CLONING AND EXPRESSION OF *gerE* AND *glmS* GENES IN *ESCHERICHIA COLI* AND *BACILLUS SUBTILIS*, AND INVESTIGATION OF THEIR POSSIBLE INTERRELATION WITH BACILYSIN BIOSYNTHESIS

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ABSTRACT

CLONING AND EXPRESSION OF *gerE* AND *glmS* GENES IN *ESCHERICHIA COLI* AND *BACILLUS SUBTILIS*, AND INVESTIGATION OF THEIR POSSIBLE INTERRELATION WITH BACILYSIN BIOSYNTHESIS

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Bacillus subtilis is the Gram-positive model bacterium that enzymatically produces the dipeptide antibiotic bacilysin. Bacilysin is the simplest bioactive peptide known composed of L-alanine at its N-terminal and L-anticapsin at its C-terminal. In a former work in our laboratory, a mutant strain of *B. subtilis*, namely OGU1 was constructed by *bacA*-targeted pMutin T3 insertion into the parental strain PY79 genome resulting in a genomic organization *bacA'::lacZ::erm::bacABCDEF* and unable to synthesize bacilysin. RT-qPCR, transcriptome, secretome, and proteome studies conducted so far revealed that there are significant differences in the expression of a vast number of genes between OGU1 and PY79, including *gerE* and *glmS* which are significantly downregulated in OGU1. In *B. subtilis*, GerE, a member of LuxR-FixJ family of transcription regulators, is expressed late during sporulation in the mother cell compartment and acts as the master protein of its own regulon. It is involved in spore germination and spore coat assembly. *glmS*, on the other hand, encodes glutamine-fructose-6-phosphate transaminase for cell wall synthesis. These two genes were cloned in *E. coli* DH5α, next subcloned and expressed in *E. coli* BL21 and *B. subtilis* OGU1. The effects of these genes were determined by comparing the parental strain PY79, the mutant strain OGU1, and the OGU1

complemented with cloned and expressed *gerE* and *glmS* with each other, respectively. Phenotypic analyses were performed, including the resistance of endospores against different chemicals, the germination profile of endospores as well as colony morphology and pigmentation of each strain. Moreover, the possible interrelations between *bac* operon and *gerE* and *glmS* functions were investigated by performing electrophoretic mobility shift assays (EMSA). These assays were conducted to elucidate the possible DNA-protein interactions between the *bac* promoter and the purified GerE and GlmS proteins as well as the promoter regions of *gerE* and *glmS* genes and the purified bacilysin. The results indicated that GerE, but not GlmS binds to *bac* promoter while bacilysin did not display any interaction with *gerE* and *glmS* promoters.

Keywords: *Bacillus subtilis*, Bacilysin, *bac operon*, Gene cloning, Phenotypic analyses, Electrophoretic Mobility Shift Assay (EMSA)

ÖZ

gerE VE *glmS* GENLERİNİN *ESCHERICHIA COLI* VE *BACILLUS SUBTILIS*E KLONLANLAMASI, EKSPRESYONU VE BASİLİSİN BİYOSENTEZİ İLE OLASI KARŞILIKLI İLİŞKİLERİNİN ARAŞTIRILMASI

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Bacillus subtilis, basilisin isimli dipeptit antibiyotiği enzimatik olarak üreten Gram pozitif model bakteridir. Basilisin, N-terminalinde L-alanin ve C-terminalinde L-antikapsinden oluşan en basit biyoaktif peptitdir. Standard suş *B. subtilis* PY79'un basilisin üretemeyen OGU1 isimli mutant türevi *bacA*-hedefli pMutinT3 insersiyonu yoluyla laboratuvarımızda daha önce oluşturulmuş, *bacA'::lacZ::erm::bacABCDEF* genomic organizasyonuna sahip olduğu gösterilmişti. Yine laboratuvarımızda yürütülmüş olan RT-qPCR, transkriptom, sekretom, and proteom çalışmaları OGU1 ve PY79 arasında gen ekspresyonu bakımından çok sayıda gen için çok önemli farklılıklar olduğunu ortaya koymuştur. Şimdiki çalışmanın konusunu oluşturan *gerE* and *glmS* transkriptlerinin ve kodladıkları proteinlerin, PY79 suşuna göre OGU1'da oldukça düşük seviyelerde ifade edildikleri gösterilmiştir. *Bacillus subtilis*'in, *gerE* ve *glmS* genleri OGU1'de negatif regüle edilen genlere örnektir. Kendi regülonunun master proteini olan *gerE*, transkripsiyon regülatörlerinin LuxR-FixJ ailesine mensuptur ve sporulasyonun geç aşamalarında ana hücre kompartmanında ifade edilir. Glutamin-fruktoz-6-phosphate transaminaz enzimini kodlayan *glmS* ise hücre duvarı sentezinden sorumludur. Bu iki gen, ayrı ayrı *E. coli* DH5α, *E. coli* BL21 ve *B. subtilis* OGU1'e klonlanmıştır. Sporların farklı

kimyasallara karşı direnci, bu sporların jerminasyon profili, koloni morfolojisi ve pigmentasyonu dahil olmak üzere çeşitli fenotipik analizler ana suş PY79, mutant suş OGU1 ve *gerE* ve *glmS* genlerini yüksek seviyede ifade eden rekombinant OGU1 suşlarında yapılarak sonuçlar karşılaştırılmıştır. *gerE* ve *glmS* genlerinin saflaştırılmış proteinleri ile basilisin operonunun promotör bölgesi ve ayrıca saflaştırılmış basilisin molekülü ile *gerE* ve *glmS* genlerinin promotor bölgeleri arasındaki olası DNA-protein etkileşimlerini aydınlatmak için elektroforetik mobilite kayma analizleri (EMSA) yapılmıştır. GerE'nin *bac* promotoruna bağlandığı gösterilmiş, GlmS ise böyle bir etkileşimde bulunmamıştır. Diğer yandan, mevcut bulgularımız basilisin molekülünün *gerE* veya *glmS* promotor bölgelerine bağlanmadığına işaret etmiştir.

Anahtar Kelimeler: *Bacillus subtilis*, Basilisin, *bac operon*, Gen Klonlama, Fenotipik Analizler, Elektroforetik Mobilite Kayma Analizi (EMSA)

To my nephew Yiğit Çakan, who is the joy of my life

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CHAPTER 1

INTRODUCTION

1.1 *Bacillus subtilis*

1.1.1 *Bacillus subtilis*: The Gram-Positive Model Organism

In 1835, Christian G. Ehrenberg, who was a German naturalist, described an organism as "*Vibrio subtilis*". Then, Ferdinand Cohn put a new name for this organism as *Bacillus subtilis*, and he also categorized it as a branch of the family Bacillaceae in 1872 (Gordon, 1981). *Bacillus subtilis*, which is one of the main branches of the genus *Bacillus*, is a Gram-positive, rod-shaped, mobile bacterium which is able to generate endospores that possess resistance against various extraneous stresses. The genus *Bacillus* includes several pathogenic bacteria such as *B. anthracis* (the causative agent of anthrax) and *B. cereus* (the causative agent of food poisoning). However, there is no pathogenic potential for *Bacillus subtilis* (Wipat and Harwood, 1999; Sonenshein *et al.*, 2002). Due to its natural transformation mechanism, *B. subtilis* has been a crucial organism for genetic manipulations and analyses way before the emergence of DNA technology (Harwood, 1992). *B. subtilis* was effectively utilized for a variety of research like quorum sensing, metabolic pathways, differentiation, and sporulation since the genetic manipulation of this organism is practically effortless. While the best-studied model organism for Gram-negative bacteria is *Escherichia coli*, *Bacillus subtilis* represents itself thoroughly as a model organism for Gram-positive bacteria. (Harwood, 1992; Zweers *et al.*, 2008).

Biotechnologically, *B. subtilis* is an essential and critical organism because the capability of producing multifarious secondary metabolites, antibiotics, and enzymes is considerably broad (Sonenshein *et al.*, 2002). For example, the probiotic strains of *B. subtilis* are capable of secreting exopolysaccharides that guard against gut inflammation (Jones *et al.*, 2014). Moreover, the capacity of secreting the products of cloned genes into culture supernatants in large quantities is better in *B. subtilis* than in *E. coli* since the inclusion bodies and pyrogenic lipopolysaccharide create problems for protein production in *E. coli* (Zweers *et al.*, 2008). Additionally, a recent study showed that the generation of nanobodies in genetically engineered recombinant host *B. subtilis* thanks to its many features such as being a stable organism, the capability of protein secretion, and the defiance against challenging situations (Yang *et al.*, 2020).

Even though *B. subtilis* is a soil bacterium, it can be found in several different environments, such as the gastrointestinal tract of humans, animals, and water sources (Nicholson, 2002). Also, *B. subtilis* is categorized as a chemoorganotroph and has a capacity for oxidizing organic compounds in order to grow. In addition to being a chemoorganotroph, *B. subtilis* is mesophilic, and this feature shows parallelism with many other members of the genus *Bacillus*. Therefore, the colonies of *B. subtilis* can be observed in optimal sizes when grown at 37°C for 16 hours with the essential nutrients and aeration (Harwood and Cutting, 1990). For many years, *B. subtilis* has been considered ~~as~~ a strict aerobic bacterium, yet the research showed that the anaerobic environment was enough for its growth. Anaerobic growth of *B. subtilis* is possible due to the fermentation or the utilization of nitrate or nitrite as the electron acceptor. When there is an activity in ResDE two-component signal transduction system, the FNR protein is expressed by this system. Subsequently, FNR protein triggers the induction of anaerobic respiration genes. This is the simplest explanation of the metabolic shift from aerobic to anaerobic (Nakano and Zuber, 1998).

B. subtilis uses several types of survival mechanisms in challenging environments like the deprivation of nutrients. These mechanisms include motility, chemotaxis,

and the generation of many hydrolases and antibiotics. However, the transcendent mechanism to survive is triggering the sporulation or deactivation (dormant) stage. The compartmentalization of mother cell and forespore cell from a single *B. subtilis* cell is the beginning of the sporulation. The mother and forespore cells each possess a copy of the bacterial chromosome. Each bacterial chromosome undergoes differential gene expression and regulation in its own compartment. As a result, the forespore is engulfed, and the programmed death of the mother cell starts. In the end, the endospore, which has a resistivity to dryness, a variety of chemicals, irradiation, and heat, is formed by this complicated procedure (Stragier and Losick, 1996).

In 1997, a group of scientists performed genome analyses in *B. subtilis* and reported its genome size as 4.2 Mb (Kunst *et al.*, 1997). Thanks to ensuing research, it was discovered that the genome involves 4106 protein-coding genes, 30 rRNA, 86 tRNA, and three small RNA genes (Ando *et al.*, 2002; Kobayashi and Ogasawara, 2002). Subsequent studies have shown that only 275 genes were detected as critical in order to grow under optimal experimental requirements despite the fact that the whole genome of *B. subtilis* is comprised of 4200 genes. Additional to the aforementioned genes, 17 sigma factors and 250 transcriptional regulators were determined in the genome (Kobayashi *et al.*, 2003).

The origin of *B. subtilis* 168, a tryptophan auxotroph strain, is *B. subtilis* Marburg with sublethal X-ray mutagenesis (Zeigler *et al.*, 2008). This strain is mainly utilized for genetic manipulation experiments and has become the standard strain (Spizizen, 1958; Harwood and Cutting, 1990). Later, the derivative of *B. subtilis* 168 emerged and is known as the prototrophic *B. subtilis* PY79 strain. This strain was widely utilized as a wild-type strain by scientists (Youngman *et al.*, 1984). Nevertheless, our lab-adapted PY79 strain possesses many differences from the reference PY79 because of repeated cultivation and continuous transpositions in our laboratory for the past forty years. The difference between the reference PY79 and our lab-adapted PY79 is that our strain has one deletion and five SNPs.

1.1.2 Primary Metabolism in *B. subtilis*

B. subtilis needs energy for the continuation of its life cycle like all living organisms. Until 1998, *B. subtilis* was categorized as a strictly aerobic bacterium for decades. However, Nakano and Zuber (1998) classified the same organism as a facultative anaerobe. To grow in anaerobic conditions, *B. subtilis* uses nitrate or nitrite as a final electron acceptor or chooses a fermentation pathway (Sun *et al.*, 1996). To put it more precisely, the metabolic activity of *B. subtilis* depends on the presence of oxygen and the type of electron acceptor.

ResD is a response regulator, and ResE is a sensor kinase, these two create a two-component signal transduction system. In *B. subtilis*, the primary regulator of anaerobic respiration is ResDE two-component signal transduction system, and this system affects crucially the early stages of anaerobic gene regulation. The oxygen limitation is the key factor for the induction of *fnr* transcription by ResDE. Transcriptional activator FNR is responsible for the activation of the respiratory nitrate reductase operon, *narGHJI*, together with the activated anaerobic respiration genes. To metabolize pyruvate, pyruvate dehydrogenase is needed, so *B. subtilis* chooses the fermentation pathway under certain conditions without any external electron acceptors, unlike many anaerobes (Nakano and Zuber, 1998).

Anaerobic and aerobic respirations cause alterations in the balance of the dynamic relationship between nitrogen and carbon metabolism. According to a comparative study about protein expression in *B. subtilis* considering aerobic growth, anaerobic, and nitrate respiration (Clements *et al.*, 2002), at least 44 proteins were induced by anaerobic fermentation, and at least 19 proteins were induced by nitrate respiration compared to aerobic growth controls. The same researchers also mentioned the upregulation of numerous metabolic proteins regarding their environments. Anaerobic growth and nitrate respiration unitedly caused the induction of some proteins; however, the induction of several other proteins was triggered by either fermentation or nitrate respiration.

The metabolic alternations mentioned earlier are very critical for the industrial applications of *B. subtilis*, because distinct environmental conditions play a key role in the synthesis and secretion of different proteases. *B. subtilis* is used for industrial purposes to collect the supplement poly-glutamic acid, the flavor agent ribose the vitamin riboflavin, and several enzymes like proteases and amylases (Schallmey *et al.*, 2004). According to another research (Renna *et al.*, 1993), the changes in environmental circumstances might result in the changes of metabolic products in *B. subtilis*. In this way, different types of industrial compounds can be secreted. *B. subtilis* is an important organism for industrial purposes since it possesses an extensive capacity for producing and secreting various industrial compounds such as enzymes and biopharmaceuticals without any challenges and effort (Westers *et al.*, 2004).

1.1.3 Secondary Metabolites and Antibiotics of *B. subtilis*

Biologically active peptides are structurally multifarious, so they possess various functions in cellular physiology. A variety of secondary metabolites with pharmacological or antimicrobial activities can be produced from *B. subtilis* due to its unique metabolism. There are two ways for the production of these peptides: non-ribosomally or ribosomal synthesis. The posttranslational modification only occurs after the production of the peptide with ribosomal synthesis, as in the case of lanthionine production. The antibiotics involving lanthionine are called lantibiotics (Schnell *et al.*, 1988; Zuber *et al.*, 1993; Weber and Marahiel, 2001).

Apart from being antimicrobial agents, there are several features of peptide antibiotics of *B. subtilis*. For instance, the role of lantibiotics in quorum sensing is to function as pheromones, or they initiate programmed cell death by acting as killing factors. Another example is that non-ribosomally synthesized lipopeptides are affiliated with the formation of biofilm and swarming motility (Stein, 2005). The structures and roles of lantibiotics and non-ribosomally manufactured peptide

antibiotics of *B. subtilis* can be observed in Table 1.1 (lantibiotics) and Table 1.2 (non-ribosomally).

Table 1.1. The roles and structures of lantibiotics of *B. subtilis* (Stein, 2005).

Antibiotic	**Role**	**Structure**
Ericin	Acting like pheromone, subtilin-like activity	Cyclic
Mersacidin	Inhibition of cell wall synthesis	Globular
Subtilin	Acting like pheromone	Cyclic
Subtilosin A	Acting against some Gram (+) bacteria	Cyclic

Table 1.2. The roles and structures of non-ribosomally produced peptide antibiotics of *B. subtilis* (Stein, 2005).

Antibiotic	**Role**	**Structure**
Bacilysin	Probable pleiotropic molecule	Linear
Bacillibactin	Siderophore	Cyclic
Fengycin	Acting on filamentous fungi specifically	Cyclic
Surfactin	Acting like detergent on biological membranes	Cyclic
Bacillomycin	Hemolytic and antifungal activity	Cyclic lipopeptide
Iturin	Hemolytic and antifungal activity	Cyclic lipopeptide
Mycosubtilin	Hemolytic and antifungal activity	Cyclic lipopeptide

1.1.4 Bacilysin: The Dipeptide Antibiotic

In 1946, a peptide antibiotic named bacilysin synthesized by *Bacillus subtilis* was discovered due to its antimicrobial activity against *Staphylococcus aureus* (Abraham *et al.*, 1946). Only two amino acids are required to produce bacilysin. The first amino acid is L-alanine, which is located at the N-terminus of the molecule. The second amino acid is L-anticapsin, a non-proteinogenic amino acid, and it is located at its C-terminus. The molecular weight of the dipeptide bacilysin molecule is only 270 Da (Walker and Abraham, 1970). *S. aureus* is not the only organism affected by bacilysin's antimicrobial activity. There are also several bacteria and a few fungi (such as *Candida albicans*) which are influenced by bacilysin's inhibitory effect on their growth. The inhibition mechanism of bacilysin can be explained as follows: when the target organism takes in the bacilysin, it is cleaved by the intracellular peptidases, releasing the anticapsin part of the molecule becomes free, which will eventually behave as a glucosamine-6-phosphate synthase inhibitor (Kenig *et al.*, 1976). Thus, the antimicrobial activity of bacilysin comes from anticapsin, and protoplast formation and subsequent cell lysis occur because of anticapsin's antimicrobial activity (Walton and Rickes, 1962).

Even though only two amino acids are required to form bacilysin, the biosynthesis of this molecule is quite complex. Prephenate is the branch point for biosynthesizing aromatic amino acids like phenylalanine and tyrosine, and prephenate provides the formation of anticapsin molecule according to molecular genetic study working with non-producing bacilysin mutant (Hilton *et al.*, 1988). *bacD* (*ywfE*) is responsible for synthesizing an enzyme (alanine-anticapsin ligase) that creates a link between anticapsin molecule and L-alanine (Sakajoh *et al.*, 1987; Tabata *et al.*, 2005). The investigation done by our research group revealed that ATP hydrolysis was not monitored for anticapsin, it took place only for L-alanine. Therefore, that anticapsin moiety is not activated as an amino acid phosphate (Yazgan *et al.*, 2001a).

In *B. subtilis* 168, the biosynthesis of bacilysin is performed by the *ywfBCDEFG* operon and the monocistronic *ywfH*, which are reidentified as *bacABCDEF* operon

and *bacG* (Inaoka *et al.*, 2003; Steinborn *et al.*, 2005). Figure 1.1 represents the composition of *bacA* operon and monocistronic *bacG* (Özcengiz and Öğülür, 2015).

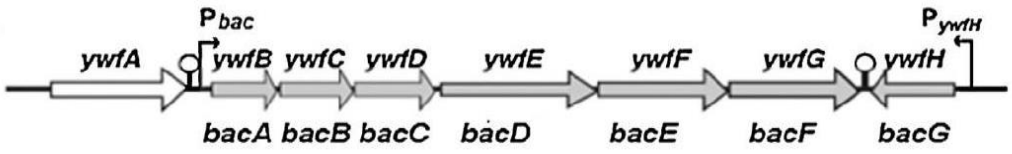

Figure 1.1 *bacA* operon and the monocistronic *bacG* with their previous *ywf* names (Özcengiz and Öğülür, 2015).

The role of each gene in this composition was examined by plenty of research. Figure 1.2 shows the pathway starting with prephenate, which is transformed into the dihydroanticapsin in several steps with the help of four enzymes (BacA, BacB, BacG, and BacF, sequentially) (Rajavel *et al.*, 2009; Mahlstedt *et al.*, 2010; Parker and Walsh, 2012). Then, dihydroanticapsin is oxidized and converted into the anticapsin form by BacC, an NAD^+ dependent alcohol dehydrogenase (Parker and Walsh, 2013). In the final step, alanine-anticapsin ligase, BacD, ligates L-anticapsin and L-alanine by creating the dipeptide bond (Tabata *et al.*, 2005). Even though BacE protein does not have any connections with the biosynthesis of bacilysin, it possesses a crucial role by sending bacilysin out, into the culture broth. In this way, the producer of bacilysin gains resistance to bacilysin (Steinborn *et al.*, 2005).

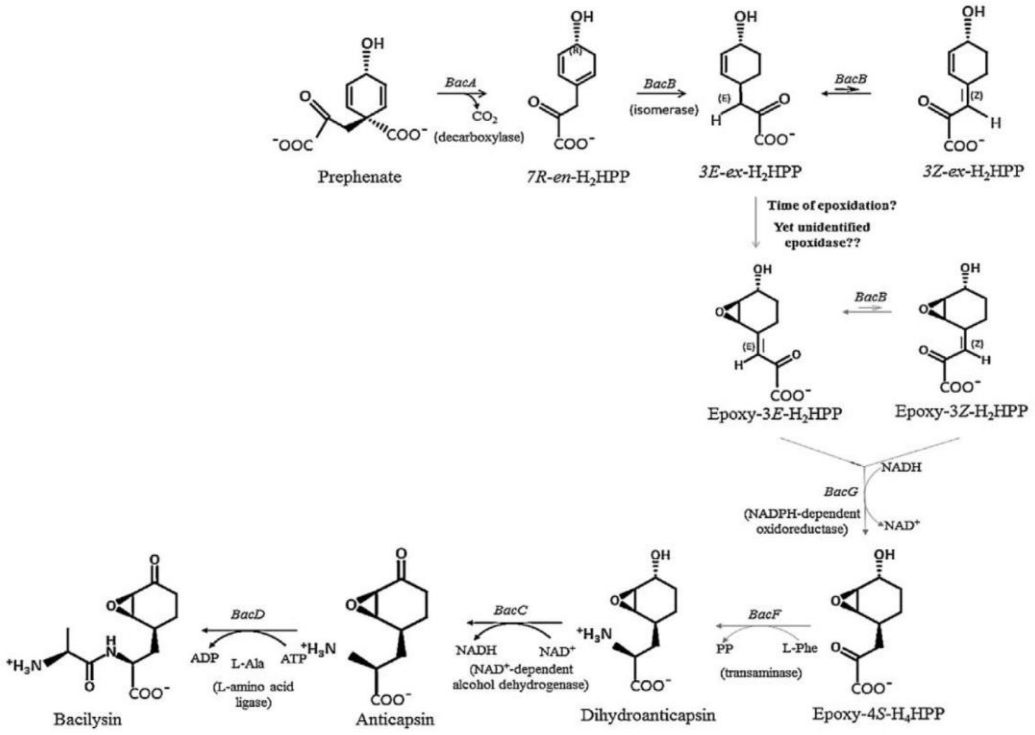

Figure 1.2 Complicated biosynthesis of bacilysin from prephenate in several steps (Parker and Walsh, 2012).

