# PHENOTYPIC RESCUE OF A BACILYSIN-SILENCED STRAIN OF *BACILLUS SUBTILIS* WITH BACILYSIN SUPPLEMENTATION

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**PHENOTYPIC RESCUE OF A BACILYSIN-SILENCED STRAIN OF *BACILLUS SUBTILIS* WITH BACILYSIN SUPPLEMENTATION**

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## ABSTRACT

### PHENOTYPIC RESCUE OF A BACILYSIN-SILENCED STRAIN OF *BACILLUS SUBTILIS* WITH BACILYSIN SUPPLEMENTATION

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*Bacillus subtilis*, the model organism for the gram positive bacteria, is distinguished for its secondary metabolite synthesis. Remarkably, most of these metabolites are small peptides which are generally synthesized non-ribosomally with multifunctional enzyme complexes. Bacilysin, a dipeptide composed of L-alanine and L-anticapsin, is the smallest peptide antibiotic which is produced by several enzymatical steps, however, its biosynthesis does not fully fit the general mechanisms of NRPSs. This small molecule acts specifically as an glucosamine-6-phosphate synthase inhibitor, thus interfering with cell wall formation in target microbes. Previously, it was found that the synthesis of bacilysin is governed by *bacA* operon and regulation of its synthesis is related with sporulation and quorum-sensing pathways. However, the effect of this dipeptide on its producer is obscure. To elucidate this effect, our research group constructed a bacilysin non-producer strain of *B. subtilis* named as OGU1. By the use of proteomic methods of 2DE MALDI-TOF MS/MS and nanoLC-MS/MS the differentially expressed proteins of OGU1 in comparison to parental strain PY79 was identified. The number of differentialy expressed proteins was rather high, being generaly underrepresented, and these proteins were mostly related with sporulation and germination pathways.

In the present study, the results of comparative proteomics were validated with RT-qPCR analysis of selected 20 genes. Certain phenotypic deficiencies/inadequacies regarding spore pigmentation, lysozyme and chloroform resistance of spores, spore coat protein profile as well as spore germination kinetics were next observed in OGU1. Protein-free and bacilysin containing culture broth concentrate supplementation to growing OGU1 cultures partially or entirely rescued all of these deficient phenotypes, indicating a pleiotropic role of bacilysin on sporulation and germination of its producer.

Keywords: *B. subtilis*, bacilysin, sporulation, germination, chemical complementation, RT-qPCR

# ÖZ

## BASİLİSİN ÜRETİMİ SUSTURULMUŞ BİR *BACILLUS SUBTILIS* SUŞUNDAKİ FENOTİPİK FARKLILAŞMANIN BASİLİSİN İLE KURTARILMASI

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Gram pozitif bakteriler için model organizma olarak kullanılan *B.subtilis*, çok sayıda antimetabolik ve farmakolojik aktiviteye sahip ikincil metabolit üretmektedir. Bu metabolitlerin büyük çoğunluğunu küçük peptidler oluşturmaktadır. Bu peptidler, genellikle ribozomal olmayan bir şekilde, peptid sentetaz isimli enzim kompleksleri tarafından sentezlenmektedir. Basilisin dipeptidi, enzimatik olarak sentezlenen biyoaktif peptidlerden biri olup L-alanine ve L-anticapsin'den oluşmakta ve *B. subtilis*'in bazı suşları tarafından sentezlenip, hücre dışına salgılanmaktadır. Basilisin dipeptidinin üretiminde rol alan proteinlerin *bacA* operonu tarafından sentezlendiği 2000'li yılların başında rapor edilmiştir. Bu molekülün üretiminin regülasyonu tam olarak kavranamamış olmakla birlikte, regülasyonun çoğunluğu algılama ve sporülasyon yolakları ile ilişkili olduğu bilinmektedir. Basilisin molekülünün hedef organizmalar üzerindeki etki mekanizması iyi bilinirken, kendi üreticisi üzerindeki olası etkileri hakkında bilinenler oldukça sınırlıdır. Bu etkileri araştırma amacıyla, grubumuz tarafından öncelikle basilisin üretemeyen OGU1 suşu oluşturulmuş, daha sonra ise ana suş PY79 ve onun basilisin üretemeyen türevi arasında arasında 2DE ve nanoLC-MS/MS yaklaşımlarını içeren karşılaştırmalı proteom analizleri yapılmıştı. Yapılan analizler sonucunda, basilisin üretemeyen

mutant suşta etkilenen protein sayısının oldukça fazla olduğu, bu proteinlerin çoğunun miktar olarak azalmış olduğu ve yine çoğunun sporülasyon ve jerminasyonda görev aldığı rapor edilmişti. Proteom analizlerinin sonuçları, şimdiki araştırmada 20 gen için gerçekleştirilen gerçek zamanlı kantitatif PCR yöntemi ile doğrulanmıştır. OGU1'in spor pigmentasyonu, sporların lizozim ve kloroform direnci, spor kılıfı protein profilleri ve jerminasyon kinetiği gibi fenotipik karakterlerinde ana suşa göre yetersizlikler belirlenmiştir. Ardından, basilisin molekülünü içeren, ancak protein içeriği uzaklaştırılmış konsantre kültür sıvısı OGU1 kültürlerine ilave edilerek fenotipik kurtarma çalışmaları yapılmıştır. Çalışmalarımız, basilisin üretemeyen OGU1 suşundaki fenotipik değişimlerin doğrudan basilisin yokluğundan kaynaklandığını ve basilisin'in üretici organizmanın sporülasyon ve jerminasyonu üzerindeki pleotrofik etkilerini doğrulamıştır.

Anahtar Kelimeler: *B. subtilis*, basilisin, sporülasyon, germinasyon, kimyasal komplementasyon, RT-qPCR

To my beloved family,

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| ABBREVIATIONS | |
|---|---|
| 2DE | : Two-Dimensional Gel Electrophoresis |
| BGSC | : Bacillus Genomic Stock Center |
| bp(s) | : Base pair(s) |
| Da(s) | : Dalton(s) |
| DPA | : Dipicolinic Acid |
| EDTA | : Ethylenediaminetetraacetic acid |
| kb | : Kilobase |
| nanoLC-MS/MS | : nanoLiquid Chromatography-Mass Spectrometry |
| NRPS | : Nonribosomal Peptide Synthetases |
| SASP | : Small Acid-Soluble Protein |
| SDS-PAGE Electrophoresis | : Sodium Dodecyl Sulphate-Polyacrylamide Gel |
| WGRS | : Whole Genome Resequencing |
| RT-qPCR | : Real Time – quantitative Polymerase Chain Reaction |
| IPTG | : Isopropyl β- d-1-thiogalactopyra |

# CHAPTER 1

# INTRODUCTION

## 1.1 *Bacillus subtilis*

### 1.1.1 *Bacillus subtilis* as a Model Organism

*Bacillus subtilis*, as an essential member of the genus *Bacillus* was first described by German naturalist Christian G. Ehrenberg in 1835 as "*Vibrio subtilis*". In 1872, it was renamed as *Bacillus subtilis* by Ferdinand Cohn and classified as a member of the family *Bacillaceae* (Gordon, 1981). Like other *Bacillus* species, it is a Gram-positive, rod-shaped motile bacterium which can produce endospores that are resistant to several external stress. Although the genus *Bacillus* has infamous pathogens like *B. anthracis* (causing anthrax) or *B. cereus* (causing food poisoning), *Bacillus subtilis* have no pathogenic potential. This microorganism also has a natural transformation system for which it was used widely for genetic manipulation and analysis before recombinant DNA technology era (Harwood, 1992). In fact, it was the first non-pathogenic microorganism to be transformed (Anagnostopoulos and Spizizen, 1961). Knowing that it could be manipulated genetically with ease, *Bacillus subtilis* is extensively used in several research areas, including differentiation, metabolic pathways, quorum sensing, and, most importantly, sporulation. While *Escherichia coli* is the best-studied model bacterium for Gram-negatives, *B. subtilis* is the model organism for Gram-positive bacteria as it is the most perfect representative.

This microorganism is also a well-known cellular factory as it can produce a variety of secondary metabolites, antibiotics, and industrial enzymes (Sonenshein *et al.*, 2002). For instance, exopolysaccharides secreted by probiotic strains of *B. subtilis* could provide protection from intestinal inflammation. (Jones *et al.*, 2014). The most common bacterial host used for production of heterologous proteins has been *E. coli*. Nevertheless, the formation of inclusion bodies due to aggregation of proteins and the presence of pyrogenic lipopolysaccharide (LPS) are the common problems for protein production in *E. coli*. On the other hand, *B. subtilis* secretes cloned gene products into culture supernatant in high amounts (Zweers *et al.*, 2008). Recently, *B. subtilis* was engineered as a versatile and stable platform for the production of nanobodies with its capability for protein secretion into culture and resistance to extreme conditions (Yang *et al.*, 2020). This study, as well as many other examples of protein production, points to the biotechnological importance of *B. subtilis* as a recombinant host.

*B. subtilis* is considered as a soil organism mostly associated with plants. Yet, it can be found in diverse environments like water sources, and the gut of various animals, including humans (Nicholson, 2002). In fact, it is considered as one of the important probiotics for humans (Hong *et al.*, 2005). *B. subtilis* oxidizes organic compounds for its growth, and is classified as a chemoorganotroph. Like many other members of the genus *Bacillus*, *B. subtilis* is mesophilic and could form optimal-sized colonies after overnight incubation at 37°C with the necessary nutrients and aeration. Although it is thought that *B. subtilis* is a strict aerobe for many years, it can also grow under anaerobic conditions (Nakano and Zuber, 1998). This is possible with the usage of nitrate or nitrite as the electron acceptor, or by fermentation. This shift in its metabolism is due to the activity of ResDE two- component signal transduction system, which will be mentioned later in detail. Briefly at this point, this two-component signal transduction system enables the expression of FNR protein, which then induces anaerobic respiration genes (Nakano and Zuber, 1998).

*B. subtilis* is a microbe that is capable of utilizing different strategies to survive in its competitive habitat. That is, when the availability of nutrients becomes limited, this bacterium searches alternative ways to survive. Induction of motility and chemotaxis, production of several hydrolases and antibiotics are just examples of these alternative ways. The ultimate survival strategy, however, is the transition into a dormant stage or induction of sporulation. This sporulation process begins with the compartmentalization of a single *B. subtilis* cell into mother and forespore cells, each carrying a copy of the bacterial chromosome. Differential gene expression and regulation in these two different compartments eventually leads to the engulfment of the forespore and finally programmed death of the mother cell. The end product of this complex process is the formation of endospore that is resistant to several sources of stress, including heat, irradiation, chemicals, and dryness (Stragier and Losick, 1996).

To understand the genomic content of this model organism better, its whole genome was analyzed by a group of scientists which was published in 1997 as a total of 4.2 Mb (4,214,810 base pairs) in size (Kunst *et al.*, 1997). The following studies showed that this genome contains 4106 protein-coding genes along with 30 rRNA, 86 tRNA (Kobayashi *et al*., 2003), and also three other small RNA genes (Ando *et al.*, 2002). Although its genome contains almost 4200 genes in total, it was shown that only 275 of them are essential for growth under optimal experimental conditions. Along with the genes mentioned above, the genome also contains approximately 250 transcriptional regulators and 17 sigma factors (Kobayashi *et al.*, 2003)

*B. subtilis* 168 is the strain that has been extensively used for genetic studies, and it is originated from *B. subtilis* Marburg with sublethal X-ray mutagenesis (Zeigler *et al.*, 2008). This strain is a tryptophan auxotroph, and it is considered as the equivalent of the famous K-12 of *E. coli* (Harwood, 1992). Another *B. subtilis* strain widely

used by the workers of the field, PY79, is a prototrophic derivative of the *B. subtilis* 168 (Youngman *et al.*, 1984).

### 1.1.2 Global Regulation of the Gene Expression in *Bacillus subtilis*

Regulation of several essential processes like antibiotic production, virulence, development, and biofilm formation in bacteria mostly occurs through the cell to cell signaling (Dunny and Winans, 1999). Quorum sensing is one and may be the most crucial example of the cell to cell signaling, and is utilized as a sensor of cell density. It is accomplished by secretion of quorum sensing molecules that are proportional to cell density, and when these molecules reach a threshold concentration, they are sensed by their producers to alter the gene expression (Fuqua *et al.*, 1994). Quorum sensing molecules and pathways differ from one microorganism to another, yet it is possible to categorize quorum sensing into two classes. The first one is the LuxI/LuxR system commonly observed in Gram-negative bacteria, in which bacteria utilize either type I autoinducers or N-acyl-L-homoserine lactone autoinducers as quorum sensing signals (Fuqua *et al.*, 1996; Fuqua and Greenberg, 1998). Gram-positive bacteria, on the other hand, utilize small peptides, also named as autoinducing peptides that often target two-component systems (Dunny and Leonard, 1997).

Peptide-based quorum sensing in Gram-positive bacteria involves at least one element of two-component systems, which are crucial for signal transduction in prokaryotic organisms. These systems contain two components, as the name implies. The first component is a histidine protein kinase, which acts as a sensor to specific signals and activates itself upon binding it by phosphorylation on its histidine residue. The second component, response regulator, is linked to the histidine kinase and activated by the phosphoryl group's translocation. Once the response regulator

is activated, it is able to regulate gene expression, which promotes the adaptation of cells to environmental signals (Stock *et al.*, 2000).

Several crucial processes like the initiation of competence (Tortosa and Dubnau, 1999), sporulation, production of secondary metabolites and antibiotics (Solomon *et al.*, 1996; Martín, 2004; Comella and Grossman, 2005) as well as the cell division (Fukuchi *et al.*, 2000) is controlled with peptide-based quorum sensing in *B. subtilis*. There are three types of signal peptides identified in *B. subtilis*. Of these, ComX is a 5 to 10 amino-acid peptide that is modified from a 55 amino acid precursor, and it acts on the cell extracellularly by interacting its receptor (Magnuson *et al.*, 1994). Another group of peptides is known as Phr peptides, which are unmodified pentapeptides, and they act on their target Rap proteins by inhibiting them after internalization into the cell (Lazazzera, 2001; Perego and Brannigan, 2001). The final group contains lantibiotic peptides like subtilin, which have both antibiotic activity and signaling activity (Kleerebezem and Quadri, 2001).

*Bacillus subtilis* is known to encode eight Phr peptides (PhrA, and PhrC to PhrK) and also eleven Rap proteins (RapA to RapK) (Lazazzera, 2001). Every Phr peptide is encoded with its corresponding Rap protein in the same operon, and mature Phr peptides have an inhibitory effect on their corresponding Rap proteins (Ogura *et al.*, 2003; Hayashi *et al.*, 2006). Competence and sporulation stimulating factor (CSF) is also known as PhrC peptide, and it has an additional inhibitory role on yet another Rap protein, RapB (Perego, 1997). There might be some other Phr peptides inhibiting Rap proteins other than their pairs (Auchtung *et al.*,2006).

After encoded as pre-Phr peptides, Phr peptides are exported and processed into their forms containing only five amino acids (Lazazzera, 2001). These small peptides are then imported into the cell with an ATP-binding cassette (ABC) transporter, known

as the oligopeptide permease (Opp) (Perego *et al.*, 1991). Imported Phr peptides then can inhibit Rap proteins' activities, which mainly includes dephosphorylation of Spo0F-P (a component of the phosphorelay controlling sporulation), and the inhibition of ComA response regulator's DNA binding to elements related to competence development (Perego, 2013). The relationship between Phr-Rap pairs and cellular processes of sporulation and competence is well depicted in Figure 1.1.

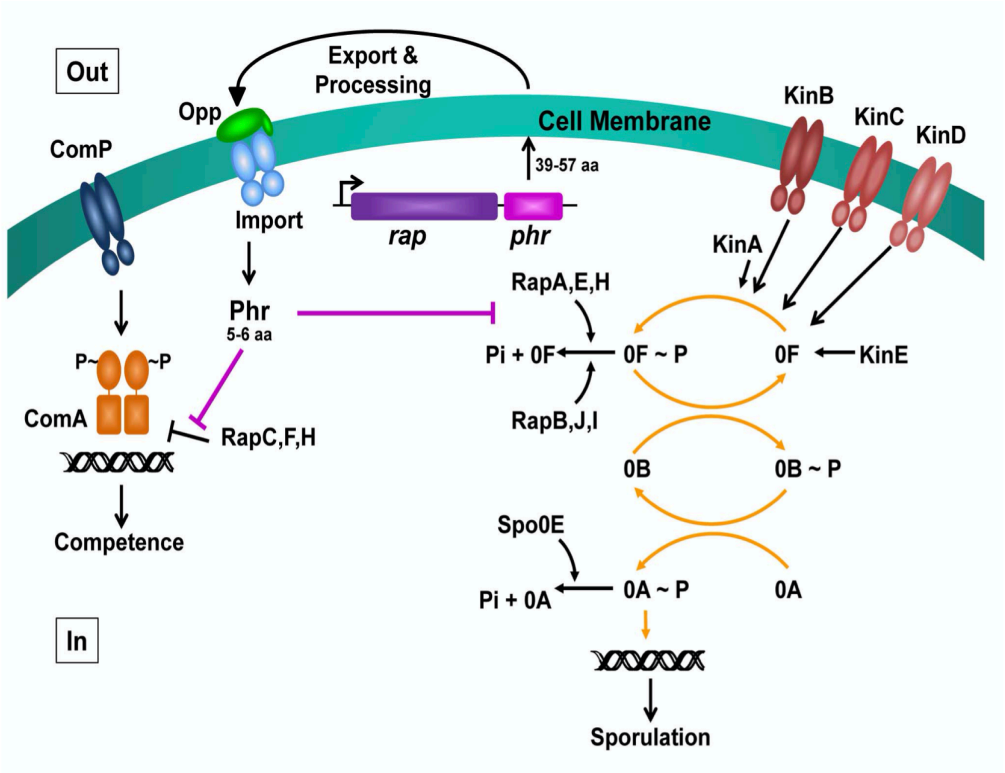


**Figure 1.1.** *B. subtilis* signaling pathways regulated by Rap-Phr interaction (Perego, 2013). RapA,B,E,H,I,J proteins dephosphorylate Spo0F-P, which is an intermediate in pathway ending with sporulation, and these Rap proteins are inhibited by corresponding Phr peptides, unblocking the sporulation process. RapC,F,H proteins inhibit the binding of the activated ComA response regulator, which halts the expression of competence related genes. Phr peptides inhibit these Rap proteins, which leads to competence development.

As Phr peptides regulate important processes of competence and sporulation, the regulation of their own expression is also very crucial for the cell. The expression of *phr* genes is commonly associated with promoters that are recognized by RNA polymerase containing alternative sigma factor $\sigma^H$, which directs transition from exponential phase to stationary phase (McQuade *et al.*, 2001; Britton *et al.*, 2002). Thus, levels of *phr* transcripts increase with the transition to the stationary phase.

Among the Phr peptide family, PhrC is the most studied peptide, and it has regulatory roles on both competence and sporulation pathways. Therefore, it is also known as competence and sporulation stimulating factor or CSF. Like other Phr peptides, CSF is first encoded as a precursor containing forty amino acids, which will be cleaved into much smaller form and exported from the cell. Mature PhrC or CSF peptide contains only five amino acids, ERGMT, and is later imported into the *B. subtilis* cells with a transporter named oligopeptide permease (Opp), which is initially known as Spo0K (Solomon *et al.*, 1996).

After its import, CSF regulates the functioning of the ComA response regulator, which controls the expression of competence related genes. This regulation occurs in a concentration-dependent manner. At relatively low extracellular concentrations, CSF inhibits its cognate RapC protein, a phosphatase that inactivates ComA (Solomon *et al.*, 1996). However, with the increased extracellular concentration, CSF can indirectly inhibit the activity of ComA by interacting and inhibiting ComP histidine kinase (Lazazzera *et al.*, 1997; Lazazzera and Grossman, 1998). CSF is also shown to inhibit ComS in the stationary phase, which results in reduced competence gene expression (Mirel *et al.*, 2000). CSF at low concentrations (at earlier stages of bacterial growth) stimulates the competence gene expression, but inhibits the same gene expression when its concentration (cell density) becomes high enough.

As mentioned before, CSF can also inhibit another Rap protein other than its cognate RapC. This Rap protein, RapB, is a phosphatase that can dephosphorylate Spo0F-P (Perego *et al.*, 1994). Spo0F is an important part of the phosphorelay that leads to the activation of Spo0A, which is an essential signal for sporulation initiation (Grossman, 1995) (Fig. 1.1). The direct inhibition of RapB or stimulation of sporulation occurs when CSF reaches high concentrations (Perego, 1997). In summary, as a peptide-quorum signal, CSF first stimulates competence by mid-exponential phase, and when cells enter the stationary phase it inhibits competence gene expression and enhances sporulation with its increased concentration (Fig. 1.2).

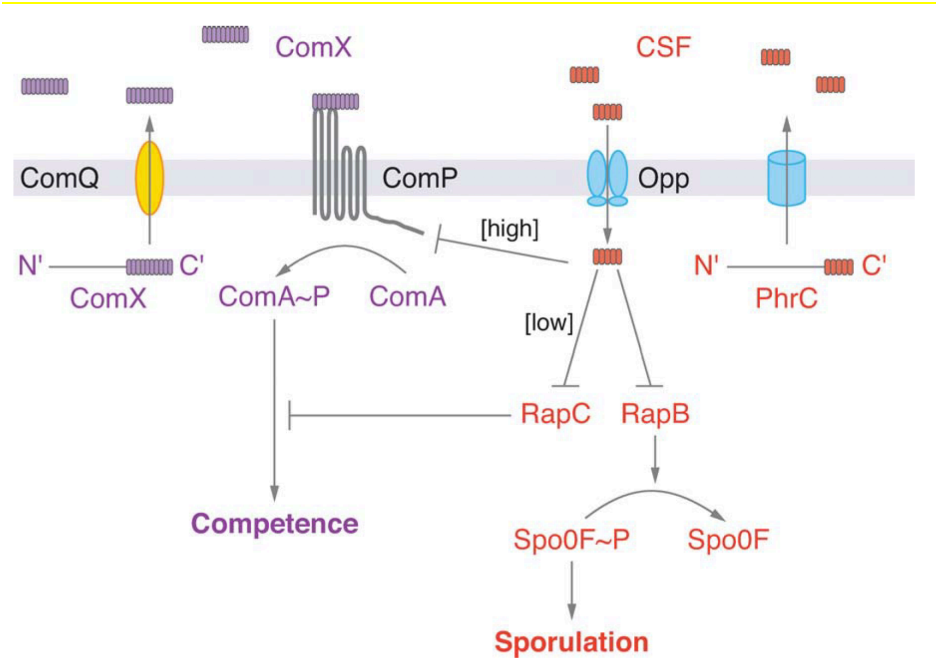


**Figure 1.2.** Regulation of competence and sporulation pathways by two different quorum sensing peptides, CSF (PhrC) and ComX (Waters and Bassler, 2005). CSF is depicted as orange, and ComX is depicted as purple.

ComX is the other famous pheromone that regulates the development of competence. The expression of both *comX* and *comQ* genes is required for the generation of this pheromone peptide (Magnuson *et al.*, 1994). *comX* encodes the precursor molecule containing 55 amino acids, yet the 3' end of this gene is highly polymorphic among the genus *Bacillus*, which results in the production of strain-specific ComX molecules containing 5-10 amino acids (Tortosa *et al.*, 2001). However, these ComX molecules share a common tryptophan residue, which is then modified by isoprenylation, increasing the hydrophobicity of the pheromone (Ansaldi *et al.*, 2002). The *comQ* gene is located upstream of the *comX* gene, and its null mutants halt the production of mature ComX (Magnuson *et al.*, 1994). Furthermore, it has putative isoprenoid domain essential for functioning, which suggests that ComQ protein is involved in the isoprenylation process (Schneider *et al.*, 2002; Okada *et al.*, 2005). Mature ComX pheromone is the ligand for the receptor kinase ComP and leads activation of this receptor upon reaching a critical concentration in the extracellular medium. This activation ends up with phosphorylation of the ComA response regulator, which enhances the expression of genes like *comS* (*srfA*) that takes a role in the development of genetic competence (Core and Perego, 2003) (Fig. 1.2).

There are at least 36 histidine kinases and 36 response regulators in *Bacillus subtilis* genome, including important pairs of CheA-CheY (chemotaxis) (Rosario *et al.*, 1994), ResD-ResE (anaerobic gene activation) (Nakano *et al.*, 1996), ComP-ComA (competence) (Grossman, 1995), DegS-DegU (degradative enzyme production) (Dartois *et al.*, 1998) and PhoR-PhoP (phosphate regulation) (Sun *et al.*, 1996) (Fabret *et al.*, 1999). Nevertheless, the most crucial regulatory pathway in *B. subtilis* is the sporulation phosphorelay, which involves five histidine kinases (KinA, KinB, KinC, KinD, and KinE) and two important response regulators of Spo0B and Spo0F (Perego and Hoch, 2002). An overview of this phosphorelay is given in Figure 1.3.

