

REGULATORY INTERACTIONS BETWEEN THE TRANSCRIPTION
FACTORS GERE AND SPOIID AND *BAC* OPERON EXPRESSION,
SPORULATION AND GERMINATION IN *BACILLUS SUBTILIS*

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FACTORS GERE AND SPOIID AND BAC OPERON EXPRESSION,
SPORULATION AND GERMINATION IN *BACILLUS SUBTILIS***

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ABSTRACT

REGULATORY INTERACTIONS BETWEEN THE TRANSCRIPTION FACTORS GERE AND SPOIIID AND *BAC* OPERON EXPRESSION, SPORULATION AND GERMINATION IN *BACILLUS SUBTILIS*

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Bacillus subtilis is a Gram-positive model organism capable of forming endospores and producing various antimicrobial secondary metabolites. One of these secondary metabolites is bacilysin, a nonribosomally synthesized dipeptide consisting of the amino acids L-alanine and L-anticapsin. Previous works by our group have shown the connection between bacilysin production and sporulation. Such that, the major sporulation regulators were shown to regulate the expression of the *bac* operon. In addition, a strain lacking *bac* operon expression, OGU1, was shown to form endospores with lowered resistances to heat, chloroform, and lysozyme as well as lowered germination rates. OGU1 was also shown to be underexpressing many major sporulation regulators suggesting a two-way connection between bacilysin production and sporulation. Two transcription factors that were downregulated in OGU1, GerE and SpoIIID were selected for this study. These genes were cloned and overexpressed in OGU1 to see if they could restore its sporulation defects where they were found to partially rescue these defects. In addition, the potential roles of these genes in *bac* regulation were studied. β -galactosidase assays were employed in OGU1 to see how GerE and SpoIIID overexpression affected *bac* expression. Disc

diffusion bioassays using *gerE* and *spoIIID* knockout strains were utilized to observe the effects of the knockouts on bacilysin production. These experiments showed conflicting results, with the β -galactosidase assay suggesting both are negative regulators and the bioassay revealing that *spoIIID* and *gerE* knockout strains might positively regulate bacilysin production. Finally, EMSAs were performed to observe whether SpoIIID and GerE can directly regulate the *bac* operon expression. Both were shown to bind *bac* operon on their own and in co-existence. Moreover, adding external bacilysin enhanced the DNA binding of both proteins.

Keywords: *B. subtilis*, Bacilysin, Sporulation, Germination, Transcription Factors

ÖZ

BACILLUS SUBTILIS'DE GERE VE SPOIIID TRANSKRİPSİYON FAKTÖRLERİNİN BAC OPERON İFADESİ, SPORULASYON VE JERMİNASYON ÜZERİNDEKİ ETKİLERİ

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Bacillus subtilis, endospor oluşturabilen ve çeşitli antimikrobiyal ikincil metabolitler üretebilen Gram-pozitif bir model organizmadır. Bu ikincil metabolitlerden biri, L-alanin ve L-antikapsin amino asitlerinden oluşan ve ribozomal olmayan şekilde sentezlenmiş bir dipeptit olan basilisindir. Grubumuz tarafından yönetilen önceki çalışmalar da basilisin üretimi ve endospor oluşumu arasındaki bağlantılar gösterilmiştir. Başlıca sporülasyon regülatörlerinin *bac* operonunun ekspresyonunu düzenlemesine ek olarak basilisin üretemeyen OGU1 suşunun sporlarının ısı, kloroform ve lizozime karşı daha düşük dirence sahip olduğu ve daha yavaş jermineasyona uğradığı gözlemlenmiştir. Ek olarak, OGU1'de sporülasyon transkripsiyonel düzenleyicileri dahil olmak üzere sporülasyonla ilgili birçok genin daha az ifade edildiği gösterilmiştir. Bu sonuçlar, basilisin üretimi ile sporlanma arasında iki yönlü bir bağlantı olduğunu göstermektedir. Şimdiki çalışma için OGU1'de gen ifadesi azaldığı gösterilen GerE ve SpoIIID sporulasyon transkripsiyon faktörleri seçilmiştir. Bu transkripsiyon faktörlerinin, sporülasyon kusurları üzerindeki olası etkilerini belirlemek amacıyla OGU1'e klonlanmaları ve aşırı ifade edilmeleri sağlanmış, GerE ve SpoIIID'nin bu sporulasyon kusurlarını

kısmen düzelttiği gösterilmiştir. Ayrıca, GerE ve SpoIIID proteinlerinin basilisin ekspresyonu üzerindeki etkilerini araştırmak amacıyla aşırı ifadeleri sağlanmış ardından OGU1'de β -galaktosidaz enzim aktivite analizleri gerçekleştirilmiştir. Ayrıca, bu genlerin basilisin üretimi üzerindeki etkilerini gözlemlemek amacıyla *gerE* ve *spoIIID* genleri susturulmuş suşlar kullanılarak disk-agar difüzyon analizleri gerçekleştirilmiştir. β -galaktosidaz enzim aktivite analizleri bu genlerin basilisin ekspresyonu üzerinde olası negatif regülatör rollerinin olduğunu ortaya koyarken, disk-agar difüzyon analizleri ise bu genlerin pozitif regülatörler olarak görev aldığı gösterilmiştir. Son olarak, EMSA deneyleri ile GerE ve SpoIIID proteinlerinin *bac* operonu ile doğrudan bağlanma gösterip göstermedikleri incelenmiş ve her iki proteinin de hem kendi başlarına, hem de bir arada *bac* operonuna doğrudan bağlandıkları kanıtlanmıştır. Buna ek olarak, basilisin ilavesinin bu proteinlerin DNA bağlanma aktivitesi üzerinde olumlu bir etkisinin olduğu da gösterilmiştir.

Anahtar Kelimeler: *Bacillus subtilis*, Basilisin, Sporulasyon, Jerminasyon, Transkripsiyon Faktörleri



To Yarco...

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LIST OF ABBREVIATIONS

ABBREVIATIONS

(p)ppGpp: Guanosyl penta- and tetraphosphate

AHL: Acyl Homoserine Lactone

AMP: Antimicrobial Peptide

bp(s): Basepair(s)

CCR: Carbon Catabolite Repression

CSF: Competence and Sporulation Factor

Da(s): Dalton(s)

DPA: Dipicolinic Acid

EMSA: Electrophoretic Mobility Shift Assay

GlcN6P: Glucosamine-6-phosphate

IPTG: Isopropyl β - d-1-thiogalactopyranoside

kb(s): Kilobase(s)

NRPS: Nonribosomal Peptide Synthetase

PTS: Phosphotransferase System

QS: Quorum Sensing

SDS-PAGE: Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis

CHAPTER 1

INTRODUCTION

1.1 *Bacillus subtilis*: A Model Organism with Biotechnological Importance

Bacillus subtilis is a Gram-positive member of the phylum Firmicutes, order Bacillales, family Bacillaceae and genus *Bacillus*. *Bacillus subtilis*, one of the earliest described bacteria, was named *Vibrio subtilis* by Christian Gottfried Ehrenberg in 1835 (Ehrenberg, 1835). *Bacillus subtilis* retrieved its currently known name by Ferdinand Julius Cohn in 1872 who identified its family as Bacillaceae (Gordon, 1981).

The members of the Bacillaceae family are mostly Gram-positive, rod-shaped heterotrophs capable of forming endospores to survive extreme conditions. The genus *Bacillus* includes several famous human pathogens such as *B. anthracis* which causes anthrax and *B. cereus* which causes food poisoning as well as the widely used insect pathogen *B. thuringiensis*. *Bacillus subtilis*, on the other hand, has no pathogenic tendencies (Priest *et al.*, 1988).

B. subtilis is most commonly found in soil, however, it can also be found in different parts of terrestrial and aquatic environments as well as a member of the microbiomes of various plants and animals (Nicholson *et al.*, 2000).

B. subtilis is a chemoorganotroph that oxidizes organic compounds in the environment to derive its energy to survive and prefers mesophilic growth conditions. Until 1998, *B. subtilis* was considered as a strict aerobe, however, it was proved that *B. subtilis* can also grow in anaerobic conditions by either using nitrate or nitrite as a terminal electron acceptor or by fermentation. (Nakano and Zuber, 1998).

In 1997, *B. subtilis* genome project was completed and constituted one of the first full-genome sequencing to be published (Kunst et al., 1997). The single chromosome genome, consisting of 4,2 million base pairs, codes for 4106 proteins, 30 rRNAs, 86 tRNAs, and a variety of other sncRNAs. Although its genome encodes over 4200 genes, only 275 of them were revealed to be essential for growth under optimal conditions. About 250 genes encode transcription factors and 17 are sigma factors (Kobayashi *et al.*, 2003).

The discovery of the ability of some strains of *B. subtilis* to develop natural competence is one of the major factors that led to the organism becoming a model organism (Anagnostopoulos and Spizizen, 1961). Natural competence enables *B. subtilis* to take up foreign DNA fragments in the environment and even integrate them into its genome.

Competence naturally occurs towards the end of the exponential growth phase; however, it can also be stimulated by artificial growth conditions in a controlled environment (Harwood, 1992). Moreover, at saturating DNA concentrations cells can take up more than one molecule of DNA, a process called congression. Congression is a valuable aspect especially in constructing new strains when the markers cannot be selected directly (Harwood and Cutting, 1990). The ease of genetic manipulations of *B. subtilis* generated interest in the organism, and many began to study many research areas including cell division, differentiation, sporulation, protein secretion, quorum sensing, and biofilm formation.

One of the major features of *B. subtilis* is its ability to form endospores to survive extreme environmental conditions for a significant amount of time (up to millions of years). When nutrients are scarce, *B. subtilis* induces motility, chemotaxis, and secretion of antibiotics as the first lines of defense. Ultimately, sporulation is initiated which ends with the transition of vegetative cells into a dormant state called a spore (or endospore). Compartmentalization of a single *B. subtilis* cell during sporulation divides it into a mother cell and a forespore. Differential expression of genes in both compartments drives the development of the forespore into a mature endospore and

leads to the release of the endospore through programmed mother cell lysis. The released endospore can resist extreme heat, chemicals, irradiation, and dryness. Upon detecting suitable environmental conditions, germination begins and spores turn back into vegetative cells (Stragier and Losick, 1996).

The use of *B. subtilis* in industry is widespread. Its ease of cultivation and genetic manipulation, its ability to convert organic substrates into biotechnological products rapidly, and its ability to secrete large amounts of protein into its culture medium are why *B. subtilis* is so prevalent in the industry (Hohmann *et al.*, 2017). Compared to its competitor *E. coli*, *B. subtilis* is advantageous as it is a non-pathogenic organism that doesn't produce endotoxins, has no significant codon bias, and is far more efficient in secreting recombinant proteins due to its lack of an outer membrane.

Many industrially important enzymes including amylase, lichenase, β -galactosidase, cellulase, alkaline serine proteases, and xylanases can be produced in vast amounts by utilizing recombinant *B. subtilis* strains (Su *et al.*, 2020).

Recombinant *B. subtilis* strains can also be used to produce riboflavin (vitamin B₂), other B vitamins (B₁, B₅, B₆, and B₇) and menaquinone-7 (vitamin K₂) at industrial scales (Shi *et al.*, 2009; Cui *et al.*, 2020). Another medically valuable compound produced is Scyllo-inositol, a potential therapeutic for Alzheimer's Disease (Yoshida & Ishikawa, 2019).

Hyaluronic acid (which is commonly used in the pharmaceutical, cosmetic, and food industries) and gamma-polyglutamic acid (which is used as a thickening, moisturizing, or antifreeze in food and cosmetics industries) are other examples of industrially valuable products produced by *B. subtilis* (Jin *et al.*, 2016; Li *et al.*, 2022).

B. subtilis also produces various secondary metabolites with useful properties including antimicrobial activity. These are discussed in detail in Section 1.3.

B. subtilis is also utilized as a probiotic for animals. Thanks to its ability to form resistant spores, it can be added to animal feeds requiring high-heat processing during preparation and can resist the low-pH environments of the gastrointestinal tract (Stülke *et al.*, 2023). Feeding livestock animals with *B. subtilis* as a microbial additive was shown to improve food digestibility, speed up animal growth, and prevent disease (Su *et al.*, 2020). Many ongoing studies are focusing on the use of *B. subtilis* as a human probiotic supplement and recent findings have identified it as one of the most promising spore-based probiotics for human use (Williams and Weir, 2024).

The strains of *B. subtilis* used for research and industrial purposes are almost all descendants of the strain *B. subtilis* 168. Strain 168 is a tryptophan auxotroph and a derivative of the original Marburg strain and was generated by X-ray mutagenesis by Burkholder and Giles in 1947. As it was highly transformable compared to the other mutant strains generated, *B. subtilis* 168 has become one of the main subjects in subsequent research worldwide (Anagnostopoulos & Spizizen 1961). When compared to the Marburg strain, *B. subtilis* 168 is incapable of multicellularity and swarming as it has mutations in two genes required for those functions (*sfp* and *swrA*) (Kearns *et al.*, 2004). 168 is also incapable of biofilm formation due to the lack of an ancestral plasmid encoding the gene *rapP* as well as mutations in *sfp*, *epsC* and *degQ* (McLoon *et al.*, 2011).

W23 is another strain of *B. subtilis*, and its origin is not well documented. When compared to strain 168, W23 shows differences in bacteriophage resistance, cell wall chemistry, enzyme kinetics and transformability (Zeigler *et al.*, 2008). The genomes of strain 168 and W23 only show 60-70% relatedness. This ratio led the classification of those strains to be classified as two subspecies as *B. subtilis* subsp. *subtilis* and *B. subtilis* subsp. *spizizenii*, respectively (Nakamura *et al.*, 1999).

Another widely used laboratory strain of *B. subtilis* is the strain PY79 which is a prototrophic derivative of strain 168 capable of producing tryptophan (Youngman *et al.*, 1984). Although it is commonly referred to as a derivative of strain 168, it is

actually a hybrid strain generated by transforming strain 168 with genomic DNA from W23. Thus, the genome of the strain PY79 contains two islands of W23 genome around *trpC* and *sacA* genes in addition to the four large deletions which add up to a total of 180 kbp missing. When compared to 168, PY79 also contains 81 SNPs (Zeigler *et al.*, 2008).

1.2 Global Regulation of Gene Expression in *Bacillus subtilis*

1.2.1 Carbon Catabolite Repression

The majority of bacteria have the ability to use several different compounds as a carbon source at the same time, however, some of those carbon sources are preferred as they are easier to access and more metabolizable for the organism, which increases competitive success over other microorganisms in the environment. Most of the time, glucose is the main carbon source that is preferred over a secondary carbon source. This preference for one specific carbon source over another is referred to as carbon catabolite repression (CCR), which defines a regulatory process where the expression of enzymes required for the metabolization of the secondary carbon source is reduced eventually. CCR is achieved by combining activities of global and operon-specific regulatory mechanisms (referred to as inducer exclusion and inducer prevention) (Görke and Stülke, 2008).

The outcomes of CCR are similar in *E. coli* and *B. subtilis*; however, the molecular mechanisms are quite different between the two organisms. In *E. coli*, the transcription of catabolic genes is inhibited through global regulatory pathways in the presence of glucose. On the other hand, the presence of glucose drives negative regulation through a repressor protein in *B. subtilis*. In both organisms, the phosphoenolpyruvate–carbohydrate phosphotransferase system (PTS) is important in the signal transduction pathway that enables CCR. In *B. subtilis*, CcpA (catabolite control protein A), HPr (histidine protein), HPrK (HPr kinase/phosphorylase), fructose-1,6-bisphosphate (FBP), and glucose-6-phosphate (Glu-6-P) are the key

players in CCR (Warner and Lolkema, 2003). Phosphorylation of HPr is the central phenomenon in signal transduction during CCR (Deutscher *et al.*, 1995). HPrK acts as a kinase when nutrients are abundant (high abundance of FBP and Glu-6-P), causing the formation of HPr(Ser-P), which acts as the cofactor of CcpA. HPr(Ser-P) binds dimeric CcpA triggering a conformational change, bringing its DNA binding domain to the surface (Jault *et al.*, 2000; Schumacher *et al.*, 2004). Then, the complex binds to operator sites of catabolic operons referred to as catabolite-responsive elements (cre) leading to the repression of catabolic genes when preferred carbon sources are available (Miwa *et al.*, 2000).

CcpA-mediated CCR was shown to affect sporulation efficiency (Miwa *et al.*, 1994). It was also shown that CcpA represses three genes involved in the regulation of sporulation (*spo0B*, *spoIIID*, and *gerE*) in the presence of glucose, thus restraining sporulation (Moreno *et al.*, 2001).

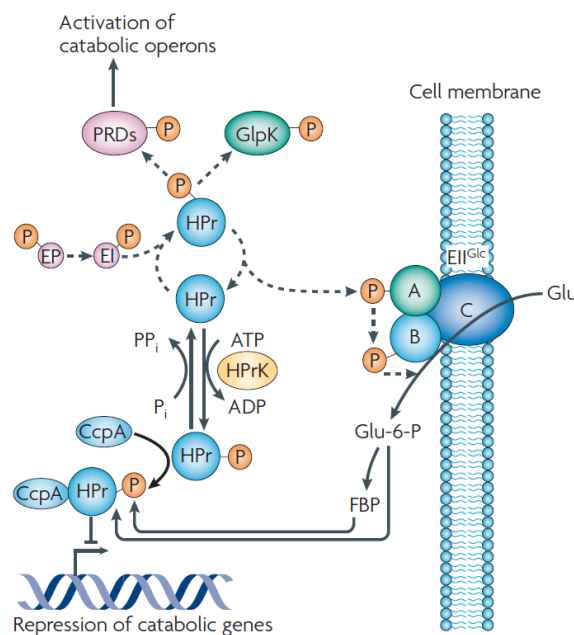


Figure 1.1: Carbon catabolite repression in *Bacillus subtilis* (Görke and Stülke, 2008)

1.2.2 Quorum Sensing

Quorum sensing (QS) modulates physiological processes by regulating gene expression in response to cell-population density in the environment (Miller and Bassler, 2001; Henke and Bassler, 2004). Until now, no quorum-sensing deficient strain of *B. subtilis* has been defined in nature, indicating the importance of the phenomenon on survival (Kalamara *et al.*, 2018). A variety of processes are regulated by QS including virulence, motility, antibiotic production, biofilm formation, competence, and sporulation. The communication between cells in the population is mediated by signal molecules called autoinducers, which are secreted to the extracellular environment (Dunny & Winans, 1999). Gram-negative and positive bacteria differ in some aspects of QS. For example, while the majority of Gram-negative bacteria use acylated homoserine lactones (AHLs), Gram-positive bacteria use oligopeptides as autoinducers. Moreover, Gram-negative bacteria employ LuxR-like proteins as receptors of autoinducers, while Gram-positive bacteria use two-component systems. A phosphorylation/dephosphorylation cascade is used by both to transmit signals (Miller and Bassler, 2001).

There are two main QS systems in *B. subtilis*, the ComQXPA system and the Rap-Phr system. In the ComQXPA system, ComX acts as the autoinducer (pheromone) which is synthesized as a 55 amino acid precursor which is modified into a 5 to 10 amino acid peptide and exported by ComQ to the outside of the cell (Solomon *et al.*, 1995; Ansaldi *et al.*, 2002). ComP acts as a sensor protein kinase that binds to extracellular ComX, leading to the phosphorylation and activation of ComA, the DNA binding response regulator of the ComP-ComA two-component signal transduction system (Roggiani and Dubnau, 1993). The ComXQPA quorum sensing system is depicted in Figure 1.2. Activated ComA positively regulates surfactin production and also increases expression of the *comS* gene, leading to the development of genetic competence (Nakano *et al.*, 1991; Turgay *et al.*, 1998). In addition, ComA activation leads to the phosphorylation of DegU, increasing the synthesis of exoproteases (Kobayashi, 2007).

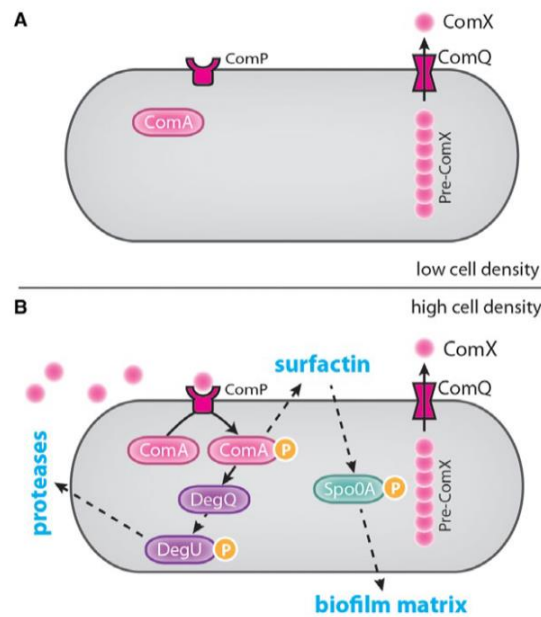


Figure 1.2 ComXQPA quorum sensing mechanism in *B. subtilis* (Kalamara *et al.*, 2018)

Another QS system in *B. subtilis* is the Rap-Phr system. Receptor-signal pairs of the Rap-Phr system can vary from strain to strain. *B. subtilis* 168 encodes eight pairs named Rap-Phr A, C, E, F, G, H, I, K, and three additional Rap proteins named RapB, D, and J (Omer Bendori *et al.*, 2015). Phr proteins are secreted as proproteins and cleaved into pentapeptides extracellularly. The most studied Phr peptide is PhrC and is also known as CSF (Competence and Sporulation Factor). After being processed, Phr proteins are taken in by an ABC transporter named oligopeptide permease (Opp) (Perego *et al.*, 1991). At low cell density, Phr peptides do not enter the cell. At high cell density, the concentration of extracellular Phr peptides increases, leading to their entry into the cell where they bind and inhibit the functions of Rap proteins (Pottathil and Lazazzera, 2003). The role of Rap proteins is to suppress the response regulators ComA~P, DegU~P, and indirectly Spo0A~P. Inhibition of Rap by Phr releases the aforementioned proteins from Rap-mediated suppression, thus, the inhibition of biofilm matrix formation, swarming motility, exoprotease secretion, competence development, and sporulation initiation is

eliminated. The Rap/Phr system in *B. subtilis* is depicted in Figure 1.3 along with the ComXQPA system (Kalamara *et al.*, 2018).

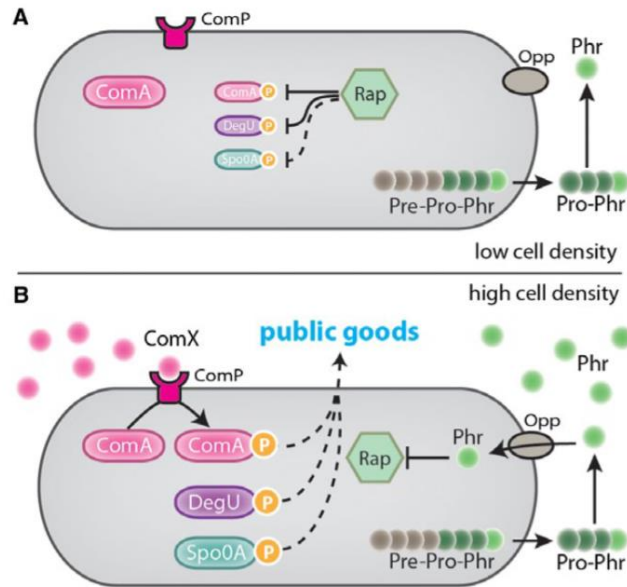


Figure 1.3 The ComXQPA and Rap/Phr quorum sensing systems in *B. subtilis* (Kalamara *et al.*, 2018)

1.2.3 Stringent Response

Stringent response is a universal response mechanism that protects bacteria from adverse environmental conditions such as nutrient limitation, exposure to antimicrobial compounds, and physicochemical stress. Stringent response is mediated by the alarmones guanosyl tetra- and pentaphosphates ppGpp and pppGpp, collectively referred to as (p)ppGpp. In *E. coli*, alarmones are synthesized by the ribosome-bound enzyme RelA and the cytosolic enzyme SpoT out of GTP and ATP. RelA is an alarmone synthase that becomes active when peptide synthesis is stalled at the ribosome due to the binding of an uncharged tRNA due to amino acid starvation (Haseltine & Block, 1973). SpoT has both hydrolase and synthase activity depending on whether conditions are stringent or relaxed (Magnusson *et al.*, 2005).

Alarmones change the transcriptional profile of the organism by directly binding RNA polymerase, repressing rRNA genes, and increasing the transcription of genes involved in amino acid synthesis and nutrient transport (Irving *et al.*, 2020).

In firmicutes, including *B. subtilis*, three genes code for (p)ppGpp synthases. The first one is RSH a bifunctional enzyme homologous to RelA/SpoT system in *E. coli*, and has both synthase and hydrolase functions. The others are RelQ and RelP (SAS1 and SAS2) which are small alarmone synthases (Atkinson *et al.*, 2011). In *B. subtilis* alarmones do not bind RNA polymerase, rather, they limit rRNA synthesis by using GTP for alarmone synthesis (Krásný & Gourse, 2004).

The decrease in GTP during alarmone synthesis causes the inactivation of a transcription factor named CodY which normally represses a variety of stationary phase genes regulating extracellular enzyme synthesis, transport systems, competence development, antibiotic synthesis, and sporulation (Sonenshein, 2005). The decrease in intracellular GTP levels also leads to upregulation of KinA and KinB. These are two kinases that phosphorylate Spo0F, which leads to the activation of Spo0A, thus stimulating the initiation of sporulation phosphorelay (Tojo *et al.*, 2013). The role of RSH and CodY in stringent response in *B. subtilis* is depicted in Figure 1.4.

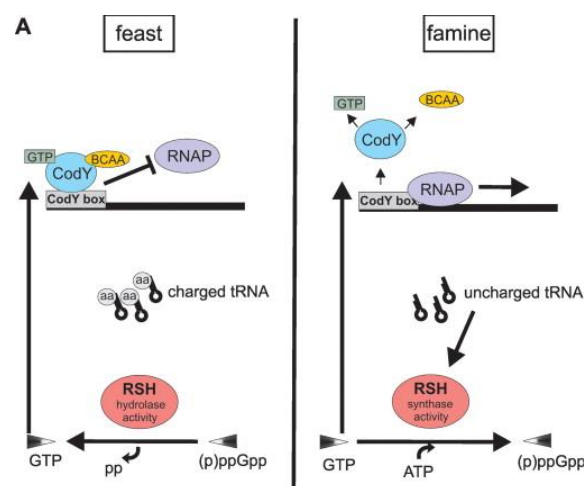


Figure 1.4 Schematic intersection of stringent response and regulation through CodY in *B. subtilis* (Geiger and Wolz, 2014)

1.3 Production of Secondary Metabolites by *B. subtilis*

Bacterial metabolism is divided into two categories: primary and secondary metabolism. In contrast to primary metabolites, secondary metabolites are not directly involved in the organism's normal growth, development, and reproduction. Secondary metabolites are usually small, uncharged, non-polar molecules that can pass through cell membranes easily and play essential roles outside of the cell at intra- and interspecies levels contributing to the fitness of their producers (Harwood *et al.*, 2018). Secondary metabolites have a wide variety of functions such as aiding in biofilm formation, attachment, swarming, acting as pheromones in quorum sensing, and recovering trace elements from the environment (Stein, 2005). Nevertheless, by far the most attractive secondary metabolites are the ones that also have antimicrobial activity. At least 4 to 5 percent of any given *B. subtilis* strain's genome is estimated to be devoted to antimicrobial compound (AMC) production. Those bioactive metabolites are schematized in Figure 1.5.

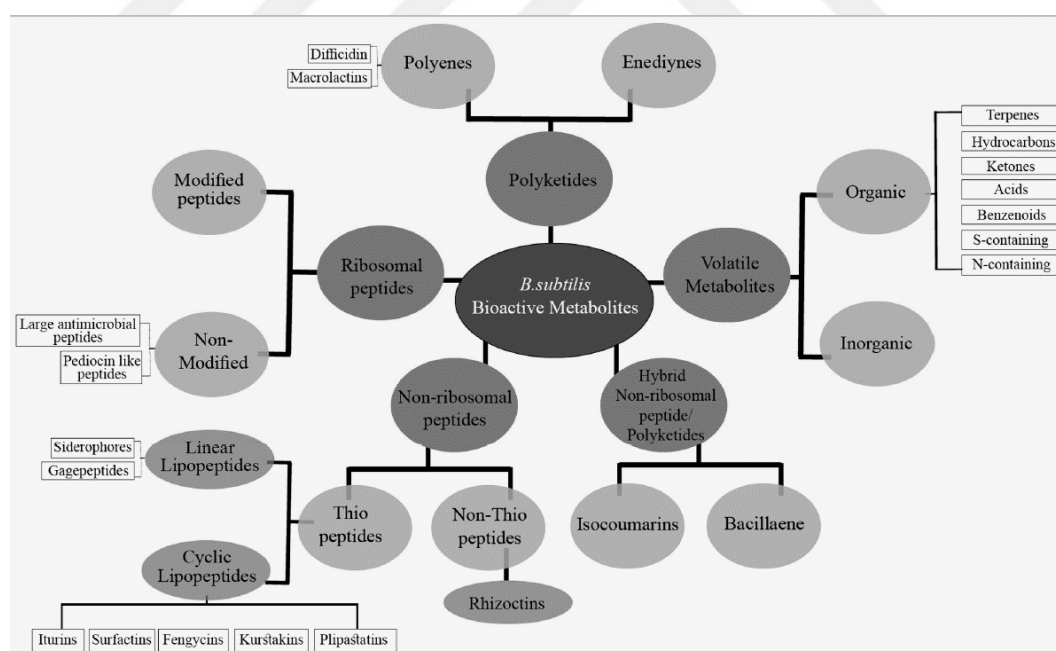


Figure 1.5 Bioactive metabolites produced by *B. subtilis* (Iqbal *et al.*, 2023)

The major group in AMCs is the antimicrobial peptides (AMP) which usually show highly rigid, hydrophobic cyclic structures. They may contain unusual constituents

such as D-amino acids and show resistance to hydrolysis by peptidases and proteases as well as resistance against oxidation (Katz and Demain, 1977).