For proper bacilysin biosynthesis, Mg^{+2} and ATP are two things needed by BacD, an alanine-anticapsin ligase, in order to operate consummately. It was shown that nitrogen sources in the medium like ammonium and casamino acids are responsible for repressing or inhibiting bacilysin production. Still, it was produced in normal levels when sucrose and glutamate were present in the medium. Nitrogen sources are not the only sources that repress or inhibit the production of bacilysin. Its production was shown to be also affected on a small scale when other carbon sources are present in the medium rather than sucrose and glucose (Özcengiz *et al.*, 1990, Özcengiz and Alaeddinoğlu, 1991). Moreover, feedback regulation, temperature, and pH also control bacilysin production (Özcengiz and Alaeddinoğlu, 1991).

Bacteria can adapt to the nutrient insufficiency in the environment due to the regulatory metabolite of the stringent response called guanosine 5'-diphosphate 3'-diphosphate (ppGpp). Like several other genes, the expression of bacilysin biosynthetic operon is regulated by the stringent response. In detail, intracellular ppGpp, GTP, and the GTP-sensing transcriptional repressor CodY are responsible for this positive regulation. The study by Inaoka *et. al.* (2003) has shown that the regulation of *bacA* operon was affected positively by high amounts of ppGpp, while increased GTP levels and CodY negatively influenced the regulation of the same operon.

To identify genes associated with the biosynthesis of bacilysin, our group applicated transposon mutagenesis. *oppA*, which has a connection with bacilysin biosynthesis, is responsible for coding the oligopeptide permease (Opp or Spo0K) protein, and this protein is crucial for quorum sensing. Another result from the same study deduced that *phrC* and *comA*-deleted strains of *B. subtilis* could not produce bacilysin, revealing the connection between quorum sensing and bacilysin biosynthesis (Yazgan *et al.*, 2001b). Another research conducted by our group has demonstrated that *comP* inactivated cells have decreased bacilysin production, and *comQ* mutants lost their ability to produce bacilysin completely (Karataş *et al.*, 2003). Additionally, the same research has revealed a link between biosynthesis of bacilysin and *srfA* operon that encodes the lipopeptide antibiotic surfactin, since the mutants with disrupted *srfA* operon were not capable of bacilysin production. Moreover, another conclusion from the same research was that the production of bacilysin did not occur when *spo0A* was blocked. Supplementary research has shown that the bacilysin production was regained when the previously studied mutated strains were additionally disrupted in *abrB*. This result indicated that the *bacA* operon is inhibited by the regulatory AbrB protein, and this inhibition can be hindered with the Spo0A, which is capable of blocking AbrB (Karataş *et al.*, 2003).

Our previous research proved that the peptide-based quorum sensing pathway positively influences bacilysin production. However, the same production is affected negatively by CodY and AbrB. Further investigations were performed to elucidate

their role in *bacA* operon on a molecular level. To do so, pMutin T3 which targeted *bacA* was inserted, and the outcome of this was *B. subtilis* OGU1 strain that is not able to generate bacilysin at all. The activity of P_{bac} could be detected by a β-galactosidase assay due to the integration of *lacZ*. The β-galactosidase assays were performed separately or conjointly to understand the impacts of mutation in 11 genes (*abrB*, *codY*, *comA*, *comP*, *comQ*, *oppA*, *phrC*, *phrF*, *phrK*, *spo0A*, and *spo0H*). The analyses concluded that there was a decrease in *bacA* operon expression for all mutants except *abrB* mutants, where increased expression of *bacA* operon was observed. In order to detect possible DNA-protein linkage, electrophoretic mobility shift assays (EMSA) were applied by using the proteins of previously mentioned 11 genes and the promoter region of bacA operon. The result proved that there was a direct binding between Spo0A, ComA, AbrB, and CodY proteins and P_{bac}, and those proteins were capable of regulating the *bacA* operon (Köroğlu *et al.*, 2011).

Research conducted by Mariappan *et al.* revealed that the regulation of *bacA* operon and *bacG* was affected positively by DegS/DegU two-component system (2012). Protease production and sporulation were negatively regulated by ScoC, which was able to bind P_{bac} and impacted its regulation also negatively (Inaoka *et al.*, 2009). Moreover, the phenotypes of *yvfI* mutants were similar to bacilysin-negative ones (Köroğlu *et al.*, 2008). Additionally, the transcriptional repressor LutR, which is involved in regulating various physiological activities, was found to be essential for bacilysin synthesis in *B. subtilis* (İrigül-Sönmez *et al.*, 2014). The regulation of *bacA* biosynthetic operon is shown in Figure 1.3 (Ertekin *et al.*, 2020).

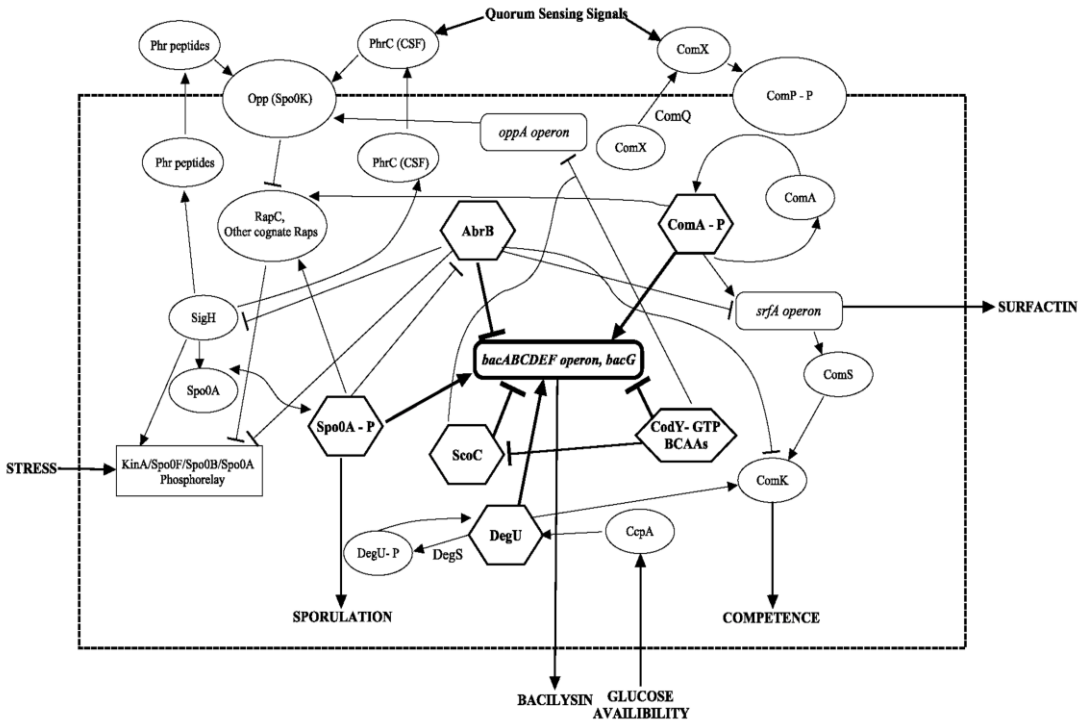

Figure 1.3 Regulation of *bacA* biosynthetic operon (Ertekin *et al.*, 2020). The meaning of arrows is activation, T-bars mean repression. Direct interactions with P_{bac} are represented with bold lines.

1.2 Sporulation in *Bacillus subtilis*

1.2.1 The Beginning of Sporulation

B. subtilis has a specialized coping mechanism against challenging circumstances such as nutrient deprivation. This mechanism includes several steps and eventually leads to endospore formation for survival (Stragier and Losick, 1996). The endospore stays in the dormant stage, and it possesses a resistance mechanism against challenging conditions such as temperature, radiation, and several chemicals due to its compact chromosomal DNA and dehydrated spore core (Nicholson *et al.*, 2000). The asymmetric division is the beginning step of the sporulation, resulting in the formation of two types of cells at one pole of the cell. The larger cell is called the

mother cell, the smaller cell is called the forespore, and a septum demarcates them from each other. The mother cell then engulfs the forespore, and gene expression begins in each cell separately and individually. Each gene expression occurs under the control of their own sigma factors. In addition to those sigma factors, the communication between the mother cell and forespore is essential for the continuation of sporulation. The outcomes of those gene expressions, which take place separately in the mother cell and forespore, are the programmed death of the mother cell and the maturation of the forespore into an endospore or spore (De Hoon *et al.*, 2010; Higgins and Dworkin, 2012). Each sigma factor that is specific to either the mother cell or forespore at different stages of forespore maturation, is represented in Figure 1.4 (Setlow and Christie, 2020).

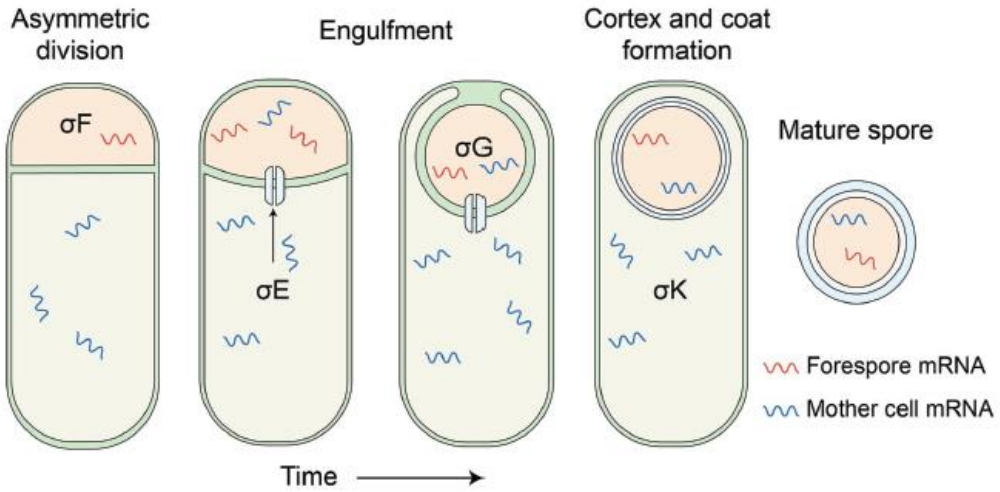

Figure 1.4 Forespore and mother cell-specific sigma factors and the steps of forespore generation (Setlow and Christie, 2020).

As the first step of sporulation, transcriptional factor Spo0A becomes activated by phosphorylation (Sonenshein, 2000). The sensory kinases KinA-E receive a signal related to induction of sporulation to activate Spo0F which subsequently activates Spo0A. The main one of those kinases is KinA, and the sporulation mechanism is

initiated immediately by over-expressed KinA (Fujita and Losick, 2005). There are some factors that are responsible for the regulation of the abovementioned kinases. For example, autophosphorylation of KinA can be blocked by the inhibitor KipI (Wang *et al.*, 1997). KipA is able to inhibit KipI since both of them are encoded by the same operon. KipA/KipI protein couple is a good example of histidine kinase regulation in *B. subtilis*. Another example is the inhibition of KinA and KinB sensory kinases by the cell cycle-related protein, Sda (Burkholder *et al.*, 2001). The expression of cell cycle-related protein Sda is a signal for the cell for DNA replication and the inhibition of the sporulation process since the cell prepares itself for cell division, meaning that the sporulation and cell division cannot take place at the same time (Veening *et al.*, 2009). Sda is similar to KipI, and both can hamper the autophosphorylation of KinA. In addition to hampering the autophosphorylation of KinA, the transmission of phosphate from KinA to Spo0F is also hampered by Sda (Cunningham and Burkholder, 2009).

Even though the transcription factor Spo0A is an essential member at the onset of sporulation, there are some other characteristic features of this transcriptional factor involved in processes such as cannibalism or biofilm formation (López and Kolter, 2010). The amount of active Spo0A or Spo0A-P is crucial for those physiological processes. For instance, the gene expression of two significant operons, *skf* (sporulation killing factor) and *sdp* (sporulation delaying protein), are triggered by active Spo0A, when the amount of Spo0A-P is relatively low. After gene expression, the products of those operons cause the death of non-resistant sister *B. subtilis* cells, which is called cannibalism (González- Pastor *et al.*, 2003). On the other hand, when the amount of Spo0A-P rises, above mentioned processes are supplanted by sporulation.

A total of 520 genes, 121 of them directly, are regulated by activated Spo0A (Molle *et al.*, 2003). Spo0A suppresses *abrB*, which is responsible for encoding a transcriptional regulator. Next, stationary phase genes, as well as *sigH* are inhibited by the transcriptional regulator encoded by *abrB*. Therefore, the indirect regulation of Spo0A takes place on σH by activating the expression of σH via boosted Spo0A

expression (Banse *et al.,* 2008). The *spoII-spoIIAB-sigF* operon is also expressed under the control of the σH. σF, anti-sigma factor SpoIIAB, and anti-anti-sigma factor SpoIIA are the products of this operon. σF, the first forespore-specific sigma factor, is critical since 50 genes and *sigG* are regulated by σF (Steil *et al.*, 2003; Wang *et al.*, 2006). Meanwhile, Spo0A-P is responsible for encoding a pro-peptide named σE, the first mother cell-specific sigma factor. This pro-peptide is cut by a protease called SpoIIGA. However, one of the earliest proteins made in the forespore under the control of σF, SpoIIR, stimulates this cleavage (Imamura *et al.*, 2008). This is one of the examples of why the communication between the mother cell and forespore is critically vital for the sporulation process. σE regulates the expression of SpoIIIAH and seven other proteins responsible for the activation of σG. Additionally, SpoIIIAH can join SpoIIQ to constitute a gate between the mother cell and the forespore (Meisner *et al.*, 2008). The second sigma factor activated in the mother cell is σK, and its expression is also regulated by σE. Similar to σE, σK is constructed as a pro-peptide, then SpoIVFB protease cleaves it to activate. σG, present in the forespore, coordinates the formation of the signaling protein SpoIVB, and the presence of SpoIVB causes elevated SpoIVFB activity (Cutting *et al.*, 1991). Despite the fact that sporulation is far more complex than what is detailed here, σH, σF, σE, σG, and σK are sequentially activated in individual compartments. The transcriptional regulatory network of endospore formation in *B. subtilis* is shown in Figure 1.5 (De Hoon *et al.*, 2010).

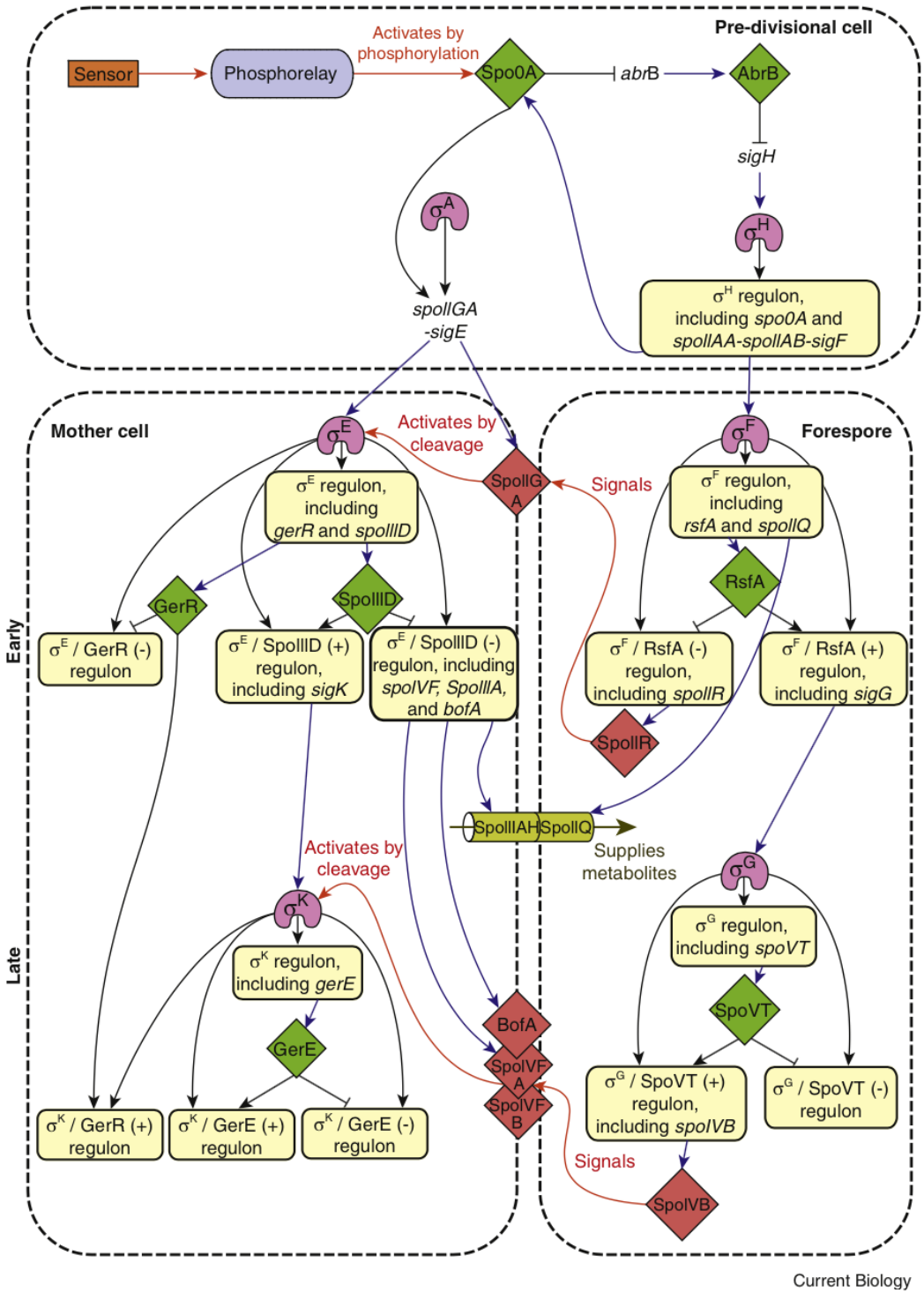

Figure 1.5 Regulatory network during endospore generation in *B. subtilis* (De Hoon *et al.*, 2010).

1.2.2 Morphology of Spores

The mature endospore of *B. subtilis* is a complex structure that tolerates extrinsic stress. Small acid-soluble proteins (SASPs) that are present in the core of the endospore are responsible for the preservation of the chromosomal DNA from any kind of damage. The forespore's cellular membrane surrounds this core structure due to the engulfment of the forespore, and this membrane is encircled by a layer known as the spore cortex, which is abundant in a high amount of peptidoglycan. On this cortex, spore coat proteins combine to form a multilayer structure (Higgins and Dworkin, 2012). Over 70 proteins are present at the inner and outer layers of this multilayer structure, and those proteins guard the spore core (Henriques *et al.*, 2004). Each region of the *B. subtilis* endospore and its whole morphology is shown in Figure 1.6 (Zeigler and Perkins, 2008).

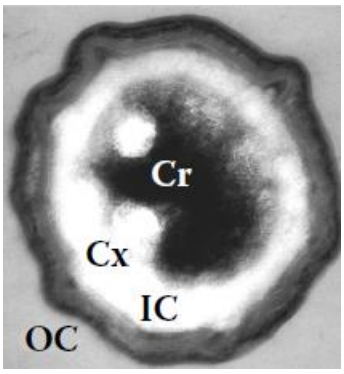

Figure 1.6 Endospore morphology in *B. subtilis* with its specific regions (Zeigler and Perkins, 2008). Cr: Spore core; Cx: Spore cortex; IC: Inner coat; OC: Outer coat.

The coat layers and the spore cortex are synthesized by the mother cell compartment. The first mother cell-specific sigma factor, σE, regulates the expression of a crucial protein SpoIVA, which is responsible for the clustering of spore coat (Wang *et al.*, 2009). The localization of SpoIVA occurs at the outer forespore membrane. At the same time, this protein interacts with SpoVM, which is a mother cell-specific protein

(Ramamurthi *et al.*, 2006). This interaction is extremely critical for the coat assembly (Ramamurthi and Losick, 2008). *σE* is responsible for the regulation of another key coat morphogenic protein, SpoVID. SpoVID's correct accumulation on the forespore membrane depends on the presence of SpoIVA (Beall *et al.*, 1993). One of the proteins required by the spore coat is called SafA, which interplays with SpoVID (Müllerová *et al.*, 2009). Research conducted by McKenney *et al.* (2010) has shown that the inner coat of the *safA*-mutated spores became slimmer and that the inner surface could not attach to the spore surface rigidly. This dysfunction eventuates because SafA has an interconnection with 16 inner coat proteins and a capacity to change the localization of those proteins. σE and σK control the formation of CotE. It is noteworthy that CotE has an indispensable role in the clustering of the inner and outer spore coats. It was shown that *cotE* mutants do not assemble correctly (Zheng *et al.*, 1988). CotE also orchestrates the clustering of other coat proteins such as CotC and CotU (Isticato *et al.*, 2010). The activation of σK triggers the inner and outer coats to finally be put together and mature, and various σK-controlled proteins, such as CotD, CotSA, CotS, CotT, CwlJ, and OxdD, unite to create the spore coat (Driks, 2004). The functionary sigma factors and sigma factor-controlled proteins in the assembly of the spore coat are displayed in Figure 1.7 (Henriques and Moran, 2007).

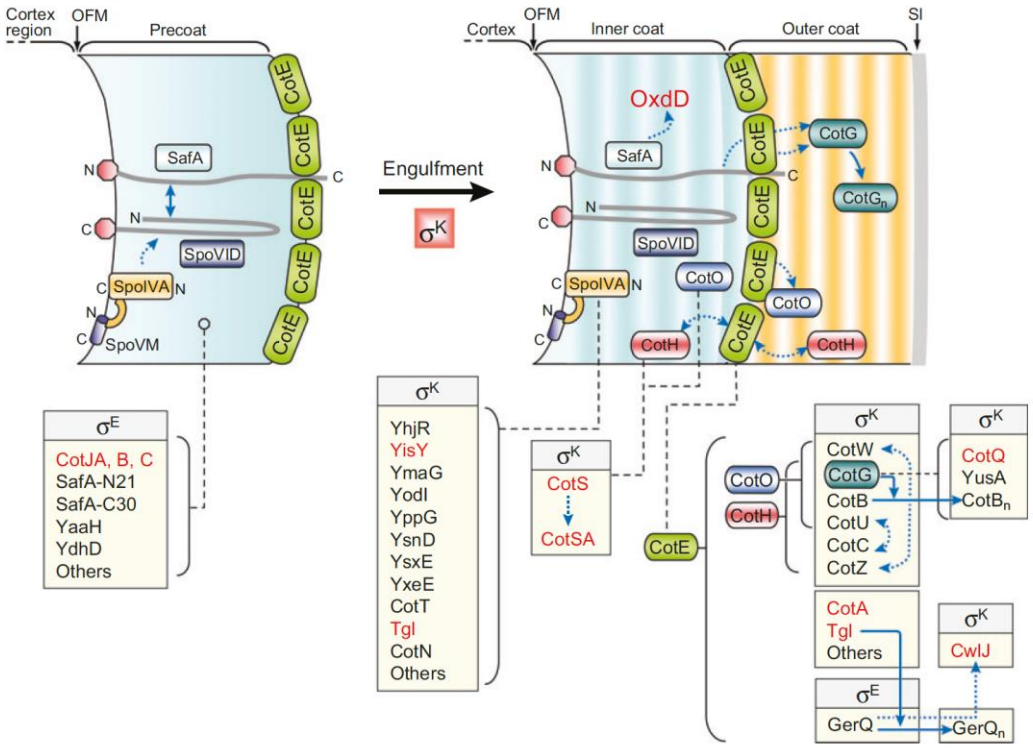

Figure 1.7 Diagram of sigma factors and their related proteins in the assembly of endospore coat (Henriques and Moran, 2007).