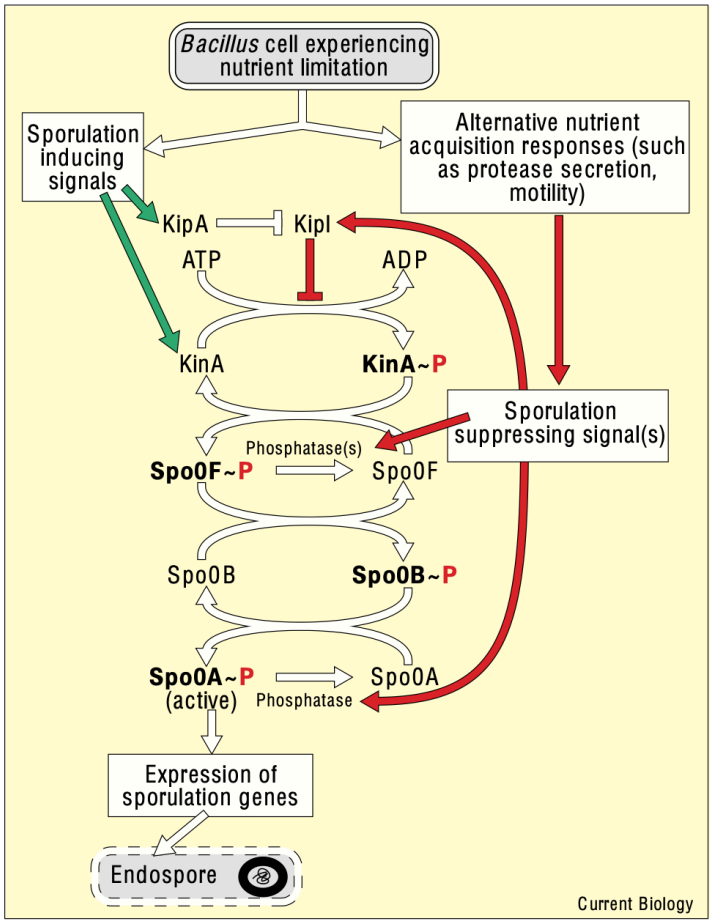


**Figure 1.3.** Sporulation phosphorelay in *B. subtilis* (Stephens, 1998). Green arrows indicate sporulation activating signals, and red arrows pointing inhibitory signals for sporulation.

Sporulation phosphorelay starts with starvation signals, which are sensed by sensory kinases. Of these kinases, KinA (also the most abundant one) and KinE are cytoplasmic, and other kinases are membrane-bound (Jiang *et al.*, 2000). Under proper conditions, this signaling pathway ends up with the activation of Spo0A, which takes a critical role in commitment to sporulation (Sonenshein, 2000). As shown in Figure 1.3, sporulation phosphorelay can be inhibited by proteins that control histidine kinases like KipI, or Rap proteins that can dephosphorylate Spo0F (Stephens, 1998). With this additional layer of regulation, sporulation occurs if and only if the conditions are suitable for a while.

Once Spo0A is activated through phosphorylation, it triggers the production of additional Spo0A proteins that will be activated, which produces an auto-stimulatory loop (Fujita and Sadaie, 1998). The transcription of *spo0A* is also regulated with a promoter that is recognized by $\sigma^H$ (Predich *et al.*, 1992). $\sigma^H$ also regulates the expression of *kinA* and *spo0F*, which are essential elements of the phosphorelay. Additionally, this sigma factor plays a role in the production of Phr peptides; therefore, it can prevent the inhibition of phosphorelay signaling. Finally, $\sigma^H$ enhances the transcription of spoIIA operon, which will encode $\sigma^F$, sporulation specific sigma factor.

In its active form, Spo0A regulates the gene expression by interacting with OA-box, the specific DNA element (Strauch *et al.*, 1990). The target genes are not only related to the sporulation process, but Spo0A also regulates many stationary phase genes. In fact, it controls the expression of a total of 520 genes, which shows the importance of this protein in the global regulation of gene expression (Liu *et al.*, 2003). From these 520 genes, 121 of them directly regulated by Spo0A, and they are mostly transcriptional regulators (Molle *et al.*, 2003).

One such transcriptional regulator is AbrB, which normally prevents the expression of crucial stationary phase genes including, *sigH*, *kinA,* and thus keeps Spo0A inactivated (Strauch, 1995). Active Spo0A negatively regulates the expression of AbrB, which enables sigH and kinA production and therefore Spo0A activation (Veening *et al.*, 2005)

### 1.1.3 Biologically Active Peptides Produced by *Bacillus subtilis*

Biologically active peptides, ranging from simple dipeptides to complicated oligopeptides having cyclic structures that are often modified, are found in various sources, including fungi, bacteria, plants, and animals. The structural diversity of these peptides enables them to have different functions in cellular physiology (Gill *et al.*, 1996). Peptide antibiotics are maybe the most important biologically active peptides among the microbial peptides known, as they can be utilized as antitumor agents, antimicrobials, immunosuppressors, and cytostatic drugs as well as promoters of seed germination and animal growth (Demain, 1980; Boman, 1995; Boman, 1996; Gill *et al.*, 1996).

As mentioned before, *B. subtilis* is capable of producing antibiotics, including a large number of peptide antibiotics. These peptide antibiotics are mostly resistant to hydrolysis by proteases and peptidases due to their rigid, hydrophobic structure and the presence of uncommon D- form of amino acids. Also, they are insensitive to oxidation as they contain already oxidized cysteine residues and/or thioether linkages (Katz and Demain, 1977). One route to the production of these peptides is the ribosomal synthesis followed by processing and post-translational modification (Zuber *et al.*, 1993). One such post-translational modification is the formation of lanthionine (nonproteinogenic amino acid formed by a cysteine and serine residue). Lanthionine-containing antibiotics are called as lantibiotics which are common to *B. subtilis* (Schnell *et al.*, 1988). Another way of peptide antibiotic production is the non-ribosomal synthesis mediated by specific enzymes, which will be mentioned in the following section (Weber and Marahiel, 2001).

Like other biologically active peptides, peptide antibiotics produced by *B. subtilis* has other functions other than being antimicrobial agents (Stein, 2005). Lantibiotics acting as pheromones or killing factors is just one example of this fact. The functions

and structures of non-ribosomal produced peptide antibiotics, and lantibiotics of *B. subtilis* are listed in Table 1.1 and Table 1.2.

**Table 1.1.** The functions and structures of non-ribosomal produced peptide antibiotics of *B. subtilis*, adapted from Stein, 2005.

| Peptide antibiotic | Function | Structure |
|---|---|---|
| Bacillibactin | Siderophore | Cyclic |
| Bacillomycin | Hemolytic activity | Cyclic lipopeptide |
| Bacilysin | Probable pleiotropic molecule (Ertekin *et al.*, 2020) | Linear |
| Fengycin | Acts on filamentous fungi | Cyclic |
| Iturin | Hemolytic activity | Cyclic lipopeptide |
| Mycosubtilin | Hemolytic activity | Cyclic lipopeptide |
| Surfactin | Acts like detergent on cellular membranes | Cyclic |

**Table 1.2.** The functions and structures of lantibiotics of *B. subtilis*, adapted from Stein *et al.*, 2005.

| Peptide antibiotic | Function | Structure |
|---|---|---|
| Ericin | Acts like pheromone | Cyclic |
| Mersacidin | Cell wall synthesis inhibition | Globular |
| Subtilin | Acts like pheromone | Cyclic |
| Subtilosin A | Acts on some Gram (+) bacteria | Cyclic |

### 1.1.4 Nonribosomal Peptide Synthesis

Peptide biosynthesis is fundamentally explained by the ribosomal synthesis; however, it is not the only mechanism. One common alternative is a template-directed nonribosomal mechanism which does not utilize nucleic acids and named as nonribosomal peptide synthesis (Süssmuth and Mainz, 2017). In this system, peptide biosynthesis occurs with megaenzymes, which are named as nonribosomal peptide synthetases (NRPSs) (Weber and Marahiel, 2001). Important antibiotics like penicillin, vancomycin as well as immunosuppressive cyclosporin A and some antitumor, antiviral, biosurfactant agents are synthesized through this alternative pathway (Marahiel *et al.*, 1997; Mootz and Marahiel, 1999; Schwarzer *et al.*, 2002; Sieber *et al.*, 2002).

In contrast to ribosomal synthesis, this system could utilize building blocks other than common 20 amino acids. That situation ends up with a massive structural diversity of peptide products composed of cyclic, branched as well as linear peptide chains with uniquely modified residues (Grünewald and Marahiel, 2006). Although this system has such a diverse set of substrates, the logic behind the peptide bond formation is common and straightforward. As the first step, substrates are converted into acyl adenylate with ATP hydrolysis, which takes place on NRPSs. Formed unstable intermediate is then translocated to another part of the enzyme, which followed by the formation of thioester linkage to enzyme-bound phosphopantetheinyl (4'-PP) cofactor. Then, this tethered amino acid is translocated to another domain where condensation takes place. Finally, mature oligopeptide is released from the machinery with the aid of the thioesterase domain of NRPSs (Marahiel *et al.*, 1997; Grünewald and Marahiel, 2006; Süssmuth and Mainz, 2017).

Nonribosomal peptide synthetases (NRPSs) are composed of repetitive units that are named as modules, and each module is responsible for incorporating one amino acid

with the system described above. Thus, the number of these modules matches the number of amino acids to be incorporated, and the number of modules in NRPS ranges from 2 to 48 (Marahiel *et al.*, 1997; Kallow *et al.*, 2002).

These modules can be further divided into domains, each responsible for unique function. Although there are a variety of different domains like cyclization (Cy), formylation (F) ketoacly reductase (KR), monooxygenase (MOx), and oxidase (Ox) domains, three domains are essential for a module (Süssmuth and Mainz, 2017). These domains are: substrate adenylation domain (A domain), PCP domain which acts as a carrier and responsible for covalent binding of adenylated amino acid, and, finally the condensation domain (C domain) where peptide-bond formation takes place (Fig. 1.4) (Weber and Marahiel, 2001).

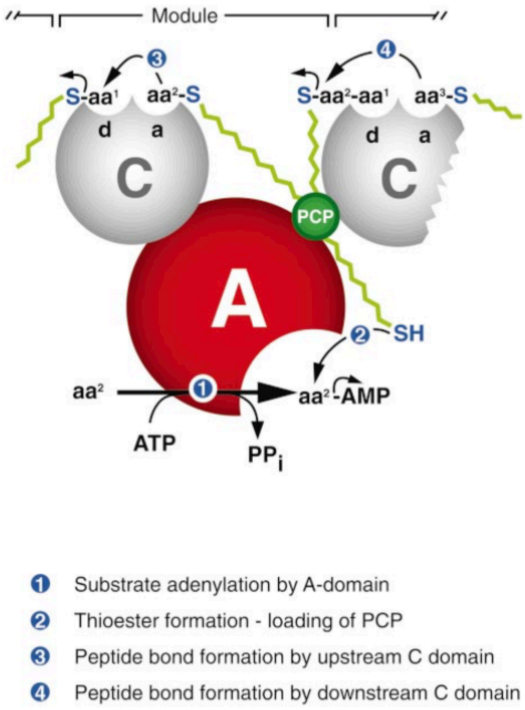


**Figure 1.4.** Composition of a module only containing essential domains (Weber and Marahiel, 2001). Each domain is represented as a colored ball, and enzymatic reactions are shown with enumerated arrows each explained above.

## 1.2 Dipeptide Antibiotic Bacilysin

Bacilysin is one of the peptide antibiotics produced by *Bacillus subtilis*, and it is discovered in 1946 with its antimicrobial activity against *Staphylococcus aureus* (Abraham *et al.*, 1946). This molecule consists of only two amino acids; L-alanine at its N-terminus and L-anticapsin, the latter being synthesized from the aromatic amino acid pathway intermediate prephenate to constitute its C-terminus. Their ligation results in the low molecular weight dipeptide of 270 Da (Walker and Abraham, 1970). The antimicrobial activity of bacilysin is not limited to *S. aureus*; it can also affect the growth of a wide range of bacteria and some fungi, including *Candida albicans* (Kenig *et al.*, 1976). Once bacilysin is transported to target microorganisms, intracellular peptidases cleave this molecule, and free anticapsin moiety acts as glucosamine-6-phosphate synthase inhibitor (Kenig *et al.*, 1976). Since this enzyme is crucial for peptidoglycan and mannoprotein synthesis, bacilysin owes its antimicrobial activity to its anticapsin component. The activity of anticapsin results in the formation of protoplasts followed by cell lysis (Walton and Rickes, 1962).

### 1.2.1 Biosynthesis of Bacilysin

Bacilysin contains only two amino acids; however, its biosynthesis is rather sophisticated. With molecular genetic studies on a mutant that cannot synthesize bacilysin, it was found that the anticapsin moiety is derived from prephenate, the branchpoint intermediate in phenylalanine and tyrosine biosynthesis in *B. subtilis* (Hilton *et al.*, 1988). This moiety is linked to L-alanine with the activity of an enzyme, alanine-anticapsin ligase which was later found to be encoded by the *bacD(ywfE)* gene (Sakajoh *et al.*, 1987; Tabata *et al.*, 2005). Our group investigated the characteristics of this linkage reaction and found that it does not entirely fit to the general mechanism described for NRPSs. That is, ATP hydrolysis was observed only

for L-alanine, but was not observed for anticapsin, and there was also no evidence of the activation of the latter as an amino acid phosphate (Yazgan *et al.*, 2001a).

The *ywfBCDEFG* operon and the monocistronic *ywfH* gene in *B. subtilis* 168 are shown to take a role in bacilysin biosynthesis and later renamed as *bacABCDEF* and *bacG* respectively (Inaoka *et al*., 2003; Steinborn *et al.*, 2005). The organization of *bacA* operon and the *bacG* is shown in Figure 1.5 (Parker and Walsh, 2012).

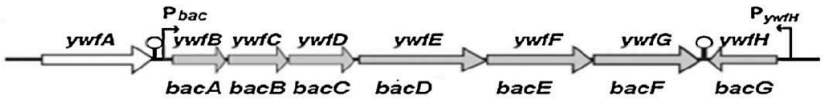


**Figure 1.5.** Organization of *bacA* operon and the *bacG* and their *ywf* synonyms (Parker and Walsh, 2012).

After identification of the *bacA* operon and *bacG* gene, the role of each gene in bacilysin biosynthesis was investigated through a series of studies. As depicted in Figure 1.6, the sequential activity of four enzymes (BacA, BacB, BacG, and BacF), converts prephenate into the dihydroanticapsin (Rajavel *et al.*, 2009; Mahlstedt *et al.*, 2010; Parker and Walsh, 2012). As the next step, BacC, an $NAD^+$ dependent alcohol dehydrogenase, oxidizes dihydroanticapsin into its final anticapsin form (Parker and Walsh, 2013). Finally, the ligation of L-anticapsin and L-alanine is accomplished by BacD (alanine-anticapsin ligase) in an ATP-dependent reaction with the formation of the dipeptide bond (Tabata *et al.*, 2005). BacE protein does not have any role in bacilysin biosynthesis but pumps bacilysin out into culture fluid, which provides resistance of the producer to bacilysin (Steinborn *et al.*, 2005).

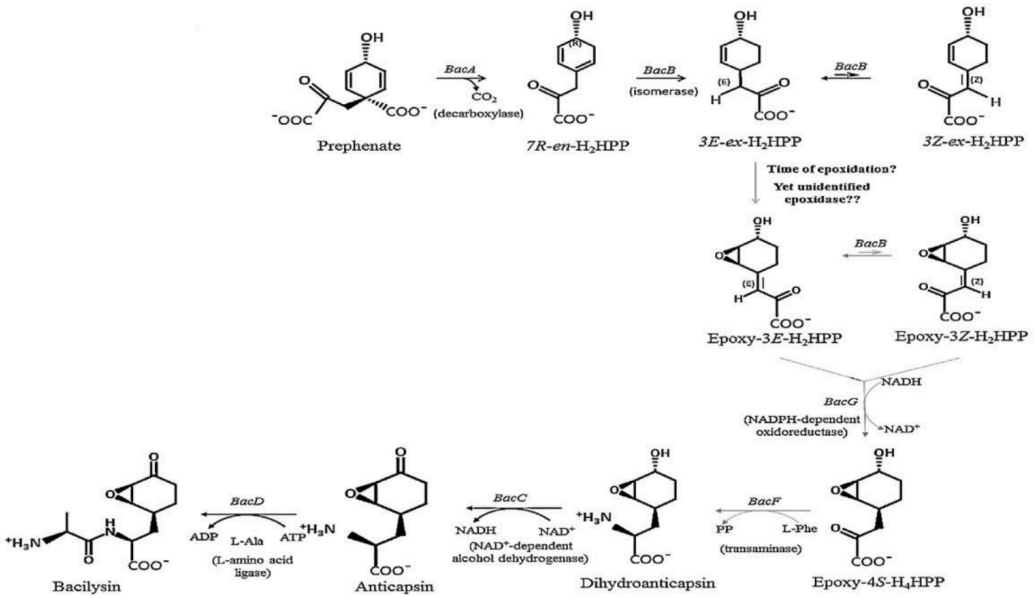


**Figure 1.6.** Detailed scheme of synthesis of anticapsin from prephenate leading bacilysin biosynthesis (Parker and Walsh, 2012).

### 1.2.2 Regulation of *bacA* Biosynthetic Operon

As the alanine-anticapsin ligase (BacD) requires $Mg^{+2}$ and ATP for functioning, their presence is essential for bacilysin production. Bacilysin is shown to be produced in a defined medium containing sucrose and glutamate, and its production is repressed or inhibited in the presence of certain nitrogen sources, including ammonium and casamino acids. Although bacilysin production is repressed with mainly nitrogen sources, carbon sources other than sucrose and glucose also result in a slightly lower titer of bacilysin (Özcengiz *et al.*, 1990, Özcengiz and Alaeddinoğlu, 1991). The production of bacilysin is also controlled by feedback regulation, as well as temperature and pH (Özcengiz and Alaeddinoğlu, 1991).

Guanosine 5'-diphosphate 3'-diphosphate (ppGpp) is a regulatory metabolite of the stringent response, which represents an adaptation of bacteria to thrive in nutrient-

limited conditions. Stringent response regulates the expression of many genes, including the expression of bacilysin biosynthetic operon. This positive regulation occurs through the intracellular ppGpp and GTP pool as well as CodY protein, a GTP-sensing transcriptional repressor. High amounts of GTP represses the expression of *bacA* operon with CodY, while increased ppGpp positively regulates the same operon (Inaoka *et al.*, 2003).

Our group employed transposon mutagenesis to identify the genes that are related to bacilysin biosynthesis. One of these genes was *oppA*, which encodes the oligopeptide permease (Opp or Spo0K) protein that is important for peptide-based quorum sensing, as explained before. In the same study, *phrC* and *comA* deleted mutant strains of *B. subtilis* were also shown to have no bacilysin produced, which reveals the role of peptide-based quorum sensing in the regulation of bacilysin biosynthesis (Yazgan *et al.*, 2001b). Furthermore, another study showed that bacilysin production is significantly decreased in *comP*- inactive mutants and wholly disrupted in *comQ* mutants (Karataş *et al.*, 2003). In the same work, it was also shown that *srfA* operon, encoding lipopeptide antibiotic surfactin, has regulatory roles on bacilysin biosynthesis as *srfA* operon-disrupted ones had no bacilysin produced. According to the same study, *spo0A*-blocked mutant strains produced no bacilysin. Nevertheless, additional disruption in the *abrB* gene in these strains resumed bacilysin biosynthesis, which implied that the regulatory AbrB protein represses *bacA* operon, and this repression can be prevented with the Spo0A which is known to inhibit AbrB (Karataş *et al.*, 2003).

The abovementioned studies show that bacilysin production is positively regulated by the peptide-based quorum sensing pathway and negatively regulated by CodY and AbrB. However, to understand their molecular role on *bacA* operon, first, the *lacZ* gene was fused to the promoter of *bacA* ($P_{bac}$) operon by our group resulting in a *B. subtilis* strain (OGU1) that is unable to produce any bacilysin detectable. With

this transcriptional-fusion, it was possible to detect the activity of the $P_{bac}$ with the β-galactosidase assay. The effects of mutations in 11 genes [*comQ* (*comX*), *comP*, *comA*, *oppA*, *phrC*, *phrF*, *phrK*, *spo0H*, *spo0A*, *abrB*, and *codY*] on $P_{bac}$, was analyzed either individually or in combinations. β-galactosidase assays showed that expression of *bacA* operon is decreased in all mutants except for *abrB* mutants in which the expression of *bacA* operon almost doubled. In the same study, electrophoretic mobility shift assay (EMSA) was employed to observe direct interaction between $P_{bac}$ and products of 11 genes mentioned. This analysis showed that AbrB, ComA, CodY and, Spo0A proteins could directly bind $P_{bac}$ and regulate the *bacA* operon (Köroğlu *et al.*, 2011).

DegS/DegU two-component system, which is essential for degradative enzyme production, was shown to positively regulate both *bacA* operon and the *bacG* gene in *Bacillus amyloliquefaciens* (Mariappan *et al.*, 2012). Another protein, ScoC (Hpr), which negatively regulates protease production and sporulation, could directly bind $P_{bac}$ and negatively regulates *bacA* biosynthetic operon (Inaoka *et al.*, 2009). In another study, *yvfI* mutants [(Δ196-314)::spc deletion or *yvfI*::Tn10::spc insertional] mutants exhibited bacilysin-negative phenotypes (Köroğlu *et al.*, 2008). Furthermore, LutR transcriptional repressor which takes a role in the control of many physiological processes was shown to be required for bacilysin production in *B. subtilis* (İrigül-Sönmez *et al.*, 2014). An overview of the bacilysin biosynthesis regulation is given in Figure 1.7 (Ertekin *et al*., 2020).

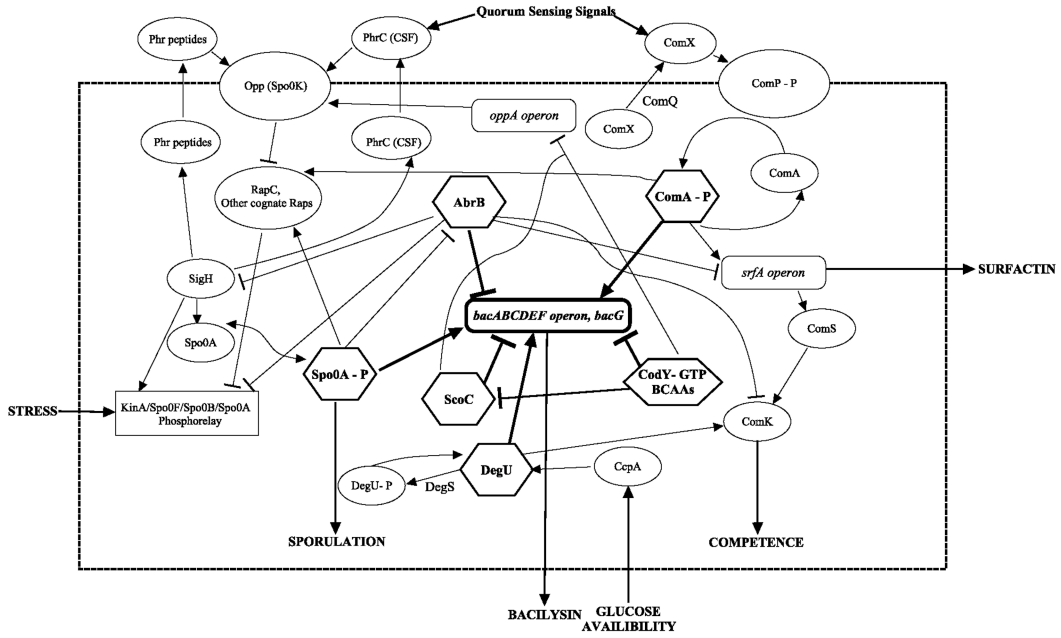


**Figure 1.7.** Regulation of *bacA* biosynthetic operon (Ertekin *et al*., 2020). Arrows indicate activation, where T-bars represent repression. The direct interactions with $P_{bac}$ are shown with bold lines.

### 1.2.3 Comparative Proteome Analyses between *bacA* operon-silenced *B. subtilis* OGU1 and *B. subtilis* PY79

As mentioned above, *B. subtilis* OGU1 is produced from *B. subtilis* PY79 with a transcriptional fusion of $P_{bac}$ and the *lacZ* gene, and it provided valuable information about the regulation of the *bacA* operon. The same strain is later utilized as the bacilysin non-producer mutant strain in comparative cytosolic proteomic analyses that employed 2DE MALDI-TOF/MS and nanoLC-MS/MS approaches (Aras-Taşkın, 2010; Demir, 2013; Ertekin *et al.*, 2020).

2DE MALDI-TOF/MS approach has identified a total of 159 differentially expressed proteins between two strains, which corresponds to 121 distinct open reading frames (ORFs). Among these 121 ORFs, 63 were underrepresented, 33 were

overrepresented and, 20 were absent in OGU1. One protein spot was detected only in OGU1 and, four proteins displayed opposing ratios in different 2DE gels covering three different pH ranges (4.5-5.5, 4-7, and 5.5-6.7) (Aras-Taşkın, 2010; Ertekin *et al.*, 2020).

The nanoLC-MS/MS was used as a complementary approach for comparative proteomic analysis of these two strains, which identified a total of 76 proteins that are differentially expressed. Of these 76 proteins, 50 were not detected in OGU1, 19 were only detected in OGU1 and, seven proteins were significantly underrepresented in OGU1 (Demir, 2013; Ertekin *et al.*, 2020).

These differentially expressed proteins belonged to different protein categories of sporulation and germination, secondary metabolite production, global regulation, stress response, and proteins of unknown functions. As its absence significantly alters the expression profile of *B. subtilis*, it would be wise to suggest that bacilysin acts as a small pleiotropic signal molecule in its producer (Özcengiz and Öğülür, 2015; Ertekin *et al.*, 2020).

Sporulation and germination was one of the major protein categories that is affected by the loss of bacilysin. A total of 36 sporulation and germination proteins were identified as significantly underrepresented or absent in OGU1 with 2DE MALDI-TOF/MS and nanoLC-MS/MS approaches (Aras-Taşkın, 2010; Demir, 2013; Ertekin *et al.*, 2020). Most of these proteins were belong to mother cell specific $\sigma^E$ and $\sigma^K$ regulons, and the importance these regulons in spore morphogenesis is introduced below. The list of underrepresented/missing proteins in OGU1 is given in Table 1.3 with their detection method and the major sporulation regulon they belong to.

