1 was the desired recombinant pMUTINT3 plasmid,

containing the 375 bp *yvgW* insert fragment inside, and we ensured that there is no change in the structure of recombinant plasmid T1 after amplification.

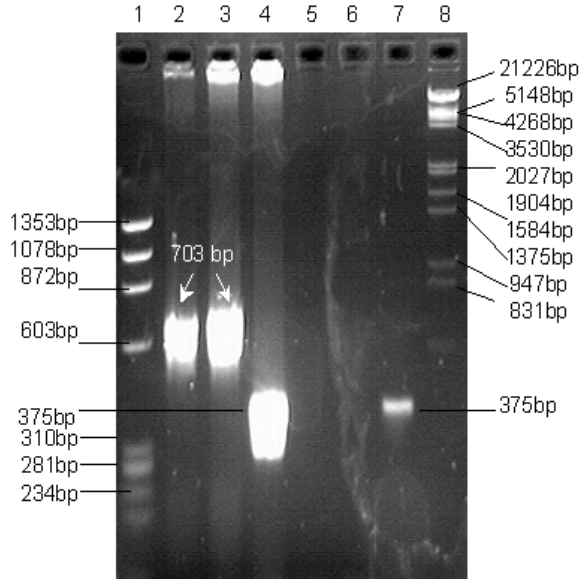


Figure 3.5: PCR reaction with primers specific to *erm* gene present on pMUTINT3 by using the pMUTINT3 (lane 2) and T1 plasmid (lane 3) as template, PCR reaction with primers specific to cloned *yvgW* fragment by using the T1 plasmid as template (lane 4), PCR control reactions with primers specific to *erm* gene present on pMUTINT3 (lane 5 and lane 6) and cloned *yvgW* fragment, and *yvgW* PCR fragments (lane 7). Markers: PhiX174 / *Bsu*RI and *Hae*III DNA fragments (lane 1) and Lambda/*Eco*RI+*Hind*III DNA fragments (lane 8).

3.2. Construction of *yvgW::lacZ* Transcriptional Fusion in *B. subtilis*

To provide construction of *yvgW::lacZ* transcriptional fusion, T1 plasmid DNA was used to transform competent cells of *B. subtilis* PY79 to erythromycin resistance. Recombinant plasmid T1 was driven into the chromosomal DNA of *B. subtilis* PY79 by a single cross over event (Campbell-like insertion). ColE1 origin of replication on pMUTINT3 enables replication in *E. coli* but not in *B. subtilis*. So that transformants would arise only by recombination between cloned *yvgW* fragment and its counterpart in the chromosome. Therefore, integration of plasmid T1 into *yvgW* gene locus by single cross over, resulted in *yvgW*::lacZ transcriptional fusion. However the selection for erythromycin resistance on LB plates containing erythromycin (1µg/ml) couldn't be achieved due to generation of the spontaneous erythromycin resistant *B. subtilis* PY79 cells. To overcome this problem the selection method for Macrolide-lincosamide-streptogramin B-resistance (MLS^R) conferred by *erm* gene was performed. Chromosomal DNA of resultant MLS^R transformant was isolated and

screened with PCR analysis by using primers specific to *erm* resistance gene on pMUTINT3 in order to verify insertion of plasmid T1 into the chromosome (Figure 3.6 lane 3). As a positive control, PCR analysis was performed by using the same primers with pMUTINT3 as template (Figure 3.6 lane 4) and to prove there is no complementary region for these primers on chromosomal DNA of wild type cells, the same PCR analysis was executed by using chromosomal DNA of *B. subtilis* PY79 as template (Figure 3.6 lane 2). As seen in figure 3.6, the sizes of the amplified fragments belonging to pMUTINT3 and to chromosomal DNA of mutant were equal. Thus, this result indicated that plasmid T1 was integrated into chromosome by Campbell recombination and corresponding MLS^R *B. subtilis* PY79 transformat was designated as AGU1.

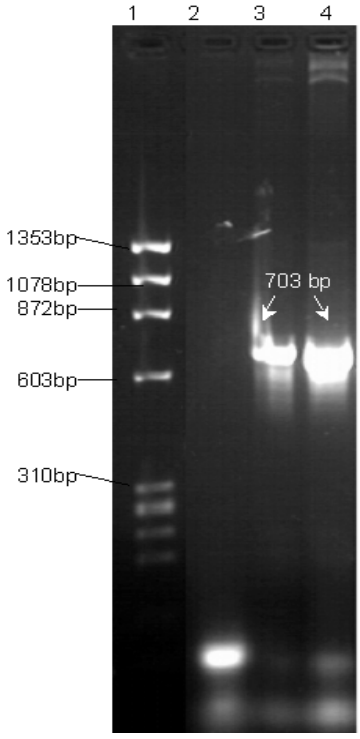


Figure 3.6: PCR reaction with primers specific to *erm* gene on pMUTINT3 by using the chromosomal DNA of MLS^R resistant mutant (lane 3) and *B. subtilis* PY79 (lane 2) pMUTINT3 (lane 4). Markers: PhiX174 / *Bsu*RI and *Hae*III DNA fragments (lane 1).

Eventually, AGU1 was screened for the insertion of plasmid T1 on *yvgW* gene locus. Since cloned *yvgW* internal fragment corresponds to 375 bp region of *yvgW* ORF, which stands between 124 bp and 498 bp, PCR analysis was managed by using primers specific to 987 bp 5`-*yvgW* gene fragment by using chromosomal DNA of *B. subtilis* PY79 and AGU1 as templates. The amplified PCR fragment from chromosomal DNA of *B. subtilis* PY79 was 987 bp long (Figure 3.7 lane 2) as

expected; while a significant amplification, other than the ones belonging to non-specific binders, couldn't be observed from PCR during which chromosomal DNA of AGU1 was used as the template (Figure 3.7 lane 3). It is open that these non-specific amplifications are generated from pMUTINT3 integrated into *yvgW* locus.