Endospores are protected against tough conditions like heat and drought by the spore cortex, which coordinates the spore core to be dehydrated. The main component present in the cortex is peptidoglycan. However, this peptidoglycan differs from the vegetative peptidoglycan since it possesses less peptide side (Popham, 2002). Moreover, peptidoglycan present in the cortex is O-acetylated, which makes the cortex less affected by antimicrobial lysozyme (Laaberki *et al.*, 2011). The formation of both spore coat and spore cortex is controlled by mother cell-specific proteins. One example is SpoVE, which is critical for the peptidoglycan polymerization (Vasudevan *et al.*, 2007). SpoVE and SpoVD can cooperate, and the expression of SpoVD is regulated by σE. This protein is important for spore cortex production (Fay *et al.*, 2010).

1.2.3 Spore Resistance

In *B. subtilis*, endospores are not enzymatically active. In other words, the DNA or macromolecules cannot be repaired. If there is any damage, they are only corrected during germination or outgrowth. Nevertheless, if the damage is unmanageable, the spore dies in germination (Setlow, 1995; Setlow *et al.*, 2002; Setlow, 2003). In order to avoid this kind of harm, spores develop special defense/resistance mechanisms for long-time survival under harsh circumstances.

One of the peculiar characteristics of bacterial endospores is their resistance to wet heat. Even though spores that undergo the vegetative stage of *B. subtilis* are immediately destroyed by 90^{o}C wet heat, non-vegetative spores are resistant to wet heat for 20 minutes (Gerhardt and Marquis, 1989). The spore core water content is a major factor in spore resistance to wet heat. A number of factors can impact this water content, and decreased water content leads to improved resistance to wet heat stress (Melly *et al.*, 2002). The decreased water content results from dipicolinic acid (DPA), which is only present at the spore core. The chelation occurs between DPA and divalent cations such as Ca^{2+} or Mg^{2+}. This interaction further increases the spore resistance by reducing water content (Gerhardt and Marquis, 1989). According to two different studies by Nicholson *et al.* and Setlow, spores without their alpha and beta SASPs are more vulnerable to wet heat, although wet heat is not linked to DNA damage in spores (2000; 2011). Additionally, *B. subtilis* endospores possess a resistance mechanism to dry heat. Nonetheless, since dry heat causes severe DNA damage unlike wet heat, this resistance is primarily mediated by major SASPs (Espitia *et al.*, 2002).

Thanks to their solid and complicated coat structures, bacterial spores also develop resistance mechanisms to a variety of chemicals and enzymes. The thick peptidoglycan part of the cell wall in Gram-positive bacteria is affected by lysozyme because it has the capacity to block peptidoglycan synthesis, thereby cell wall synthesis. Lysozyme can also impact the peptidoglycan in the spore cortex, so each spore coat protein present in the spore coat is vitally important for lysozyme

resistance. For instance, *cotE* null-mutated spores do not have an outer spore coat which leads to more susceptibility to lysozyme since CotE is one of the essential agents for the generation of the outer coat (Zheng *et al.*, 1988). The lysozyme resistance of mutants that express *cotE* at subsequent phases was almost the same as the wild type, but they lack an outer coat. This result indicates that CotE may have other roles in lysozyme resistance (Costa *et al.*, 2007). In addition to the outer coat and its proteins, there are inner coat proteins, such as CotH play a critical role in lysozyme resistance (Naclerio *et al.*, 1996). Spores can resist certain chemicals such as organic solvents, oxidizing agents, and alkylating agents due to the integrity and complexity of their inner and outer coatings (Setlow, 2011). The inner and outer coats guard the spore by detoxifying and prohibiting the entry of those chemicals into the sensitive interior of the spore. Moreover, the spore's inner membrane hinders penetration even more since it has a remarkably poor permeability to tiny and hydrophobic molecules (Gerhardt *et al.*, 1972).

1.2.4 Germination

B. subtilis endospores continuously monitor the environment in which they exist. Germination could happen under appropriate circumstances, followed by the formation of vegetative cells (Paidhungat and Setlow, 2002). While monitoring their environments, spores can detect some agents such as germinants that are generally purine derivatives, amino acids, and sugar. In addition to those types of-germinants, *B. subtilis* spores can germinate when they encounter an extraordinary mixture like AGFK (Asparagine, Glucose, Fructose, and Potassium ion) (Setlow, 2003). According to Hudson *et al.*, the GerA family of germinant receptors, which are formed in the late stages of sporulation and positioned in the endospore's inner layer, are extremely sensitive to mentioned germinants (2001). These germinant receptors set off a cascade of physiological reactions for germinant attachment. The onset of these reactions includes the delivery of monovalent cations like H^+, Na^+, K^+, and Zn^{2+} to the outside by the spore core, and those cations rearrange the pH of the

environment by boosting it from 6.5 to 7.7. This pH alteration is essential for enzymatic activity (Jedrzejas and Setlow, 2001). Subsequently, the spore core also delivers dipicolinic acid (DPA) with chelated Ca^{2+}, and this structure switches places with water. Consequently, refractivity and wet heat resistance are reduced; however, enzymes are still inactive (Cowan *et al.*, 2003). Cortex lytic enzymes are triggered to be activated by the production of DPA-Ca^{2+}, and the peptidoglycan found in the spore cortex starts to break down. Water is taken into the core because of this breakdown, which leads to the enlargement of the germ cell wall, the start of enzymatic activity, and finally, outgrowth (Setlow *et al.*, 2001).

The two critical enzymes formed during sporulation, SleB and CwlJ, are responsible for the breakdown of the spore cortex in *B. subtilis*. For complete germination, one of these enzymes is sufficient to trigger the process. Nevertheless, Makino and Moriyama (2002) have proven the importance of both enzymes as the double mutant spores could not break down the spore cortex. Muramic-deltalactam is an essential molecule for the proper function of these enzymes since the breakdown of the cell wall could not occur due to the absence of this molecule (Moir *et al.*, 2002). Even though CwlJ is only present at the spore coat, SleB is present at many subcellular locations like the coat outer membrane, cortex, and coat layers (Chirakkal *et al.*, 2002). Some critical proteins are also needed for mentioned enzymes to unite and function correctly: YpeB is a necessary protein for SleB; while GerQ is the main protein for CwlJ to function appropriately (Boland *et al.*, 2000; Ragkousi *et al.*, 2003). However, the SleB activation system is obscure in that it may be associated with alterations in the spore cortex content (Tovar-Rojo *et al.*, 2002).

Not only can nutritional germinants induce the germination process, but also several non-nutrient germinants such as lysozyme, DPA-Ca^{2+}, and high pressure can. The spore cortex could be broken down by lysozyme to initiate germination, but there are some conditions. Before adding lysozyme, a pre-treatment is performed to dispose of spore coats. Moreover, the lysozyme concentration should be minimal to avoid any cell wall deterioration. If those conditions are provided, then lysozyme can be a potential germinant. Research conducted by Paidhungat *et al.* concluded that

exogenous DPA-Ca^{2+} initiates the germination process by direct or indirect activation of CwlJ, not SleB or any other germinant receptor (2001). In addition to lysozyme and DAP-Ca^{2+}, high pressure is another non-nutrient germinant. Germinant receptors are activated to initiate germination under low pressures (Wuytack *et al.*, 2000). However, the activation of receptors is omitted under high pressures. The direct delivery of DPA-Ca^{2+} takes place due to high pressures without activating any receptors (Paidhungat *et al.*, 2002). In *B. subtilis*, both nutrient and non-nutrient germination operations of spores are shown in Figure 1.8 (Setlow, 2003).

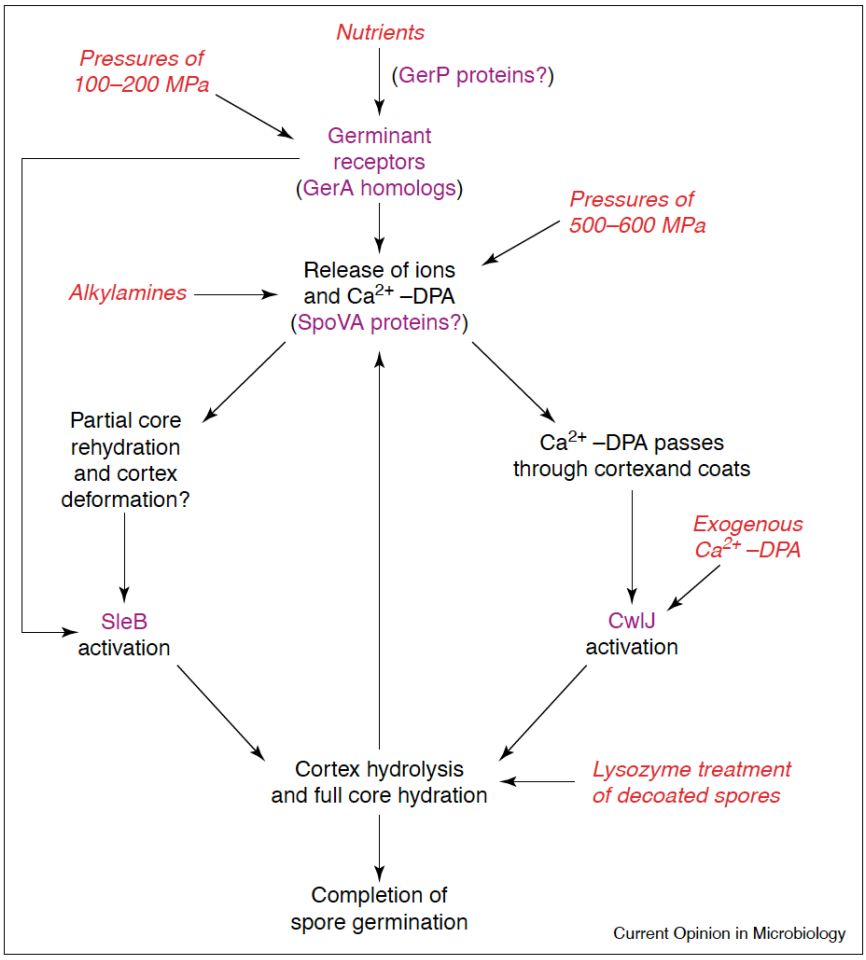

Figure 1.8 The representation of nutrient and non-nutrient germination processes of *B. subtilis* spores (Setlow, 2003).

1.2.5 Transcriptional Regulator: *gerE*

One of the essential proteins for the sporulation process in *B. subtilis* is GerE. GerE is one of the members involved in the hierarchical order of sporulation and regulates the genes that code the sporulation proteins. GerE is part of the σK regulon, one of four mother cell-specific regulatory factors. In addition, it has its own regulon (GerE regulon) (Zheng and Losick, 1990; Hämmerle *et al.*, 2014). GerE regulon, which has the capability of DNA-binding, activates three operons and 30 genes and suppresses 13 genes and two operons. GerE regulon primarily activates or suppresses the *cot* genes that encode the structural components of the spore coat (http://subtiwiki.uni-goettingen.de). It has been proven that GerE, responsible for the synthesis and combination of spore coat proteins, interacts with many operons, genes, and proteins, affecting their expression negatively or positively. GerE generally controls the expression of spore coat proteins. For instance, the expression of *cotA*, *cotE*, *cotM* and *oxdD* genes are negatively regulated by GerE (Zheng *et al.*, 1992; Henriques *et al.*, 1997; McKenney *et al.*, 2012; Driks and Eichenberger, 2016; Lin *et al.*, 2020). On the other hand, it positively affects the expression of genes such as *cotB, cotC*, and *cotD* (Zheng *et al.*, 1992; Cangiano *et al.*, 2010; Driks and Eichenberger, 2016). In addition, it is involved in the activation of the coat protein expression, operon *cotYZ*, which forms the outermost part of the spore coat (McKenney *et al.*, 2012), in the regulation of CotG, CotS, CotV, CotW, CotX, CotY proteins (Cangiano *et al.*, 2010; Ducros *et al.*, 2001), and in the regulation of *cotZ* expression (McKenney *et al.*, 2012). Furthermore, *gerE* is capable of silencing *σK*, which indirectly lowers its own gene expression. Therefore, *gerE* creates a feedback loop by regulating its own gene expression (Ducros *et al.*, 2001). Structurally, it has been proven that *gerE* contains an HTH (Helix-Turn-Helix) that binds to DNA, and GerE dimerizes. It has been determined that a mutation in *gerE* causes the production of spores with abnormal spore coats, making these spores extremely sensitive to lysozyme. Additionally, it has been observed that spores with this mutation cannot enter the germination process properly (Ducros *et al.*, 1998; Ducros *et al.*, 2001).

1.3 Peptidoglycan Synthesis: *glmS*

Glucosamine (GlcN) and its acetylated derivative N-acetylglucosamine (GlcNAc) are essential molecules used for peptidoglycan synthesis, which is part of the cell wall in *B. subtilis. glmS* possesses a critical role in the GlcNAc synthesis pathway since the enzyme product of *glmS*, GlcN synthase, converts fructose-6-phosphate to GlcN-6-phosphate, which is extremely essential for the cell wall (Liu *et al.*, 2013). The mRNA of *glmS* consists of the *glmS* ribozyme domain and the open reading frame (ORF) that synthesizes the GlmS protein, GlcN synthetase. Because GlmS is a ribozyme, it has a feedback mechanism. This complex mechanism works as follows: When GlcN6P accumulates in the cytoplasm, it binds to the *glmS* ribozyme region, revealing its self-cleaving activity. As a new 5′-OH appears in the cleaved mRNA, RNase J1 recognizes the 5′-OH portion and fragments the GlmS mRNA. Elimination of *glmS* mRNA results in a reduction in *glmS* activity and hence GlcN6P synthesis (Niu *et al.*, 2018). Therefore, the expression of *glmS* occurs only in the absence of GlcN6P (Ertekin *et al.*, 2020).

1.4 Electrophoretic Mobility-Shift Assay

To identify the linkage between DNA and proteins, an experimental procedure named the electrophoretic mobility shift assay (EMSA) was developed (Fried & Crothers, 1981). The principle of this method is that free nucleic acids are more mobile than nucleic acids attached to proteins when they are loaded on a gel. In this way, the DNA-protein complexes are detected due to their slow movement. The identification of linkage between transcription factors and DNA-binding proteins is one of the major purposes of the EMSA. This method has undergone many modifications since its invention, but they all hold to the same fundamental principle (Holden and Tacon, 2011). The working principle of mobility shift assay is shown in Figure 1.9 (“EMSA – Profacgen”, 2022).

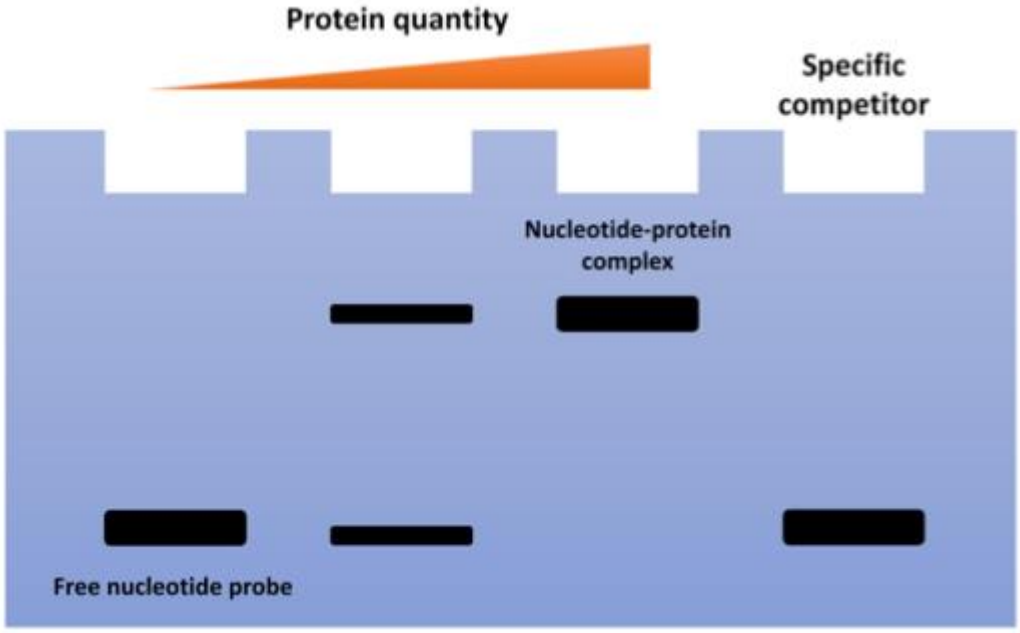

Figure 1.9 Working principle of EMSA ("EMSA – Profacgen", 2022).

There are many advantages and disadvantages of the mobility shift assay. This method is easy to execute but also versatile enough to handle various binding conditions such as temperature, pH, pressure, and reducing agents. The method's sensitivity increases when radioisotope-labeled nucleic acids are utilized as they enable the mobility shift assay to be carried out with low nucleic acid and protein concentrations and small specimen volumes. The size or structure of the nucleic acid is insignificant since all nucleic acids, such as short length or thousand base paired nucleic acids, single-stranded or duplex/triplex nucleic acids, are suitable for this method. Furthermore, mobility shift assays can be perfectly operated by using several different types of proteins like small oligopeptides or transcription complexes, purified proteins, or unprocessed cell extracts.

On the other hand, there are some limitations during the electrophoresis process. For instance, specimens are not in chemical equilibrium, which may result in a quick or delayed severance of the DNA-protein complex. Moreover, a protein-nucleic acid

complex's movement is influenced by a variety of variables in addition to the protein's size (Hellman and Fried, 2007).

1.5 The Aim of the Present Study

The prototrophic strain *B. subtilis* PY79 and its *bacABCDEF* operon silenced derivative, bacilysin non-producing strain OGU1 were comparatively examined in our previous transcriptomic, proteomic, secretomic, and real-time quantitative PCR studies. According to those studies, consistent alterations in the levels of key proteins involved in sporulation, germination, global regulation, stress response, and several different pathways in OGU1 have been detected (Aras-Taşkın, 2010; Demir, 2013; Tekin-İşlerel, 2017; Ertekin *et al.*, 2020; Kocabay, 2021). In the context of consistent alterations from our previous studies, two critical genes were selected due to their functions in the sporulation pathway and cell wall synthesis. The first gene, *gerE*, is a member of LuxR-FixJ family of transcription regulators that is expressed during sporulation and helps in spore coat assembly. Even though GerE was not detected in the previous proteomic study, transcriptomic and RT-qPCR analyses revealed that *gerE* was downregulated 2.74-fold and 3.2-fold, respectively. N-acetylglucosamine, a crucial component of peptidoglycan and consequently the cell wall, is synthesized by an enzyme that is produced by the second gene, *glmS*. According to transcriptomic, proteomic, and RT-qPCR analyses, *glmS* was downregulated by 1.20-fold, 2.06-fold, and 6.25-fold, respectively. These two genes were cloned into our mutant strain OGU1. Then, phenotypic analyses were performed, including the resistance of spores, the germination kinetics of these spores, colony morphology, and pigmentation of each strain between the parental strain PY79, mutant strain OGU1 and two recombinant OGU1 strains to distinguish the effects of those genes. In addition to phenotypic analyses, the proteins of cloned genes were expressed and purified via *E. coli* BL21 (DE3) strain, and mobility shift assays were executed to elucidate the possible DNA-protein binding between the purified proteins and the promoter region of bacilysin. Moreover, mobility shift assays were performed

between the bacilysin dipeptide and the promoter regions of cloned genes. These experiments in this study provide a better understanding of the pleiotropic effect of bacilysin by mobility shift assays and phenotypic analyses comparing the bacilysin-producing strain, non-producing mutant strain, and two recombinant strains.

CHAPTER 2

MATERIALS AND METHODS

2.1 Bacterial Strains and Plasmids

The names, characteristics, and sources of bacterial strains in this study are listed in Table 2.1. The prototrophic derivative of the standard strain of *B. subtilis* 168 is *B. subtilis* PY79 (Youngman *et al.*, 1984), which was used as the bacilysin producer in this study. The non-bacilysin-producer strain that was constructed by our group from PY79 is *B. subtilis* OGU1 (*bacA::lacz::erm*), which was used for cloning experiments and phenotypic analyses. Additionally, *E. coli* DH5α was used for cloning experiments, and *E. coli* BL21 was used for protein over-expression and purification experiments. The names, sizes, markers, and sources of the plasmids in this study were listed in Table 2.2. Moreover, the plasmids that were used for cloning and expression procedures are presented in Appendix A.

Table 2.1 Bacterial strains and their characteristics

Strain	**Source and Reference**	**Characteristics**
E. coli DH5α	American Type Culture Collection	F-ϕ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)U169 *supE44*λ*-thi-1 gyrA recA1 relA1 endA1 hsdR17*
E. coli BL21 (DE3)	Novagen, Merck (Germany)	F– *ompT gal dcm lon hsdS*$_B$ (r_B^- m_B^-) λ(DE3 [*lacI lacUV5-T7 gene 1 ind1 sam7 nin5*])
B. subtilis PY79	P. Youngman	Prototrophic derivative of *B.subtilis* 168

B. subtilis OGU1	In our lab	*bacA'::lacZ::erm::bacABCDEF*
B. subtilis gerE-overexpressing OGU1	In this study	
B. subtilis glmS-overexpressing OGU1	In this study	

Table 2.2 Plasmids used for cloning and expression experiments

Plasmid Name	**Markers**	**Size**	**Source and Reference**
pGEM®-T Easy	*amp* (Amp^r), lacZ	3.0 kb	Promega Inc. (Madison, WI)
pHT08	*amp* (Amp^r), *cm* (Cm^r)	8.0 kb	MoBiTec (Goettingen, Germany)

2.2 Culture media

The components of culture media and media preparations are given in Appendix B.

2.3 Buffers and solutions

The components of buffers and solutions are stated in Appendix C.

2.4 Chemicals and enzymes

The enzymes and chemicals used in this study were listed in Appendix D with their suppliers.

2.5 Maintenance and Growth of Bacterial Strains

B. subtilis strains (PY79, OGU1, *glmS*-overexpressing OGU1, and *gerE*-overexpressing OGU1) were grown in PA medium and Luria-Bertani (LB, Merck, Germany) agar plates. Erythromycin (final concentration 1 μg/mL) and lincomycin (final concentration 25 μg/mL) were added to the solid media for colony selection in OGU1, and chloramphenicol (final concentration 25 μg/mL) was added into the solid and liquid media for both *glmS*-overexpressing OGU1, and *gerE*-overexpressing OGU1 regardless of the experiment. All the agar media were kept at 4°C and a single colony from each medium was transferred into another sterile medium monthly to prevent any contamination and the death of the strains. In any experiment that included *B. subtilis* strains, cells were taken from solid media and inoculated into 15-20 mL of PA medium (Perry and Abraham, 1979) (pH 7.4) (Appendix B) in order to make seed cultures. After inoculation, the cultures were incubated at 37°C (200 rpm) for 16 hours. The overnight cultures were used to culture cells at larger volumes of PA medium, with the initial optical density to be set as 0.1 at 595 nm (OD595). In addition to *B. subtilis* strains, two different strains of *E. coli* were used for different purposes: DH5α strain was used for cloning experiments, and BL21 strain was used for protein overexpression and purification. Both *E. coli* strains were grown in LB agar and LB liquid culture. For cloning experiments in *E. coli* DH5α, the solid media were supplemented with ampicillin (final concentration 100 μg/mL). Like *B. subtilis* strains, the agar plates for *E. coli* were kept at 4°C and transferred into new plates monthly. For all strains of *B. subtilis* and *E. coli*, 15% glycerol solution was mixed with the pellet of grown cells in 2 mL Eppendorf tubes to be stored at -80°C for further usage and long-term storage.