**Table 1.3.** Sporulation and germination proteins identified with 2DE MALDI-TOF/MS and nanoLC-MS/MS approaches (Aras-Taşkın, 2010; Demir, 2013; Ertekin *et al.*, 2020).

| No | Locus Name | Gene Product | Detection Method | Main Regulon* |
|---|---|---|---|---|
| **1** | BSU10790 | AsnO | nanoLC-MS/MS | SigE |
| **2** | BSU37920 | GerQ (YwdL) | 2DE MALDI-TOF/MS | SigE |
| **3** | BSU19330 | SodF | nanoLC-MS/MS | SigE |
| **4** | BSU09400 | SpoVR | nanoLC-MS/MS | SigE |
| **5** | BSU37900 | SpsB[c,g,m] | nanoLC-MS/MS | SigE |
| **6** | BSU37830 | SpsJ[c,g,m] | nanoLC-MS/MS | SigE |
| **7** | BSU37820 | SpsK[c,g,m] | nanoLC-MS/MS | SigE |
| **8** | BSU09780 | YheC[c,j] | nanoLC-MS/MS | SigE |
| **9** | BSU19710 | YodQ | nanoLC-MS/MS | SigE |
| **10** | BSU35240 | CtpB[e] | nanoLC-MS/MS | SigE |
| **11** | BSU00160 | YaaH[b,j] | 2DE MALDI-TOF/MS | SigE |
| **12** | BSU25540 | Gpr[d,e,k] | nanoLC-MS/MS | SigF |
| **13** | BSU29500 | (GerW) YtfJ | nanoLC-MS/MS, 2DE MALDI-TOF/MS | SigF |
| **14** | BSU08820 | KatA[r] | nanoLC-MS/MS | SigF |
| **15** | BSU22930 | SleB | nanoLC-MS/MS | SigG |
| **16** | BSU16790 | TepA[k] | nanoLC-MS/MS | SigG |
| **17** | BSU09180 | YhcQ | 2DE MALDI-TOF/MS | SigG |
| **18** | BSU22920 | YpeB | nanoLC-MS/MS | SigG |
| **19** | BSU33540 | YvaB[p] | 2DE MALDI-TOF/MS | SigG |
| **20** | BSU18360 | GalM(YoxA)[b] | 2DE MALDI-TOF/MS | SigH |

**Table 1.3.** (continued)

| | | | | |
|---|---|---|---|---|
| **21** | BSU28000 | MinC[a,f,h,i,l] | 2DE MALDI-TOF/MS | SigH |
| **22** | BSU27990 | MinD[a,f,i,l] | 2DE MALDI-TOF/MS | SigH |
| **23** | BSU19780 | CgeA[m,n] | nanoLC-MS/MS | SigK |
| **24** | BSU06300 | CotA[m] | nanoLC-MS/MS, 2DE MALDI-TOF/MS | SigK |
| **25** | BSU36050 | CotB | nanoLC-MS/MS | SigK |
| **26** | BSU17030 | CotE[c,m] | nanoLC-MS/MS | SigK |
| **27** | BSU30920 | CotI | nanoLC-MS/MS | SigK |
| **28** | BSU30910 | CotSA[m] | nanoLC-MS/MS | SigK |
| **29** | BSU17670 | CotU[m,n] | nanoLC-MS/MS | SigK |
| **30** | BSU17410 | CwlC | nanoLC-MS/MS | SigK |
| **31** | BSU04110 | LipC[g,m,n,o,s] | nanoLC-MS/MS | SigK |
| **32** | BSU18670 | OxdD[m] | nanoLC-MS/MS | SigK |
| **33** | BSU16730 | DpaA (SpoVFA) | 2DE MALDI-TOF/MS | SigK |
| **34** | BSU16740 | DpaB (SpoVFB) | nanoLC-MS/MS | SigK |
| **35** | BSU07280 | YfnG[c,m] | nanoLC-MS/MS | SigK |
| **36** | BSU10950 | YitD[c] | nanoLC-MS/MS | SigK |

*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

## 1.3 Sporulation in *Bacillus subtilis*

### 1.3.1 Entry into Sporulation and Sporulation Process

Under optimal conditions, *B. subtilis* cells can propagate by binary fission which takes place almost twice an hour. However, when nutritional sources become scarce, *B. subtilis* has alternative ways to strive. The formation of the endospore is the ultimate way of survival under such stressful conditions and occurs after a series of events, which takes almost 10 hours (Stragier and Losick, 1996). The endospore is metabolically dormant, and most importantly, it is capable of resisting extremes of chemicals, temperature, and ionizing radiation with its dehydrated spore core and compact chromosomal DNA (Nicholson *et al.*, 2000). The sporulation process starts with an asymmetric division that occurs at one pole of the cell. This asymmetric division results in two different cells separated with a septum; the larger, mother cell, and the smaller, forespore. Eventually, forespore becomes engulfed within the mother cell, and two parallel, complex sets of gene expression take place in these two different cells. This gene expression is controlled with alternative sigma factors, and signaling between the mother cell and forespore is crucial for sporulation. These two parallel sets of gene expression lead to the programmed death of the mother cell, and maturation of forespore into endospore or spore (De Hoon *et al.*, 2010; Higgins and Dworkin, 2012). The morphological and genetic inequality of the mother cell and the forespore are depicted in Figure 1.8 (Higgins and Dworkin, 2012).

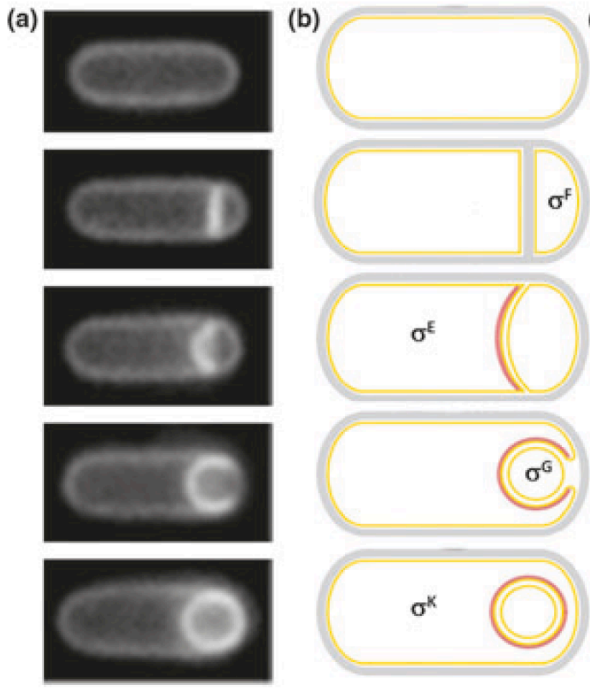


**Figure 1.8.** Morphological (a) and genetic (b) differences between the mother cells and the forespore (Higgins and Dworkin, 2012).

One unique property of the sporulation is the unequal distribution of chromosomal DNA between the forespore and the mother cell. After the asymmetric division, the complete copy of the chromosome is localized in the mother cell. However, the forespore had only about one-third of its chromosome (the origin-proximal region) captured after the division. The chromosome is attached to cell pole with the action of RacA protein (Ben-Yehuda *et al.*, 2003), and for sporulation to proceed the rest of the chromosome should be translocated into the forespore. This translocation is mediated by SpoIIIE protein found in the center of the septum, which pumps the rest of the chromosome into the forespore (Ptacin *et al.*, 2008). After successful asymmetric division, mother cell membranes move and encircle the forespore eventually. This process is named as engulfment and has similarities to phagocytosis. Nevertheless, in the engulfment process, a layer of peptidoglycan encircles the forespore, which will be later modified for proper membrane movement (Pogliano *et al.*, 1999). At the end of the engulfment, the forespore is surrounded by two

membranes. Later, the spore cortex is produced between these two membranes, while spore coat proteins assemble on this cortex to produce a multi-layer structure (Higgins and Dworkin, 2012). As the last step, the mother cell is lysed with a programmed gene expression which releases the mature spore that is metabolically dormant and resistant to external stress (Nicholson *et al.*, 2000).

As mentioned previously, sporulation initiation occurs with the activation of transcription factor Spo0A with phosphorylation through phosphorelay signaling (Sonenshein, 2000). KinA-E sensory kinases sense sporulation inducing signals and activates Spo0F, which will eventually activate Spo0A. Of these kinases, KinA is the major one, and its overexpression is enough to induce the sporulation process (Fujita and Losick, 2005). As these sensory kinases determines the fate of the sporulation, they are tightly regulated by a range of factors. One such factor is KipI, an inhibitor that suppresses the autophosphorylation of KinA (Wang *et al.*, 1997). This inhibitor is encoded in the same operon with KipA protein, which inhibits KipI. Thus, KipI/KipA pair represents one mechanism of histidine kinase regulation in *B. subtilis*. RapA, RapB, RapE, and RapH proteins are crucial for the regulation of the sporulation initiation, as they target phosphorylated Spo0F (Smits *et al.*, 2007). The initiation of sporulation is also tightly regulated with cell cycle cues. One such regulator is Sda protein, which is shown to inhibit major sensory kinase KinA as well as KinB (Burkholder *et al.*, 2001). This protein could block the autophosphorylation of KinA, just like KipI, but it could also inhibit the phosphate transfer from KinA to Spo0F (Cunningham and Burkholder, 2009). As the expression of *sda* correlates with the initiation of chromosomal DNA replication, it prevents sporulation initiation during replication. In other words, cell-cycle dependent *sda* expression prevents the formation of polyploid spores (Veening *et al.* 2009).

The transcription factor Spo0A is crucial for the initiation of sporulation, yet it also has roles in other physiological processes like cannibalism or biofilm formation

(López and Kolter, 2010). This transcription factor regulates different sets of genes, and this regulation depends on the level of active Spo0A or Spo0A-P. As an example, after reaching a relatively lower threshold, Spo0A-P induces *sinI* expression, which derepresses matrix genes, including *sinR* (Fujita *et al.*, 2005). At these levels, active Spo0A also induces the expression of two critical operons of *skf (*sporulation killing factor) and *sdp* (sporulation delaying protein). These operons encode two factors responsible for the killing of sister *B. subtilis* cells that are not resistant (since they do not express these operons), and this process is known as cannibalism (González-Pastor *et al.*, 2003). These alternative processes to cope with stress is replaced with a commitment to sporulation when Spo0A-P reaches higher levels.

Activated Spo0A regulates a total of 520 genes, and 121 are directly regulated (Molle *et al.*, 2003). *abrB* is one of the genes that is repressed by Spo0A-P, and it encodes for a transcriptional regulator that represses stationary phase genes, including *sigH*. Hence, Spo0A indirectly derepresses the expression of $\sigma^H$ by further increasing the expression of Spo0A (Banse *et al.*, 2008). The $\sigma^H$ also regulates the expression of the *spoII-spoIIAB-sigF* operon. This operon encodes for anti-sigma factor SpoIIAB, anti-anti-sigma factor SpoIIA, and, most importantly, $\sigma^F$, which is the first forespore specific sigma factor regulating about 50 genes including *sigG* (Steil *et al.*, 2003; Wang *et al.*, 2006). On the other hand, $\sigma^E$ is the first mother cell-specific sigma factor, and it is encoded under the control of Spo0A-P as a pro-peptide. This precursor is cleaved with SpoIIGA protease, and this cleavage is triggered by SpoIIR protein, which is one of the first proteins synthesized in forespore under the regulation of $\sigma^F$ (Imamura *et al.*, 2008). The activation of $\sigma^G$ is dependent on eight proteins that are expressed under the control of $\sigma^E$, including SpoIIIAH, which forms a channel between forespore and mother cell upon interacting with SpoIIQ (Meisner *et al.*, 2008). $\sigma^E$ also controls the expression of $\sigma^K$, which is the second sigma factor activated in the mother cell. Like $\sigma^E$, $\sigma^K$ is produced as a precursor that is cleaved by SpoIVFB protease. The activity of SpoIVFB protease is enhanced with the presence of SpoIVB, which is a signaling protein that is produced under the control of $\sigma^G$

(Cutting *et al.*, 1991). Although the regulation of sporulation is much more complicated than what is described here, it follows sequential activation of $\sigma^H$, $\sigma^F$, $\sigma^E$, $\sigma^G$ and $\sigma^K$ in separate compartments. This transcriptional regulatory network is depicted in Figure 1.9 (De Hoon *et al.*, 2010).

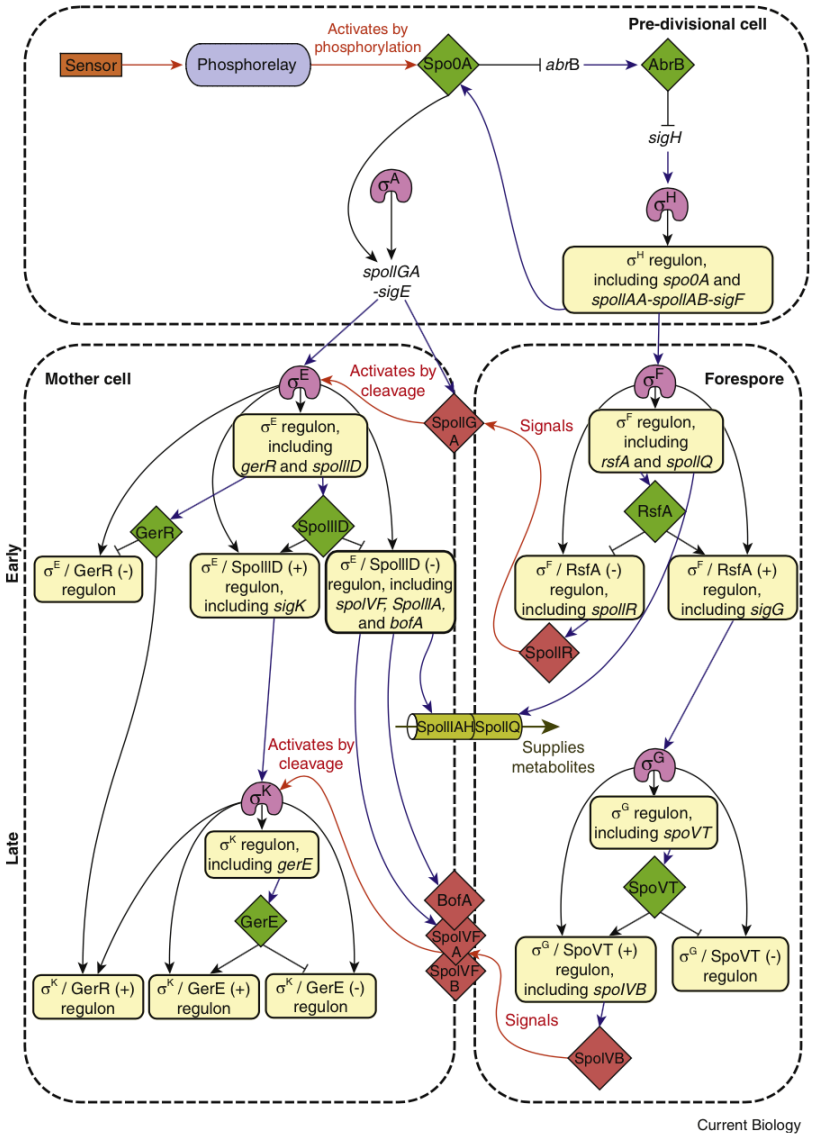


**Figure 1.9.** Modular transcriptional regulatory network of endospore formation in *B. subtilis* (De Hoon *et al.*, 2010)

### 1.3.2 Spore Morphology of *Bacillus subtilis*

The mature endospore of *B. subtilis* is a complex structure that endures external stress. In its core, chromosomal DNA is protected from damage with the aid of small acid-soluble proteins or SASPs. This core structure is enclosed with the cellular membrane of forespore, and this membrane is further surrounded by a layer that is rich in peptidoglycan and named as spore cortex. Two coat layers (inner and outer) that contain more than 70 proteins (Henriques *et al.*, 2004) encircles the spore cortex and further protect the spore core. The complex structure of the *B. subtilis* endospore is shown in Figure 1.10 (Henriques and Moran, 2007).

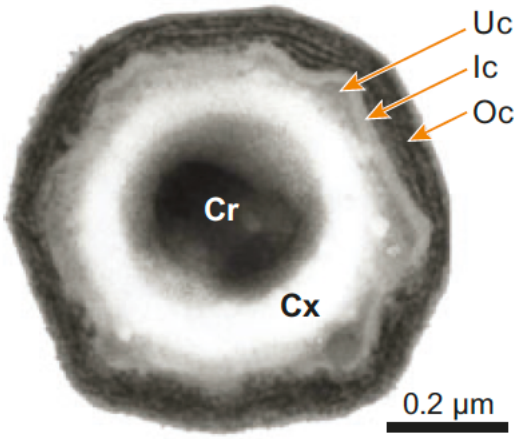


**Figure 1.10.** Morphology of the *B. subtilis* endospore (Henriques and Moran, 2007). The spore core (Cr), spore cortex (Cx), inner coat (Ic), outer coat (Oc), and also undercoat (Uc) regions are shown.

Synthesis of the spore cortex and also coat layers are mainly executed by the mother cell. SpoIVA is an essential protein for spore coat assembly, and it is produced under the control of $\sigma^E$, the first mother-cell specific sigma factor (Wang *et al.*, 2009). This protein localizes near outer forespore membrane with interacting another mother-cell specific protein SpoVM (Ramamurthi *et al.*, 2006) and forms a scaffold for coat assembly by hydrolyzing ATP (Ramamurthi and Losick, 2008). This scaffold

formation marks the outer membrane as both the site of the spore coat attachment and also the cortex (Catalano *et al.*, 2001). SpoVID is another key coat morphogenic protein regulated with $\sigma^E$, and it requires SpoIVA for proper localization on the forespore membrane (Beall *et al.*, 1993). SafA is a necessary protein for the spore's encasement, and it interacts with SpoVID protein (Müllerová *et al.*, 2009). It alters the localization of about 16 inner coat proteins, and *safA* null mutant spores have an inner coat that is often thinner and does not bind the spore surface tight (McKenney *et al.*, 2010).

CotE is an essential morphogenic protein that plays a role in the assembly of both inner and outer layers of the spore coat. However, its presence is vital for the outer coat to assemble as *cotE* mutant spores could not form this assembly (Zheng *et al.*, 1988). Its production is controlled both by $\sigma^E$ and later by $\sigma^K$, and it directs the assembly of other important coat proteins like CotC and CotU (Isticato *et al.*, 2010). Final assembly of the inner and outer coat and their maturation takes place with the activation of $\sigma^K$, and several $\sigma^K$ controlled proteins including CotT, CotD, CotSA, CotS, CwlJ, and OxdD become assembled to form spore coat (Driks, 2004). The assembly of the spore coat is depicted in Figure 1.11 (Henriques and Moran, 2007).

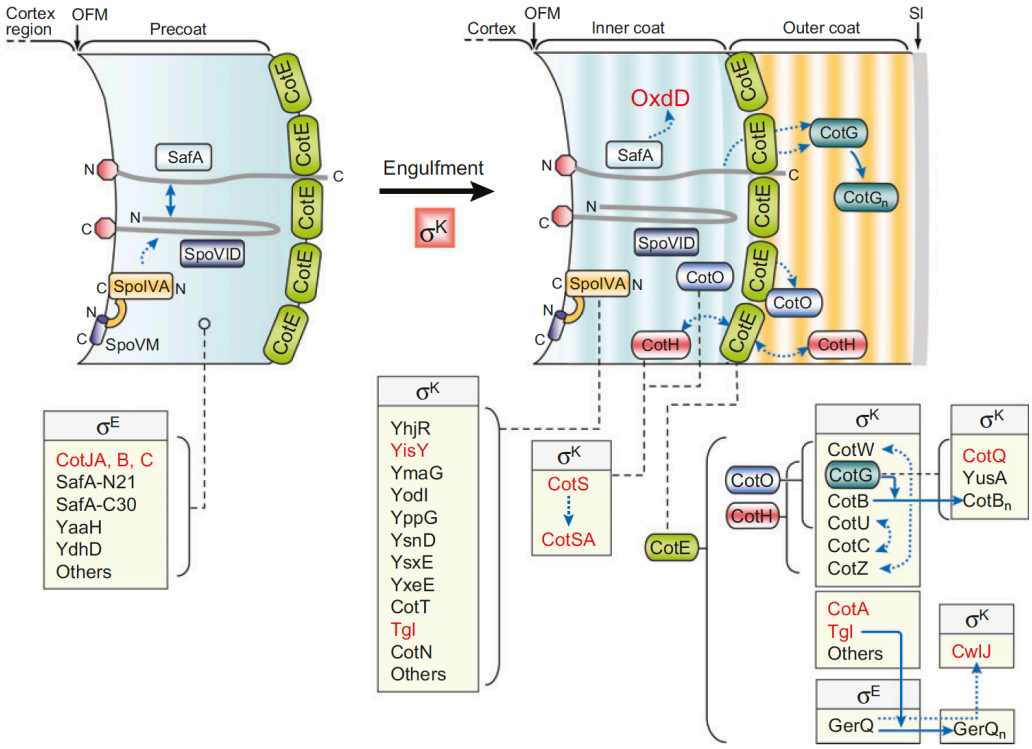


**Figure 1.11.** Assembly of the *B. subtilis* endospore coat (Henriques and Moran, 2007).

The spore cortex mediates the dehydration of the spore core, which protects endospores from heat and desiccation. The cortex structure mainly comprises peptidoglycan that has fewer peptide side chains than vegetative peptidoglycan (Popham, 2002). The spore cortex's production is mediated by the activity of mother-cell specific proteins, just like the spore coat. One such protein is SpoVE, and it takes role in peptidoglycan polymerization from the cytoplasmic precursors (Vasudevan *et al.*, 2007). It interacts with another $\sigma^E$-controlled protein on the forespore membrane, SpoVD, which is also required for the synthesis of the spore cortex (Fay *et al.*, 2010). Peptidoglycan in the *B. subtilis* spore cortex was found to be O-acetylated, which reduces the sensitivity to the antimicrobial lysozyme (Laaberki *et al.*, 2011).

### 1.3.3 Spore Resistance

Endospores of *B. subtilis* lack any enzymatic activity, including repair of DNA or other macromolecules. Any damage could be repaired only upon the germination or outgrowth of the spore. However, if the damage on the spore is overwhelming, the germinated spore eventually dies (Setlow, 1995; Setlow *et al.*, 2002; Setlow, 2003). To reduce such damage on macromolecules and to survive under stressful conditions for a long time, specific resistance mechanisms have evolved in spores.

Wet heat resistance is one of the unique properties of bacterial endospores. Spores of *B. subtilis* could resist to wet heat (at 90ºC) for almost 20 minutes, whereas its vegetative form would be killed within a minute (Gerhardt and Marquis, 1989). The resistance of spores to wet heat is mainly determined by the water content of the spore core. This water content could vary by a set of factors, and lower water content result in higher resistance against wet heat stress (Melly *et al.*, 2002). Dipicolinic acid or shortly DPA is the main factor that results in lower water content, and it is only found in the spore core. The spore core is rich in divalent cations like $Ca^{2+}$ or $Mg^{2+}$ mostly chelated with DPA which also increases the resistance by lowering the water content (Gerhardt and Marquis, 1989). Wet heat is not associated with DNA damage in spores, yet it was shown that spores lacking their major SASPs (alpha and beta types) are more susceptible to wet heat (Nicholson *et al.*, 2000; Setlow, 2011). The endospores of *B. subtilis* are also resistant to dry heat, but in contrast to wet heat, the resistance mechanism mainly involves major SASPs as dry heat damage the DNA significantly (Espitia *et al.*, 2002).

Bacterial spores are also resistant to several enzymes and chemicals due to their complex and rigid spore coats. One of these enzymes is lysozyme, which targets peptidoglycan synthesis and therefore interferes mostly with Gram-positive cell wall synthesis. Lysozyme targets the spore cortex that is rich in peptidoglycan, and the

importance of spore coat in lysozyme resistance was shown with the characterization of individual spore coat proteins. Because CotE takes a role in the formation of the outer spore coat, *cotE* null mutant lacking outer coat was shown to be less resistant to lysozyme (Zheng *et al.*, 1988). However, in mutants that express *cotE* gene at later stages, not having any outer coat either, the lysozyme resistance was similar to the wild type suggesting additional roles of CotE in lysozyme resistance (Costa *et al.*, 2007). The inner coat layers of the spore and inner coat proteins like CotH were also shown to have functions in lysozyme resistance (Naclerio *et al.*, 1996). The inner and outer coats are crucial for resistance to a variety of chemicals, including alkylating agents, oxidizing agents, and organic solvents (Setlow, 2011). These layers act as an armor, they prevent penetration of the chemicals into sensitive inner structures and meanwhile detoxify some of these chemicals. The inner membrane of the spore further prevents the penetration as it shows extremely low permeability to hydrophobic, and small hydrophilic molecules (Gerhardt *et al.*, 1972).

### 1.3.4 Spore Germination

Endospores of *B. subtilis* survey their surroundings, and if the conditions are suitable, they could germinate, which ultimately results in the production of vegetative cells (Paidhungat and Setlow, 2002). Spores sense the presence of certain factors, named germinants that are usually low molecular nutrients like sugars, amino acids and purine derivates. There are also unique combinations of nutrients that are well-known to induce the germination process, and AGFK (asparagine, glucose, fructose, and potassium ion) is such a mixture for *B. subtilis* spores (Setlow, 2003). These nutritional germinants are immediately sensed by the GerA family of germinant receptors located in the inner membrane of the endospore and produced in late sporulation (Hudson *et al.*, 2001). Although the binding mechanism and the site of germinants are unclear, these germinant receptors initiate a series of physiological events upon nutritional germinant binding. First, monovalent cations of $H^+$, $Na^+$, $K^+$ and also $Zn^{2+}$ are released from the spore core, which increases the pH from 6.5 to

7.7, a pH that is necessary for enzyme action (Jedrzejas and Setlow, 2001). This event is followed by the release of Dipicolinic acid (DPA) with chelated $Ca^{2+}$ from the spore core, and DPA-$Ca^{2+}$ is replaced with water, which decreases the wet heat resistance along with refractility but not enough for any enzyme action (Cowan *et al.*, 2003). Upon the release of DPA-$Ca^{2+}$, the enzymatic activity of cortex lytic enzymes is induced, and the peptidoglycan spore cortex is degraded. This degradation results in water uptake into the core, which expands the germ cell wall and allows enzymatic action and outgrowth (Setlow *et al.*, 2001).