AMPs can be divided into two main categories: ribosomal peptides (RPs) and non-ribosomal peptides (NRPs). The ribosomal synthesis of antimicrobial peptides is followed by post-translational modifications (Zuber *et al.*, 1993). The formation of lanthionine, a nonproteinogenic amino acid, is an example of such modifications. Lantibiotics are antibiotic peptides containing lanthionine and they constitute a major group among RPs (Schnell *et al.*, 1988).

There are four classes of lantibiotics, however only two of them are known to be produced by *B. subtilis*. Type I or type A lantibiotics mostly show linear structures and lyse Gram-positive cells by forming pores in their cytoplasm. Type II or Type B lantibiotics are more likely to have globular structures. Lantibiotics are generally more effective against Gram-positive bacteria than Gram-negative bacteria due to their outer membranes (Barbosa *et al.*, 2015).

A list of lantibiotics produced by *B. subtilis* and their antimicrobial activity is given in Table 1.1 adapted from Iqbal *et al.*, 2023.

Table 1.1 Lantibiotics produced by *B. subtilis* and their antimicrobial activities

Lantibiotics	Antimicrobial activity		
	Gram-positive	Gram-negative	Anti-fungal
Entianin	+	-	-
Ericin A	+	-	-
Erisin S	+	-	-
Subtilin	+	-	-
Mersacidin	+	-	-
Sublancin 168	+	-	-
Lichenicidin	+	-	-
Subtilosin A	+	+	-
Subtilomycin	+	+	+
Amylolysin	?	?	+

1.3.1 Non-Ribosomal Peptide Synthesis and Non-Ribosomal Peptides (NRPs)

The second major class of antimicrobial peptides (AMPs) can be considered as non-ribosomal peptides (NRPs) which are synthesized by non-ribosomal peptide synthases (NRPSs). NRPs can be divided into two classes depending on whether they are synthesized through a thio-template mechanism or not. The first class is thiotemplate NRPs which can be further divided into sub-groups as cyclic and linear lipopeptides and the second class is non-thiotemplate NRPs (Caulier *et al.*, 2019).

NRPSs are mega-enzymes that directly produce their products using macromolecular protein complexes instead of nucleic acid templates. NRPSs are composed of different modules with different catalytic activities, and each NRPS complex consists of a loading (or initiation) module, a variable number of elongation modules, and a termination module (Harwood *et al.*, 2018). Each module also has different domains depending on their catalytic activity. The loading module has an adenylation domain that selects the first amino acid building block and turns it into an aminoacyl adenylate by hydrolyzing an ATP. The products of the A domain are then transferred and attached via a thioester bond to a peptidyl carrier protein (PCP) also referred to as the T domain. The following elongation modules have A and T domains in addition to a condensation (C) domain responsible for peptide bond formation between amino acids on its T domain and the peptidyl intermediate bound to the T domain of the previous module. The termination (Tc) module has an additional domain named the thioesterase (TE) domain which releases the newly produced NRP from the NRPS (Donadio *et al.*, 2007). In addition to those three fundamental modules, additional modules can also be present. These modules can carry out specific modifications via their special domains M, R, E, and Cy, which are responsible for amino acid methylation, reduction, epimerization, and heterocyclization, respectively (Donadio *et al.*, 2007). Not being dependent on genetically coded 20 amino acids, their modular nature and the fact that multiple

combinations of catalytic modules are possible enable NRPSs to synthesize a wide diversity of NRPs.

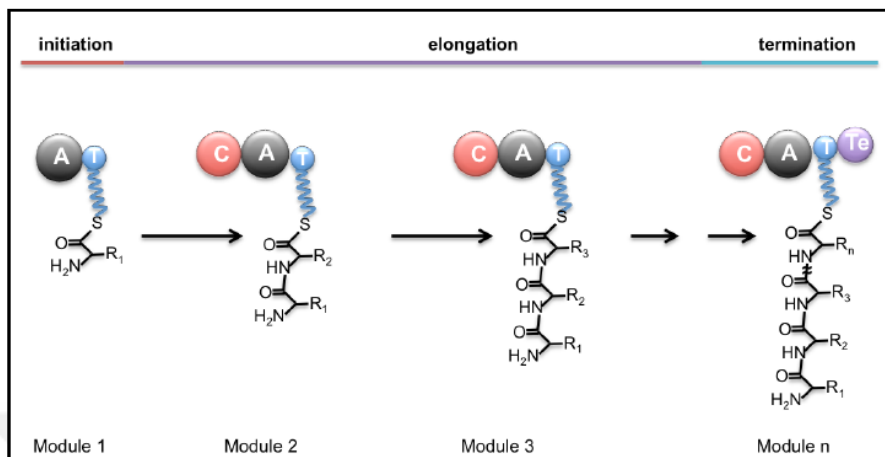


Figure 1.6 Non-ribosomal peptide synthesis (NRPS) in *B. subtilis* (Harwood *et al.*, 2018) A, adenylation domain; C, condensation domain; T, peptidyl carrier protein; Te, termination domain

Surfactins, iturins, and fengycins are three families of cyclic lipopeptides produced by *B. subtilis*. At least 13 surfactins, 14 iturins, and 8 fengycins are known to be produced by *B. subtilis* (Caulier *et al.*, 2019).

The surfactin group is the most extensively studied class of lipopeptides due to their extraordinary surfactant power (Wang *et al.*, 2015). The surfactin group has approximately 20 different members that show a wide variety of functions including emulsifying, foaming, antibacterial, antifungal, and antiviral features (Peypoux *et al.*, 1999). These compounds show even antitumor, anticoagulant, and hypocholesterolemic properties (Cao *et al.*, 2019). Regulation of surfactin biosynthesis is highly related to the competence development pathway, both can be induced at the same time via quorum sensing. Although there aren't any known secretion systems for surfactins, they may passively diffuse through cell membranes (Stein, 2005).

The Iturin family is composed of cyclic heptapeptides, four iturins, five bacillomycins, and mycosubtilin (Wang *et al.*, 2015). They are best known for their

potent antifungal activity and relatively low antibacterial activities, however, no antiviral activity has been observed (Moyne *et al.*, 2001; Walton and Woodruff 1949; Kumar *et al.*, 2012). The low toxicity, low allergic effects, and high biodegradability alongside their wide antifungal spectrum make the iturin family promising antifungal agents (Ye *et al.*, 2012).

Fengycins are cyclic decapeptides showing potent antifungal activity, especially against filamentous fungi, but no antibacterial and antiviral activities have been observed until now (Ongena *et al.*, 2005).

Other NRPs that are not members of surfactin, iturin, or fengycin families are listed in Table 1.2.

Table 1.2 Other classes of NRPs and their antimicrobial activity (Caulier *et al.*, 2019)

NRP Subgroup	Compound	Antimicrobial activity		
		Antibacterial	Antifungal	Antiviral
Dipeptide	Bacilysin	+	+	-
Dipeptide	Chlorotetain	-	+	-
Siderophores	Bacillibactin	-	+	+
Polypeptide	Bacitracin A, F	+	-	-
Polypeptide	Mycobacillin	-	+	-
Dipeptide	Rhizocticin A	-	+	-
Nonapeptide	Locillomycins	+	-	+
Gageopeptide	Gageotetrine A-C	-	+	-

1.4 Bacilysin

Bacilysin is an NRP synthesized by *B. subtilis* which was discovered in 1946 by Abraham *et al.* as an antibiotic effective against *S. aureus* (Abraham *et al.*, 1946). Bacilysin was independently discovered by Foster and Woodruff later in 1946 and was named bacillin by them. (Foster and Woodruff, 1946).

Bacilysin is a dipeptide with molecular formula C₁₂H₁₈N₂O₅ and has a molecular mass of 270 Da. It consists of only two amino acids, L-alanine at its N-terminus and

L-anticapsin at its C-terminus, making it the smallest currently known peptide antibiotic (Walker & Abraham, 1970).

In 1949, the production of bacilysin by *B. subtilis* strain A14 as a highly heat-stable molecule was reported, and it was not able to be extracted using organic solvents (Newton, 1949). In 1965, bacilysin was isolated from the culture filtrate of *B. subtilis* A14 (Rogers *et al.*, 1965) and acid hydrolysis exposed its composition of L-alanine and L-anticapsin (Walker & Abraham, 1970). In 1973, another antibiotic named tetaine was shown to be produced by *Bacillus pumilus* B-180. In 1975 bacillin, tetaine, and bacilysin were shown to be identical compounds (Atsumi *et al.*, 1975). Other early studies revealed various features of bacilysin such as high heat tolerance, stability between pH 1.4 to 12.0 for four hours at 20°C, insolubility in organic solvents, and antimicrobial activity against a wide range of bacteria, fungi, and algae (Newton, 1949).

To exert its antimicrobial activity bacilysin has to be hydrolyzed into its amino acid moieties upon entering cells (Kenig *et al.*, 1976). L-anticapsin is a modified unusual amino acid that inhibits glucosamine 6-phosphate (GlcN6P) synthase by forming irreversible covalent bonds and blocks glucosamine and N-acetyl glucosamine synthesis which are required for bacterial peptidoglycan and fungal mannoprotein synthesis (Milewski *et al.*, 1986). The impairment of microbial cell wall synthesis eventually leads to cell lysis and cell death. Although L-anticapsin is adequate on its own for antimicrobial effect, bacilysin was shown to be transported into cells more easily. Moreover, while anticapsin can form a C–S bond with Cys1 of GlcN6P synthase, bacilysin cannot (Wang *et al.*, 2018).

A wide range of microorganisms susceptible to bacilysin, including bacterial species *E. coli*, *S. aureus*, *S. pyogenes*, and the fungi *C. albicans* and *S. cerevisiae*.

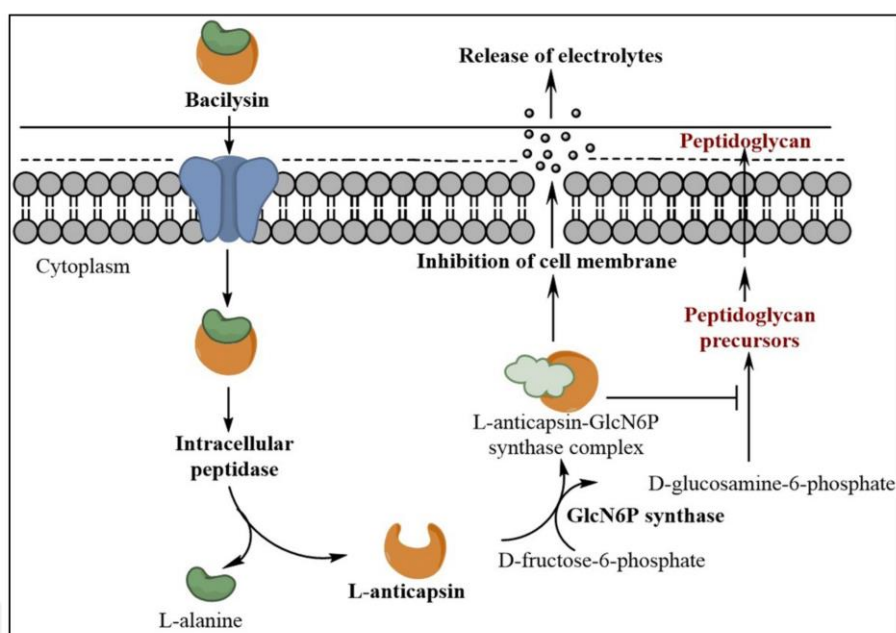


Figure 1.7 Mode of action of bacilysin (Islam *et al.*, 2022)

1.4.1 Biosynthesis of Bacilysin

The biosynthesis and export of bacilysin depends on the proteins encoded by the *bacABCDEF* operon and the *bacG* gene. These genes were originally named *ywfBCDEFG* and *ywfH* and were renamed after their role in bacilysin synthesis was identified (Inaoka *et al.*, 2003; Steinborn *et al.*, 2005). Bacilysin biosynthesis is composed of two major steps; the first is the production of L-anticapsin and the second is its ligation to L-alanine to form bacilysin. BacABCDFG are required to convert prephenate, an intermediate compound of phenylalanine and tyrosine synthesis, to L-anticapsin in a series of reactions (Hilton *et al.*, 1988; Parker and Walsh, 2013). Then, anticapsin is ligated to alanine by the ligase encoded by *bacD* (Tabata *et al.*, 2005). Previous studies conducted by our group showed that BacD has unusual properties for an NRPS, as ATP hydrolysis was only observed for alanine but not for anticapsin, and only alanine was activated as an amino acid phosphate (Yazgan *et al.*, 2001b). BacE is not involved in bacilysin synthesis, but it

acts as a bacilysin efflux pump and provides bacilysin resistance to its producer (Steinborn *et al.*, 2005).

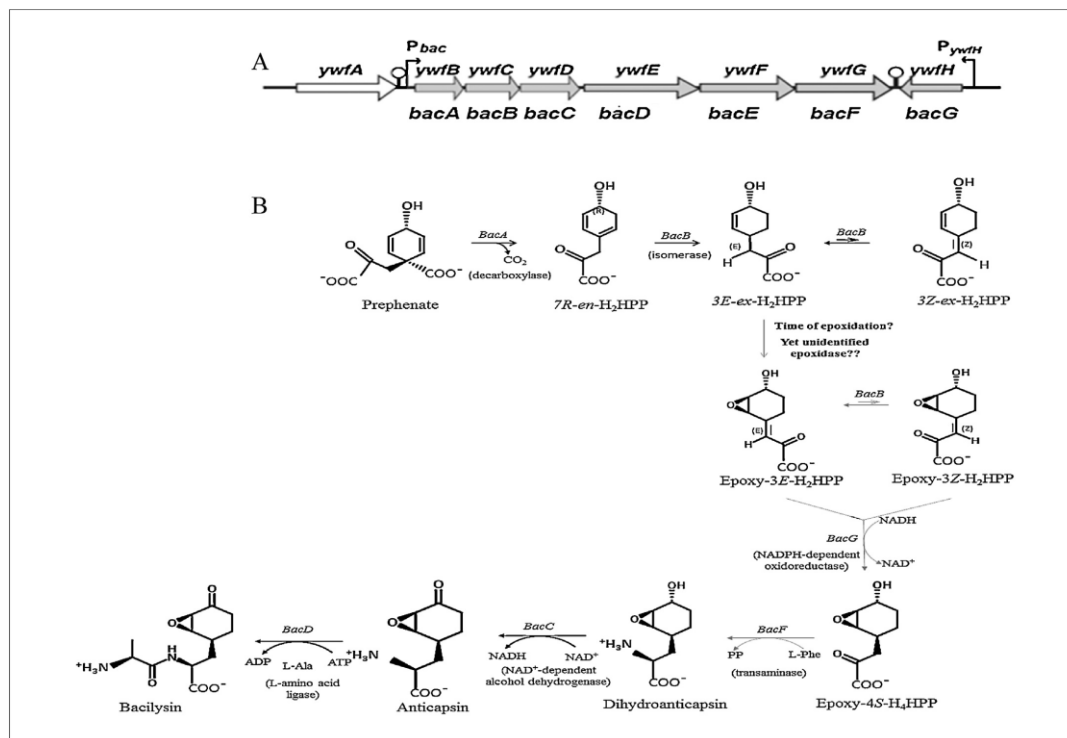


Figure 1.8 Bacilysin operon (A) and its biosynthetic pathway (B) (Özcengiz and Ögölür, 2015)

1.4.2 Regulation of the *bac* Operon

Regulation of bacilysin production through the *bac* operon has been extensively studied. Bacilysin production is strongly affected by growth conditions and the composition of the medium. The type of carbon source in the growth medium greatly affects bacilysin production. While glucose and maltose are the ideal carbon sources, xylose, and ribose were shown to negatively affect bacilysin production if present in the early phases of growth. Nitrogen sources are another important factor, such that, while glutamate and aspartate are ideal nitrogen sources, alanine, casamino acids, phenylalanine, and ammonium salts have antagonistic effects on bacilysin production. Additionally, ferric citrate and phosphate also inhibit bacilysin production when present in high concentrations. Manganese is an essential

ingredient because it is the cofactor of the alanine-anticapsin ligase encoded by *bacD* and its optimal concentration is 660 μ M (Özcengiz *et al.*, 1990; Başalp *et al.*, 1992). The ideal temperature for bacilysin production was found to be 32.5°C, where production reaches its maximum. The ideal pH was found to be 6.8, while both lower and higher pH conditions adversely affect bacilysin biosynthesis (Özcengiz & Alaeddinoğlu, 1991b).

The transcriptional regulators controlling bacilysin production have also been studied by our group. In a study conducted by Yazgan *et al.* in 2001, transposon mutagenesis was employed to create bacilysin non-producer derivatives of PY79 followed by the identification of silenced genes responsible for the bacilysin-negative phenotype (Yazgan *et al.*, 2001a) *oppA* was one of the genes found to be involved in bacilysin biosynthesis which encodes for an oligopeptide permease which has significant roles in the quorum sensing pathway. This finding suggested a strong relation between bacilysin biosynthesis and the quorum sensing pathway. For further investigation, peptide pheromones PhrA and PhrC (CSF), which are internalized by the Opp system and the response regulator ComA-deleted mutants were constructed. As a result, *phrC* and *comA* were found to be essential for bacilysin production as strains lacking these genes produced no bacilysin. Deletion of the transition state regulator *abrB* resulted in increased production of bacilysin. When *spo0A/abrB* double mutants were examined, however, the bacilysin-negative phenotype observed in *spo0A* mutants was shown to be suppressed. This suggests the repressive role of AbrB on some gene(s) involved in *bac* operon expression which can be relieved by Spo0A, a known inhibitor of AbrB.

Another study by our group examined the relation between bacilysin production and 11 genes (*comQ*, *comX*, *comP*, *comA*, *oppA*, *phrC*, *phrF*, *phrK*, *spo0H*, *spo0A*, *abrB*, and *codY*) in more depth. The direct binding of AbrB, CodY, ComA, and Spo0A to the promoter region of *bac* operon *in vitro* was proved by employing EMSA. Moreover, a *bac* operon-blocked strain, namely OGU1, carrying a *lacZ* fusion expressed under *bac* promoter was constructed and enabled the tracking of operon

activity via β -galactosidase assays. Those assays demonstrated that *comQ*, *comX*, *comP*, *comA*, *oppA*, *phrC*, *phrF*, *phrK*, *spo0H* and *spo0A* mutants of *B. subtilis* displayed greatly reduced *bac* operon expression while *abrB* and *codY* mutants were not affected. It was also shown that AbrB and CodY can bind P_{bac} simultaneously (Köroğlu *et al.*, 2011).

DegU and its kinase DegS together form a two-component response system that is activated under the quorum sensing pathway which mainly regulates extracellular degradative enzyme production and swarming motility. In 2012, Mariappan *et al.* showed that DegU positively regulates bacilysin biosynthesis by activating *bacG* expression. ScoC, a known antagonist to DegU which represses functions activated by it, was found to repress the *bac* operon expression via direct binding (Barbieri *et al.*, 2016). LutR, a transcription factor important in swarming motility, was also shown to be required for bacilysin synthesis although the standard strain 168 and its derivative PY79 do not display swarming behaviour (Köroğlu *et al.*, 2008).

This complex regulatory network of bacilysin production is depicted in Figure 1.9.

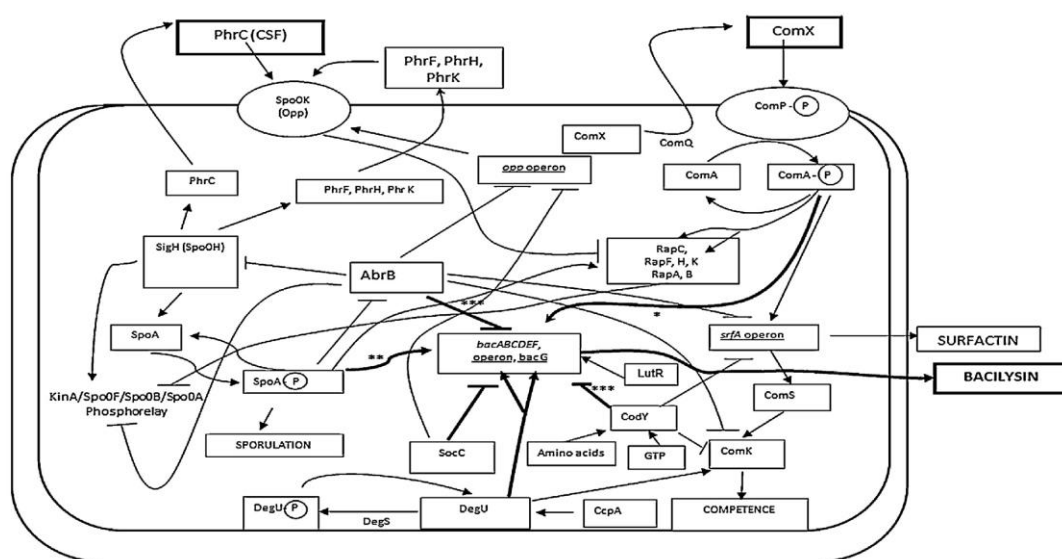


Figure 1.9 Major regulators of the *bac* operon (Özcengiz & Ögülür, 2015).

1.4.3 Bacilysin Non-producer Strain OGU1 and the Pleiotropic Effects of Bacilysin

In the light of previous studies, it can be stated that bacilysin appears to have more functions than being just an antibiotic. It seems to act like a pheromone affecting gene expression in *B. subtilis*. The importance of major sporulation regulators Spo0A and Spo0H was mentioned in section 1.4.2 whose linkage to bacilysin production was also revealed in much early studies (Özcengiz & Alaeddinoğlu, 1991a). Those studies showed reduced resistance of spores to heat, lysozyme, and chloroform in a bacilysin-negative strain of PY79. Interestingly those defects were relieved by the external addition of bacilysin. Later on, alterations in the morphology of sporulating colonies and changes in germination kinetics were depicted in another bacilysin non-producer strain, namely OGU1 (Ertekin *et al.*, 2020). Collectively, those results implied the reciprocal relationship between bacilysin biosynthesis and sporulation.

OGU1, a derivative of PY79, was developed by our group via fusing pMutin T3 plasmid into *bac* operon resulting in *lacZ-bac* fusion. As mentioned in the previous section this feature has been used for tracking the expression of the *bac* operon via β -galactosidase assays provided that the expression of the *lacZ* gene contained in pMutin T3 is regulated by the *bac* promoter. Also, the expression of the *bac* operon was put under the control of P_{spac} due to fusion, making it possible to induce its expression with lactose or IPTG. While the mRNA synthesis from the operon was observed upon induction, bacilysin production was completely disabled in OGU1 which was proved using various analyses such as UPLC-MS (Ertekin *et al.*, 2020).

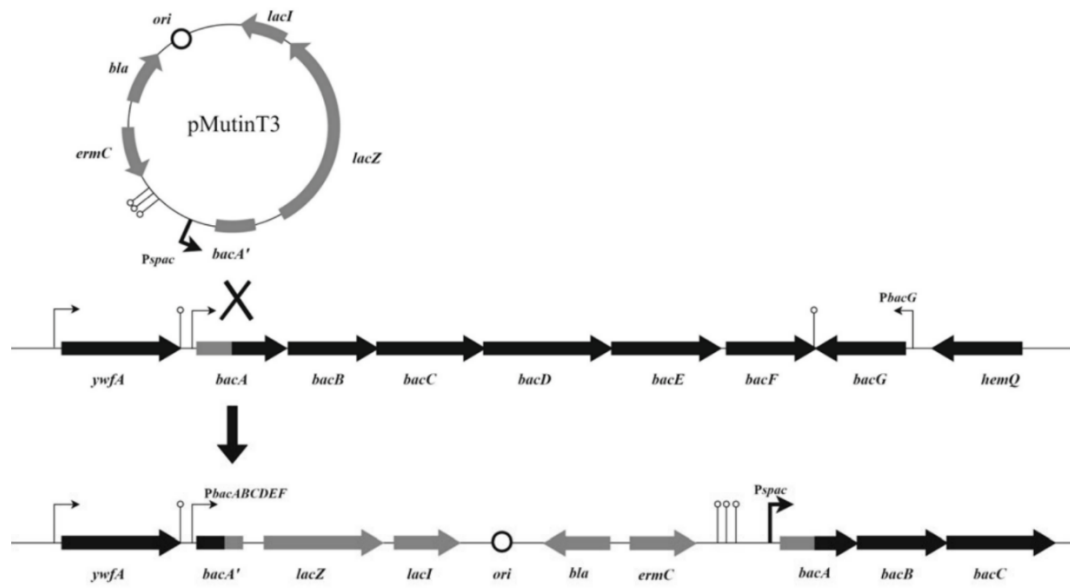


Figure 1.10 Diagram of *bacA*-targeted pMutin T3 and its insertion into the genome of OGU1 (Ertekin *et al.*, 2020).

To understand the regulatory role of bacilysin, transcriptomic and proteomic analyses were carried out to detect differentially expressed genes and proteins in OGU1 compared to its parental strain PY79. A gel-based method, 2DE MALDI-TOF MS revealed 150 differentially expressed proteins from 121 different ORFs, 63 of these were downregulated, 33 were upregulated and 20 were completely missing in OGU1. One protein, however, was found only in OGU1. NanoLC-MS was also employed as a complementary approach which identified 76 differentially expressed proteins, 50 of them were only found in PY79, 19 were only found in OGU1 and the remaining 7 were downregulated in OGU1 (Ertekin *et al.*, 2020).

The results of those proteomic studies were also confirmed by quantitative PCR analyses comparing OGU1 and PY79. A good correlation between these analyses was observed (Ertekin *et al.*, 2020).

Over and underrepresented proteins were categorized according to their cellular functions. It was found that overrepresented proteins of OGU1 mainly take a role in metabolism and stress response while underrepresented proteins contain many sporulation- and germination-related genes in addition to metabolism-related genes.

Whole genome sequencing analysis confirmed that the only genetic difference between OGU1 and PY79 is in the *bac* operon. Thus, these collectively indicate the strong possibility of bacilysin being a pleiotropic peptide acting both as an antibiotic and a signal molecule (Ertekin *et al.*, 2020).

When combined with previous findings showing defects in spores of bacilysin non-producer strains, the importance of bacilysin concerning sporulation is further highlighted upon finding many underrepresented genes. A list of significantly underrepresented proteins and the sporulation sigma regulons they belong to is given in Table 1.3.

Table 1.3 Sporulation and germination-related proteins identified to be underrepresented in OGU1 (Ertekin *et al.*, 2020).

Regulon	Protein	Product and/or Function
SigE	AsnO	Asparagine synthase (glutamine-hydrolysing)
	GerQ (YwdL)	Spore coat protein /spore germination
	SodF	Superoxide dismutase/ detoxification of oxygen radicals
	SpoVR	spore cortex synthesis
	SpsB ^{c,g,m}	spore coat polysaccharide synthesis
	SpsJ ^{c,g,m}	dTDP -glucose-4,6-dehydratase/ rhamnose biosynthesis, spore coat polysaccharide synthesis
	SpsK ^{c,g,m}	dTDP -4-dehydrorhamnose reductase/rhamnose biosynthesis, spore coat polysaccharide synthesis
	YheC ^{c,j}	ATP-binding spore coat protein
	YodQ	Putative deacylase
	CtpB ^e	Carboxy-terminal processing serine protease, cleaves SpoIVFA/ processing of pro-SigK; control of SigK activation
	YaaH ^{b,j}	N-acetylglucosaminidase/ survival of ethanol stress, protection of the spore
SigF	Gpr ^{d,e,k}	Germination protease/ degradation of SASPs
	YtfJ	Germination
	KatA ^r	Major catalase in spores; detoxification of hydrogen peroxide

Table 1.3 (continued).