2.6 Genomic DNA Isolation

The isolation of the genomic DNA from *B. subtilis* was performed by following the procedure of Owen and Borman (1987) with minor modifications. *B. subtilis* cells were inoculated into 100 mL PA medium and were grown at 37°C, 200 rpm for 16 hours. 3 mL from overnight cultures were taken and centrifuged at 6000 rpm for 10 minutes. After centrifugation, the supernatant was discarded, and 1 mL of SET buffer was used to resuspend the pellet by pipetting. 23 μL of lysozyme (50 mg/mL) to burst the cells and 6 μL of RNase (10 mg/mL) to disintegrate the RNAs were added into the mixture, and the mixture was put in the incubator (37°C) for one hour. Following the incubation, 29 μL of proteinase K (20 mg/mL) to digest proteins and 120 uL of 10% SDS to denature secondary and non-disulfide-linked tertiary protein structures were mixed by inversion, and the mixture was put into the water bath (55°C) for two hours with occasional inversions. Next, the mix was aliquoted into two sterile 2.0 mL Eppendorf tubes, and the samples were incubated at 37°C for cooling down. After aliquoting the mixture, 200 μL from 5 M NaCl was added into each tube, and the samples were blended gently. 500 μL of chloroform was added in order to separate organic and aqueous phases. The samples were mixed again by inversion for half an hour at room temperature. Next, the samples were centrifuged at room temperature at 6000 rpm for 20 minutes. After centrifugation, the supernatants including the bacterial genomic DNA were transferred into sterile 1.5 mL Eppendorf tubes. Three volumes of cold absolute ethanol, which was previously stored at -20°C, were mixed with the supernatant to precipitate the genomic DNA, and the specimens were placed at -20°C for 30 minutes, followed by centrifugation at 6000 rpm for 20 minutes at 4°C. The supernatant was removed, 1 mL of 70% ethanol was used to rinse the pellet, and the pellet was centrifuged again at 6000 rpm for 2 minutes. The supernatant was removed again, and the pellet was air-dried for 10 minutes. Following air-drying, 50 μL of TE buffer (Appendix C) was gently mixed with the pellet. BioDrop Touch UV/Vis spectrophotometer (BioDrop Ltd., UK) was used to examine the concentration and the purity of specimens after the

isolation of bacterial genomic DNA. The isolated genomic DNAs were loaded and run on 1% agarose gel to evaluate the integrity of the genomic DNA.

2.7 Primer Design

The whole genome sequence of *B. subtilis* was used to design forward and reverse primers of *gerE* and *glmS* (Gene ID: 937462, Gene ID: 938736, respectively). The exact sequence was also used for designing the promoter regions of *bacA* operon, *gerE*, and *glmS*, which were later used for EMSA experiments.

An important aspect of designing primers is adding the restriction cut sites. For this purpose, the restriction enzyme cut site of *Bam*HI (5'-GGATCC-3') was inserted at the beginning of forward primers of both *gerE* and *glmS*. At time same time, the restriction enzyme cut site of *Xba*I (5'-TCTAGA-3') was inserted into the reverse primers of the same sequences. The primers used in PCR are shown in Table 2.3.

Table 2.3 Designed Primers. Cut sites are underlined and in bold letters.

Gene Name	Primer Name	Sequence	PCR Product Size
gerE	gerE_FP	5' AAGGG**GGATCC**TACTTGAAGGAGA AAG 3'	255 bp
gerE	gerE_RP	5' GGAATAC**TCTAGA**GGATTAAAGCTC TAGCTC 3'	
glmS	glmS_FP	5' GAGG**GGATCC**AATATGTGTGGAAT CGTA 3'	1831 bp

glmS	glmS_RP	5' GGGGTTAA**TCTAGA**ATTACTCCACA GTAAC 3'	
Promoter of *gerE*	P_gerE_FP	5' GTGGCAAGCCAGCAATGCGAT 3'	415 bp
Promoter of *gerE*	P_gerE_RP	5' TTCGAACACTTCTCTTTCTCT 3'	
Promoter of *glmS*	P_glmS_FP	5' ACGGGATTATTGCTTTACCTA 3'	404 bp
Promoter of *glmS*	P_glmS_RP	5' TGGATTCCCTGTTCGTTGGCA 3'	
Promoter of *bacA* operon	P_bacA_FP	5' GCTATGCAGCTGTCGGAT 3'	382 bp
Promoter of *bacA* operon	P_bacA_RP	5' GATCGCGGATCCTTATGCGTACTCA CTGCTTGT 3'	

2.8 Polymerase Chain Reactions (PCR)

To amplify the genes of interest via PCR, Phire Green Hot Start II PCR Master Mix (Thermo Scientific, USA) was used. For the amplification of promoter regions, PCR mixture was manually prepared without using any PCR master mix. The mixtures and conditions for both are shown in Tables 2.4, 2.5, and 2.6.

To verify the sizes of PCR products, 1% agarose gel was prepared to load and run each PCR product to measure the size of the fragments. Next, PCR clean-up kit was used to purify the size-verified bands for further experiments (ligation or EMSA).

Table 2.4 PCR mixture for *gerE* and *glmS* by using Phire Green Master Mix

Component of Mixture	**Amount**
2X Phire Green Hot Start II PCR Master Mix	10 µl
Forward and Reverse Primers	1 µl from each primer 10 µM stock
Genomic DNA	0.5 µg
Nuclease free water	Complete to total volume
Total Volume	20 µl

Table 2.5 PCR mixture for promoters of *gerE*, *glmS*, and *bacA*

Component of Mixture	**Amount**
10X PCR Buffer	5 µl
10 mM dNTP mix	2 µl from each primer 10 µM stock
Forward and Reverse Primers	3 µl
$MgCl_2$	4 µl
Taq Polymerase	1 µl
Genomic DNA	0.5 µg
Nuclease free water	Complete to total volume
Total Volume	50 µl

Table 2.6 PCR Conditions

Product	**Primers**	**Conditions**
gerE	gerE_FP and gerE_RP	Initial denaturation: 30 s at 98 °C Denaturation: 5 s at 98 °C Annealing: 5 s at 54 °C Extension: 10 s at 72 °C Final extention: 1 min at 72 °C (40 cycles)
glms	glmS_FP and glmS_RP	ID: 30 s at 98 °C

		D: 5 s at 98 °C A: 5 s at 59 °C E: 10 s at 72 °C FE: 1 min at 72 °C (40 cycles)
Promoter *bacA*	P_bacA_FP and P_bacA_RP	ID: 1 min at 95 °C D: 10 s at 95 °C A: 15 s at 50-60 °C E: 1 min at 72 °C FE: 3 min at 72 °C
Promoter *gerE*	P_gerE_FP and P_gerE_RP	ID: 1 min at 95 °C D: 10 s at 95 °C A: 15 s at 50-60 °C E: 1 min at 72 °C FE: 3 min at 72 °C
Promoter *glmS*	P_glmS_FP and P_glmS_RP	ID: 1 min at 95 °C D: 10 s at 95 °C A: 15 s at 50-60 °C E: 1 min at 72 °C FE: 3 min at 72 °C

2.9 Agarose Gel Electrophoresis

1.3 gr agarose powder and 130 mL TAE Buffer (Appendix C) were mixed to prepare 1% agarose gel, and ethidium bromide (final concentration 0.5 μg/mL) was put in the gel before it was completely solidified. 6X loading dye was stirred with DNA samples before loading the samples onto the gel. Electrophoresis was executed at 90 Volts for one hour. Vilber Lourmat Gel Imaging System (Vilber Lourmat Sté, 46 France) was used to observe the DNA bands. In order to confirm the size of DNA bands, GeneRuler 1 kb Plus DNA ladder (ThermoFisher Scientifics, MA, USA)

(Appendix E) was used. After electrophoresis, the DNA bands were cut from the gel and purified by using Gel Extraction Kit (Macherey-Nagel, Germany) for further cloning experiments.

2.10 Ligation Reactions

Ligation reactions between PCR products and pGEM-T Easy Vector (Promega) were performed by following the manufacturer's protocol. The components of the reaction and the amounts of each ingredient based on the mentioned protocol are shown in Table 2.7. The details of ligation between the desired genes and expression vector pHT08 were also shown in Table 2.8. After preparing the ligation mixture, the reaction tubes were put at +4°C for 16 hours.

Table 2.7 Reaction mixture for ligation into pGEM-T Easy Vector

Ingredient	**Volume**
2X Ligase Buffer	5 µl
pGEM-T Easy Vector	1 µl
Insert DNA	50 ng
T4 DNA Ligase	1 µl
Nuclease Free Water	Complete to total volume
Total volume of reaction mixture	10 µl

Table 2.8 Reaction mixture for ligation into pHT08 Expression Vector

Ingredient	**Volume**
10X Ligase Buffer	1.5 µl
pHT08 Expression Vector	2 µl

Insert DNA	500 ng
T4 DNA Ligase	1 µl
Nuclease Free Water	Complete to total volume
Total volume of reaction mixture	15 µl

2.11 Preparation and Transformation of Competent *E. coli* cells

The competent *E. coli* strains DH5α and BL21 (DE3) were used for cloning experiments and protein expression, respectively. Competent cells of both strains were prepared by following the procedure by Hanahan (1985). Both strains were grown at 37°C (200 rpm) overnight. Next, two sterile 250 mL LB media were mixed with overnight-grown DH5α and BL21 cultures separately by setting the initial OD_{600} value as 0.1. Freshly prepared cultures of DH5α and BL21 were put into the shaker incubator (200 rpm, 37°C) for growth until their OD_{600} values reached a range between 0.4-0.6. Then, the cultures were placed on ice for 15 minutes and centrifuged at 3500 rpm for 5 min at 4 °C. The supernatants were discarded, and the previously cooled Buffer-1 (Appendix C) was used to dissolve the remaining cell pellets. The mixtures were then centrifuged at 3500 rpm for 5 min at 4 °C to harvest the cells. After the second centrifugation, the pellets were dissolved by 8 mL of previously cooled Buffer-2 (Appendix C). When the pellets were completely dissolved, 100 µl of aliquots were taken and put at -80°C for further usage.

Competent *E. coli* cells were placed on ice for 10 minutes to thaw slowly. After thawing, 85 µl of competent cells were transferred to sterile microcentrifuge tubes on ice, 15 µl of ligation products were added onto competent cells, and the mixtures were flicked gently. Then, the mixtures were kept on ice for 20-30 minutes. Heat shock was applied to the cells in the water bath (previously arranged at 42 ^{o}C) for 45-50 seconds to take in the desired plasmid. Following heat shock, the cells were instantly put back onto the ice for 2-5 minutes. Next, the transformed cells were gently stirred with 900 µl sterile LB medium, and the mixtures were put into the

shaker incubation (37 ^{o}C, 150 rpm) for 90 minutes. When the incubation was completed, the mixtures were centrifuged at 3000 rpm for 10 minutes, and 900 µl of supernatant was thrown away. The remaining supernatant was used to dissolve the cell pellet. Lastly, LA plates involving proper antibiotics [100 µg/ml ampicillin for pGEM-T Easy Vector and pHT08 Vector] were used to grow 100 µl of remaining transformed cells by spreading. Blue-white colony selection was made to detect the presence of recombinant pGEM-T Easy Vector. In addition to 100 µg/ml ampicillin, 80 µg/ml X-gal and 0.5 mM IPTG were added to LA plates for blue-white colony selection.

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

Competent cells of *B. subtilis* were prepared and completely transformed based on the procedure by Klein *et al.* (1992). For this purpose, HS and LS (Appendix C) media were required. Initially, cells were incubated overnight at 37°C in 3 mL HS medium by shaking (250 rpm). Afterwards, 1 mL from the overnight culture was taken and added to 20 mL of fresh LS medium. This new cell culture was incubated at 30°C (100 rpm) until the OD_{600} value hit 0.55. Then, 1 mL of competent cells and 1–10 µL of desired plasmid DNA were mixed in a 2 mL Eppendorf tube. Competent cells and DNA mixture were shaken at 250 rpm for two hours at 37°C. Next, they were harvested by centrifugation for 15 minutes at 5000 rpm. After centrifugation and the resuspension, cells were plated onto LB agar plates with specific antibiotic chloramphenicol (25 µg/mL) and grown at 37°C overnight.

2.13 Plasmid Isolation

pGEM-T Easy and pHT08 vectors were isolated from *E. coli* strains using GeneJET Plasmid Miniprep Kit (Thermo Scientific, USA). The isolation of pHT08 vector from *B. subtilis* strains was performed using NucleoSpin Plasmid Mini Kit (Macherey Nagel, Germany). After the isolation, the concentration and the purity of

the plasmids were estimated. The presence of isolated plasmids was evaluated by agarose gel electrophoresis. Finally, isolated plasmids were put at -20°C for further usage.

2.14 Restriction Enzyme Digestion

Restriction enzyme digestion with specific enzymes was applied to the isolated plasmids in order to see the desired gene product on the gel. *BamHI* and *XbaI* were used to cut the isolated plasmids for both genes, *gerE,* and *glmS*. The enzymes, the enzyme buffer, plasmids, and the nuclease-free water were mixed according to the manufacturer's procedure, and the mixture was put at 37°C incubator for one and a half hours. After incubation, the mixture was loaded into the agarose gel to determine whether the desired gene was integrated with the plasmid or not.

2.15 Overexpression of Proteins

E. coli BL21 (DE3) cells containing desired gene (either *gerE* or *glmS*) within the expression vector pHT08 were grown as a seed culture in 50 mL sterile LB with ampicillin (final concentration 100 μg/ml) in the shaker incubator (200 rpm, 37°C, 16 hours). Next, 3 mL from the seed cultures were taken and added into a new, sterile 250 mL LB with ampicillin. This new culture was grown at the same conditions (200 rpm, 37°C) until OD_{600} = 0.6. The overexpression of desired proteins was inititated by IPTG induction. First, 50 mL of culture was stored as the negative control. After adding IPTG, the culture was grown under the same conditions for 6 hours to overexpress the desired proteins. At the third, fourth, fifth, and sixth hours, 50 mL from IPTG-induced culture was taken and stored. The samples were centrifuged (6000 g, 4°C) for 15 minutes, and the supernatant was removed. The pellets were resuspended using 5 mL equilibration buffer (Appendix C). After resuspension, the samples were frozen and thawed three times. Then, CP70T Ultrasonic Processor (Cole-Parmer, Vernon Hills, IL) was used for the sonication step to lyse the cells

mechanically. The sonication step was performed six times, 10 seconds at 60% amplitude, while the samples were stood on ice. Following sonication, the samples were put at centrifugation (15000 rpm, 4°C) for 20 minutes to remove cellular debris. The samples' optical densities at 280nm were gauged. Next, the samples were loaded onto an SDS-PAGE gel and run to verify overexpressed proteins of interest. The same procedure was applied for the overexpression of *gerE* and *glmS* in OGU1.

2.16 Purification of Proteins

His60 Ni Gravity Columns (Takara, USA) containing immobilized nickel ions were utilized to purify 8X His-tagged proteins. Firstly, 5 mL of equilibration buffer was passed from the column. Next, previously filtered cell lysate was put into the column and waited for 1 hour by gently shaking the column to facilitate the binding between nickel ions and the poly-histidine tail of desired protein. After incubation, the column was rinsed with 20 mL wash buffer to remove untagged proteins. Then, the elution buffer, including imidazole (Appendix C), was used to elute and purify the target protein. The optical density of each elute was measured at 280 nm, and each elute was loaded onto SDS-PAGE to ensure that the protein was purified. The purified protein samples were put at -20°C for later use in EMSA analyses.

2.17 Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Laemmli's (1970) procedure was followed for the preparation of SDS-polyacrylamide gels. The ingredients and the amount of each ingredient are shown in Table 2.9. 6X sample loading buffer (Appendix C), which helps monitor the specimens, was mixed with the specimens before loading them onto the SDS-polyacrylamide gel. The specimens were loaded into wells of the gel, and the gel was run at 95V in 1X running buffer (Appendix C) for 2 hours using a Mini-Protean electrophoresis apparatus (Bio-Rad). When the loading dye was observed at the end

of the gel, the electrophoresis was stopped, and the gel was taken for staining with Coomassie Brillant Blue.

Table 2.9 The ingredients for preparing SDS- polyacrylamide gels.

	Stacking Gel 0.125 M Tris, pH 6.8	**Separating Gel 0.375 M Tris, pH 8.8**
Monomer Concentration	4.5%	12%
Acrylamide/Bisacrylamide	0.65 mL	4 mL
dH_2O	3.05 mL	3.35 mL
1.5 M Tris-HCl, pH 8.8	-	2.5 mL
0.5 M Tris-HCl, pH 6.8	1.25 mL	-
10% (w/v) SDS	50 µL	100 µL
10% Ammonium persulphate	25 µL	50 µL
TEMED	5 µL	5 µL
Total Volume	5 mL	10 mL

2.18 Coomassie Brillant Blue Staining of Polyacrylamide Gels

SDS-PAGE gels were stained using Coomassie Blue R-250 (Appendix C) for 30-45 minutes. Next, the gel was put into the destaining solution (Appendix C) to remove the excess dye for 1-2 hours. When the protein bands could be seen clearly, the gel was taken from the destaining solution for visualization.

2.19 Sporulation and Germination Procedures

2.19.1 Mature Spore Purification

B. subtilis PY79, OGU1, *glmS*-overexpressing OGU1, and *gerE*-overexpressing OGU1 strains were grown in PA medium as seed cultures, as previously described. Next, the strains were transferred onto a freshly prepared, sterile Difco sporulation medium (DSM). Overnight cultures in PA medium were used to set the initial OD_{595} to 0.1. After arranging the OD_{595} value of the cultures in the Difco sporulation medium, the cultures were put in the 37°C shaker incubator (200 rpm) for 72 hours for complete sporulation. Then, the procedure by Nicholson and Setlow (1990) was applied to 72 hours cultures to purify spores. 50 mL were taken from each culture and centrifuged at 14000 rpm for 10 minutes at 4°C. The supernatants were discarded, and each pellet was rinsed with 10 mL 1 M KCl/0.5 M NaCl solution (Appendix C) and centrifuged again. Pellets were dissolved using 10 mL of Tris-Cl buffer (50 mM, pH 7.2) (Appendix C) containing 50 µg/mL lysozyme. The mixtures were incubated at 37°C for 1 hour. After the incubation, spores were rinsed with different solutions (1 M NaCl solution, deionized water, 0.05% SDS solution, TEP buffer (50 mM Tris-Cl, pH 7.2 with 10 mM EDTA and 2 mM PMSF) (Appendix C) and deionized water, respectively) and cleansed by multiple centrifugations (14000 rpm, 10 minutes) after each solution. After a final rinse with deionized water, the purified spores were dissolved with cold deionized water and stored at -20°C for further usage. Every 15 days, the purified spores were centrifuged and dissolved with sterile, cold deionized water for storage.

2.19.2 Determination of Spore Resistance

The procedures by Nicholson and Setlow (1990) were applied to detect the mature spores' resistance against numerous agents.

2.19.2.1 Chloroform Resistance Assay

450 µL of purified spores were mixed with 50 µL chloroform in a sterile Eppendorf tube. Negative control was prepared by mixing 450 µL of purified spores and 50 µL of sterile potassium phosphate buffer (10 mM, pH 7.4 with 50 mM KCl and 1 mM MgSO4) (Appendix C). After mixing both tubes carefully, they were kept at room temperature for 10 minutes. Following the incubation, 10-fold serial dilutions were prepared by using potassium phosphate buffer. 100 µL from each dilution were cultivated on LB agar plates in triplicates, and the plates were incubated overnight at 37°C for 16 hours. Next, the colony number of each plate was counted, and the survival ratio was calculated as the ratio of CFU/mL between the chloroform treatment and the control.

2.19.2.2 Wet Heat Resistance Assay

500 µL of purified spores were put in the 80°C water bath for 30 minutes to determine the effect of heat. Meanwhile, 500 µL of purified spores were kept at room temperature as a negative control. After preparing 10-fold serial dilutions in potassium phosphate buffer, 100 µL from each dilution were cultivated on LB agar plates in triplicates. The survival ratio was calculated as the ratio of CFU/mL between the heat treatment and the control.

2.19.2.3 Lysozyme Resistance Assay

100 µL of the lysozyme solution (final concentration 250 µg/mL) were added to 400 µL of purified spores and mixed gently. As a negative control, 100 µL of potassium phosphate buffer was mixed with the purified spores. Both specimens were waited at 37°C for 10 minutes. 10-fold serial dilutions were prepared after incubation. 100 µL from each dilution were cultivated onto LB agar plates in triplicates. The survival

ratio was calculated as the ratio of CFU/mL between the lysozyme treatment and the control.

2.19.2.4 Statistical Analyses

Mean values of the survival ratios were compared by ordinary one-way ANOVA in the GraphPad Prism 8.0 software (GraphPad, CA, USA). Multiple comparisons were made by Tukey's Honest Significance Test (HSD) in the same software when there was a significant difference between the mean values. The level of the significance was stated as * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$), and **** ($p < 0.0001$) in the graphs.

2.19.3 Morphology of Sporulating Colonies

B. subtilis PY79, OGU1, *glmS*-overexpressing OGU1, and *gerE*-overexpressing OGU1 strains were grown in PA medium as seed cultures like previously described. Then, fresh sterile PA medium was used to dilute the overnight cultures: 99 µL PA medium was mixed with 1 µL overnight culture for each strain, separately. After mixing thoroughly, 1 µL was taken from each dilution and placed on different Difco sporulation medium agar plates to observe the colony morphology of each sporulating *B. subtilis* strains. DSM agar plates were put in at 30°C incubator for three weeks. Each day, the plates were scanned with Epson Perfection V750 Pro scanner (Epson Corp, Japan) to distinguish the pigmentation and colony morphology differences for each strain.

2.19.4 Spore Germination

The germination profile of the purified spores was calculated according to Nicholson and Setlow (1990). In order to do that, 10 mM Tris-Cl (pH 8.4) (Appendix B) was mixed with the purified spores to arrange the final OD_{585} around 0.8. Spores were

put in a water bath (70°C) for half an hour for activation. After the activation was complete, 500 μL of spores were immediately mixed with either minimal (Spizizen Minimal Medium, SMM) (Appendix B) or complex (2xYT medium) (Appendix B) germination media. Then, the OD_{585} value of the mixture was checked and recorded as t_0. After measuring t_0, mixtures were put at 37°C incubator for 150 minutes, and OD_{585} was checked at ten minutes intervals. Each OD_{585} value was normalized according to t_0 and expressed as relative OD_{585} percentage with standard deviation.