In *B. subtilis*, CwlJ and SleB are two essential enzymes that play a role in spore cortex degradation. The presence of either of them is sufficient for complete germination; however, double mutant spores cannot degrade the spore cortex (Makino and Moriyama, 2002). Both of these enzymes require muramic-delta-lactam for enzymatic activity; therefore, they could not degrade the cell wall which lacks this modification (Moir *et al.*, 2002). These enzymes are produced during sporulation, and CwlJ is only located in the spore coat, while SleB is found in coat layers, cortex, and also outer membrane (Chirakkal *et al.*, 2002). Both of these enzymes require certain proteins for proper assembly and functioning. For SleB, the required protein is YpeB, while GerQ (yet another coat protein) is essential for CwlJ functioning (Boland *et al.*, 2000; Ragkousi *et al.*, 2003). CwlC is known to be activated by $Ca^{2+}$-DPA released from the spore core (Paidhungat *et al.*, 2001). On the other hand, the exact mechanism for SleB activation is not clear, and it could be related to the changes in spore cortex content (Tovar-Rojo *et al.*, 2002).

Although the germination is triggered with nutritional germinants, there are also a variety of non-nutrient germinants like lysozyme, high pressures, certain salts, and exogenous DPA-$Ca^{2+}$. As lysozyme could degrade the spore cortex, it is a potential germinant. However, for lysozyme to act on the spore cortex, spore coats should be removed with a pretreatment, and lysozyme concentration should be low enough to

prevent any cell wall degradation. Exogenous DPA-$Ca^{2+}$ is another non-nutrient spore germinant that does not require any germinant receptor or SleB, showing that it directly or indirectly activates CwlJ for germination (Paidhungat *et al.*, 2001). Furthermore, high pressures could induce germination in *B. subtilis* spores. At relatively lower pressures (about 100-200 megapascals (MPa)), germination is initiated with the activation of germinant receptors (Wuytack *et al.*, 2000). Nevertheless, higher pressures of 500-600 MPas bypasses the receptor activation and directly cause the release of DPA-$Ca^{2+}$ (Paidhungat *et al.*, 2002). The germination process in *B. subtilis* is summarized in Figure 1.12 (Setlow, 2003).

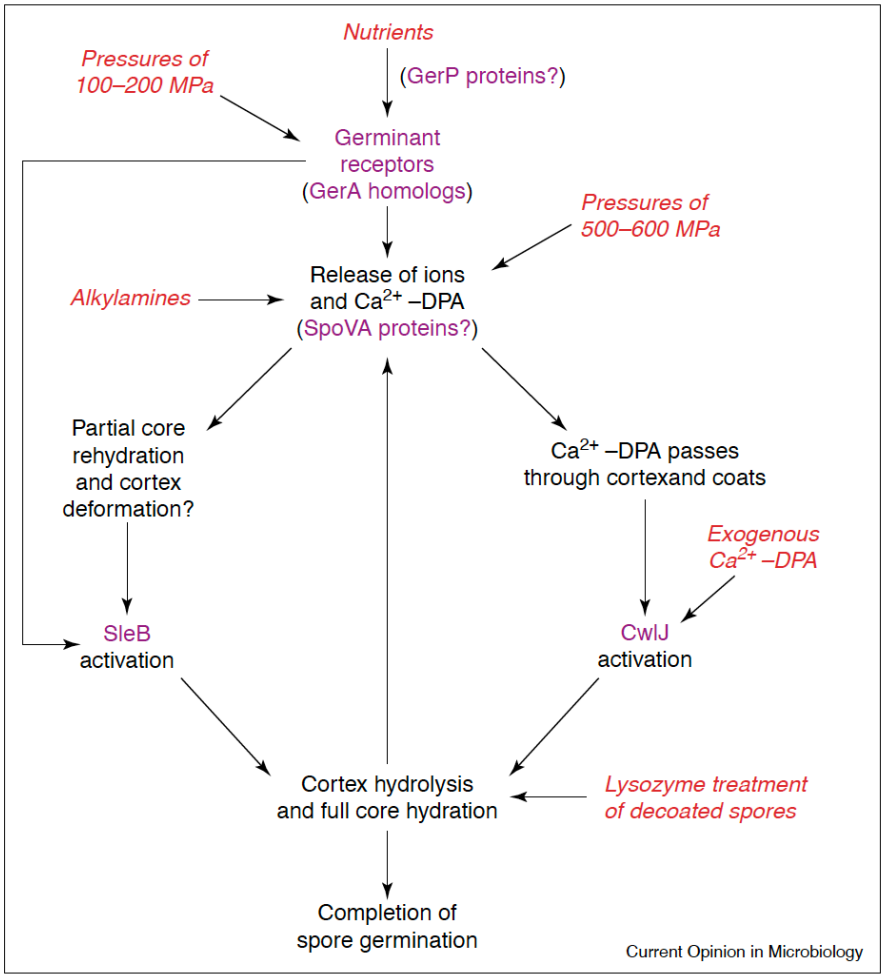


**Figure 1.12.** Nutrient and non-nutrient spore germination processes in *B. subtilis* (Setlow, 2003).

## 1.4 Chemical Complementation

Genetic complementation is the conversion into the wild type phenotype in the presence of two different genetic information sources, usually two different mutations. In this process, each mutant provides the necessary information that other mutant does not have. For complementation to occur, these mutations should not be on the same gene. Based on this principle, complementation tests (or cis-trans test) are developed to test whether two different mutations are located on the same gene or not, and these tests are carried on fungi, bacteria, and also *Drosophila*

*melanogaster* (Horowitz, 1991). These tests have also been useful for understanding the molecular genetics of T4 bacteriophage and provided information about significant processes (Epstein *et al.*, 1963). Functional complementation, on the other hand, is defined as the restoration of the function of a mutant with an observable phenotype. This restoration is commonly done with the introduction of wild type genetic elements and used to identify the functions and roles of many genes of a range of organisms, from microorganisms to humans. However, if the phenotype is the result of a complex gene expression process or requires post-translational modification, the introduction of a genetic element will not be enough to restore. Therefore, the addition of the product itself could be an alternative way to functional complementation in such situations (Vogt and Lazo, 2005).

One example of the chemical complementation is related to the mutation in the *gltB* gene in *B. subtilis*. The knockout mutant of *gltB* is shown to have a defect in biofilm formation and decreased production of gamma-polyglutamate and three lipopeptide antibiotics (Zhou *et al.*, 2016). As it was unclear that the exact reason for these defects is *gltB* mutation or not, Zhou *et al.* (2016) attempted to restore the defect in biofilm formation by adding gamma-polyglutamate to the cultures. By doing that, they indirectly showed the role of GltB in biofilm formation and gamma-polyglutamate production required for biofilm formation. Another study of chemical complementation is related to bacillithiol production in *B. subtilis*. Fang and Dos Santos (2015) identified a *B. subtilis* mutant that lacks bacillithiol production, and this mutant was more sensitive to metal toxicity and oxidative stress. With the external addition of bacillithiol disulfide, they managed to rescue mutant phenotype and prove the role of bacillithiol in Fe-S metabolism and stress resistance (Fang and Dos Santos, 2015).

## 1.5 Whole Genome Resequencing (WGRS)

Although the double helix structure of DNA is solved as early as 1953, the first breakthrough in DNA sequencing came in two decades later with the development of Sanger's chain-termination technique that employs dideoxynucleotides (ddNTPs). These molecules lack 3' hydroxyl group, and they prevent further DNA synthesis upon incorporated in the newly synthesis strand. Usage of radioactively labeled ddNTPs in DNA synthesis, therefore, generates a mixture of DNA fragments, with each possible length possible which could be separated on polyacrylamide gels that are used for inferring nucleotide sequences. This method is improved in the following years, which allowed automated DNA sequencing machines that can read nearly one kilobase (kb) (Heather and Chain, 2016). To analyze longer fragments than that, overlapping DNA fragments are sequenced separately and then assembled into one long sequence named "contig" in silico (Anderson, 1981).

The first example of next-generation sequencing is the "pyrosequencing" that utilizes a luminescence method for measuring pyrophosphate synthesis during DNA synthesis. One of the four types of nucleotides added to the wells, and if these nucleotides were incorporated into the newly synthesized strand, the released pyrophosphate is detected with emitted light produced by luciferase (Nyrén and Lundin, 1985). Pyrosequencing is later developed and licensed to 454 Life Sciences. Another example of the second-generation DNA sequencing is the "bridge amplification", commonly used by Illumina sequencer. This method uses adapter-ligated DNA fragments which are loaded into nanowells and fold-into bridge-like structure. Then, these bridges were amplified by PCR utilizing a reversible 3'flourescent blocker that enables the addition of only one nucleotide at a time. After each round, the wavelength of the fluorescent tag is detected, and the incorporated nucleotide is recorded (Turcatti *et al.*, 2008).

With the advances in DNA sequencing technology, the number of sequenced genomes increases enormously each day. Eventually, these ultrahigh throughput next-generation technologies became suitable for the detection of genetic variation in large genomes. This technique is known as whole-genome resequencing and relies on comparison of the newly sequenced genome with a reference genome. In WGRS, the genome to be analyzed is first sequenced with one of the next-generation techniques mentioned above. Then, the raw sequences produced by the sequencer are filtered and processed to remove any duplicate reading. Finally, these reads are aligned to the reference genome, and the variants are annotated according to the reference genome (Li *et al.*, 2009).

## 1.6 Mass Spectrometry Analysis

Identification of proteins and determination of their chemical structures are crucial for the life sciences. Several methods have been developed for these, including chemical and enzymatic methods and mass spectrometry (Domon and Aebersold, 2006). Edman degradation is one of the oldest methods used for peptide and protein identification, and it employs labeling and cleavage of N-terminal amino acids. This stepwise degradation from N-terminus is proposed by Edman in 1950, and later it was automated in 1967. As this degradation starts at N-terminus, it is not useful for the amino acid identity of proteins having modified residues at their N-terminus. This method is also not useful for the identifying of disulfide bridges, which gives essential information about the structure of the protein (Han *et al.*, 1985).

Although mass spectrometry was described by physicists in as early as 1880s, it became a powerful tool for the life sciences in the last three decades. This method relies on the formation of gas-phase ions, which then be separated according to their mass-to-charge ratio (m/z). After this mass analysis, a spectrum will be produced containing m/z values at is the x-axis, and the ions count at the y-axis. This spectrum,

called the mass spectrum, can provide valuable information about protein structure (amino acid sequences or peptide masses). Therefore, it can also be useful for identifying unknown samples with their mass spectrum searched in peptide and protein databases (El-Aneed *et al.*, 2009).

Before the formation of gas-phase ions, the biological sample to be analyzed must be adequately prepared. That is, the molecule must be charged and dry for MS analysis (Graves and Hystead, 2002). For peptide identification from one- or two-dimensional gels, peptide extracted from the gel should be digested with certain proteases like trypsin. This digestion results in both more efficient sample recovery from the gel and more information in MS analysis (Andersen and Mann, 2000).

Formation of gas-phase ions could be done with several techniques; however, Matrix-Assisted Laser Desorption/Ionization (MALDI) and Electrospray Ionization are the most common ones. Shortly, in MALDI, the sample to be analyzed is dissolved in an organic compound called the matrix, which later dried on a metal plate in tiny volumes. These plates are then bombarded with ultraviolet (UV) light, which causes desorption and ionization of the sample (Jurinke *et al.*, 2004). On the other hand, ESI uses electrical energy to transform ions in a solution into their gaseous form. Both of these two methods produce gaseous ions without any change in the sample's integrity, which is crucial for mass information (Ho *et al.*, 2003).

The mass analyzer part of the mass spectrophotometer is responsible for the separation of gaseous ions with respect to their mass-to-charge ratio (m/z). There are several different mass analyzers varying in size, resolution, and mass range. Four of these mass analyzers are commonly used: quadrupole, quadrupole ion trap (QIT), Fourier transform ion cyclotrone resonance (FT-ICR), and time of flight (TOF) (El-Aneed *et al.*, 2009). Among them TOF is maybe the simplest one, which determines

m/z ratio by measuring the time that is required for an ion to travel the length of the flight tube. There are also tandem quadrupole systems which contains three quadrupoles in which further fragmentation of ions occurs with collision. This system usually donated as MS/MS (Ho *et al.*, 2003). Mass analyzer gives a mass spectrum which then could be compared with proteins and peptide databases with the aid of a software, resulting in partial or whole identification of peptide or protein.

## 1.7 The Aim of the Present Study

In the context of two earlier M. Sc. theses, differentially expressed proteins of the bacilysin non-producer *B. subtilis OGU1* have been compared to its parental strain *B. subtilis* PY79 were determined by using complementary proteomic approaches, 2DE MALDI-TOF/MS and nanoLC-MS/MS (Aras-Taşkın, 2010; Demir, 2013) ), and these studies were validated in this study with RT-qPCR analysis of selected 20 genes. The abundance of the sporulation- and germination- related proteins were found to be significantly decreased in OGU1, which suggested a pleiotropic effect of bacilysin in its producer. In the present study, phenotypic analysis of OGU1 with respect to sporulation, particularly for colony pigmentation, resistance to lysozyme and chloroform as well as germination response was performed. It was next aimed to restore these phenotypic changes by using the broth concentrate of PY79 as a chemical complementation agent. The broth concentrate of OGU1 was also used as the negative control, and the phenotypes of four different sources of spores were assessed simultaneously.

# CHAPTER 2

# MATERIALS AND METHODS

## 2.1 Bacterial Strains

*B. subtilis PY79*, the prototrophic derivative of the standard strain *B. subtilis* 168 (Youngman *et al.*, 1984), was utilized as the bacilysin producer in this study. As the bacilysin non-producer, *bacA::lacz::erm B. subtilis* OGU1 that was constructed by our group from PY79 (Köroğlu *et al.*, 2011) was used as the bacilysin non-producer organism. *S. aureus* ATCC 9144 was employed as the test (assay) organism for bacilysin bioactivity determinations.

## 2.2 Culture Media

The composition and preparation of culture media used in this study are given in Appendix A.

## 2.3 Buffers and Solutions

The composition and preparation of buffers and solutions used in this study are given in Appendix B.

## 2.4 Chemicals and Enzymes

The chemicals and enzymes used in this study are given in Appendix C.

## 2.5 Maintenance and Growth of Bacterial Strains

*B. subtilis* strains and *S. aureus* ATCC 9144 were grown in the liquid Luria-Bertani (LB) medium and kept on Luria-Bertani (LB) agar plates. However, for the selection of OGU1, LB agar plates were supplemented with antibiotics erythromycin and lincomycin at the final concentrations of 1 μg/mL and 25 μg/mL, respectively. All solid cultures were stored at 4°C and subcultured monthly. For the long-term storage, 20% of glycerol stocks were prepared for each strain and kept at -80°C.

Regardless of the experiment, *B. subtilis* strains maintained on LB agar plates were first inoculated into 15 mL of PA medium (Perry and Abraham, 1979) (pH 7.4) (Appendix A), and these cultures were grown at 37°C (200 rpm) for 16 h to generate seed cultures. These cultures were then used to inoculate larger volumes of cultures with an initial optical density of 0.1 at 595 nm ($OD_{595}$). The nature of cultures and their growth conditions varied with the experiment; therefore, these will be explained in the following headings with details. *S. aureus* ATCC 9144 was grown in LB medium at 37°C (180 rpm) for 16 h to be used in bacilysin bioactivity assay.

## 2.6 Whole Genome Resequencing (WGRS)

### 2.6.1 Genomic DNA Isolation

Genomic DNA of *B. subtilis* strains was isolated by a procedure developed for rapid bacterial chromosomal DNA purification (Owen and Borman, 1987) with minor modifications.

First, *B. subtilis* strains were grown in 100 ml of PA medium at 37°C (200 rpm) for 16 h. 2 mL of these overnight cultures were centrifuged at 6000 rpm for 10 minutes. After the supernatant was discarded, the pellet was resuspended in 1 mL of the SET

buffer (Appendix B) by vortexing. 20 µL of lysozyme (100 mg/mL) and 6 µL of RNase (10 mg/mL) were added into the resuspension, and the mixture was incubated for one hour at 37°C water bath. Then, 20 µL of proteinase K (20 mg/mL) and 100 uL of 10% SDS were added, and the mixture was incubated for two hours at 55°C water bath with occasional inversions. After this incubation, the mixture was divided into two separate sterile 2.0 mL micro-volume centrifuge tubes for each strain. Next, 200 µL of 5 M NaCl solution was added and the samples were mixed gently with inversions. As the next step, 500 µL of chloroform solution was added, and the samples were mixed again with inversions for almost 30 minutes at room temperature. Samples were centrifuged at 6000 rpm for 15 minutes, and the supernatants containing genomic DNA were transferred into new 1.5 mL micro-volume centrifuge tubes. Three volumes of cold ethanol (absolute, ≥ 99.8%, stored at -20°C beforehand) were added onto the supernatant and mixed shortly to precipitate genomic DNA. Then, the samples were stored at -20°C for at least 30 minutes which followed by centrifugation at 6000 rpm for 15 minutes at 4°C. As soon as the supernatant was discarded, the pellet was rinsed with 750 µL of 70% ethanol and centrifuged at 6000 rpm for 2 minutes. After removing the supernatant and air-drying, the pellet was resuspended in 50 µL of TE buffer (Appendix B). The isolated genomic DNA was then run on 0.8% agarose gel to check the integrity of genomic DNA. The concentration and purity of isolated DNA were checked with BioDrop Touch UV/Vis spectrophotometer (BioDrop Ltd., UK).

### 2.6.2 Agarose Gel Electrophoresis

0.8% agarose gel was prepared with TAE buffer (Appendix B), and ethidium bromide was added with a final concentration of 0.5 µg/mL in the molten gel. While loading the samples to gel, loading dye (6X) was mixed with DNA samples. Electrophoresis was performed at 100 Volts for 50 minutes. The DNA bands were photographed with Vilber Lourmat Gel Imaging System (Vilber Lourmat Sté,

France). GeneRuler 1 kb DNA ladder (ThermoFisher Scientifics, MA, USA) (Appendix D) was used to check the size of genomic DNA bands.

### 2.6.3 Whole Genome Resequencing and Identification of Variants

Genomic DNA from *B. subtilis* PY79 and OGU1 were shipped to South Korea for whole-genome resequencing analysis, which was made by Macrogen Inc. (Seoul, South Korea). After quality control of DNA isolates, the sequencing library was prepared with random fragmentation of sample followed with 5' and 3' adapter ligation. These adapter-ligated fragments were then amplified into distinct, clonal clusters through bridge amplification. These templates were sequenced with Illumina Truseq in 2x 150 bp paired-end settings. This sequencing resulted in raw data production with an average coverage of 1-2 Gb per sample.

Adapter trimming and quality filtering were performed to decrease possible bias in the analysis. Then, the processed data were aligned to the reference genome of the Bacillus Genetic Stock Center (BGSC) deposited *B. subtilis* PY79 (with GenBank accession no. CP006881.1, the same strain that was used in our lab over almost 30 years). This alignment, SNPs, and small indel analyses were performed with the Burrows-Wheeler Aligner software package (Schroeder and Simmons, 2013). The genomic data of OGU1 and its parental PY79 were deposited [with GenBank accession no: QLNM00000000.1 (for OGU1) and WOWX00000000 (for PY79)]. To remove duplicated reads and find further variant information Picard and SAMTools programs were used. Identified variants were then annotated, and their locations were marked.

## 2.7 IPTG induction of OGU1 cultures

*B. subtilis* OGU1 cells growing in 100 mL of PA medium were induced with 1 mM of IPTG at 15$^{th}$ hour of the growth. These induced cells were then collected at 16$^{th}$ hour of growth with centrifugation at 4000 rpm for 10 minutes.

## 2.8 Chemical Complementation of OGU1 with Bacilysin Concentrate

### 2.8.1 Preparation of Bacilysin Broth Concentrate

To obtain bacilysin dipeptide in a protein-free and concentrated form as "bacilysin broth concentrate", first *B. subtilis* PY79 cells were grown in 100 mL of PA medium for 16 h at 37°C (200 rpm) starting with an initial $OD_{595}$ around 0.1 as previously described. Then, broth or bacilysin concentrate was prepared by following the procedure described by Roscoe and Abraham (1966). Initially, broth containing bacilysin molecule was separated from bacterial cells with centrifugation at 10000 rpm for 10 minutes. Then, this supernatant was mixed with acetone with a final concentration of 70% (v/v) and centrifuged at 10000 rpm for 5 minutes. This acetone addition and centrifugation, known as acetone precipitation, was repeated one more time. Following that, the supernatant fluid was concentrated by using a rotary evaporator (Büchi R-200) in Central Laboratory at Biological Sciences Department, METU. The concentrate was then sterilized by double filtration with membrane filters (having 0.45 and 0.22 μm pore sizes) and stored at -20°C. As a negative control, the broth concentrate of the *B. subtilis* OGU1 was also prepared with the same method.

### 2.8.2 UPLC-MS Analysis

To check the presence of bacilysin molecule in broth concentrates of *B. subtilis* strains, ultra-performance liquid chromatography-mass spectrometry (UPLC-MS)

analysis was performed in MOBGAM/ITU, Istanbul. First, 200 µL of broth concentrates were extracted with 50 µL butanol, which was followed by lyophilization. Before analysis, lyophilized samples were dissolved in 10 µL of the solvent (H2O: 1% formic acid + 5% acetonitrile). 7.5 µL of this was injected into an Acquity Peptide BEH C18 column (Waters Corp, MA, USA) (2.1 mm x 100 mm), which is equilibrated with the solution (1% of acetonitrile, 0.1% formic acid and water) with 0.2 mL/minute flow rate and the temperature of the column kept at 65°C. Elution was performed with a gradient of solution A (0.1% formic acid) and solution B (acetonitrile). The gradient of these solutions was: 1 minute with 1% of solution B, 10 minutes with 1-40% of solution B, 1 minute with 40-80% of solution B which is kept for 2 minutes, 50 seconds with 80-1% of solution B and finally 10 minutes of 1% solution B re-equilibration before next sample. The analysis was performed at the positive detection mode. In this analysis, Acquity UPCL H-Class and Synapt G2-Si HDMS systems were used (Waters Corp, MA, USA).

### 2.8.3 Chemical Complementation

The bacilysin concentrate was added to 100 mL of growing cultures of OGU1 (either in PA or sporulation medium (DSM) (Appendix A)) at 7$^{th}$ hour of the incubation. The final concentration of bacilysin was adjusted to 150 Units/mL, and the incubation was continued at the same conditions. As the negative control, the broth concentrate of OGU1 was added at the same volume to another OGU1 culture simultaneously.

### 2.8.4 Determination of Growth and Bacilysin Levels

For the determination of growth and bacilysin levels, the samples were drawn from *B. subtilis* PY79, OGU1, and also OGU1 cultures supplemented with broth concentrates, which were all growing in PA medium at 37°C (200 rpm). First

samples were drawn at the 3rd hour of growth, and samples were collected until 17th hour at two hours intervals.

Bacterial growth was measured as the absorbance of the samples at 595 nm with the Multiscan Spectrophotometer (ThermoFisher Scientifics, MA, USA). Before the measurement, cultures were diluted with fresh PA medium in order to obtain appropriate absorbance values. Then, these dilution factors were multiplied with $A_{595}$ values.

Bacilysin activity in collected samples was determined with paper disc-agar diffusion assay, as described in Özcengiz *et al*. (1990). First, collected culture fluids were separated from bacterial cells with centrifugation at 10000 rpm for 10 minutes, which was followed by membrane filtration. Then, 20 µL of these culture fluids were added onto sterile paper discs, which were then dried at 50°C for five minutes. After adding 20 µL acetone, these disks were allowed to air-dry near the flame. Meanwhile, the assay microorganism, *S. aureus* ATCC 9144 grown overnight in LB medium was incorporated into the molten bioassay medium described by Mah *et al*. (1967) (Appendix A) at a final concentration of approximately 8 x $10^4$ CFU/mL. Treated paper-discs were then placed onto these agar plates carefully. After overnight incubation at 37°C, the diameter of inhibition zones was recorded. For every time point, the bioassays were with two technical replicates.

As a pure bacilysin molecule is not available, erythromycin was used to generate a standard curve for relative antibiotic activity estimation (Sakajoh *et al*., 1987). For this purpose, different concentrations of erythromycin (ranging 25 to 200 µg/mL or 0.5 – 4 µg/disc) were applied to paper discs. The inhibition zone generated by 1.25 µg/mL of erythromycin was arbitrarily defined as the inhibition zone produced by

one unit/mL of bacilysin. Accordingly, a calibration curve was generated from the erythromycin standards (Fig. 2.1).