SigG	SleB	Spore cortex lytic enzyme/ degradation of spore cortex, germination
	TepA ^k	Orphan ClpP-like germination protease/degradation of SASP
	YhcQ	Unknown
	YpeB	Germination protein/ essential for SleB assembly in spores
	YvaB ^p	Azoreductase/ sporulation protein; resistance to 2-methylhydroquinone
SigH	GalM (YoxA) ^b	Aldolase-1-epimerase (mutarotase)
	MinC ^{a,f,h,i,l}	Cell-division inhibitor (septum placement)/ destabilizes Z ring placement
	MinD ^{a,f,i,l}	Septum placement/ cell division inhibitor
SigK	CgeA ^{m,n}	Spore crust protein/maturation of the outermost layer of the spore
	CotA ^a	Laccase, bilirubin oxidase, spore coat protein (outer)/ resistance of the spore
	CotE ^{c,m}	Outer spore coat morphogenetic protein/ controls the assembly of the outer spore coat
	CotI	Spore coat protein/ spore envelope
	CotSA ^m	Spore coat protein/ resistance of the spore
	CotU ^{m,n}	Spore coat protein/ resistance of the spore
	CwlC	N-acetylmuramoyl-L-alanine amidase/ mother cell lysis
	LipC ^{g,m,n,o,s}	Spore coat phospholipase B/ spore germination
	OxdD ^m	Oxalate decarboxylase/ inner spore coat protein; protection of the spore
	DpaA (SpoVFA)	Dipicolinate synthase (subunit A)/ dipicolic acid production
	DpaB (SpoVFB)	Dipicolinate synthase (subunit B)/ dipicolic acid production
	YfnG ^{c,m}	Similar to CDP-glucose 4, 6-dehydratase
	YitD ^c	Unknown

* A member of other regulon(s) as well: ^a SigA, ^b SigB, ^c SigE, ^d SigF, ^e SigG, ^f SigH, ^g SigK, ^h SigM, ⁱ SigV, ^j SpoIIID, ^k SpoVT, ^l ComK, ^m GerE, ⁿ GerR, ^o KipR, ^p MhqR, ^r PerR, ^s TnrA.

In addition to the differential expression of genes in OGU1 compared to PY79, various phenotypical defects in OGU1 were also recorded. However, those defects were partially restored by the external addition of the bacilysin into the culture fluid of OGU1. Such that, decreased chloroform and lysozyme resistance of spores, dark pigmented colonies, decreased germination efficiency and the expression of some downregulated genes of OGU1 appeared to partially resemble those of the parent strain PY79 upon the addition of bacilysin (Ertekin *et al.*, 2020). Experimental results showing the effects of bacilysin on OGU1 are depicted in Figure 1.11.

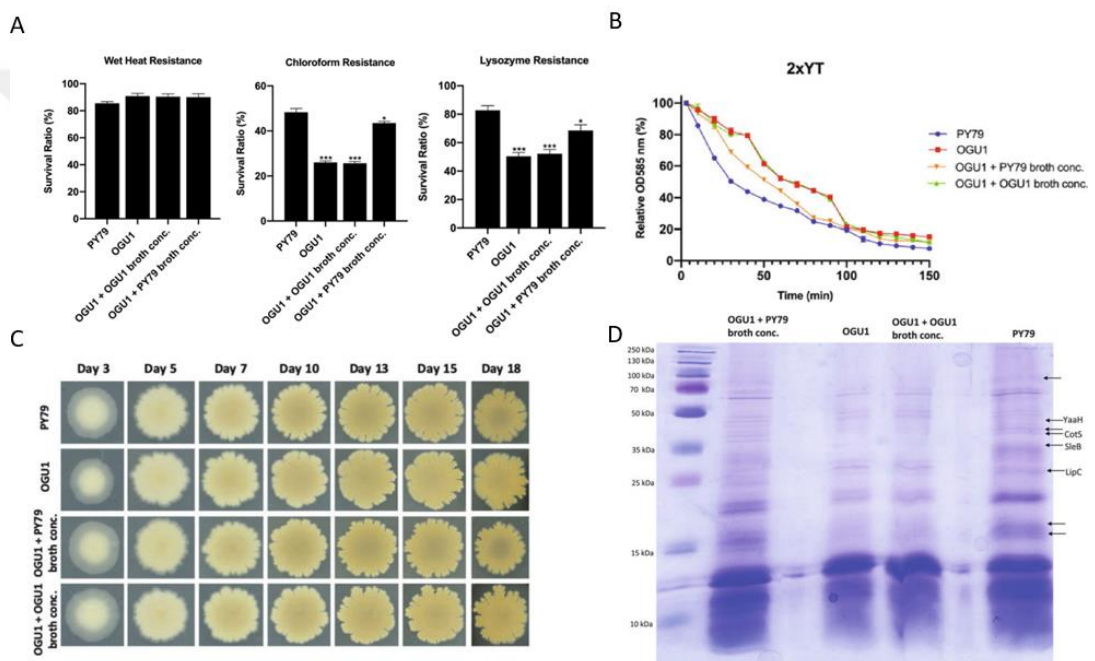


Figure 1.11 Chloroform, wet heat and lysozyme resistance (A), Germination assay (B) and Colony morphology (C) and SDS-PAGE of extracted spore coat proteins (D) of *B. subtilis* endospores comparing PY79, OGU1, and OGU1 treated with broth concentrate from PY79 (containing bacilysin) and OGU1 (no bacilysin) (Ertekin *et al.*, 2020)

1.5 Sporulation and Germination in *B. subtilis*

Sporulation is a survival mechanism that enables some species of bacteria to endure adverse environmental conditions. The stages of sporulation are depicted in Figure 1.12. The initiation of sporulation is followed by an asymmetrical cell division that divides the cell into the forespore and mother cell. Upon completion of mature endospore formation, the mother cell lyses and releases the endospore. Endospores can turn back into vegetative cells through a process called germination.

Endospore formation is triggered mainly by nutrient deprivation as well as other forms of stress. Metabolically dormant endospores do not require nutrients for maintenance. In addition, they are highly resistant to a wide variety of hazards including heat, radiation, organic solvents, and hydrolytic enzymes (Nicholson *et al.*, 2000). Endospores can remain intact for thousands of years and once environmental conditions improve, germination occurs and reverts endospores into vegetative cells (Setlow, 2003).

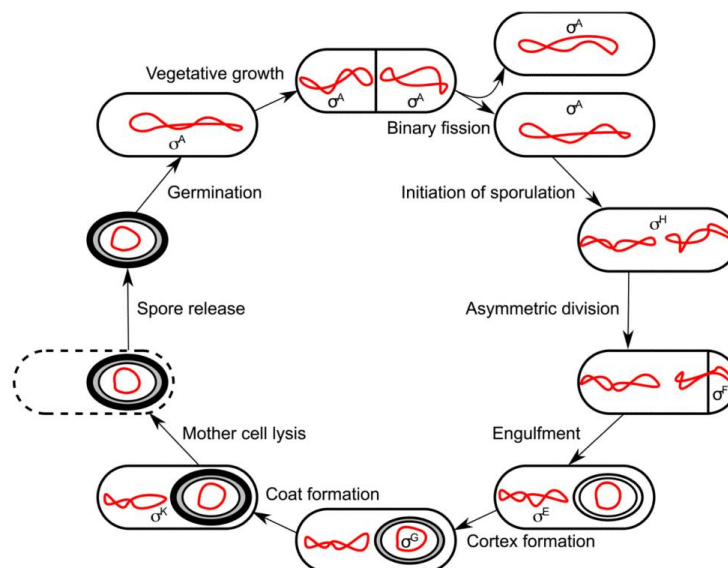


Figure 1.12 The stages of *B. subtilis* life cycle demonstrating sporulation, germination, and vegetative growth (de Hoon *et al.*, 2010)

1.5.1 Initiation of Sporulation

B. subtilis is one of the bacterial species capable of forming endospores. Sporulation is a complex cascade of alternative sigma factors, transcriptional factors, and structural genes. The formation of an endospore takes approximately 8 to 10 hours in *B. subtilis* and demands a lot of energy (Stragier and Losick, 1996). Thus, *B. subtilis* uses sporulation as a last resort of defense when other options such as competence development and cannibalism are not enough to survive (González-Pastor *et al.*, 2003; Veening *et al.*, 2006).

Initiation of sporulation is controlled by the master regulator transcription factor Spo0A and the alternative sigma factor Spo0H (σ^H). To be activated, Spo0A needs to be phosphorylated by a phosphorelay involving multiple kinases named KinA-E. These sensory kinases are tightly regulated by various mechanisms. For example, depletion of GTP during stringent response causes inactivation of CodY which normally inhibits KinA-E. KipI/KipA is another example of the kinase regulatory mechanisms where KipI acts as an inhibitor of autophosphorylation of KinA and KipA is the repressor of KipI (Wang *et al.*, 1997). KinA is one of the major kinases in phosphorelay whose autophosphorylation eventually leads to Spo0A activation.

Upon release of repression on those kinases, the phosphorylation of Spo0F takes place. Spo0F can be dephosphorylated by RapA, B, and E, which are inhibited by the pheromones PhrA, C, and E highlighting the importance of quorum sensing in the initiation of sporulation. Spo0F-P transfers its phosphate group to Spo0B which then phosphorylates Spo0A to activation (Burbulys *et al.*, 1991). Spo0A-P represses AbrB thus inhibition on *sigH* is eliminated which results in the upregulation of KinA, Spo0A, and Spo0F. In addition, Spo0A-P increases the expression of KinB, thus inducing a positive feedback loop making initiation of sporulation irreversible (Fürbaß *et al.*, 1991; Hoch, 1993). Spo0A in its active form takes a role in the regulation of 520 genes, 121 of which are regulated directly (Molle *et al.*, 2003). At lower concentrations of active Spo0A, it induces other processes including biofilm formation and cannibalism rather than sporulation (López and Kolter, 2010). At

higher levels of Spo0A-P, genes responsible for stress response mechanisms are replaced by the genes related to the initiation of sporulation, the last line of defense (Fujita *et al.*, 2005). Our previous studies indicated that mRNA levels of Spo0A in OGU1 are only 36% of that of PY79. On the other hand, those two strains showed no significant difference in the levels of Spo0A protein (Ertekin *et al.*, 2020).

σ^H (*sigH*, *spo0H*) is an alternative sigma factor that is expressed continuously in the absence of AbrB (Weir *et al.*, 1991). σ^H upregulates over 45 genes including *spo0A*, *kinA*, *spo0F*, *phrC*, *E*, *F*, *G*, *I*, and *K* as well as other sporulation-related genes needed in later stages such as *spoIIAB*, *spoIIAA*, and *sigF* (Britton *et al.*, 2002). There are some genes that require both Spo0A and σ^H for expression such as *spoIIA*. Moreover, induction of both Spo0A and σ^H can enforce the initiation of sporulation during vegetative growth (Karaki *et al.*, 2023). These suggest that Spo0A and σ^H work together at the initiation of sporulation (Buckner & Moran, 1998). The mRNA expression levels of σ^H were found to have decreased to 18% of the original in OGU1 when compared to PY79 but showed no significant change in protein concentration (Ertekin *et al.*, 2020).

Initiation of sporulation is also shown to be regulated by cell cycle checkpoint proteins. One example is Sda which was shown to inhibit the autophosphorylation of KinA and KinB, moreover, it also blocks phosphate transfer between KinA and Spo0F. (Burkholder *et al.*, 2001; Cunningham and Burkholder, 2009). As such, initiation of sporulation is blocked during DNA replication which otherwise might end up with polyploid or DNA-damaged spores (Veening *et al.* 2009).

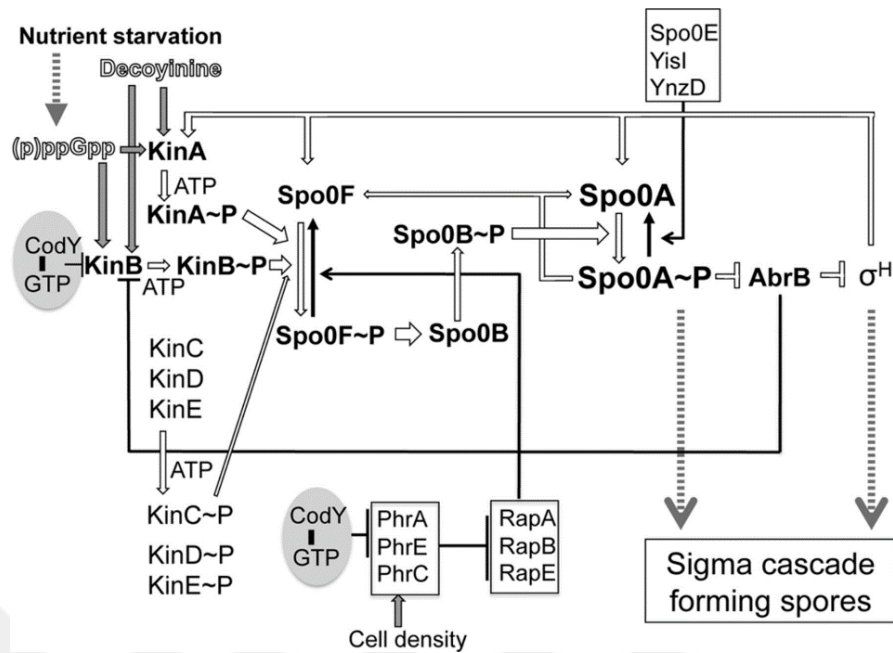


Figure 1.13 Sporulation initiation phosphorelay (Tojo *et al.*, 2013)

1.5.2 Stages of Sporulation and Sporulation Regulatory Proteins

Stage I of sporulation (spoI) is generally referred to as “axial filamentation” where duplicated chromosomes form a single chromatin filament in the middle of the cell. However, as axial filamentation is not solely a characteristic of sporulating cells, some parties do not consider it an official sporulation stage (Piggot & Coote, 1976). Stage II is known as septum formation (or asymmetric cell division) between the forespore and the mother cell.

Activation of Spo0A and σ^H induces the asymmetrical division which separates cells into two parts; the smaller forespore and the larger mother cell which have different fates at the following stages of the sporulation. As such, the endospore develops into a mature spore while the mother cell engulfs and supplies nutrients to the developing endospore. At the end, the mother cell lyses and the mature endospore is released. Although both cells carry the same genetic information, they must express genes in different orders for compartment-specific activities to occur. The expression of different gene sets must also be regulated according to the early and late stages of

sporulation within the same compartment. There are four sigma factors working in the regulation of sporulation after the asymmetric division of the cell: σ^F , σ^E , σ^G , and σ^K . These sigma factors are the major regulators of both regulatory and structural genes during endospore formation.

Stage II – Asymmetric Cell Division

The septum formation starts as a medial septum which then turns into a polar septum that divides the cell into a forespore and mother cell. This process is accepted as a morphological hallmark of sporulation. An increase in division regulator protein FtsZ which is under the regulation of spo0A and σ^H and the production of SpoIIE which deploys FtsZ to polar sites and triggers the switch from medial to polar septum formation (Carniol *et al.*, 2005). The asymmetric division of the cell is followed by the activation of forespore-specific sigma factor σ^F . At stage 0, σ^F is produced under the control of Spo0A-P, however, this sigma factor is held in an inactivated state by the anti-sigma factor SpoIIAB until the completion of asymmetric division. Next, SpoIIE accumulates at the polar septum and performs a second function that leads to the activation of the anti-anti-sigma factor SpoIIAA which inhibits SpoIIAB and thus relieves the inhibition on σ^F (Duncan *et al.*, 1995). Interestingly, production of SpoIIE takes place both in the forespore and the mother cell but σ^F activation only takes place in the forespore, though the mechanism is still not well understood. The expression levels of σ^F do not show significant differences between OGU1 and PY79, both on transcriptional and protein levels (Ertekin *et al.*, 2020).

During asymmetric septation, the polar septum splits the axial filament which causes just one-third of the chromosome to enter the forespore at the beginning (Wu and Errington, 1998). Before the remaining chromosome is pulled into the forespore by the DNA translocase SpoIIIE, the forespore is only able to express genes located on the part of the chromosome localized within the forespore, causing genetic asymmetry between compartments (Wu and Errington, 1994; Fiche *et al.*, 2013). This asymmetry is thought to play a role in the arrangement of compartment-specific gene activation during sporulation (Frandsen *et al.*, 1999).

The activation of the second sigma factor of sporulation, σ^E , is also dependent on Spo0A-P (Pedrido *et al.*, 2013). Compared to PY79, OGU1 has been found to have significantly more (over 2.5 times) σ^E mRNA, however, protein concentrations show no significant difference between the two strains (Ertekin *et al.*, 2020). Similar to σ^F , σ^E is produced under the regulation of Spo0A-P before the asymmetric division and is found in both compartments at the beginning (Fujita and Losick, 2002). However, unlike σ^F , σ^E is produced as a pro- σ^E precursor that is only activated in the mother cell compartment. Processing of pro- σ^E to its mature form is dependent on spoIIGA which requires SpoIIR for activation (Jonas *et al.*, 1988; Hofmeister *et al.*, 1995). SpoIIR is synthesized under the control of σ^F in the forespore and secreted to the intermembrane space where it activates SpoIIGA. However, SpoIIR needs to be acylated to gain activator function which requires *de novo* fatty acid synthesis (Diez *et al.*, 2012). At the beginning of the asymmetric division, the forespore is not capable of *de novo* fatty acid synthesis as the required genes remain at the protruding part of the chromosome. It is hypothesized that σ^E activation only occurs in the mother cell as *de novo* fatty acid synthesis only occurs there which enables acetylation of SpoIIR residing solely on the mother cell site of the septum where it can activate SpoIIGA and eventually σ^E (Pedrido *et al.*, 2013). The transcription of engulfment-related genes belonging to the σ^E regulon initiates the third stage of sporulation.

Stage III – Engulfment

Asymmetric cell division is followed by engulfment, where the polar septum curves around the forespore and results in a double-membrane bound forespore within the cytosol of the mother cell. Protein machinery composed of SpoIID, SpoIIM and SpoIIP is responsible for the degradation of peptidoglycan needed for septal wall thinning (Abanes-De Mello *et al.*, 2002). Then, a mother cell protein called SpoIIAH and a forespore protein called SpoIIQ directly interact at intermembrane space where they perform “zippering” action to separate compartments (Blaylock *et al.*, 2004; Doan *et al.*, 2005). For the full release of the forespore, engulfing

membranes must undergo fission which is mediated by a mother cell-specific protein FisB. FisB causes membranes to destabilize by interacting with cardiolipin which is thought to accumulate at the site and leads to the scission of membranes (Doan *et al.*, 2013). After the forespore becomes a free-floating cell in the mother cell cytosol, SpoIIAA-SpoIIAH and SpoIIQ proteins, are produced under the control of σ^E and σ^F respectively. These proteins are needed for the activation of the forespore-specific σ^G . Compared to PY79, the transcript level of σ^G in OGU1 is 30% lower (Ertekin *et al.*, 2020). Transcriptional regulation of σ^G is under control of σ^F which is also a forespore-specific sigma factor. Although the activation of σ^F occurs after asymmetric division, the activation of σ^G does not take place until the completion of engulfment. This is caused by the decreased metabolic capacity of the forespore after engulfment (Camp and Losick, 2009; Doan *et al.*, 2009). The aforementioned proteins, SpoIIAA-SpoIIAH and SpoIIQ form a channel between the mother cell and the forespore that enables the transfer of nutrients required for gene expression in the forespore (Meisner *et al.*, 2008; Camp and Losick, 2009). It is thought that the timing and place location of σ^G activation is due to gene expression resuming in the forespore upon the arrival of metabolites. However, recent observations showed that the precise timing of σ^G activation may also depend on the fact that complete translocation of the chromosome also occurs at the end of engulfment (Regan *et al.*, 2012).

Another mother cell-specific sigma factor, σ^K , is produced after the activation of σ^G as an inactive pro-protein similar to σ^E . Pro- σ^K is cleaved and activated by an intermembrane protein called SpoIVFB (Lu *et al.*, 1995). Initially, SpoIVFB is held in an inactive state by a complex composed of SpoIVFA and BofA which are also located in intermembrane space (Resnekov and Losick, 1998). Relief of inhibition on SpoIVFB depends on a σ^G -activated protein, SpoIVB. SpoIVB can cleave SpoIVFA at multiple sites, at the same time, it can also activate the protease CtpB that cleaves SpoIVFA to release SpoIVFB in an active form (Campo and Rudner, 2006).

Stages IV-V – Coat and Cortex Assembly

The mature spore is enclosed within two concentric shells which act as an “armor” protecting the endospore from harsh environmental conditions (Setlow, 2006). The outer shell is named “coat” and consists of almost seventy different proteins. The inner layer is called the “cortex” which is made of specialized peptidoglycan (Henriques and Moran, 2007).

The coat is composed of four layers ordered from inner to outer as follows: basement layer, inner coat, outer coat, and crust (McKenney *et al.*, 2010). One of the first proteins participating in basement layer formation is SpoVM, which is produced by the mother cell. This protein can recognize the outer surface of the recently engulfed forespore due to its positive membrane curvature (Levin *et al.*, 1993; van Ooij and Losick, 2003; Ramamurthi *et al.*, 2009). SpoVM draws another protein named SpoIVA which is the major building block of the basement layer (Price and Losick, 1999). SpoIVA hydrolyses ATP for self-assembly, eventually forming a layer for other coat proteins to bind (Ramamurthi and Losick, 2008; Castaing *et al.*, 2013).

For the proper formation of the final product, the construction of each layer must be tightly regulated by a wide variety of genes. For instance, *spoIVA* knockout mutants showed malformation of the basement layer which subsequently caused disrupted formation of the following layers (Roels *et al.*, 1992. *safA*, *cotE*, or *cotZ* and *cotY* mutants showed defects in the formation of their inner coat, outer coat, and crust respectively (Chada *et al.*, 2003; Imamura *et al.*, 2011).

The assembly of the coat mainly occurs in the mother cell side of the forespore surface which is predominantly mediated by mother cell-specific genes. However, it was proven that the SpoIIAH-SpoIIQ complex is also necessary for the successful assembly of the coat which suggests that the forespore may also act in the coordination of coat assembly (McKenney and Eichenberger, 2012). Although the coat and the cortex are separated by the outer forespore membrane, deletion of either *spoIVA* or *spoVM* not only prohibits coat assembly but also distorts cortex formation,

suggesting that coat and cortex assembly are somehow related (Roels *et al.*, 1992; Levin *et al.*, 1993).

The cortex consists of two layers: an inner germ cell wall and an outer cortex. Together, these two layers reside between the inner and outer forespore membranes. The cortex consists of a differentiated type of peptidoglycan that specializes in protecting the spore from heat and dehydration. The germ cell wall is significantly thinner than the cortex and is structurally more similar to the wall of vegetative cells (Tipper and Linnett, 1976). However, peptidoglycan of the cortex is highly different from the vegetative cell wall such that it shows a decreased density of crosslinking between glycan chains caused by several small penicillin-binding proteins and additionally contain muramic lactam provided by CvlD and PdaA which are enzymes that turn muramic acid to muramic lactam (Popham and Setlow, 1993; Warth and Strominger, 1972; Popham *et al.*, 1999; Gilmore *et al.*, 2004).

Although the exact role of the special peptidoglycan of the cortex is not understood clearly, it is thought that due to the low density of crosslinking, the spore becomes structurally more flexible enabling it to contract and expand more easily in response to environmental changes (Ou and Marquis, 1970).

The synthesis of the cortex and vegetative cell wall peptidoglycans use similar mechanisms. Cortex peptidoglycan precursors are produced in the cytosol of the mother cell by Mur proteins which also have roles in vegetative cell wall peptidoglycan precursor modification. Regulation of *mur* expression is positively controlled by σ^K during sporulation (Vasudevan *et al.*, 2007). Modification of peptidoglycan precursors is followed by their attachment to the outer membrane of the forespore mediated by the formation of lipid intermediates which are then flipped into the intermembrane space residing between the outer and inner membranes of the forespore by the functioning of a Lipid II flippase.

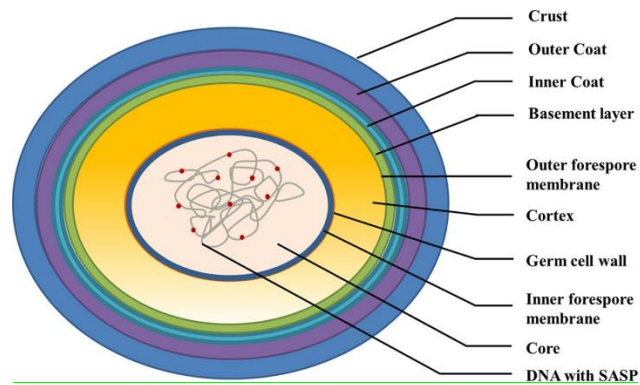


Figure 1.14 Schematic representation of spore coat and cortex of *B. subtilis* (Djouiai *et al.*, 2018)

Stages VI-VII – Endospore Maturation and Release

Stage VI of sporulation is referred to as endospore maturation which takes place in the mother cell. During maturation, the forespore gains typical endospore properties such as resistance, metabolic dormancy, and germinability. Spore resistance develops stepwise such that resistance against organic solvents is established first, then, resistance to 65°C is followed by 80°C, and at last resistance to lysozyme is established (Jenkinson *et al.*, 1980). Forespores in stage IV can germinate rapidly in rich media however, stage VI spores germinate only in response to the presence of L-alanine and germination takes a relatively longer time (Dion & Mandelstam, 1980).

Stage VII is the final stage of the sporulation process where the mature endospore is released from the mother cell. The lysis of the mother cell requires N-acetylmuramoyl-L-alanine amidases such as CwlC and CwlH both of which are dependent on the late mother cell-specific sigma factor σ^K (Smith & Foster, 1995; Nugroho *et al.*, 1999).

1.5.3 Spore Resistance

As mentioned in previous sections, *B. subtilis* spores show great resistance to various extreme environmental conditions. However, as those spores are metabolically inert, they lack any enzymatic activity otherwise responsible for repairing damaged cell parts such as DNA. For this reason, damage can only be repaired upon germination if it is not beyond repairability due to extreme distortion (Setlow, 2003).

Endospores can resist wet heat at 90°C for 20 minutes which would kill vegetative cells within a minute (Gerhardt & Marquis 1998). Resistance against wet heat depends on the water content of the spore core. As the water content decreases, heat resistance increases (Melly *et al.*, 2002). One of the several mechanisms adapted by spores to lower water content is the presence of dipicolinic acid (DPA). DPA can only be found in spore core where it chelates Ca^{2+} and Mg^{2+} to increase resistance by lowering water content (Gerhardt & Marquis 1998). Another protection mechanism against damage caused by heat is the presence of SASPs (especially alpha and beta types) bound to DNA. Although dry heat was shown to harm endospore DNA far more than wet heat, SASPs were shown to provide resistance to both types of heat (Nicholson *et al.*, 2000; Espitia *et al.*, 2002).

In addition to heat, endospores are also highly resistant to degradative enzymes and harmful chemicals thanks to their multilayered and rigid spore coat. Lysozyme is one such harmful enzyme that targets and breaks down peptidoglycan. In spores, the cortex is the main target of lysozyme as it is rich in peptidoglycan. For this reason, the coat enclosing the cortex should have all spore coat proteins owing different roles. For example, CotE null mutants lacked their outer spore coat and were shown to be less resistant to lysozyme (Zheng *et al.*, 1988). Interestingly, forced expression of CotE at later stages in those mutants rescued lysozyme susceptible phenotype, suggesting additional roles of CotE for lysozyme resistance (Costa *et al.*, 2007). Inner coat proteins, such as Coth, were also shown to be required for lysozyme resistance (Naclerio *et al.*, 1996).

Resistance against other chemicals including organic solvents, oxidizing, and alkylating agents also depends on the inner and outer layers of the spore coat (Setlow, 2012). The dual function of the spore coat enables the blocking of the penetration of harmful chemicals to the inner parts of the spore and uses detoxifying enzymes localized in the spore coat to neutralize any penetrating chemicals. The plasma membranes of endospores are also adapted to block the penetration of those chemicals since they are less permeable to hydrophobic molecules than usual membranes (Gerhardt *et al.*, 1972).

1.5.4 Germination

Germination in *B. subtilis* is stimulated by the response of germination receptors to the nutrients in the environment and if other conditions are suitable, germination proceeds to form a vegetative cell (Paidhungat *et al.*, 2002). Germination receptors are produced towards the end of sporulation, and they can detect a variety of nutrients such as amino acids, sugars, and nucleotides. Different nutrient combinations that induce germination response are known. One of these is AGFK, which is composed of asparagine, glucose, fructose, and potassium (Setlow *et al.*, 2003). AGFK can initiate a germination response through the GerA family of receptors that reside on the inner membrane of the endospore (Hudson *et al.*, 2001). The activation of receptors triggers the opening of SpoVA which then releases the content of the spore core (Christie & Setlow, 2020). First, cations such as H^+ , Na^+ , K^+ and Zn^{+2} are released, which results in an increase of the spore core pH from 6.5 to 7.7. This pH change activates enzymes otherwise inhibited at low pH. The release of cations is followed by the release of DPA with chelated Ca^{2+} from the spore core which is replaced by the intake of water, providing the core with partial hydration. The release of DPA also triggers several enzymes responsible for the degradation of the cortex, enabling further water uptake. As water becomes more abundant, enzymatic activity also accelerates (Setlow *et al.*, 2001). Eventually, the hydration of the core causes reduced resistance against wet heat (Setlow *et al.*, 2003).

One of the main steps in germination is the hydrolysis of the spore cortex which is mediated by the enzymes CwlJ and SleB. *cwlJ-sleB* double mutants show improper germination due to their inability to degrade the spore cortex (Makino & Moriyama 2002). Both enzymes are produced during sporulation and localize in the coat, cortex, and membrane (Chirakkal *et al.*, 2002). CwlJ and SleB depend on GerQ and YpeB respectively for localization (Ragkousi *et al.*, 2003; Boland *et al.*, 2000).

Germination can also be initiated by non-nutrient compounds in addition to the nutrient germinants. For instance, lysozyme is capable of triggering germination by degrading the cortex, however, the spore coat must be removed beforehand for lysozyme to exert its function. Additionally, the lysozyme concentration in the environment should not pass a threshold due to the possibility of the degradation of the cell wall of newly germinated vegetative cells.