2.20 Preparation of Bacilysin Broth Concentrate

The bacilysin broth concentrate, which contains the bacilysin dipeptide in concentrated form without any other proteins, was collected. *B. subtilis* PY79 cells were inoculated in 100 mL of PA medium at 37°C, shaken at 200 rpm for 16 hours with an initial OD_{595} around 0.1, as described in Section 2.5. Following cell growth, the procedure of Roscoe and Abraham (1966) was applied to collect the bacilysin concentrate: Overnight-grown PY79 cells were centrifuged at 10000 rpm for 10 minutes to separate supernatant containing the bacilysin dipeptide from cells. Next, the supernatant was collected and mixed with acetone with a final concentration of 70% (v/v). The mixture of supernatant and acetone was centrifuged at 10000 rpm for 5 minutes. The supplementation of acetone and the centrifugation of supernatant/acetone mixture is known as acetone precipitation. This acetone precipitation step was performed once again. A rotary evaporator (Büchi R-200) from Central Laboratory at Biological Sciences Department, METU, was used to concentrate the supernatant/acetone mixture. Finally, 0.45 and 0.22 μm filters were used to sterilize the concentrate, and the sterilized concentrate was stored at -20°C.

2.21 Mobility Shift Assays (EMSAs)

In order to perform mobility shift assays, promoter regions of *gerE*, *glmS*, and *bacA* were amplified; the proteins GerE and GlmS were purified; the bacilysin broth

concentrate was obtained, all as described in previous procedures in 2.8, 2.16, and 2.20.

EMSAs for GerE-*bacA* promoter and GlmS-*bacA* promoter were performed separately by preparing a variety of mixtures, and the protein amounts (GerE and GlmS) varied in those mixtures from 0 to 16 mM, whereas the amount of DNA fragment (*bacA* promoter) was stable as 1 μg. EMSAs for bacilysin broth concentrate-*ger*E promoter and bacilysin broth concentrate-*glmS* promoter were also performed separately, and the volume of bacilysin broth concentrate in the reaction mixture ranged 0-80 μL, while the DNA fragments (*gerE* and *glmS* promoters) as 1 μg. The components of the reaction mixture are as follows: Binding buffer (60 mM HEPES, 20 mM Tris-HCl, 0.3 M KCl, 5 mM EDTA, 60% glycerol), 0.1 M DTT, 1% BSA, DNA from herring sperm (1 μg/μl), 25 mM $MgCl_2$, nuclease-free water, varying amounts of protein (either GerE, GlmS or bacilysin broth concentrate depending on the analysis), the stable amount of DNA fragment as 1 μg (either promoter of *gerE*, *glmS*, or *bacA* depending on the analysis). The prepared mixtures were kept at room temperature for 30 minutes. After incubation, 10X loading dye buffer (250 mM Tris-HCl, 40% glycerol, 0.2% bromophenol blue) was added into each reaction mixture. Before loading the samples, 5% native polyacrylamide gel was pre-run for 30 minutes, 200V at 4°C. EMSA was performed in 1X TGE (Tris-Glycine-EDTA) buffer containing 43 mM imidazole and 35 mM HEPES (Molle *et al.*, 2003b) at 200V for 3-3.5 hours at 4°C. After electrophoresis, the gel was soaked in SYBR™ Gold Nucleic Acid Gel Stain (1/10000, v/v) (ThermoFisher Scientific) for 1 hour at room temperature for staining the nucleic acids, and the gel was visualized with a UV-transilluminator.

CHAPTER 3

RESULTS AND DISCUSSION

3.1 Genomic DNA Isolation from *B. subtilis* PY79

The genomic DNA of *B. subtilis* PY79 is required for the amplification of desired genes. The purity and integrity of the genomic DNA (gDNA) are critically essential. For this reason, A_{260}/A_{230} and A_{260}/A_{280} ratios were checked. A_{260}/A_{230} ratio was 2.031, which was in the range of expected ratios (2.0-2.2). The expected ratio for A_{260}/A_{280} is approximately 1.8, and the observed ratio was 1.869. In order to check the integrity of the genomic DNA from PY79, the genomic DNA was loaded into the agarose gel and run. In Figure 3.1, pure and intact genomic DNA can be observed.

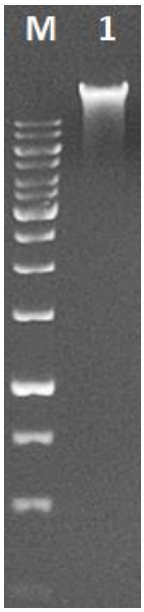

Figure 3.1 Genomic DNA of *B. subtilis* PY79. M: GeneRuler 1 kb DNA ladder, Lane 1: pure and intact gDNA from PY79

3.2 PCR Amplifications

The desired DNA regions (*gerE*, *glmS*, promoter of *bacA*, promoter of *gerE*, and promoter of *glmS*) could be amplified by PCR using the genomic DNA of *B. subtilis* PY79.

3.2.1 *gerE*

The open reading frame of *gerE* with specific restriction enzyme cut sites on both ends was amplified by PCR, using the genomic DNA of PY79 as a template. After PCR amplification, the PCR product was loaded into agarose gel and run. The size of *gerE* was checked. The band was between 200 and 300 bp as expected, since *gerE* is 255 bp long. Figure 3.2 shows the amplified PCR product of *gerE*.

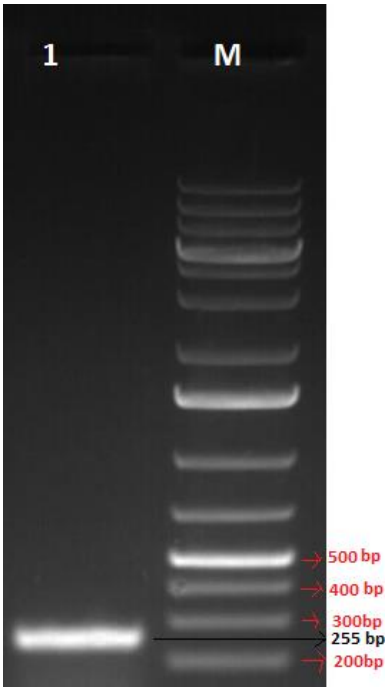

Figure 3.2 Verification of PCR amplification of *gerE* gene. Lane 1: Amplified *gerE*, M: GeneRuler 1 kb Plus DNA ladder.

3.2.2 *glmS*

The 1831 bp-long *glmS* with specific restriction enzyme cut sites on both ends was amplified by PCR. The size of the desired *glmS* gene was. The band was between 1500 and 2000 bp as expected, since the open reading frame of *glmS* is 1831 bp long. Figure 3.3 shows the amplified PCR product of *glmS*.

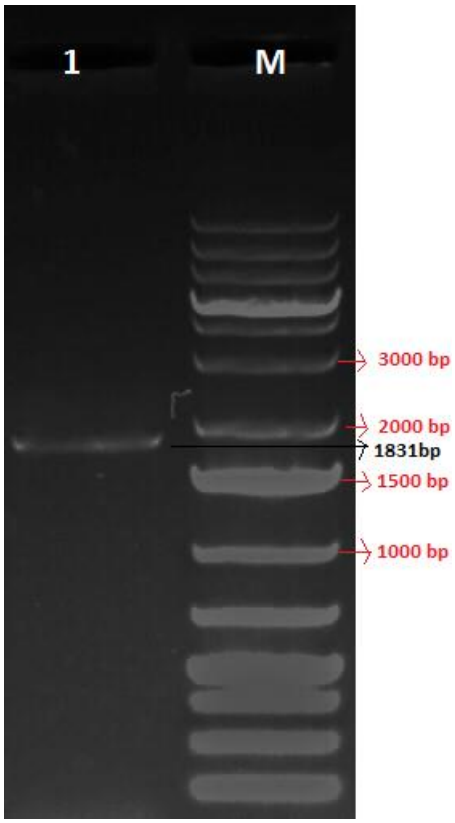

Figure 3.3 Verification of the PCR amplification of *glmS*. Lane 1: Amplified *glmS*, M: GeneRuler 1 kb Plus DNA ladder.

3.2.3 Promoter of *bacA* operon

The primers of the promoter region of the *bacA* operon were designed as 382 bp in length. After gradient PCR amplification, the size was evaluated to be observed as expected, 382 bp, which was between 300 and 400 bp.

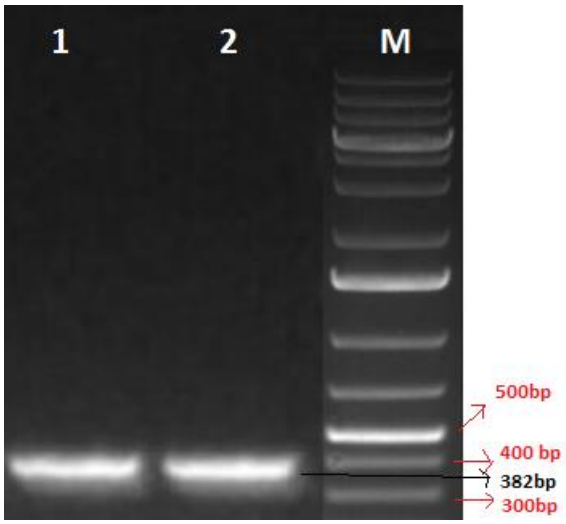

Figure 3.4 Verification of the gradient PCR amplification of the promoter of *bacA* operon (P_{bac}). Lane 1: Amplified P_{bac}, Lane 2: Amplified P_{bac}, M: GeneRuler 1 kb Plus DNA ladder.

3.2.4 Promoter of *gerE*

The primers for the promoter region of *gerE* were designed to be 415 bp in length. The size was checked in agarose gel after gradient PCR, and the size of the desired region was observed as expected.

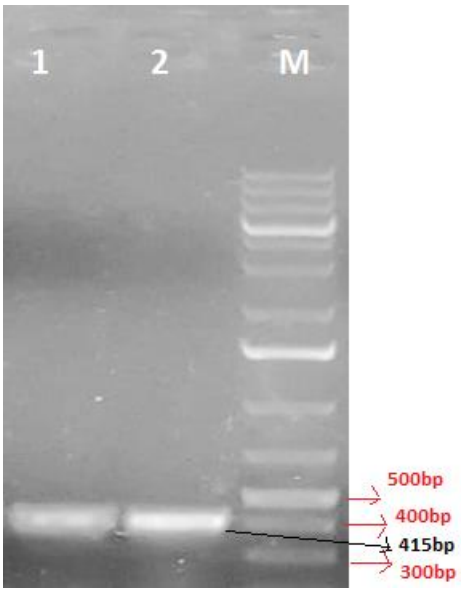

Figure 3.5 Verification of gradient PCR amplification of promoter of *gerE*. Lane 1: Amplified *gerE* promoter, Lane 2: Amplified *gerE* promoter, M: GeneRuler 1 kb Plus DNA ladder.

3.2.5 Promoter of *glmS*

The gradient PCR was performed to amplify the *glmS* promoter. After PCR, the size was determined. The size of the designed primers was 404 bp, and the bands were detected at near 400 bp using GeneRuler 1 kb Plus DNA ladder, as shown in Figure 3.6.

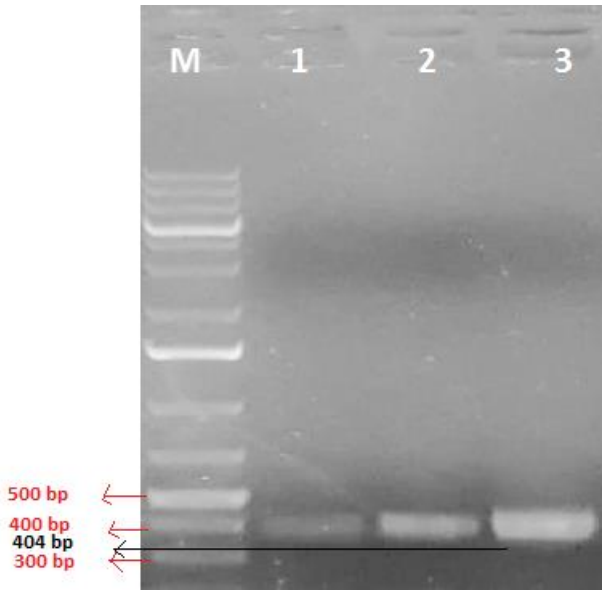

Figure 3.6 Gradient PCR amplification of *glmS* promoter. M: GeneRuler 1 kb Plus DNA ladder, Lane 1: Amplified *glmS* promoter, Lane 2: Amplified *glmS* promoter, Lane 3: Amplified *glmS* promoter.

3.3 Cloning *gerE* and *glmS* into pGEM-T Easy Vector

Following a successful PCR amplification, the desired genes, *gerE* and *glmS* with the cut sites for restriction enzymes *Xba*I and *BamH*I, were separately cloned into pGEM-T Easy vector by ligation according to the manufacturer's procedure. The vectors were transformed into *E. coli* DH5α strain. After the transformation was complete, vectors containing the desired genes were isolated and were both single and double cut by the restriction enzymes *Xba*I and *BamH*I. Due to this procedure, the genes that were inserted into pGEM-T Easy vector could be verified in the agarose gel.

3.3.1 Validation of *gerE* in pGEM-T Easy Vector

After PCR amplification, *gerE* was cut and extracted from the agarose gel to be ligated with pGEM-T Easy vector according to the supplier's procedure. The next day, a transformation procedure was applied to the ligation mixture and DH5α strain. Following transformation, blue-white colony selection was performed to detect the recombinant plasmids. After blue-white colony selection, recombinant colonies were grown on LB agar plates and LB liquid cultures with the proper antibiotic for further selection. Then, the recombinant plasmid containing *gerE* gene was isolated from the liquid culture to apply the restriction enzyme cut. The recombinant plasmid was cut by both *Xba*I and *BamH*I to check the size of the desired gene in the agarose gel. Figure 3.7 verifies that *gerE* was successfully subcloned into the pGEM-T Easy vector.

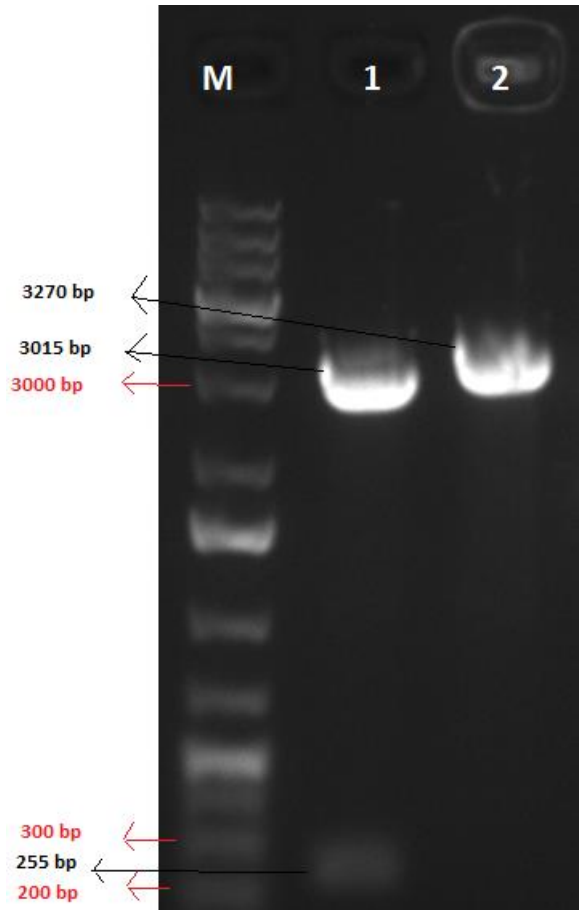

Figure 3.7 Verification of subcloning *gerE* into pGEM-T Easy vector. M: GeneRuler 1 kb Plus DNA ladder, Lane 1: *BamH*I-*Xba*I digested pGEM-T Easy vector and *gerE*, Lane 2: *BamH*I digested pGEM-T Easy vector containing *gerE*.

3.3.2 Validation of *glmS* in the pGEM-T Easy Vector

After PCR amplification, ligation, and transformation procedures, which were also applied previously for *gerE*, the recombinant plasmid was isolated to determine the presence of *glmS* in pGEM-T Easy vector. The isolated recombinant plasmid was cut with the restriction enzymes *Xba*I and *BamH*I. The insert size was verified as 1831 bp long *glmS*, and Figure 3.8 depicts that *glmS* was subcloned into the vector successfully.

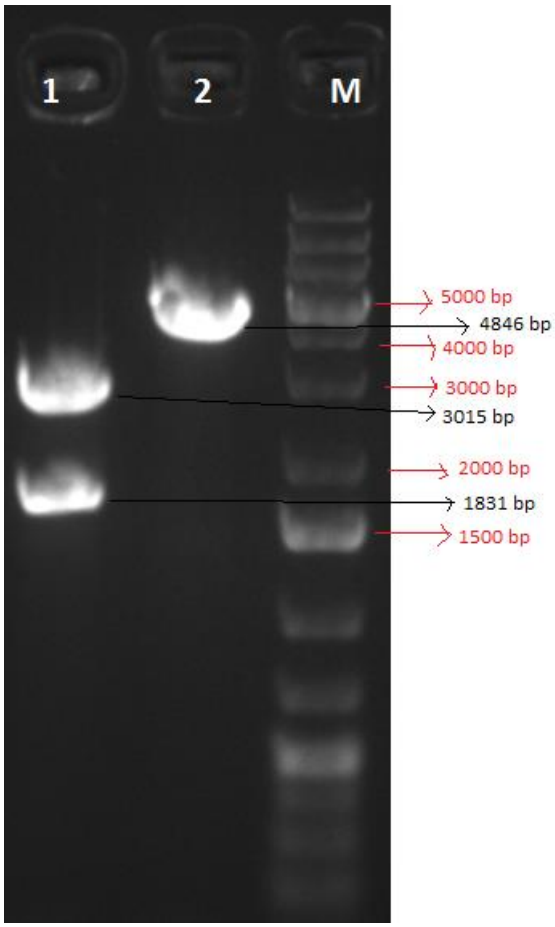

Figure 3.8 Verification of subcloning *glmS* into pGEM-T Easy vector. Lane 1: *BamH*I-*Xba*I digested pGEM-T Easy vector and *glmS*, Lane 2: *BamH*I digested pGEM-T Easy vector containing *glmS*, M: GeneRuler 1 kb Plus DNA ladder.

3.4 Cloning *gerE* and *glmS* into the expression vector pHT08

After subcloning the desired genes into pGEM-T Easy vector, *gerE* and *glmS* were cloned into expression vector pHT08 as the protein product of each gene should be expressed via this expression vector in *E. coli* BL21 (D3) for later use in EMSA experiments. Furthermore, cloning these genes into pHT08 was essential for comparative phenotypic analyses between PY79, OGU1 and recombinant OGU1 strains.

3.4.1 Validation of *gerE* in the expression vector pHT08

The gene was obtained from an agarose gel after the recombinant pGEM-T Easy vector was cut with enzymes. The gel extraction procedure for *gerE* was applied to remove excessive gel and salts. The extracted *gerE* was ligated with the expression vector pHT08, which was also cut with *Xba*I and *Bam*HI. After ligation and transformation, the recombinant pHT08 vector was isolated and cut again with the same enzyme to control whether *gerE* was subcloned successfully. The size of the expression vector is 7991 bp, and the size of *gerE* is 255 bp. Figure 3.9 showed the bands of the single-cut recombinant vector (total size 8246 bp) and double-cut recombinant vector.

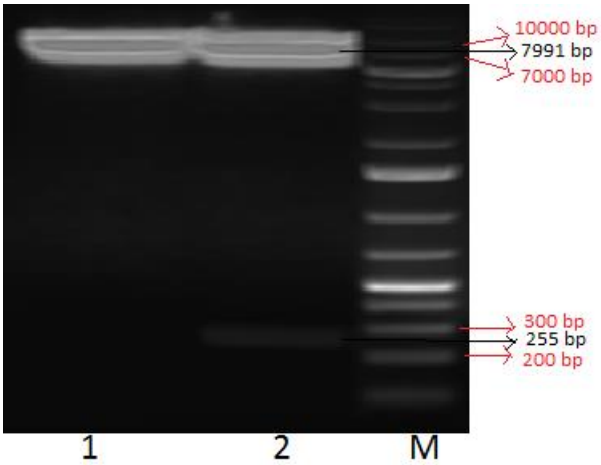

Figure 3.9 Verification of cloning *gerE* into the expression vector pHT08. M: GeneRuler 1 kb Plus DNA ladder, Lane1: *BamH*I-*Xba*I digested pHT08 and *gerE*, Lane2: *BamH*I digested pHT08 containing *gerE*.

3.4.2 Validation of *glmS* in the expression vector pHT08

All the procedures performed in section 3.4.1 were also executed for *glmS* to subclone the gene into the expression vector pHT08. After doing gel extraction,

ligation, transformation, plasmid isolation, and enzyme cut, the recombinant expression vector could be observed in agarose gel. The size of *glmS* is 1831 bp, and the size of the expression vector is 7991 bp. Therefore, the size of the recombinant vector is 9822 bp. In Figure 3.10, a single and double cut of the recombinant vector is shown.

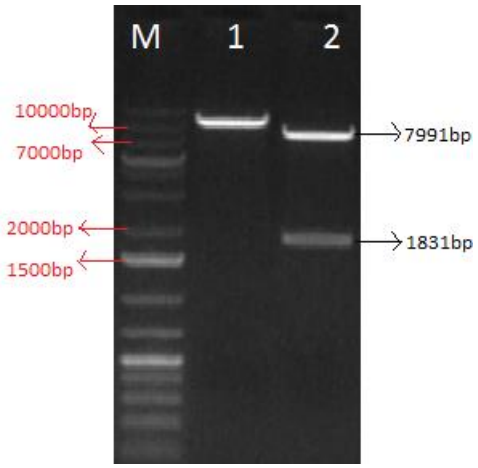

Figure 3.10 Verification of cloning *glmS* into expression vector pHT08. M: GeneRuler 1 kb Plus DNA ladder, Lane 1: *BamH*I digested pHT08 containing *glmS*, Lane 2: *BamH*I-*Xba*I digested pHT08 and *glmS*.

3.5 Expression of GerE and GlmS in *E. coli* BL21 strain

After subcloning *gerE* and *glmS* into the expression vector pHT08, the recombinant plasmids were transferred into *E. coli* BL21 strain that was used for the expression of proteins. BL21 strains containing the recombinant plasmids were grown in liquid LB with the required antibiotic and induced with IPTG for the overexpression of proteins. After IPTG induction, the samples were collected at different hours. Total protein extraction procedure was applied for both GerE and GlmS to determine the overexpressed proteins by comparing proteins of non-induced culture and IPTG-induced culture in SDS-PAGE.

3.5.1 Expression of GerE and GlmS in *E. coli* BL21

For the overexpression of the desired proteins, GerE (≈11 kDa with His-Tag) and GlmS (≈68 kDa with His-Tag), recombinant *E. coli* BL21 strains were induced with IPTG. The non-induced and induced cell lysates were loaded and run on an SDS-PAGE gel to observe the overexpressed proteins. The overexpressed proteins were indicated with arrows on Figure 3.11. GerE and GlmS expressions were shown at the same gel to distinguish the differences between *gerE*-overexpressing BL21 strain and *glmS*-overexpressing BL21 strain. The band for GlmS protein indicated with arrow did not present in the cell lysate of *gerE*-overexpressing BL21 or the band for GerE protein indicated with arrow did not present in the cell lysate of *glmS*-overexpressing BL21.