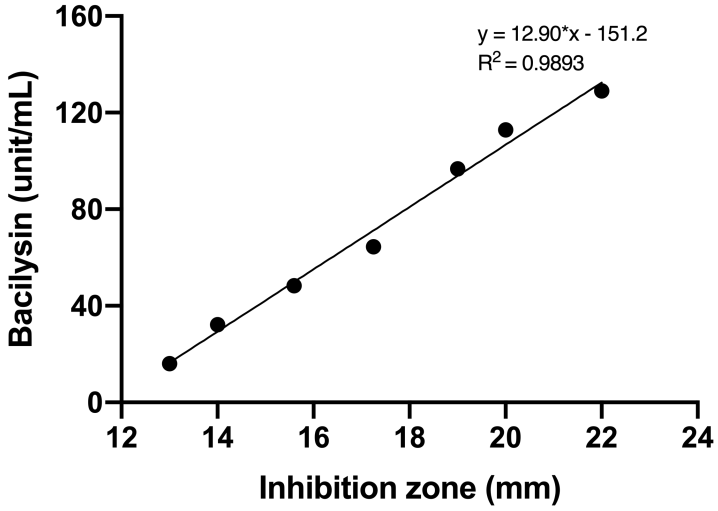


**Figure 2.1.** Calibration curve for bacilysin activity based on erythromycin standards

## 2.9 Sporulation and Germination Procedures

### 2.9.1 Mature Spore Purification

For the induction of sporulation, three *B. subtilis* OGU1 (two of them to be supplemented with broth concentrates) and one PY79 culture were prepared in 100 mL of Schaeffer's sporulation medium or Difco sporulation medium (DSM) (Schaeffer *et al.*, 1965) (Appendix A) with an initial $OD_{595}$ of 0.1. These four cultures were grown at 37°C (200 rpm) for 72 h for complete sporulation.

Spore purification was performed with the procedure that was proposed by Nicholson and Setlow (1990). 40 mL of *B. subtilis* cultures grown in DSM medium were first harvested with centrifugation at 14000 rpm for 10 minutes (4°C). The pellet was washed with 10 mL 1 M KCl/0.5 M NaCl solution (Appendix B) and centrifuged again. Then, the pellet was resuspended in 10 mL of Tris-Cl buffer (50

mM, pH 7.2) (Appendix B) with 50 µg/mL lysozyme, and incubated at 37°C for one hour. Following that incubation, spores were cleaned with alternate centrifugations (14000 rpm, 10 minutes) and washed sequentially with the followings: NaCl solution (1 M), deionized water, SDS solution (0.05%), TEP buffer (50 mM Tris-Cl, pH 7.2 with 10 mM EDTA and 2 mM PMSF) (Appendix B) and finally three washes with deionized water. After the final wash, spores were resuspended in cold deionized water and stored at -20°C. These spores were periodically, twice a month, centrifuged and resuspended in fresh and cold deionized water for storage.

### 2.9.2 Determination of Spore Resistance

In order to determine the resistance of mature spores for various agents, the protocols described by Nicholson and Setlow (1990) were used.

#### 2.9.2.1 Chloroform Resistance Assay

In a sterile 1.5 mL micro-volume centrifuge tube, 450 µL of purified spores and 50 µL of chloroform were mixed in a vortex. In another tube, 450 µL of spores were mixed with 50 µL of sterile potassium phosphate buffer (10 mM, pH 7.4 with 50 mM KCl and 1 mM $MgSO_4$) (Appendix B) as negative control. Then, these tubes were incubated at room temperature for 10 minutes. Next, 10-fold serial dilutions of the tube contents in potassium phosphate buffer were prepared and 100 µL of these dilutions were plated on LB agar plates in triplicates. After overnight incubation at 37ºC, colonies were counted and the survival ratio was calculated as the ratio of CFU/mL between the chloroform treatment and the control.

#### 2.9.2.2 Heat Resistance Assay

For heat resistance assay, 500 µL of purified spores were heated at 80ºC for half an hour. As a negative control, another 500 µL of spores were kept at room temperature.

These samples were then serially diluted in potassium phosphate buffer and 100 µL aliquots from these dilutions were plated onto LB agar in triplicates. The survival ratio was calculated as the ratio of CFU/mL between the heat treatment and the control.

#### 2.9.2.3 Lysozyme Resistance Assay

400 µL of purified spores were mixed with 100 µL of the lysozyme solution (final concentration 250 µg/mL) and kept at 37ºC for 10 minutes. As a negative control, 100 µL of potassium phosphate buffer was used instead of the lysozyme solution. After 10 minutes of incubation, 10-fold serial dilutions were made with potassium phosphate buffer and 100 µL aliquots from these dilutions were plated onto LB agar plates in triplicates. The survival ratio was calculated as the ratio of CFU/mL between the lysozyme treatment and the control.

#### 2.9.2.4 Statistical Analysis

The mean values of the survival ratios were compared with the ordinary one-way ANOVA in GraphPad Prism 8.0 software (GraphPad, CA, USA). When the difference between mean values was significant, Tukey's Honest Significance Test (HSD) was applied for multiple comparisons in the same software. The level of the significance was stated as * ($p < 0.05$), ** ($p < 0.01$) and *** ($p < 0.001$) in the graphs.

### 2.9.3 Morphology of Sporulating Colonies

Cultures after overnight growth in PA medium (including supplemented ones) were diluted 100 times in fresh PA medium. Then, 1 µL of these dilutions were spotted on DSM agar plates to characterize colony morphologies of sporulating *B. subtilis* cells. These plates were incubated at 30ºC for 18 days. Images of these plates were

taken daily with Epson Perfection V750 Pro scanner (Epson Corp, Japan) to record the pigmentation and colony morphologies.

### 2.9.4 Spore Germination

The germination profile of the purified spores was assessed as in Nicholson and Setlow (1990). First, spores were diluted with 10 mM Tris-Cl (pH 8.4) (Appendix B) to give a final $OD_{585}$ around 0.8. Then, these spores were activated with 30 minutes incubation at 70ºC. Immediately after this activation, 500 µL of spores were mixed with either minimal (Spizizen Minimal Medium, SMM) (Appendix A) or complex (2xYT medium) (Appendix A) germination media. As soon as these were mixed, $OD_{585}$ was measured and recorded as $t_o$. These solutions were incubated at 37ºC for 150 minutes, and $OD_{585}$ was measured at every 10 minutes. Measurements were normalized according to $t_o$, and expressed as relative $OD_{585}$ percentages with standard deviations. A 60% decrease in relative $OD_{585}$ was accepted as the time required for germination of whole spores as described by Atluri *et al.*, 2006.

## 2.10 Analysis of Spore Coat Proteins

### 2.10.1 Extraction of Spore Coat Proteins

For the extraction of spore coat proteins from mature spores, the procedure described by Takamatsu *et al.* (2000) was used. Purified spores were centrifuged at 14000 rpm for 10 minutes, and washed with 10 mM sodium phosphate buffer (pH 7.2) (Appendix B). Then, the centrifugation was repeated and pellets were resuspended in 100 µL of sodium phosphate buffer supplemented with 1% lysozyme (called lysozyme buffer). After incubation at room temperature for 10 minutes, spores were washed again with 10 mM sodium phosphate buffer (pH 7.2). Then, spore pellets were resuspended in 100 µL of SDS-PAGE loading buffer (Appendix B) and boiled for 10 minutes to solubilize spore coat proteins. The concentration of spore coat

proteins was determined by measuring the absorbance of the sample at 280 nm ($OD_{280}$).

### 2.10.2 SDS-PAGE and Coomassie Blue R-250 Staining

The original procedure of Laemmli (1970) was modified to prepare sodium dodecyl sulfate-polyacrylamide gels. The contents of these gels are given in Table 2.1. As the samples were already dissolved in loading buffer, they were directly loaded into the gel. PageRuler Plus Prestained Protein Ladder (ThermoFisher Scientifics, MA, USA) (Appendix D) was used as the protein ladder, and the gel was run at constant 100 V in 1X SDS-PAGE running buffer (Appendix B) in a Mini-Protean electrophoresis apparatus (Bio-Rad Labs. Inc., CA, USA) until the dye front was at the end of the gel.

**Table 2.1.** Content of SDS-polyacrylamide gels

| | **The Stacking Gel** 0.125 M Tris, pH 6.8 | **The Separating Gel** 0.375 M Tris, pH 8.8 |
|---|---|---|
| Monomer concentration | **4.5%** | **15%** |
| Acrylamide/bis-acrylamide solution (30 %) | 0.975 mL | 5 mL |
| $dH_2O$ | 4.575 mL | 2.35 mL |
| 0.5 M Tris-HCl (pH 6.8) buffer | 1.875 mL | - |
| 1.5 M Tris-HCl (pH 8.8) buffer | - | 2.5 mL |
| 10% (w/v) SDS solution | 75 µL | 100 µL |
| 10% ammonium persulphate (APS) solution | 37.5 µL | 50 µL |
| N,N,N',N'-Tetramethyl ethylenediamine (TEMED) | 7.5 µL | 5 µL |
| Total Monomer | 7.5 mL | 10 mL |

Following the electrophoresis, the gel was stained in 200 mL of Coomassie Blue R-250 stain (Appendix B) for 3 hours at room temperature with shaking. Then, the gel was destained with the destaining solution (Appendix B) for at least 24 hours.

### 2.10.3 Sample Preparation and Analysis with Mass Spectrometry

Tryptic in-gel digestion of selected bands from SDS-PAGE gels was done in Genome and Stem Cell Center (GenKök), Kayseri. First, the selected protein bands were excised from the gel with a scalpel on a sterile glass surface. Then, these bands were chopped into smaller pieces (1x1 mm) and transferred into low-protein binding collection tubes (1.5 mL). A 700 µL of washing solution (Appendix B) was added into each tube, and these tubes were incubated at 37ºC for 15 minutes (900 rpm) for decolorization. After a quick spin-down, the washing solution was removed from the tube. This washing step was repeated until the gel pieces were completely destained. These pieces were then dried with Eppendorf Concentrator plus (Eppendorf AG, Germany) at 30ºC for at least half an hour. As the next step, dried gel pieces were covered with 100 µL of 1x Trypsin solution (Appendix B) and incubated for 15 minutes. After this incubation, excess trypsin solution was removed, and swollen gel pieces were incubated at 37ºC overnight.

For the peptide recovery, 50 µL of solution A (3% acetonitrile, 0.4% formic acid) was added onto swollen gel pieces. Samples were sonicated in an ice-cold water bath for 5 minutes, which was followed by incubation on ice for another 5 minutes. Then, the sonication was repeated, and samples were centrifuged at 10000 rpm for 30 seconds. The supernatant was transferred into a new low-binding collection tube. With the addition of 50 µL of solution B (50% acetonitrile, 0.4% formic acid), sonification, and centrifugation steps were repeated, and the supernatant was transferred into the same low-binding collection tube. The same steps were repeated

after the addition of solution C (100% acetonitrile), and the supernatant was collected into the collection tube. This collected supernatant containing the peptides of interest was dried with Eppendorf Concentrator plus (Eppendorf AG, Germany) at 30ºC. These samples were stored at -20ºC until usage.

Dried samples were dissolved in 10 µL of sample solution (30% acetonitrile, 0.1% trifluoroacetic acid). Then, this sample was mixed with saturated alpha-cyano-4-hydroxy cinnamic acid (HCCA) matrix (1/10, sample vol./matrix vol.). 0.5 µL of this mixture was dried on the ground steel plate with the dried droplet method (Karas and Hillenkamp, 1988). Mass spectrometric analysis of these plates was done with Ultraflextreme MALDI-TOF (Bruker Daltonics, MA, USA) at the HUNITEK center of Hacettepe University, Turkey. The device was calibrated with Bruker Peptide Calibration Mix (ranging from 700 – 3500 Da), and the analysis was done at reflection positive mode. Mass spectra were gathered by using Bruker flexAnaysis program.

### 2.10.4 Protein Identification

The mass spectra (including peak lists for each band analyzed) were analyzed with MASCOT software (Matrix Science Inc., MA, USA) using its Peptide Mass Fingerprint (PMF) and MS/MS Ion Search engines against *B. subtilis* PMF. MASCOT output (including protein accession numbers) was searched in the SwissProt/UniProtKB database to identify proteins (http://www.uniprot.org/).

## 2.11 RNA Isolation and Real Time Quantitative PCR

After collecting the cells into 2 mL microcentrifuge tubes with centrifugation at 4000 rpm for 10 minutes, total RNA of 16$^{th}$ hour cultures of *B. subtilis* PY79 and OGU1, as well as the IPTG-induced OGU1, were isolated by Macherey-Nagel Nucleospin®

RNA isolation kit. Manufacturer's instructions were modified at two steps for better field: (i) in the cell lysis step, 450 µL of RA1 buffer was used, (ii) 450 µL of 70% (v/v) ethanol was used in the following step. Total RNA samples were eluted with nuclease-free water and stored at -80°C until further usage. 1 µg of total RNAs was used in cDNA synthesis with BioRad iScript® cDNA kit, which contains random hexamer and oligo(dT) primers. The reaction setup for cDNA synthesis was: (i) annealing of primers at 25°C for 5 minutes, (ii) extension of cDNA strands at 46°C for 30 minutes, and finally, (iii) inactivation of the reverse transcriptase enzyme at 95°C for 5 minutes. Newly synthesized cDNA samples were stored at -20°C until further usage. Real-time quantitative PCR (RT-qPCR) reaction was performed using BioRad SYBR Green Supermix (2x) (BioRad) with 200 nM of forward and reverse primers and 1 uL of cDNA. For the no-template control sample, 1 uL of nuclease-free water was used instead of cDNA. The list of primers used in RT-qPCR reactions is given in Table 2.2. The reaction was performed in a BioRad CFX Connect Real-Time PCR machine, and Ct values were calculated using a relative standard curve. Relative fold change was calculated by the ΔΔCt method with corrected efficiencies (Rao *et al.*, 2013) of primers. The relative mRNA expression of RNA polymerase beta subunit coding *rpoB* was selected as house-keeping control (reference gene). The student's t-test was performed to examine the significance of relative fold changes obtained. The application of this test, as well as the plotting of RT-qPCR data, were made with GraphPad Prism 8.0 software (GraphPad, CA, USA). The level of the significance was stated as * ($p < 0.05$), ** ($p < 0.01$) and *** ($p < 0.001$) in the graphs.

**Table 2.2.** Primer sets used for real-time quantitative PCR.

| Gene | Sense Primer(5′-3′) | Antisense Primer(5′-3′) |
|---|---|---|
| *rpoB* | TGAACATCGGGCAGGTATTG | GTTTCCCAGACATCCTCTTCTC |
| *dpaA* | GGAACGATCATGCTGGCTAT | AACTTCTTGCCCCCACTTTT |
| *dpaB* | TTTTATTCCATTCGGGCAAG | GTGCATGAGTGCCTTTTCAA |
| *cotA* | GCCGCTTCTTATCACAGACC | CCGGAACGATTGAAGGATTA |
| *ytfJ* | CGAAAAGAAATCGGAAGACG | ATTCTGATGCCTGTCGATCC |
| *yhcQ* | TCAAAACAGGAAGCGAACCT | ATTTGTCGGCTGATGATGCT |
| *yxbC* | GAACTCGCTGTAAATCATCAATCC | GGTCAATGTCTCTGCGTTCT |
| *cheV* | AGTTTGGCGTGGGTGAAA | CTTCTACATGCTGATGGGAGTG |
| *resD* | TGAAGCGGGAACAGATGATT | GAGGTTTGGGATGCTCTTCT |
| *degU* | GCGGTTAAAGTAGTGGCTGA | TGTTGAGGGTGTGCAGAAA |
| *degS* | CAGAGGACGGATTCCAAGAAA | CAAGGGCCATCGGTCTTAAA |
| *yugI* | GCTGCTCAAGTAAGCGAAGA | TAAGGTCTTTGCGGTTGGAC |
| *dnaK* | ATTCAGGGCGGCGTTATC | TGTTGCGGTCGATCAGTTT |
| *yceE* | GGCATCACAGTGACCATTCA | CTCCCAAATCGAAGCGAAGA |
| *ytkL* | ACCTTGCTTTCCTTCCGATT | TTCGATGACCGGGAATGTAT |
| *yxbC* | GAACTCGCTGTAAATCATCAATCC | GGTCAATGTCTCTGCGTTCT |
| *albE* | TCACTGATCCCTTCTCCTACTC | GAATCGCCTTTATCCTGTCTCTC |
| *ispA* | AGCTTCCTGAGGGCATTAAA | GTCGCAGCCTGTGTCTAATA |
| *oppD* | CTGCTGGAATTAGTCGGTATCC | GCAATGACAACCCTCTGTCT |
| *glmS* | GCTCGTGAATACCTGACTGTATC | CCTTCTGCCTGGATGTAAGAAA |
| *glnA* | GACCTCGGCTTCAGTGATTT | TCCGCCTTTGTCGTTTAGTT |
| *bacA* | CTTCTACAAGGGCTGAACAGTAA | GCATGTGGGACGATGGTATATT |
| *bacB* | CTGATGATGACTGTCGGAGATG | TCTTGGTCTGTGTCGTTTCTT |
| *bacC* | ATGTGGTTGTGGCGGATATT | TCGTCTGTGATGTCCGTTTG |
| *bacD* | CATAACAAGCCTGAGGAAGAGG | CGCCATCGGAGCAATGAATA |
| *bacE* | GTCATGTCGTCCTTGGAATCA | CAGATCCGCCTAACAGAAAGAG |
| *bacF* | GGGAATACGGAGTGACAATCAA | GATTCGGGACTAAAGCGATGT |
| *bacG* | TGTCGGAAATCTGTGGAAAGAA | GGAGCAAGCTGGATGGAAATA |

# CHAPTER 3

# RESULTS AND DISCUSSION

## 3.1 Whole Genome Resequencing (WGRS) Analyses of *B. subtilis* PY79 and OGU1

Stochastic mutations occur in cells of every organism as the DNA replication process is not error-free. Most of these errors can be fixed with proofreading and DNA repair mechanisms, yet, with every cell division, some mutations could be inherited into the offspring (Crow, 1997). The rate of these mutations is nearly 9 x $10^{-9}$ per base pair per cell division for sporulating *B. subtilis* cells (Maughan and Nicholson, 2004). *B. subtilis* contains 4.2 Mb (4,214,810 base pairs) in its whole genome (Kunst *et al.*, 1997), thus it is expected to undergo 0.038 mutations in its genome at every cell division.

*B. subtilis* PY79 has been extensively used in our laboratory for nearly 40 years and subjected to many consecutive passagings. Likewise, its bacilysin non-producer derivative *B. subtilis* OGU1 was constructed in 2008 and extensively used for almost 12 years. This extensive usage and consecutive passaging could alter the genomes of PY79 and OGU1. Therefore, in order to confirm that proteomic and phenotypic changes observed OGU1 strain were solely due to the loss of bacilysin, whole-genome resequencing analyses of our lab-adapted PY79 and also OGU1 were performed against the reference genome of Bacillus Genetic Stock Center (BGSC)-deposited *B. subtilis* PY79. http://subtiwiki.uni-goettingen.de/ was the website extensively used to address several indel and base substitution mutations revealed by WGRS analysis.

### 3.1.1 Quality Control of Genomic DNA Isolates

The genomic DNA isolate should be both pure and intact for a successful whole-genome analysis. The purity of the genomic DNA obtained from lab-adapted PY79 and OGU1 was assessed with A260/A230 and A260/A280 ratios, which are helpful for detection any residual guanidine and protein contamination, respectively. Pure DNA samples have an A260/A230 ratio in the range of 2.0-2.2 and an A260/A280 ratio of approximately 1.8. The A260/A230 ratios of PY79 and OGU1 genomic DNA isolate were 2.121 and 2.134, and their A260/A280 ratios were 1.831 and 1.856, respectively. Genomic DNAs were also run on an agarose gel, and as can be seen in Figure 3.1, both were intact.

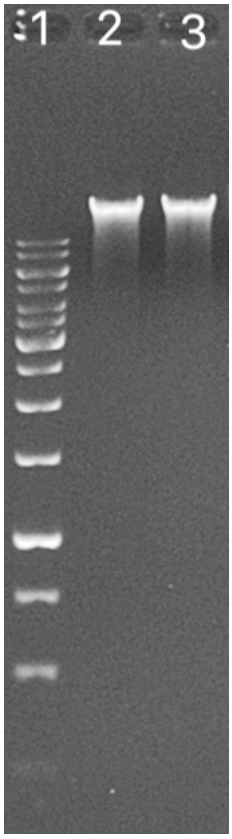


**Figure 3.1.** Genomic DNA of PY79 and OGU1. Lane 1: Marker (GeneRuler 1 kb DNA ladder), Lane 2: Genomic DNA of PY79, Lane 3: Genomic DNA of OGU1.

### 3.1.2 Identification of Gene Variants

Whole genome resequencing analyses revealed a total of ten gene variations in our lab-adapted PY79 and OGU1. Three base substitutions and one deletion conserved in both strains, two base substitutions were unique to OGU1, and also two insertions, one deletion and one duplication were unique to our lab-adapted PY79. The nature and the possible effects of these mutations will be discussed in the following sections.

#### 3.1.2.1 Common Gene Variants of *B. subtilis* PY79 and OGU1

As mentioned above, the exact three base substitutions and also one deletion were identified in both our lab-adapted PY79 and OGU1. These mutations must have occurred in the genome of PY79 before the construction of OGU1, and the latter inherited all of them. The position of these mutations in the genome, their nature and outcomes are given in Table 3.1.

**Table 3.1.** The position and nature of mutations conserved in PY79 and OGU1 with their outcomes.

| Position | Reference | Alteration | Gene Name | Amino Acid Change | Type of Mutation |
|---|---|---|---|---|---|
| 1705156 | G | A | *ylxY(swsB)* | $P^{264}K$ | Missense |
| 3439792 | ATTTTTTTTT | ATTTTTTTT | *swrAA* | -1 Frameshift | Deletion |
| 3740961 | C | T | intergenic region | - | - |
| 3801088 | G | A | *yxkH* | $V^{168}M$ | Missense |

One of the three base substitutions was identified in the intergenic region between *ywcC* and *slrA* genes. *slrA* gene encodes the anti-repressor SlrA that antagonizes the transcriptional regulator SinR, which controls the biofilm formation by repressing

*eps* and *yqxM* operons (Kobayashi, 2008). *ywcC* gene encodes for the repressor YwcC that represses SlrA production, therefore negatively regulates biofilm formation (Chai *et al.*, 2009). The intergenic region contains a non-coding RNA, *S1474*, positioned at 5'UTR of the *slrA* gene (Zhu and Stülke, 2018). However, the base substitution identified in this study is not related to this non-coding RNA, hence would not affect either *slrA* or *ywcC* genes since it does not encode a known transcript.

Another base substitution was identified to be within *ylxY* gene, which encodes YlxY protein. This protein was renamed recently as SwsB, and it was shown that the presence of SwsB is required for CwlJ-dependent germination in *B. subtilis*. In fact, spores lacking SwsB had a 4% reduced germination when compared to the wild type (Amon *et al.*, 2020). The mutation identified was a missense mutation, and it changed the 264th residue from proline into lysine, which could alter the structure and the functioning of SwsB significantly. Nonetheless, as this mutation is conserved in both strains, any possible functional change in SwsB would not affect bacilysin biosynthesis.

The third base substitution was identified to occur in the *yxkH* gene, which encodes a putative polysaccharide deacetylase. The exact function of its product and also its regulation is not yet known and remains to be elucidated (Yoshida *et al.*, 2000). The mutation identified was again a missense mutation, and it altered the 168th residue of the gene product from valine into methionine. As the exact function of the gene product is unknown, this mutation's possible role is also hard to determine.

The *swrAA* gene encodes for a swarming motility protein that activates DegU, and is one of the genes that are responsible for swarming motility in *B. subtilis* (Kearns and Losick, 2003). This gene is active in undomesticated *B. subtilis* strains which

can swarm; but it is inactive in the laboratory strains like *B. subtilis* 168 that are unable to swarm. This inactivity in laboratory strains is due to a single base pair insertion of thymidine which results in nine consecutive thymidine (Kearns *et al.*, 2004). Interestingly, the deletion of thymidine detected in this study appears to happen at the same site, resulting in a wild type sequence.

#### 3.1.2.2 Unique Gene Variants of *B. subtilis* PY79

The whole-genome resequencing analysis of our lab-adapted PY79 compared to BGSC-deposited PY79 also revealed some unique mutations which has most likely occurred after the construction of OGU1. The position of these mutations in the genome, their nature and the outcomes are given in Table 3.2.

**Table 3.2.** The position and nature of mutations that are unique to PY79 with their outcomes.

| Position | Reference | Alteration | Gene Name | Amino Acid Change | Type of the Mutation |
|---|---|---|---|---|---|
| 1472755 | CGAAGA GGAAGA GGAAGA | CGAAGAGGA AGAGGAAGA GGAAGA | *fruA* | $E^{160}$ duplication, $N^{162}$ insertion | - |
| 1472770 | AGA | AGAGGACGA | | | |
| 2311880 | GAAAAA AAA | GAAAAAAA | *bmrU* | -1 Frameshift | Deletion |
| 2995871 | CGGGG | CGGGGG | *rrnB* | - | Insertion |

*fruA* encodes for FruA protein responsible for fructose uptake into the cell and phosphorylation of fructose into fructose-1-phosphate. As a protein of phosphotransferase system, FruA is indirectly involved in carbon catabolite repression (Reizer *et al*., 1999) and is a multi-pass membrane protein that contains a total of 635 amino acids and three domains (Marchadier *et al.*, 2011). Of these

domains, the PTS EIIA type-2 domain is positioned from the 5$^{th}$ to 149$^{th}$ residue, and upon its phosphorylation on histidyl residue (the 68$^{th}$), it transfers the phosphoryl group to the PTS EIIB type-2 domain. This domain spans from the 172$^{nd}$ to 267$^{th}$ residue, and it transfers the phosphoryl group to the substrate. The final domain, the EIIC type-2 domain positioned from the 301$^{st}$ residue to 635$^{th}$ residue, and it forms the translocation channel for fructose. Our mutated FruA protein contains additional two amino acids due to duplication of the $E^{160}$, which is immediately followed by the insertion of $N^{162}$. However, these residues do not belong to any aforementioned functional domain. Because protein domains are self-folding independent of the rest of the protein, the mutations detected in this gene would have no effect on the function of the protein.

*bmrU* encodes for a multidrug resistance protein, and its expression is under the control of $\sigma^B$. This protein does not have any identified regulatory role or pleiotropic role in *B. subtilis* (Petersohn *et al.*, 2001). Therefore, it is likely that the detected deletion in *bmrU* has resulted in a defective BmrU protein with no pleiotropic effect in our lab-adapted *B. subtilis* PY79.