Another factor that can trigger germination is extracellular DPA-Ca²⁺ which can activate CwlJ to stimulate cortex degradation (Paidhungat *et al.*, 2001). Furthermore, high pressure (100-200 megapascals) may trigger germination by activating germination receptors (Wuytack *et al.*, 2000). Even higher pressures (500-600 megapascals) may bypass receptor activation and directly trigger germination by releasing DPA from the spore core (Paidhungat *et al.*, 2002).

1.6 Mother Cell Sporulation Transcription Factors GerE and SpoIIID

1.6.1 GerE

GerE is one of the major transcription factors of the sporulation process. It is a late mother cell-specific protein whose expression is under the control of σ^K and its expression is observed approximately 120 minutes after the initiation of sporulation (Zheng and Losick, 1990; Tu *et al.*, 2021). It consists of 74 amino acids making GerE the smallest member of the LuxR-FixJ family of transcription factors (Crater *et al.*,

2001). The DNA binding ability of GerE comes from a helix-turn-helix (HTH) domain (Ducros *et al.*, 2001). In addition, this protein can also be found and bind DNA in dimeric form (Ducros *et al.*, 1998).

GerE has its own regulon (GerE regulon) where it activates 3 operons and 30 genes while repressing 2 operons and 13 genes. One gene repressed by GerE is *sigK* which is the sigma factor controlling GerE expression. For this reason, GerE indirectly regulates its own expression negatively (Ducros *et al.*, 2001). The major role of GerE is regulating the final stages of coat deposition and maturation, thus, the majority of its regulon consists of spore coat proteins (Ducros *et al.*, 2001). *cotG*, *cotC*, *cotD*, *cotU*, and *cotX* are examples of upregulated coat protein-coding genes, while *cotA*, *cotB*, *cotE*, *cotH*, *cotM*, and *cotT* are downregulated (Zheng *et al.*, 1992; Henriques *et al.*, 1997; Driks and Eichenberger, 2016; Lin *et al.*, 2020). As expected, *gerE* mutants form endospores with spore coat abnormalities which were shown to be highly sensitive to lysozyme. Furthermore, those spores were also shown to struggle in germination processes (Ducros *et al.*, 1998; Ducros *et al.*, 2001).

GerE is regulated both at posttranscriptional and posttranslational levels. The stability of *gerE* mRNA depends on the binding of CitB, an aconitase involved in the citric acid cycle, during iron deprivation (Alén & Sonenshein, 1999). Moreover, the GerE protein can be stabilized by SpoVIF so that its accumulation in the mother cell is accelerated (Cangiano *et al.*, 2010).

Although GerE was not encountered in proteomic studies conducted by our group, transcriptomic and qPCR analyses showed an 85% decrease in *gerE* mRNA levels in OGU1 compared to PY79 (Ertekin *et al.*, 2020).

1.6.2 SpoIIID

SpoIIID is one of the two early mother cell specific proteins under the regulation of σ^E . *spoIIID* codes for a transcription factor with its own regulon where 4 operons and 12 genes are activated and 8 operons and 13 genes are repressed. SpoIIID

consists of 93 amino acids and it resembles *gerE* as it contains a helix-turn-helix domain. In addition to this similarity, SpoIIID also contains another DNA-binding domain in its C-terminal, allowing it to bind DNA as a monomer (Stevens & Errington, 1990; Himes *et al.*, 2010). The upregulation of *spoIIID* starts approximately 60 minutes after sporulation initiation and decreases gradually after 180 minutes (Tu *et al.*, 2021).

Transcriptional regulators upregulated by SpoIIID include *gerR* (the other early mother cell-specific transcription factor) *sigK* and *gerE* (Kunkel *et al.*, 1990; Stevens and Errington, 1990). The majority of SpoIIID-activated genes are responsible for endospore coat formation including *cotA*, *cotB*, *cotF*, *cotT*, *cwlJ*, and *spoVM* (Eichenberger *et al.*, 2004). *spoIVFA-spoIVFB* and *bofA* are examples of downregulated genes that regulate σ^K activity.

spoIIID is a part of the *usd-spoIIID-mbl* operon. The transcription of *spoIIID* depends on active σ^E . In addition, *spoIIID* is regulated on the mRNA level by *usd*, which resides upstream on its operon. The translation of *spoIIID* relies on the translation of *usd* such that when *usd* is not translated, its mRNA sequence forms a stem loop that blocks *spoIIID* translation (Decatur *et al.*, 1997). The downregulation of *spoIIID* occurs during the switch from early to late sporulation i.e., when the mother cell switches from σ^E to σ^K control. If the expression of *spoIIID* is maintained after the switch, defects in spore formation are observed (Wang *et al.*, 2007). The importance of SpoIIID in sporulation was also shown by Eichenberger *et al.*, where *spoIIID* knockouts showed a 100,000-fold decrease in sporulation efficiency (Eichenberger *et al.*, 2004). Much like GerE, SpoIIID could not be encountered in proteomic studies, however, it showed a 25% decrease in mRNA level when OGU1 and PY79 were compared (Ertekin *et al.*, 2020).

1.7 The Aim of the Present Study

Earlier studies by our research group have shown the importance of bacilysin production for proper sporulation and germination in terms of spore resistance and germination efficiency by examining a bacilysin-silenced strain. The positive effect of bacilysin on those defects was also demonstrated as well, collectively suggesting a reciprocal relationship between sporulation and bacilysin synthesis (Özcengiz & Alaeddinoğlu, 1991a; Ertekin *et al.*, 2020). Further investigation of this relationship revealed the roles of various sporulation transcription factors on the expression of *bac* operon (Köroğlu *et al.*, 2011). Most recently, transcriptomic and proteomic studies on PY79 and its *bac* silenced derivative OGU1 discovered that many sporulation-related genes, more remarkably sporulation-specific transcription factors are downregulated at both transcript and protein levels in the absence of bacilysin (Ertekin *et al.*, 2020).

In this study, two sporulation transcription factors, GerE and SpoIIID, were chosen to investigate their relation to bacilysin biosynthesis and the defective phenotypes observed in OGU1. To understand the effects of those genes on bacilysin production four sets of experiments were conducted: Firstly, the genes were cloned into OGU1 and the effects of their overexpression on the activity of *lac*-fused *bac* operon were measured via β -galactosidase assay. Secondly, the amount of bacilysin production from *gerE* and *spoIIID* knockout strains was measured via a disk-diffusion bioassay to observe the effects of their absence. Thirdly, the genes were cloned into *E. coli* BL21 to obtain vast amounts of purified proteins which were then used in EMSA experiments to observe in-vitro DNA-binding between GerE, SpoIIID and the *bac* operon. Additionally, the EMSA experiments were repeated with an addition of bacilysin to observe whether its presence changes the DNA-binding activity of these proteins. Finally, a variety of phenotypic assays were employed to observe whether overexpression of GerE and SpoIIID in OGU1 could rescue the defects in spore resistance, germination rate, and sporulating colony morphology.

CHAPTER 2

MATERIALS AND METHODS

2.1 Bacterial Strains

Bacterial strains used in this study, their sources, and their characteristics are listed in Table 2.1. *E. coli* DH5 α was used in cloning procedures as a carrier for plasmids. *E. coli* BL21 (DE3) was used to overexpress proteins of interest. *B. subtilis* 168 is the standard strain of the organism and was used as the genomic DNA source for PCR and as the control organism in bioassay experiments. PY79 is a prototrophic derivative of *B. subtilis* 168, which was used to prepare bacilysin broth concentrates and as the positive control for phenotypic assays (Youngman *et al.*, 1984). OGU1 is the bacilysin non-producing strain constructed by our group via insertional inactivation of bacilysin operon (*bacA'*::*lacZ*::*erm*::*bacABCDEFG*) of its mother strain PY79 (Köroğlu *et al.*, 2011). OGU1 was used in cloning procedures as well as phenotypic analyses. Knockout strains BKK28410 *trpC2*, (Δ *gerE*::*kan*) and BKK36420 (*trpC2*, Δ *spoIIID*::*kan*) were derived from *B. subtilis* 168 by Koo *et al.* (2017) and were gathered from BGSC (Bacillus Genetic Stock Center, USA). *S. aureus* ATCC 9144 was used as the assay organism as it is susceptible to bacilysin.

Table 2.1 The bacterial strains used in this study

Strain	Source	Characteristics
<i>E. coli</i> DH5a	American Type Culture Collection	F- endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG Φ 80dlacZ Δ M15 Δ (lacZYA-argF)U169, hsdR17(rK- mK+), λ -
<i>E. coli</i> BL21 (DE3)	Novagen, Merck (Germany)	F- ompT gal dcm lon hsdSB (rB- mB-) λ (DE3 [lacI lacUV5-T7 gene 1 ind1 sam7 nin5])
<i>E. coli</i> BL21 (DE3)+ <i>gerE</i>	This Study	<i>E. coli</i> BL21 (DE3) transformed with pET28a(+) cloned with <i>gerE</i>
<i>E. coli</i> BL21 (DE3)+ <i>spoIIID</i>	This Study	<i>E. coli</i> BL21 (DE3) transformed with pET28a(+) cloned with <i>spoIIID</i>
<i>B. subtilis</i> 168	BGSC	trpC2
<i>B. subtilis</i> PY79	P. Youngman	Prototrophic derivative of <i>B. subtilis</i> 168
<i>B. subtilis</i> OGU1	Previous Studies	bacA':::lacZ::erm::bacABCDEF
<i>B. subtilis</i> OGU1+ <i>gerE</i>	This Study	OGU1 transformed with pHT08 cloned with <i>gerE</i>
<i>B. subtilis</i> OGU1+ <i>spoIIID</i>	This Study	OGU1 transformed with pHT08 cloned with <i>spoIIID</i>
<i>B. subtilis</i> BKK28410	BGSC	trpC2, Δ <i>gerE</i> ::kan
<i>B. subtilis</i> BKK36420	BGSC	trpC2, Δ <i>spoIIID</i> ::kan
<i>S. aureus</i> ATCC 9144	ATCC	Strain of <i>S. aureus</i> susceptible to bacilysin

2.2 Plasmids

A list of the commercial and constructed plasmids used in this study is given in Table 2.2. Schematic representations of those plasmids are given in Appendix A.

Table 2.2 Plasmids used and constructed in this study

Plasmid	Markers	Size	Source
pET28a(+)	kan (KanR)	~5.3 kb	Novagen (Darmstadt, Germany)
pHT08	amp (AmpR), cm (CmR)	~8.0 kb	MoBiTec (Goettingen, Germany)
pET28a(+)-GerE	kan (KanR)	~5.5 kb	This Study
pET28a(+)-SpoIIID	kan (KanR)	~5.5 kb	This Study
pHT08-GerE	amp (AmpR), cm (CmR)	~8.2 kb	This Study
pHT08-SpoIIID	amp (AmpR), cm (CmR)	~8.2 kb	This Study

2.3 Culture Media

Components and preparations of culture media are given in Appendix B.

2.4 Buffers and Solutions

Components and preparations of buffers and solutions are given in Appendix C.

2.5 Chemicals, Enzymes, and Kits

The chemicals, enzymes, and kits are given in Appendix D.

2.6 Growth and Maintenance Conditions of Bacterial Strains

E. coli, *B. subtilis*, and *S. aureus* strains that do not harbor plasmids were grown in liquid Luria-Bertani medium and LB agar plates. *E. coli* strains containing recombinant pHT08 plasmids were supplemented with ampicillin at a final

concentration of 100 µg/mL, *B. subtilis* strains containing pHT08, on the other hand, were supplemented with chloramphenicol (25 µg/mL final concentration). *E. coli* strains harboring pET28a(+) derivatives and knockout *B. subtilis* strains (BKK28410 and BKK36420) were supplemented with kanamycin with a final concentration of 30 µg/mL.

B. subtilis 168 and its knockout strains were supplemented with tryptophan (final concentration 50 µg/mL) when grown in PA medium.

All liquid cultures were grown overnight at 37°C with constant shaking at 150 rpm, if not mentioned otherwise. All solid cultures were inoculated into agar plates and grown overnight at 37°C, afterwards, those plates were kept at 4°C and passaged once a month. For long-term storage, 1.4 mL of liquid cultures were centrifuged at 1000g for 10 minutes. The supernatant was discarded, 500 µL of 10% glycerol was added, and the pellet was resuspended by pipetting. The stocks were stored at -80°C.

2.7 Isolation of Genomic DNA

For the isolation of genomic DNA, a modified version of the method developed by Owen and Borman (1987) was used. 2 mL overnight culture of *Bacillus subtilis* 168 was taken into microcentrifuge tubes and centrifuged at 6000 rpm for 10 minutes. The supernatant was discarded, and the pellet was resuspended in 1 mL SET buffer (Appendix C) via vortex. 23 µl lysozyme (50 mg/mL) and 6 µl RNase (10 mg/mL) were added to lyse the cells and degrade the RNAs. The mixtures were incubated at 37°C for 45 minutes. After the incubation, each tube was supplemented with 29 µl of Proteinase K (20 µg/mL) and 120 µl of 10% SDS, mixed by inversion. The tubes were incubated at 55°C for 2 hours to allow the denaturation of proteins. Samples were divided into two new 2 mL microcentrifuge tubes. 200 µL of 5M NaCl solution and 500 µL of chloroform were added to each tube and mixed by inversion for 30 minutes at room temperature. Then, the tubes were centrifuged at 6000 rpm for 15 minutes. Two separate phases appeared in each tube and the upper phase was taken

into a new tube. 1.4 mL of ice-cold absolute ethanol was added. Samples were chilled at -20°C for 30 minutes and centrifuged at 6000 rpm for 15 minutes at 4°C. The supernatant was discarded, and the pellet was resuspended with 1 mL of 70% ethanol. Samples were centrifuged at 6000 rpm for 2 minutes. The supernatant was discarded, and the pellet was air-dried for 10 minutes. The dried pellet was resuspended in 50 µl of TE buffer (Appendix C) by pipetting. The concentration and purity of the isolated genomic DNA was measured by using BioDrop Touch UV/Vis (BioDrop Ltd., UK). The purity and integrity of genomic DNA samples were also checked by running a 1% agarose gel. The samples are stored at -20°C for future use.

2.8 Primer Design

The whole genome sequence of *B. subtilis* 168 (NC_000964.3) was used as the reference for the designing of forward and reverse primers of cloned genes *gerE* and *spoIIID* (gene IDs are BSU_28410 and BSU_36420, respectively) and promoter regions of genes to be used in EMSA experiments.

The primer sequences of the genes to be cloned into pET28a(+) were designed to include BamHI (5'-GGATCC-3') cut site for forward and HindIII (5'-AAGCTT-3') for reverse primers. Genes to be cloned pHT08 were designed to include BamHI for forward and XbaI (5'-TCTAGA-3') for reverse primers. To ensure proper processing of PCR products by restriction enzymes, a minimum of 5 extra bases were added to the designed primers.

The primers for promoter regions that were going to be used in EMSA experiments of *bac* operon, *cotB*, *bofA*, *abrB*, *gerE* and *spoIIID* were designed according to literature (Köroğlu *et al.*, 2011; Eichenberger *et al.*, 2004)

First, each primer was dissolved in the appropriate amount of TE buffer considering the producer's (Oligomer, Türkiye) manual. Then each sample was diluted tenfold in nuclease-free water and stored at -20°C for further use.

Table 2.3 Primers used in this study. Cut sites are indicated.

Product Name	Primer Name	Primer Sequence (5'-3')	Product Size
SpoIIID pET28a	SpoIIID- pET_F	AGG <u>GGATCC</u> GGTGTGCACGATT A	319 bp
	SpoIIID- pET_R	GGCAATGCC <u>AAGCTT</u> TTACGAT TGCTGAAC	
GerE pET28a	GerE- pET_F	GGGTTAG <u>GGATCC</u> TTGAAGGAG AAAGAA	249 bp
	GerE- pET_R	TACCGG <u>AAGCTT</u> TTAAAGCTCT AGCTC	
SpoIIID pHT08	SpoIIID- pHT_F	GAGGTCG <u>GGATCC</u> GTGCACGA TTACAT	310 bp
	SpoIIID- pHT_R	GGCAATGCCT <u>TCTAGA</u> TTACGAT TGCTGAAC	
GerE pHT08	GerE- pHT_F	GGAGGG <u>GGATCC</u> TACTTGAAG GAGAAAGAA	256 bp
	GerE- pHT_R	TCAGGCT <u>TCTAGA</u> TTACGTTTCT TGAGC	
P_{bac}	pbac_F	GCTATGCAGCTGTCGGAT	383 bp
	pbac_R	GCTGCGTACTCACTGCTTG	
P_{cotB}	pcotB_F	TATGAACGGATTAGGCCG	537 bp
	pcotB_R	TCATCAGCATCGAGCATTTCTC AGTC	
P_{bofA}	pbofA_F	ACTTGAAGGAAGACGTGA	592 bp
	pbofA_R	TGATAAATTGCTTAATGACGAC	
P_{abrB}	pabrB_F	ACTTTGGTTCGAAAGTCT	371 bp
	pabrB_R	CAACATAGATTTCAAGAGCAT	
$P_{spoIIID}$	pSpoIIID _F	AAGTTCGTTTCACCCCTTGTCC	324 bp
	pSpoIIID _R	CACCAAATTCCTTCGCAATGAC AC	
P_{gerE}	pGerE_F	CGTGATGGTTCAAGTCTCAAAG G	303 bp
	pGerE_R	CGCTTGCAATCTCCTTTGTTG	

2.9 Polymerase Chain Reaction

NEB OneTaq® DNA Polymerase (New England Biolabs, USA) was used for polymerase chain reactions (PCR) during the study. Reaction mixture components, their amounts, and reaction conditions were determined according to the manufacturer's manual.

Table 2.4 PCR Reaction Mixture

Components	Amount
5X OneTaq® Standard Reaction Buffer	4 µL
OneTaq® DNA Polymerase	0.1 µL
<i>B. subtilis</i> 168 Genomic DNA	1 µL
Forward Primer (FP)	0.4 µL
Reverse Primer (RP)	0.4 µL
dNTP	0.4 µL
Nuclease Free Water	13.7 µL
Total reaction volume	20 µL

Table 2.5 PCR Reaction Conditions

Step	Temperature	Duration	Cycles
Initial Denaturation	94°C	30s	1
Denaturation	94°C	30s	30x
Annealing	Variable	60s	
Extension	68°C	60s	
Final Extension	68°C	5 min	1
Hold	4°C	∞	

Gradient PCR was done to decide the optimal annealing temperatures for each primer pair and in the following experiments, those exact temperatures were set. PCR

products were checked and confirmed for size, integrity, and purity via agarose gel electrophoresis.

2.10 PCR Cleanup

GeneJET Gel Extraction Kit (Thermo Scientific, USA) was used to clean up the PCR samples that were confirmed by agarose gel electrophoresis. The purity and concentration of cleaned-up samples were measured using BioDrop Touch UV/Vis (BioDrop Ltd., UK).

2.11 Plasmid Isolation

In order to isolate the plasmids Monarch® Plasmid Miniprep Kit (New England Biolabs, USA) was used. Overnight-grown cultures of *E. coli* and *B. subtilis* in LB with the presence of appropriate antibiotics were collected. The isolation was conducted as suggested by the manufacturer's manual except for one additional step that requires the addition of lysozyme to *B. subtilis* samples for better lysis of cells. After the resuspension of *B. subtilis* cultures in the Resuspension Buffer, 100 µL lysozyme (45 mg/mL) was added to tubes and mixed thoroughly, then the samples were incubated at 37°C for 30 minutes. The procedure continued as described in the kit's manual.

2.12 Restriction Enzyme Digestion

The same restriction enzyme digestion procedure was applied to both isolated plasmids and cleaned-up PCR products. BamHI-HF (High-Fidelity) and HindIII-HF enzyme combination was used for the fragments aimed to be cloned to pET28a(+) and BamHI-HF-XbaI combination for pHT08. All enzymes were supplied from New England Biolabs, USA and the reaction mixtures were prepared as advised by the manufacturer. The reaction was completed at 37°C for 1 hour.

Table 2.6 Restriction Digestion Mixture

Component	Amount
DNA (plasmid or linear)	1 µg
10X CutSmart Buffer	5 µL
HinDIII-HF or XbaI	1 µL
BamHI-HF	1 µL
Nuclease Free Water	To complete 50 µL
Total volume	50 µL

2.13 Agarose Gel Electrophoresis

1% agarose gel was prepared by dissolving 1.3 g agarose in 130 mL of TAE buffer (Appendix C) in a 250 mL flask. The mixture was heated in a microwave until it completely dissolved. Melted agarose was cooled down before adding ethidium bromide (final concentration of 0.5 µg/mL). DNA samples were supplemented with 6X Purple Loading Dye (New England Biolabs, USA) before being loaded to gel. GeneRuler 1 kb Plus DNA ladder (Thermo Scientific, MA, USA) was used as the reference for the size determination of sample bands. Electrophoresis was performed at 90 V for 1.5 hours. DNA bands were visualized under UV light, and then, Vilber Lourmat Gel Imaging System (Vilber Lourmat Sté, 46 France) was used to get digital images.

2.14 Gel Extraction

In order to get DNA fragments with sticky ends, restriction digested plasmid or PCR samples were run on agarose gel, and gel extraction was performed by using GeneJET Gel Extraction Kit (Thermo Scientific, USA) to the interested bands with confirmed sizes. The concentration and purity of extracted DNA samples were measured using BioDrop Touch UV/Vis (BioDrop Ltd., UK).

2.15 Ligation Reaction

Ligation reaction between gel-extracted *gerE* or *spoIIID* and gel-extracted plasmids (pET28a(+)) or pHT08) was performed by using NEB T4 DNA Ligase (New England Biolabs, USA). The ligation reaction mixtures (Table 2.7) were prepared as advised by the manufacturer and kept at 16°C overnight to completion. Three different insert-to-vector mass ratios were determined between 3:1 and 15:1 and separate reactions were performed for each.

Table 2.7 Ligation Reaction Mixture

Components	Amounts
Vector DNA	50-100 ng
Insert DNA (1 kb)	Varies with the ratio
10X T4 DNA Ligase Buffer	2 µL
T4 DNA Ligase	1 µL
Nuclease Free Water	Complete to 20 µL
Total	20 µL

2.16 Preparation of Competent *E. coli*

Competent *E. coli* DH5α and *E. coli* BL21 (DE3) were prepared using the procedure described by Hanahan (1983). 3 mL of overnight cultures were taken into 250 mL fresh LB medium. Cells were grown at 37°C (150 rpm) until their OD₆₀₀ reached between 0.4 and 0.6. 50 mL of those cultures were taken, put on ice for 15 minutes, and centrifuged at 3500 rpm for 5 minutes at 4°C. Supernatants were discarded, pellets were dissolved in 20 mL of ice-cold Buffer-1 (Appendix C) and centrifugation was performed at the same conditions. Pellets were resuspended gently in 8 mL of ice-cold Buffer-2 (Appendix C). 100 µL aliquots were taken into 1.5 mL tubes and stored at -80°C.

2.17 Transformation of Competent *E. coli*

Competent *E. coli* cells were taken from -80°C and were left to thaw slowly on ice. Thawed cells were mixed gently by tapping with either 15 µL of ligation product or 3 µL of plasmid and the mixture was put on ice for 20 minutes. Heat shock was applied in a water bath at 42°C for 50 seconds and cells were rested on ice for 5 minutes. 1 mL fresh LB medium was added to tubes and cells were left to recover at 37°C 100 rpm for 1.5 hours. At the end of incubation, tubes were centrifugated at 1000 g for 10 minutes. 1 mL of the supernatant was removed, and cells were resuspended in the remaining supernatant by pipetting and then spread on an LB agar plate supplemented with relevant antibiotics. Plates were incubated at 37°C overnight.

2.18 Colony PCR

Colony PCR was performed to test whether candidate transformant colonies contain plasmid with the desired insert. Up to 8 transformant colonies were selected from agar plates and each was added to separate reaction mixtures in PCR tubes. NEB OneTaq® DNA Polymerase (New England Biolabs, USA) was used and PCR reaction mixtures and conditions are given in Table 2.8 and Table 2.9, respectively. PCR products were examined by agarose gel electrophoresis to confirm the existence of desired *gerE* or *spoIIID* inserts.

Table 2.8 Reaction Mixture of Colony PCR

Components	Amounts
OneTaq® DNA Polymerase	0.2 µL
5X OneTaq® Standard Reaction Buffer	10 µL
Forward Primer	0.8 µL
Reverse Primer	0.8 µL
dNTP	0.8 µL
Nuclease Free Water	29.4 µL
Total	50 µL

Table 2.9 Conditions of Colony PCR

Steps	Temperature	Duration	Cycles
Initial Denaturation	94°C	120 s	1
Denaturation	94°C	30 s	30x
Annealing	Primer Dependent	1 min	
Extension	68°C	1 min	
Final Extension	68°C	5 min	1
Hold	4°C	∞	

2.19 Plasmid Sequencing

All recombinant plasmids (both cloned to pET28a(+) and pHT08) were sequenced by İNTERGEN (Türkiye) to confirm the correctness of their insert and to check for the presence of any mutation. The expected plasmid sequence was created by using Benchling (USA) and it was aligned with the acquired sequencing data, then the alignment was visualized using IGV (Robinson *et al.*, 2011).

2.20 Transformation of *B. subtilis*

Transformation of *Bacillus subtilis* OGU1 was done following the procedure described by Anagnostopoulos and Spizizen (1961) which utilizes its natural competence ability. 20 mL of SP medium (Appendix B) was inoculated and incubated at 37°C 150 rpm overnight. Then, 400 µL overnight culture was added to 20 mL of fresh SP medium. After OD₆₀₀ of culture reached around 0.7, it was grown for one more hour. In a 2 mL tube, 500 µL of the culture was mixed with 15 µL of pHT08 containing either *gerE* or *spoIIID*, isolated from *E. coli* DH5a. The tube was incubated at 37°C 150 rpm for 90 minutes to allow recovery of the cells. Next, the tube was centrifuged at 1000g for 10 minutes and 400 µL of supernatant was discarded. The pellet was resuspended in the remaining 100 µL of supernatant and spread on LB agar containing chloramphenicol.

2.21 Overexpression of Proteins in Recombinant *E. coli* BL21 (DE3) and OGU1

Recombinant *E. coli* BL21 (DE3) and OGU1 cells harboring pET28a(+) and pHT08 plasmids, respectively, were grown overnight in 20 mL 2xYT medium supplemented with relevant antibiotics at 37°C 150 rpm. Then flasks containing 100 mL of fresh 2xYT were inoculated by adjusting their OD₆₀₀ to 0.05. Cultures were induced by adding 100 µL of sterile 1 M IPTG when their OD₆₀₀ reached about 0.6. As a negative control, each sample had a replicate that had not been supplemented with IPTG. 50 mL of cultures were collected into 50 mL centrifuge tubes from each sample at 0, 2, 4, and 6 hours and centrifuged at 6000g for 15 minutes. The supernatants were discarded completely, and pellets were stored at -80°C.

2.22 Protein Extraction

Pellets were dissolved in 2 mL of LEW buffer (Appendix C) by vortex. Tubes were put in -80°C for 15 minutes to freeze completely, then, vortexed until complete thawing. After three repeats of the freeze-thaw cycle, tubes were placed on ice.

For the further breakdown of the cells, sonication was applied using CP70T Ultrasonic Processor (Cole-Parmer, USA). *E. coli* BL21 cells were sonicated for 10 seconds, and allowed to cool down for 30 seconds and this cycle was repeated until a total of 10 rounds of sonication were completed. *B. subtilis* cells, on the other hand, were subjected to 40 seconds of sonication and left for 60 seconds to cool, with three rounds of sonication in total.

Samples were centrifuged at 15000 g at 4°C for 15 minutes after sonication. The soluble part, containing the total soluble protein extracted from cells, was filtered using a 0.45 µm nylon or polyamide filter. Filtered protein extracts were stored at -20°C until further use.

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

Polyacrylamide gels were prepared according to Laemmli (1970), and the gel compositions are given in Table 2.10. Mini-Protean electrophoresis apparatus (Bio-Rad, USA) was used for preparation and running of the gels. 6X Laemmli buffer was added to samples before loading into gels. Samples were run alongside a pre-stained protein ladder. Samples were run in 1X SDS-PAGE Running Buffer at 100 V for approximately 2 hours.

Table 2.10 SDS-Polyacrylamide Gel Compositions (For 2 Gels)

Components	Stacking Gel (4%)	Separating Gel (18%)
Acrylamide/Bisacrylamide (30%)	650 μ L	6 mL
Distilled Water	3 mL	1.25 mL
1.5 M Tris-Cl pH 8.8	-	2.5 mL
0.5 M Tris-Cl pH 6.8	1.25 mL	-
10% SDS	50 μ L	100 μ L
10% APS	50 μ L	100 μ L
TEMED	5 μ L	10 μ L
Total	5 mL	10 mL

Coomassie Blue R-250 Staining Solution (Appendix C) was used to stain proteins in gels. Gels were placed on an orbital shaker at 50 rpm for 30 minutes for staining. Then, gels were taken into a new container with Destaining Solution (Appendix C) and left on an orbital shaker at 50 rpm until protein bands became visible.

2.24 Protein Purification

Recombinant proteins, GerE and SpoIIID, over-expressed from pET28a(+) by *E. coli* BL21 (DE3) which had a 6xHis-Tag on their N-terminals were purified using Ni-TED 2000 Packed Columns (MACHERY-NAGEL, Germany). The purification protocol was performed as suggested by the manufacturer. Extracted total protein samples were loaded into a purification column, and flow-through, wash, and elution solutions were collected, and then visualized using SDS-PAGE. The concentration of eluted proteins was measured at OD₂₈₀. Purified protein samples were stored at -20°C.

2.25 Preparation of PY79 Broth Concentrate

PY79 broth concentrate, a solution containing bacilysin in high concentrations, was prepared as described by Roscoe and Abraham (1966). 20 mL of PA medium (Perry and Abraham, 1979) (Appendix B) was inoculated with *B. subtilis* PY79 and grown overnight at 37°C 150 rpm. The seed culture, then, was used to inoculate 150 mL of fresh PA medium with an OD₆₀₀ to 0.1 and grown for 16 hours.

The culture was collected into 50 mL centrifuge tubes and cells were pelleted at 10000 rpm for 10 minutes. Acetone precipitation was performed as; 15 mL of supernatant was taken in a new tube and mixed with 35 mL of pure acetone, followed by centrifugation at 10000 rpm for 5 minutes. This step was repeated one more time and the supernatant was taken into a volumetric flask. In order to separate acetone from the mixture by evaporation Büchi R-200 rotary evaporator (Büchi, Switzerland) was utilized at Central Laboratory at the METU Biological Sciences Department. Acetone-free broth concentrate was sterilized using a 0.22 µm cellulose acetate filter and stored at -20°C for future use.

2.26 Bacilysin Disc Diffusion Bioassay

A disc diffusion bioassay was done to compare bacilysin production levels of *gerE* and *spoIIID* mutant *B. subtilis* strains (BKK28410 and BKK36420, respectively) to their parent strain *B. subtilis* 168. *B. subtilis* 168 and the mutant strains were grown in PA medium supplemented with tryptophan and appropriate antibiotics at 37°C 150 rpm overnight. Then, 20 mL of fresh PA medium was inoculated to OD₆₀₀ to 0.1 and grown at the same conditions for 24 hours. At 4, 8, and 24 hours of growth as well as at every hour from 12 to 20, 1 mL of cells were taken into 2 mL centrifuge tubes and centrifuged at 10000 rpm for 10 minutes. 200 µL of supernatants were collected into sterile PCR tubes and 20 µL of it was added into sterile blank antibiotic discs. Discs were dried at 50°C for 15 minutes. Next, to sterilize the discs, 20 µL pure acetone was added to each and dried at 50°C for 15 minutes.

S. aureus ATCC 9144 was used as the assay organism due to its susceptibility to bacilysin. It was grown in LB medium and mixed with molten bioassay medium agar described by Mah *et al.* (1967) (Appendix B) with its final CFU set to be 10^5 , such that 7.5 mL of cells with OD₆₀₀ of 1 were added to 75 mL molten agar. Dried discs were placed on the solidified agar plate and incubated at 37°C overnight. Afterwards, the diameters of the inhibition zones were measured and compared to each other.

2.27 β -Galactosidase Assay

β -Galactosidase enzyme activity assay was done in order to examine the effect of cloned genes *gerE* and *spoIIID* on the expression levels of *bac* operon in *B. subtilis* OGU1 by using the procedure described by K  ro  lu *et al.* (2011). The OGU1 and recombinant OGU1 strains were grown in 20 mL PA medium overnight at 37°C 150 rpm with the relevant antibiotics and then transferred to 100 mL of fresh PA as its initial OD₆₀₀ to be 0.1. Samples were collected at 4, 8, 12-20, and 24 hours, between 12 and 20 hours, samples were taken at each hour. Meanwhile, OD₆₀₀ was periodically measured until they reached about 0.6, to induce *gerE* and *spoIIID* expression in pHT08 harboring OGU1 cultures using 100 μ L of 1M IPTG. At each time point, 1 mL of culture was taken to OD₆₀₀ measurement and another 1 mL was collected into 1.5 mL Eppendorf tubes for assay. Tubes were centrifuged at 13000 rpm for 5 minutes; the supernatant was discarded, and pellets were resuspended in 500 μ L ice-cold 25 mM Tris-Cl (pH 7.4) (Appendix C) by vortex. Centrifugation was applied with the same settings. Pellets were dissolved in 640 μ L Z-Buffer (Appendix C) and 160 μ L of lysozyme (2.5 mg/mL) (Appendix C) was added and vortexed. Tubes were incubated at 37°C for 5 minutes in a water bath. 8 μ L of 10% Triton-X100 (Appendix C) was added to tubes and tubes were incubated again. To start the reaction, 200 μ L ONPG (4 mg/mL) (Appendix C) was added and mixed by inversion, and the tubes were carefully observed for yellow color formation. As soon as the color formation was established, 400 μ L of Na₂CO₃ (1 M) (Appendix C) was added to stop the reaction, and time was recorded immediately. Tubes were

centrifuged at 13000 rpm for 5 minutes. Supernatants were collected and OD₄₂₀ and OD₅₅₀ measurements were taken. OD₆₀₀, OD₄₂₀ and OD₅₅₀ measurements and reaction time values were used to calculate the Miller Units of samples as described by Miller (1972).

2.28 Sporulation and Germination in Recombinant Strains

2.28.1 Enumeration of Heat-Resistant Spores

All knockout and recombinant pHT08 carrying *B. subtilis* strains were grown in 50 mL of DSM (Schaeffer, Millet, & Aubert, 1965) (Appendix B) at 37°C 150 rpm for 16 hours in the presence of appropriate antibiotics. OD₆₀₀ of OGU1/*gerE* and OGU1/*spoIIID* cultures were monitored periodically and were supplemented with 50 µL of IPTG for induction when OD₆₀₀ reached about 0.6. At the end of the 16 hours of incubation, 500 µL samples were taken from each culture as two replicates. Experimental samples were incubated at 85°C for 20 minutes in a water bath, meanwhile, control samples were kept at room temperature for the same duration. Samples were serially diluted tenfold in PBS and spread on LB agar containing no antibiotics. Plates were incubated overnight at 37°C and colonies were counted. CFU percentage ratios between heat and control samples were calculated.

2.28.2 Mature Spore Purification

B. subtilis strains were grown in 50 mL DSM overnight at 37°C 150 rpm with relevant antibiotics and were transferred to 100 mL of fresh DSM to an initial OD₆₀₀ of 0.1. Cultures were grown under the same conditions for 72 hours. Recombinant pHT08 carrying *B. subtilis* strains were induced with IPTG as described in section 2.28.1.

Purification of mature spores was done as described by Nicholson and Setlow (1990). 30 mL of cultures were taken into Oak Ridge tubes and centrifuged at 14000 rpm for 10 minutes at 4°C. Supernatants were discarded and pellets were resuspended in 10 mL of 1M KC/0.5M NaCl (Appendix C). Tubes were centrifuged at the same settings. Supernatants were discarded and pellets were resuspended by vortex in 10 mL Tris-Cl (50 mM pH 7.2) with 50 mg/mL lysozyme and incubated at 37°C in a water bath for 1 hour to allow lysis of the cells. Centrifugation was applied under the same conditions. After the discarding of supernatants, pellets were washed with 10 mL of the mentioned sterile solutions in the written order: 1 M NaCl, deionized water, 0.05% SDS, and TEP Buffer (50 mM Tris-Cl, pH 7.2, 10 mM EDTA, and 2 mM PMSF). Afterward, the pellets were washed three more times with sterile deionized water. The final pellets were resuspended in 10 mL of sterile deionized water and tubes were stored at -20°C.

Spore assays including resistance against wet heat, lysozyme, and chloroform were performed as described by Nicholson and Setlow (1990).

2.28.3 Wet Heat Resistance Assay

500 µL aliquots of mature spores of each strain were taken into 1.5 mL tubes twice, one tube for the assay and the other for control. The assay group was incubated at 80°C for 30 minutes in a water bath, meanwhile, the control group was kept at room temperature. All samples were serially diluted tenfold in PBS and spread on LB agar containing no antibiotics. Plates were incubated overnight at 37°C and colonies were counted. CFU percentage ratios between heat and control samples were calculated.

2.28.4 Chloroform Resistance Assay

450 µL aliquots of mature spores of each strain were taken into 1.5 mL tubes twice, one tube for the assay and the other for control. The assay group was supplemented with 50 µL chloroform, meanwhile, the control group was given the same amount of

sterile PBS instead of chloroform. Both groups were incubated at room temperature for 10 minutes. All samples were serially diluted tenfold in PBS and spread on LB agar containing no antibiotics. Plates were incubated overnight at 37°C and colonies were counted. CFU percentage ratios between chloroform-treated and control samples were calculated.

2.28.5 Lysozyme Resistance Assay

400 µL aliquots of mature spores of each strain were taken into 1.5 mL tubes twice, one tube for the assay and the other for control. The assay group was supplemented with 100 µL lysozyme (final concentration 250 µg/mL), meanwhile, the control group was given the same amount of sterile PBS instead of lysozyme. Both groups were incubated 37°C for 10 minutes. All samples were serially diluted tenfold in PBS and spread on LB agar containing no antibiotics. Plates were incubated overnight at 37°C and colonies were counted. CFU percentage ratios between lysozyme-treated and control samples were calculated.

2.28.6 Spore Germination Assay

Spore germination assay was performed by following the methods described by Nicholson and Setlow (1990). OD₅₈₅ of purified spores was measured and the required samples were diluted with 10 mM Tris-Cl (pH 8.4) to an OD₅₈₅ of 0.8. Samples were heated at 70°C for 30 minutes in a water bath. The heated samples were mixed with 20 mL of preheated at 37°C 2xYT medium and the first OD₅₈₅ measurements were recorded as t_0 . The mixtures were incubated at 37°C and samples were taken every 10 minutes for OD₅₈₅ measurements for a total of 150 minutes. Spore to germinated cell ratio was accepted as 100% at t_0 and OD₅₈₅ values at each time point were then calculated as percentages, accordingly. A 60% decrease in OD₅₈₅ relative to t_0 was accepted as the time required for the germination of all spores as described by Atluri *et al.* (2006).

2.28.7 Colony Morphology

Knockout and recombinant *B. subtilis* strains were grown in 20 mL of PA medium overnight at 37°C 150 rpm with appropriate antibiotics. The cultures were diluted a hundredfold in PA medium, and 1 µL of the dilution was dropped in the middle of the DSM agar supplemented with antibiotics and IPTG for required strains. The plates were incubated at 30°C for 15 days, their images were taken daily by using Epson Perfection V750 Pro scanner (Epson Corp, Japan) to monitor the changes in colony morphology and pigmentation.

2.28.8 Statistical Analysis

Statistical analyses of spore count and resistance experiments were performed using GraphPad Prism (GraphPad, USA). The means of the number of surviving colonies were compared using an ordinary one-way ANOVA test. Significant differences between multiple mean values were compared using Tukey's Honest Significance Test (HSD). In the figures, the level of the significance was denoted as * ($p < 0.05$), ** ($p < 0.01$), and *** ($p < 0.001$).

2.29 Electrophoretic Mobility Shift Assay (EMSA)

Electrophoretic Mobility Shift Assay (EMSA) was performed to examine the direct interactions between purified recombinant proteins GerE and SpoIIID and *bac* promoter. Also, bacilysin broth concentrate was tested against the promoter regions of *gerE*, *spoIIID*, *cotB*, *abrB*, and *bofA*. Protocols described by Köroğlu *et al.* (2011) were followed throughout the experiment.

EMSAs with purified GerE were conducted using P_{cotB} for positive control, P_{abrB} for negative control and P_{bac} . EMSAs with purified SpoIIID were conducted using P_{bofA} for positive control, P_{cotB} for negative control, and P_{bac} . Those experiments were also repeated by replacing nuclease-free water in reaction mixtures with bacilysin broth

concentrate to investigate the effect of bacilysin on binding affinity between proteins and promoter regions. EMSAs with bacilysin broth concentrate were conducted with P_{bac} , P_{gerE} , $P_{spoIIID}$, P_{cotB} , P_{abrB} , and P_{bofA} in order to examine whether bacilysin can bind to these promoter regions on its own.

A gradient concentration of proteins ranging from no protein to the maximum possible amount depending on purification yield was used in protein-promoter EMSAs. Bacilysin-promoter EMSAs were performed using bacilysin in two concentrations, one containing no bacilysin and the other at the maximum concentration possible. Independent of the experiment, the initial concentration of promoter DNA added was approximately 40 $\mu\text{g/mL}$. Ingredients and required amounts for sample preparation are given in Table 2.11. The reaction mixtures were incubated at room temperature for 45 minutes once prepared.

Polyacrylamide gels were prepared as described in Table 2.12. Mini-Protean electrophoresis apparatus (Bio-Rad, USA) was used to run the gels and all runs were performed in a cold room at 4°C. Pre-run was applied in cold 1xTBE at 80 V for 90 to 120 minutes to eliminate impurities enclosed in gels. Incubated samples were mixed with 10X EMSA loading dye before loading to gels. Samples were run at 80V for 150 to 180 minutes.

DNA bands were stained using SYBRTM Gold Nucleic Acid Gel Stain (ThermoFisher Scientific) for 30 minutes at room temperature with constant shaking on an orbital shaker. For the visualization of the gels, Vilber Lourmat Gel Imaging System (Vilber Lourmat Sté, 46 France) was used.

Table 2.11 Ingredients for EMSA Samples (1 Reaction Tube)

Ingredient	Amount
Binding Buffer (5x)	4 μ L
BSA (1 mg/mL)	2 μ L
DNA from Herring Sperm (50 mg/mL)	1 μ L
25 mM MgCl	4 μ L
0.1 M DTT	0.2 μ L
Promoter DNA (~40 mg/mL)	2 μ L
Purified Protein	0-6.8 μ L
NF Water/Bacilysin broth Concentrate	0-6.8 μ L (Complete to 20 μ L)
Total	20 μ L

Table 2.12 Ingredients of 5% polyacrylamide gel for EMSA

Ingredients	Amount
Distilled water	15.3 mL
40% acrylamide/bisacrylamide	2.5 mL
10X Buffer (TBE)	2 mL
10% APS	200 μ L
TEMED	25 μ L

CHAPTER 3

RESULTS

3.1 Genomic DNA Isolation from *B. subtilis* 168

Genomic DNA of *B. subtilis* 168 is isolated and used as the template for amplifying genes *gerE* and *spoIID* and promoter regions for EMSAs by PCR. The integrity and purity of isolated genomic DNA were checked by running agarose gel and visualizing under UV light can be seen in Figure 3.1.

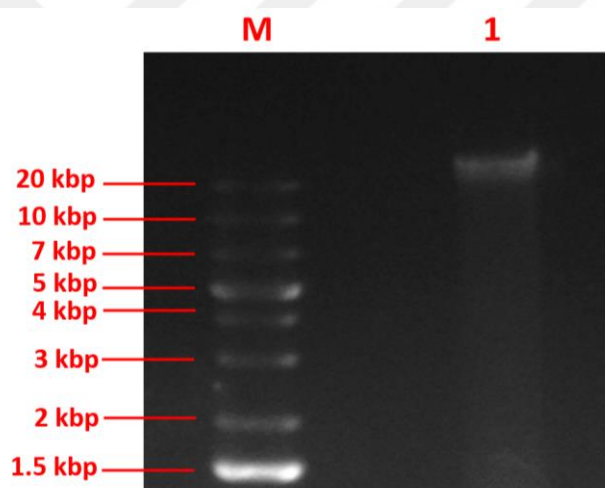


Figure 3.1 Genomic DNA isolated from *B. subtilis* 168; M: GeneRuler 1kb Plus, Lane 1: pure and intact gDNA isolated from *B. subtilis* 168

3.2 Polymerase Chain Reactions

PCR is used to amplify genes of interest (*gerE* and *spoIID*) and promoter regions (P_{bac} , P_{gerE} , P_{spoIID} , P_{abrB} , P_{cotB} and P_{bofA}) by using *B. subtilis* 168 genomic DNA.

3.2.1 *gerE* and *spoIIID* in pHT08

gerE and *spoIIID* to be cloned into pHT08 were designed to include the whole coding region with BamHI at the 5' end and XbaI at the 3' end. PCR products were run on agarose gel to confirm the sizes, integrity and purity of the cloned genes. The band of the *gerE*/pHT08 PCR product was observed between 200-300 bp as expected as the product was designed to be 256 bp. The *spoIIID*/pHT08 PCR product was observed just above 300 bp and the expected size of 310 bp was confirmed. The agarose gel image of these PCR products is given in Figure 3.2.

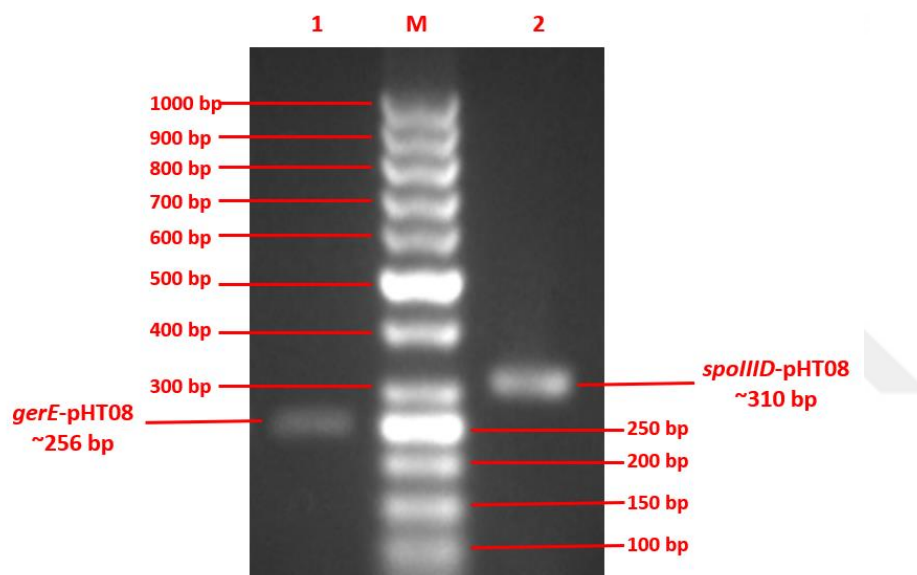


Figure 3.2 Verification of PCR products M: SiZer-50 plus DNA ladder, Lane 1: *gerE*-pHT08, Lane 2: *spoIIID*-pHT08.

3.2.2 *gerE* and *spoIIID* in pET28a(+)

gerE and *spoIIID* to be cloned into pET28a(+) were designed to include the whole coding region with BamHI and HindIII at their 5' and 3' ends respectively. PCR products were run on an agarose gel to confirm the size, integrity, and purity of the cloned genes. The band of the *gerE*/pHT08 PCR product was observed between 200-

300 bp as expected as the product was designed to be 249 bp. The *spoIIID*/pHT08 PCR product was observed just above 300 bp and the expected size of 319 bp was confirmed. The agarose gel image of the PCR products is given in Figure 3.3.

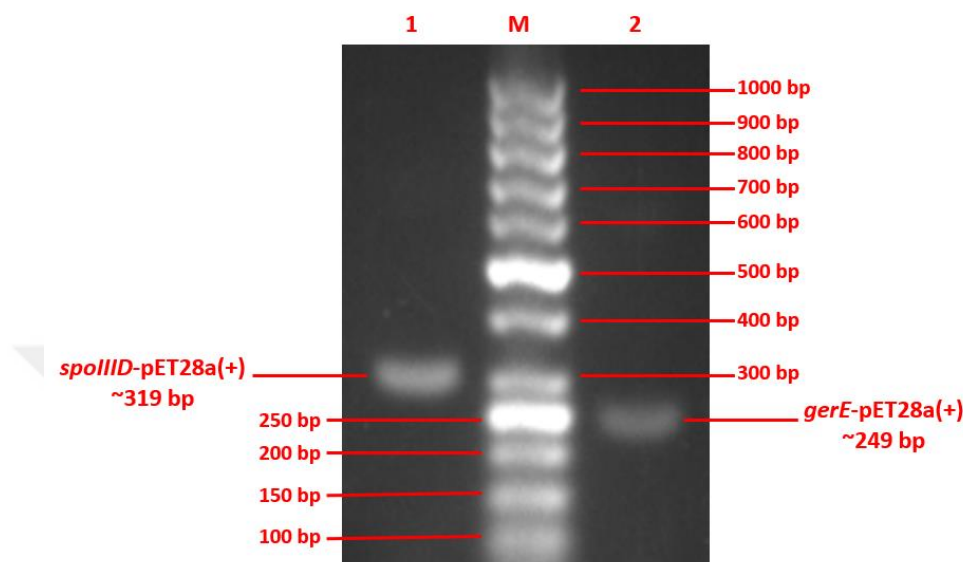


Figure 3.3 Verification of PCR products M: SiZer-50 plus DNA ladder, Lane 1: *spoIIID*-pET28a(+), Lane 2: *gerE*-pET28a(+).

3.2.3 Promoter Regions for EMSAs

PCR-amplified promoter regions P_{bac} , P_{gerE} , $P_{spoIIID}$, P_{abrB} , P_{cotB} and P_{bofA} were run and visualized on an agarose gel to confirm size, purity and integrity of the products. The expected sizes of promoters were as follows: P_{bac} : 383 bp, P_{gerE} : 303 bp, $P_{spoIIID}$: 324 bp, P_{abrB} : 371 bp, P_{cotB} : 537 bp, P_{bofA} : 592 bp. Figure 3.4 shows their sizes and purity after being run on agarose gel.

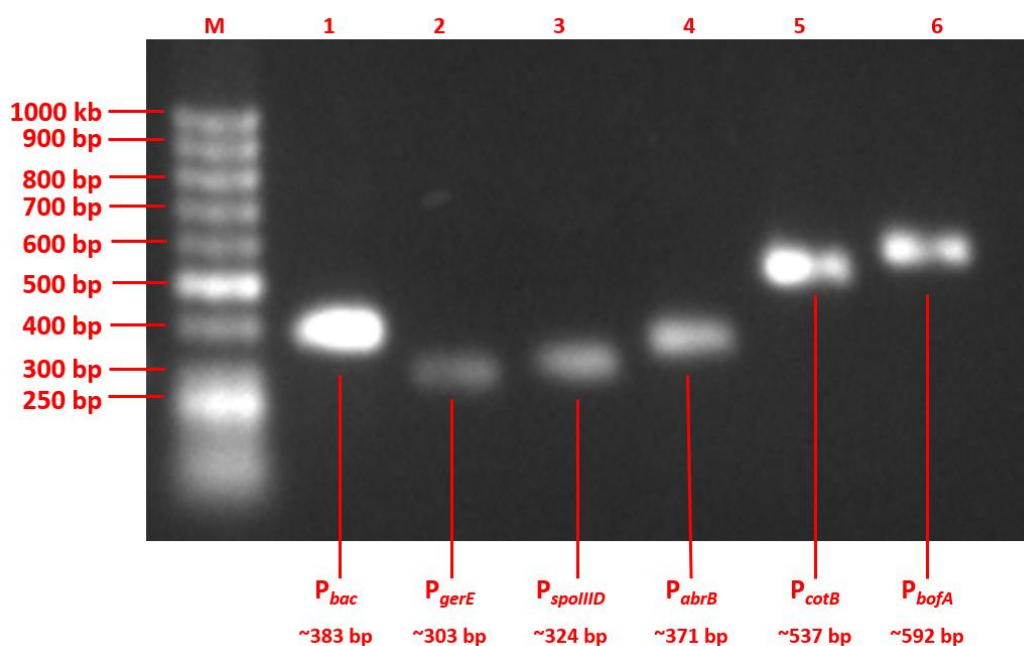


Figure 3.4 Verification of PCR products of promoters for EMSAs M: SiZer-50 plus DNA ladder, Lane 1: P_{bac}, Lane 2: P_{gerE}, Lane 3: P_{spoIIID}, Lane 4: P_{abrB}, Lane 5: P_{cotB}, Lane 6: P_{bofA}.

3.3 Cloning of *gerE* and *spoIIID* into pET28a(+)

After the completion of PCR, both the PCR products of *gerE* and *spoIIID* and the pET28a(+) vector were digested with BamHI and HindIII. After running cut samples on agarose gel, the PCR products and plasmid were extracted from the gel. Genes and plasmids with matching sticky ends were ligated and the insert carrying plasmids were transformed into *E. coli* DH5 α .

The presence of plasmids with desired inserts in transformant colonies was checked via the colony PCR technique. Confirmed colonies were selected for future use. For further confirmation, plasmids were isolated from those colonies and were cut with restriction enzymes. The products of the cut were run on agarose gel and plasmid and insert bands with the correct sizes were confirmed as *gerE*: 225 bp and *spoIIID*: 282 bp. Figure 3.5 shows their correct sizes and purity after being run on an agarose gel.

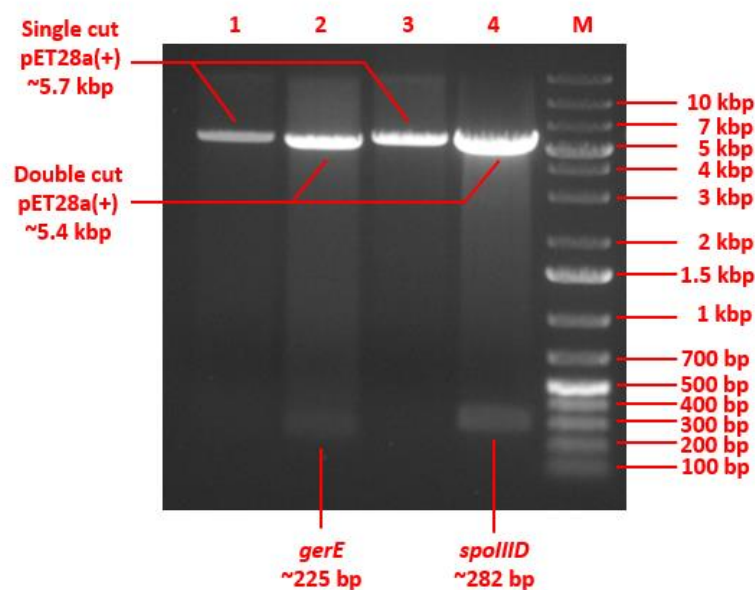


Figure 3.5 Verification of *gerE* and *spoIIID* inserts in pET28a(+) isolated from *E. coli* DH5a M: GeneRuler 1kb Plus DNA ladder, Lane 1: *gerE*-pET28a(+) single cut, Lane 2: *gerE*-pET28a(+) double cut, Lane 3: *spoIIID*-pET28a(+) single cut, Lane 4: *spoIIID*-pET28a(+) double cut.

3.4 Overexpression of GerE and SpoIIID in *E. coli* BL21 (DE3)

Recombinant GerE (10.4 kDa) and SpoIIID (12.4 kDa) were overexpressed by inducing *E. coli* BL21 (DE3)+*gerE* and *E. coli* BL21 (DE3)+*spoIIID* using IPTG, and whole cell lysates extracted from these cells were run on SDS-PAGE alongside a control lysate. The size and presence of the overexpressed proteins in the lysates were confirmed by comparing their sizes to a prestained protein marker. The lysates and the recombinant proteins visualized using SDS-PAGE are shown in Figure 3.6.

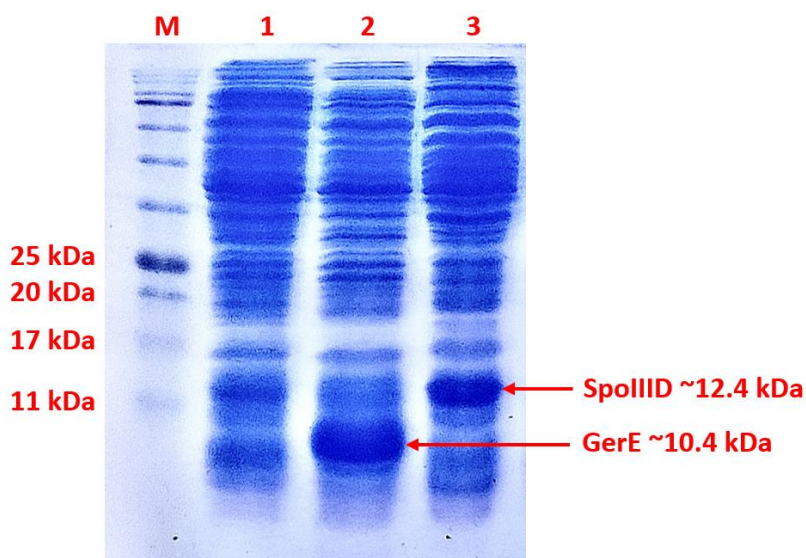


Figure 3.6 Lysates from *E. coli* BL21 (DE3)+*gerE* and *E. coli* BL21 (DE3)+*spoIIID*. M: EcoTech ClearBand prestained protein marker, Lane 1: Uninduced *E. coli* BL21(DE3) lysate, Lane 2: Induced *E. coli* BL21+*gerE*, Lane 3: Induced *E. coli* BL21+*spoIIID*.

3.5 Purification of GerE and SpoIIID

Recombinant, His-tagged GerE and SpoIIID were purified using IMAC. The cell lysate used as well as the flowthrough, wash, and elutes collected using the columns were run using SDS-PAGE, and the size and purity of the purified recombinant proteins were confirmed. Both GerE and SpoIIID were purified, and their SDS-PAGE gels are shown in Figures 3.7 and 3.8.

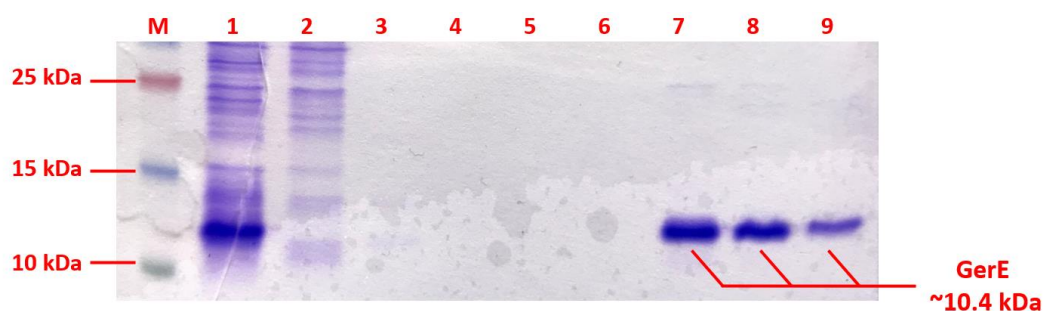


Figure 3.7 Purification of GerE. M: PageRuler plus prestained protein ladder, Lane 1: Induced *E. coli* BL21 (DE3)+*gerE* lysate, Lane 2: Flowthrough, Lane 3-9: Elutes 1-7

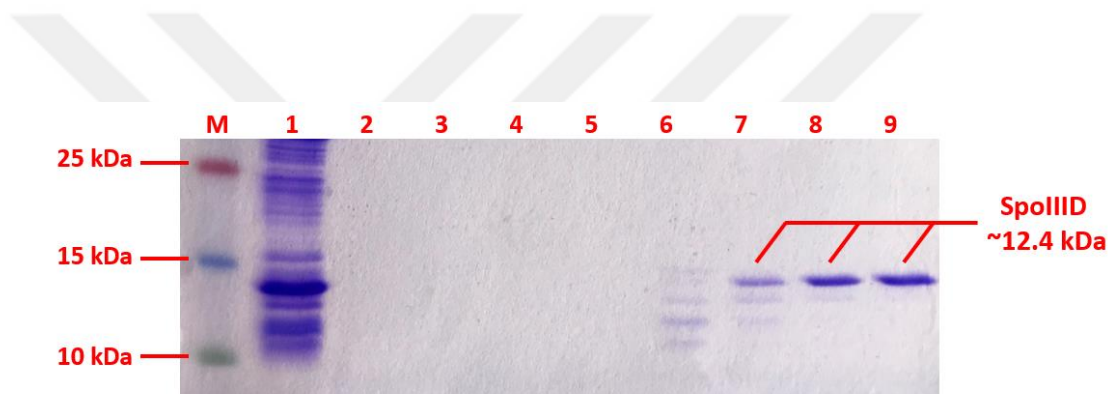


Figure 3.8 Purification of SpoIIID. M: PageRuler plus prestained protein ladder, Lane 1: Induced *E. coli* BL21 (DE3)+*spoIIID* lysate, Lane 2-9: Elutes 1-8

3.6 Cloning of *gerE* and *spoIIID* into pHT08

After the completion of PCR, both PCR products of *gerE* and *spoIIID* and the pHT08 vector were digested with BamHI and XbaI. After running cut samples on agarose gel, the PCR products and plasmid were extracted from the gel. Genes and plasmids with matching sticky ends were ligated and the insert carrying plasmids were transformed into *E. coli* DH5 α .

The presence of plasmids with desired inserts in transformant colonies was checked via the colony PCR technique. Confirmed colonies were selected for future use. For

further confirmation, plasmids were isolated from those colonies and were cut with restriction enzymes. The products of the cut were run on agarose gel and plasmid and insert bands with the correct sizes were confirmed as *gerE*: 225 bp and *spoIIID*: 282 bp. Figure 3.9 shows their correct sizes and purity after being run on an agarose gel.

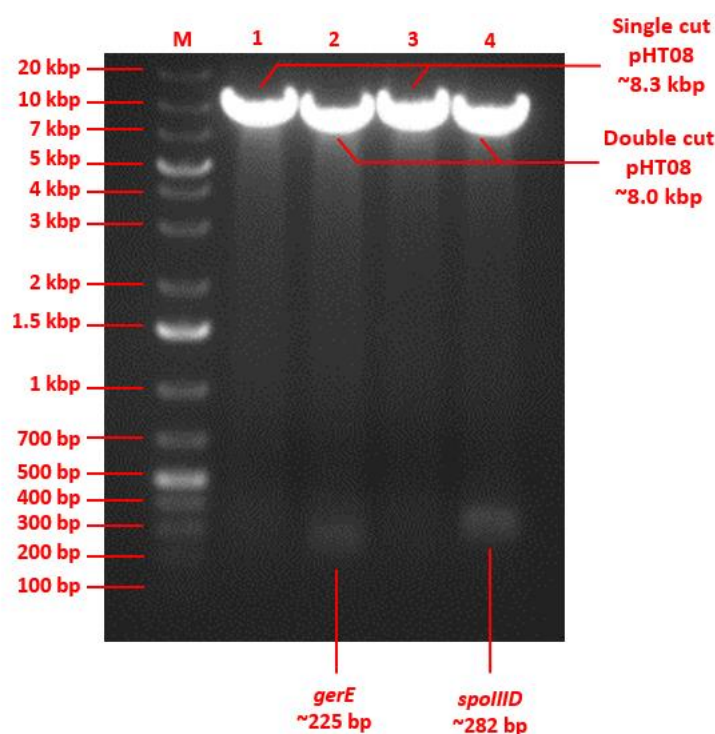


Figure 3.9 Verification of *gerE* and *spoIIID* inserts in pHT08 isolated from *E. coli* DH5a M: GeneRuler 1kb Plus DNA ladder, Lane 1: *gerE*-pHT08 single cut, Lane 2: *gerE*-pHT08 double cut, Lane 3: *spoIIID*-pHT08 single cut, Lane 4: *spoIIID*-pHT08 double cut.

3.7 Cloning of pHT08-*gerE* and pHT08-*spoIIID* into *B. subtilis* OGU1

pHT08 plasmids confirmed to be carrying *gerE* and *spoIIID* inserts were transformed into *B. subtilis* OGU1. Plasmids were then isolated from transformant colonies and cut using BamHI and XbaI. The products of the cut were run on agarose gel and plasmid and insert bands with the correct sizes were confirmed as *gerE*: 225 bp and

spoIIID: 282 bp. Figure 3.10 shows their correct sizes and purity after being run on an agarose gel.

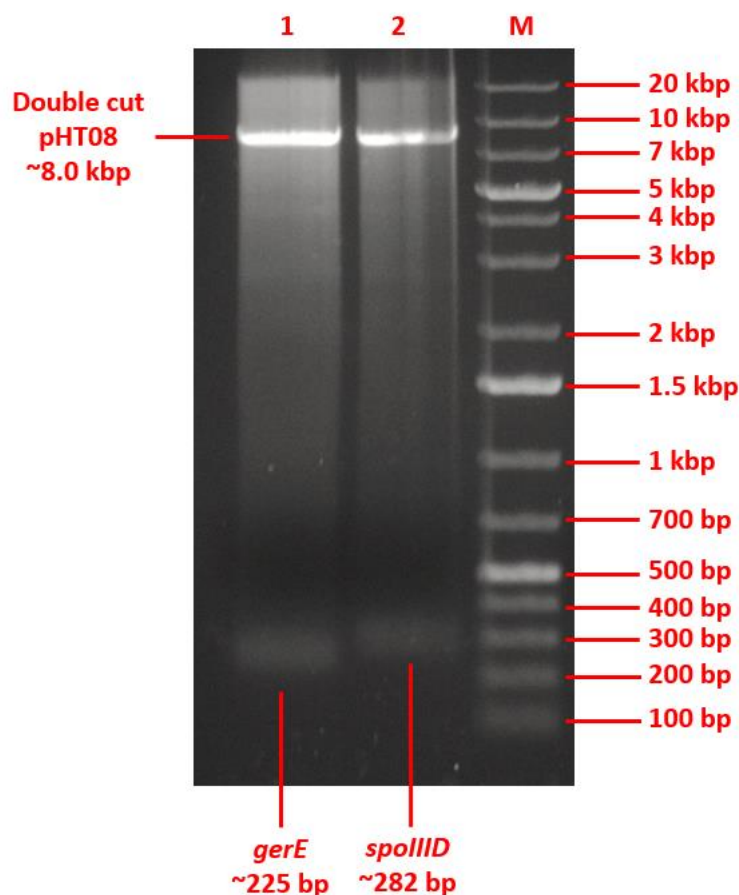


Figure 3.10 Verification of *gerE* and *spoIIID* inserts in pHT08 isolated from *B. subtilis* OGU1 M: GeneRuler 1kb Plus DNA ladder, Lane 1: *gerE*-pHT08 double cut, Lane 2: *spoIIID*-pHT08 double cut.

3.8 Overexpression of GerE and SpoIIID in *B. subtilis* OGU1

Recombinant GerE (10.4 kDa) and SpoIIID (12.4 kDa) were overexpressed by inducing *B. subtilis* OGU1+*gerE* and OGU1+*spoIIID* using IPTG, and whole cell lysates extracted from these cells were run on SDS-PAGE alongside a control lysate.

The size and presence of the overexpressed proteins in the lysates were confirmed by comparing their sizes to a prestained protein marker. The lysates and the recombinant proteins visualized using SDS-PAGE are shown in Figure 3.11.

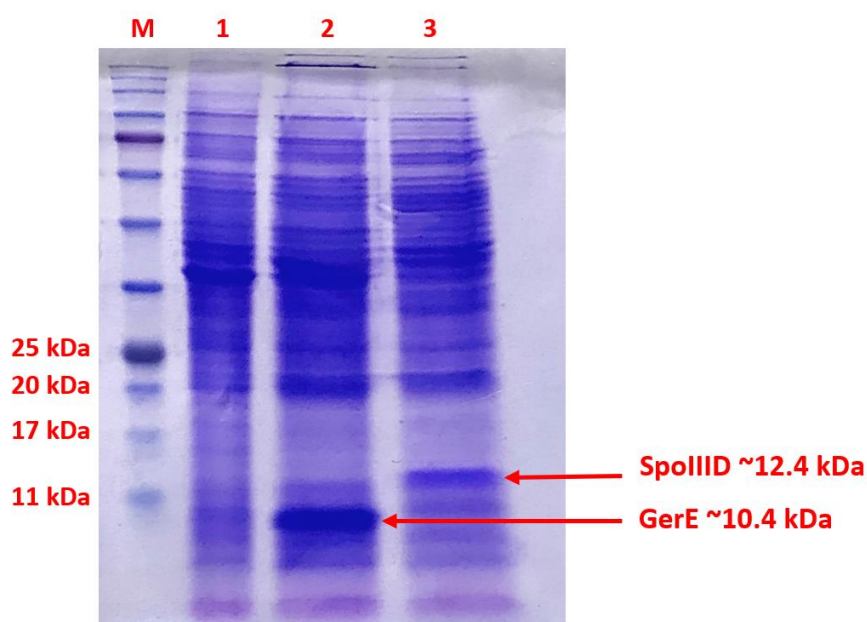


Figure 3.11 Lysates from OGU1+*gerE* and OGU1+*spoIIID*. M: EcoTech ClearBand prestained protein marker, Lane 1: Uninduced OGU1 lysate, Lane 2: Induced OGU1+*gerE*, Lane 3: Induced OGU1+*spoIIID*

3.9 Phenotypic Analyses

Previous studies by our group (Ertekin *et al.*, 2020) have shown that when compared to PY79, the bacilysin mutant strain OGU1 shows defects in spore resistances to heat, chloroform, and lysozyme as well as spore germination and sporulating colony morphology and that adding external bacilysin can partially rescue these defects.

To see whether these defects can be rescued by the overexpression of *gerE* and *spoIIID*, these phenotypic analyses were performed once again, this time using

OGU1 strains overexpressing *gerE* and *spoIIID* as well as *gerE* and *spoIIID* knockout derivatives of strain 168.

3.9.1 Viable Spore Count

B. subtilis strains 168, PY79, OGU1, OGU1+*gerE*, OGU1+*spoIIID*, BKK28410 and BKK36420 were grown overnight in DSM and were subjected to wet heat to determine the percentage of viable, heat-resistant endospores formed by each strain. The results of this experiment are shown in Figure 3.12.

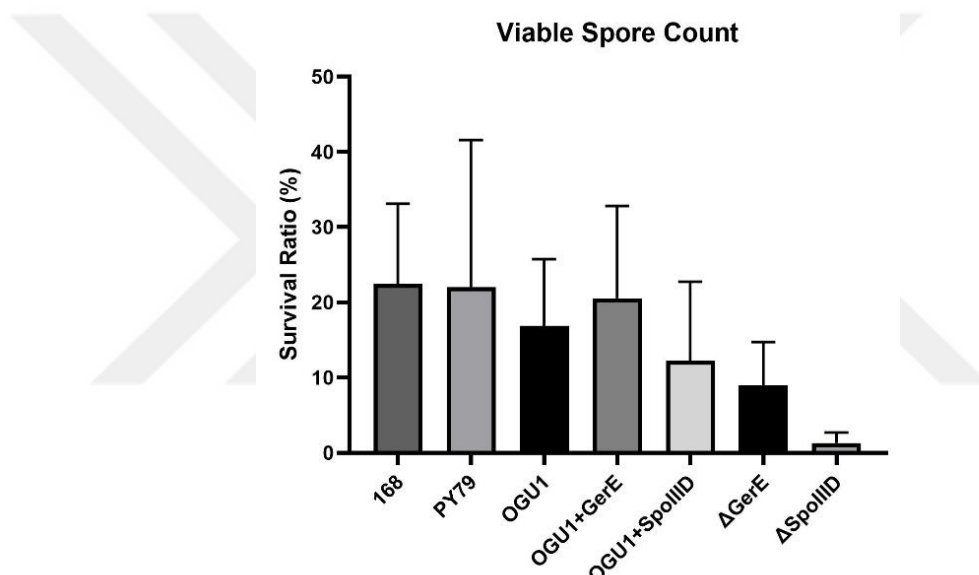


Figure 3.12 Survival ratios of cells grown in DSM overnight after the heat treatment. The significance levels, compared to OGU1, are represented as * ($p < 0.05$), ** ($p < 0.01$) and *** ($p < 0.001$).

OGU1 appears to form slightly less viable spores than the bacilysin-positive strains 168 and PY79. Overexpression of *gerE* appeared to increase the spore count to levels similar to those of wild-type strains, while *spoIIID* overexpression appeared to lower the spore count further. It is to be noted that both the *gerE* mutant BKK28410 and the *spoIIID* mutant BKK36420 formed fewer endospores than the other strains with BKK36420 having less than 2% of its cells forming endospores.

3.9.2 Heat Resistance

Endospores purified from *B. subtilis* strains 168, PY79, OGU1, OGU1+*gerE*, OGU1+*spoIIID*, BKK28410, and BKK36420 were grown overnight in DSM and were subjected to wet heat at 80°C for 30 minutes to determine the percentage spores resistant to wet heat. The results of this experiment are shown in Figure 3.13.

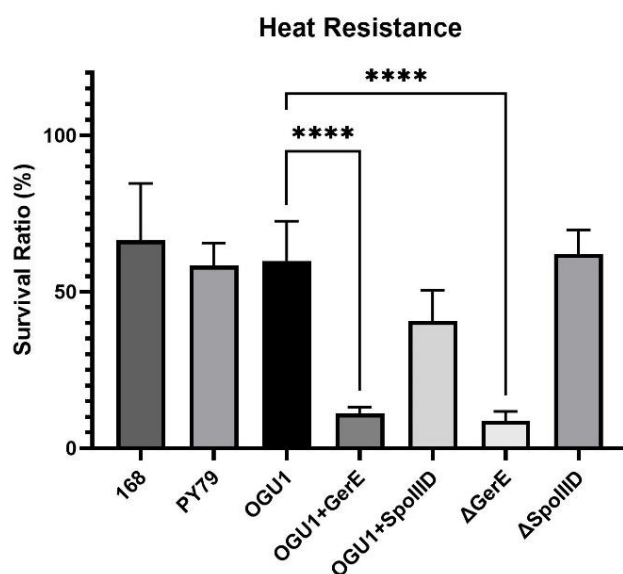


Figure 3.13 Survival ratios mature purified endospores after the heat treatment. The significance levels, compared to OGU1, are represented as * ($p < 0.05$), ** ($p < 0.01$) and *** ($p < 0.001$).

OGU1 appears to have similar levels of heat resistance when compared to the bacilysin-positive strains 168 and PY79. Overexpression of *gerE* appeared to significantly lower the heat resistance of OGU1, while *spoIIID* overexpression appeared to lower heat resistance in a non-significant manner. The *gerE* mutant BKK28410 also showed significantly lower levels of heat resistance while the *spoIIID* mutant BKK36420 similar amounts of heat resistance when compared to the wild-type strains.

3.9.3 Chloroform Resistance

Endospores purified from *B. subtilis* strains 168, PY79, OGU1, OGU1+*gerE*, OGU1+*spoIIID*, BKK28410, and BKK36420 were grown overnight in DSM and were subjected to chloroform for 10 minutes to determine the percentage spores resistant to chloroform. The results of this experiment are shown in Figure 3.14.

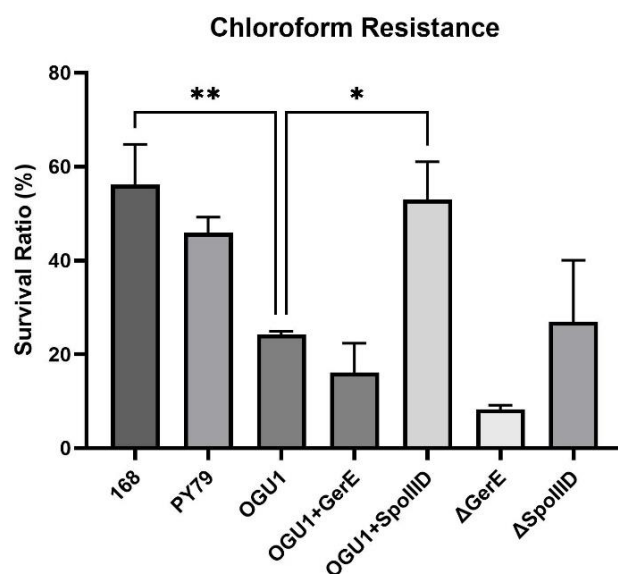


Figure 3.14 Survival ratios mature purified endospores after the chloroform treatment. The significance levels, compared to OGU1, are represented as * ($p < 0.05$), ** ($p < 0.01$) and *** ($p < 0.001$).

OGU1 shows less chloroform resistance than the bacilysin-positive strains 168 and PY79, with the difference compared to 168 being significant compared to OGU1. Overexpression of *gerE* appeared to slightly lower the chloroform resistance of OGU1, while *spoIIID* overexpression appeared to significantly increase chloroform resistance. The *gerE* mutant BKK28410 also showed lower levels of chloroform resistance while the *spoIIID* mutant BKK36420 showed similar amounts of chloroform resistance compared to OGU1.

3.9.4 Lysozyme Resistance

Endospores purified from *B. subtilis* strains 168, PY79, OGU1, OGU1+*gerE*, OGU1+*spoIIID*, BKK28410 and BKK36420 were grown overnight in DSM and were subjected to lysozyme for 10 minutes to determine the percentage of the lysozyme-resistant spores. The results of this experiment are shown in Figure 3.15.

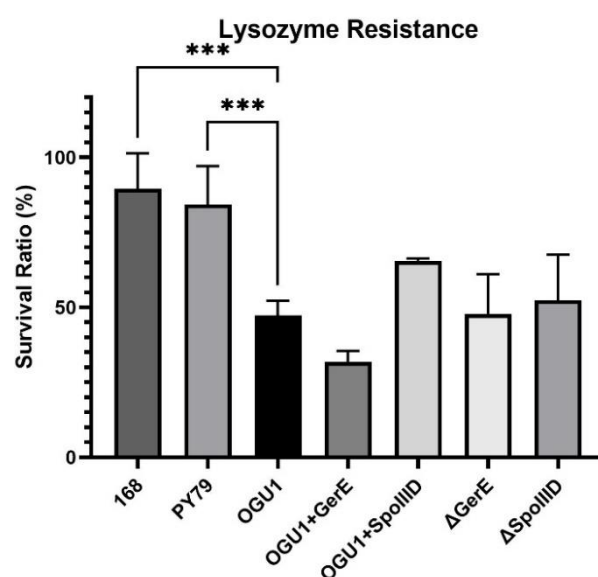


Figure 3.15 Survival ratios mature purified endospores after the lysozyme treatment. The significance levels, compared to OGU1, are represented as * ($p < 0.05$), ** ($p < 0.01$) and *** ($p < 0.001$).

OGU1 showed significantly less lysozyme resistance than the bacilysin-positive strains 168 and PY79. Overexpression of *gerE* appeared to slightly lower the lysozyme resistance of OGU1, while *spoIIID* overexpression appeared to increase the lysozyme resistance. The *gerE* mutant BKK28410 and the *spoIIID* mutant BKK36420 both displayed levels of lysozyme resistance comparable to that of OGU1.

It is worth noting that the concentration of purified spores which could be obtained from the mutant strains BKK28410 and especially BKK36420 was far lower than

the other strains tested. Therefore their resistance levels may not be statistically significant to accurately reflect the true resistances of endospores formed by those strains.

3.9.5 Germination Assay

The germination kinetics of endospores purified from *B. subtilis* strains 168, PY79, OGU1, OGU1+*gerE*, OGU1+*spoIIID*, BKK28410, and BKK36420 were determined as described by Atluri *et al.* (2006). Activated endospores inoculated into 2xYT had their OD₅₈₅ measured over 150 minutes and were considered to be fully germinated when their OD₅₈₅ reached 60% of the initial value. The graph of the OD₅₈₅ of each strain is shown in Figure 3.16.

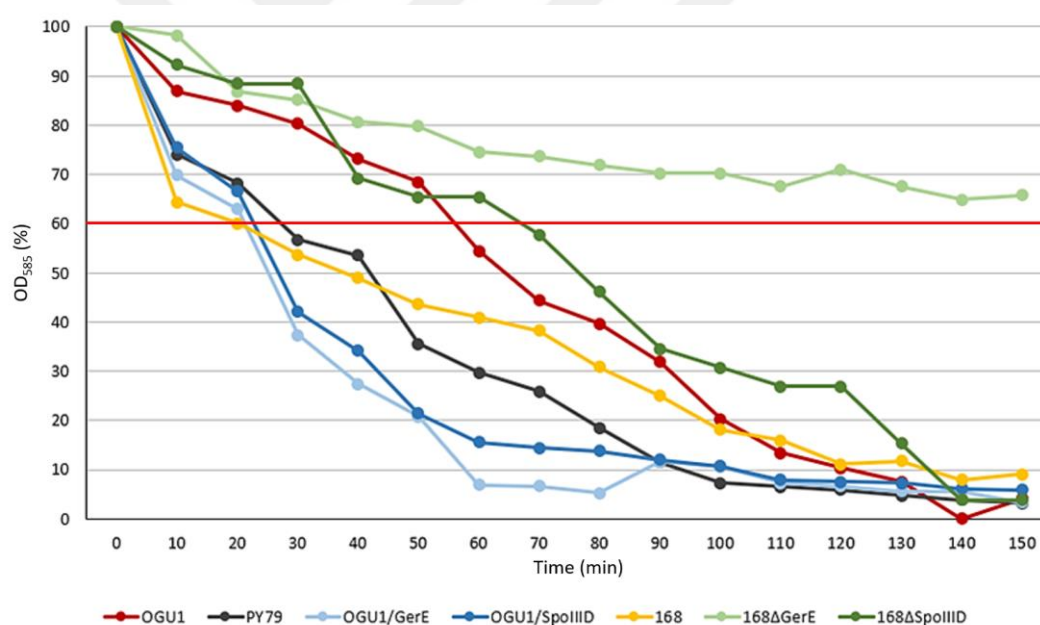


Figure 3.16 Germination kinetics of OGU1 (red), PY79 (black), OGU1+*gerE* (light blue), OGU1+*spoIIID* (dark blue), 168 (yellow), BKK28410 (light green) and BKK36420 (dark green).

OGU1 appeared to germinate slower than the wild-type strains PY79 and 168. *gerE* and *spoIIID* overexpression both significantly increase the germination speed of

OGU1, causing it to germinate as fast as the wild-type strains 168 and PY79. The *gerE* mutant BKK28410 was incapable of germination within 150 minutes while the *spoIIID* mutant BKK36420 appeared to germinate slightly slower than OGU1 (Fig. 3.16).

3.9.6 Colony Morphologies

The morphology and pigmentation of sporulating colonies formed by *B. subtilis* strains 168, PY79, OGU1, OGU1+*gerE*, OGU1+*spoIIID*, BKK28410, and BKK36420 on DSM agar were photographed 3 times a week for 15 days. The results are presented in Figures 3.17 and 3.18.

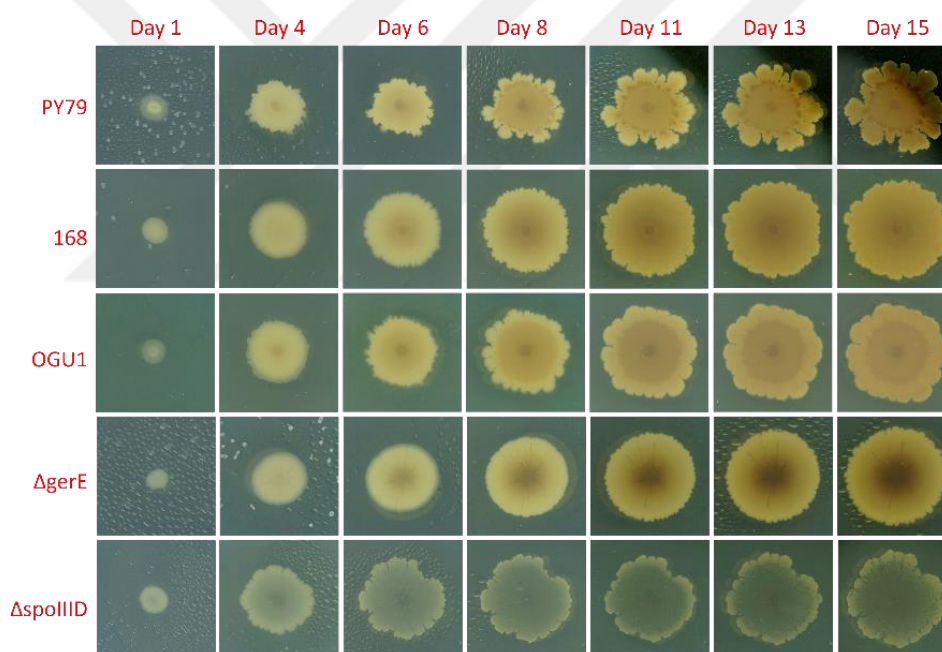


Figure 3.17 Colony morphologies of PY79, 168, OGU1, BKK28410 and BKK36420 grown on DSM agar for 15 days.

When compared to the colony formed by PY79, 168 formed a less wrinkled colony with a smoother gradient of pigmentation. Despite being derived from PY79, OGU1 also showed less wrinkles and had lighter pigmentation than PY79. The colony formed by the *gerE* mutant BKK28410 had an edge even smoother than that formed

by 168, however, it had a much more pigmented center. This pigmented center may be due to the coat enzyme CotA, which is repressed by GerE and is known to give endospores a brown pigmentation. The colony formed by the *spoIIID* mutant BKK36420 had significantly altered morphology: its edges were wrinkled, however the core of the colony appeared to be nearly translucent. This may be due to the fact that *spoIIID* mutants are considered oligosporous, and therefore the cells in the center may not be able to survive.

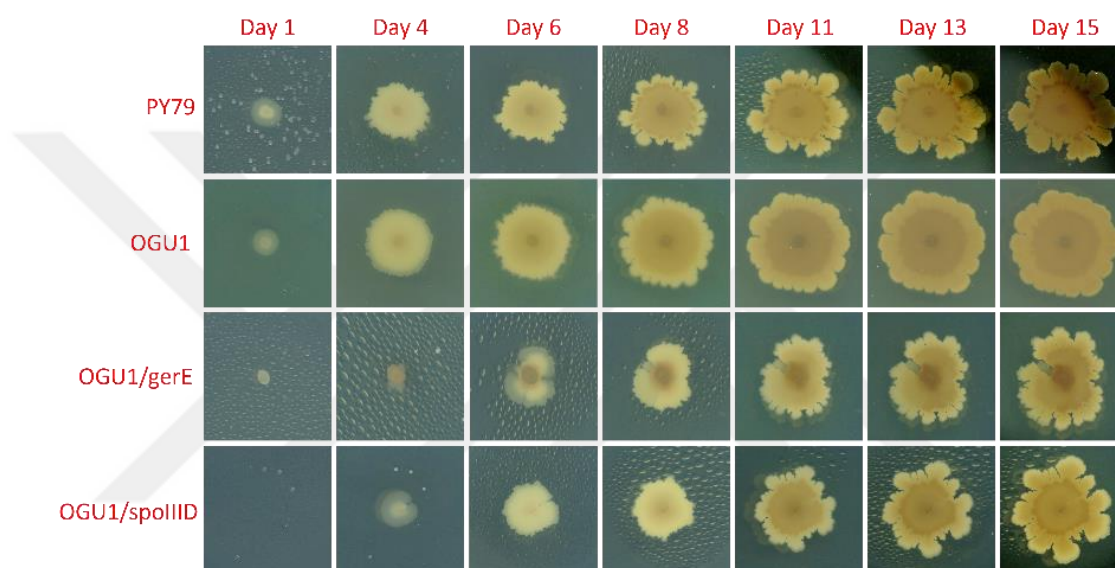


Figure 3.18 Colony morphologies of PY79, OGU1, OGU1+*gerE* and OGU1+*spoIIID* grown on DSM agar for 15 days.

The overexpression of *gerE* appeared to increase the wrinkles of the colony formed by OGU1, and also significantly darkened pigmentation in the center of the colony. The size of the colony was also smaller when compared to those formed by the other strains. The overexpression of *spoIIID* in OGU1 appeared to increase both the amount of wrinkles and the pigmentation, causing the colony to appear more like that of PY79, suggesting that *spoIIID* overexpression may be able to rescue the colony morphology defect caused by a lack of bacilysin.

3.10 β -Galactosidase Assay

The effects of *gerE* and *spoIIID* overexpression on the expression of the *bac* operon were observed using a β -galactosidase assay utilizing the *lac-bac* fusion found in OGU1. OGU1 as well as OGU1 strains overexpressing *gerE* and *spoIIID* were grown in PA medium and β -galactosidase activity was measured and calculated regularly. In addition, the OGU1 strains carrying plasmids were induced at OD₆₀₀ 0.6 at approximately 6 hours using IPTG.

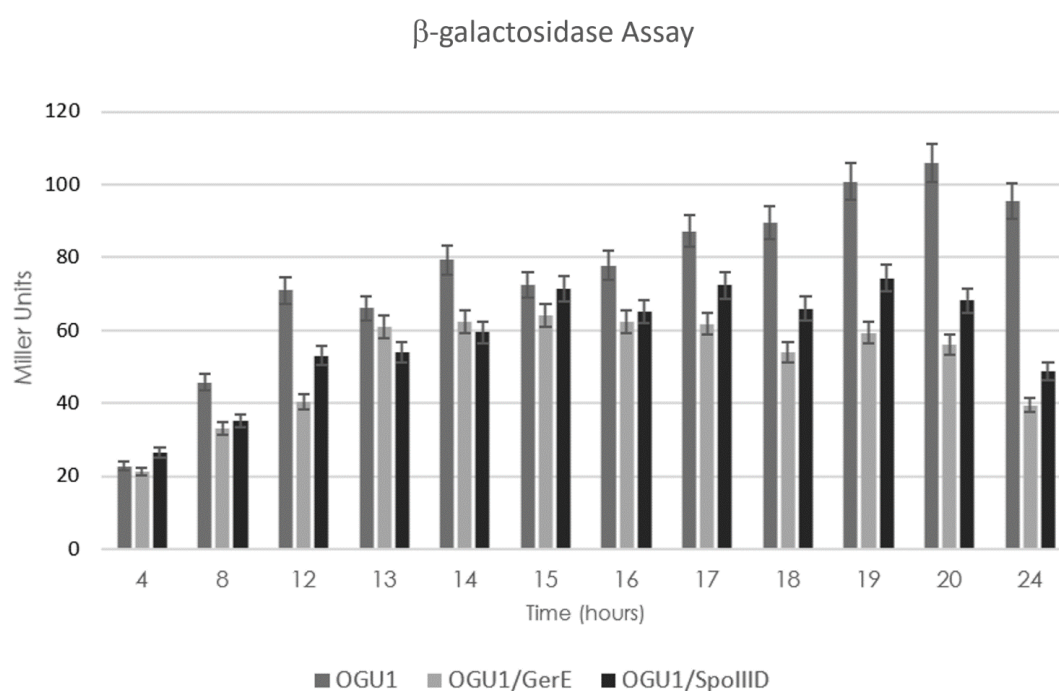


Figure 3.19 β -galactosidase activities of OGU1, OGU1+*gerE*, and OGU1+*spoIIID* over time.

The overexpression of both *gerE* and *spoIIID* appeared to lower the expression of the *lac*-fused *bac* operon in OGU1, suggesting that both proteins may be negative regulators of bacilysin production (Fig. 3.19).

3.11 Bioassay

To further understand the roles of GerE and SpoIIID on the regulation of bacilysin production, bacilysin bioassays using *S. aureus* as a test organism were utilized. Strains 168, PY79, and OGU1 were compared to the *gerE* mutant BKK28410 and the *spoIIID* mutant BKK36420. The results are shown in Figure 3.20.

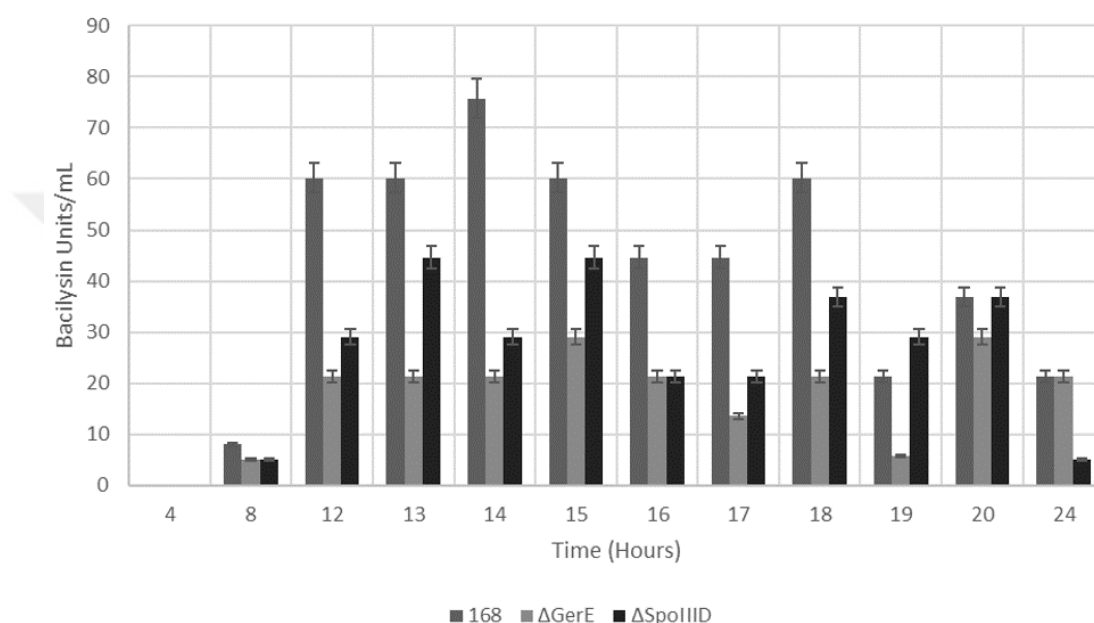


Figure 3.20 Bacilysin bioassay using broths from *B. subtilis* 168, BKK28410 (ΔgerE) and BKK36420 ($\Delta\text{spoIIID}$).

In bioassays, BKK28410 (ΔgerE) and BKK36420 ($\Delta\text{spoIIID}$) were found to produce less bacilysin than their wild-type strain 168 between hours 12 to 18. After the 18th hour, inhibition zones show inconsistencies which may be due to differing growth rates and sporulation capabilities between the strains.

3.12 Electrophoretic Mobility Shift Assays

3.12.1 EMSAs with GerE

EMSAs using purified GerE were used to investigate whether GerE binds and regulates *bac* directly. Experiments used increasing concentrations of purified GerE alongside constant amounts of the *bac* promoter to observe potential binding. In addition, the promoter P_{cotB} was used as a positive control while the promoter P_{abrB} was used as a negative control.

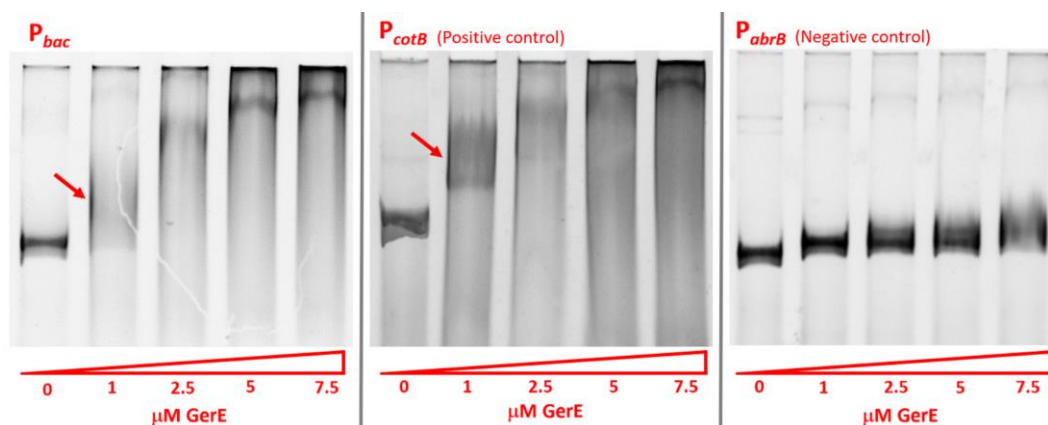


Figure 3.21 EMSAs using purified GerE for P_{bac} , as well as its positive and negative controls P_{cotB} , and P_{abrB} , respectively.

GerE appeared to bind all three promoters used, although the shifts in the negative control P_{abrB} were far weaker than those observed in the positive control P_{cotB} and the test promoter P_{bac} . The existing shift indicated that GerE directly binds to the promoter of the *bac* operon. The minor shift detected for the negative control P_{abrB} with the highest concentration (7.5 μM) of purified GerE was likely due to nonspecific binding (Fig 3.21)

3.12.2 EMSAs with SpoIIID

EMSAs using purified SpoIIID were used to investigate whether SpoIIID binds and regulates *bac* directly. Experiments used increasing concentrations of purified SpoIIID alongside constant amounts of the *bac* promoter to observe potential binding. In addition, the promoter P_{bofA} was used as a positive control while the promoter P_{cotB} was used as a negative control.

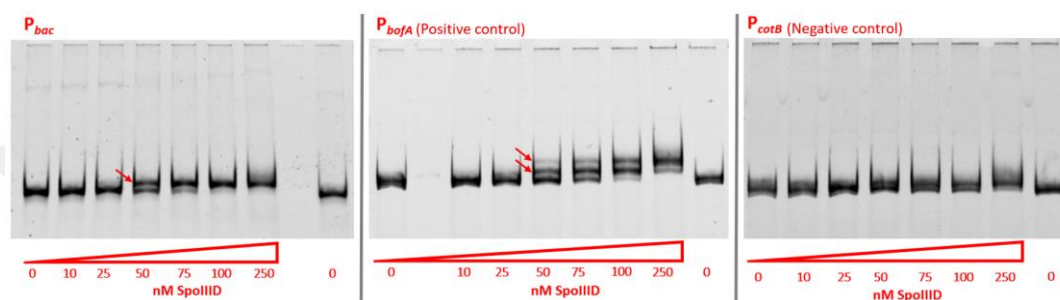


Figure 3.22 EMSAs using purified SpoIIID for P_{bac} as well as its positive and negative controls P_{bofA} and P_{cotB} , respectively.

SpoIIID, at a concentration of 50 nM appeared to cause one band shift in P_{bac} , suggesting it binds the *bac* promoter. The positive control P_{bofA} also shifted at the same concentration of SpoIIID, however, it showed up to three bands per lane. This is because P_{bofA} has two binding sites for SpoIIID (Halberg and Kroos, 1994). SpoIIID did not appear to cause any shifts for its negative control P_{cotB} . Compared to GerE, SpoIIID was able to bind P_{bac} at much lower concentrations, suggesting that SpoIIID may have a higher affinity for P_{bac} as compared to GerE (Fig. 3.22).

3.12.3 EMSA for Possible GerE-SpoIIID Co-binding to P_{bac}

Since it was found that both GerE and SpoIIID bind P_{bac} , EMSAs using both proteins at once were performed to observe if their P_{bac} binding sites do overlap. These experiments employed a constant amount of one protein with an increasing amount of the other, with both combinations.

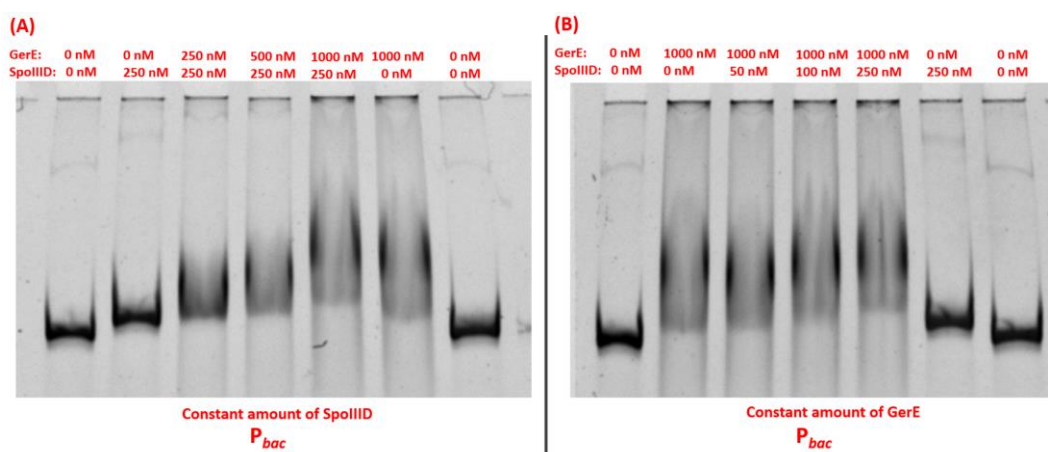


Figure 3.23 EMSAs for P_{bac} using (A) a constant concentration of purified SpoIIID with increasing concentrations of GerE, and (B) a constant concentration of purified GerE with increasing concentrations of SpoIIID.

Both proteins were capable of binding P_{bac} simultaneously, as increasing the concentration of either shifted already shifted bands even further. This suggested that the binding sites for GerE and SpoIIID on P_{bac} do not overlap (Fig. 3.23).

3.12.4 GerE and SpoIIID EMSAs in the Presence of Bacilysin Broth Concentrate

To see if the presence of bacilysin alters the DNA binding dynamics of GerE and SpoIIID, bacilysin was incorporated into the relevant EMSAs.

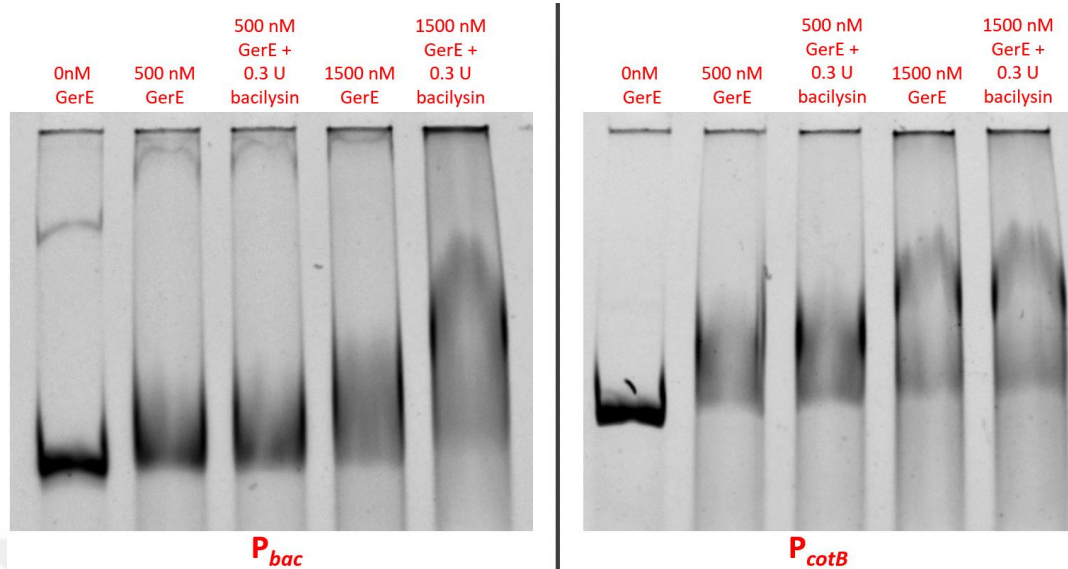


Figure 3.24 EMSAs for P_{bac} and P_{cotB} using increasing concentrations of purified GerE with and without bacilysin addition.

While 0.3 U bacilysin did not appear to alter the binding of 1500 nM GerE to its positive control P_{cotB} , it caused a stronger shift when added at the same amount to 1500 nM GerE for binding to P_{bac} . This suggested that bacilysin may enhance the DNA-binding capability of GerE to the promoter of its biosynthetic operon (Fig. 3.24).

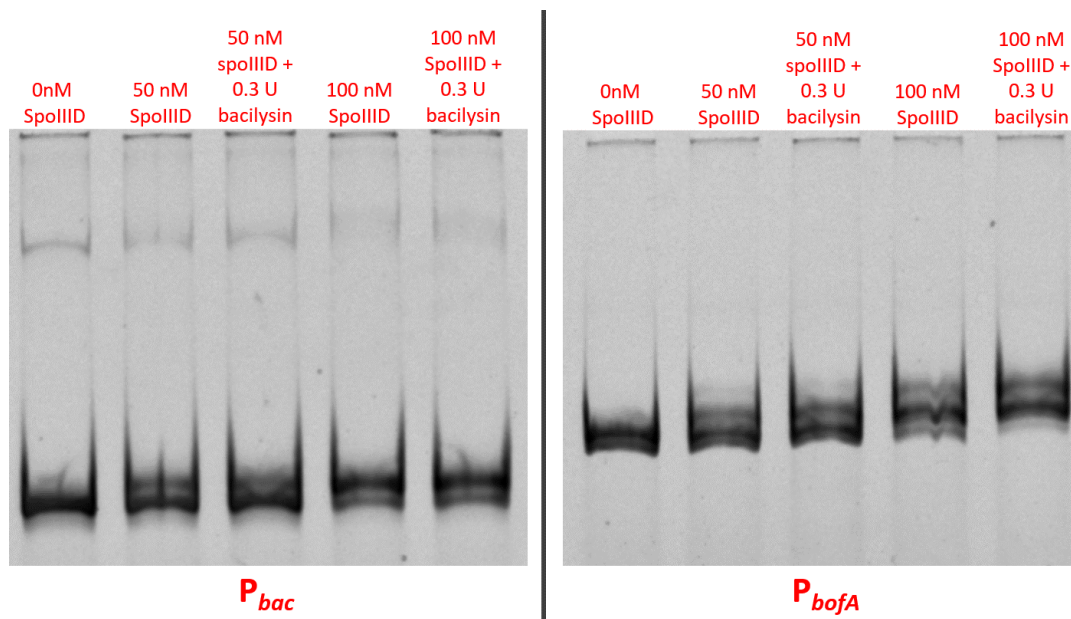


Figure 3.25 EMSAs for P_{bac} and P_{bofA} using increasing concentrations of purified SpoIIID with and without bacilysin addition.

On the other hand, bacilysin did not appear to affect the binding of SpoIIID to P_{bac} . However, bacilysin enhanced the binding of SpoIIID to its positive control P_{bofA} when added to 100 nM of SpoIIID. This suggested that bacilysin may promote the DNA binding capability of SpoIIID to certain promoters (Fig. 3.25).

3.12.5 EMSAs for Testing the Activity of Bacilysin to Bind Certain Promoters

To see if bacilysin can bind DNA on its own to regulate genes, bacilysin broth concentrates were mixed with the promoters $P_{spoIIID}$, P_{gerE} , P_{bac} , P_{cotB} , P_{abrB} , and P_{bofA} .

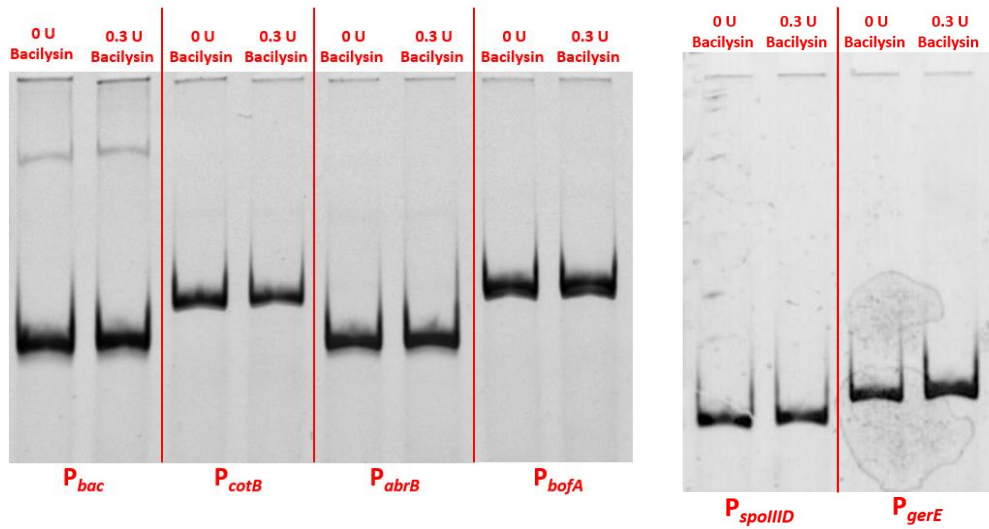


Figure 3.26 EMSAs for testing the activity of bacilysin to bind on its own to P_{bac} , P_{cotB} and P_{abrB} , P_{bofA} , P_{gerE} and $P_{spoIIID}$.

These EMSAs displayed no shifts, suggesting that bacilysin alone does not bind to the promoters tested (Fig 3.26).

CHAPTER 4

CONCLUSION

- Our research group has focused on the bacilysin-negative derivative of PY79 known as OGU1. Studies on OGU1 have shown that the strain shows lower levels of spore resistance, slower germination, altered sporulating colony pigmentation, and that it underexpresses several sporulation-related genes when compared to its parent strain PY79. These defects in OGU1 could also be rescued by supplementing OGU1 with external bacilysin. This study chose two sporulation transcription factors important in forming robust, resistant and germinable endospores found to be downregulated in OGU1, *GerE* and *SpoIIID*.
- *gerE* and *spoIIID* were overexpressed in OGU1 using the *E. coli/B. subtilis* shuttle vector pHT08 to see if their overexpression could rescue OGU1's defects in its viable spore count as well as resistances to wet heat, chloroform, and lysozyme. It was found that overexpressing *gerE* slightly increased the viable spore count of OGU1, which is lower than the viable spore count of the wild-type 168 and PY79. *spoIIID* overexpression, on the other hand, appeared to slightly lower the viable spore count of OGU1. OGU1 endospores do not show reduced wet heat resistance compared to the wild-type strains 168 and PY79. The overexpression of *gerE* appeared to significantly lower wet heat resistance in OGU1. The overexpression of *spoIIID* also appeared to lower wet heat resistance, but the change was not significant compared to OGU1. OGU1 shows significantly less chloroform resistance than the wild-type strains 168 and PY79. *gerE* overexpression slightly lowered chloroform resistance, while *spoIIID* overexpression significantly increased chloroform resistance, causing it to reach levels similar to those of wild-type strains. OGU1 also shows significantly less

lysozyme resistance than the wild-type strains 168 and PY79. *gerE* overexpression appeared to have little effect on lysozyme resistance, slightly lowering it compared to OGU1. *spoIIID* overexpression increased lysozyme resistance, though the change was not statistically significant. These results show that *gerE* overexpression partially rescues the low viable spore count, but has negative effects on spore resistance, while *spoIIID* overexpression appears to at least partially rescue the chloroform and lysozyme resistance defects found in the bacilysin-negative strain OGU1.

- The *gerE* and *spoIIID* mutants BKK28410 and BKK36420 were also compared to OGU1 and the other strains to compare their viable spore counts and spore resistances. Both the *gerE* mutant BKK28410 and the *spoIIID* mutant BKK36420 formed less viable spores than the other strains with BKK36420 producing far less viable spores compared to any other strain. The *gerE* mutant BKK28410 showed far lower wet heat resistance than the other strains while the *spoIIID* mutant BKK36420 showed similar levels of resistance to the wild-type strains 168 and PY79 as well as OGU1. The *gerE* mutant BKK28410 showed far less chloroform resistance than any other strain while the *spoIIID* mutant BKK36420 showed similar levels of chloroform resistance to OGU1, about half the chloroform resistance of the wild-type strains. Both the *gerE* mutant BKK28410 and the *spoIIID* mutant BKK36420 showed similar levels of lysozyme resistance to OGU1, being lower than the resistances of the wild-type strains 168 and PY79. Overall, while it produced far fewer viable endospores, the resistances of the *spoIIID* mutant BKK36420 appear similar to those of OGU1. It is worth noting that the concentration of purified spores which could be obtained from the mutant strains BKK28410 and especially BKK36420 were far lower than the others, thus their resistance levels may not accurately reflect the true resistance of endospores formed by those strains.
- Another defect found in OGU1 is in germination, as it germinates more slowly than the wild-type strains 168 and PY79. *gerE* and *spoIIID* were

overexpressed to see if they could rescue the slow-germinating phenotype of OGU1. When overexpressed, both *gerE* and *spoIIID* sped up the germination times of OGU1 to be slightly faster than PY79. The *spoIIID* mutant BKK36420 was found to germinate slightly slower than OGU1 while the *gerE* mutant BKK28410 appeared unable to germinate within 150 minutes. These results show that both GerE and SpoIIID are important to germination and that their overexpression can rescue the germination defect found in bacilysin-negative strains.

- OGU1 is also known to exhibit altered colony morphology and pigmentation whilst sporulating, forming colonies which are less wrinkled and pigmented when compared to its parent strain PY79. This decrease in pigmentation is likely caused by the lower levels of *cotA* expression in OGU1. CotA is an enzyme which is known to give endospores a dark brown color. The overexpression of *gerE* appeared to increase the number of wrinkles and cause the core of the colony to have abnormally dark pigments. The colony formed by *gerE*-overexpressing OGU1 also appeared to be smaller than colonies formed by other strains. Meanwhile, the overexpression of *spoIIID* increased both wrinkles and colony pigmentation, causing the colony to have pigmentation similar to that of PY79, suggesting a potential rescue. The strain 168 also differs from PY79, having a less wrinkled colony with its pigments forming a gradient rather than an abrupt ring. The *gerE* and *spoIIID* mutant derivatives of strain 168, BKK28410 and BKK36420 showed altered colony morphology as well: BKK28410 formed a smooth colony with a very heavily pigmented core, which may be because the *cotA* repressor *gerE* is knocked out. Meanwhile, BKK36420 showed wrinkled edges and a nearly transparent core. This may be because *spoIIID* mutants are oligosporous, and the cells in the center may be unable to form spores and survive.
- Bioassays were performed to compare the levels of bacilysin production in strains 168 and the *gerE* and *spoIIID* mutants BKK28410 and BKK36420. Both mutant strains appeared to produce less amounts of bacilysin when

compared to 168, suggesting that their deletions may have a negative effect on bacilysin production.

- β -galactosidase assays were done using the *lac*-fused *bac* operon of OGU1. The β -galactosidase activity of OGU1 was compared to those of OGU1 overexpressing *gerE* and OGU1 overexpressing *spoIIID*. Both *gerE* and *spoIIID* overexpression appeared to lower β -galactosidase activity in OGU1, suggesting that they may have repressive roles on the *bac* operon.
- To see whether GerE and SpoIIID could simultaneously bind P_{bac} , EMSAs using both proteins at once were performed. It was observed that they could both cause further shifts when added to one another, suggesting that the binding sites of GerE and SpoIIID on P_{bac} do not overlap.
- To see whether the presence of bacilysin affected the DNA binding activities of GerE and SpoIIID, the proteins were added to EMSAs alongside bacilysin broth concentrate obtained from PY79. In the presence of bacilysin, GerE could cause a larger shift when binding to P_{bac} . While bacilysin did not alter the binding of SpoIIID to P_{bac} , it did enhance the binding of SpoIIID to its positive control P_{bofA} . These results suggest that bacilysin can enhance the DNA-binding affinities of GerE and SpoIIID.
- To see whether bacilysin could directly bind DNA to alter gene expression, EMSAs were performed using the promoters $P_{spoIIID}$, P_{gerE} , P_{bac} , P_{cotB} , P_{abrB} , and P_{bofA} and bacilysin. Bacilysin did not appear to cause any shifts, suggesting that it does not bind DNA directly by itself.

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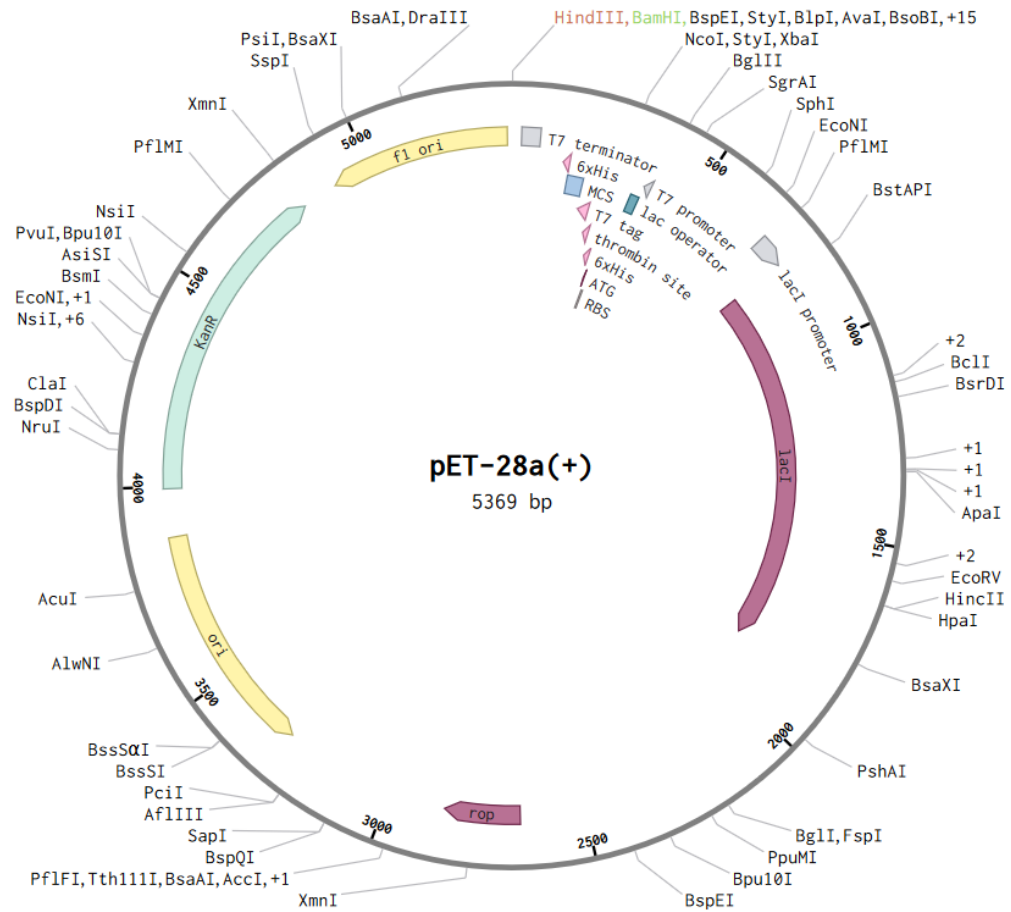
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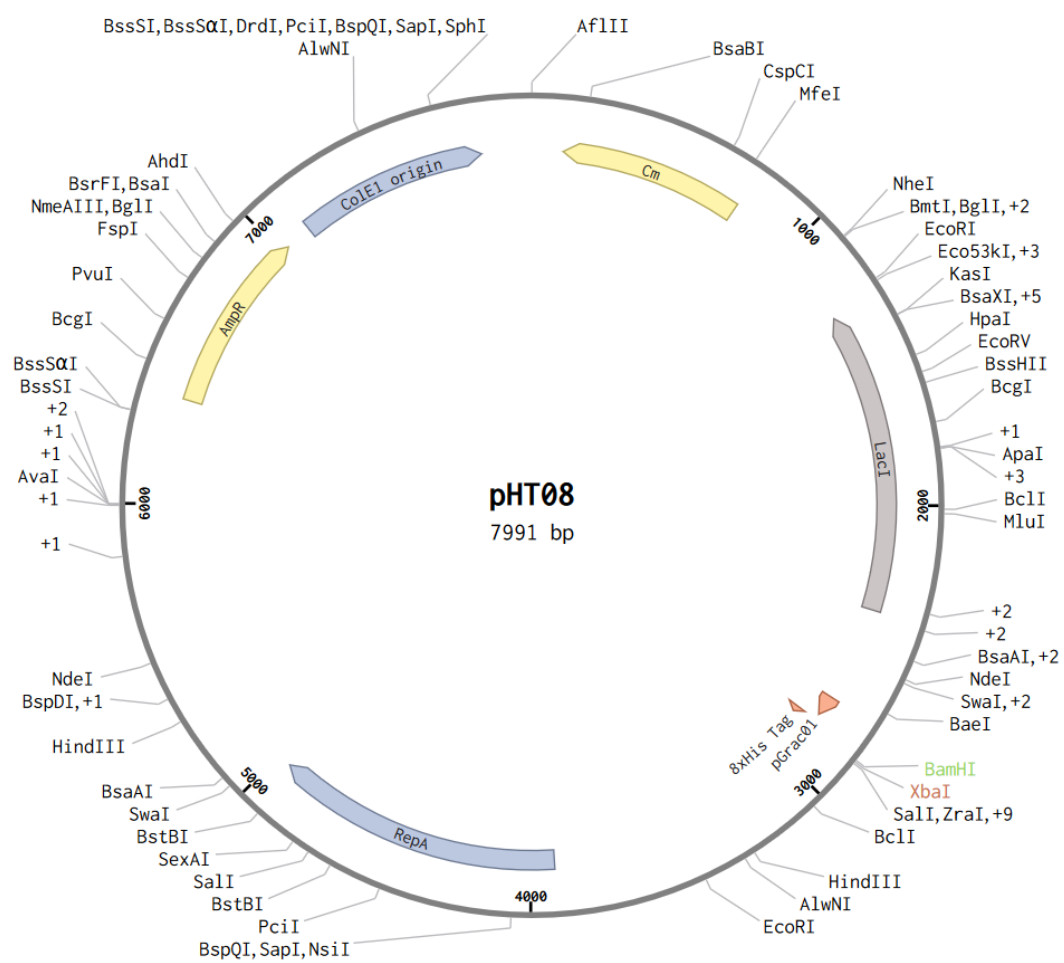
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APPENDICES

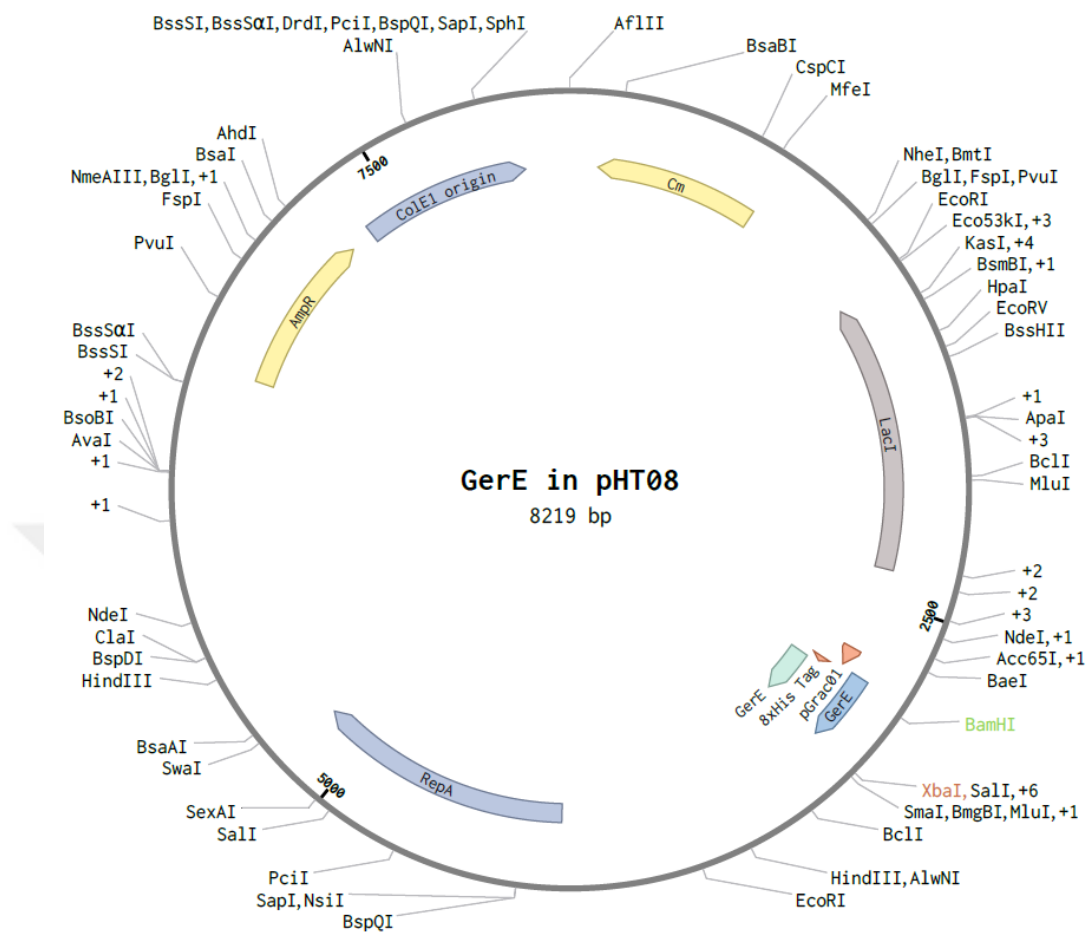
A. Plasmids



pET28a(+) *E. coli* expression vector (Novagen, Germany)



pHT08 *E. coli*/*B. subtilis* expression shuttle vector (MoBiTec, Germany)



pHT08-*gerE* *E. coli*/*B. subtilis* expression shuttle vector

B. Compositions and Preparation of Culture Media

LB Broth, Miller

Yeast extract	5 g/L
Peptone from casein	10 g/L
NaCl	10 g/L
Agar (optional)	14 g/L
Glucose (optional)	10 g/L

Dissolve in distilled water, adjust pH to 7.0 using NaOH or HCl.

Complete volume with distilled water, sterilize by autoclaving for 20 minutes at 121°C.

2xYT Medium

Yeast extract	10 g/L
Peptone from casein	16 g/L
NaCl	5 g/L

Dissolve in distilled water, adjust pH to 7.0 using NaOH or HCl.

Complete volume with distilled water, sterilize by autoclaving for 20 minutes at 121°C.

Perry and Abraham (PA) Medium

Potassium Buffer Solution

KH ₂ PO ₄	1 g
KCl	0.2 g

Dissolve in 800 mL distilled water, adjust pH to 7.4 with KOH.

Complete volume to 900 mL with distilled water, sterilize by autoclaving for 20 minutes at 121°C.

Sucrose Solution

Sucrose	10 g
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MgSO ₄ 7H ₂ O	0.5 g
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Dissolve in 80 mL distilled water, sterilize by autoclaving for 20 minutes at 121°C.

Trace Elements Solution

CaCl ₂ 2H ₂ O	0.01 g
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Ammonium molybdate (NH ₄) ₆ Mo ₇ O ₂₄	0.01 g
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MnCl ₂ 2H ₂ O	0.08 g
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ZnSO ₄ 7H ₂ O	0.01 g
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CuSO ₄ 5H ₂ O	0.001 g
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Dissolve in 100 mL distilled water, sterilize using 22 mm cellulose acetate filters.
Store at 4°C.

Glutamate Solution

Monosodium Glutamate H ₂ O	5 g
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Dissolve in 10 mL distilled water, sterilize using 22 mm cellulose acetate filters.
Store at 4°C.

Ferric Citrate Solution

Ferric citrate 0.3 g

Dissolve in 20 mL distilled water, sterilize using 22 mm cellulose acetate filters.

Store at 4°C.

Combine the solutions after cooling to obtain 1 L of PA medium.

Potassium Buffer Solution 900 mL

Sucrose Solution 80 mL

Trace Elements Solution 1 mL

Glutamate Solution 8 mL

Ferric Citrate Solution 10 mL

Supplement with tryptophan, 50 mg/mL for tryptophan auxotrophs.

Tryptophan 0.5 g

Dissolve in 10 mL distilled water, sterilize using 22 mm cellulose acetate filters.

Store at 4°C.

SP Medium

SP Salts

K₂HPO₄ 14 g

KH₂PO₄ 6 g

Trisodium citrate 2H₂O 1.38 g

Dissolve in 1 L distilled water, sterilize by autoclaving for 20 minutes at 121°C.

Glucose Solution (50% w/v)

Glucose 5 g

Dissolve in 10 mL distilled water, sterilize using 22 mm cellulose acetate filters.

Store at 4°C.

Casein Solution (5% w/v)

Peptone from casein 0.5 g

Dissolve in 10 mL distilled water, sterilize using 22 mm cellulose acetate filters.

Store at 4°C.

Tryptophan Solution

Tryptophan 0.05 g

Dissolve in 10 mL distilled water, sterilize using 22 mm cellulose acetate filters.

Store at 4°C.

Ammonium Ferric Citrate Solution

Ammonium Ferric Citrate 0.22 g

Dissolve in 10 mL distilled water, sterilize using 22 mm cellulose acetate filters.

Store at 4°C.

Monosodium Glutamate Solution (40% w/v)

Monosodium Glutamate 3 g

Dissolve in 10 mL distilled water, sterilize using 22 mm cellulose acetate filters.

Store at 4°C.

Magnesium Sulfate Solution

MgSO₄ 7H₂O 1.2 g

Dissolve in 10 mL distilled water, sterilize using 22 mm cellulose acetate filters.
Store at 4°C.

Combine the solutions to obtain 100 mL of SP medium.

SP salts	92.15 mL
Glucose solution	4 mL
Casein solution	2 mL
Tryptophan solution	1 mL
Ammonium ferric citrate solution	50 mL
Monosodium glutamate solution	500 mL
Magnesium sulfate solution	300 mL

Difco Sporulation Medium (DSM)

Nutrient broth	8 g
KCl	1 g
MgSO ₄ 7H ₂ O	0.12 g
Agar	14 g

Dissolve in 800 mL distilled water, adjust pH to 7.6 with NaOH.

Complete volume to 1 L with distilled water, sterilize by autoclaving for 20 minutes at 121°C.

Then, supplement with 1 mL of each of the following trace element solutions after the main medium cools.

1 M Calcium Nitrate Solution:

$\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$ 2.36 g

Dissolve in 10 mL distilled water, sterilize using 22 mm cellulose acetate filters.
Store at 4°C.

10 mM Manganese Chloride 2

$\text{MnCl}_2 \cdot 2\text{H}_2\text{O}$ 0.0161 g

Dissolve in 10 mL distilled water, sterilize using 22 mm cellulose acetate filters.
Store at 4°C.

1 mM Ferrous Sulfate

$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ 0.00278 g

Dissolve in 10 mL distilled water, sterilize using 22 mm cellulose acetate filters.
Store at 4°C.

Bioassay Medium**10x Glucose Solution**

Glucose 1 g

Na_2HPO_4 0.33 g

KH_2PO_4 0.1 g

NaCl 0.1 g

Trisodium citrate $2\text{H}_2\text{O}$ 0.05 g

Dissolve in 100 mL distilled water, adjust pH to 7.1 with NaOH or HCl. Sterilize by autoclaving for 20 minutes at 121°C.

100x Yeast Extract Solution

Yeast extract 0.5 g

Dissolve in 100 mL distilled water, sterilize using 22 mm cellulose acetate filters.
Store at 4°C.

100x Magnesium Sulfate Solution

MgSO₄ 7 g

Dissolve in 100 mL distilled water, sterilize by autoclaving for 20 minutes at 121°C.
Store at 4°C.

100x Ferrous Sulfate Solution

FeSO₄ 0.1 g

Dissolve in 100 mL distilled water, sterilize using 22 mm cellulose acetate filters.
Store at 4°C.

10x Glutamate Solution

Monosodium Glutamate H₂O 2.4 g

Dissolve in 100 mL distilled water, sterilize using 22 mm cellulose acetate filters.
Store at 4°C.

12.5x Amino Acid Solution

Histidine	0.125 g
Leucine	0.125 g
Methionine	0.125 g
Phenylalanine	0.125 g
Proline	0.125 g
Threonine	0.125 g
Tryptophan	0.125 g
Tyrosine	0.125 g
Valine	0.125 g

Dissolve in 400 mL distilled water, sterilize using 22 mm cellulose acetate filters.
Store at 4°C.

Agar Solution

Agar	0.75 g
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Dissolve in 51.75 mL distilled water, sterilize by autoclaving for 20 minutes at 121°C
right before use.

Combine the solutions to obtain 75 mL of bioassay medium.

10x glucose solution	7.5 mL
100x yeast extract solution	0.75 mL
100x magnesium sulfate solution	0.75 mL
100x ferrous sulfate solution	0.75 mL
10x glutamate solution	7.5 mL
12.5x amino acid solution	6 mL
Agar solution	51.75 mL

C. Buffers and Solutions

TE Buffer

Tris-HCl	7.88 g
EDTA	1.46 g

Dissolve in 400 mL distilled water. Adjust pH to 8 with NaOH. Complete volume to 0.5 L with distilled water. Sterilize by autoclaving for 20 minutes at 121°C. Store at 4°C.

10x TAE Buffer

Trizma base	48.4 g
Glacial acetic acid	11.4 mL
EDTA	3.72 g

Dissolve in 800 mL distilled water. Adjust pH to 8.2 with NaOH. Complete volume to 1 L with distilled water.

Buffer 1 (Competent *E. coli* preparation)

CaCl ₂ 2H ₂ O	0.0147 g
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Dissolve in 10 mL distilled water, sterilize using 22 µm cellulose acetate filters. Prepare right before use.

Buffer 2 (Competent *E. coli* preparation)

CaCl ₂ 2H ₂ O	0.022 g
-------------------------------------	---------

Dissolve in 2 mL distilled water, sterilize using 22 µm cellulose acetate filters. Prepare right before use.

LEW Buffer

NaH ₂ PO ₄ H ₂ O	6.89 g
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NaCl	17.5 g
------	--------

Dissolve in 800 mL distilled water. Adjust pH to 8.0 using NaOH. Complete volume to 1 L with distilled water. Store at 4°C.

Elution Buffer

NaH ₂ PO ₄ H ₂ O	6.89 g
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NaCl	17.5 g
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Imidazole	17 g
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Dissolve in 800 mL distilled water. Adjust pH to 8.0 using NaOH. Complete volume to 1 L with distilled water. Store at 4°C.

6x Laemmli Buffer

Trizma base	1.47 g
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SDS	1.5 g
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Glycerol	15 mL
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Bromophenol blue	7.5 mg
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2-mercaptoethanol	2.25 mL
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Complete to 20 mL distilled water, adjust pH to 6.8 with NaOH. Complete to 25 mL with distilled water. Store 1 mL aliquots at -20°C.

Acrylamide/Bisacrylamide (30%)

Acrylamide 290 g

N,N'-methylbisacrylamide 10 g

Dissolve in 600 mL distilled water. Complete volume to 1 L with distilled water.

Filter using filter paper. Store at 4°C in a dark bottle.

1.5 M Tris-Cl pH 8.8

Trizma base 181.5 g

Dissolve in 850 mL distilled water and adjust pH to 8.8 using HCl. Complete volume to 1 L. Store at 4°C.

0.5 M Tris-Cl pH 6.8

Trizma base 60.5 g

Dissolve in 850 mL distilled water and adjust pH to 6.8 using HCl. Complete volume to 1 L. Store at 4°C.

10% SDS Solution

SDS 10 g

Dissolve in 80 mL distilled water and complete volume to 100 mL.

10% APS Solution

APS 1 g

Dissolve in 10 mL distilled water. Store 1 mL aliquots at -20°C.

10x SDS-PAGE Running Buffer

Trizma base	30.3 g
Glycine	144.4 g
SDS	10 g

Dissolve in 800 mL distilled water. Complete volume to 1 L.

Coomassie Blue R-250 Staining Solution

Coomassie blue R-250	1 g
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Dissolve in 300 mL methanol. Add 650 mL distilled water and 50 mL glacial acetic acid.

Destaining Solution

Add 50 mL methanol and 50 mL glacial acetic acid to 400 mL distilled water.

25 mM Tris-Cl pH 7.4

Trizma base	0.303 g
-------------	---------

Dissolve in 80 mL distilled water and adjust pH to 7.4 using HCl. Complete volume to 100 mL using distilled water. Store at 4°C.

Z-Buffer

Na_2HPO_4	5.33 g
NaH_2PO_4	3.12 g
KCl	0.373 g
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	0.123 g

Dissolve in 400 mL distilled water and adjust pH to 7 using HCl and NaOH. Complete volume to 500 mL using distilled water. Add 270 μL of 2-mercaptoethanol per 100 mL of Z-buffer right before use. Store at 4°C.

2.5 g/mL Lysozyme Solution

Lysozyme	25 g
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Dissolve in 10 mL distilled water. Store at -20°C.

10% Triton X-100 Solution

Dilute 10 μL Triton X-100 in 990 μL distilled water

4mg/mL ONPG Solution

ONPG	0.04 g
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Dissolve in 10 mL distilled water. Store at 4°C.

1 M Na_2CO_3 Solution

Na_2CO_3	1.06 g
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Dissolve in 10 mL distilled water. Store at 4°C.

1 M KCl/0.5 M NaCl Solution

KCl	7.45 g
NaCl	2.922 g

Dissolve in 100 mL distilled water. Sterilize by autoclaving at 121°C for 20 minutes.

50 mM Tris-Cl pH 7.2 with 50 mg/mL Lysozyme

Trizma base	0.606 g
-------------	---------

Dissolve in 80 mL distilled water. Adjust to pH 7.2 using NaOH and HCl. Add 5 g lysozyme. Complete to 100 mL using distilled water. Sterilize by autoclaving at 121°C for 20 minutes. Sterilize using 22 µm cellulose acetate filters.

1 M NaCl Solution

NaCl	5.844 g
------	---------

Dissolve in 100 mL distilled water. Sterilize by autoclaving at 121°C for 20 minutes.

0.05% SDS Solution

Dilute 500 µL of 10% SDS in 99.5 mL distilled water. Sterilize using 22 µm cellulose acetate filters.

TEP Buffer

Tris-HCl	0.785 g
EDTA	0.292 g
PMSF	0.0348 g

Dissolve Tris-HCl and EDTA in 80 mL distilled water. Adjust pH to 7.2 using NaOH and HCl. Complete to 100 mL using distilled water, then add PMSF.

Phosphate Buffered Saline (PBS)

Na ₂ HPO ₄	1.44 g
KCl	0.2 g
KH ₂ PO ₄	0.24 g

Dissolve in 800 mL distilled water and adjust to pH 7.6 using NaOH or HCl. Complete to 1 L using distilled water. Sterilize by autoclaving for 20 minutes at 121°C. Store at 4°C.

250 µg/mL Lysozyme Solution

Lysozyme	2.5 mg
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Dissolve in 10 mL distilled water. sterilize using 22 µm cellulose acetate filters. Store at -20°C.

10 mM Tris-Cl pH 8.4

Tris-HCl	0.157 g
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Dissolve in 80 mL distilled water and adjust to pH 8.4 using NaOH. Complete to 100 mL using distilled water. Sterilize by autoclaving for 20 minutes at 121°C. Store at 4°C.

Acrylamide/Bisacrylamide 40%

Acrylamide	190 g
N,N'-methylbisacrylamide	10 g

Dissolve in 300 mL distilled water. Complete volume to 500 mL with distilled water. Filter using filter paper. Store at 4°C in a dark bottle.

10x EMSA Loading Dye

Tris-HCl	0.4 g
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Bromophenol blue	0.02 g
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Dissolve in 4 mL distilled water and adjust pH to 7.5. Complete to 6 mL using distilled water and add 4 mL glycerol. Store 1 mL aliquots at -20°C.

10x TBE Buffer

Trizma base	108 g
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Boric acid	55 g
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EDTA	3.92 g
------	--------

Dissolve in 800 mL distilled water. Adjust to pH 8.3 using glacial acetic acid. Complete volume to 1 L.

5x Binding Buffer (NaCl)

Tris-HCl	1.575 g
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NaCl	0.292 g
------	---------

EDTA	0.185 g
------	---------

Dissolve in 20 mL distilled water and adjust pH to 7.4 using NaOH. Complete to 40 mL using distilled water and add 60 mL glycerol for a total of 100 mL. Store at 4°C.

5x Binding Buffer (KCl)

HEPES	1.43 g
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Tris-HCl	0.315 g
----------	---------

KCl	2.236 g
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EDTA	0.3722 g
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Dissolve in 20 mL distilled water and adjust pH to 8 using NaOH. Complete to 40 mL using distilled water and add 60 mL glycerol for a total of 100 mL. Store at 4°C.



D. Chemicals, Enzymes and Kits Buffers and Solutions

Chemicals:

Acrylamide: Sigma

Agar-agar: Merck

Agarose: Sigma

Ammonium Ferric Citrate: Sigma

Ammonium persulfate (APS): BioRad

Ampicillin Sodium Salt: Applichem

Boric Acid: Merck

Bovine Serum Albumin: Sigma

Bromophenol Blue: Merck

CaCl₂ 2H₂O: Merck

Ca(NO₃)₂ 4H₂O: Merck

Chloramphenicol: Sigma

Chloroform: Merck

Coomassie Blue R-250: Fluka

CuSO₄ 5H₂O: Merck

dNTPs: ThermoScientific

EDTA: Sigma

Ethanol: Merck

Ethidium Bromide: Sigma

Ferric Citrate: Sigma

FeSO₄ 7H₂O: Merck

Glacial Acetic Acid: Merck

Glucose: Merck

Glycerol: Merck

Glycine: Merck

HCl: Merck

HEPES: Sigma

Histidine: Merck

IPTG: Cayman

Kanamycin Sulphate: Sigma

KCl: Sigma

K₂HPO₄: Merck

KH₂PO₄: Merck

KOH: Merck

Leucine: Merck

Methanol: Merck

Methionine: Merck

Monosodium Glutamate H₂O: Merck

MnCl₂ 2H₂O: Merck

MgSO₄ 7H₂O: Sigma

N,N'-methylbisarcylamide: Sigma

Na₂CO₃: Merck

Na₂HPO₄: Merck

NaH₂PO₄ H₂O: Merck

NaCl: Merck

NaHCO₃: Merck

NaOH: Merck

ONPG: Sigma

Peptone From Casein: Merck

Phenylalanine: Merck

PMSF: Sigma

Proline: Merck

SDS: Merck

Skim Milk Powder: Sigma

Sucrose: Merck

TEMED: BioRad

Threonine: Merck

Trisodium Citrate 2H₂O: Merck

Tris-HCl: AppliChem

Triton X-100: Sigma

Trizma base: Sigma

Tryptophan: Merck

Tween-20: Merck

Tyrosine: Merck

Valine: Merck

Yeast Extract: Merck

ZnSO₄ 7H₂O: Sigma

2-mercaptoethanol: Merck

Enzymes:

BamHI-HF: NEB

HinDIII-HF: NEB

Lysozyme: Applichem

OneTaq DNA Polymerase: NEB

Proteinase K: MACHERY-NAGEL

RNase A: EcoTech

T4 DNA Ligase: NEB

XbaI: NEB

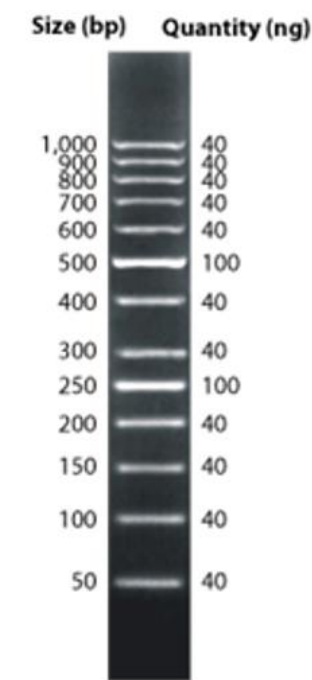
Kits:

GeneJET Gel Extraction Kit: ThermoScientific

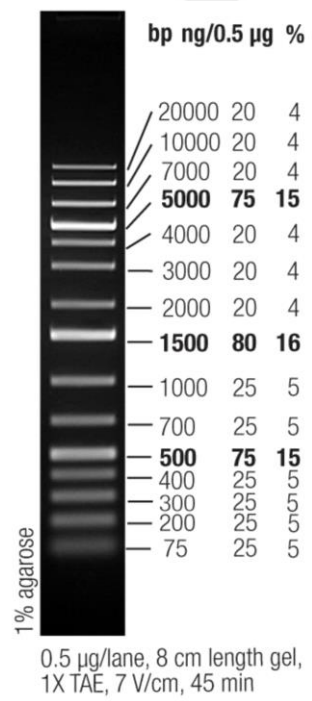
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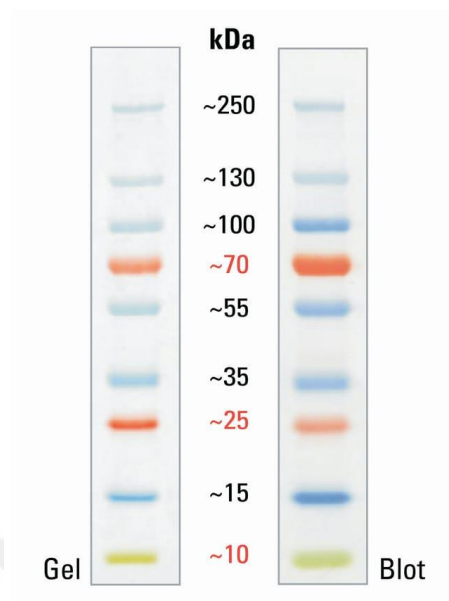
Markers:



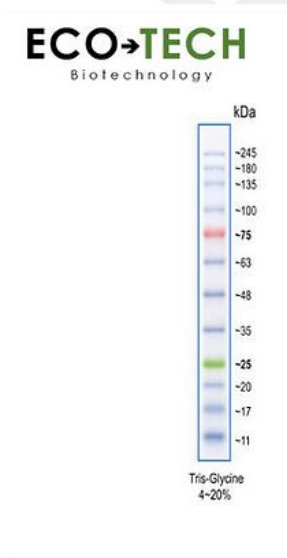
SiZer-50 plus DNA ladder



GeneRuler 1kb Plus DNA ladder



PageRuler plus prestained protein ladder



EcoTech ClearBand prestained protein marker