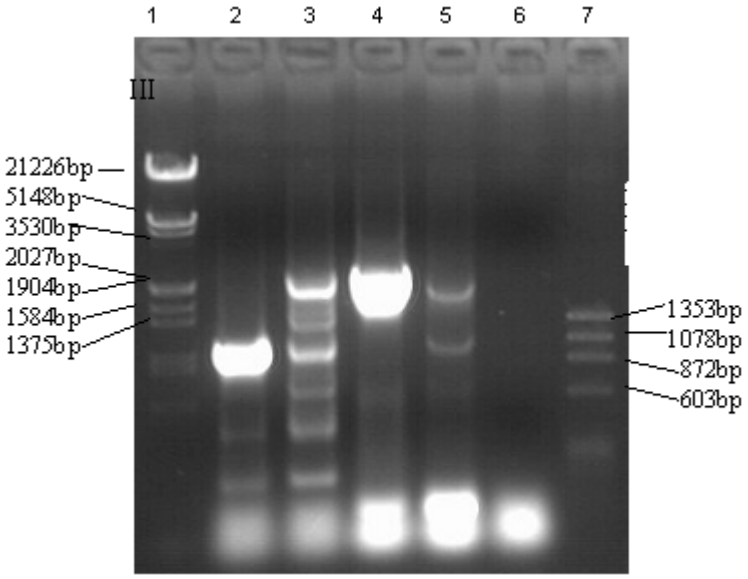


Figure 3.7: PCR reaction with primers specific to upstream of *yvgW* gene (987 bp 5`-*yvgW*); PY79 (lane 2) and AGU1 (lane 3), and to entire *yvgW* gene (2106 bp long); PY79 (lane 4) and AGU1 (lane 5). PCR control reaction (lane 6). Markers: PhiX174 / *Bsu*RI and *Hae*III DNA fragments (lane 1) and Lambda/*Eco*RI+*Hind*III DNA fragments (lane 7).

As a second verification, another PCR analysis was carried out to amplify total 2106 bp *yvgW* gene by using chromosomal DNA of *B. subtilis* PY79 and AGU1 as template. PCR analysis of *B. subtilis* PY79 resulted in 2106 bp long DNA fragment (Figure 3.7 lane 4), as anticipated, however no significant amplification was obtained from PCR analysis of AGU1. As a consequence of the insertion of plasmid T1, that is 9209 bp long, into *yvgW* gene locus, the size of the total gene has increased up to 11315 bp. On the other hand, amplification of DNA fragments higher than 4-5 kb long by *Taq* Polymerase is unperformable under normal PCR conditions. Thus, the amplification of 11315 bp long DNA fragment couldn't be achieved as expected (Figure 3.7 lane 5). The amplified DNA bands were an outcome of non-specific binders, just like the same bands could be observed on lane 3. Based on these results we concluded that plasmid T1 was integrated into *yvgW* gene on chromosome of *B. subtilis* PY79 and a *yvgW*::*lacZ* transcriptional fusion marked by *erm* resistance gene was constructed (Figure 3.8).

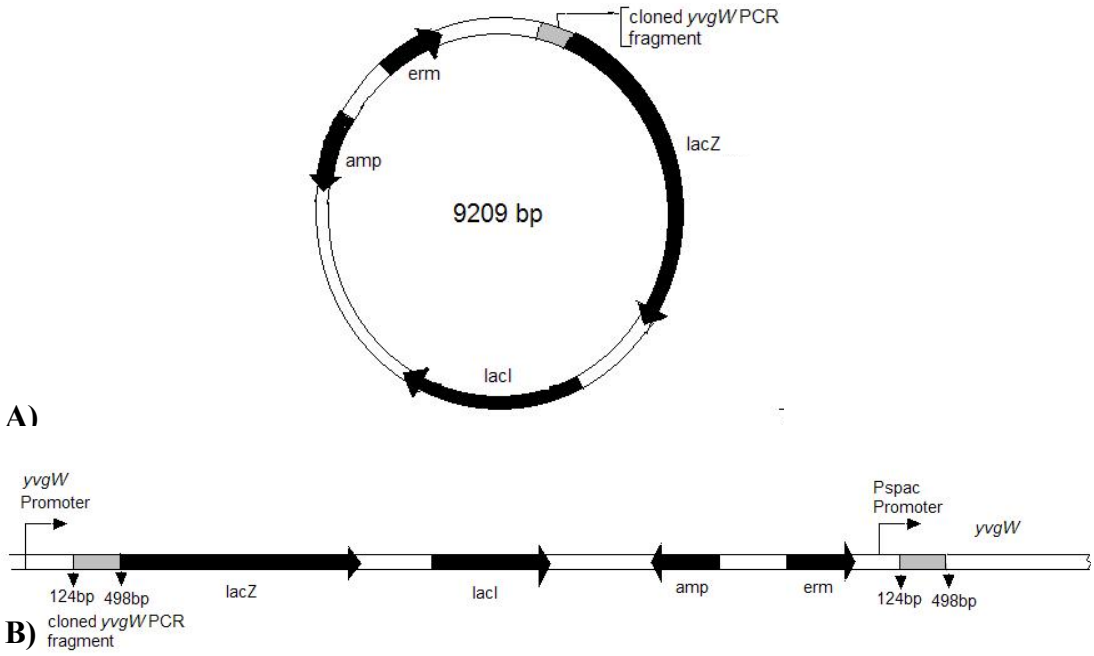


Figure 3.8: A) The structure of Plasmid T1 that consists of cloned 375 bp *yvgW* PCR fragment and 8834 bp pMUTINT3 vector. **B)** The result of integration of Plasmid T1 into *yvgW* gene locus, found on *Bacillus subtilis* PY79 chromosome.

3.3. Induction of *yvgW::lacZ* Fusion by Cd^{+2} and Zn^{+2}

It is reported that *yvgW* encoding CPx-type ATPase play a significant role in Cd (II) resistance and transcription of *yvgW* is selectively induced by Zn (II) and Co (II) as well as Cd (II) ions in *B. subtilis* (Solovieva and Entian, 2002; Gaballa and Helman, 2003). Therefore transcriptional *yvgW::lacZ* fusion mutant AGUI was tested for its response to Cd (II) and Zn (II) through *β*-galactosidase assays to evaluate whether *yvgW*::*lacZ* transcriptional fusion was properly constructed and functional. Since the integration of plasmid T1 into *yvgW* gene locus by a single cross over could be either forward or reverse orientation. Under this respect, AGU1 and PY79 strains were treated with 5μM Cd^{+2} and 0.1mM Zn^{+2} when cells enter their mid-exponential phase of their growth. After 1-hour incubation *β*-galactosidase specific activity was measured. As expected, this test was resulted with apparently induced *β*-galactosidase activity in response to Cd^{+2} and Zn^{+2} ions (Figure 3.9).

Cd^{+2} and Zn^{+2} treated AGU1 had a high beta-galactosidase specific activity that was 19,374 and 96.669 miller units, respectively when compared with wild type strain *B. subtilis* PY79 and uninduced AGU1 that showed 0,13 and 0,8 *β*-galactosidase specific activity in miller units, respectively (Figure 3.9).

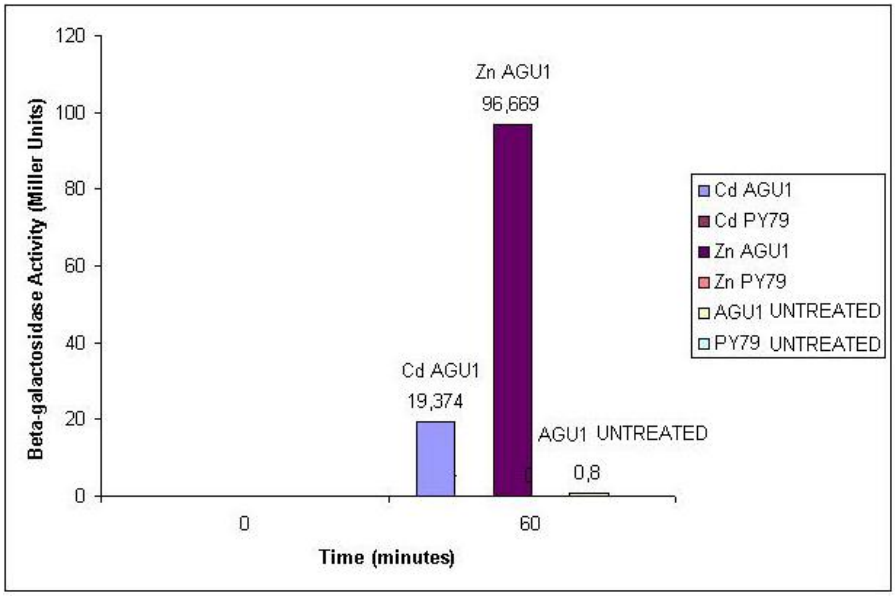


Figure 3.9: Induction of *yvgW::lacZ* fusion by $Cd^{+2}$and Zn^{+2}.

These results indicate that integrated plasmid T1 on *yvgW* gene locus was in its forward orientation, which enable the expected *yvgW::lacZ* transcriptional fusion and corresponding mutant strain AGU1 can be used to identify the expression time of *yvgW* gene in *B. subtilis* by *β*-galactosidase assay.

A powerful method to determine the time period of the expression of a gene in *B. subtilis* is the use of a reporter gene fusion, which will be expressed together with the promoter region of the regarded gene. This technique is being used successfully for the expression analysis of sporulation specific genes in *Bacillus subtilis* with the *lacZ* reporter gene, which provides a measurable *β*-galactosidase activity (Nicholson & Setlow, 1990). Through monitoring the *β*-galactosidase levels, samples were assayed with ONPG (o-nitrophenyl-*β*-D-galactosidase), which is a substrate of *β*-galactosidase enzyme that gives bright yellow color after digestion.

3.4. The Expression of *yvgW* in *B. subtilis* During Growth

To monitor the possible *yvgW* gene expression during the growth, AGU1 and *B. subtilis* PY79 standing as the control strain to analyze the background *β*-galactosidase level were grown in Luria Bertani medium and samples (1ml) of cultures were assayed for *β*-galactosidase, using ONPG as substrate. When cultures arrived to their mid-log growth phase, samples were started to be taken at each hour and continued for 6 hour until cultures reached to stationary phase. As in seen in

Figure 3.10, growth profiles of AGU1 and *B. subtilis* PY79 were in parallel form implying that cultures were synchronized. This was facilitated by streaking cultures at the same time and treating them in exactly the same fashion. When cultures were synchronized, *β*-galactosidase specific activity was comparable reflecting expression time of the gene of interest (Nicholson & Setlow, 1990). While *β*-galactosidase activity was very close to background levels (detected as 1 Miller Unit), during logarithmic growth phase the *β*-galactosidase activity of AGU1 increased rapidly (detected as 7 Miller Unit) at the end of exponential phase. *β*-galactosidase activity was accumulated apparently in early stationary phase detected after 6-7 hr with the maximum *β*-galactosidase activity (ca 10 Miller unit). These data indicate that there is no significant *yvgW* gene expression during exponential phase of growth but its transcription is induced at the end of the logarithmic growth phase and accumulated during the transition to stationary phase. The possible reason of this slight activity of *yvgW* generated during the transition to stationary phase could be a part of general stress response against the accumulation of toxic elements or compounds resulted from normal metabolic pathways of bacteria. Since *yvgW* gene has been previously found to be induced by heat shock, ethanol stress and disulfide stress as a part of general stress response (Helman *et al*, 2001; Petersohn *et al*. 2001 and Leichert *et al*. 2003).

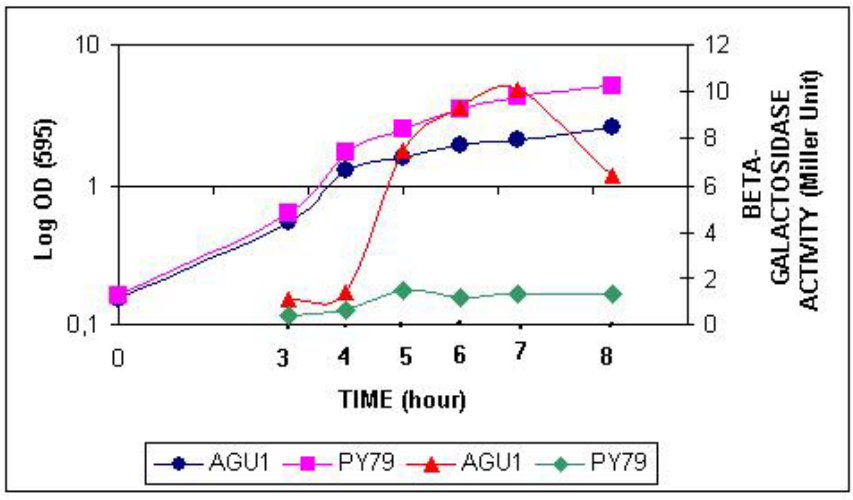
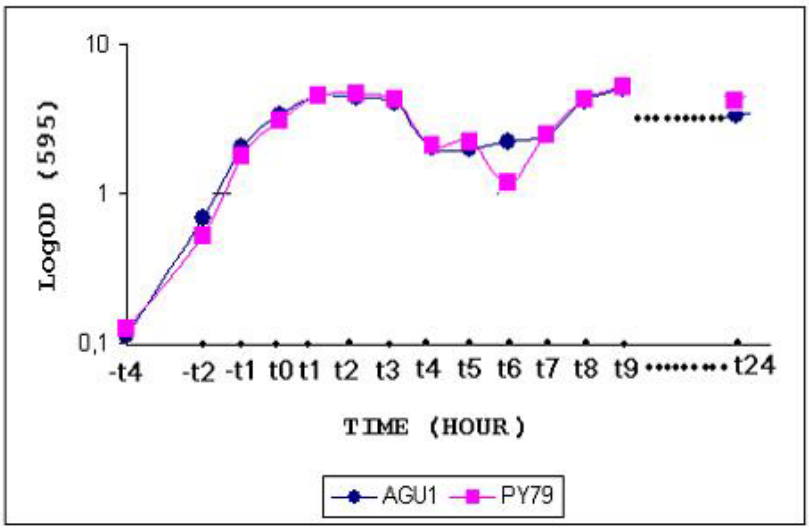


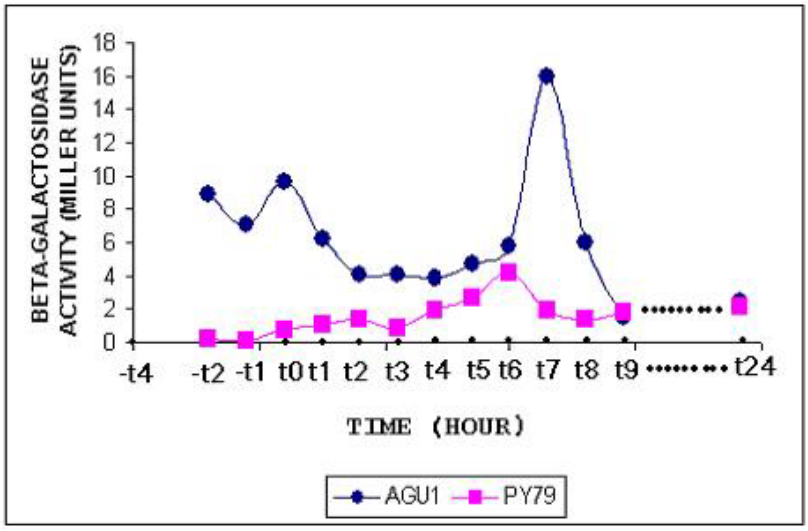
Figure 3.10: Growth profiles of AGU1 and *B. subtilis* PY79 and expression of transcriptional *yvgW::lacZ* fusion in Luria Bertani medium. AGU1 and *B. subtilis* PY79 cells were grown in LB medium, and samples were taken at each hour and assayed for *β*–galactosidase. The symbols used for growth profiles of the strains; (●) AGU1 (*yvgW::lacZ::erm*) and (■) PY79 (wild type), and for the *β*–galactosidase activity of the strains; (▲)AGU1 (*yvgW::lacZ::erm*) and (◆)PY79 (wild type).

3.5. The Expression of *yvgW* in *B. subtilis* During Sporulation

To analyze expression time of *yvgW* during sporulation, AGU1 and *B. subtilis* PY79 were directed to sporulation in Difco Sporulation Medium, which offers limited growth substrates that induces sporulation by exhaustion of the nutrients. After incubation of AGU1 and *B. subtilis* PY79 in DSM, samples were started to be taken at hourly intervals during the mid-log phase of their growth (Figure 3.11.A) 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. Although a slight *yvgW*-directed *β*-galactosidase was detected as 9 Miller unit during vegetative growth in DSM which might be resulted from the metal ion composition of DSM medium, *yvgW*-directed *β*-galactosidase activity was induced at about 5 hr into sporulation and accumulated significantly (detected with the maximum *β*–galactosidase activity as 16 miller unit) after seventh hour of sporulation (Figure 3.11.B) which was greater than the *β*-galactosidase activity detected in LB medium. These results revealed that *yvgW* expression is sporulation specific and seems to be a cell-compartment specific-mother cell or forespore since it is known that genes expressed after t_2-t_3 may be expressed in only one of these compartment (Nicholsan and Setlow, 1990).



A)

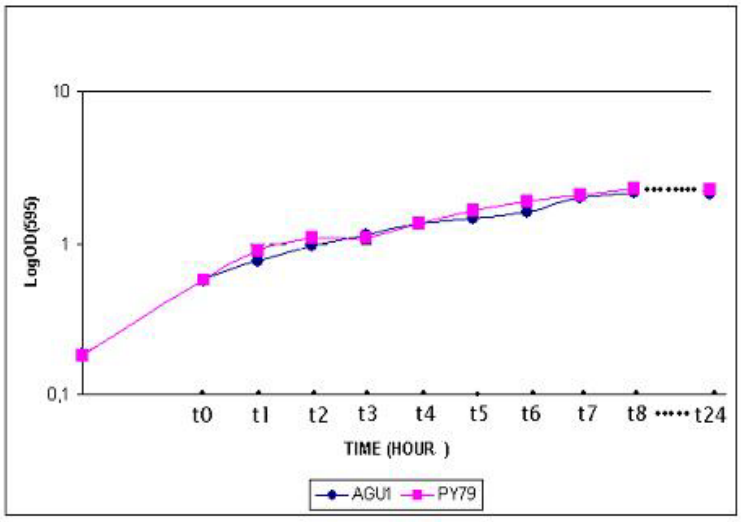


B)

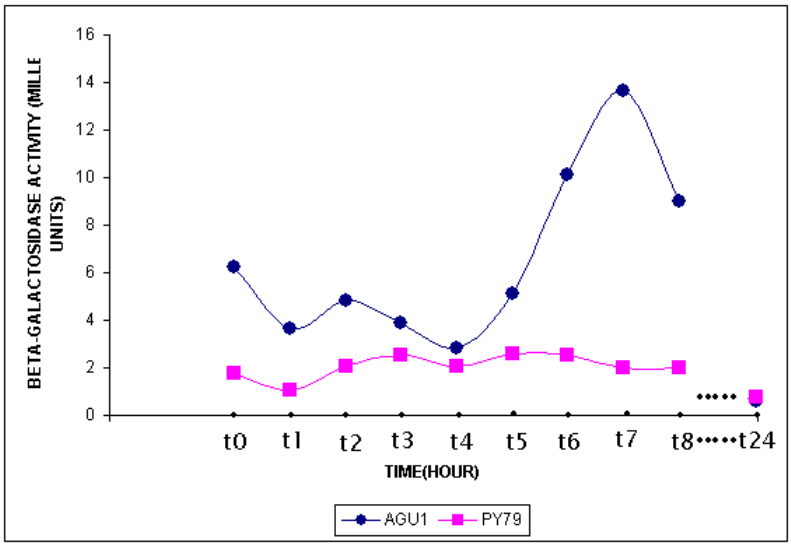
Figure 3.11:A) Growth profiles of AGU1 and *B. subtilis* PY79 in DSM during sporulation. **B)** Expression of transcriptional *yvgW::lacZ* fusion during sporulation. AGU1 and *B. subtilis* PY79 cells were sporulated in Difco Sporulation Medium, and samples were taken at each hour and assayed for β–galactosidase. Time zero represents initiation of sporulation. The symbols used for the strains are; (●) AGU1 (*yvgW::lacZ::erm*) and (■) PY79 (wild type).

Note that it is difficult to certain "Time zero", the beginning of sporulation, precisely in DSM. Since it is arbitrarily defined for experimental convenience as either the time at which cultures cease to grow exponentially in exhaustion medium. However exact knowledge of the time of expression of a sporulation specific gene is important because of the fact that it provides suggestive information about how the gene is regulated, which sporulation sigma factors might enhance the transcription of the gene and it may well also indicate if expression of the gene is cell-compartment specific. Therefore AGU1 and PY79 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 hourly 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 is determined after incubating cultures until OD_{595} at 0.5-0.8 in growth medium and resuspending them in resuspension medium. The time of resuspension reflects the beginning of sporulation. The growth patterns of AGU1 and *B. subtilis* PY79 (Figure 3.12.A) were more similar than the ones exhibited in DSM suggesting that, resuspension method

was a more powerful approach, for the aim of synchronizing cells and deciding exact point of initiation of sporulation. The expression of *yvgW* in SM was showed to be induced at about 5th hour of its sporulation with a maximum *β*-galactosidase activity (approximately 16 Miller units) detected at 7th hour of sporulation (Figure 3.12.B), confirming the result of *β*-galactosidase assay performed in DSM (Figure 3.11.B).



A)



B)

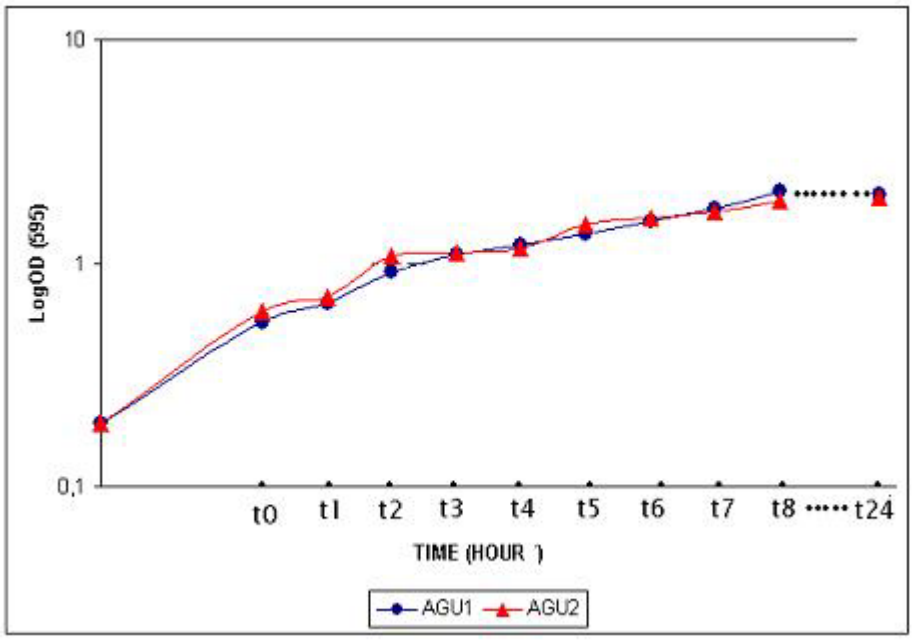
Figure 3.12: A) Growth profiles of AGU1 and *B. subtilis* PY79 in SM medium during sporulation. AGU1 and *B. subtilis* PY79 cells were sporulated by the resuspension method, and samples were taken at each hour and assayed for β–galactosidase. **B)** Expression of transcriptional *yvgW::lacZ* fusion during sporulation. Time zero is the time of resuspension of the culture to initiate sporulation. The symbols used for the strains are; (●) AGU1 (*yvgW::lacZ::erm*) and (■) PY79 (wild type).

These results verified 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. 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).

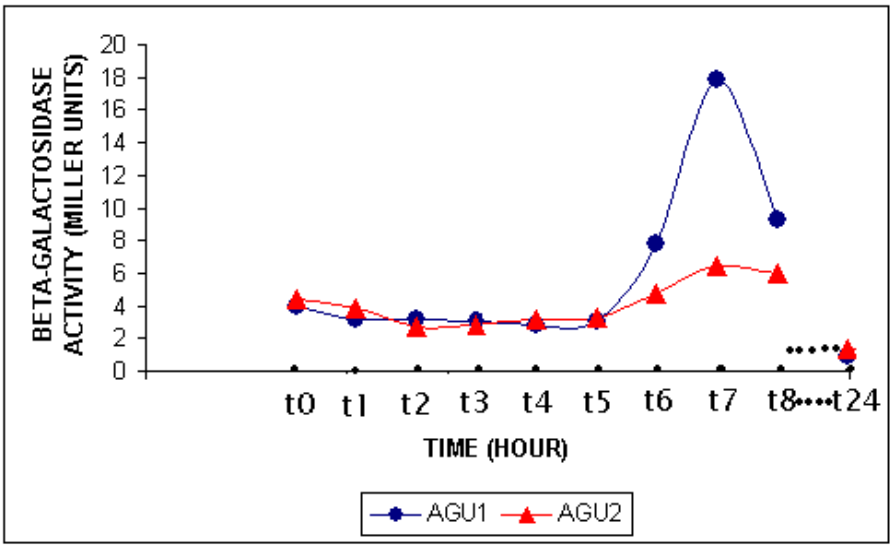
3.6. Sigma factor dependence of *yvgW* expression

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. 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, we first examined the dependence of *yvgW* expression on sporulation specific σ^G factor by constructing a new mutant strain containing transcriptional *yvgW::lacZ* fusion and deletion on *spoIIIG* locus that encodes sigma G marked by *cat* resistance gene and then analyzing the expression of transcriptional *yvgW::lacZ* fusion in *spoIIIG* mutant background. Firstly, chromosomal DNA of RL560 (*B. subtilis* PY79 Δ*spoIIIG::cat*) was used to transform AGU1 and possible transformants were selected against Cm^R and Erm^R. During transformation, deletion of *spoIIIG* gene in AGU1 was revealed by double cross over in a way that, *spoIIIG* was replaced by the *cat*-cassette belonging to RL560. Ten 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 *spoIIIG*. All of them were *spo*$^-$, therefore one of them was chosen randomly and called as AGU2 that contains transcriptional *yvgW::lacZ* fusion and deletion on *spoIIIG* gene with *cat* gene. In order to observe the effect of deletion of *spoIIIG* gene on expression of *yvgW* gene, AGU1 and AGU2 strains were induced to sporulaton in SM medium and *β*-galactosidase activity was measured during sporulation process. As seen on figure 3.13.A, AGU1 and AGU2 exhibited similar growth pattern, indicating that they were in the same stage of sporulation. Therefore, the *β*-galactosidase activity of AGU1 and AGU2 were comparable to each other giving idea about the sigma G activity on the expression of *yvgW*. The deletion of *spoIIIG* gene had significant effect on

yvgW::lacZ expression and caused a reduction of the level of *yvgW* driven *lacZ* expression of about 66.7% relative to the wild-type level (Figure 3.13.B). These data 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 it should be analyzed 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 and σ^K mutants' background. Additionally, the structural genes of sigma factors σ^G, *spoIIIG,* replaced under the control of the IPTG-inducible *spac* promoter should be introduced into a strain containing a transcriptional *yvgW::lacZ* fusion and as well as a mutation in *spoIIIG* to elucidate the effect of induction of σ^G synthesis on the expression of *yvgW::lacZ.*



A)



B)

Figure 3.13: A) Growth profiles of AGU1 and AGU2 in SM medium during sporulation. AGU1 and *B. subtilis* PY79 cells were sporulated by the resuspension method, and samples were taken at each hour and assayed for β–galactosidase. **B)** Expression of transcriptional *yvgW::lacZ* fusion during sporulation. Time zero is the time of resuspension of the culture to initiate sporulation. The symbols used for the strains are; (●) AGU1 (*yvgW::lacZ::erm*) and (▲) AGU2 (Δ*spoIIIG::cat::erm*).

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, the expression of transcriptional *yvgW::lacZ* fusion in sporulation specific sigma factors σ^F, σ^E and σ^K mutants background will be analyzed to certain the the role of σ^G in controlling *yvgW* expression. Then, green fluorescent protein (GFP) will be used for a fusion construction in order to detect the localization of the *yvgW* gene product within the cell. Later, *yvgW* knockout mutants will be constructed, to visualize effect of deletion of *yvgW* vegetative cells and spores. Finally, *yvgW* promoter region used during sporulation will be identified by primer extension method.

4. CONCLUSION

To examine the expression of *yvgW*, the transcriptional *yvgW::lacZ* fusion was constructed in *B. subtilis* PY79 by cloning of *yvgW* internal fragment into pMUTINT3 integrated by Campbell-like insertion mechanism into the *yvgW* locus, resulting in AGU1 strain and *yvgW*-driven *lacZ* expression was analyzed during growth and sporulation.

To elucidate the possible *yvgW* expression during vegetative growth, AGU1 and *B. subtilis* PY79 standing as the control strain to analyze the background β-galactosidase level were grown in Luria Bertani medium and *yvgW*-directed β-galactosidase activity in these cells were measured. There was no significant *yvgW* gene expression during exponential phase of growth but it's transcription was induced at the end of the logarithmic phase and a low level activity accumulated during the transition to stationary phase presumably because of the generation of stress condition due to accumulation of the toxic compounds produced in normal metabolism of bacteria.

AGU1 strain and *B. subtilis* PY79 standing as the control strain were induced to sporulation to analyze the *yvgW* expresion during sporulation. *yvgW*-directed *β*-galactosidase activity was initiated at about t_5 of sporulation and accumulated significantly after t_7 of sporulation. These results revealed that *yvgW* expression is sporulation specific and could be a cell-compartment-specific- mother cell or forespore-.

Because of the timing of *yvgW* expression corresponding to late sporulation process, the dependence of *yvgW* expression on forespore-specific sigma factor σ^G was tested by constructing a new mutant strain containing transcriptional *yvgW::lacZ* fusion and deletion on *spoIIIG* locus that encodes σ^G and then analyzing the expression of transcriptional *yvgW::lacZ* fusion in *spoIIIG* mutant background. The finding that *yvgW* is expressed specifically in the forespore compartment of the sporulation cell and *yvgW* transcription is controlled by mainly by σ^G.

REFERENCES

Abanes-De Mello, A., Sun, Y., L., Aung, S., Pogliano, K., 2002. A cytoskeleton-like role for the bacterial cell wall during engulfment of the *Bacillus subtilis* forespore, Genes Dev. **16,** 3253–3264.

Appleby J, Parkinson J, Bourret R., 1996. Signal transduction via the multistep phosphorelay: not necessarily the road less travelled, Cell, **86,** 845-848.

Asai, K., Takamatsu, H., Iwano, M., Kodama, T., Watabe, K., Ogasawara N., 2001. The *Bacillus subtilis yabQ* gene is essential for formation of the spore cortex Microbiology **147,** 919–927

Burbulys, D., Trach, K. A. & Hoch, J. A., 1991. Initiation of sporulation in *B. subtilis* is controlled by a multicomponent phosphorelay, Cell, **64**, 545–552

Duncan, L., Losick, R., 1993. SpoIIAB is an anti-σ factor that binds to and inhibits transcription by regulatory protein σ^F from *Bacillus subtilis,* Proc. Natl. Acad. Sci., **90,** 2325-2329.

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

Eichenberger, P., Jensen, S., T., Conlon, E., M., Ooij, C., Silvaggi, J., Gonza´lez-Pastor, J., Fujita, M., Ben-Yehuda, S., Stragier, P., Liu, J., S., Losick, R., 2003. The sigma E Regulon and the Identification of Additional Sporulation Genes in *Bacillus subtilis*, Molecular Biology, **327,** 945–972

Errington, J., 1993. *Bacillus subtilis* sporulation: regulation of gene expression and control of morphogenesis, Microbiology Reviews, **57,** 1–33.

Errington, J., 1996. Determination of cell fate in Bacillus subtilis, Trends in Genetic, **12,** 31-34.

Errington, J., 2003. Regulation of endospore formation in *Bacillus subtilis*, Nature Reviews of Microbiology, **1,** 117-126.

Fabret, C., Feher, V., A., Hoch, J., A., 1999. Two-component signal transduction in *Bacillus subtilis*: how one organism sees its world, Journal of Bacteriology, **181,** 1975–1983.

Fawcett, P., **Eichenberger, P., Losick, R., & Youngman, P.,** 2000. The transcriptional profile of early to middle sporulation in *Bacillus subtilis*, Proc. Natl. Acad. Sci., ***97*,** 8063–8068.

Frandsen, N., Bara´k, I., Karmazyn-Campelli, C., Stragier, P., 1999. Transient gene asymmetry during sporulation and establishment of cell specificity in *Bacillus subtilis,* Genes and Development, **13,** 394–399.

Fujita, M., Losick, R., 2003. The master regulator for entry into sporulation in *Bacillus subtilis* becomes a cell-specific transcription factor after asymmetric division. Genes and Development, **17**, 1166–1174.

Fukushima, T., Yamamoto, H., Atrih, A., Foster, S., J., Sekiguchi, J., 2002. A Polysaccharide Deacetylase Gene (*pdaA*) Is Required for Germination and for Production of Muramic δ-Lactam Residues in the Spore Cortex of *Bacillus subtilis,* Journal of Bacteriology, **184,** 6007–6015

Gaballa, A., Helmann, J., D., 2003. Bacillus subtilis CPx-type ATPases: Characterization of Cd, Zn, Co and Cu efflux systems, BioMetals, **16,** 497–505.

Garsin, D., A., Duncan, L., Paskowitz, D., M., Losick, R., 1998. The kinase activity of the antisigma factor SpoIIAB is required for activation as well as inhibition of transcription factor sF during sporulation in Bacillus subtilis. Journal of Molecular Biology, **284,** 569–578

Grossman A., Losick R., 1997. Extracellular control of spore formation in *Bacillus subtilis*, Proc Natl Acad Sci *USA*, **85,** 4369-4373.

Haldenwang, W., G., 1995. The sigma factors of *Bacillus subtilis*, Microbiology Reviews, **59,** 1-30.

Harwood, C. R., Cutting S. M., 1990. Molecular Biological Methods for *Bacillus* (pp. 1-3). A Wiley- Interscience Publication.

Hoch, J.A., Silhavy, T.J., 1995. Two-Component Signal Transduction, pp. 13-14, Washington, DC: American Society for Microbiology Press.

Karow, M., L., Glaser, P., Piggot, P., J., 1995. Identification of a gene, *spoIIR*, that links the activation of σ^E to the transcriptional activity of σ^F during sporulation in *Bacillus subtilis*, Proc. Natl Acad. Sci. USA, **92**, 2012–2016.

Kobayashi, K., Ogasawara, N., 2002. Genome biology of a model bacterium, *Bacillus subtilis*. International Congress Series, **1246,** 15–25.

Kobayashi, K., Ehrlich, S., D., Albertinid, A., Amatid, G., Andersene, K., K., Arnaudf, M., Asaig, K., Ashikagah, S., Aymerichi, S., Bessieresj, P., Bolandk, F., Brignelll, S., C., Bronm, S., Bunain, K., Chapuisb, J., Christianseno, L., C., Danchinp, A., De´ barbouille´ , M., Dervynb, E., Deuerlingq, E., Devinee, K., Devinee, S., K., Dreesenp, O., Errington J., Fillingeri, S., Fosterk, S., J., Fujitas,

Y., Galizzid, A., Gardanf, R., Eschevinsm, C., Fukushimat, T., Hagau, K., Harwoodl, C., R., Heckerv, M., Hosoyaw, D., Hullop, M., F., Kakeshitan, H., Karamatax, D., Kasaharaa, Y., Kawamurah, F., Kogah, K., Koskiy, P., Kuwanaz, R., Imamuraw, D., Ishimaruw, M., Ishikawat, S., Ishios, I., Le Coqi, D., Massonaa, A., Maue, C., Meimam, R., Melladobb, R., P., Moirk, A., Moriyaa, S., Nagakawas, E., Nanamiyah, H., Nakaia, S., Nygaardo, P., Oguracc, M., Ohananq, T., O'Reillye, M., O'Rourkek, M., Pragail, Z., Pooleyx, H., M., Rapoportf, G., Rawlinsr, J., P., Rivasbb, L., A., Rivoltax, C., Sadaieu, A., Sadaieg, Y., Sarvasy, M., Satow, T., Saxildo, H., H., Scanlane, E., Schumannq, W., Seegersaa, J., F., M., L., Sekiguchit, J., Sekowskap, A., Se´ roraa, S., J., Simondd, M., Stragierdd, P., Studerx, R., Takamatsuz, H., Tanakacc, T., Takeuchiw, M., Thomaidesr, H., B., Vagnerb, V., Dijlm, J., M., Watabez, K., Wipatl, A., Yamamotot, H., Yamamotos, M., Yamamotos, Y., Yamanen, K., Yataee, K., Yoshidas, K., Yoshikawau, H., Zuberv, U., Ogasawaraa, N., 2003. Essential Bacillus subtilis genes **,** Proc Natl Acad Sci USA, **100,** 4678–4683.

Kunst, F. *et al.,*1997. The complete genome sequence of the gram-positive bacterium *Bacillus subtilis*, Nature*, **390,*** 249–256.

Kroos, L., Zhang, B., Ichikawa, H., Nicco Yu, Y., T., 1999. Control of σ factor activity during *Bacillus subtilis*, Molecular Microbiology, **31,** 1285–1294.

Kroos L., Nicco Y., Y., Mills, D., Ferguson-Miller, S., 2002. Forespore Signaling Is Necessary for Pro-σ^K Processing during *Bacillus subtilis* Sporulation Despite the Loss of SpoIVFA upon Translational Arrest, J. Bacteriology, **184,** 5393–5401

Klein, C., Kaletta, Schnell, N., Entian, K., D., 1992, Analysis of genes involved in biosynthesis of the lantibiotic subtilin, Appl. Eviron. Microbiol., **58,** 132-142

LeDeaux, J., Yu, K., Grossman, A., 1995. Different roles for KinA, KinB, and KinC in the initiation of sporulation in *Bacillus subtilis*, Journal of Bacteriology, **177:**861-863.

Leichert, L., I., O., Scharf, C., Hecker, M., 2003. Global Characterization of Disulfide Stress in Bacillus subtilis, **185,** 1967–1975

Lewis, P., J., Partridge, S., R., Errington, J., 1994. σ factors, asymmetry, and the determination of cell fate in *Bacillus subtilis*. Proc. Natl Acad. Sci. USA, **91**, 3849–3853.

Londoño-Vallejo, J., A., Stragier, P., 1995. Cell-cell signalling pathway activating a developmental transcription factor in *Bacillus subtilis*, Genes and Development, **9**, 503–508.

Losick, R., Stragier, P., 1992. Crisscross regulation of cell-type-specific gene expression during development in *B. subtilis*, Nature, **355,** 601-604.

Mason, J., M., Hackett, R. H., Setlow, P., 1998. Regulation of expression of genes coding for small, acid-soluble proteins of Bacillus subtilis spores: studies using lacZ gene fusions. J. Bacteriol. **170,** 239–244.

Miller, J., 1972. Experiments in Molecular Genetics, Cold Spring Harbor Laboratuary: Cold Spring Harbor.

Nicholson W.L., Setlow, P., 1990. Sporulation, germination and outgrowth. In C.R. Harwood & S.M.Cutting (Editors), *Molecular Biological Methods for Bacillus* (pp.391- 429). Chichester, England: John Wiley & Sons.

Pedersen, L., B., , Ragkousi, K., Cammett, T., J., Melly, E., Sekowska, A., Schopick, E., Murray, T., Setlow, P., 2000. Characterization of *ywhE*, which encodes a putative high-molecularweight class A penicillin-binding protein in *Bacillus subtilis,* Gene, **246,** 187–196

Perego, M., Hanstein, C.G., Welsh, K.M., Djavakhishvili, T., Glaser, P., and Hoch, J.A., 1994. Multiple protein aspartate phosphatases provide a mechanism for the integration of diverse signals in the control of development in *Bacillus subtilis*. Cell, **79,** 1047–1055.

Perego, M., 1997. A peptide export-import control circuit modulating bacteria development regulates protein phosphatases of the phosphorelay, Proc Natl Acad Sci USA, **94,** 8612-8617.

Perego, M., and Hoch, J.A., 1996. Protein aspartate phosphatases control the output of two-component signal transduction systems, Trends in Genetic, **12,** 97–101.

Piggot, P. J., and R. Losick., 2001. Sporulation genes and intercompartmental regulation, pp. 483–517, in A. L. Sonenshein, J. A. Hoch, and R. Losick, eds. *Bacillus subtilis* and its closest relatives. ASM Press, Washington, D.C.

Seavers, P. R., Lewis, R. J., Brannigan, J. A. Wilkinson, A. J., 2001.Crystallization and preliminary X-ray analysis of the sporulation factor SpoIIAA in its native and phosphorylated forms, Acta Cryst D, **57,** 292-295.

Serrano, M., Coˆrte, L., Opdyke, J., Charles, P., Moran, Jr., A., Henriques, O., 2003. Expression of *spoIIIJ* in the Prespore Is Sufficient for Activation of sigma G and for Sporulation in *Bacillus subtilis,* Journal of Bacteriology, **185,** 3905–3917

Solovieva, I., M., **Entian, K., D.,** 2002. Investigation of the *yvgW Bacillus subtilis* chromosomal gene involved in Cd^{+2} ion resistance, FEMS Microbiology Letters, **208,** 105 – 109.

Sonenshein, A.L, Hoch , J.A., Losick, R., 2002. *Bacillus subtilis* and its closest relatives: from genes to cells (pp. 1–7). Washington DC: American Society for Microbiology Press.

Stephens, C., 1998. Bacterial sporulation: A question of commitment?, Current Biology, **8,** 45-48.

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

Sterlini, J.M., Mandelstam, J., 1969. Commitment to sporulation in *Bacillus subtilis* and its relationship to the development of actinomycin resistance, Biochemical Journal, **113,** 29–37.

Stragier, P., Losick, R., 1996. Molecular genetics of sporulation in *Bacillus subtilis,* Annual Review of Genetic, **30,** 297-341.

Sun, Y.-L., Sharp, M., D., Pogliano, K., 2000. A dispensable role for forespore-specific gene expression in engulfment of the forespore during sporulation of Bacillus subtilis. J. Bacteriol. **182,** 2919–2927.

Trach, K, Hoch J., 1993. Multisensory activation of the phosphorelay initiating sporulation in *Bacillus subtilis*: identification and sequence of the protein kinase of the alternate pathway. Molecular Microbiology, **8,** 69-79.

Yazgan, A., Özcengiz, G., Marahiel, M., A., 2001. Tn10 insertional mutations of *Bacillus subtilis* that block the biosynthesis of bacilysin. Biochimica et Biophysica Acta*,* **1518,** 87-94.

Wakeley, P., Hoa, N., T., Cutting, S., 2000. BofC negatively regulates SpoIVB-mediated signalling in the *Bacillus subtilis* sigma K-checkpoint, Molecular Microbiology, **36,**1415-1424

Webb, C. D., Teleman, A., Gordon, S., Straight, A., Belmont, A., Lin, D. C., Grossman, A.D., Wright, A., Losick, R., 1997. Bipolar localization of the replication origin regions of chromosomes in vegetative and sporulating cells of *B. subtilis.* Cell, **88,** 667-674

(http://www1.qiagen.com/HB/PCRCloning)

(http://users.path.ox.ac.uk/~erring/index.htm)

(http://www.mun.ca/biochem/courses/4103/figures/Spo0A.GIF)

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 1000ml and then autoclaved for 15 minutes.

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 1000ml and then autoclaved for 15 minutes

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 (1000ml)

Tryptone	16 g
Yeast Extract	10 g
NaCl	5 g

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

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 minutes 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 distelled 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.

APPENDIX B

Compositions of Buffers and Solutions

TAE Buffer (50X)

Tris base (2 moles)	242 g
Glacial acetic acid (57.1 ml)	57.1 ml
EDTA (100mL 0.5M)	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 500ml 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 1000ml 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
Taq polymerase	Fermantas
T4 DNA Ligase	Fermentas

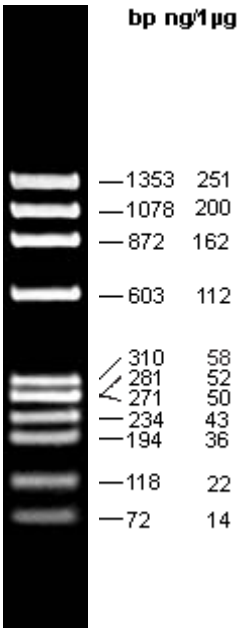
Chemical	**Supplier**
Agar	Sigma
Agarose	Sigma
β-mercapthoethanol	Merck
Calcium chlorid ($CaCl_2$)	Merck
D(+)-Glucose monohydrate	Merck
Iron(III) sulfate – 7 – hydrate ($FeSO_4.7H_2O$)	Riedel-de Haën
L- Argininemonohydrochlorid	Merck
L-Histidinmonohydrochlorid	Merck
L-Tryptophan	Merck
Lysozyme	Sigma
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

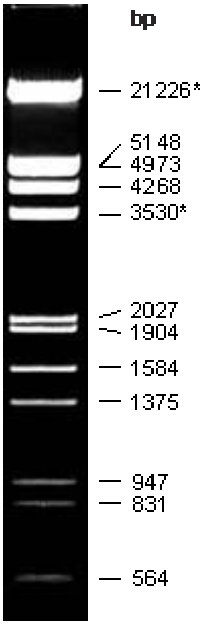
MARKERS……Fermentas

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



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

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

```
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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
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2161 gttaaaataa agaagtgggt caattgcgat ccgcttc
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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 (1.5ml)

Transillumunator: Biorad UV transilluminator 2000

Vortexing machine: Heidolph Raax top

Waterbaths: Memmert wb-22

RESUME

Ayça Gülçin ÜLGEN was born in LÜLEBURGAZ in 1981. After getting her high school diploma from Lüleburgaz High School in 1998, she started to study in İstanbul University, Department of Biology in 1998. She graduated in 2002 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.