Another mutation specific to our lab-adapted PY79 has occurred in the 16S rRNA coding gene as a base pair insertion, with no effect on essential cellular processes.

#### 3.1.2.3 Unique Gene Variants of *B. subtilis* OGU1

The whole-genome resequencing analysis of OGU1 revealed unique gene variants other than the inserted pMutin T3 vector sequence. To verify that those mutations has no relation with the proteomic and phenotypic differences in this bacilysin-silenced mutant, sporulation and germination in particular, the nature and possible effects of OGU1 specific mutations should be carefully examined. The position of

these mutations in the genome, their nature, and the outcomes are presented in Table 3.3.

**Table 3.3.** The position and nature of mutations that are unique to OGU1 with their outcomes.

| Position | Reference | Alteration | Gene Name | Amino Acid Change | Type of Mutation |
|---|---|---|---|---|---|
| 598957 | A | G | *rrnE* | - | - |
| 937764 | A | G | *prkA* | $R^{525}R$ | Silent |

Of two base substitution mutations, the one was identified in the *rrnE* gene that encodes for 16S rRNA. This mutation should have no effect on translation machinery, as the cells of OGU1 have no defect in their growth or survival.

The second base substitution was identified in the *prkA* gene that encodes for the PrkA serine/threonine protein kinase. PrkA is localized in the spore coat, and its absence leads to several defects in sporulation like the delay in the entry and the decrease in total spore number (Eichenberger *et al.*, 2003; Pompeo *et al.*, 2016). Although the exact mechanism is not known yet, PrKA increases the expression of $\sigma^K$, and its downstream genes, by directly or indirectly inhibiting the transcriptional repressor ScoC (Yan *et al.*, 2015). Otherwise, this mutation would complicate the present study, but fortunately, the base substitution in *prkA* results in a silent mutation.

In order to obtain OGU1, the pMutin T3 vector containing 420 bp of the *bacA* gene (designated as *bacA'*) was integrated into the genome of PY79 with a single cross-over event (Köroğlu *et al.*, 2011). The exact genetic outcome of this integration was also revealed with the whole genome resequencing analysis. The resulting genomic organization contains a *bacA':lacZ* fusion under $P_{bac}$ and the intact *bacA* operon

under the control of $P_{spac}$, a weak promoter that could be induced with IPTG (Yansura and Henner, 1984; Vavrová *et al.*, 2010) (Fig 3.2). The IPTG induction of OGU1 has resulted in 3- to 7-fold increase (1.2- to 1.7-fold increase with respect to PY79) in transcripts of *bacA, bacB, bacC, bacD, bacE* and *bacF* (Fig 3.3). On the other hand, the transcript level of *bacG* was not affected by this induction as it has its own promoter (Fig 3.2). Although *bacA* operon transcripts were overrepresented significantly with IPTG induction, no bacilysin was detectable by either bacilysin bioassay and UPLC-MS analysis (Fig 3.6, Fig 3.7) (Ertekin *et al.*, 2020). The sequences of the structural genes of the operon were completely identical to the reference sequences, thus the exact molecular mechanism causing this defect could not be elucidated. Aslight alteration in the ribosome-binding site could be one of the logical explanations. Overall, IPTG induction of OGU1 could not bring about genetic complementation of bacilysin production.

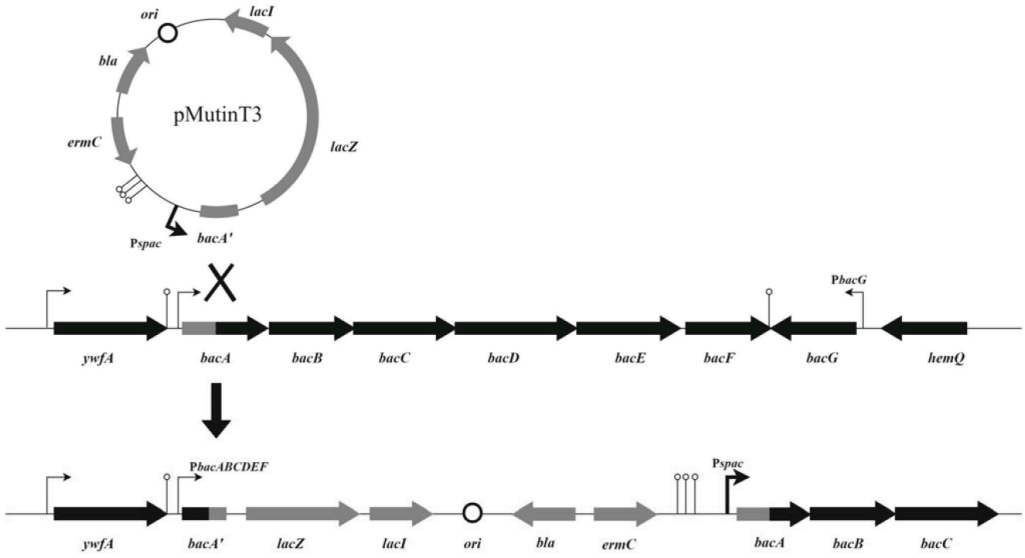


**Figure 3.2.** The genomic organization of the *bacA* operon and its flanking regions in OGU1 following the integration of the pMutin T3 vector (Ertekin *et al.*, 2020).

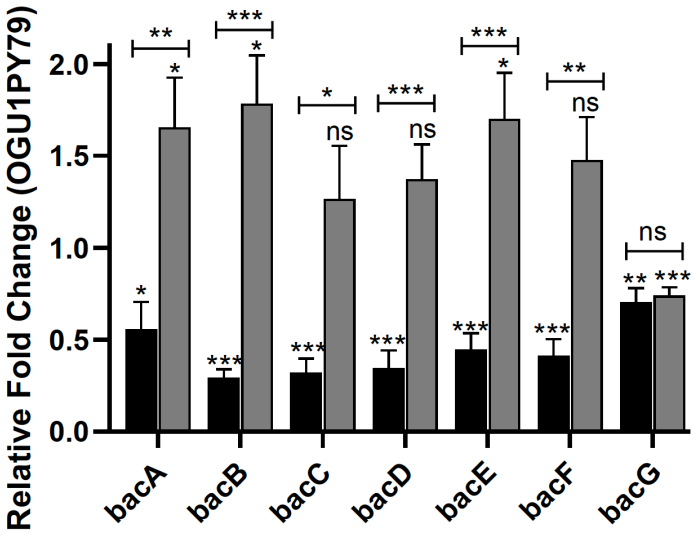


**Figure 3.3.** RT-qPCR results of *bacA* operon genes with (black bars) and without IPTG induction (grey bars) of OGU1 cells. The significance levels are represented as ns (no significance, $p > 0.05$), * ($p < 0.05$), ** ($p < 0.01$) and *** ($p < 0.001$). The significance levels shown on the brackets refer to a comparison between the expression in induced and uninduced cells whereas those shown just on the top of grey bars refer to a comparison between PY79 and IPTG-induced OGU1 cells.

## 3.2 Analysis of Bacilysin Broth Concentrates

Acetone precipitation is a popular method that results in aggregation of proteins by reduced hydration. Although proteins are insoluble in acetone, many small molecules like bacilysin could not be precipitated by acetone treatment (Burgess, 2009). Therefore, acetone precipitation aids us to get rid of proteins in broth concentrates that would interfere with the complementation process. Nonetheless, acetone-treated broth concentrate of PY79 would not have only bacilysin molecule, but also ComX and CSF which are small signal peptides of sporulation. Therefore, as a negative control for all complementation experiments, the broth concentrate of bacilysin non-producer OGU1 was also used in parallel.

Ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) was utilized to identify the presence or the absence of bacilysin molecule in broth concentrates, and also the culture supernatant of IPTG-induced OGU1. Bacilysin dipeptide has a molecular weight of 270.28 g/mol, and it gives a typical peak with the *m/z* of 271.1 in mass spectrometry (Walker and Abraham, 1970). When the broth concentrates of PY79, and OGU1 were analyzed, this peak was only detected in PY79 broth concentrate (Fig. 3.4, Fig. 3.5, Fig. 3.6). These results prove that acetone precipitation did not alter the presence of bacilysin in PY79's broth concentrate, and the broth concentrate of OGU1 could be used as the negative control as it did not have any detectable bacilysin molecule.

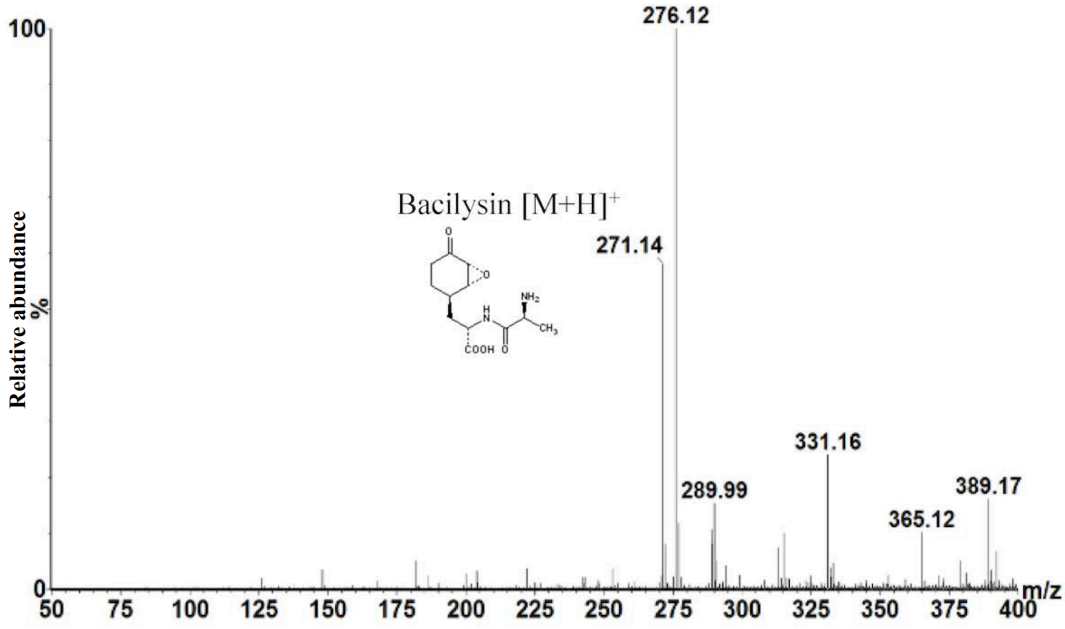


**Figure 3.4.** UPLC- mass spectrum of the broth concentrate of *B. subtilis* PY79.

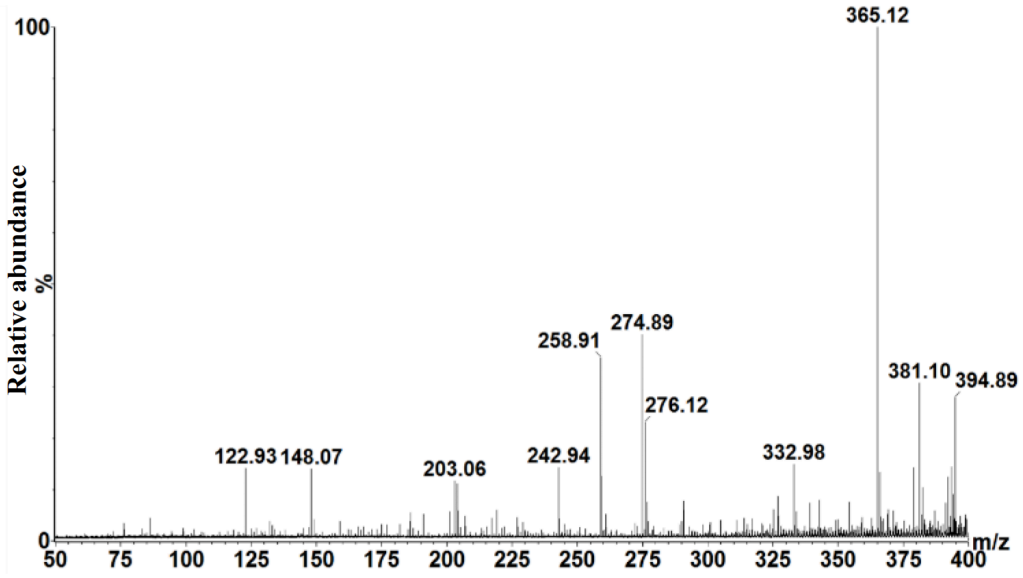


**Figure 3.5.** UPLC- mass spectrum of the broth concentrate of *B. subtilis* OGU1.

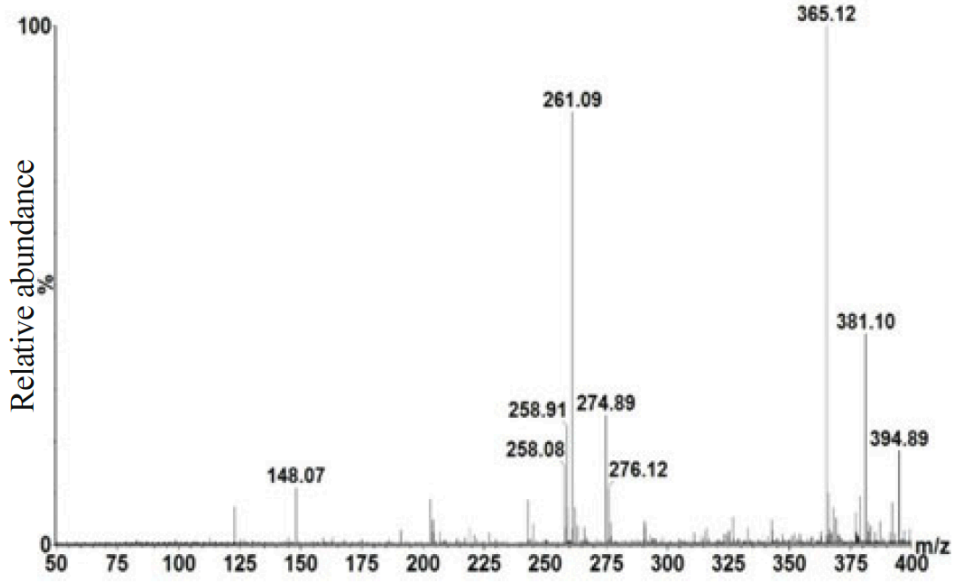


**Figure 3.6.** UPLC- mass spectrum of the culture supernatant of IPTG-induced *B. subtilis* OGU1.

For the complete functional complementation of OGU1 to occur, the bacilysin should be in its biologically active form. Therefore, the biological activity of broth concentrates and the culture supernatant of IPTG-induced OGU1 was tested with the paper-disc diffusion assay against *S. aureus* ATCC 9144. This assay contained two

technical replicates, and inhibition zones were only observed for broth concentrate of PY79. The diameter of these zones were 23.5 and 24 millimeters, which were then converted into bacilysin activity (U/ml) according to erythromycin standard curve given in Figure 2.1. The average bacilysin activity was 158.3 U/mL for the 16$^{th}$ hour broth concentrate of PY79, and this activity was consistent with those reported previously (Özcengiz *et al.*,1990; Köroğlu *et al.*, 2011). As seen in Figure 3.7, there was no inhibition zone for broth concentrate of OGU1, which further proved the inability of this strain to synthesize bacilysin.

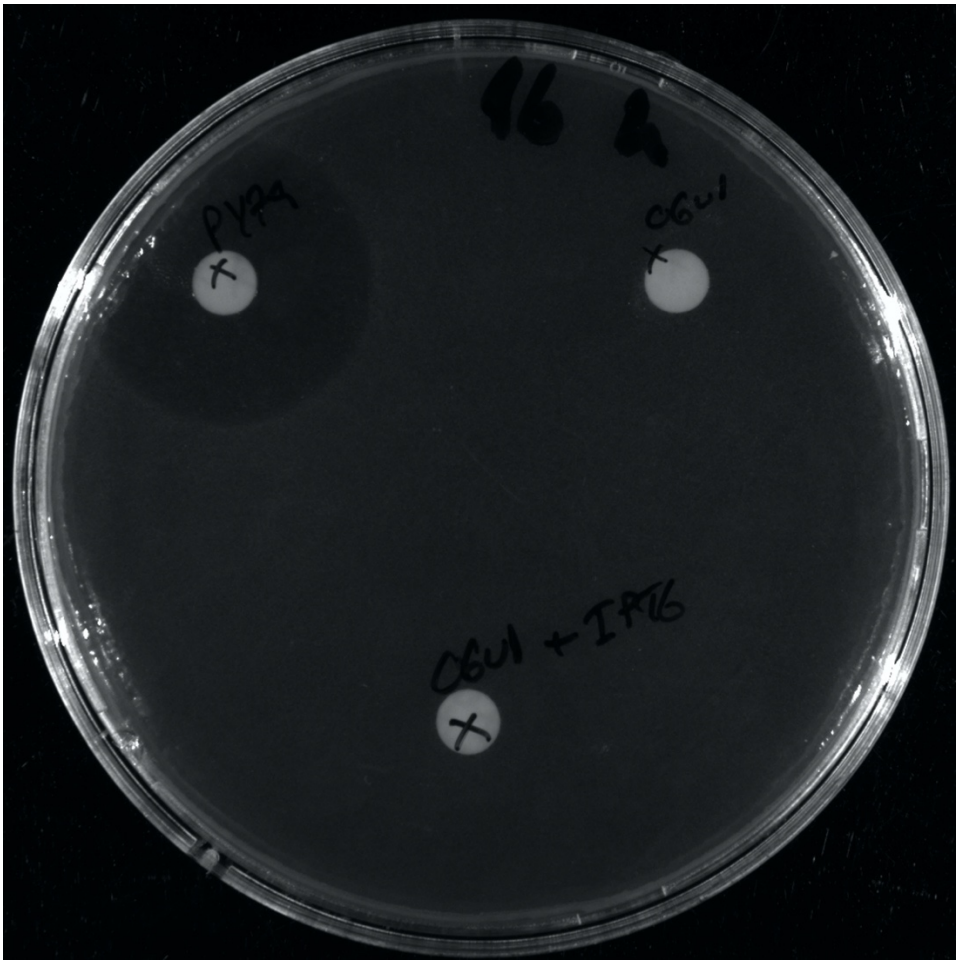


**Figure 3.7.** Bacilysin bioassay with the 16$^{th}$ hour broth concentrates of PY79 and OGU1.

## 3.3 The Effect of Broth Concentrate Supplementations on Growth of *B. Subtilis* OGU1 and Bacilysin Levels in Culture Fluid

In an earlier study (Özcengiz and Alaeddinoğlu, 1991) the broth concentrates containing bacilysin were added at different times of incubation (0, 3, 5, and 7 h) into the growing cultures of *B. subtilis* 168 in PA medium to investigate a possible feedback effect of bacilysin on its biosynthesis. The addition of broth concentrates provided 150 Unit/mL bacilysin (an amount that is typically produced at the beginning of the transition phase) to the growing cultures, and it slightly reduced the cultural growth when it was added at the $5^{th}$ hour of incubation or earlier. The addition of bacilysin after transition phase was not also suitable in the present study, as the sporulation event starts to take place upon entry into the stationary phase (Sonenshein, 2000). Hence the addition of broth concentrates was made at $7^{th}$ hour in order not to disturb the growth pattern and to provide bacilysin before the onset of stationary phase. The final activity of bacilysin added to OGU1 culture was 150 Unit/mL to mimic its production by PY79. The negative control, the broth concentrate of OGU1, was added at the same volume.

The growth curves of PY79, and three parallel OGU1 cultures, two of them supplemented with the broth concentrate of PY79 and its own, are shown in Figure 3.8.

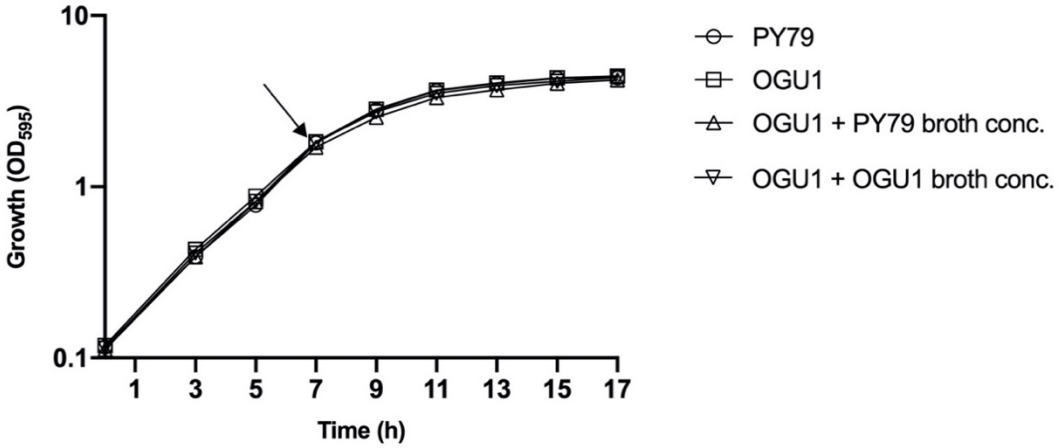


**Figure 3.8.** Growth curves of three parallel *B. subtilis* OGU1 cultures (two of them supplemented with PY79 or OGU1 broth concentrate) and *B. subtilis* PY79 in PA medium. The time of addition of broth concentrates is indicated by an arrow.

As seen in Figure 3.8, the growth pattern of OGU1 was not only identical to that of its parental strain PY79, but also was not affected by the addition of broth concentrates. The result of bacilysin bioassays is given in Figure 3.9.

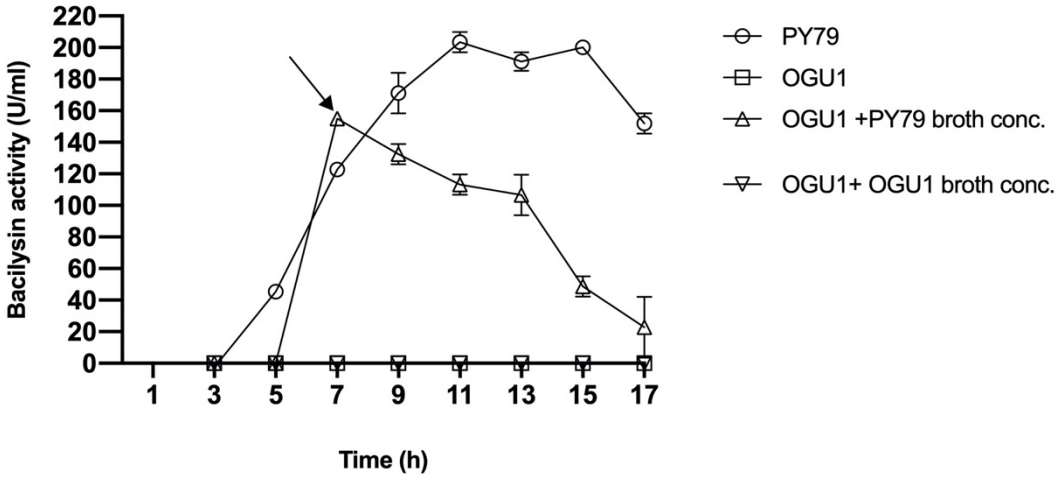


**Figure 3.9.** Bacilysin titers (U/mL) of three parallel *B. subtilis* OGU1 cultures (two of them supplemented with PY79 or OGU1 broth concentrate) and *B. subtilis* PY79 in PA medium. The time of addition of broth concentrates is indicated by an arrow.

The bacilysin activity in *B. subtilis* PY79 reaches its maximum upon entry into stationary phase (Özcengiz *et al.*, 1990; Köroğlu *et al.*, 2011), The pattern was the same in the present study (Fig. 3.9). The bacilysin activity reached its maximum of nearly 200 U/mL at $11^{th}$ of the growth which started to decline beyond 15 hours incubation.

In OGU1 cultures with or without OGU1 broth concentrate supplementation, there was no detectable bacilysin activity. On the other hand, in OGU1 culture supplemented with PY79 broth concentrate, the bacilysin activity was measured as 150 U/mL upon supplementation, as planned. However, bacilysin activity consistently decreased with time. Because, OGU1 is unable to produce any bacilysin, its gradual decrease in culture supernatant could not be compensated as in PY79 cultures.

The fate of externally-added bacilysin molecules remains to be elucidated. If we attribute a function to the molecule as a pleitropic regulator and/or a small signalling peptide, some of the molecules should be imported by *B. subtilis* OGU1 cells where, they interact with genetic elements or important regulatory proteins, as in PY79. Fluorescent labels are commonly used for tracking peptides, proteins, and also single amino acids (Sahoo, 2012), and if the unique L-anticapsin residue could be labeled, it would give crucial information regarding the fate of the bacilysin molecules added into OGU1 cultures. On the other hand, it is not likely that the molecule disintegrates by the time since it is known to be highly stable, even resistant to cleavage by proteases (Özcengiz and Öğülür, 2015).

## 3.4 Phenotypic Rescue of Defected Spore Phenotype of *B. subtilis* OGU1

Although the sporulation process results in almost the same sporulation efficiency of 90% for both OGU1 and PY79, OGU1 spores had several deficiencies regarding spore morphology, resistance, and germination. These defects and their complementation with bacilysin broth concentrate are discussed below.

### 3.4.1 Complementation of Spore Pigmentation

The *cotA* gene, initially identified as the *pig* gene, encodes for the 65 kDa CotA protein that locates at the outer spore coat, and its expression is regulated by $\sigma^K$ (Donovan *et al.* 1987). The absence of CotA protein leads to the loss of characteristic brownish pigmentation of spores but does not directly affect the resistance (Iichinska, 1960). CotA is shown to be a copper-dependent laccase that could react with a specific substrate, syringaldazine. This reaction results in melanin-like brown spore pigment production, which could provide UV resistance to mature spores (Hullo *et al.*, 2001).

CotA protein was significantly underrepresented in the 16$^{th}$-hour proteome of OGU1 compared to PY79, with both nanoLC-MS/MS and 2DE MALDI-TOF/MS analyses (Table 1.3) (Aras-Taşkın, 2010; Demir, 2013; Ertekin *et al.*, 2020). In fact, 2DE MALDI-TOF/MS analysis showed that the expression of this protein is decreased by 11.3-fold (Aras-Taşkın, 2010). As the relationship between the CotA protein and brown pigmentation is well known, OGU1 is expected to have spores that do not have proper pigmentation. In order to compare this possible brownish pigmentation, 1 µL of diluted cultures of PY79, OGU1, and also broth-concentrate supplemented OGU1 were spotted on the solid sporulation media. The pigmentation of spores which was recorded for 18 days are shown in Figure 3.10.

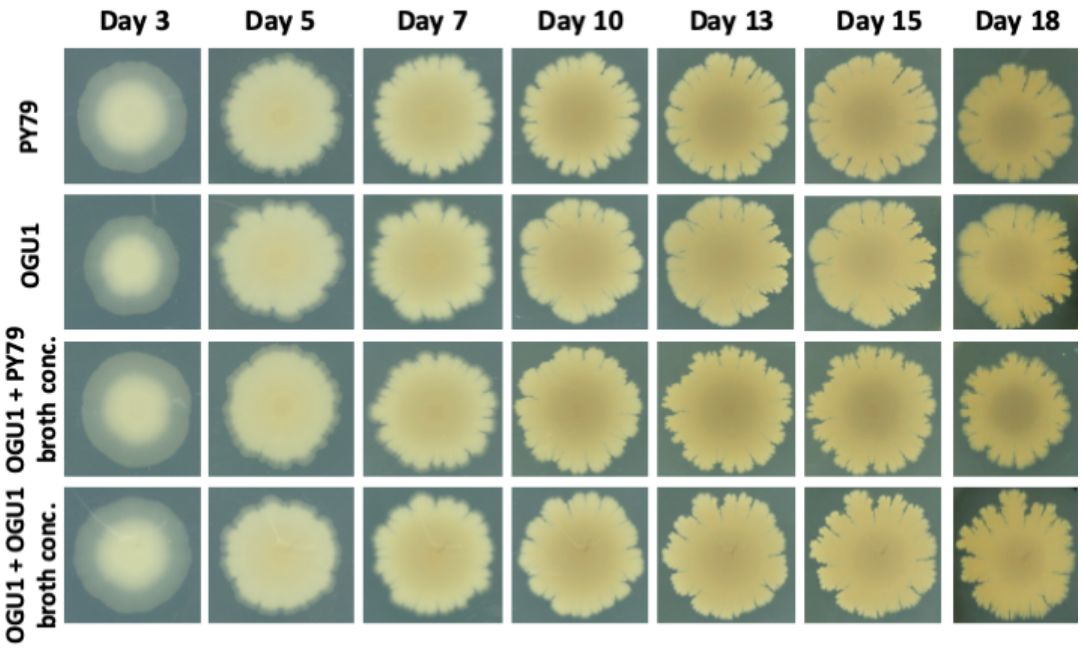


**Figure 3.10.** The pigmentation of diluted spots on solid sporulation media over a time period of 18 days.

As seen in Figure 3.10, the brownish pigmentation was initially observed on the 10$^{th}$ day in PY79, and became the most pronounced on the 13$^{th}$ day. On the other hand, in OGU1, this unique pigmentation did not occur most probably due to insufficient abundance of CotA protein. The defected pigmentation phenotype was almost wholly cured with the addition of bacilysin containing broth concentrate, while supplementation of OGU1 broth concentrate did not change the phenotype. Since the only difference between these concentrates was the presence of bacilysin molecules, we concluded that bacilysin plays a role in the regulation of gene expression of *cotA* that eventually results in formation of brownish pigment. However, the exact nature and timing of this regulation resulting in CotA expression remain to be further investigated.

### 3.4.2 Complementation of Spore Coat Protein Profile

The endospore of *B. subtilis* has two layers of spore coat that are crucial for resistance to external stress, and these layers contain more than 70 proteins in their final structure (Henriques *et al.*, 2004). The presence of a group of these proteins, spore coat morphogenetic proteins including CotE, SafA, SpoIVA, and SpoIVD, is essential for the proper assembly of spore coat layers and their loss results in a defected spore coat structure (Plomp *et al.*, 2014). The synthesis of spore coat proteins mainly occurs in the mother cell compartment, and their regulation occurs through mother cell-specific sigma factors of $\sigma^E$ and $\sigma^K$ as previously mentioned (Steil *et al.*, 2005).

Gel-based and non-gel-based proteomic approaches have been used by our group to analyze the effect of the loss of bacilysin in the *B. subtilis* OGU1 (Aras-Taşkın, 2010; Demir, 2013). These cytosolic proteomic analyses were made at the $16^{th}$ of growth as the bacilysin production peaks at that time (Köroğlu *et al.* 2011). According to these analyses, one of the most affected protein groups was sporulation and germination with a total of 36 proteins that are either identified as absent or significantly underrepresented in OGU1 (Aras-Taşkın, 2010; Demir, 2013; Ertekin *et al.*, 2020) (Table 1.3). Most of these identified proteins belonged to mother cell-specific $\sigma^E$ (11 proteins) and $\sigma^K$ (14 proteins) regulons.

The list of sporulation and germination related proteins that are underrepresented in OGU1 included many coat proteins, and also spore cortex synthesis proteins. Of these significantly underrepresented spore coat proteins, CotE is a well-characterized spore coat morphogenic protein, and it controls the assembly of other spore coat proteins to inner and outer coat (Isticato *et al.*, 2010). The CotE protein presence is vital for the assembly of the outer coat as *cotE* mutant spores of *B. subtilis* were shown to lack this layer (Zheng *et al.*, 1988). As CotE was significantly

underrepresented, and also other essential spore coat proteins, namely, CotA, CotB, CotI, CotSA, CotU, CgeA, and OxdD, were missing in OGU1; the spores of this strain were expected have defected spore coat structure. In order to analyze this defect and its complementation, coat proteins of PY79, OGU1, and broth concentrate supplemented OGU1 spores were solubilized and then analyzed with SDS-PAGE. The results of this analysis and identification of missing bands were presented in the following sections.

#### 3.4.2.1 Solubilization of Spore Coat Proteins and SDS-PAGE Analysis

The spore coat of *B. subtilis* mainly contains highly hydrophobic proteins in its inner and outer layers. Solubilization of these coat proteins requires harsh chemical treatments, which mostly leaves 20-30% of coat proteins insoluble (Pandey, 1980). The protocol described by Takamatsu and his colleagues (2000), which was utilized in this study, offers solubilization of not all but of the majority the spore coat proteins. The solubilized proteins of the spore coat were separated with SDS-PAGE, and the result is given in Figure 3.11.

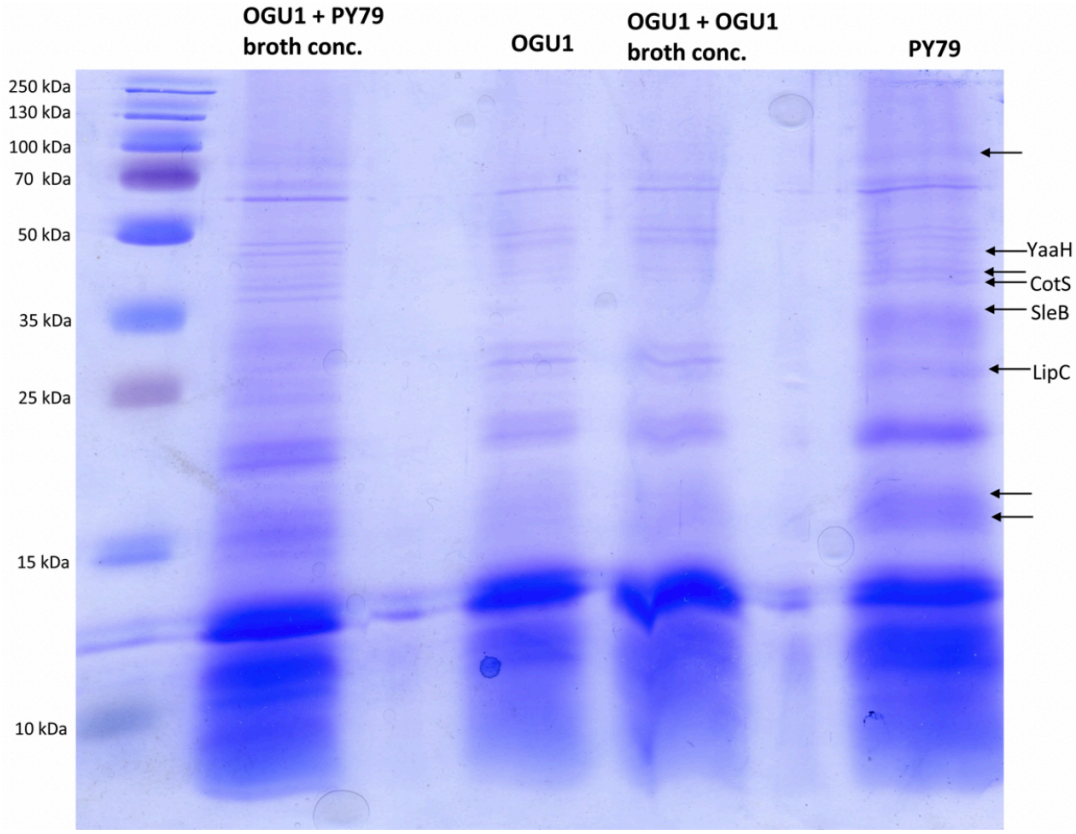


**Figure 3.11.** SDS-PAGE of the solubilized spore coat proteins. From left to right: Lane 1: Marker (PageRuler Plus Prestained Protein Ladder), Lane 2: Spore coat profile of OGU1 supplemented with PY79 broth concentrate, Lane 3: Spore coat profile of OGU1, Lane 4: Spore coat profile of OGU1 supplemented with OGU1 broth concentrate, Lane 5: Spore coat profile of PY79.

As seen in Figure 3.11, the spore coat of OGU1 had at least eight proteins that are either underrepresented or missing, which are marked with an arrow, compared to its parental strain PY79. Since all spore coat proteins could not be solubilized, this SDS-PAGE analysis misrepresents the actual number of spore coat proteins that are missing or underrepresented in OGU strain. Still, the spore coat profile of the OGU1 was defected which was probably due to the very low abundance of morphogenic CotE protein that plays a crucial role in spore coat assembly (Isticato *et al.*, 2010).

The defected profile was almost completely cured with the supplementation of bacilysin containing broth concentrate but not with OGU1's, its own, which provided the strongest evidence for the role of bacilysin in differentiation and sporulation of *B. subtilis*. Although the specific molecular interaction between the bacilysin and sporulation process is still unclear, the presence of the bacilysin molecule is undoubtedly essential for the formation of a proper spore coat. As the formation of the spore coat is regulated with mother-cell specific sigma factors of $\sigma^E$ and $\sigma^K$, and also underrepresented sporulation proteins in OGU1 were mostly belonged to these regulons, we believed in an interaction between bacilysin and the mother cell-specific gene expression.

#### 3.4.2.2 Identification of In-Gel Digested Proteins

The eight protein bands marked with an arrow in Figure 3.11 were cut from the gel for in-gel digestion. After a successful in-gel digestion procedure, these peptide fragments were subjected to MALDI-TOF/MS analysis for proper identification. The mass spectra were analyzed with the MASCOT software, and possible protein matches were ordered for their protein score. The protein score was defined as -10*log(p), where p is the probability of this match. Protein scores higher than 49 (occurs when $p<0.05$) were accepted as significant matches.

Unfortunately, the protein scores of four digested bands were below 49; therefore, these proteins were not identified successfully. This might be due to several problems that occurred in the in-gel digestion procedure. As there were too many protein bands in our gel, which are very close to each other, the separation of the selected band was not easy. Therefore, neighboring protein samples might contaminate our sample to be analyzed, reducing the protein score significantly.

YaaH was one of the identified spore coat proteins that were underrepresented in OGU1 spores. This protein is localized at the inner spore coat of *B. subtilis,* and this localization depends on the presence of spore morphogenic protein SafA (Imamura *et al.*, 2010). YaaH is also known as SleE, and it is expressed during sporulation under the control of the mother cell-specific sigma factor $\sigma^E$ (Kodama *et al.*, 1999). It acts as an N-acetylglucosaminidase, utilizes the fragments generated by cortex-lytic enzymes of SleB and CwlJ; therefore, it is characterized as a cortex fragment lytic enzyme (Üstok *et al.*, 2015). This protein was 2.6-fold underrepresented in the 16$^{th}$-hour proteome of OGU1 (Table 1.3) (Aras-Taşkın, 2010), which supports our finding herein. As this protein plays a role in cortex degradation, its low expression in OGU1 might impair the germination process.

Another identified protein was SleB, one of the major cortex-lytic enzymes that play a crucial role in the germination process (Makino and Moriyama, 2002). This protein is produced during sporulation in a $\sigma^G$ dependent manner, and it requires another protein, YpeB, for proper functioning (Moriyama *et al.*, 1999; Boland *et al.*, 2000). Both YpeB and SleB were identified as underrepresented proteins in OGU1 (Table 1.3) (Aras-Taşkın, 2010; Demir; 2013), which might explain the late germination response observed in OGU1.

LipC was another identified protein that plays a role in the germination process. Unlike SleB and YaaH (SleE), LipC is not responsible for the lysis of the spore cortex, but it is a phospholipase B that plays an essential role in the degradation of outer spore membrane during germination (Masayama *et al.*, 2010). This protein is localized at the inner spore coat with the functioning of SpoIVA and produced at late sporulation under the control of mother cell-specific sigma factor $\sigma^K$. The expression of LipC is essential for the germination process, as *lipC* mutant spores are defective germination stimulated with L-alanine (Masayama *et al.*, 2007). LipC was not detected in the 16$^{th}$-hour proteome of OGU1 with the nanoLC/MS-MS approach

(Table 1.3) (Demir, 2013), further explaining the germinations defect in OGU1 spores.

CotS was another spore coat protein identified to be absent in OGU1 spores, as seen in Figure 3.11. This protein is expressed during the late stages of sporulation, and the mother cell-specific sigma factor σK regulates its expression. It is localized at the outer part of the spore coat with the aid of CotE protein, and it provides resistance to chemicals and lysozyme (Takamatsu *et al.*, 1998). Although CotS was not detected in proteomic studies of our group, another coat protein CotSA, which requires CotS for proper assembly and simultaneously produced with CotS was detected as one of the underrepresented proteins in OGU1 (Table 1.3) (Takamatsu *et al.*, 1999; Demir, 2013). Overall, we would conclude that identified proteins were functioning either in germination or spore resistance, and they were complemented upon the addition of bacilysin containing broth concentrate.

### 3.4.3 Complementation of Spore Germination

A total of eight germination related proteins were identified as absent or significantly underrepresented in OGU1 with our group's proteomic studies (Table 1.3) (Aras-Taşkın, 2010; Demir, 2013). Of these proteins, YaaH, LipC, and SleB were also not abundant in mature spores of OGU1 spores, but they were complemented upon external bacilysin addition (Fig. 3.11). Heat-activated spores were germinated in a complex (2xYT) and a minimal (SMM) medium to analyze the effect of bacilysin addition on germination. The germination is monitored with $OD_{585}$, which falls almost 60% upon complete germination of endospores (Atluri *et al.*, 2006). The results of these germination assays were given in Figure 3.12 and Figure 3.13.

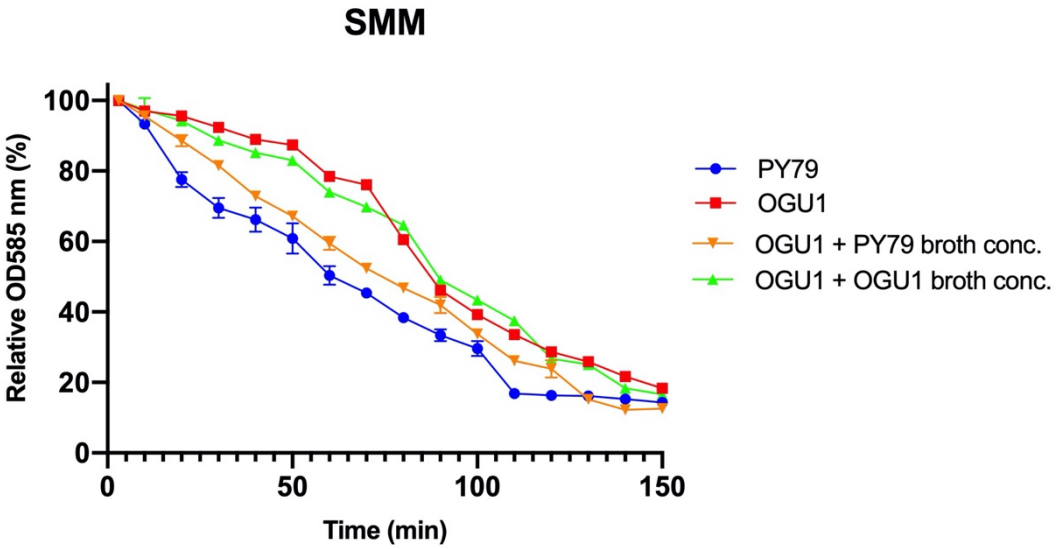


**Figure 3.12.** Germination response of activated endospores in the minimal (SMM) medium. Two biological replicates were used for each measurement.

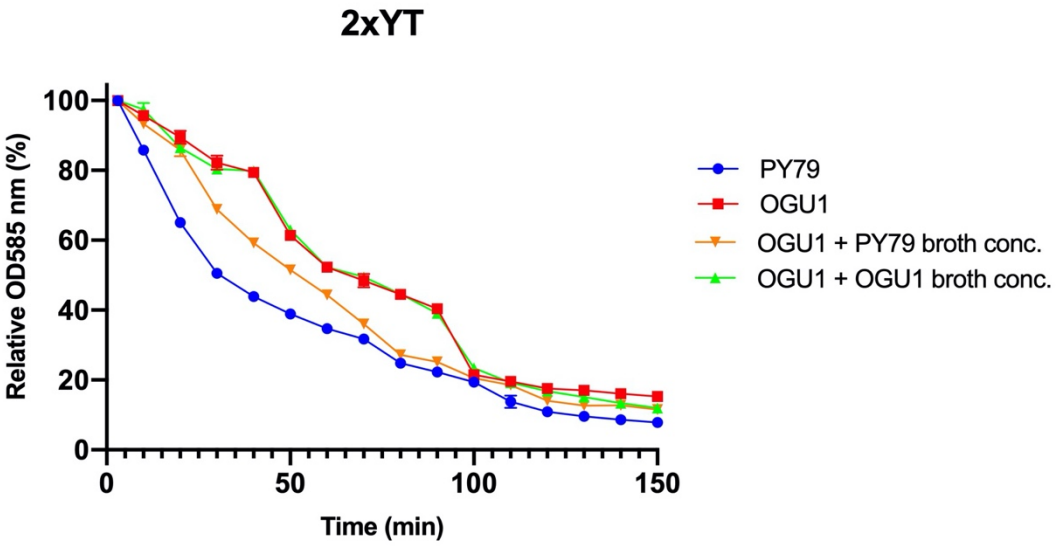


**Figure 3.13.** Germination response of activated endospores in the complex (2xYT) medium. Two biological replicates were used for each measurement.

As seen in the figures above, OGU1 spores were showed a delay in germination response in both media. This delay was 25 minutes in SMM, and 30 minutes in 2xYT medium compared to PY79 spores. This delay was restored with bacilysin containing broth concentrate, but not with OGU1's broth concentrate as expected. Nonetheless, this restoration was not complete as the bacilysin supplemented spores still exhibited 10 and 15 minutes of germination delays, in SMM and 2xYT, respectively. As the abundance of YaaH, SleB, and LipC proteins in the spore coat was almost entirely complemented with bacilysin addition (Fig. 3.11), the delay observed in bacilysin supplemented spores was most likely due to other five underrepresented germination proteins in OGU1 that might not be complemented. Their abundance at mature spores should be analyzed for a proper conclusion.

As mentioned before, YpeB is essential for the assembly and proper functioning of the cortex lytic enzyme SleB (Boland *et al.*, 2000). Although the abundance of SleB in the spore coat seems to be complemented, it might not be wholly functional if the expression of YpeB was not changed with bacilysin addition. On the other hand, GerQ has required for the proper functioning of another critical cortex lytic enzyme, CwlC (Ragkousi *et al.*, 2003). Even though CwlC was not detected in our proteomic analysis as a differentially represented protein in OGU1, it might not be functional without a proper abundance of the GerQ protein, which would significantly delay the germination of the mutant.

Almost 20% of the spore core protein content is made up of small acid-soluble proteins (SASPs), and SASPs play a role in resistance to wet heat and UV radiation (Setlow, 2007). During the germination process, these proteins are degraded with specific proteases, which free the spore DNA for transcription (Setlow, 1988). One of such proteases is Gpr, which is produced as an inactive precursor during late sporulation, and is activated upon hydration of the spore core (Sanchez-Salas and Setlow, 1993). Although the degradation of SASPs occurs mainly with Gpr protease,

other proteases also contribute to this process. TepA, also known as YmfB, is such a protease involved in SAPS degradation. Both double and single null mutants of *gpr* and *tepA* were shown to delay germination response (Traag *et al.*, 2013).

GerW, also known as YtfJ, was another germination related protein that is found to be underrepresented in OGU1. *gerW* deficient spores were shown to have no germination response against L-alanine (Kuwana and Takamatsu, 2013). However, this finding is disputed with a study that reports that loss of *gerW* in *B. subtilis* does not affect either receptor-dependent or independent germination process (Cruz-Mora *et al.*, 2015).

### 3.4.4 Complementation of Spore Resistance

In the 16$^{th}$ hour proteome of OGU1, several spore coat proteins, namely, CotA, CotB, CotI, CotSA, CotU, CgeA, and OxdD, were identified as absent. The abundance of the CotE protein, essential coat morphogenic protein for outer spore coat assembly, was very low in OGU1. Furthermore, vital spore coat polysaccharide synthesis proteins of SpsB, SpsJ, SpsK, and also spore cortex synthesis protein SpoVR were missing in OGU1 (Table 1.3) (Aras-Taşkın, 2010; Demir, 2013). These findings suggested a defective spore coat and cortex structure, which could directly alter the resistance of the mature spores to external stress. Therefore, heat, chloroform, and lysozyme resistance of mature spores of PY79, OGU1, and bacilysin broth supplemented OGU1 were assessed. The spores of OGU1 were more sensitive to chloroform and lysozyme compared to PY79, which was almost entirely cured with the addition of bacilysin containing broth concentrate. The results of these assays are further discussed below.

### 3.4.4.1 Heat Resistance

Mature endospores resuspended in deionized water were subjected to 80ºC for half an hour to assess the wet heat resistance. The result of this assay is given in 3.12.

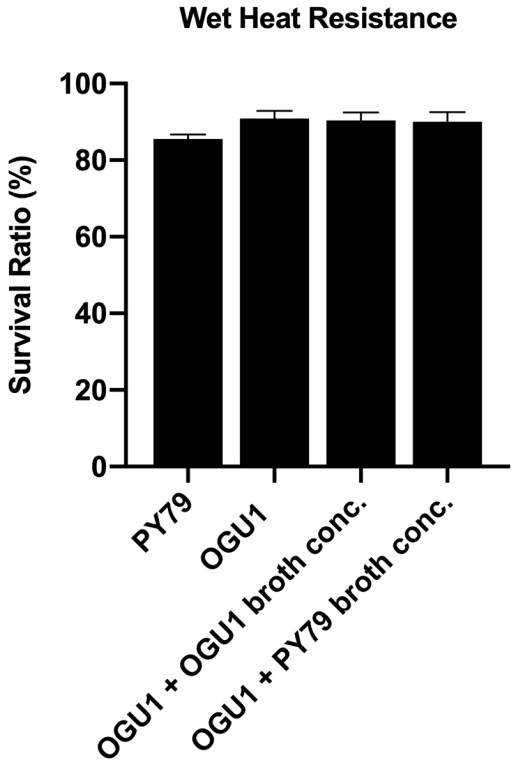


**Figure 3.14.** Survival ratios of mature endospores after wet heat treatment

As seen in Figure 3.14, the wet-heat resistance of OGU1 spores was similar to those of PY79, and this situation was not altered with the supplementation of bacilysin. One-way ANOVA analysis of the results showed that the mean values of survival ratios were not significantly different ($p = 0.1176$).

The resistance of endospores to wet heat treatment is related to the spore core's very low water content. Dipicolinic acid or DPA is the main element that lowers the water content, and divalent cations like $Ca^{2+}$ or $Mg^{2+}$, which are found in spore core as

chelated with DPA and also contribute heat resistance (Gerhardt and Marquis, 1989). The abundance of dipicolinate synthase subunits A and B (DpaA and DpaB) were found to be underrepresented in OGU1 at 16$^{th}$ hour (Table 1.3) (Aras-Taşkın, 2010; Demir, 2013). However, the total amount of the DPA in mature spores was almost the same (15.828 ± 1.758 and 16.139 ± 1.438 µg per OD600, for PY79 and OGU1, respectively) (Ertekin *et al.*, 2020). Therefore, as the DPA levels were not altered in OGU1, the wet heat resistance of this strain no different.

#### 3.4.4.2 Chloroform Resistance

The results of the chloroform resistance assay on mature endospores are given in Figure 3.15.

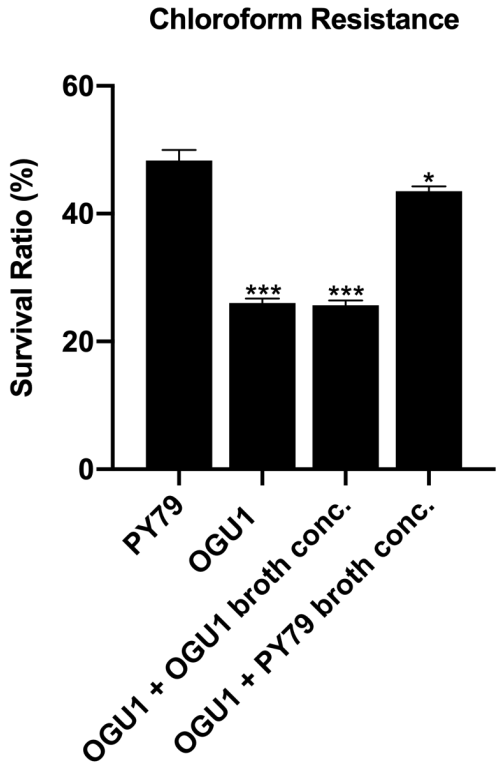


**Figure 3.15.** Survival ratios of mature endospores after the chloroform treatment. The significance levels, compared to PY79, are represented as ns (no significance, $p > 0.05$), * ($p < 0.05$), ** ($p < 0.01$) and *** ($p < 0.001$).

As seen in Figure 3.15, the spores of OGU1 were significantly susceptible to the chloroform treatment as compared to PY79. The resistance to organic solvents is mediated by the spore coat's inner and mostly outer layer, which acts like an armor that prevents the penetration (Setlow, 2011). Several outer coat proteins (CotA, CotB, CotI, CotS, CotSA, and CotU) were missing, and also CotE that plays a role in the assembly of outer spore coat was significantly underrepresented in OGU1 (Table 1.3) (Aras-Taşkın, 2010; Demir, 2013). Therefore, the armor against chloroform was defected in OGU1, and this defect was probably the main reason for the reduction in survival ratio.

Bacilysin containing broth concentrate supplemented OGU1 spores, on the other hand, displayed a better survival ratio compared to OGU1. This situation might be explained with the complementation of most spore coat proteins in the presence of bacilysin, as observed in Figure 3.11.

#### 3.4.4.3 Lysozyme Resistance

The result of the lysozyme resistance assay on mature endospores is presented in Figure 3.16.

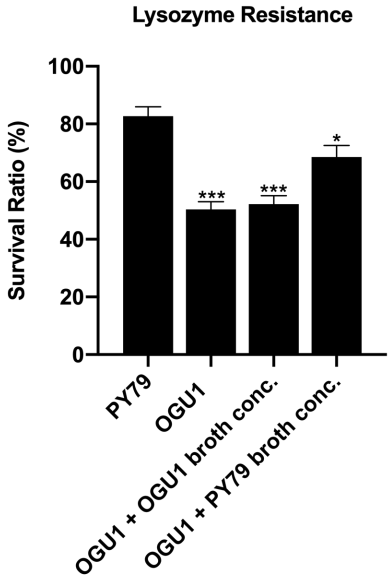


**Figure 3.16.** Survival ratios of mature endospores after lysozyme treatment. The significance levels, compared to PY79, are represented as ns (no significance, $p > 0.05$), * ($p < 0.05$), ** ($p < 0.01$) and *** ($p < 0.001$).

As seen in Figure 3.16, the mature spores of OGU1 displayed a significant susceptibility to lysozyme treatment compared to PY79. Lysozyme targets the spore cortex content rich in peptidoglycan; therefore, a proper spore coat structure provides inherent resistance to this enzyme activity. OGU1 has spores with a defected outer spore coat, which explains the susceptibility to lysozyme treatment (Fig. 3.11). As in chloroform resistance, this lower survival ratio is complemented with the supplementation of bacilysin-containing broth concentrate, but not with OGU1's own broth concentrate, which further supported the findings related to spore coat profile. However, the bacilysin-complemented spores were still not resistant to lysozyme as much as PY79's spores ($p = 0.030$). That might be due to the role of CotE in lysozyme resistance. As a morphogenic coat protein, CotE is essential in the formation of the outer spore coat, but it also has a separate role in lysozyme resistance (Costa *el al.*, 2007).

## 3.5 Validation of Previous Comparative Proteome Analyses between *B. subtilis* OGU1 and *B. subtilis* PY79 with Real Time Quantitative PCR

The data generated by proteomic approaches usually rely on mass spectrometry, which produces a massive fragment ion spectra pool. Identification and quantification of peptides from this pool present computational and statistical challenges; therefore, quantitative proteomics data should be validated with other techniques (Nesvizhskii *et al.*, 2007). Although Western blotting is commonly used as a validation tool for quantitative proteomics data, the RT-qPCR could also be used for validation as it provides complementary information about protein levels (Handler *et al.*, 2018). In this study, expression levels of 20 selected genes, mostly in *B. subtilis* OGU1 were investigated with RT-qPCR technique to validate previous quantitative proteomic work done by our group. The results of RT-qPCR analyses were given as fold change and depicted separately according to the functions of the genes. The results of sporulation and germination related genes (*dpaA, dpaB, cotA, ytfJ, yhcQ* and *yxbC*), two-component systems related genes (*cheV*, *resD*, *degU* and *degS*), stress response related genes (*yugI*, *dnaK*, *yceE* and *ytkL*) and others (*yxbC*, *albE*, *ispA*, *oppD*, *glmS* and *glnA*) are given Figure 3.17, 3.18, 3.19 and 3.20, respectively.

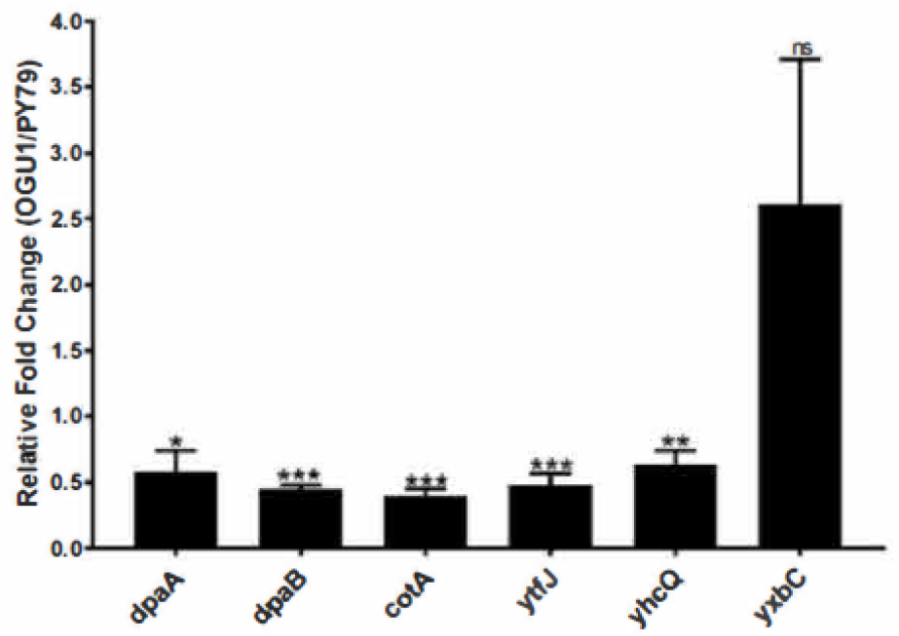


**Figure 3.17.** RT-qPCR results of the selected sporulation and germination genes. The significance levels are represented as ns (no significance, $p > 0.05$), * ($p < 0.05$), ** ($p < 0.01$) and *** ($p < 0.001$).

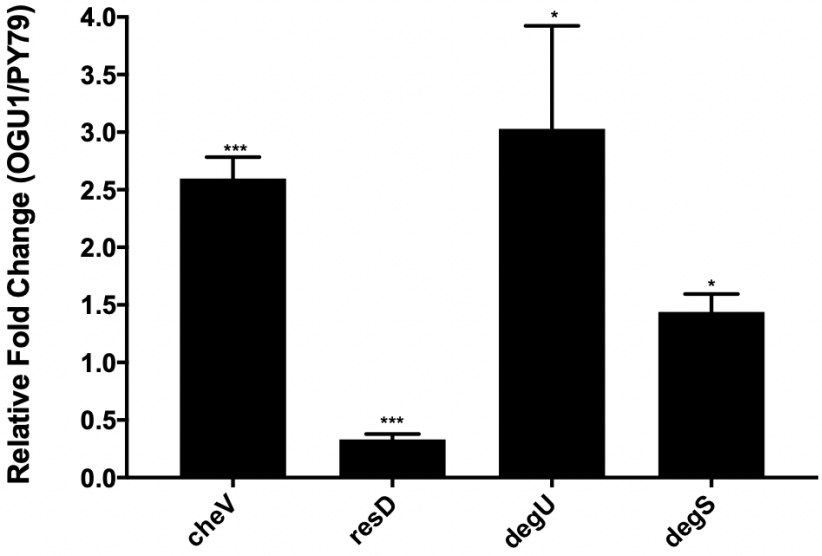


**Figure 3.18.** RT-qPCR results of the selected two-component system genes. The significance levels are represented as ns (no significance, $p > 0.05$), * ($p < 0.05$), ** ($p < 0.01$) and *** ($p < 0.001$).

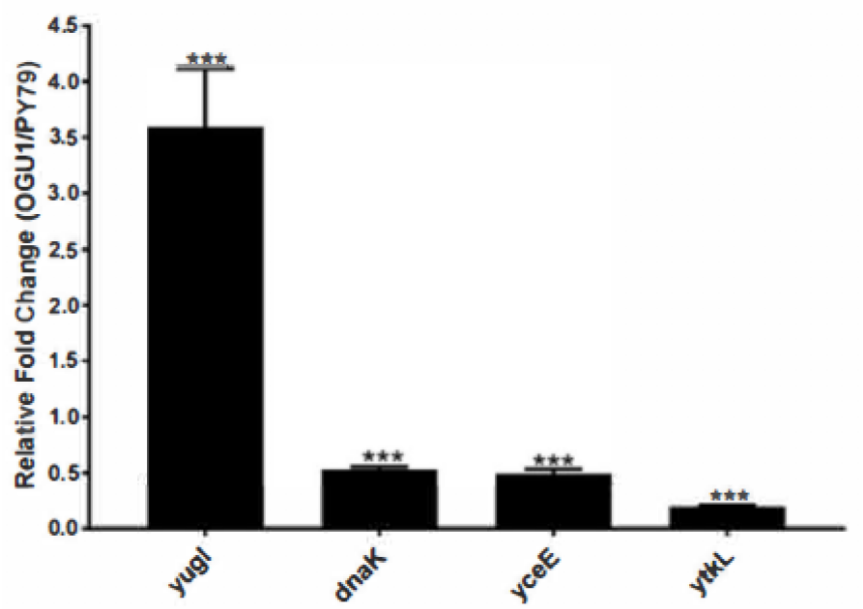


**Figure 3.19.** RT-qPCR results of the selected stress response genes. The significance levels are represented as ns (no significance, $p > 0.05$), * ($p < 0.05$), ** ($p < 0.01$) and *** ($p < 0.001$).

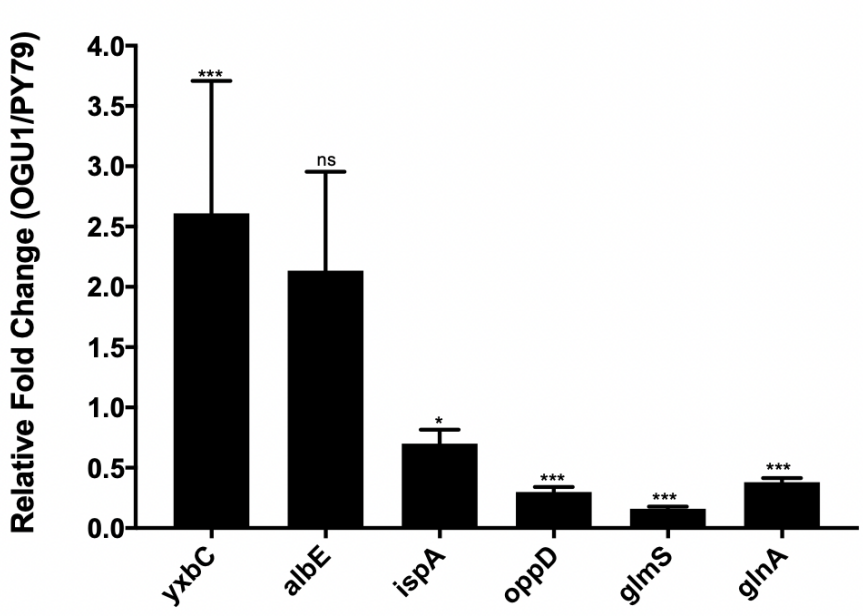


**Figure 3.20.** RT-qPCR results of the selected other genes. The significance levels are represented as ns (no significance, $p > 0.05$), * ($p < 0.05$), ** ($p < 0.01$) and *** ($p < 0.001$).

The expression levels of 17 genes out of 20 were consistent with previous proteomic studies of our group; therefore, we would conclude that RT-qPCR experiments were sufficient for validation of our former results. Additionally, the transcripts of *degS* was overrepresented in *B. subtilis* OGU1, while our proteomic studies did not detect its protein form. DegS-DegU is a well-known two-component system responsible for degradative enzyme production (Dartois *et al.*, 1998). Therefore, the increase in *degS* transcript was not unexpected as the response regulator of this pair, DegU, was identified as overrepresented by proteomic studies as well as RT-qPCR (Aras-Taşkın, 2010; Demir, 2013) (Fig. 3.18).

Surprisingly, the transcript levels of *ytkL* were found to be significantly downregulated in OGU1 (Fig. 3.19). In contrast, its product, the general stress protein YtkL, was found to be 3.4 times overrepresented in the same strain with 2DE MALDI-TOF/MS (Aras-Taşkın, 2010). Another discrepancy in mRNA and protein levels was observed in *yxbC,* which encodes YxbC protein with unknown function under conditions that trigger sporulation (Molle *et al.*, 2003). While YxbC was found to be overrepresented 7.7 times in OGU1 with 2DE MALDI-TOF/MS (Aras-Taşkın, 2010), this overrepresentation was not significant at a transcript level (Fig. 3. 17).

Although the total DPA levels were the same for PY79 and OGU1, as well as their heat resistances, the transcript levels of dipicolinate synthase subunits A and B (*dpaA* and *dpaB*) were found to be underrepresented, just like their protein levels (Fig. 3.17) (Table 1.3) (Aras-Taşkın, 2010; Demir, 2013). Moreover, the downregulation in the transcripts of *cotA* further explains the pigmentation loss observed in OGU1 spores (Fig. 3.10). As this pigmentation was restored with the bacilysin broth concentrate addition as well as the missing coat proteins in OGU1, the mRNA level of *cotA*, as well as the transcripts of other coat proteins, can also be analyzed after complementation.

# CHAPTER 4

# CONCLUSION

- Whole-genome resequencing (WGRS) analysis of the *B. subtilis* OGU1 and also *B. subtilis* PY79, the latter being extensively used in our laboratory for almost 40 years, has revealed unique mutations for each strain as well as mutations shared by both strains. Mutations that were conserved in these strains were found to have no effect to complicate any comparative studies between these strains, including our previous proteomic analyses and the present work.

- The unique mutations of OGU1 were either silent or in the gene that encodes 16S ribosomal RNA, which would not have a significant effect as the translation mechanism was not altered. Similarly, one of the unique mutations of PY79 was in another gene that encodes the 16S ribosomal RNA that also would not affect any comparisons. Other unique mutations of PY79 most probably resulted in defective FruA and BmrU proteins. As these proteins do not have any known pleiotropic roles in *B. subtilis,* and they are not related to sporulation and germination, these mutations would not affect the comparisons made herein.

- The whole-genome resequencing (WGRS) analysis of OGU1 also revealed the exact sequence of the disrupted *bacA* operon and also the flanking regions. In this new analysis, the *bacA* operon was the in frame and under the IPTG-inducible P*spac* promoter. The IPTG induction resulted in a significant increase in *bacA* operon transcripts; however, it did not result in any bacilysin production. Therefore, the IPTG induction was not sufficient for the functional complementation.

- Acetone-treated broth concentrates were subjected to the UPLC-MS analysis for the molecular detection of the bacilysin molecule. The bacilysin was only detected in the broth concentrate of PY79, which was also shown to be active through bacilysin activity assay. As the broth concentrates could also contain small signaling molecules that could directly affect sporulation process, the broth concentrate of OGU1 was used as a negative control. Both of these broth concentrates did not alter the growth of OGU1 upon supplementation. The bacilysin activity in OGU1 that is supplemented with PY79's bacilysin broth concentrate consistently decreased with time, which implied that the bacilysin supplementation did not trigger any bacilysin production. This finding ruled out our former hypothesis about a possible autoregulatory role of bacilysin.

- According to our group's previous proteomic finding, the major protein group that was affected by the loss of bacilysin belonged to sporulation and germination. A total of 36 proteins related to these functions were identified with complementary proteomic approaches, and these proteins mostly belonged to mother cell-specific regulons of $\sigma^E$ and $\sigma^K$. Of these proteins, CotA was known to play a role in brownish pigmentation of mature spores. As CotA was significantly underrepresented in OGU1, this pigmentation was defective in mature spores of OGU1. However, bacilysin containing broth supplementation on growing OGU1 culture almost entirely rescued this pigmentation phenotype, indicating a role of bacilysin on the expression of sporulation and germination proteins like CotA. The supplementation of OGU1's own broth concentrate did not alter the pigmentation profile, further suggesting that bacilysin is necessary for biosynthesis and proper assembly of spore coat proteins.

- As the spore coat profile of mature spores directly defines the resistance of spores to external stress, the resistance to heat, chloroform, and lysozyme was also analyzed. The spores of OGU1 were resistant to heat as much as PY79, as their DPA levels were almost the same. On the other hand, OGU1 spores were significantly susceptible to chloroform and lysozyme treatment most possibly due to a defective outer coat structure. With bacilysin, the spore survival rate were almost restored.

- MALDI-TOF/MS analysis successfully identified four proteins (YaaH, CotS, SleB and LipC) three of them being crucial for the germination process and already reported as underrepresented in our proteomic works. Due to the absence of these germination proteins as well as the others reported herein, the spores of OGU1 displayed a significant delay in germination. This delay was at least partially overcome with the supplementation of bacilysin, further pointing to a role of bacilysin in the regulation of sporulation and germination.

- RT-qPCR analysis of selected 20 genes was employed to validate the former comparative proteomics done by our group. Of these 20 genes, the transcript levels of 17 were consistent with the former studies, while only the level of *ytkL* was opposing to its protein levels. *degS*, which was not identified in former studies, was also shown to be upregulated just like its partner *degU*. *cotA*, encoding the spore pigmentation protein CotA, was another gene that was significantly downregulated in OGU1.

- Overall, it could be concluded that bacilysin plays an essential role in morphogenesis and resistance of the mature endospores and also in the germination process. Further studies remain to be undertaken to gain a better understanding of how bacilysin regulates these processes at a molecular level and/or its possible mediators.

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# APPENDICES

## A. Compositions and Preparation of Culture Media

### Perry and Abraham (PA) Medium (pH 7.4)

| | |
|---|---|
| $KH_2PO_4$ | 1 g/L |
| KCl | 0.2 g/L |
| $MgSO_4.7H_2O$* | 0.5 g/L |
| Glutamate.Na.$H_2O$ | 4 g/L |
| Sucrose* | 10 g/L |
| Ferric citrate** | 0.15 g/L |

Trace elements**

| | |
|---|---|
| $CoCl_2.6H_2O$ | 0.0001 g/L |
| Ammonium molybdate | 0.0001 g/L |
| $MnCl_2.4H_2O$ | 0.001 g/L |
| $ZnSO_4.7H_2O$ | 0.0001 g/L |
| $CuSO_4.5H_2O$ | 0.00001 g/L |

*Autoclave separately

**Filter sterilization

**Luria Bertani (LB) Medium**

| | |
|---|---|
| Tryptone | 10 g/L |
| Yeast Extract | 5 g/L |
| NaCl | 5 g/L |
| Agar | 15 g/L |
| | (add before autoclave for LB agar medium) |

Distilled water was added up to 1000 mL and then autoclaved for 15 minutes.

**Schaeffer's Sporulation Medium or Difco Sporulation Medium (DSM) (pH 7.6)**

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

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

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

*Filter sterilization

**Bioassay Medium (pH 7.1)**

| | |
|---|---|
| $Na_2HPO_4.2H_2O$ | 3.3 g/L |
| $KH_2PO_4$ | 1 g/L |
| NaCl | 1 g/L |
| Glucose* | 10 g/L |
| $MgSO_4.7H_2O$* | 0.7 g/L |
| $Na_3$.Citrate.$2H_2O$ | 0.5 g/L |
| Glutamic acid.Na.$H_2O$** | 2.4 g/L |
| 13 amino acid*** | 0.0125 g/L (each) |
| $FeSO_4.7H_2O$** | 0.01 g/L |
| Yeast Extract**** | 0.05 g/L |
| Agar**** | |

*Autoclave glucose and $MgSO_4.7H_2O$ together, separately

**Filter Sterilization

***Arginine, cystidine, glycine, histidine, leucine, methionine, phenylalanine, proline, threonine, tryptophane, tyrosine, valine, alanine (all in L-form)

****Autoclave separately

**Spizizen Minimal Medium (SMM)**

| **5x SM base medium** | **200 mL/L** |
|---|---|
| $K_2HPO_4$ | 43.75 g |
| $KH_2PO_4$ | 18.75 g |
| $Na_3$.Citrate.$2H_2O$ | 3.125 g |
| $MgSO_4.7H_2O$ | 0.625 g |

Dissolved in 500 mL of distilled water and autoclaved for 15 minutes.

| **1000x Trace Element Solution** | **1 mL/L** |
|---|---|
| $CaCl_2$ | 0.55 g |
| $MnCl_2.4H_2O$ | 0.1 g |
| $ZnCl_2$ | 0.17 g |
| $CuCl_2.6H_2O$ | 0.043 g |
| $CoCl_2.6H_2O$ | 0.06 g |
| $Na_2MoO_4.2H_2O$ | 0.06 g |

Dissolved in 100 mL of distilled water, and applied filter sterilization.

| **100x Fe-Citrate Solution** | **10 mL/L** |
|---|---|
| $FeCl_3.6H_2O$ | 0.0135 g |
| $Na_3$.Citrate.$2H_2O$ | 0.1 g |

Dissolved in 100 mL of distilled water, and applied filter sterilization.

**50% Glucose Solution** **10 mL/L**

| | |
|---|---|
| Glucose | 50 g |

Dissolved in 100 mL of distilled water, and autoclaved.

**N-source solutions**

| | |
|---|---|
| **40% (w/v) Glutamate sol.** | **25 ml/L (final conc. 1%)** |
| **50% (w/v) Glutamine sol.** | **20 mL/L (final conc. 1%)** |
| **10% (w/v) $(NH4)_2SO_4$ sol.** | **20 mL/L (final conc. 0.2%)** |

Volume was adjusted to 1 L with distilled water.

**2xYT Medium**

| | |
|---|---|
| Tryptone | 16 g/L |
| Yeast Extract | 10 g/L |
| NaCl | 5 g/L |

Distilled water was added up to 1000 mL and then autoclaved for 15 minutes.

## B. Compositions of Buffers and Solutions

**Tris-Cl Solution (50 mM, 1000 mL, pH 7.2)**

Tris base 6.06 g

Dissolve in 1000 mL distilled water and adjust pH to 7.2 with HCl (1 M).

**Tris-Cl Solution (10 mM, 1000 mL, pH 8.4)**

Tris base 1.21 g

Dissolve in 1000 mL distilled water and adjust pH to 8.4 with HCl (1 M).

**TE Buffer (pH 8.0)**

10 mM Tris-Cl

1 mM EDTA

pH is set to 8.0 and sterilized with autoclave for 15 minutes.

**SET Buffer**

| | |
|---|---|
| Sucrose | 25 g/L |

0.05 M Tris-Cl Solution (pH 8.0)

0.5 M EDTA (pH 8.0)

Sterilized with autoclave for 15 minutes.

**TAE Buffer (50x)**

| | |
|---|---|
| Tris base | 242 g |
| Glacial acetic acid | 57.1 mL |
| 0.5 M EDTA (pH 8.0) | 100 mL |

Adjust the final volume to 1000 mL with distilled water.

**Low Melting Agarose Gel (0.8%)**

| | |
|---|---|
| Agarose | 0.8 g |
| TAE buffer (1X) | 100 ml |

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

**1 M KCl/0.5 M NaCl Solution**

| | |
|---|---|
| KCl | 7.45 g |
| NaCl | 2.92 g |

Dissolve in 100 mL distilled water and sterilize with autoclave for 15 minutes.

**TEP Buffer (pH 7.2)**

50 mM Tris-Cl

10 mM EDTA

2 mM PMSF

pH is set to 7.2, PMSF added the latest.

**Potassium Phosphate Buffer (10 mM, pH 7.4)**

| | |
|---|---|
| $K_2HPO_4$ | 0.61 g |
| $KH_2PO_4$ | 0.21 g |

These were dissolved in 450 mL distilled water and the solution was autoclaved. After cooling sterile KCl (final conc. 50 mM) and $MgSO_4$ (final conc. 1 mM) solutions were added.

**Sodium Phosphate Buffer (10 mM, pH 7.2)**

| | |
|---|---|
| $Na_2HPO_4.7H_2O$ | 0.89 g |
| $NaH_2PO_4.H_2O$ | 0.23 g |

These were dissolved in 475 mL distilled water and pH was arranged to 7.2 with using HCl or NaOH. Volume completed to 500 mL with distilled water and the solution was sterilized by autoclave for 15 minutes.

**Acrylamide/Bis-acrylamide Solution**

| | |
|---|---|
| Acrylamide | 46 g |
| N.N'-Methylene-bis Acrylamide | 4 g |

Distilled water to 500 mL. