ISTANBUL TECHNICAL UNIVERSITY ★ INSTITUTE OF SCIENCE AND TECHNOLOGY

CHARACTERIZATION OF THE *bacA* MUTANT SPORES

M.Sc. Thesis by
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June 2010

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bacA MUTANT SPORLARI KARAKTERİZASYONU

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Elif Saygılı

Biyolog

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ABBREVIATIONS

AGFK : AsparagineGlucose Fructose KCl
CSF : Competence Stimulating Factor
DPA : Dipicolinic Acid
DSM : Difco Sporulation Medium
DTT : Dithiothreitol
EDTA : Ethylenediaminetetraacetic acid
GPR : Germination Protease
LB : Luria-Bertani Liquid Medium
RNAP : RNA Polymerase
SASP : Small, Acid-Soluble Protein
SDS-PAGE : Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SMM : Spizizen's Minimal Medium
TAE : Tris-Acetate-Edta
TE : Tris-Edta Buffer
UVP : Ultraviolet transilluminator

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CHARACTERIZATION OF THE *bacA* MUTANT SPORES

SUMMARY

Upon nutrient depletion, *Bacillus subtilis* cells produce endospores which are metabolically dormant and resistant to heat, desiccation, radiation, toxic chemicals and pH extremes. When conditions turn favorable for growth, spore germinates and goes through outgrowth, and ultimately it is converted back into a vegetatif cell. During the germination process, *B. subtilis* loses of its resistance properties and subsequently, germination is followed by outgrowth when biosynthetic activity is resumed and actively dividing cells regenerated. There are some elements playing the important roles in spore's resistance and germination properties. Those are small acid-soluble proteins (SASPs), spore coat proteins and dipicolinic acid (DPA). SASPs are the predominant spore core proteins providing the spores resistant to UV light, oxidizing agents, desiccation and heat. They also provide the amino acids essential for protein synthesis during germination process. DPA plays a important role in spore dehydration and provide the spore with a good stability during germination, leading to spontenous germination. Furthermore, there are more than 40 types of coat proteins in *Bacillus subtilis* most of which are exposed to posttranslational modifications and assembled together to create the endurance providing shield of spores, the spore coat.

For many years, peptide antibiotics have been doubted to be important compounds for sporulation process. Previous research has implied that bacilysin, a dipeptide antibiotic composed of L-alanine and L-anticapsin, might be a control element in the sporulation process. Under light of this finding, the objective of the present study was aimed to elucidate the possible action sites of bacilysin in the sporulation process. For this purpose, the major spore properties of the bacilysin defective OGU1 strain, was compared with wild type PY79 spores. In this study, both mutant type and wild type spores were treated with lysozyme, heat and chlorofom. Only heat resistance rate is similar in two types spores, on the other hand, OGU1 spores are more sensitive against lysozyme and chlorofom treatments. Subsequently, both SASPs and spore coat proteins were extracted from spores of both wild type PY79 and bacilysin defective strains. Three major types of SASPs, α/β-type and γ-type were demonstrated to be expressed at an equal level by both PY79 and OGU1 spores on a low pH PAGE. On the other hand, PY79 spores were examined to differ from OGU1 spores by a small number of spore coat proteins. These differences were manifested in lysozyme and chloroform sensitivity of OGU1 spores. Also to achieve molecular characterization of *bacA* mutant spores, germination and outgrowth characteristics of OGU1 and PY79 spores were performed in order to explore more details about the functional role of *bacA* in the sporulation process. Our data suggests that, they have displayed similar germination and outgrowth properties not only in rich medium 2x YT and but also in SMM medium. Level of DPA was also

investigated in *bacA* spores and compared to wild type spores, to be found that *bacA* spores have the same DPA level with the wild type spores. Therefore, the reason of the same heat resistance profile of OGU1 and PY79 spores was considered to be a result of the same DPA accumulation or synthesis in both mutant type and wild type.

bacA MUTANT SPORLARI KARAKTERİZASYONU

ÖZET

Besin tükenmesi halinde, *Bacillus subtilis* hücreleri metabolik açıdan faaliyet göstermeyen, ısıya, kuraklığa, radyasyona toksik kimyasallara ve yüksek pH seviyelerine dirençli endosporlar oluşturular. Koşullar büyüme için tekrar elverişli hale geldiğinde spor çimlenir ve çimlenme sonrası büyüme evresi olan outgrowth aşamasına geçer ve tekrar vejetatif hücreye dönüşür. Çimlenme evresi boyunca *B. subtilis* hücreleri dirençlilik özelliklerini kaybederler ve ardından çimlenmeyi, biyosentetik aktivetenin kaldığı yerden devam ettiği, "outgrowth" evresi takip eder ve aktif olarak bölünebilen hücre tekrar oluşur. Spor dirençliliğine ve sporun çimlenme profiline etki eden başka elementler bulunmaktadır. Bunlar küçük asitte çözünebilen proteinler (SASP), spor ceket proteinleri ve dipikolinik asittir (DPA). Küçük asitte çözünebilen proteinler, sporlarda en fazla karşılaşılan spor çekirdek bölgesi proteinleridir ve spora, UV'ye, oksitleyici ajanlara, kuraklığa ve ısıya karşı direnç sağlarlar. SASP' ler aynı zamanda çimlenme prosesi boyunca, protein sentezinde esansiyel olan amino asitleri sağlarlar. Dipikolinik asit ise spor oluşumu sırasında önemli fonksiyonlar (spor çekirdeğinde dehidrasyon sayesinde metabolik hızın düşmesi ve kristalleşme gibi) üstlenmektedir. Dipikolinik asitin bir diğer fonksiyonu spora çimlenme sırasında dayanıklılık sağlamasıdır. Bunlara ek olarak, *Bacillus subtilis*'ta 40'ın üzerinde spor ceketi proteini bulunmakta olup bunların çoğu posttranslasyonel modifikasyonlara maruz kalırlar ve daha sonra bir araya gelerek spora dayanıklılık sağlayan spor ceketini oluştururlar.

Yılllardır peptid antibiyotiklerinin sporlanma prosesi için önemli olgular olduklarından şüphelenilmiştir. Önceki bir çalışma basilisinin (L-alanin ve L-anticapsinden oluşmuş dipeptid antibiyotik) sporlanmada kontrol elemanı olabileceğini gösterilmiştir. Bu bulgunun ışığı altında, bu çalışmanın amacı basilisinin sporlanmadaki olası etki bölgelerinin açığa çıkarılmasıdır. Bu amaçla, basilisin defektif OGU1 sporlarının temel spor özellikleri yabanil suş olan PY79 sporlarınınki ile karşılaştırılmıştır. Bu çalışmada, hem mutant suş hem yabanil suş sporları lizozim, ısı ve kloroform ile işleme tutulmuştur. Sadece ısı direnci oranı iki suşta da benzer çıkmıştır. Onun dışında OGU1 sporları lizozim ve kloroform işlemlerine karşı daha hassastır. Bunu takiben, hem SASP hem spor ceketi proteinleri, hem yabanil suş olan PY79'dan hem basilisin defektif suştan ekstrakte edilmiştir. 3 ana tip küçük asitte çözünebilen protein türü olan α/β-tipi ve γ-tipinin, düşük pH'lı poliakrilamid jel elektroforezi kullanılarak iki suşta da eşit seviyelerde eksprese edildikleri gösterilmiştir. Diğer yandan PY79 sporları, OGU1 sporlarından az sayıda spor ceketi proteiniyle ayrılır. Bu farklılık, OGU1 sporlarının lizozim ve klorofoma karşı olan hassasiyetinde açıkça görülmektedir. Yine, *bacA* mutant sporlarının moleküler karakterizasyonlarını ortaya koyabilmek amacıyla OGU1 ve

PY79 sporlarının çimlenme ve outgrowth karakteristikleri incelenmiş ve bu sayede *bacA* geninin sporlanmadaki fonksiyonu detaylı olarak gözlemlenmiştir. Sonuçlarımız göstermiştir ki, OGU1 ve PY79 sporları yanlızca zengin besiyeri olan 2x YT'de değil aynı zamanda SMM besiyerinde de benzer çimlenme ve outgrowth özellikleri göstermiştirlerdir. DPA seviyeleri yine *bacA* sporlarında incelenmiş ve yabanil suşla karşılaştırılmıştır. *bacA* sporlarının yabanil suşla aynı miktarda DPA içerdiği bulunmuştur. Bu nedenle, OGU1 ve PY79 sporlarının benzer ısı dirençlerinin, aynı DPA miktarı yada sentezinin sonucu olabileceği düşünülmektedir.

1. INTRODUCTION

1.1. *Bacillus subtilis*

The best characterized member of Gram-positive bacteria is *Bacillus subtilis*. *Bacillus subtilis* is a an aerobic, endospore-forming, rod-shaped bacterium and it's commonly found in soil, water sources and in association with plants. *B. subtilis* and its close relatives have capacity to secrete enzymes such as amylases and proteases, because of these enzymes they are important source for industry and much of the commercial interest in these bacteria arises from their capacity to secrete these enzymes at gram per litre concentrations. It has therefore been used for the study of protein secretion and for development as a host for the production of heterologous proteins (Harwood, 1992).

In 1997, the complete genome sequence of *B. subtilis* was published by an international consortium that included Japanese and European laboratories (Kunst et al., 1997). In its genome database 4106 protein-coding genes, 86 tRNA genes, 30 rRNA genes and three small stable RNA genes are annotated on the 4215 kb genome (http://genolist.pasteur.fr/SubtiList/) and more genes coding stable small RNA have recently been found (Ando et al., 2002).

Under conditions of nutrient deprivation, *Bacillus subtilis* stops growing and initiates responses to restore growth by increasing 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 induced to form chemically, irradiation and desiccation resistant endospores (Stragier and Losick, 1996). As an alternative developmental process, *B. subtilis* is also able to differentiate into a physiological state, the competent state, that allows it to undergo genetic transformation (Solomon and Grossman, 1996).

In 1958, transformable characteristic of *Bacillus subtilis* 168 was reported and following this information, this strain has became the most useful strain for genetic

researches based on this organism (Spizen, 1958; Harwood et al., 1990). Therefore, *Bacillus subtilis* PY79 has found its place as the wild type strain of this project as a consequence of its being prototrophic derivative of *Bacillus subtilis* 168.

1.2. Sporulation in *Bacillus subtilis*

In order to survive, an organism consistently has to adapt to ever-changing conditions to its environment. The key feature of its adaptability is the capacity to monitor the environment and respond to changes in, including nutrient availability, temperature and cell density. As long as nutrients are available, *B. subtilis* cells grow and divide as fast as possible. However, when the conditions deteriorate, in other words, under conditions of nutrient deprivation, Gram positive soil bacteria, *B. subtilis* cells cease growing exponentially and enter stationary phase. (Hoch, 1993; Grossman, 1995; Stragier and Losick, 1996; Burkholder and Grossman, 2000; Sonenshein, 2000b).

During this transition, a variety of alternative responses can occur, including the activation of flagellar motility to seek new food sources by chemotaxis, the production of antibiotics to destroy individuals from same or different species because of the limited resources, the secretion of hydrolytic enzymes to scavenge extracellular proteins and polysaccharides. Another example of responses is the induction of 'competence' for uptake of exogenous DNA for consumption, with the occasional side–effect that new genetic information is stably integrated. Among these responses, sporulation is the last one and is suppressed until alternative responses prove inadequate (Grossman and Losick, 1997).

1.2.1. Initiation of sporulation

In *B. subtilis,* sporulation starts by the differentiations in cell division cycle. During vegetatif cell cycle, in other words, during exponential growth, cells divide in the middle to produce two identical daughter cells. In sporulating cells, on the other hand, septum formation is asymetrically. The developping cell (sporangium) is partitioned into large and small compartments. The large compartment is called as the mother cell and the small compartment is called as forespore (prespore). Both mother cell and prespore receive a chromosome.

The next stage of development is the engulfment of the forespore by the mother cell which results with the pinching off of the prespore by the mother cell. At this point, the forespore is a free protoplast within the mother cell. As a cell-within-a-cell spores of *Bacillus* are known as endospores. In subsequent stages, a variety of complex biosynthetic and morphogenic changes take place in the mother cell, in the forespore, and in the space between the forespore and the mother cell, as a result, the forespore matures into spore.

The proteinaceous coat which is produced by the mother cell, assembles on the outside surface of the mother cell membrane around the forespore.The coat consists of a lamellar inner layer and an electron-dense outer layer and provides a thick, protective barrier that encases the mature spore. Notwithstanding, forespore produces small acid soluble proteins known as SASP. The forespore chromosome's is coated by some of these proteins and is packaged, thanks to this doughnut-like structure, it gains the resistance to ultraviolet radiation. The production of a thin layer of peptidoglycan known as the germ cell wall on the surface of the forespore membrane, is followed by the synthesis of a thick layer of peptidoglycan known as the cortex. Both the germ cell wall and the cortex is tought to be involved in maintaining the dehydrated and heat-resistant state of the spore.

Finally, after about 6-8 hours of development, with the completion of the maturation, the fully ripined spore is liberated by lysis of the mother cell. Thus, the mother cell is mortal in that it undergoes programmed cell death, whereas the forespore is immortal and it becomes the mature spore (Stragier and Losick, 1996).

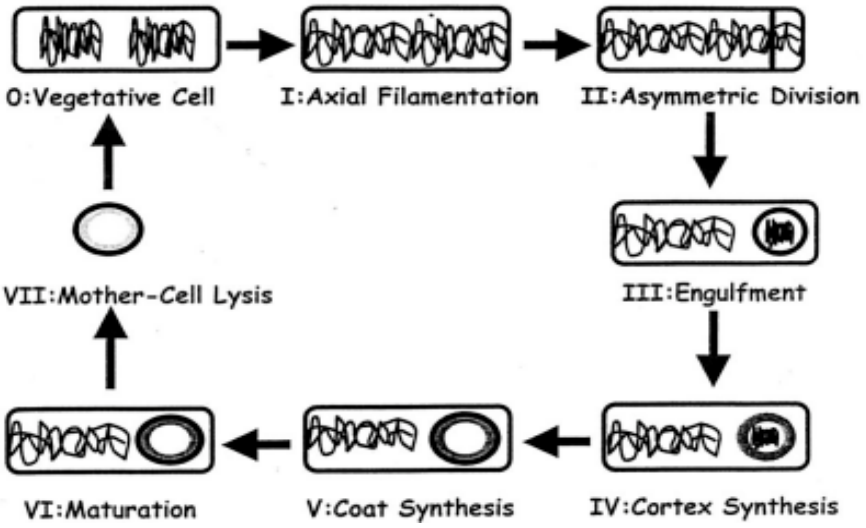

Figure 1.1 : Schematic representations of the stages of sporulation.These morphological events are divided into seven stages and they are showed by Roman numerals. (Hilbert and Piggot, 2004).

1.2.2. Localizations and functions of the RNA polymerase σ factors

Activation of alternative RNA polymerase sigma σ factors effect global changes in gene expression and morphological changes that occur during sporulation (Piggot et al.,1976; Piggot et al., 2002). In response to starvation, σ^A, the major sigma factor present in growing cell, and σ^H**,** a minor abundance σ factor, direct transcription of genes whose product redirect septum formation from the mid-cell to a polar position and partition one copy of the chromosome to the larger mother cell and the other copy to the smaller forespore (Kroos et al.,1999). Expression of the factors important for axial filament formation, asymmetric cell division, and compartmentalization of gene expression are directly affected by the activation of the σ^H, (and the response regulator spo0A) in the predivisional cell.

After asymmetric division, the activation of σ^F in the prespore is rapidly followed by the activation of σ^E in the mother cell. Engulfment of the prespore by the mother cell results in synthesis of the late-compartment specific sigma factors. σ^G becomes active in the engulfed forespore, in which it controls transcription of genes encoding proteins that condense and protect the chromosome and prepare the spore to germinate when nutrients become available. Shortly after the activation of σ^G, σ^K becomes active in the mother cell.

These late stages of cell-specific gene expression controls core and cortex synthesis, spore maturation, and mother cell lysis (Hilbert and Piggot, 2004; Kroos et. al., 1999).

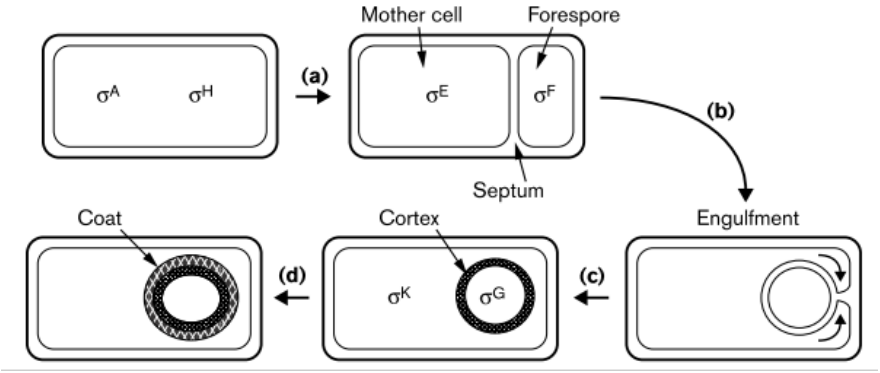

Figure 1.2: Morphological changes during sporulation and the approximate time and localizations of active σ factors. **a)** Genes under the control of σ^A and σ^H, RNAP cause asymmetric polar septum formation. **b)** The septum migrates, engulfing the forespore in a double membrane. **c)** σ^G RNAP becomes active in forespore, singaling the activation of , σ^K in the mother cell. Cortex is synthesized between the membranes, and surrounds the forespore. **d)** Genes under the control of , σ^K RNAP produces proteins in the mother cell that assemble on the surface of the forespore to produce the spore coat (Kroos et al., 1999).

1.2.3 The Sporulation signal transduction system

1.2.3.1. The phosphorelay mechanism

Under conditions of poor growth conditions, *Bacillus subtilis* cells sporulate and then, under more favorable conditions, the genetic matter in the spore is used to produce a new bacterium. It uses very complex molecular machinery to decide whether to sporulate or to divide. A backbone of this molecular mechanism is the phosphorelay (Hoch and Silhavy, 1995).

The phosphorelay is a expanded version of two-component system and bacteria commonly uses the phosphorelay for monitoring and responding to the environment. A typical two-component system is divided into two domains:histidine kinase and response regulator, which is activated by phosphorylation to carry out transcription mission. The histidine kinase usually acts as a signal sensor that respond to the initiating signal by autophosphorylating a histidine residue by transferring the γ - phosphoryl gorup from bound ATP. Sensor kinases consist also two domains:its N-terminal signal detection domain is connected to a kinase domain. The kinase domain

is made up of a phosphotransferase subdomain containing the active histidine and an ATP-binding subdomain. Response regulator transcription factors are also divided into two domain:the C-terminal DNA-binding domain and N-terminal receiver domain which accepts the phosphoryl gorup. The histidine kinase transfers the phosphoryl gorup to an aspartate on the N-terminal domain of the response regulator and becomes dephosphorylated. Phosphorylation of the receiver domain generally augments the DNA-binding affinity of the second domain. In the two-component system, therefore phosphorylation of the substrate is two-step process, on the other hand in Ser/Thr/Tyr kinases, the phosphoryl group is transferred directly form ATP to the substrate proteins.

In phosphorelay mechanism, phosphotransfer becomes an even more elaborate process involving four steps. The initiation of sporulation of *Bacillus subtilis* is controlled by the phosphorelay mechanism which is depicted in Fig 1.3. (Varughese, 2003).

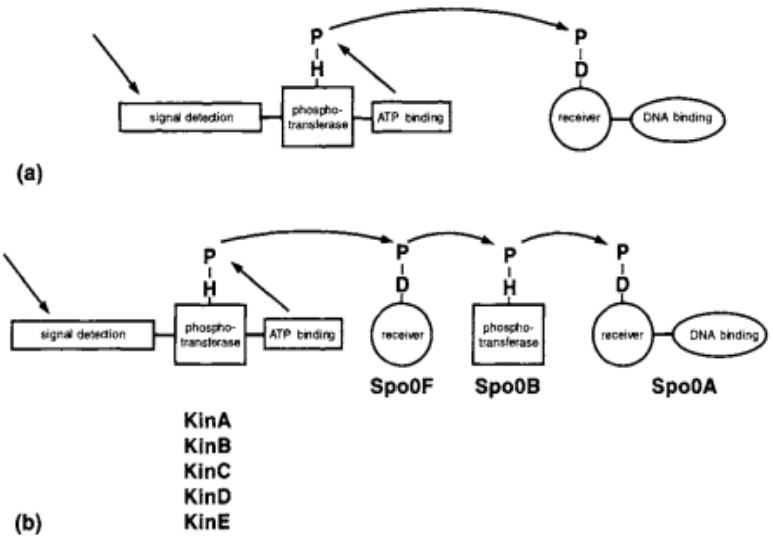

Figure 1.3: Schematic view of domain organization of a typical two-component system and the sporulation phosphorelay signal transduction system.(a) Signal is recognized by the kinase and then, it induces transfer of the γ-phosphate of ATP and phosphorylation of the phosphotransferase domain. In a two-component system, kinase donates the phosphoryl group to an aspartate on a response regulator/transcription factor.(b) In the phosphorelay, histidine kinases pass the phosphoryl group to an intermediate response regulator, Spo0F, and subsequently to the response regulator/transcription factor, Spo0A , via a phosphotransferase, Spo0B. During sporulation five different histidine kinases are used: KinA,KinB,KinC,KinD,KinE (Varughese, 2003).

Sporulation is not controlled by a simple two-component system. Spo0A and Spo0H encode transcription factors that are required for the initiation of sporulation. DNA binding protein that functions as a transcriptional activator and repressor, is encoded by Spo0A, and Spo0H (also called as SigH) encodes a sigma factor σ^H, of RNA polymerase. Asymmetric division and transcription of the genes required for cell-type specific gene expression need both Spo0A and Spo0H (Burkholder and Grossman, 2000).

Spo0A lies at the end of a series of inter-protein phosphotransfer reactions, in other words phosphorelay (Burbulys et al., 1991). Spo0A obtains phosphate from a multicomponent phosphotransfer pathway, the phosphorelay. Histidine kinases autophosphorylate, and phosphate is transferred first to Spo0F. Spo0B is simply an intermediary, receiving its phosphate from the Spo0F protein, which is in turn phosphorylated by one of the related histidine kinases (Stephens , 1998). Finally from Spo0B to Spo0A (Burkholder and Grossman, 2000). Phosphorylation increases the affinity of Spo0A for its DNA binding sites and may enhance its interactions with RNA polymerase holoenzyme (Baldus et al., 1995; Bird et al., 1996; Bramucci et al., 1995; Buckner and Moran, 1998; Buckner et al., 1998; Hatt and Youngman, 1998; Rowe-Magnus and Spiegelman, 1998;Schyns et al., 1997; Spiegelman et al., 1995). Spo0A-P activates the expression of most of its target genes, often by interacting directly with the sigma subunit of RNA polymerase, but inhibits the expression of several genes. The locations of Spo0A binding sites within a promoter region determine whether Spo0A-P acts as an activator or repressor of transcription (Grossman and Burkholder, 2000).

One of the important roles of phosphorylated Spo0A (Spo0A-P) is to repress abrB, a gene that encodes an unstable repressor of numerous stationary phase genes, some of which are required for sporulation (Strauch, 1993).

1.2.3.2. Sensor kinases

Five histidine kinases are functional in phosphorelay mechanism. KinA has the major role in at the onset of sporulation. KinA cytosolic protein, whereas KinB and KinC are both integral membrane proteins (Burkholder and Grossman, 2000). The activities of KinA, KinB, and KinC vary with the growth phase and nutrient conditions, and each kinase probably responds to a different environmental or

intracellular signal. KinA and KinB are required for efficient sporulation , on the other hand KinC contributes little to sporulation but is required for Spo0A-dependent regulation of abrB during exponential growth under some conditions (LeDeaux et al., 1995).

The alternative sigma factor σ^H, is required for full expression and activation of Spo0A as well as for formation of the polar septum and the initiation of cell-type-specific gene expression. σ^H**,** enhances the activation of Spo0A by inducing the expression of high levels of Spo0A from the sporulation-specific promoter of spo0A, the sensor kinase KinA and the phosphorelay protein Spo0F, and extracellular signaling factors that indirectly promote activation of Spo0A (Grossman, 1995; Hoch, 1995)

Like Spo0A, σ^H plays important roles in the development of competence. One role of σ^H common to both sporulation and competence is inducing the expression of extracellular signaling factors that mediate quorum sensing (Lazazzera et al., 1999).

1.2.3.3. Rap phosphateses

RapA, RapB and RapE , cause dephosphorylation of Spo0F–P, and this action is inhibited by cognate pentapeptides, some or all of which may act as cell density signals. Initially, the Rap proteins, which are similar to each other, were all tought to function as phosphotases. However, study of RapC (assosiated with competence) has proved that, it is not a phosphotase, but rahter inactivates its target protein, Com-P by binding to the protein. The RapA, RapB and RapE bind to the Spo0F-P and they cause dephosphorylation of it. The regulating pentapeptides for the Rap proteins, A, B and E are derived from processing of the exported products of the phrA, phrC and phrE genes, respectively. Transcription of the phrA and phrC is repressed by CodY (Molle et al., 2003). CodY is the key sensor of guanine nucleotide levels (Ratnayake-Lecamwasam et al., 2001). A decrease in the concentration of GTP and GDP is critical to the initiation of sporulation.The fall in guanine nucleotide levels relieves the CodY-mediated repression of phrA and phrC and also of kinB, encoding a kinase for Spo0F (Molle et al., 2003). Hence CodY links a fall in guanine nucleotide levels to activation of the phosphorelay. All this regulations and signal transduction system is depicted in figure 1.4.

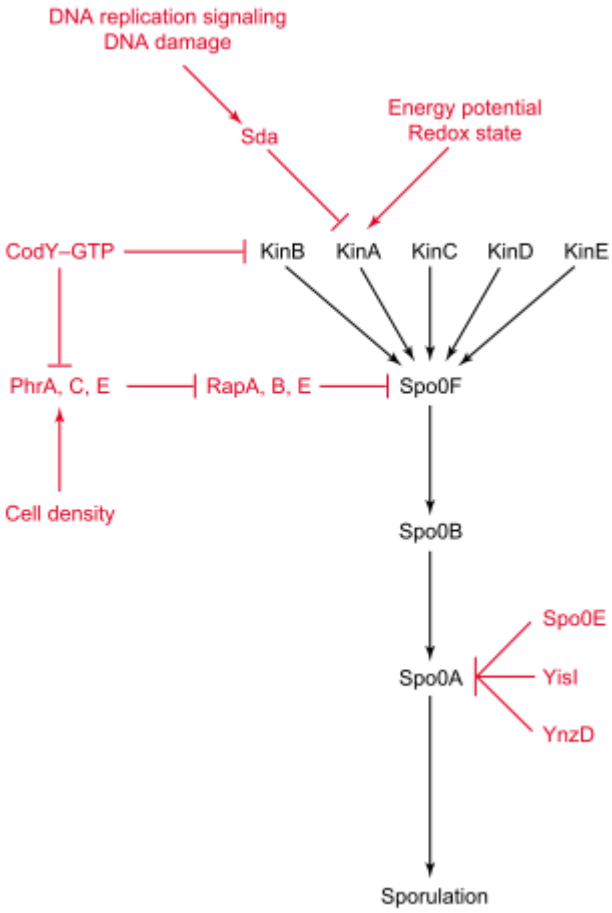

Figure.1.4: Signal Transduction and Regulation Mechanism in sporulation (Piggot and Hilbert, 2004

1.2.4. Compartmentalized gene expression

Three critical engulfment proteins, SpoIID, SpoIIM and SpoIIP are all produced in the mother cell and localize to the sporulation septum. It is believed that these membrane bound proteins hydrolyze peptidoglycan, thereby driving membrane migration around the prespore. These proteins also prevent a second asymmetric division in the mother cell (Piggot and Hilbert, 2004).

After asymmetric division and before the chromosome has completely partitioned into prespore , σ^F becomes active in the prespore. In the pre-divisional cell, σ^F is inactive by the anti-sigma–factor SpoIIAB; and this inhibition is reversed by the anti-anti-sigma factor SpoIIAA. SpoIIAA is regulated by its phosphorylation state; it is inactive when phosphorylated by SpoIIAB (kinase a well as an anti-sigma) and active when it is dephosphorylated by SpoIIE. SpoIIE localizes to asymmetric division interacts with FtsZ; it is tought to sense asymmetric division and activate σ^F

in response. Interaction of SpoIIAA with non-binding SpoIIAB induces release of σ^F from the other SpoIIAB by steric displacement (Ho et al., 2003).

σ^E becomes active in the mother cell after the completion of the activation of σ^F in the prespore. The master response regulator Spo0A greatly enhances expression of the gene encoding pro-σ^E, SpoIIGB. It has been previously shown that asymmetric division is followed by a burst of Spo0A activity in the mother cell. As a result, the level of pro- σ^E is higher than in the prespore. It's important to note that, both before and after engulfment, the main sigma factor σ^A continues to be active in both the mother cell and the prespore (Li et al., 2001).

Before the completion of engulfment, σ^G is synthesized in the pre-engulfment prespore and held inactive. It was previously thought that SpoIIAB causes inhibiton. SpoIIAB causes inhibition of σ^G by binding it whereas inhibition is reversible by dephosphorylated SpoIIAA, which is present in the prespore before engulfment (Evans et al., 2003).

Furthermore, the late mother cell sigma factor σ^K is synthesized as an inactive precursor. Pro- σ^K is activated by the putative protease SpoIVFB, and the reaction is intibited by SpoIVFA and BofA (which is illutrated in Fig 1.5). SpoIVFA brings BofA and SpoIVFB together in a multimeric complex localized to the outer prespore membrane and causes inhibition (Rudner and Losick, 2002).

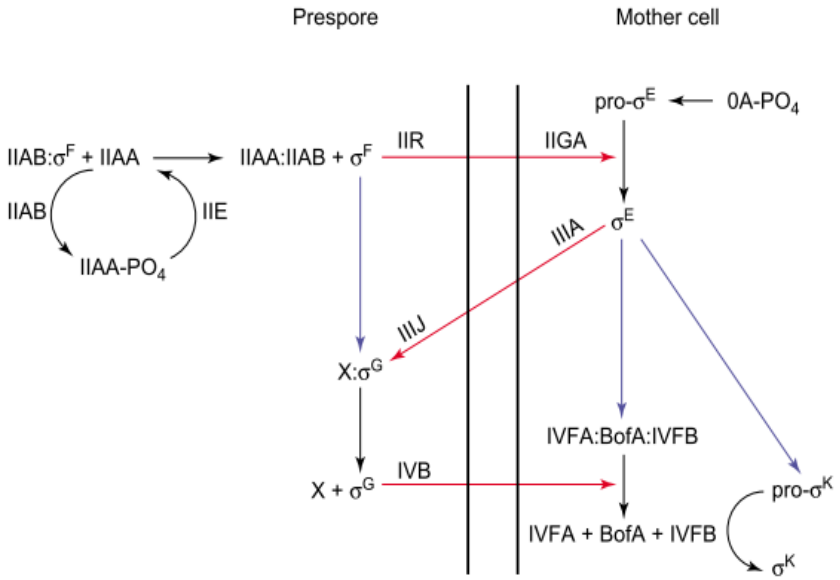

Figure 1.5: Compartmentalized gene expression and signal system between the compartments. All Spo proteins are represented by only their roman numeral. Intercompartmental signals are illustrated by red arrows, blue arrows show intracompartmental transcription activation, intracompartmental posttranslational activation is indicated by black arrows and two dark lines illustrate the membranes separating the two compartkments. (Piggot and Hilbert, 2004)

1.3. Spore Germination in *Bacillus subtilis*

Spores of *Bacillus subtilis* which are produced in a sporulation , are metabolically dormant and resistant to heat , desiccation, radiation, toxic chemicals and pH extremes. Dormant spores are also able to monitor its environment, and when conditions are favorable for growth, spores germinate (Paidhungat and Setlow, 2002) Germination may be defined as the loss of spore resistance properties and it is followed by a period of outgrowth, when biosynthetic activity is resumed and an actively dividing rod-shaped cell is regenerated (Setlow, 1984).

Bacillus subtilis endospores consist have a unique structure that determines their extreme resistance properties. The structure of *Bacillus subtilis* endospores consist three distinct features, i.e. spore coat, cortex and core. The spore cortex, which is required for spore dormancy and heat resistance, consists of a thick layer of peptidoglycan of a spore-specific structure. Despite their resistance and dormancy, the dormant spore monitors its environment, and when conditions are again favorable for growth, the spore germinates and goes through outgrowth, ultimately being converted back into a growing cell (Chirakkal et al., 2002). Resuscitation of spores is

associated with a loss of phase brightness caused by hydration of all internal structural compartments which results in a phase dark, germinated spore when viewed under phase contrast optics of a light microscope. Germination of a spore population is a diverse, heterogeneous process and it is necessary to correlate the characteristic changes in a population of germinating spores with the behavior of individual spores in the same population (Vary and Halvorson, 1965).

1.3.1. Germinant receptors

Nutrient germinants bind to receptors located in the spore's inner membrane. In *B. subtilis* these receptors are encoded by the homologous tricistronic *gerA*, *gerB* and *gerK* operons (termed gerA operon homologs) expressed in the forespore late in sporulation. The gerA and gerB genes, at least, are expressed in the developing forespore (Feavers et al., 1990; Corfe et al., 1994). Under the control of σ^G-containing RNA polymerase and of SpoVT (Bagyan et al., 1996) a regulatory protein homolog of AbrB. Like the spo loci, the ger loci are scattered the chromosome of *B. subtilis*, rather than being clustered in one place (Moir et al., 1994). In *B. subtilis* spores, the GerA receptor recognizes L-alanine, whereas the GerB and GerK receptors are required together for germination with AGFK (Paidhungat and Setlow, 2002; Moir et al., 2002).

The gerA operon represents the first described of a large family of related operons in spore formers. All three genes of gerA operon are likely to encode membrane-associated components-the GerAA protein has a predicted domain of five or six membrane-spanning segments, as well as a large N-terminal hydrophilic domain; the GerAB protein resembles an integral membrane protein with 10 transmembrane helices; and the GerAC protein,a hydrophilic gene product, is predicted to have a prelipoprotein signal sequence (Zuberi et al., 1987) , suggesting that it is transported across a membrane, the signal peptide cleaved, and the protein then anchored to the outer surface of the membrane via an N-terminally attached lipid moiety. As the gerB and gerK operons encode homologs of gerA (Corfe et al., 1994; Irie et al., 1996), the response of spores to different germinants has evolved as a consequence of gene and operon duplication and divergence; the requirement for all three protein components encoded by a particular operon, implies that the genes within an operon have co-evolved, and therefore that the gene products are likely to interact.

In addition to the gerA operon homologs, there are several other genes whose products play roles in *B. subtilis* spore germination (Paidhungat and Setlow, 2001). These include gerF, which is a prelipoprotein diacylglycerol transferase necessary for nutrient receptor function (Igarashi et al., 2004; Igarashi and Setlow., 2005) and its product adds diacylglycerol to membrane proteins, probably including the proteins encoded by the B cistrons of the *gerA* operon homologs; *gerC*, which encodes an enzyme of quinone biosynthesis; and gerD . Spores of gerD and gerF mutants have a defect early in germination, before the release of the spore's large depot of pyridine-2,6-dicarboxylic acid (DPA)(Pelczar et al., 2007). Transcription of gerD is at the same time as that of the gerA, gerB, and gerK operons and is induced only in the developing forespore compartment of the sporulating cell under the control of the forespore-specific sigma factor for RNA polymerase , σ^G (Kemp et al., 1991). The amino acid sequence of GerD (Yon et al., 1989) includes a short N-terminal hydrophobic region that is likely a signal peptide (SP), followed by a recognition sequence for diacylglycerol addition to a specific cysteine residue. The presence of these sequence features suggests that GerD is a membrane protein, and its forespore-specific expression is most consistent with its location being the spores's inner membrane (Pelczar et al., 2007).

1.3.2. Nutrient and non-nutrient germinants

Spores in nature germinate probably only in response to nutrients, termed germinants. These germinants are generally single amino acids, sugars or purine nucleosides, but there are also combinations of nutrients that trigger spore germination, one being a mixture of asparagine, glucose, fructose and K+ (AGFK) that triggers *B. subtilis* spore germination (Paidhungat and Setlow, 2001). In addition to nutrients, spore germination is also triggered by variety of non-nutrients germinants (Gould, 1969), including lysozyme, Ca^{2+} -DPA, cationic surfactants, high pressures and salts. The individual components of the nutrient germination can be bypassed by these various non-nutrients. Firstly, lysozyme is a potential non-nutrient germinant because it dagrades the cortex of most spores. Germination by the lysozyme is dependent of the being removed of the spore coat. With this pretreatment, spores become ready to germinate by lysozyme. Germination by exogenous Ca^{2+} -DPA is also dependent via some direct or indirect activation of CwIJ. Moreover, germination induced by dodecylamine causes rapid Ca^{2+} – DPA

release from the spores which are not able to degrade their cortex and it does not require spore's germinant receptors, whereas either CwIJ or SleB is reqiured for this type of germination. Probably, dodecylamine causes to opening the spore's channels for Ca^{2+} -DPA. Another non-nutrient-germinant is pressure. High pressures (100-600 megaPascals) can germinate many species of spores (Paidhungat and Setlow, 2002; Gould, 1969). Under the conditions of low pressures, activation of the germinant receptors cause spore germination. However, at higher pressures (500-600Mpa) , spores that lack nutrient receptors trigger germination rapidly and this proves that these pressures somehow open the spore's Ca^{2+} -DPA channels (Paidhungat et al., 2002).

1.3.3 Subsequent steps in germination

The process of germination consists interaction of chemical germinants with specific receptors in the spore, and the transduction of this signal in some way. Initially, germinating spore releases H^+, monovalent cations and Zn^{2+} (Swerdlow et al., 1981) probably from the spore core. Simultaneously, release of H^+ rises the pH of the spore core from 6.5 to 7.7 and this change is essential for spore metabolism once spore core hydration levels are high enough for enzyme action (Jedrzejas and Setlow, 2001). In a second stage, the germinating spore releases the spore core's large depot (10% of spore dry wet) of prydine-2,6-dicarboxylic acid (dipicolinic acid DPA) and its associated divalent cations, predominantly Ca^{2+} . Third, DPA is replaced by water, as a result , core hydration is increased and causing some decrease in spore wet-heat resistance. Although this initial increase in core hydration is not sufficient for protein mobility or enzyme action in the spore core (Setlow et al., 2001; Cowan et al., 2003). Fourth, cortex lytic enzymes become activated and this is followed by the degradation of the protective spore peptidoglycan cortex and fifth, spore core swelled through further water uptake and germ cell wall is expanded (Setlow et al., 2001). Only after this further increase in core hydration does protein mobility in the core return, thus allowing enzyme action (Setlow et al., 2001; Cowan et al., 2003). As of this stage, germination process has finished. These events take place without detectable energy metabolism, and copmrise the process of germination (Paidhungat and Setlow, 2002). Germination process is divided into two stages(figure 1.6) stage I comprises the first three steps of the process , and stage II coprises the fourth and fifth steps. The two stages can be separated experimentally by either chemical

treatments or mutations (Paidhungat and Setlow, 2001; Setlow et al., 2001; Setlow et al., 2002). After completion of stage II, the initiation of enzyme action in the spore core allows initiation of spore metabolism, followed by the macromolecular synthesis that converts the germinated spore into a growing cell (Paidhungat and Setlow, 2001). This period is also called as outgrowth.

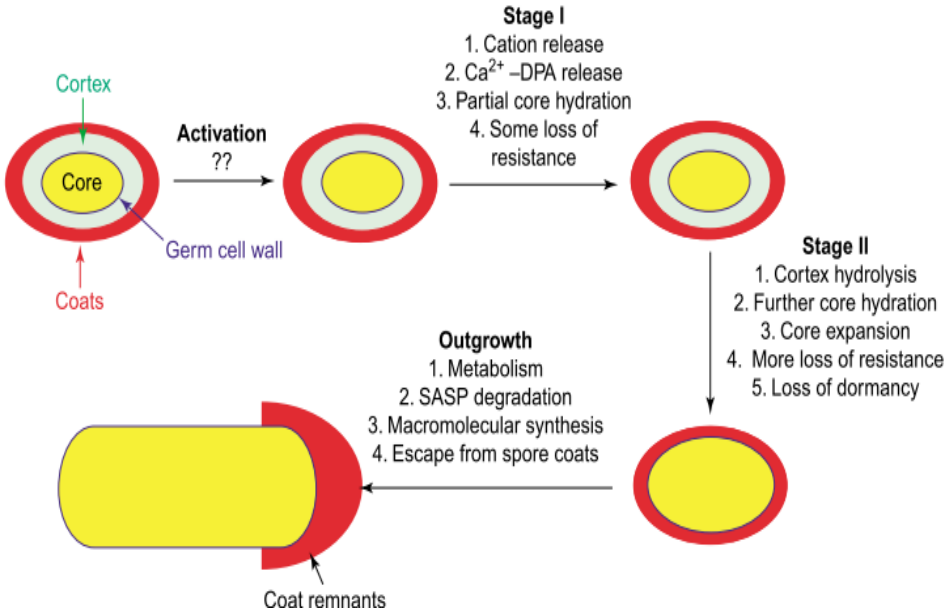

Figure 1.6: Events in spore germination.

Spore germination is divided into two stages as cortex hydrolysis is not required for stage I. SASP degradation denotes the hydrolysis of the large depot of small, acid-soluble spore proteins (SASP) that make up 10-20% of the protein in the spore core.

One type of SASP, the α/β-type, saturates spore DNA and prevents many types of DNA damage (Setlow, 2000). Whereas metabolism and SASP degradation (which require enzyme action in the spore core) are shown as taking place only after stage II is complete, these events may begin partway through stage II when the core water content has risen sufficiently for enzyme action. Also shown in figure1.6 is that the spore's germ cell wall must expand significantly to complete stage II of germination (Paidhungat and Setlow, 2001). The events in stage I may take only seconds for an individual spore, although there may be a lag of several minutes after addition of a germinant before these events begin, and spore cortex degradation may take several minutes for an individual spore. However, because of significant variation between individual spores, particularly in the times for the initiation of the first events in stage I after addition of a germinant, these events may take many minutes for a spore

population (Setlow, 2003).

1.3.4. Ion/ DPA channels

Early in spore germination, DPA and associated cations are released after the release of cations.This release suggests that one or more channels for these ions must be opened in the inner spore membrane upon binding of a germinant to its receptors. Also , there must be mechanism during sporulation for the uptake of DPA into the forespore form its site of synthesis in the mother cell compartment. Probably DPA uptake requires energy but there is no obvious need for energy in the release of DPA and cations in germination. Unfortunately, proteins which are responsible in these ion movement are not known (Setlow, 2003).

1.4. Functions of DPA and SASP's of *Bacillus subtilis*

Normally, spores of *Bacillus* and *Clostridium* species contain approximately 10% of their dry weight dipicolinic acid, DPA (Murrell, 1967; Murrell and Warth, 1965; Setlow, 1994). DPA is synthesized late in sporulation in the mother cell compartment of the sporulating cell but accumulates only in the developing forespore (Daniel and Errington, 1993; Setlow, 1981). The great majority of the spore's DPA is in the spore core and it is associated with divalent cations , predominantly with Ca^{2+} , Mg^{2+} and Mn^{2+}. (Murrell, 1967; Murrell and Warth, 1965; Setlow, 1983; Setlow, 1994) . DPA and its associated divalent cations are excreted in the first minutes of spore germination (Setlow, 1981; Setlow, 1983).

Since DPA is found only in dormant spores of *Bacillus* and *Clostridium* species and since these spores differ in a number of properties from vegetative cells, especially in their dormancy and heat resistance, it is not suprising that DPA and divalent cations are functional in some of the spore's unique properties.

DPA is synthesized from an intermediate in the lysine pathway and the functional enzyme is DPA synthetase (Daniel and Errington, 1993). In *Bacillus subtilis* two cistron of SpoVF operon encode this enzyme which is expressed only in the mother cell compartment of the sporulating cell, the site of DPA synthesis.

Germination protease (GPR) initiates the degradation of the spore's depot of small, acid soluble spore proteins (SASPs) during germination and DPA does allosterically modulate with GPR. GPR is synthesized as an inactive zymogen (called as P46)

during sporulation and approximately 2h later in sporulation, P46 autoprocesses to a smaller active form (called as P41). DPA stimulates allosterically this conversion of P46 to P41 and only the physiological DPA isomer is effective (Illades-Aguiar and Setlow, 1994; Sanchez-Salas and Setlow, 1993).

Acidification and dehydration of the spore core also stimulates the activation of the zymogen and together these conditions ensure that P41 is generated only late in sporulation, when the conditions in the spore core preclude enzyme action (Illades-Aguiar and Setlow, 1994; Sanchez-Salas and Setlow, 1993).

As a result, GPR's SASP substrates, which are synthesized in parallel with P46, are stable in developing and dormant spore. This is important for spore survival, as some major SASP (α/β-type) are essential for the protection of spore DNA from a variety of types of damage, while degradation of both the α/β-type SASP and the other major SASP (γ) provides amino acids for protein synthesis early in spore germination (Setlow, 1988; Setlow, 1994; Setlow, 2000).

1.5. Spore Coat Proteins of *Bacillus subtilis*

Bacillus subtilis spores includes three distinct structures and it is possible to observe by transmission electron microscopy (Aronson and Fitz-James, 1976). The core is the central part of the spore and includes chromosomal DNA. Proteins which play important role in the protection of nucleotides and during the process of germination and outgrowth, thought to exist in the core. On the outside of the core, there is a thin peptidoglycan layer which is called as cortex, includes some proteins involved in germination.The outermost layer is called the spore coat and is composed of two layers, the inner coat and the outer coat. The spore coat have many variously sized proteins and they are functional in cell development and morphogenesis (Driks, 2001). As mentioned before, *Bacillus subtilis* spores can withstand extremes of heat, radiation, chemical treatments and time. Fundemental to spore resistance and germination is the coat, a tough proteinaceous multilayered shield that, at a minimum, provides mechanical integrity and excludes large toxic molecules while allowing small nutrient molecules to access germination receptors beneath the coat. The coat also appears to house enzymes with direct roles in germination and, perhaps detoxification (Bagyan and Setlow, 2002; Hullo et al., 2001).

Spore coat serves two main roles; First, it provides protection from against bactericidial enzymes and chemicals, such as lysozyme and chloroform. Second, the coat influences the spore's ability to monitor its environment and to germinate to appropriate germinants (Aronson and Fitz-James, 1976; Henriques and Moran, 2000).

A least a dozen spore coat proteins are synthesized during the later stages of development and they confer resistance to lysozyme and various solvent. Spore coat proteins consist several types of post-translational modifications, including glycosylation, proteolytic processing and crosslinking (Henrique et al., 2000). SodA, OxdD (YoaN), YabG, and TgI are important for post-translational modifications. SodA is superoxide dismutase and required for the assembly of the coat protein CotG into the insoluble spore matrix (Henrique er al., 1998), while OxdD (YoaN) is an oxalate decarboxylase and associates with the spore coat structure (Costa et al., 2004). TgI (transglutaminase) is a spore coat protein that is involved in the modification of some spore coat proteins in *B. Subtilis* (Kuwana et al., 2006; Ragkousi and Setlow, 2004). YabG is a protease in the spore coat of *B. Subtilis* (Takamatsu et al., 2000; Takamatsu et al., 2000). SigK-containing RNA polymerase transcribes *yabG* gene which encodes a protease involved in the modification of CotF, CotT, SafA (YrbA), SpoIVA, YeeK and YxeE during sporulation (Takamatsu et al., 2000; Takamatsu et al., 2000).

There is little known concerning assembly of the coat, altough several of the structural genes for coat proteins (*cot genes*) have been characterized. Inactivation of any one of six cot genes*(cotA, cotB, cotC, cotD, cotF, and cotT*) have no major effect in spore resistance properties (Aronson et al., 1989; Bourne et al., 1991; Cutting et al., 1991; Donowon et al., 1987). However, there is an exception with morphogenetic proteins, SpoIVA and CotE. They have especially striking effects on coat assembly. Without SpoIVA, the coat detaches from the spore and it forms a swirl in the mother cell cytoplasm (Piggot and Coote, 1976; Roels et al., 1992). SpoIVA designates the spore surface as the site of all subsequent deposition of coat protein and connects the coat to the spore. SpoIVA is located at an ideal place within the spore and it coordinates the synthesis of the coat and the peptidoglycan, as it resides at or very close to the membrane separating these two structures.

On the other hand, in the absence of CotE protein the outer coat fails to form, because CotA forms a sheel around the spore well before most coat proteins are synthesized (Driks, 1999). CotE directs the deposition but not the synthesis, of at least eight proteins, most of which reside in the outer coat (Zheng et al., 1988; Little and Driks, 2001).

cotA, cotB, cotC, cotD, cotF, and *cotT* genes are transcribed by RNA polymerase containing σ^K, which is produced exclusively in the mother cell compartment of the sporangium late in development (Driks and Losick, 1991; Kunkel et al., 1990; Stragier er al., 1989). The coat proteins are synthesized in the mother cell and assembled around the forespore. In addition to σ^K, *cotB* and *cotC* expression is dependent on the DNA-binding protein encoded by gerE (Sandman et al., 1988; Zheng et al.,1992; Zheng and Losick, 1990).

1.6. *bac* Operon and Bacilysin : A Dipeptide Antibiotic

Certain strains of *Bacillus subtilis* produce and secrete extracellularly bacilysin - a simple peptide antibiotic. Bacilysin is 125 kDa and has basic structure consisting of L-alanine at N-terminus and L-anticapsin at C terminus, which is an unusual aminoacid for C terminus. Antibiotic production affects bacteria and fungi, especially *Candida albicans* (Walker and Abraham, 1970).

The special peptide permease system transports antibiotic into susceptible cells, and subsequently, antibiotic is hydrolyzed to L-alanine and L-anticapsin by peptidases. Glucosamine synthetase activity, which is necessary for bacterial peptidoglycan and fungal mannopretein biosynthesis, is prevented by intracellular anticapsin (Perry and Abraham 1979; Chmara et al., 1981). Also, the basis of antibiotic activity is anticapsin (Whitney et al., 1972). As a result of this blockage protolasting occurs and host cells lysis (Whitney and Funderburk, 1970; Kenig et al., 1976; Chmara et al., 1982; Chmara, 1985; Milewski, 1993). On the basis of its target, anticapsin becomes specifically antagonized by glucosamine or N-acetylglucosamine (Walton and Rickes, 1962; Kenig and Abraham, 1976).

Moreover, when growth conditions (such as glucose or casaminoacids) are available, or when physiological factors (such as pH or temperature) are favorable for *Bacillus*

subtilis, bacilysin biosynthesis is inhibited (Özcengiz et al., 1990; Özcengiz and Alaeddinoğlu, 1991; Basalp et al., 1992).

Additionally, bacilysin biosynthesis is under the control of quorum-sensing mechanism via the OppA (Spo0K), transporter element and Opp-imported peptide pheromone PhrC which is essential for sporulation and competence development (Yazgan et al., 2001). As mentioned above, bacilysin biosynthesis is under the control of both nutritional and feedback regulation. Besides, ComQ/ComX, PhrC (CSF), ComP/ComA and also which is responsible in their transporter, Spo0K (Opp), products or *srfA, spoOA, spoOH* and *abrB* genes are all functional in quorum sensing mechanism and they play key role in bacilysin biosynthesis (Karatas et al., 2003).

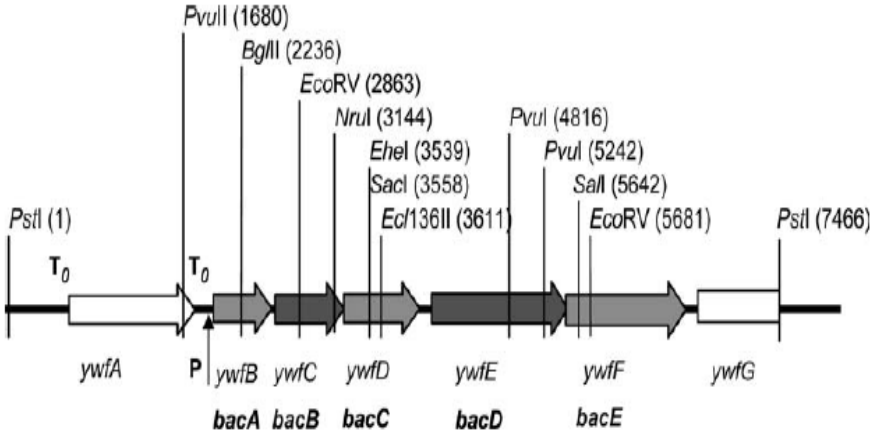

Figure 1.7 : The bacilysin gene cluster organisation, *bacABCDE,* relative to openreading frames *ywfABCDEFG* of *Bacillus subtilis* 168. DNA sequence is among 3875148–3867678 bp in the chromosome obtained from SubtiList database R16.1 (Kunst et al., 1997). Proposed terminator (T0) elements are indicated according to the SubtiList database. Sigma A promoter (P) elements_35 (TTGACA) and _10 (TAAAATt) were detected 56 bp and 33 bp upstream of the ATG codon of the bacA gene (Steinborn et al., 2005).

The *ywfBCDEFG* gene cluster of *B. subtilis* has biosynthetic core function on bacilysin production and it is renamed as *bacABCDE* (Steinborn and Hofemeister, 1998/2000; Inaoka et al., 2003). Each gene of this gene cluster has specific role; proteins which are functional in anticapsin biosynthesis are synthesized by *bacABC (ywfBCD), bacD (ywfE)* is functional in the amino acid ligation of anticapsin to alanine and *bacE* play role in self-protection from bacilysin. Also anticapsin production needs aminotransferase and prephenate dehydratase and both of them are

encoded by ywfB and ywfG (Hilton et al., 1988; Inaoka et al., 2003; Steinborn et al., 2005).

Large multienzyme complex functioning in a thiotemplate mechanism in a pathway initiated by a protein template is specific to prokaryotic organisms for producing antimicrobial peptide synthesis but still ribosomal peptide synthesis, driven by aminoacyl tRNA synthetase, is conserved for all cellular organisms. Biosynthesis mechanism of bacilysin formation is not completely matched with non-ribosomal peptide synthetase (NRPS) mechanism since adenylation and thiolation are evident only for L-alanine and not for L-anticapsin (Marahiel, 1997; vonDöhren et al., 1999). Furthermore, *ywfE* is recently announced as a novel gene synthesizing L-amino acid ligase belonging to ATP-dependent carboxylate-amine/thiol ligase superfamily, widely known to contain enzymes catalyzing the formation of various types of peptide.

1.7. The Aim of the Present Project

The relationship between bacilysin production and sporulation in *Bacillus subtilis* was previously detected by Özcengiz and Alaeddinoğlu in 1991. Bacilysin-negatif strain (bac^-) NG79 was found to be oligosporogenous, meaning that they produce spores at a low frequency and showed defective profiles in their heat, lysozyme, chloroform resistance and dipicolinate accumulation profiles. Furthermore, the transfer of the *bac* locus to the bacilysin-negative mutant NG79 was shown to ameliorate its sporulation ability. Moreover, external bacilysin addition to the cultures of the bac^- strain, for the ultimate purpose of understanding if the ability to produce bacilysin is directly responsible for the sporulation defect, has shown that bacilysin-negatif strain has restored its sensitive profile (Özcengiz and Alaeddinoğlu, 1991). Finally, all of these studies have been supposed that bacilysin might be a control element in sporulation process. However, NG79 used as bacilysin deficient strain in the former study was created by NTG treatment, therefore beside to *bac-1* lesion, it is likely to be found another genetic lesions which could cause to the sporulation deficient phenotypes. Altough this possibility seems to be excluded with later study in which the external addition of bacilysin to the cultures of the bacilysin nonproducer strain was resulted with the restoration of the deficient profiles of the mutant spores, the relationship between the bacilysin and sporulation has been still

obscured because of the used impure bacilysin (broth concentrate) in this study. Therefore, the objective of the present study was aimed to clarify the possible action sites of bacilysin in the sporulation process**.** For this purpose, the major spore properties of the bacilysin defective *B. subtilis* OGU1 strain was compared with wild type PY79 spores. The bacilysin biosynthetic operon, *bacABCDE,* in *OGU1 strain* was previously disrupted by using the *B. subtilis* integration vector pMUTIN T3 and thus bacilysin deficient strain OGU1 (*bacA::lacZ::erm)* was generated. In this study, both wild type PY79 spores and *bacA* mutant OGU1 spores were treated with lysozyme, heat and chloroform to investigate resistance profiles of *bacA* spores. Subsequently, to observe the effect of spore coat proteins on spore resistance properties and germination process, spore coat proteins were extracted from both mutant type and wild type spores. Small acid-soluble proteins were also compared from OGU1 and PY79 spores. The level of dipicolinic acid was also explored in OGU1 spores and compared to wild type spores. Furthermore, germination patterns in two different media and outgrowth characteristics of mutant type and wild type cells were analyzed for the ultimate purpose of gaining more insights about the functional role of *bacilysin* in the sporulation process.

2. MATERIALS and METHODS

2.1. Materials

2.1.1. Bacterial Strains

Strains used in this project are listed in Table 2.1. For the construction of *bacA-lacZ* transcriptional vector, integrative vector pMutinT3 (Figure. 2.1) was used. pGEM-T vector was used for cloning of PCR products (Figure 2.2).

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

Strain or plasmid	Relevant Genotype, phenotype, and/or characteristics	Construction, source or reference
B. subtilis PY79	wild type, BSP cured prototrophic derivative of *B.subtilis* 168	P.Youngman
OGU1	[*lac*Iq Tn10(Tet^r)], *mcrA* Δ(*mrr-hsdRMS-mcrBC*), *f80lacZΔM15 ΔlacX74*, *deoR, recA1, araD139 Δ(ara-leu)7697, galU, galK,rpsL (Strr), ,*	M.A.Marahiel

2.1.2 Bacterial culture media

Composition and preparation of culture media are given in the Appendix

2.1.3. Buffers and solutions

Composition and preparation of culture media are given in the Appendix A.

2.1.4. Chemicals and enzymes

The chemicals and enzymes that were used are given in the Appendix C.

2.1.5. Laboratory equipment

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

2.1.6. Maintenance of bacterial strains

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

2.2. Mature Spore Isolation

Cells were grown in DSM at 200 rpm and harvested at the end of the 72h by centrifugation (10 000 g, 10 min, 4°C). Spores were purified through washes in ice cold deionized water four times, and lysozyme was added (0.1 mg/ml). After addition of lysozyme, cells were incubated overnight at 4°C. Following this step, spores were collected once again and washed two times by centrifugation (10 000 g, 10 min, 4°C) with ice cold deionized water.

2.3. Germination and Outgrowth of *Bacillus subtilis* Spores

For the germination assay, spores were not treated with lysozyme. Spores were purified through washes in ice cold deionized water and they were suspended in ice cold deionized water and stored at 4°C overnight. The next day, spores were centrifuged once again. Purified spores in water were diluted to an OD600 of 0.4 in 2xYT and to an OD585 of 0.8 in SMM, following this step, purified spores in 2xYT and SMM were heat activated at 70 °C for 15 min, and then, cooled down. After 5 min of adaptation at 37°C, germination agents of either L-alanine (10 mM) or AGFK (3.3 mM L, asparagine, 5.6 mM D-glucose, 5.6 mM D-fructose, 10 mM KCl) was added to the medium in order to provide germination initiation. Furthermore,

germination was monitored by measurement of loss in optical density at 585 nm at 37°C for 400 min with 10 minute intervals.

2.4. Spore Resistance

Cells were grown in DSM at 37°C for 3 days and spores were purified through washes and cells were diluted serially 10-fold in 0,85% saline solution and 0.1 ml aliquots of dilutions were plated on LB agar plates for total viable cell count. Afterwards these diluted cells were heated at 80°C for 20 min and plated once again for total viable cell count. For the chloroform and lysozyme treatments, samples from the cultures were taken, diluted serially 10-fold in 0,85% saline solution and plated. Then, new samples were taken from the cultures and treated with lysozyme (final concentration, 0.25 mg/ml) at 37°C for 15 min or with 10% v/v chloroform at room temperature with repeated wortex for 15 min. Following the treatments, lysozyme or chloroform-treated cultures were serially diluted and plated on LB agar medium containing plates. All the plates were incubated overnight at 37°C.

2.5. Assay for Dipicolinic Acid (DPA)

Purified spores were diluted to OD_{600} of 2,5 in cold deionized water and 2 ml from these suspensions were harvested by 1 min of centrifugation at 13.000 rpm and pellets were suspended in 1 ml of deionized water. Following this step, tubes were capped and placed in a boiling waterbath for 20 min.The sample was cooled on ice 15 min. Subsequently, samples were centrifuged for 2 min and their supernatants were saved. Assay reagent, composed of 25 mg L-cystein, 170 mg iron sulfate, 80 mg ammonium sulfate in 25 ml of 50 mM sodium acetate (pH 4.6 with glacial acetic acid) was prepared and 0,2 ml of this reagent was mixed with 0.4 ml of the supernatants taken and 0.4 ml of dH2O. These mixtures were centrifuged again for 2 min and their optical densities at 440 nm were measured against a blank prepared from 0.8 ml of dH2O and 0.2 ml of assay reagent. Aiming the determination of exact DPA concentration (µg/ml), a calibration curve was prepared and treated similarly with pure DPA using standards of 0, 10, 20, 40, 70 and 100 µg DPA/ml.

2.6 Isolation and Extraction of the Spore Coat Proteins

Approximately 5 x 10^9 spores were suspended in ST solution. The suspension was incubated at 70°C for 30 min in ST (1% w/v SDS, and 50mM dithiothreitol) solution. The suspension were centrifuged at 10000g for 10 min and following this step, the supernatant was saved. In order to proceed with the dialysis procedure, low molecular weight cut-off dialysis tubes were prepared. For this purpose, tubing was cut into 18-24 inch lenghts. 2% (w/v) sodium bicarbonate /1mM EDTA pH:8 solution was prepared. Tubing was boiled in this solution for 10 minutes. The tubing was rinsed two times with distilled water. Subsequently, tubing was boiled for 10 min in a solution of 1mM EDTA. The tubing was rinsed with distilled water again and finally, the tubing was allowed to cool and stored at 50% ethanol with tubing submerged. Before use, tubing was washed out with distilled water. Consequently, the saved supernatant was used to fill these already prepared low molecular weight cut-off dialysis tubes and the tubes were submerged in 0.5M sodium acetate/acetic acid buffer (pH 5.0) for overnight. 0.5M sodium acetate/acetic acid buffer (pH 5.0) was changed with deionized water at 4 °C. Finally, the dialyzed material was lyophilized and was dissolved in TMS for subsequent analysis by SDS-PAGE. To run the gel, 0,002% bromophenol blue is added in samples and they were incubated for 5 minutes at 100 °C. The samples were applied to the gel and was run the gel at 25mA until the dye is 0.5-1.0 cm from the bottom. Gels were stained for 16h, following this step destained until the background is colorless.

2.7. Extraction of SASPs From Spores

For SASP extraction, lyophilized spores were used. 20 mg dried cells were broken at room temperature in a dental amalgamator using 200mg otoclaved glass beads. Ten 1-min periods of shaking were sufficient to distrupt >90% of all cells and mature spores. The 1-min periods of shaking were separated by 1-min periods with the machine at rest. Furthermore, the dry powder was removed from the amalgamator capsule and it was extracted on ice with 2.5 ml ice-cold 0.3 M HCl in a 15 ml falcon. After 30 min on ice with intermittent vortex mixing the suspension was centrifuged at 40000g for 25min at 4 °C. The supernatant fluid was saved in new falcon and the pellet was re-extracted with an additional 1.5 ml of 0.3M HCl. The pellet was incubated 10 nimutes on ice with repeated vortex and then it was centrifuged again at

4000g for 25 minutes. The supernatant was saved. After this step, ultrafiltration tubes were used (3000 cut-off) for the extraction. Centrifugation (2500 g, 20 min) was continued until the supernatant fluids were finished. Finally, the supernatant was washed five times by %1 acetic acid buffer. After repeated washes, the supernatant was saved in 2 ml ependorf tubes and lyophilyzed. The lyophilized material was dissolved in 100µl of 8 M urea for analysis by polyacrylamide gel electrophoresis at low pH. SASP analysis was routinely carried out by polyacrylamide gel elecrophoresis at low pH, which separates all three major *B. subtilis* SASPs. These gels were run with the electrode polarity set approximately for the fact that SASPs are positively charged in this gel system and will run towards the cathode. SASPs were stacked in this gel system at 20 mA constant current, and run at 50mA. The upper gel was polymerized for 30-60 min in strong sunlight; on a cloudy day a long-wavelength transilluminator can be used to promote polymerization. Gels were stained for 45 min with gentle agitation, and destained overnight..

3. RESULTS AND DISCUSSION

To examine the expression of *bacA* operon, a *B. subtilis* strain, OGU1 was previously constructed by İsmail Öğülür as a part of his master thesis project at Middle East Technical University, containing a transcriptional *bacA-lacZ* fusion at *bacA* locus. For this purpose, the *B. subtilis* integration vector pMUTIN T3 was used. Upon the integration of pMUTIN T3 vector into the target gene, while inactivated the target gene *lacZ* reporter gene becomes transcriptionally fused to the target gene, allowing its expression profile to be monitored. For integration of pMUTIN T3 vector into *B. subtilis* PY79 *bacA* gene locus, initially a 420 bp long fragment, 5'- *bacA* gene fragment was amplified by PCR using the chromosomal DNA of wild type strain *B. subtilis* PY79 as a template. 437 bp *bacA* PCR fragment was purified from the gel and ligated into pGEM-T vector and ligation product was used for the transformation of *E. coli* DH5α cells.

Recombinant pGEM-T plasmid was isolated from the transformant *E. coli* cells for cloning of *bacA* gene into the integrative plasmid pMUTIN T3 vector. The recombinant pMUTIN T3 plasmid of an expected size (9254 bp) was isolated from the transformant. Furthermore, it was concluded that the plasmid constructed was a desired recombinant containing the 420 bp *bacA* insert fragment in its structure. The structure of the resulting recombinant pMUTIN T3 plasmid vector is shown in Fig.3.1.

For permanent construction of *bacA::lacZ* transcriptional fusion in *B. subtilis*, the recombinant pMUTIN T3 plasmid DNA was used to transform *B. subtilis* PY79 to erythromycin resistance gene (Erm R) by integration through a single cross-over event at *bacA* locus (Campbell-like insertion). ColE1 origin of replication on pMUTIN T3 enables replication in *E. coli* but not in *B. subtilis*. The transformants would arise only by recombination between cloned *bacA* fragment and its counterpart in the chromosome. Therefore, integration of plasmid into *bacA* gene locus by single crossover resulted in *bacA::lacZ* transcriptional fusion.

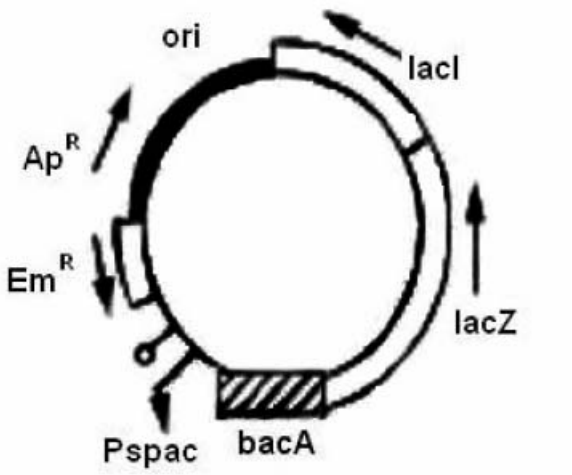

Figure 3. 1. *bacA::lacZ* fusion construct in pMUTIN T3.

3.1. Properties of *bacA* Spores

In this study, to understand the effect of the disruption of the *bac operon* on the spores properties of OGU1 spores and PY79 were treated with heat, lysozyme and chloroform (Table 3.1).

Table 3.1: Heat, chloroform and lysozyme resistance of PY79 and OGU1 spores

Strain	Treatment[a]	Cell titer before treatment[b] (cfu/ml)	Cell titer after treatment (cfu/ml)	Survival frequency[c]
PY79	heat	509 x 10^7	460x 10^7	0.90
OGU1	heat	400 x 10^7	376x 10^7	0.94
PY79	chloroform	441.5 x 10^7	207 x 10^7	0.46
OGU1	chloroform	502.5 x 10^7	142.5 x 10^7	0.28
PY79	lysozyme	372.5x 10^7	315 x 10^7	0.84
OGU1	lysozyme	549.5x 10^7	317.5 x 10^7	0.57

a. heat treatment: incubation of the sample at 80^{o}C for 25 min chloroform treatment: with 10% v/v chloroform at room temperature for 15 min lysozyme treatment: with lysozyme (final concentration, 0.25 mg/ml) at 37^{o}C for 15 min.

b. cfu/ml of culture following growth for 72h at 37^{o}C in DSM.

c. Survival is calculated by dividing cfu/ml after treatment to cfu/ml before treatment

Firstly, OGU1 and PY79 spores were treated with 10% (v/v) chloroform at room temperature for 15 min. PY79 spores show 46% resistance to chloroform, whereas the rate of OGU1 spores is 28%. Furthermore, OGU1 and PY79 spores were treated

with lysozyme (final concentration, 0.25 mg/ml) at 37°C for 15 min. As a result, PY79 spores were found to be more resistant than mutant type. When the survival frequencies of PY79 and OGU1 spores were compared after the incubation at 80°C for 20 min, OGU spores were heat-resistant just-like PY79 spores. According to our results, wild type spores PY79 differ from OGU1 spores (*bacA::lacZ::erm)* with its lysozyme and chloroform resistance properties. Due to these explored differences, OGU1 spores and PY79 spores were further compared according to their coat proteins profile (Fig 3.2).

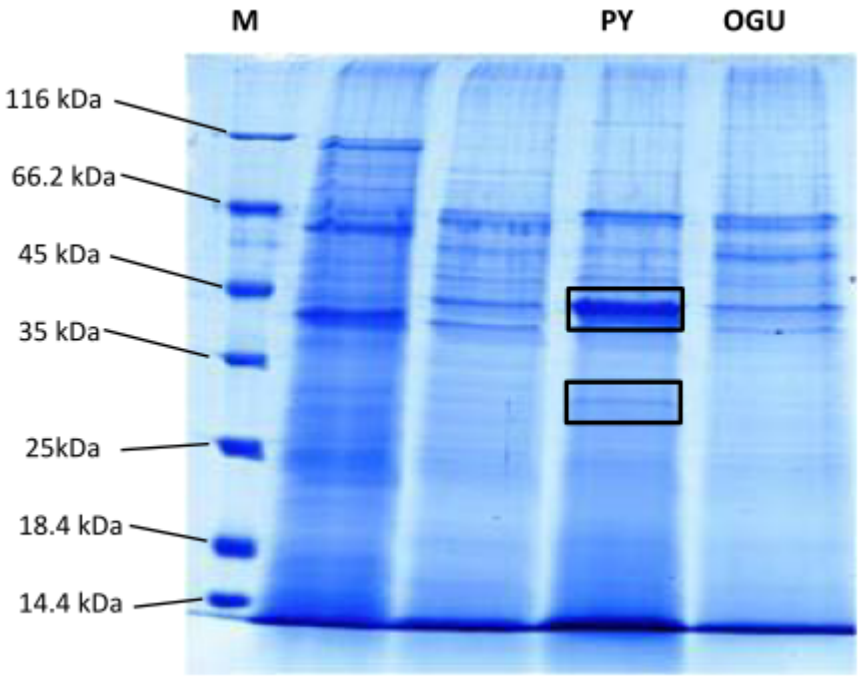

Figure 3.2: Coat proteins of a wild-type strain, PY79 and mutant type OGU1 (*bacA::lacZ::erm)*. Coat proteins were extracted by treatment with a buffer containing SDS-DTT. The extracted proteins were resolved in 12% polyacrylamide gel containing SDS, and the gel was stained with Coomassie brilliant blue.

Bacterial endospores are encased within a complex multilayered protein structure known as the coat, There are two major layers in the coat, a lightly staining lamellar inner layer and a darkly staining outer layer (Aronson and Fitz-James, 1976; Warth et al., 1963). The morphogenetic coat protein CotE directs the assembly of most, if not all, outer coat proteins and some of the inner coat proteins (Driks and Setlow, 2000). Additional morphogenetic proteins, such as SafA (YrbA) and SpoVID, further

guide coat protein deposition (Beall et al., 1993; Ozin et al., 2000; Ozin et al., 2001; Takamatsu et al., 1999). Spore coat serves two main roles. First, it protects against bacteriocidal enzymes and chemicals, such as lysozyme and chloroform, thus contributing to the spore's resistance properties and viability. Second, the coat influences the spore's ability to monitor its environment and to germinate within minutes of exposure to appropriate germinants (Aronson and Fitz-James, 1976; Henriques and Moran, 2000). The coat is critical for resistance properties as well as germination. It provides mechanical integrity, excluding large toxic molecules, on the other hand, it allows small nutrient molecules to penetrate and interact with the germination receptors located toward the spore interior (Moir et al., 2002; Paidhungat and Setlow, 2002). The coat comprises approximately 40 proteins species that do not resemble one another (Kuwana et al., 2007).

According to our SDS-PAGE results, PY79 spores differ from OGU1 spores with its two protein bands at the expression levels. First band stands between 45 kDa and 35 kDa. Spore coat proteins of *Bacillus subtilis* were identified by Lai et al in 2003, and molecular mass of CotG protein was depicted as 40 kDa. CotG protein is required for the incorporation of CotB into the coat and *cotG* gene seems to be divergent from *cotH*. Actually, the production of coat proteins are under the control of a regulatory cascade of four transcription factors and they act in the mother cell of the sporangium. These transcription factors are σ^E, SpoIIID, σ^K, and GerE (Zheng and Losick, 1990).

On the other hand, both *cotB* and *cotG* genes are expressed later under the dual control of σ^K and GerE (Ichikawa and Kroos, 2000; Nacleiro et al., 1996; Sacco et al., 1995;Zheng and Losick, 1990; Zheng et al., 1992). Furthermore, both CotG and CotB are located in the outer coat (Sacco et al., 1995; Zheng et al., 1988). It has previously been showed by Sacco et al in 1995 that, CotG has a morphogenic role in the assembly of CotB. The molecular mass of YtaA is 41 kDa and is regaraded as a coat protein based on significant similarity to CotS (Blast score of 3E-25), as already noted in the *B. subtilis* genome sequence annotation, and its gene being adjacent to, and oriented away from, the operon harboring *cotS*.

There are also other spore coat proteins depicted in subtiList, including CotB, CotH, CotSA, and CotS that have molecular masses approximately 40 kDa. CotB is

encoded downstream of *cotH.* The localization of CotB is depicted as 42.9 kDa (Isticato et al., 2001). *cotH* gene is divergent from *cotG,* upstream of *cotB. cotH* codes for a 42.8kDa protein, apparently located in the inner layer of the coat. Altough, *cotH* null mutations show pleiotropic effect on several components of the outer coat and cause a germination-deficient phenotype (Nacleiro et al., 1996). CotSA is encoded from *cotSA cotS* operon and *cotSA* gene is divergent from *cotI (ytaA).* Finally, CotS is encoded from *cotSA cotS* operon.

Second band stands between 35 kDa and 25 kDa. Molecular mass of YckK protein, that resembles to an ABC transporter, was also demonstrated by Lai et al in 2003 as 31 kDa. Also in 2002, Kuwana et al. have pointed out that YrbA (SafA) protein's molecular mass was 29 kDa and it was noted that YrbA was a coat protein and it was involved in coat assembly. YrbA forms a complex with SpoVID and is localized to the cortex-coat interface in mature spores (Ozin et al., 2000). Furthermore, YrbA is required for spore resistance and/or germination (Kodama et al., 1999).

Despite the fact that the spore coat provide many resistance properties to spores, it is not involved in maintaining dormancy and heat resistance, since decoated spores are still dormant and heat resistant (Cassier& Ryter 1971; Warth, 1978). Consistently, this current results showed that, even there are differences at expression levels of two protein bands, OGU1 and PY79 spores display approximately same resistance to heat treatment. On the other hand, it is known that spore coat contributes to the protection against lysozyme and chloroform (Aronson and Fitz-James, 1976). Therefore, lysozyme and chloroform resistance rates of wild type and mutant type differing from each other have consisted with the data that show coat protein profile difference.

The different expression level of gene between 35 and 45 kDa, is thought to belong to one of these six coat proteins, including CotG, YtaA, CotB, CotH, CotS, CotSA. The second band is between 25 and 35 kDa and, Yckk or YrbA is thought to be possible coat protein because of their appropriate molecular mass. Lower expression levels of these possible coat proteins in OGU1 spores might cause to sensitivity of them against lysozyme and chloroform. On the other hand, most structural coat proteins, including CotA, CotB, CotC, CotD and CotF are functionally redundant and the absence of any one of them does not cause evident phenotypic alterations (9-14-CotB makalesi). According to this data, CotB might not be our interested protein.

Altough, as mentioned before by Nacleiro et al in 1996, *cotH* null mutation causes germination-deficient phenotype, whereas the germination profiles of the OGU1 and PY79 spores are similar with each other.

On the basis of dipicolinic acid assay, PY79 spores were found to synthesize about 16.139 ± 1.438 µg/OD_{600} DPA, and OGU1 mutant spores synthesized about 15.828 ± 1.758 µg/ OD_{600} DPA (Fig. 3.3).

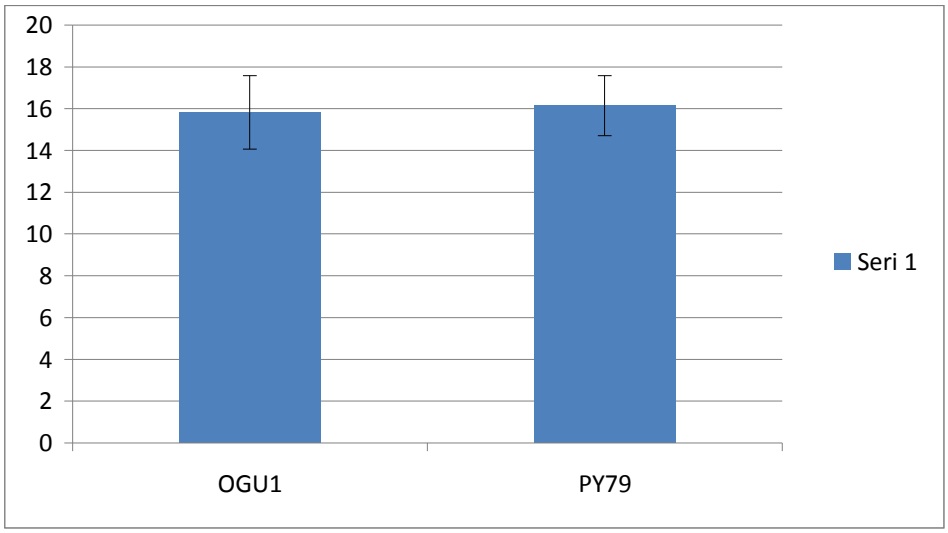

Figure 3.3: DPA contents of OGU1 and PY79 spores.

In *B. subtilis*, the specifically blockage of DPA synthesis results in DPA-less spores with decreased resistance to wet heat by the increased core hydration (Balassa et al.; 1979, Coote, 1972) which is the major determinant of spore heat resistance (Todd et al., 1986; Popham et al., 1995).Furthermore, DPA plays more important direct role in protecting spore DNA against several types of damage (Gerhardt and Marquis, 1989; Paidhungat et al., 2000; Setlow et al., 2006; Setlow, 2006). Similarity of heat resistance of PY79 and OGU1 can be consequence of the same levels of DPA synthesis and/or accumulaton in the spores.

For the comparison of germination properties, pure spores from OGU1 and PY79 were treated with different germination agents at different media. Firstly, spores were made grown in rich medium 2x YT, germination and outgrowth patterns did not differ from each other; even if spores were treated with different germination agents (Figure 3.4B, 3.5B). Following similar profile observations, spores were made germinate in minimal medium SMM and PY79 and OGU1 spores were found to germinate in a similar pattern when exposed to two different germination agents, L-

alanine or AGFK (Figure 3.4A, 3.5A). Spore germination is a process that includes three stages: activation, which is followed by germination and finally outgrowth. Outgrowth is the process during which anabolic reactions starts to take place of catabolic ones, so that spores regain their vegetative forms. Consequently, outgrowth is validated as the initial optical densities of purified spores are restored at the end of the second stage of germination. Therefore, when the outgrowth of OGU1 and PY79 spore cells was visualized either in 2x YT or SMM media , it was noticed that OGU1 cells return to their vegetative state approximately at the same time with wild-type germinating spores.

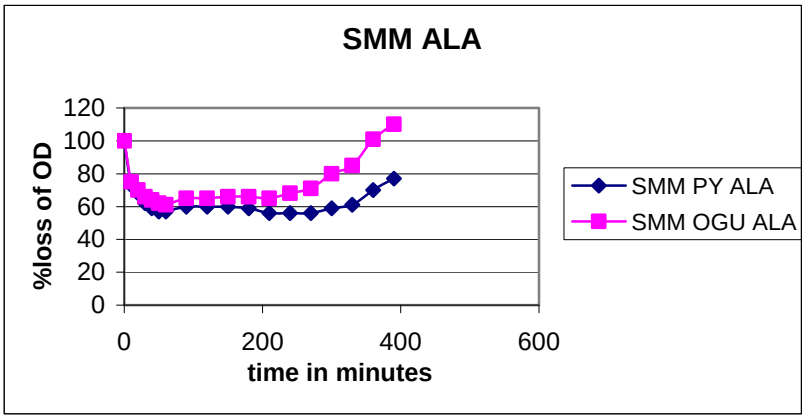

Figure 3.4.(A) Germination and outgrowth profiles of OGU1 and PY79 spores with L-alanine in SMM medium. Germination and outgrowth profile of OGU1 and PY79 spore cells were drawn through measurement of loss in optical density at 585 nm at 37^{o}C for 90 min at 10 min intervals and further measurements continued until outgrowth is observed at 30 min intervals.

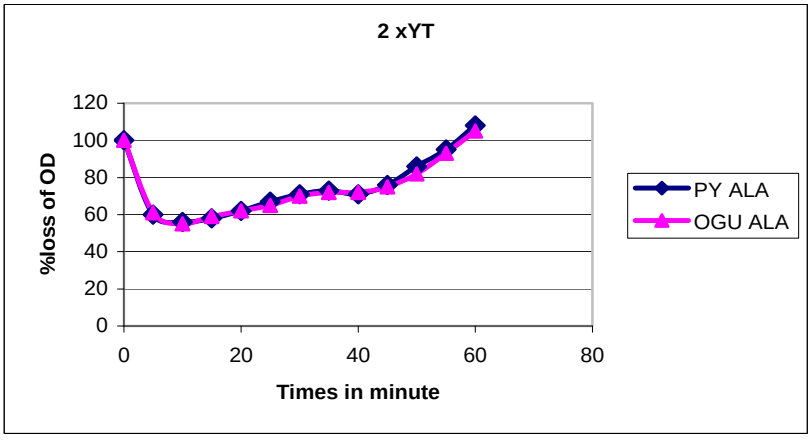

Figure 3.4. (B) Germination and outgrowth profiles of OGU1 and PY79 spores with L-alanine in 2 x YT medium

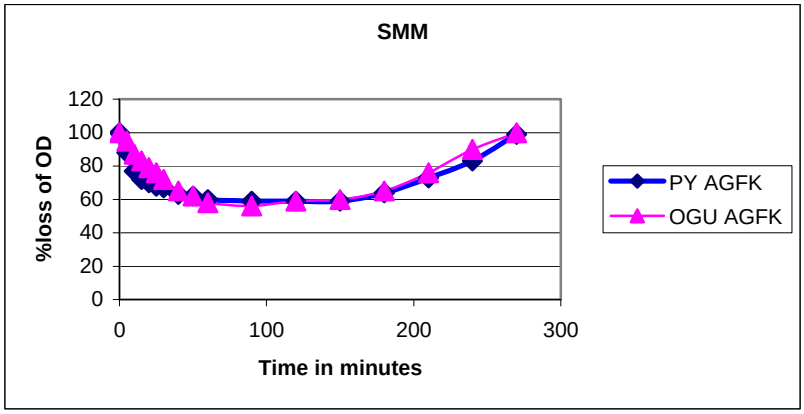

Figure 3.5. A: Germination and outgrowth profiles of OGU1 and PY79 spores with AGFK (3.3 mM L-asparagine, 5.6 mM D-glucose, 5.6 mM D-fructose, 10 mM KCl) in SMM medium.

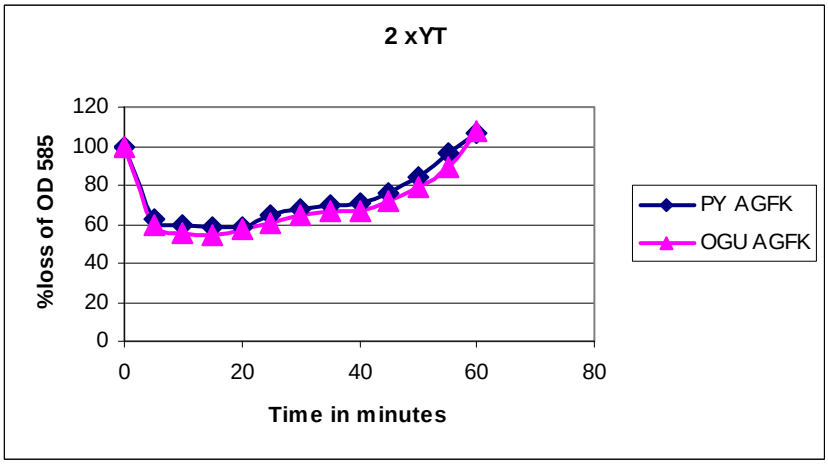

Figure 3.5.B: Germination and outgrowth profiles of OGU1 and PY79 spores with AGFK (3.3 mM L-asparagine, 5.6 mM D-glucose, 5.6 mM D-fructose, 10 mM KCl) in 2 x YT medium.

To determine the effect of *bacilysin*, on the small, acid-soluble proteins, SASPs were extracted from both OGU1 and PY79 spores (Fig 3.6). There are three major SASP in spores of *B.subtilis,* one γ-type SASP and two α/β-type SASP; all these proteins comprise 8-15% of the protein in spores of *Bacillus* species (Setlow, 1988). In our result, three major types of SASP of PY79 and OGU1 *(bacA::lacZ::erm)* spores are shown. The only known function of a spore's γ-type SASP is to be degraded early in spore germination, thus providing amino acids for protein synthesis during this period of development, and α/β-type SASP also serve this function (Setlow, 1988, 1995). At the same time, SASP (the α/β –type) are essential for the protection of spore DNA from a variety of types of damage , while degradation of both the α/β –type SASP and the other major SASP(γ) provides amino acids for

protein synthesis early in spore germination (Setlow, 1988; Setlow, 1994; Setlow, 2000). α/β-type SASP are DNA-binding proteins that saturate the spore's chromosome, and protect the spore's DNA from a variety of types of damage; this DNA protection by α/β-type SASP is a significant component of spore resistance to a variety of treatments, including heat, oxidizing agents and UV radiation (Setlow,1995). According to our results, the presence of equal levels of α/β- type SASPs in OGU1 *(bacA::lacZ::erm)* and PY79 can be result of the show similar resistance properties of these two types of spores to heat and estimated UV radiation, since α/β- type SASPs are the major determinant of spore UV and heat resistance (Setlow, 1988 and Setlow, 2000).

SASP-γ plays no role in spore resistance but SASP-γ is an amino acid storage protein and its degradation helps support protein synthesis early in spore germination and outgrowth. SASP-γ^- spores are retarded in spore outgrowth (Hackett and Setlow, 1988). Our results suggest that OGU1 *(bacA::lacZ::erm)* and PY79 spores have similar levels of γ-type SASP. Consistently, similar outgrowth profiles of PY79 and OGU1 spores as mentioned before in the germination assay data, can be result of the equal levels of γ-type SASP of PY79 and OGU1 spores.

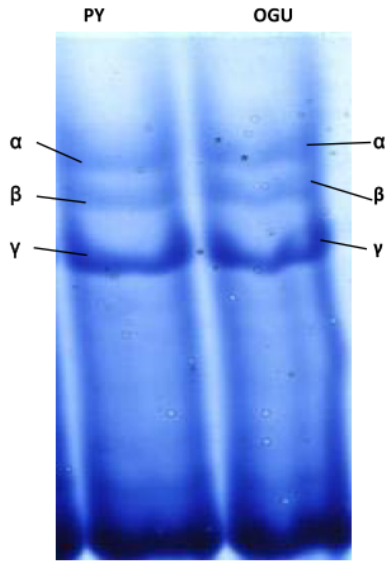

Figure 3.6: Levels of SASP-α ,-β ,-γ in *(bacA::lacZ::erm)* OGU1 and PY79 strains.SASP's were extracted from purified spores, lyophilization is followed by ultrafiltration and then spores were lyophilized again for the second time.Samples were subjected to PAGE at low pH, and the gel was stained with Coomassie Brilliant Blue R.

4. CONCLUSION

Present study was focused on the characterization of *bacA* mutant spores. To understand the effect of the disruption of *bacABCDE operon* on the spore properties, spores were treated with 10% (v/v) chloroform, subsequently, with lysozyme (final concentration, 0.25 mg/ml) at 37^{o}C for 15 min and finally spores were compared after the incubation at 80^{o}C for 20 min. As a result OGU1 spores are more sensitive to chloroform and lysozyme treatments than PY79 spores. In order to back up this obtained data of resistance properties, spore coat proteins were extracted from both OGU1 and PY79. PY79 differs from OGU1 with two more expressed protein bands. One of the two bands was observed to be between 45 kDa and 35 kDa and the location of the other band was between 35 kDa and 25 kDa. There are six possible coat proteins which are approximately 40kDa; CotB, CotH, CotSA, CotS, CotG, YtaA and two spore coat proteins which are approximately 30kDa. Except CotB and CotH, the others can be our interested coat proteins which are responsible to provide resistance against lysozyme and chloroform to spores. Owing to the fact that observed similar heat resistance properties, DPA content was analyzed in both mutant type and wild type. PY79 spores were found to synthesize about 16.139 ± 1.438 μg/OD_{600} DPA, and OGU1 mutant spores synthesized about 15.828 ± 1.758 μg/ OD_{600} DPA. Similarity of heat resistance of PY79 and OGU1 was concluded to be a consequence of the same levels of DPA synthesis and/or accumulaton in the spores.

Subsuquently, small-acid soluble proteins (SASPs) of OGU1 and PY79 spores were compared. Three major types of SASP were shown on the gel. α/β -type SASPs are the major determinants of spore UV and heat resistance. Equal level of these two types of SASPs in both OGU1 and PY79 strongly suggest that *bacA* spores are resistant to heat just as PY79 spores. The only known function of a spore's γ-type SASP is to be degraded early in spore germination, thus providing amino acids for protein synthesis during this period of development. For the comparison of germination properties and their outgrowth profile, pure spores from OGU1 and

PY79 were treated with different germination agents at different media. But, there is no difference between their outgrowth in both 2x YT and SMM medium. However, according to our results, the same levels of γ-type SASP also confess germination assay data.

As a result, the effect of the *bac* operon was observed in spore coat proteins such that mutant strain spores were more sensitive to lysozyme and chloroform treatments than PY79. Consequently, in this study it was demostrated that bacilysin seemes to play a regulatory role in the synthesis of spore coat proteins.

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APPENDICES

APPENDIX A : Compositions and Preparation of Culture Media

APPENDIX B: Reagents and Solutions for Gel Electrophoresis of SASPs at Low pH
Reagents and Solutions for SDS-PAGE Analysis of Spore Coat Proteins

APPENDIX C: Enzymes and Chemicals

APPENDIX D: Laboratory Equipment

APPENDIX E: *bacA* Gene Sequence

APPENDIX A

Compositions and Preparation of Culture Media

Luria Bertani (LB) Medium (1000ml)

Tryptone	10 g/L
Yeast Extract	5 g/L
NaCl	5 g/L

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

Luria Bertani (LB) Agar Medium (1000 ml)

Tryptone	10 g/L
Yeast Extract	5 g/L
$NaCl_2$	5 g/L
Agar	15 g/L

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

SMM (1000 ml)

$(NH_4)_2SO_4$	2 g
K_2HPO_4	14 g
KH_2PO_4	6 g
Na_3.citrate.$2H_2O$	1 g
$MgSO_4.7H_2O$	0,2 g

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

50% glucose	10 ml
L-tryptohan(3mg/ml)	10 ml

2xYT Medium (1000 ml)

Tryptone	16 g
Yeast Extract	10 g
NaCl	5 g

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

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

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

Nutrient Broth	8 g
KCl (10% w/v)	10 ml
$MgSO_4.7H_2O$ (1.2%)	10 ml
NaOH (1M)	0.5 ml

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

$Ca(NO_3)_4$ (1M)	1 ml
$MnCl_2$ (0.01M)	1 ml
$MnCl_2$ (0.01M)	1 ml (resuspend before use)
$FeSO_4$ (1mM)	1 ml (resuspend before use)

% 1.5 Agar was added if necessary before autoclaving.

APPENDIX B

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

Solution needed:

A. 1M KOH	24.0 ml
glacial acetic acid	11.2 ml
tetramethylenediamine (TEMED)	2.3 ml
H_2O	62.5 ml

B. 1M KOH	24.0 ml
glacial acetic acid	1.44 ml
TEMED	0.23 ml
H_2O	74.33 ml

C. acrylamide	28 g
bis-acrylamide	0.74 g
H_2O	to 100 ml

(filter solution C through Whatman #1 paper)

D. riboflavin	0.4 g
H_2O	100 ml

F. ammonium persulfate	0.14 g
H_2O	100 ml

Running Buffer (8X)

BETA-alanine	31.2 g

Glacial acetic acid	8.0 ml
H_2O	to 1 litre
(dilute 1/8 before use)	

Sample Diluent

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

Gel Stain

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

Destaining Solution

Methanol	75 ml
Glacial acetic acid	50 ml
H_2O	875 ml

Preparation of gels

	Solution	**Relative amount**
Lower resolving gel	A	1
	C	2
	F	4
	H_2O	1

Upper stacking gel	B	1
	D	2
	E	4
	H_2O	1

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

Solutions

A. acrylamide	30.0 g
bis-acrylamide	0.5 g
dH_2O	to 100 ml

(filter solution A through Whatman #1 paper)

B. acrylamide	10.0 g
Bis-acrylamide	0.76 g
dH_2O	to 100 ml

(filter solution B through Whatman #1 paper)

C.1.5 M Tris.Cl, pH 6.8	100 ml
SDS	0.4 g

D. 0.5 M Tris.Cl, pH 6.8	100 ml
SDS	0.4 g

E. ammonium persulfate	100 mg
dH_2O	to 1 ml

F. tetramethylenediamine (TEMED)

Running Buffer (8X)

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

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

Gel Stain

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

Gel destain

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

Gel storage

Ethanol	5 ml
Glacial acetic acid	10 ml
dH_2O	85 ml

Gel formulation

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

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

APPENDIX C

ENZYMES AND CHEMICALS

Enzyme	Supplier
*Bam*HI	MBI Fermentas
*Hin*dIII	MBI Fermentas
Lysozyme	AppliChem
Proteinase K	Sigma
RNAse A	Sigma
*Sac*I	MBI Fermentas
*Xba*I	MBI Fermentas
Taq DNA Polymerase	MBI Fermentas
T4 DNA Ligase	MBI Fermentas

Chemical	Supplier
Agar	Sigma
Agarose	Sigma
β-mercapthoethanol	Merck
Calcium chlorid ($CaCl_2$)	Merck
Choloroform	Merck
D(+)-Glucose monohydrate	Merck
Glucose	Merck
Glycerol	Merck
HCl	Merck

Iron(III) sulfate – 7 – hydrate ($FeSO_4.7H_2O$)	Riedel-de Haën
Isopropanol	Merck
KH_2PO_4	Merck
K_2HPO_4	Merck
KCl	Merck
L- Argininemonohydrochlorid	Merck
L-Histidinmonohydrochlorid	Merck
L-Tryptophan	Merck
Lysozyme	Sigma
Methanol	Merck
Mercaptoethanol	Sigma
MgCl2.6H2O	Merck
MnCl2.4H2O	Merck
Magnesium sulphate ($MgSO_4.7H_2O$)	Riedel-de Haën
Na_2CO_3	Merck
$Na_2SO_4.10H_2O$	Merck
Na_3citrate.$2H_2O$	Merck
N-acetlyglucoseamin	Sigma
Natrum hydroxid (NaOH)	Riedel-de Haën
Natrium sulfate (Na_2SO_4)	Riedel-de Haën
Nutrient broth	Merck
ONPG	Sigma
Phenol-chloroform-isoamylalchol	Sigma
Phenylalanin	Sigma
Potassium chloride (KCl)	Riedel-de Haën
SDS	Merck
Spermidine	Sigma
Sodium carbonate (Na_2CO_3)	Riedel-de Haën
Sodium chloride (NaCl)	Riedel-de Haën
Sodium hydrogen phosphate($Na_2HPO_4.7H_2O$)	Merck
Sucrose	Merck
Tris (hydrocymethyl) aminomethane	Merck
Triton-X100	Sigma

Tryptone	Sigma
Tryptophane	Sigma
X-Gal	MBI Fermentas
Yeast Extract	Sigma

APPENDIX D

LABORATORY EQUIPMENT

Autoclave: Tuttnauer Systec Autoclave (2540 ml)

Balances: Precisa 620C SCS

Precisa 125 A SCS

Centrifuge: Beckman Coulter, Microfuge 18

Centrifuge rotor: F241.5P

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

-20°C Arçelik 209lt

+4°C Arçelik

Electrophoresis equipments: E – C mini cell primo EC320

Gel documentation system: UVI PHotoMW Version 99.05 for Windows

Incubators: Nüve EN400

Nüve EN500

Orbital shaker incubators: Sertomat S – 2

Thermo 430

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

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

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

pH meter: Mettler Toledo MP220

Spectrophotometer: PerkinElmer Lambda25 UV/VIS Spectrometer

Thermomixer: Eppendorf thermomixer comfort (1.5 ml)

Transillumunator: Biorad UV transilluminator 2000

Vortexing machine: Heidolph Raax top

Waterbaths: Memmert wb-22

Amalgamator: YDM

Ultrafiltration tube: VIVASPIN

Lyophilizator : ALPHA 1-2 LD plus

Power supply: Bio-Rad

Dialysis Tubing Cellulose Membrane: Sigma-Aldrich

SDS-PAGE Apparatus: Bio-Rad

Polyacrylamide Gel Electrophoresis Apparatus: Bio-Rad

GS-800 CALIBRATED DENSITOMETER: Bio-Rad

APPENDIX E

bacA gene sequence

```
  1 - atg att ata ttg gat aat agc att cag aca
 31 - aaa aaa aga act gat tca cta tcc aaa ctc
 61 - att aca gtc aac acg ctc ggc cct gag ggg
 91 - aca agc agt gag tac gca gca aag cat ttc
121 - att tcc aat ttt act ctt cta caa ggg ctg
151 - aac agt aaa ttg tcc ttg cat gat act ttt
181 - gaa tcg tgc atc gaa agg acg ctc caa agc
211 - ccg ctg gaa tat acc atc gtc cca cat gct
241 - tac gat ggc att aag cat ttc tac atg agg
271 - ccg gat ttg cag cta ttg cag atc ttc agg
301 - tgc gat aca ccg atg tac ggc ctg gct gtt
331 - cgt cct gat ttt gaa ttc aga gac gat atg
361 - ctt gat aca tct gtt att gtt tca cat cct
391 - tca cct att aat tta ata aaa tat ttt acc
421 - cgc aaa gat gtt cgt ttc aaa cta gtc aat
451 - tca acc agt caa gcc gca aga aaa gta aaa
481 - gaa ggt ttg tat gac att gcc tta acc aat
511 - gag ctt gca cgg caa aag tac ggg cta acc
541 - ttt gta aaa aca ttc aaa agc att cca atg
571 - agc tgg tca tta ttt gga aaa gga gac gtt
601 - gat gat gaa aac
```

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