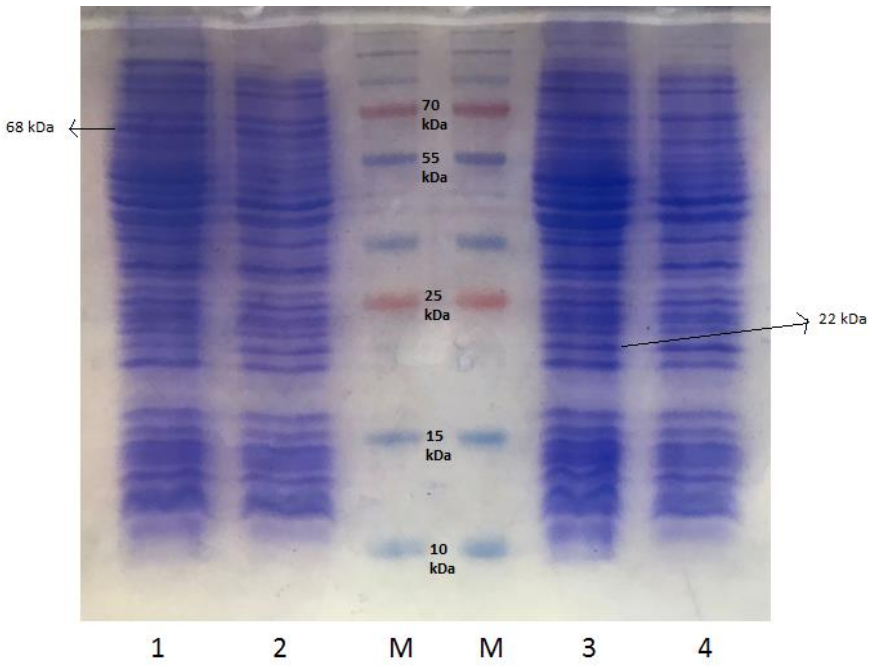

Figure 3.11 Overexpression of GlmS and GerE in *E. coli* BL21. Lane 1: IPTG-induced lysate of BL21 containing *glmS*, Lane 2: Non-induced lysate of BL21 containing *glmS*, M: GeneRuler 1 kb Plus DNA ladder, Lane 3: IPTG-induced lysate from BL21 containing *gerE*, Lane4: Non-induced lysate of BL21 containing *gerE*.

3.6 Purification of GerE and GlmS from *E. coli* BL21

The overexpressed proteins were purified by using His60 Ni Gravity Columns (Takara, USA), and the purified elutes were kept at -20^{o}C for further EMSA experiments.

3.6.1 Verification of purified GerE

After protein purification from the IPTG-induced sixth-hour sample, flow through and various collected elutes were run on an SDS-PAGE gel to see whether the protein was purified or not. The size of GerE is normally 8.43 kDa. However, since the protein was expressed with His-tag, which is critical for the purification procedure, the size of the desired protein is ≈ 11 kDa. According to previous studies, GerE can form a dimer in solution (Ducros *et al.*, 1998, Ducros *et al.*, 2001. Figure 3.12 shows the dimerized form of purified GerE (≈ 22 kDa).

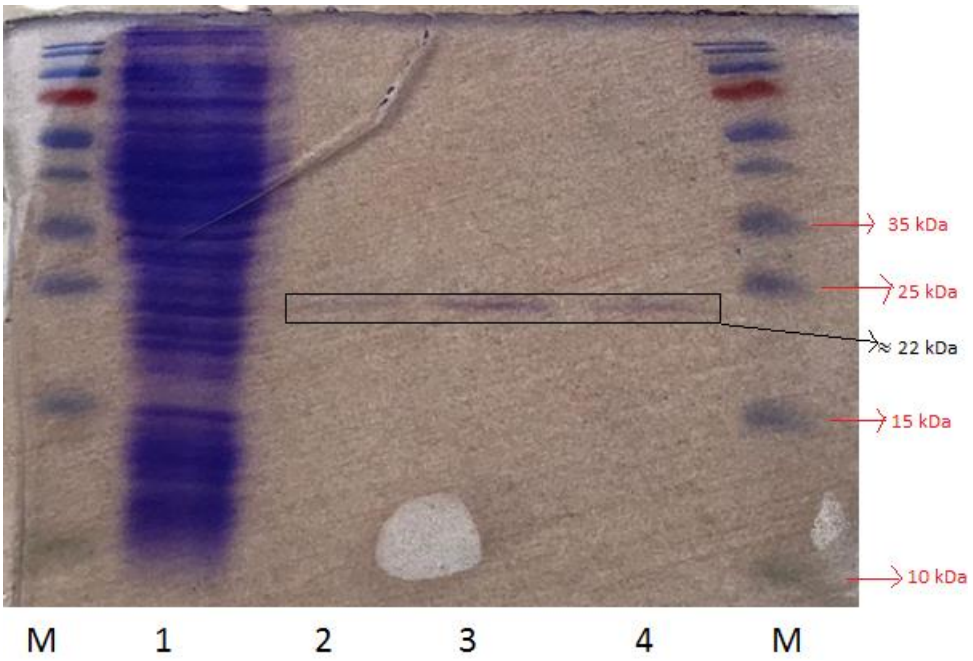

Figure 3.12 SDS-PAGE result for purified GerE (in dimer form). M: PageRuler™ Prestained Protein Ladder, Lane 1: IPTG-induced *E. coli* BL21 lysate, Lanes 2-4: Purified GerE (dimer form) from different elutes.

3.6.2 Verification of purified GlmS

IPTG induction, total protein expression, and protein purification from the sixth-hour sample were also performed for GlmS. The size of GlmS is normally 65.16 kDa. However, as the protein was expressed with His-tag, the size of the desired protein increased to ≈ 68 kDa. The purified GlmS (≈ 68 kDa) is shown in Figure 3.13.

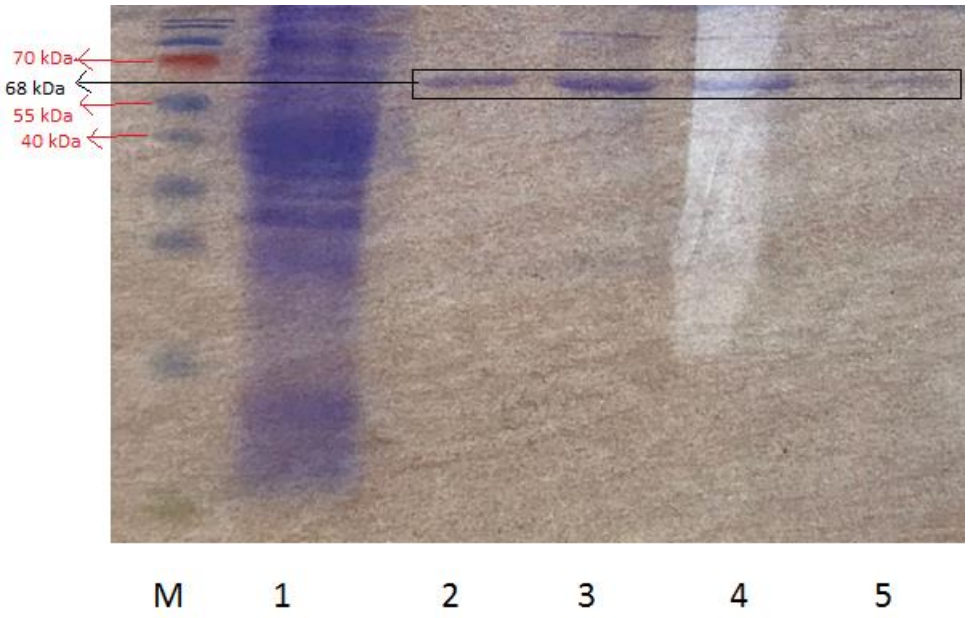

Figure 3.13 SDS-PAGE result for purified GlmS. M: PageRuler™ Prestained Protein Ladder. Lane 1: IPTG-induced *E. coli* BL21 lysate, Lanes 2-5: Purified GlmS from different elutes.

3.7 Expression of GerE and GlmS in *B. subtilis* OGU1

gerE and *glmS*, which were subcloned into the expression vector pHT08, were also transformed into competent *B. subtilis* OGU1 cells. The recombinant OGU1 strains contained the desired genes were induced with IPTG for protein overexpression. Samples were collected at different hours after IPTG induction. The total protein extraction procedure was applied to samples to see the expression of desired genes' products. Total protein extraction samples from different hours were loaded on an SDS-PAGE gel to distinguish the difference and to detect overexpressed GerE and GlmS.

3.7.1 Overexpression of GerE in OGU1

The same procedures performed for the expression of GerE in *E. coli* BL21 were also applied in order to overexpress GerE in OGU1. After IPTG induction and total protein extraction, the samples were loaded and run on an SDS-PAGE gel. The result of SDS-PAGE and the overexpression of GerE are shown in Figure 3.14. The size of GerE with His-Tag is almost 11 kDa, and the overexpressed protein band was observed near the 10 kDa band of PageRuler™ Unstained Protein Ladder.

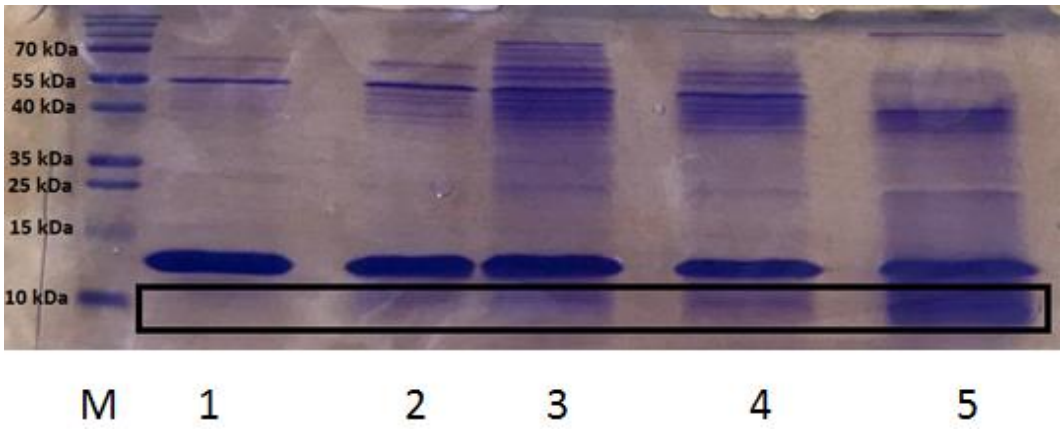

Figure 3.14 Overexpressed GerE protein in *B. subtilis* OGU1. M: PageRuler™ Unstained Protein Ladder, Lane 1: Control, non-IPTG induced cell lysate, Lanes 2-5: IPTG-induced cell lysate from different hours.

3.7.2 Overexpression of GlmS in OGU1

After the same procedures, the SDS-PAGE result demonstrated the overexpression of GlmS in OGU1 (Figure 3.15). The total size of GlmS is 68 kDa, and the overexpressed protein band was observed near the 70 kDa band of PageRuler™ Unstained Protein Ladder.

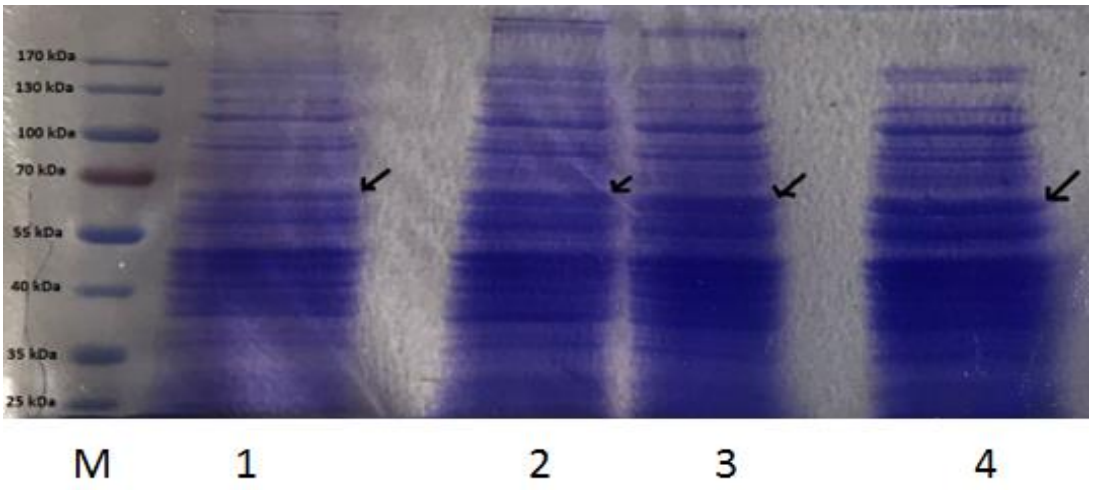

Figure 3.15 Overexpressed GlmS in *B. subtilis* OGU1. M: PageRuler™ Unstained Protein Ladder, Lane 1: Control, non-IPTG induced cell lysate, Lanes 2-4: IPTG-induced cell lysate from different hours.

3.8 Phenotypic Analyses

In our previous research (Ertekin *et al.*, 2020), several phenotypic analyses were performed to understand the pleiotropic effect of bacilysin. Germination kinetics, the resistance, pigmentation, and morphology of the spores were studied by comparing parental *Bacillus subtilis* strain PY79, its mutant strain OGU1, OGU1 strain, which was supplemented with PY79 broth concentrate (bacilysin), and OGU1 strain which was supplemented with OGU1 broth concentrate. Based on these analyses, the addition of bacilysin into OGU1 cultures positively affected the resistance and the pigmentation of spores. In other words, the survival rate of spores for bacilysin-supplemented OGU1 was improved, with the pigmentation phenotype also being restored. These results suggested that bacilysin might have a critical role in the expression of proteins related to sporulation and germination, such as CotA. Based on the analyses mentioned earlier, the same experiments were repeated by comparing PY79, OGU1, *gerE*-overexpressing OGU1, and *glmS*-overexpressing OGU1 to

detect the effects of cloned genes since *gerE* is a transcriptional regulator (regulon) which controls over 80 genes and *glmS* is critical for the synthesis of peptidoglycan.

3.8.1 Spore Pigmentation

The outer spore coat protein CotA (65 kDa) is encoded by *cotA* (previously known as *pig*), whose expression is controlled by σK (Donovan *et al*., 1987). The disappearance of the unique brownish pigmentation of spores in *Bacillus subtilis* is associated with the lack of CotA, but the absence of CotA does not directly impact their resistance (Iichinska, 1960). The copper-dependent laccase CotA is demonstrated to react with the particular substrate syringaldazine. This process causes the development of brown pigmentation of spores (Hullo *et al*., 2001).

Our previous works have shown that *cotA* was downregulated, and CotA was underrepresented in OGU1 (Aras-Taşkın, 2010; Demir, 2013; Ertekin et al., 2020; Kocabay, 2021). GerE is responsible for regulating the expression of several genes, including the *cot* genes (Zheng *et al*., 1992; Cangiano *et al*., 2010; Driks and Eichenberger, 2016). In order to determine the effects of cloning *gerE* and *glmS* into OGU1, spores' pigmentation was observed, and the collective result is represented in Figure 3.16.

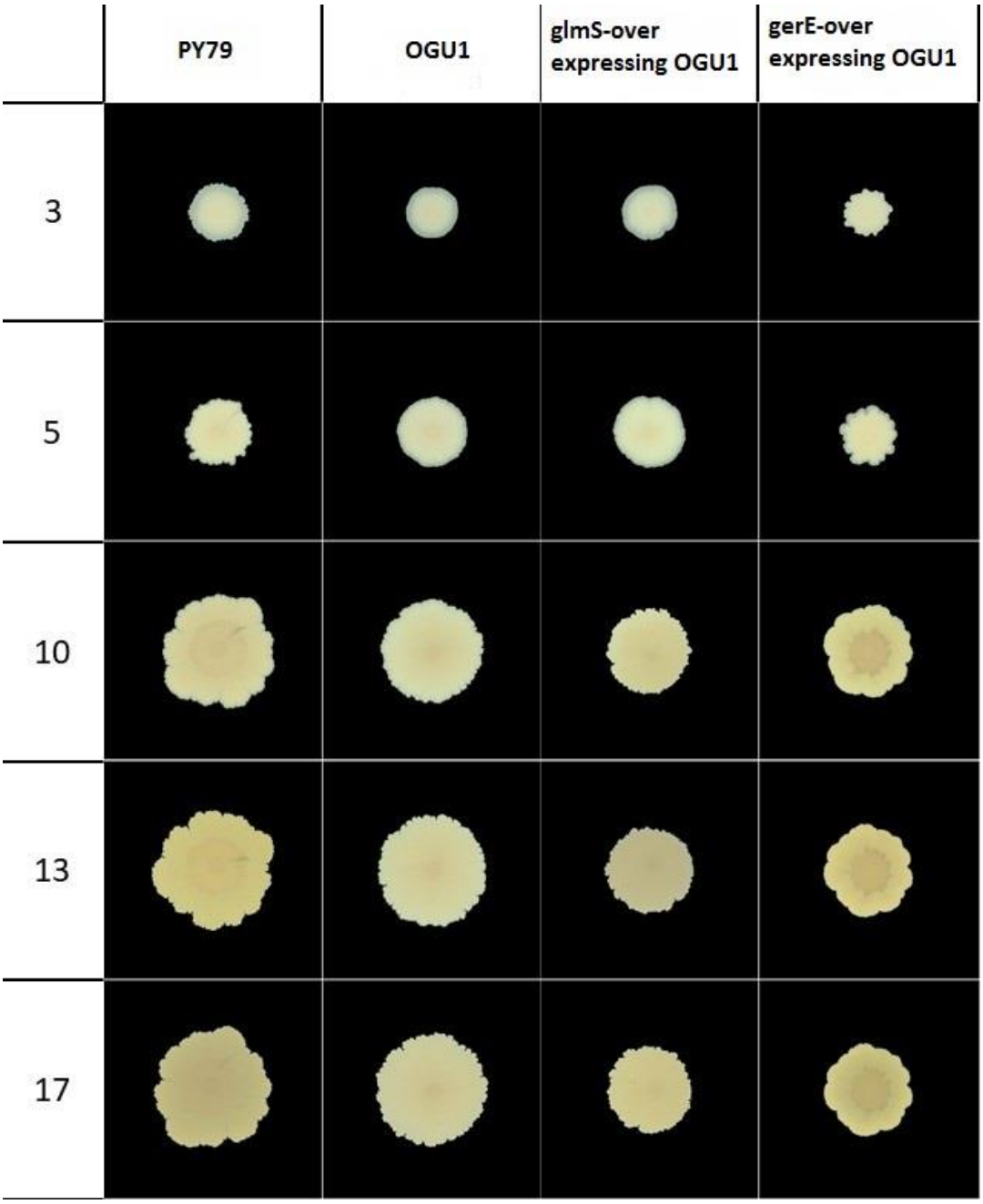

Figure 3.16 Pigmentation of PY79, OGU1, *glmS*-overexpressing OGU1, and *gerE*-overexpressing OGU1 over three weeks.

Brownish pigmentation, which was absent in OGU1 due to the downregulation of *cotA*, was restored in *gerE*-overexpressing OGU1 spores. Interestingly, this was not expected since *cotA* was found to be repressed by GerE (Eichenberger *et al.*, 2004).

CotA interacts with CotY, CotZ, and CgeA (Krajčíková *et al*., 2017). *cotY*, *cotZ*, and *cgeA* were reported to be downregulated in our previous studies, and those genes are activated by GerE (Zhang *et al*., 1994; Roels and Losick, 1995). Cloning *gerE* might indirectly influence the interaction between CotA, CotY, CotZ, and CgeA by activating *cotY*, *cotZ*, and *cgeA* even though CotA was found to be underrepresented in the proteomic studies. No specific difference in the pigmentation between OGU1 and *glmS*- overexpressing OGU1 was detected, meaning that cloning *glmS* does not affect the colony pigmentation of spores in *B. subtilis*.

3.8.2 Spore Germination

Based on our previous proteomic, RNA-seq, and RT-qPCR studies (Aras-Taşkın 2010; Demir, 2013; Ertekin, 2020; Kocabay, 2021), genes and their proteins involved in the germination process were found to be missing, underrepresented or downregulated. Several works indicated that mutations in *gerE* cause a deficiency in entry to germination. In other words, spores with severe coat assembly abnormalities due to mutations in *gerE* have a poor germination profile (Moir, 1981; Driks, 1999; Ghosh *et al.*, 2008). In addition, a mutation in *cotD* influenced the germination profile of spores (Donovan *et al.*, 1987), and *cotD* is one of the *cot* genes activated by *gerE* (Table 3.1). In light of the previous findings of both our research group and others, the germination profile of PY79, OGU1, *gerE*-overexpressing OGU1, and *glmS*-overexpressing OGU1 were investigated to detect the effects of cloned genes in the germination process. Measuring OD_{585} was critical to assess the germination process as a 60% decrease in OD_{585} points to complete germination (Atluri *et al.*, 2006). The graph of the germination profile of each strain is given in Figure 3.17.

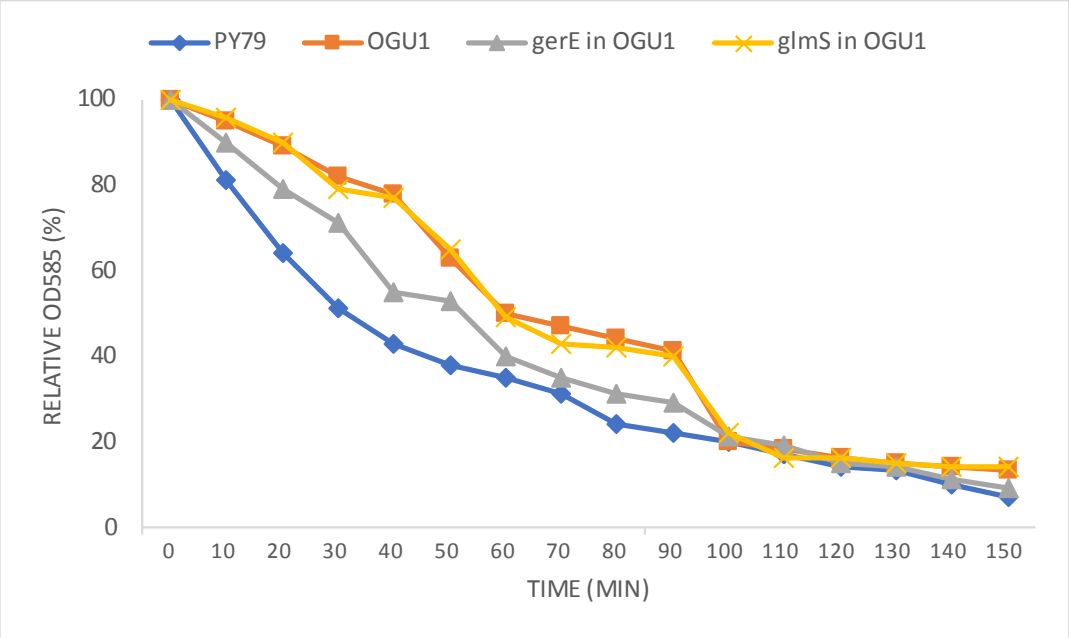

Figure 3.17 Germination response of activated endospores in the complex (2xYT) medium.

The germination delay in OGU1 was proven before (Ertekin *et al.*, 2020), and the same result was obtained in this study. The similarity of germination kinetics of OGU1 and *glmS*-overexpressing OGU1 suggested that there might be no effect of cloning *glmS*, since the only protein that interacts with GlmS is its activator GlmR (Patel *et al.*, 2018). *glmR* (*yvcK*) was found to be 2.66-fold downregulated in our transcriptome study (Kocabay, 2021). This might be why cloning *glmS* does not affect the germination profile of OGU1. On the other hand, the germination kinetics of *gerE*- overexpressing OGU1 was found to be improved compared to OGU1 and *glmS*-overexpressing OGU1, but not PY79. Germination kinetics was partially rescued due to cloning *gerE* since previous studies found that *gerE* mutations affected the germination of spores negatively (Moir, 1981; Driks, 1999; Ghosh *et al.*, 2008). However, the germination profile of *gerE*-overexpressing OGU1 was not similar to PY79. *gerE* did not cause fully rescue of germination kinetics in OGU1 since 7 germination related genes (*gerT*, *gerPE*, *gerPB*, *gerPF*, *gerPA*, *gerPD*, and *gerPC*) are repressed by GerE (Ferguson *et al.*, 2007) and these genes were already

found to be downregulated in our RNA-seq analysis (2.11, 2.23, 2.29, 2.34, 2.90, 2.48, and 2.16-fold, respectively).

3.8.3 Spore Resistance

nanoLC-MS/MS and 2DE MALDI-TOF/ MS analyses (Aras-Taşkın, 2010; Demir, 2013) revealed 36 germination and sporulation proteins were missing or inadequately represented in OGU1. Most importantly, these proteins are regulated by the mother cell-specific σE and σK regulons. Previous research by our group indicated that OGU1 spores might be less resistant to external stress due to a malformed spore coat and cortex, and the research conducted by Ertekin *et al.* (2020) proved this suggestion. *gerE* is a transcriptional regulator of a subset of σK-dependent late spore coat genes, and it positively or negatively regulates most of the genes involved in the resistance of the spore, spore crust assembly, protection of the spore, legionaminic acid synthesis, germination, and more. The impact of cloning *gerE* into OGU1 was the focus of research because it controls a number of spore coat proteins. The effect of cloning *glmS* into OGU1 was also investigated, as *glmS* is a crucial component involved in peptidoglycan biosynthesis. Assays for spore resistance to several conditions such as wet heat, chloroform, and lysozyme were investigated by comparing survival rates of spores of PY79 with OGU1, *gerE*-overexpressing OGU1, and *glmS*-overexpressing OGU1. The results of these assays are covered in more detail below.

3.8.3.1 Heat Resistance

Mature endospores were heated in a water bath 80^{o}C for 30 minutes to investigate the wet heat resistance. The graph for wet heat resistance is shown in Figure 3.18.

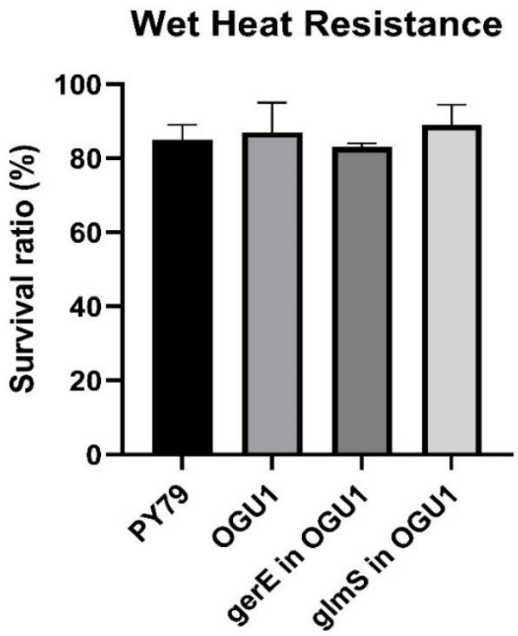

Figure 3.18 Survival ratios of mature endospores after wet heat treatment.

Previous research proved that the wet heat resistance of PY79 spores was almost identical to that of OGU1 spores, and bacilysin did not influence the resistance of spores against wet heat (Ertekin *et al.*, 2020). The extremely low amounts of water in the spore core render endospores highly resistant to wet heat. Divalent cations like Ca^{2+} or Mg^{2+} in the spore core can chelate with dipicolinic acid (DPA). Only DPA or chelated DPA are the primary components that minimize the water amount in the spore core (Gerhardt and Marquis, 1989). Even though DpaA and DpaB, which are the subunits of dipicolinate synthase, were underrepresented in OGU1 (Aras-Taşkın, 2010; Demir, 2013), the overall quantity of DPA was nearly identical for PY79 and OGU1 strains (Ertekin *et al.*, 2020). Therefore, the wet heat resistance of PY79 and OGU1 strains are almost the same. Neither *gerE* nor *glmS* are associated with the dipicolinate synthase subunits or any other protein involved in wet heat resistance, resulting in the similarity of wet heat resistance of *gerE*-overexpressing OGU1 and *glmS*-overexpressing OGU1 with PY79 and OGU1.

3.8.3.2 Chloroform Resistance

Mature endospores were treated with chloroform to assess chloroform resistance, and the survival ratios were calculated by comparing with the non-treated control group. The graph for chloroform resistance is shown in Figure 3.19.

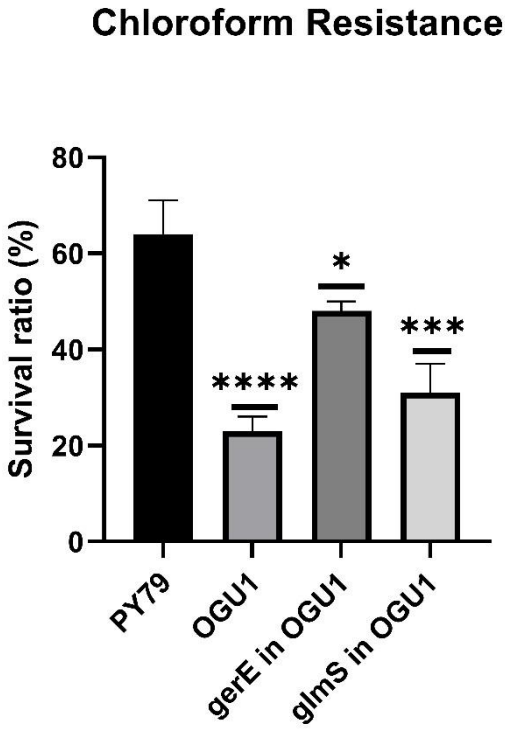

Figure 3.19 Survival ratios of mature endospores after chloroform treatment.

Consistent with previous research (Ertekin *et al.*, 2020), the spores of OGU1 were less resistant to the chloroform than PY79. A variety of spore coat proteins such as CotA, CotS, CotSA, and more were absent in our proteomic studies (Aras-Taşkın, 2010; Demir, 2013). Moreover, our transcriptomic research and RT-qPCR analyses revealed that several spore coat genes were downregulated in OGU1 as compared to PY79 (Ertekin, 2020; Kocabay, 2021). Cloning *glmS* did not dramatically affect the

resistance of spores. Impaired inner and outer spore coats did not protect the spores of *glmS*-overexpressing OGU1 against chloroform. Additionally, the spores of *gerE*-overexpressing OGU1 were less resistant to the chloroform in comparison to PY79. GerE is responsible for the repression of 7 spore coat genes that are involved in spore resistance: *cotA, cotB, cotM, cotP*, *cotT, cotH,* and *cotE* (Henriques *et al.*, 1997; Eichenberger *et al.*, 2004; Baccigalupi, 2004). Those genes were found to be downregulated in our RNA-seq findings (Kocabay, 2021). Therefore, cloning *gerE* did not cause full recovery in the spore resistance of *gerE*-overexpressing OGU1 against chloroform. On the other hand, the chloroform resistance of spore for *gerE*-overexpressing OGU1 is higher than OGU1 and *glmS*-overexpressing OGU1. *gerE* activates several spore coat-related genes, and all of those genes were found to be downregulated in our transcriptomic study, and genes activated by *gerE* are generally involved in resistance, spore crust assembly, and protection of spore, showed in Table 3.1 (Takamatsu *et al.*, 2009; Arrieta-Ortiz *et al.*, 2015; Kuwana *et al.*, 2007; Bagyan *et al.*, 1998; Kodama *et al.*, 2011; Isticato *et al.*, 2008; Zheng *et al.*, 1992; Sacco *et al.*, 1995; Eichenberger *et al.*, 2004; Zhang *et al.*, 1994).

Table 3.1 The list of genes activated by *gerE*

Locus ID	**Genes *gerE* Activates**	**The function of Gene**	**Transcriptome Results (log2FC)**
BSU30910	*cotSA*	resistance of the spore	-3.029305002 (downregulated)
BSU11770	*cotW*	resistance of the spore	-3.058089973 (downregulated)
BSU36070	*cotG*	resistance of the spore	-3.738947742 (downregulated)
BSU11780	*cotV*	resistance of the spore	-3.185708334 (downregulated)

BSU17700	*cotC*	resistance of the spore	-1.687394298 (downregulated)
BSU30900	*cotS*	resistance of the spore	-2.817540406 (downregulated)
BSU17670	*cotU*	resistance of the spore	-3.757632879 (downregulated)
BSU22200	*cotD*	resistance of the spore	-2.929044578 (downregulated)
BSU11760	*cotX*	spore crust assembly	-3.13229105 (downregulated)
BSU11750	*cotY*	spore crust assembly	-2.462447242 (downregulated)
BSU11740	*cotZ*	spore crust assembly	-2.709945029 (downregulated)
BSU34520	*cotQ*	protection of the spore	-3.052331315 (downregulated)
BSU09958	*sscA*	spore coat assembly	
BSU32640	*sspG*	protection of spore DNA	-4.570978523 (downregulated)
BSU11320	*yjzB*	protection of the spore	-2.502830148 (downregulated)
BSU30890	*ytxO*	protection of the spore	-2.69116532 (downregulated)
BSU39580	*yxeE*	protection of the spore	-2.139798261 (downregulated)
BSU14970	*ylbD*	protection of the spore	-2.492036884 (downregulated)
BSU22250	*yppG*	protection of the spore	-2.222208392 (downregulated)

BSU17310	*ymaG*	protection of the spore	-2.578236994 (downregulated)
BSU06850	*yeeK*	protection of the spore	-1.756197693 (downregulated)

3.8.3.3 Lysozyme Resistance

To observe the lysozyme resistance of spores for each strain, spores were treated with lysozyme, and the survival ratios were calculated. The graph for lysozyme resistance is shown in Figure 3.20.

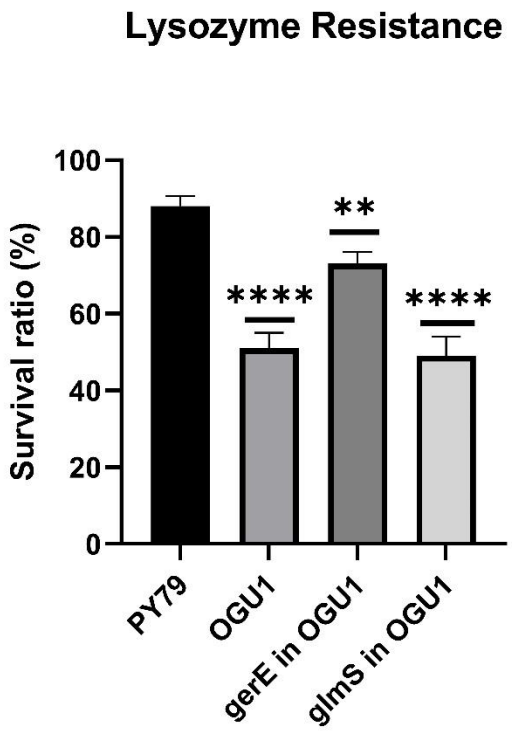

Figure 3.20 Survival ratios of mature endospores after lysozyme treatment.

Ertekin *et al.* proved that the spores of OGU1 were less resistant to lysozyme than spores of PY79 due to impaired outer spore coat (2020). This result was demonstrated again in this study, as shown in Figure 3.20. Moreover, cloning *gerE* into OGU1 positively affected the resistance of spores to lysozyme compared to OGU1 and *glmS*-overexpressing OGU1. The genes activated by *gerE* involved in resistance, spore crust assembly, and protection of spore, previously mentioned in Table 3.1. In addition, a mutation in *gerE* negatively affects the resistance of spores to lysozyme (Riesenman and Nicholson, 2000). Nevertheless, the spore resistance of *gerE*-overexpressing OGU1 is still lower than PY79. CotE, which has an important role in the outer spore coat generation and lysozyme resistance (Costa *et al.*, 2007), and *cotE* is one of the *cot* genes repressed by *gerE* (Eichenberger *et al.*, 2004). Another research suggested that CotH and CotO are critical for lysozyme resistance (Henriques and Moran, 2007) and *cotH* is repressed by *gerE* (Eichenberger *et al.*, 2004). Lysozyme disrupts the peptidoglycan of the cell wall in Gram-positive bacteria to prevent peptidoglycan production, thereby cell wall synthesis. Each spore coat protein is crucial for lysozyme resistance since lysozyme can also affect the peptidoglycan within the spore cortex. For example, CotE is one of the crucial components for the development of the outer coat. *cotE*-null spores lack the outer spore coat making them more susceptible to lysozyme (Zheng *et al.*, 1988). Furthermore, cloning *glmS* into OGU1 did not alter the resistance of spores. Even though cloning *glmS* might restore the peptidoglycan synthesis in the cell wall and the spore cortex, the peptidoglycan is still susceptible to lysozyme since there are several *cot* genes that are probably underregulated and Cot proteins that are defective in *glmS*-overexpressing OGU1, like OGU1 itself.

3.9 Electrophoretic Mobility Shift Assays

3.9.1 EMSA for purified GerE and promoter of *bacA* operon

EMSA was performed to detect possible interaction between GerE and promoter of *bacA* operon (P_{bac}). The amount of promoter DNA (P_{bac}) was stable. On the other hand, the amount of purified protein was gradually increased for each well. 0 μM, 1 μM, 2 μM, 4 μM, 8 μM, 16 μM, and 20 μM purified protein were used for each well respectively, as can see in Figure 3.21. No GerE or sperm DNA (competitive DNA) were used for negative control, so there was no binding of DNA-protein complex. Competitive DNA was not added to the last well to observe the binding of purified protein and promoter DNA.

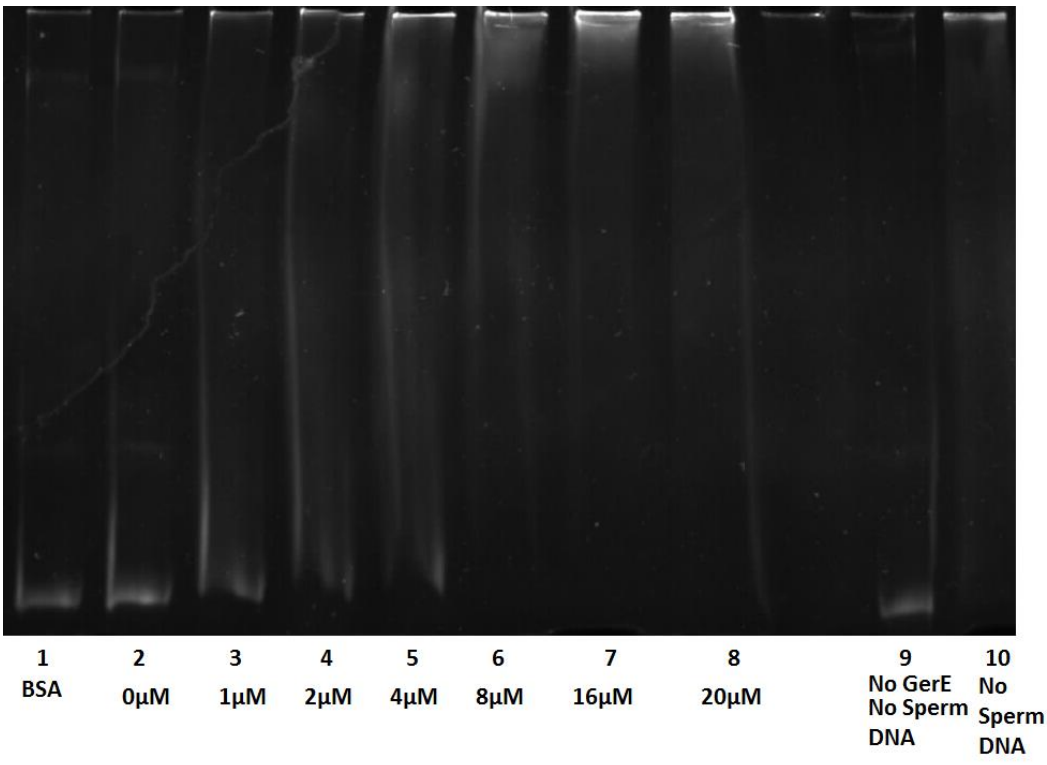

Figure 3.21 EMSA for purified GerE and promoter of *bacA* operon (P_{bac}). GerE concentration was increased for each well.

The binding of GerE and P_{bac} was observed. GerE has a DNA-binding site (helix-turn-helix) and it was previously proven that GerE controls the expression of *cot* genes by binding with its HTH motif to those genes' promoter sites (Zheng *et al*., 1992).

3.9.2 EMSA for bacilysin dipeptide and the promoter of *gerE*

The binding of purified GerE and P_{bacA} was detected. To determine the interrelation of bacilysin biosynthesis with *gerE*, bacilysin dipeptide and the promoter of *gerE* were used for EMSA. The amount of *gerE* promoter was stable for each well, and the amount of bacilysin dipeptide was continuously boosted. 0 μL, 10 μL, 20 μL, and 40μL purified bacilysin was used for each well respectively, as seen in Figure 3.22.

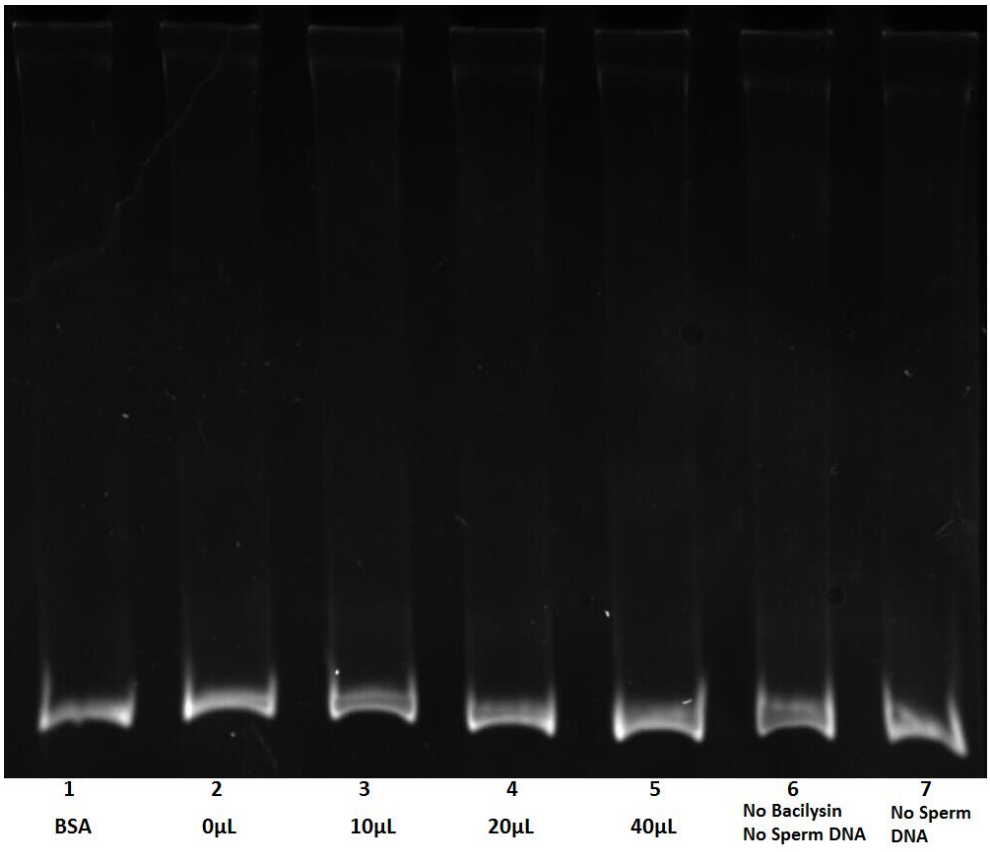

Figure 3.22 EMSA for purified bacilysin and promoter of *gerE*. The amount of bacilysin was increased for each well.

No binding was detected between bacilysin and *gerE* promoter. In other words, there was no interrelation between bacilysin and *gerE* promoter, even though GerE-P_{bac} linkage was determined previously.

3.9.3 EMSA for purified GlmS and the promoter of *bacA* operon

To determine the possible linkage between P_{bac} and purified GlmS protein, EMSA was performed. The concentration of P_{bac} was the same for each well. On the other hand, the amount of purified protein was gradually increased for each well. 0 μM, 1 μM, 2 μM, 4 μM, 8 μM, and 16 μM purified protein were utilized for each well respectively. No GlmS or sperm DNA (competitive DNA) were used for negative control, so the binding of the DNA-protein complex was not expected for this well due to the lack of DNA fragments. No competitive DNA was added to the next well, as the purpose of EMSA is to identify the purified protein-promoter linkage, not purified protein-competitive DNA.

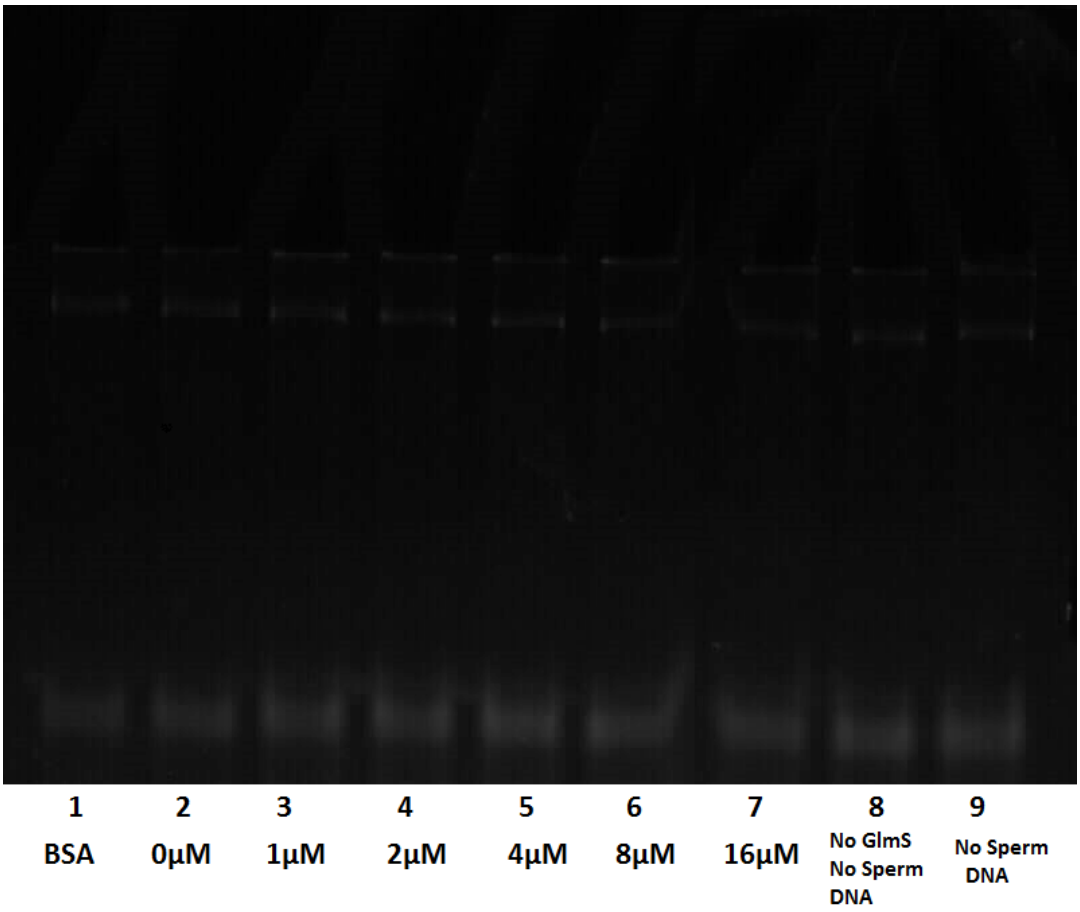

Figure 3.23 EMSA for purified GlmS and promoter of *bacA* operon (P_{bac}). The concentration of GlmS was increased for each well.

No binding was observed between GlmS and P_{bac}. GlmR, which enhances GlmS activity, is the only reported protein that links with GlmS (Patel *et al*., 2018). Lack of GlmR might be the reason for the absence of connection between GlmS and P_{bac}. GlmS, unlike GerE, does not have DNA-binding site that might explain the lack of linkage between GlmS and P_{bac}.

3.9.4 EMSA for bacilysin dipeptide and promoter of *glmS*

In order to investigate the interrelation between bacilysin biosynthesis and *glmS*, EMSA was performed by using bacilysin and the promoter of *glmS*. The amount of *glmS* promoter was the same for each well. The volume of bacilysin was gradually

increased. 0 µL, 10 µL, 20 µL, and 40µL purified bacilysin was used for each well respectively, as shown in Figure 3.24.

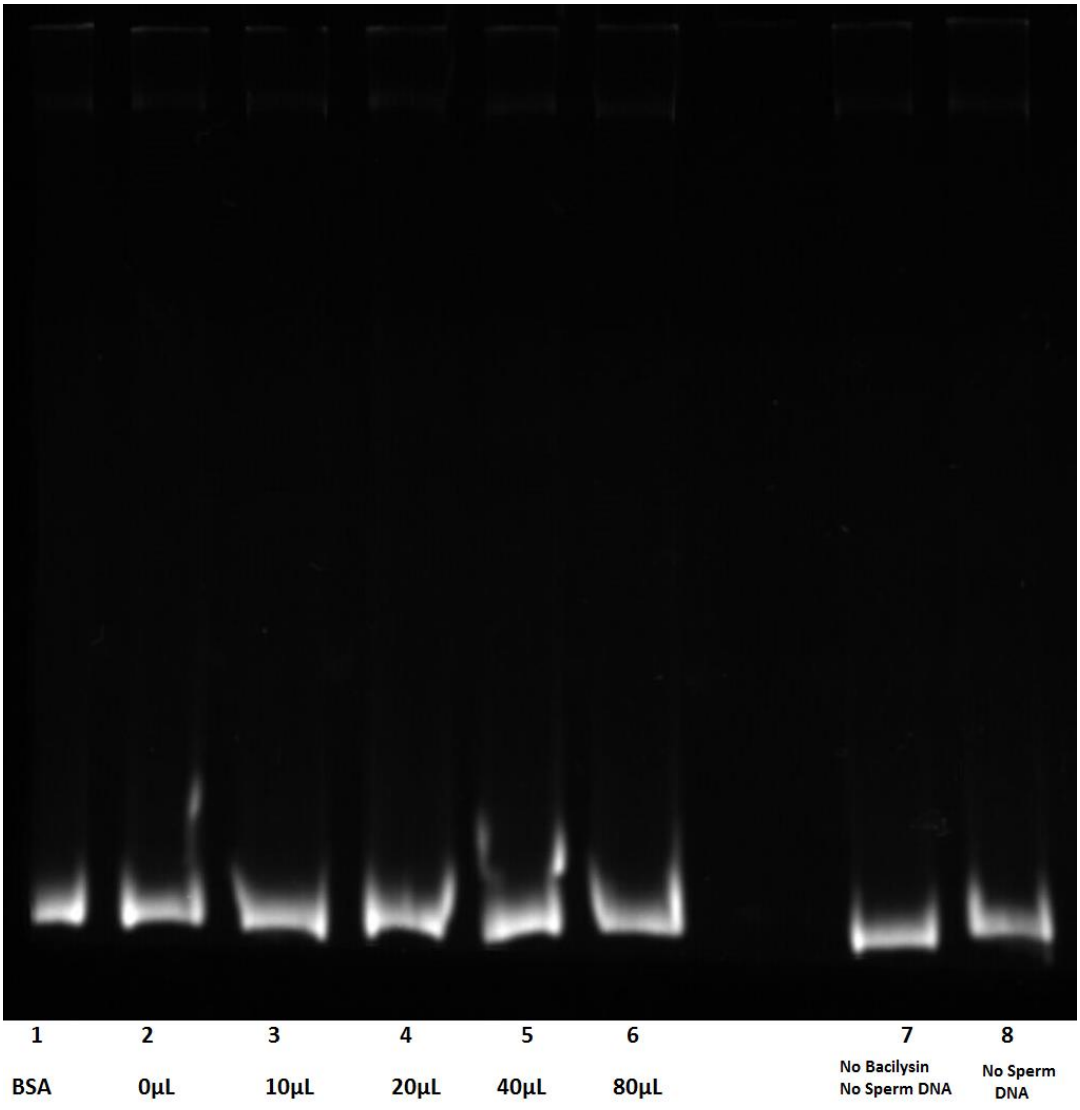

Figure 3.24 EMSA for purified bacilysin and promoter of *glmS*. The amount of bacilysin was increased for each well.

EMSA results shows that there was no binding between bacilysin and *glmS* promoter. It was expected since there was no linkage between GlmS and P_{bac}.

CHAPTER 4

CONCLUSION

- Prior to this study, the proteomic, secretomic, RT-qPCR, and RNAseq analyses were conducted in parallel between the parental organism *Bacillus subtilis* PY79 and its mutant strain OGU1. These analyses determined a broad range of genes as significantly underexpressed in OGU1 compared to PY79. Based on these earlier studies, two of the crucial genes, *gerE*, and *glmS*, were chosen for their functions in the sporulation pathway and cell wall synthesis. Proteomic analysis was not able to determine GerE (8.43 kDa), but 2.74-fold and 3.2-fold downregulations were detected in the transcriptomic and RT-qPCR analyses, respectively. Moreover, 6.25 fold, 2.06 fold, and 1.20 fold underrepresentations were found in our previous RT-qPCR, proteomic, and transcriptomic studies for GlmS (65.16 kDa).
- These two genes were cloned into OGU1 and expressed to reveal the impacts of the genes by comparing the respective recombinant strains with parental strain PY79 and the mutant strain OGU1. The spores of each strain (two recombinant strains, parental strain, and mutant strain) were isolated and their resistance to lysozyme, chloroform, and wet heat was determined. Even though the spore resistance of PY79 was the highest, the spore resistance of *gerE*-overexpressing OGU1 was higher than OGU1. Moreover, the pigmentation profiles of *gerE*-overexpressing OGU1 and the standard strain PY79 were almost the same. This result was expected, since *gerE* master regulator is known to interact with several other genes by regulating them positively or negatively, particularly the spore coat (*cot*) genes. The effects of cloning and expression of *gerE* in OGU1 were almost similar to those obtained from chemical complementation with bacilysin, which revealed the pleiotropic impact of the latter. Lastly, cloning and expression of *glmS* had

no effect on spore resistance, germination and pigmentation properties of spores.

- Based on earlier findings in our study, the connection between *gerE* and bacilysin biosynthesis was a new object of curiosity. To understand this possible connection better, mobility shift assays (EMSAs) were performed with the purified GerE and the promoter region of *bacA* operon. EMSAs revealed that there is a linkage between GerE and *bacA* promoter. After discovering this connection, a possible interaction between bacilysin and the *gerE* promoter became our focus of interest. Mobility shift assays were next performed by using bacilysin concentrate and the promoter region of *gerE*, however there was no evidence for binding of bacilysin to *gerE* promoter. Finally, no interaction was detected in EMSAs between either GlmS-*Pbac*, and bacilysin-*glmS* promoter.

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APPENDICES

A. Appendix

STRUCTURES OF PLASMID VECTORS AND SIZE MARKERS

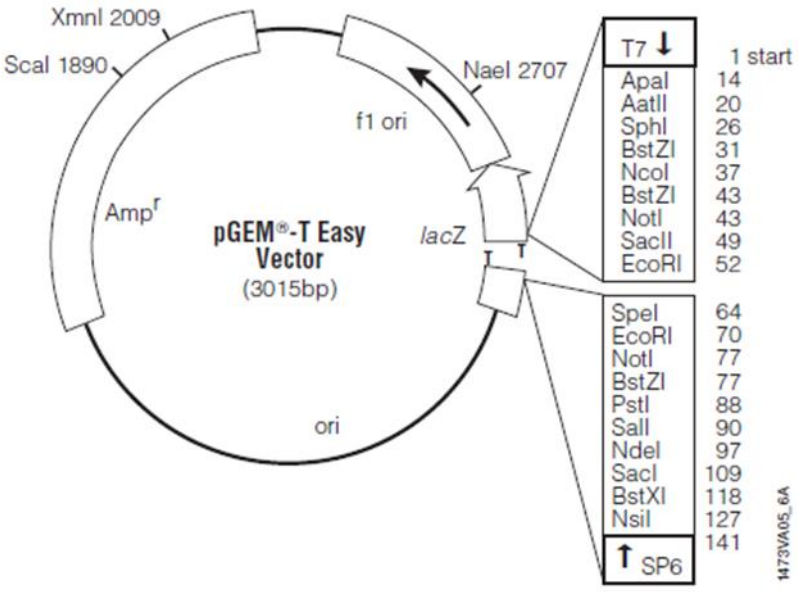

Figure A.1 pGEM®-T Easy Cloning Vector (Promega #A1360)

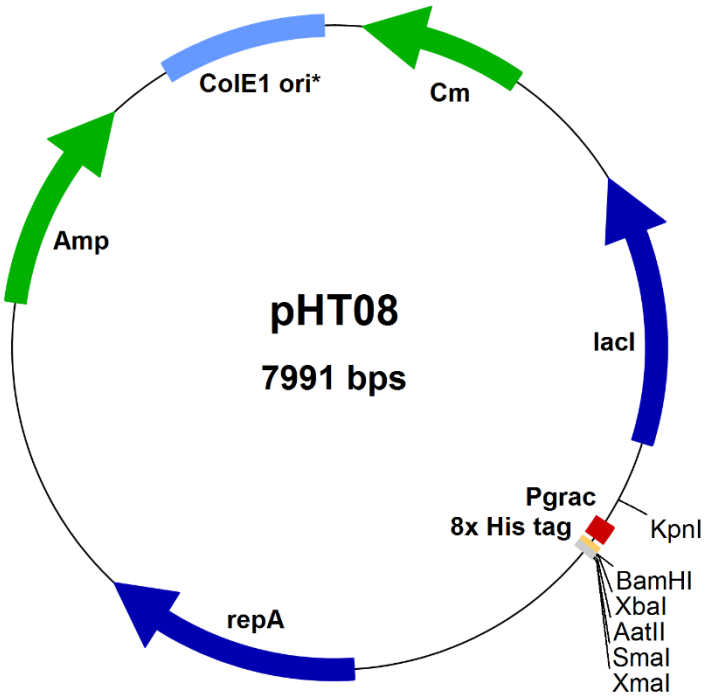

Figure A.2 pHT08 *Bacillus subtilis*/*E. coli* expression (shuttle) vector

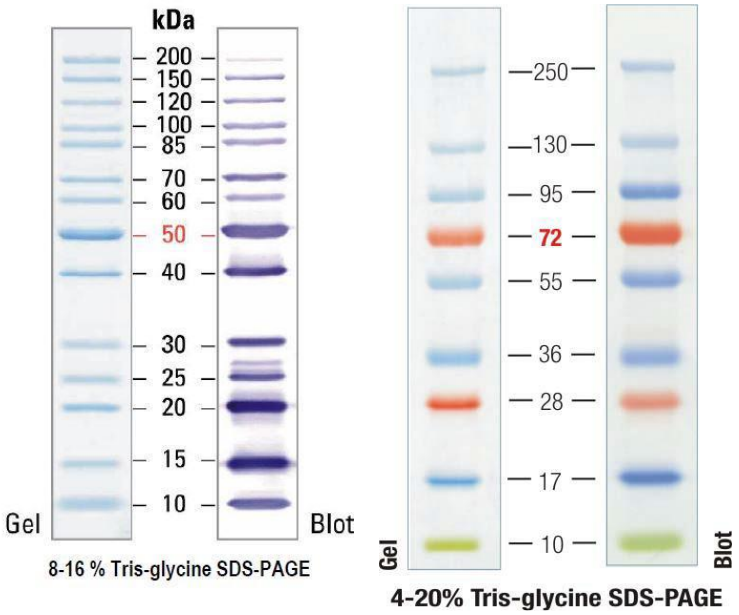

Figure A.3 PageRuler™ Plus Prestained Protein Ladder (Thermo Scientific #26619) (A) and PageRuler™ Unstained Protein Ladder (Thermo Scientific #26614) (B).

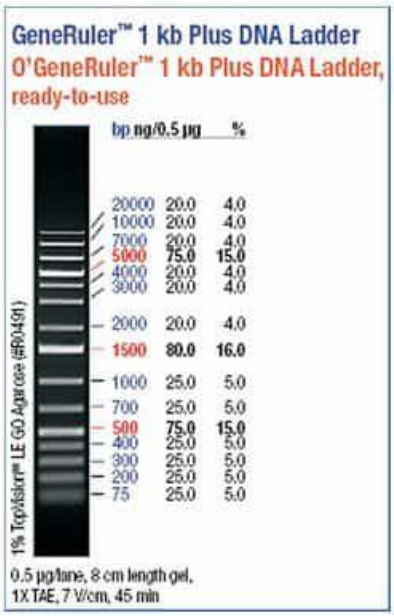

Figure A.4 1 kb plus DNA Ladder (GeneRuler # SM1333)

B. Appendix

COMPOSITION AND PREPARATION OF CULTURE MEDIA

B1. Perry and Abraham (PA) Medium (pH = 7.4)

- KH_2PO_4 — 1 g/L
- KCl — 0.2 g/L
- $MgSO_4.7H_2O$* — 0.5 g/L
- Glutamate.$Na.H_2O$ — 4 g/L
- Sucrose* — 10 g/L
- Ferric citrate** — 0.15 g/L
- Trace Elements**
  - $CoCl_2.6H_20$ — 0.0001 g/L
  - Ammonium molybdate — 0.0001 g/L
  - $MnCl_2.4H_20$ — 0.001 g/L
  - $ZnSO_4.7H_2O$ — 0.0001 g/L
  - $CuSO_4.5H_2O$ — 0.00001 g/L

*Autoclave separately
**Filter sterilization

B2. Luria Broth:

- Tryptone — 10g
- Yeast Extract — 5g
- NaCl — 10g
- Distilled water — up to 1000 mL

Final pH is 7.0; sterilized at 121^{o}C for 15 minutes.

B3. Luria Agar:

- Tryptone — 10g
- Yeast Extract — 5g
- NaCl — 10g
- Agar — 15g
- Distilled water — up to 1000 mL

Final pH is 7.0; sterilized at 121^{o}C for 15 minutes.

B4. Schaeffer's Sporulation Medium or Difco Sporulation Medium (DSM) (pH = 7.6)

- Nutrient Broth 8 g/L
- 10% (w/v) KCl 10 mL/L
- 1.2% (w/v) $MgSO_4.7H_2O$ 10 mL/L
- Agar (add before autoclave for solid media) 15 g/L

Volume was adjusted to 1 L with distilled water, pH was adjusted to 7.6 with 1 M NaOH. After cooling following were added:

- 1 M $Ca(NO_3)_4$* 1 mL/L
- 0.01 M $MnCl_2$* 1 mL/L
- 1 mM $FeSO_4$* 1 mL/L

*Filter sterilization

C. Appendix

SOLUTIONS AND BUFFERS

C1. Agarose Gel Electrophoresis

C1.1. TAE Buffer (50X)

- Tris-base 242 g
- Glacial acetic acid 57.1 mL
- EDTA (0.5 M, pH 8.0) 100 mL
- Distilled water up to 1000 mL

C1.2. Loading Buffer (10X)

- Bromophenol blue (w/v) 0.25%
- Xylene cyanol FF (w/v) 0.25%
- Sucrose (w/v) 40%

C2. SDS-Polyacrylamide Gel Electrophoresis (PAGE)

C2.1. Acrylamide/Bis

- Acrylamide 146 g
- N.N'-Methylene-bis acrylamide 4 g

- Distilled water up to 500 mL

C2.2. Tris HCl (1.5 M)

- Tris-base 54.45 g
- Distilled water 150 mL

pH is adjusted to 8.8 with HCl, distilled water to 300 ml and stored at 4°C.

C2.3. Tris HCl (0.5 M)

- Tris-base 6 g
- Distilled water 50 mL

pH is adjusted to 6.8 with HCl, distilled water to 100 mL and stored at 4°C.

C2.4. Running Buffer (10X)

- Tris-base 30 g
- Glycine 144 g
- SDS 10 g
- Distilled water up to 1000 mL

C2.5. Sample Loading Buffer (6X)

- Tris-HCl (1 M, pH 6.8) 3.5 mL
- Glycerol 3.6 mL
- SDS 1.03 g
- β-mercaptoethanol 0.5 mL
- Bromophenol blue 0.0013 g
- Distilled water up to 10 mL

C2.6. Fixation Solution

- Ethanol 40%
- Glacial acetic acid 10%
- Distilled water 50%

C2.7. Coomassie Blue R-250 Stain

- Coomassie Blue R-250 — 0.25 g
- Methanol — 125 mL
- Glacial acetic acid — 25 mL
- Distilled water — 100 mL

C2.8. Destaining Solution

- Methanol — 100 mL
- Glacial acetic acid — 100 mL
- Distilled water — 800 mL

C3. *Bacillus subtilis* competent cell preparation

C3.1. HS-Medium

- 10 X-S-Base — 10 mL
- Glucose, 50% (w/v) — 1 mL
- Yeast Extract, 10% (w/v) — 1 mL
- Casamino acid, 2% (w/v) — 1 mL
- Arginine, 8% (w/v) + Histinde, 0.4% (w/v) — 10 mL
- Tryptophane, 0.5% (w/v) — 1 mL
- Phenylalanine, 0.3% (w/v) — 1.5 mL

Total volume was completed to 100 mL with sterile dH_2O.

C3.2. LS-Medium

- 10 X-S-Base — 2 mL
- Yeast Extract, 10% (w/v) — 0.2 mL
- Casamino acid, 2% (w/v) — 0.1 mL
- Tryptophane, 0.5% (w/v) — 20 µL
- Phenylalanine 0.3% (w/v) — 30 µL
- 50 mM Spermidine — 0.2 mL
- 1 M $MgCl_2$ — 50 µL
- Glucose, 50% (w/v) — 0.2 mL

Total volume was made up to 20 Ml with sterile dH_2O.

C3.3. 10 X-S-Base

- $(NH_4)_2SO_4$ 20 g/L
- K_2HPO_4 140 g/L
- KH_2PO_4 60 g/L
- Na_3citrate.$2H_2O$ 10 g/L

They were autoclaved together and allowed to cool to 50^oC and then supplemented with 1 mL of sterile 1 M MgSO4.

C4. Protein Purification

C4.1. LEW (Lysis- Wash) Buffer (pH 8.0)

- Urea 8 M
- NaCl 300 mM
- NaH_2PO_4 50 mM

C4.2. LEW (Elution) Buffer (pH 8.0)

- Urea 8 M
- NaCl 300 mM
- NaH_2PO_4 50 mM
- Imidazole 250 mM

C5. *E. coli* Competent Cell Preparation

C5.1.Buffer I

- RuCl 100 mM
- Kac 30 mM
- $CaCl_2$ 10 mM
- Glycerol 15%

pH is adjusted to 5.8 with dilute acetic acid, and the filter is sterilized.

C5.2. Buffer II

- $CaCl_2$ 75 mM
- RuCl 10 mM
- MOPS 10 mM
- Glycerol 15%

pH is adjusted to 6.5 with 0.2 M KOH, and the filter is sterilized.

C6. IPTG (Isopropyl-β-D-thiogalactoside) for Colony Selection

• IPTG	100 mg
• Distilled water	1 ml

The solution was filter sterilized and stored at –20°C.

C7. X-Gal (5-bromo-4-chloro-3-indolyl-B-D-galactoside)

• X-Gal	20 mg
• Dimethylformamide	1 mL

The solution was stored at –20°C, protected from light.

D. Appendix

SUPPLIERS OF CHEMICALS, ENZYMES AND KITS

D1. Chemicals	**Suppliers**
Acrylamide	Sigma
Agar-agar	Sigma
Agarose	Sigma
Ammonium persulfate	BioRad
Ampicillin	Sigma
Anti-mouse IgG	Sigma
Bovine serum albumin	Sigma
Bromophenol blue	Merck
$CaCl_2.2H_2O$	Merck
Coomassie Blue G-250	Sigma
Coomassie Blue R-250	Sigma
Dimethylformamide	Merck
dNTPs	ThermoScientific

DTT	Merck
EDTA	Sigma
Ethanol	Merck
Ethidium bromide	Sigma
Fetal bovine serum	Biochrom
Formaldehyde	Merck
Glacial acetic acid	Merck
Glycerol	Merck
Glycine	Merck
H_2SO_4	Merck
HCl	Merck
IPTG	Cayman
Isopropanol	Merck
Kanamycin	Sigma
KCl	Merck
KH_2PO_4	Merck
Luria Broth	Merck
Methanol	Merck
$MnCl_2$	Merck
MOPS	Merck
N.N-Methylene-bis acrylamide	Merck
Na_2CO_3	Merck
Na_2HPO_4	Merck
NaCl	Merck
$NaHCO_3$	Merck
NaOH	Merck

Non-essential amino acids	Biochrom
Penicillin/streptomycin	Biochrom
Phenol/chloroform/isoamylalcohol	Merck
Phosphoric acid	Merck
Potassium acetate	Merck
RuCl	Merck
SDS	Merck
Skim milk	Sigma
Streptavin-HRP	BPS
Sucrose	Merck
TEMED	Biorad
TMB	ThermoScientific
Tris-base	Merck
Tris-HCl	Merck
Tween-20	Merck
Urea	Merck
X-gal	ThermoScientific
Xylene cyanol FF	Merck
2-mercaptoethanol	Merck

D2. Enzymes

*Bam*HI	NEB
*Xba*I	NEB
T4 DNA ligase	ThermoScientific
Taq DNA polymerase	ThermoScientific

D3. Kits

AP Conjugate Substrate Kit	Biorad
Gel Extraction Kit	ThermoScientific
pGEMT Easy Vector System	Promega
Plasmid Mini Kit	ThermoScientific
Takara Ni60 colums	Takara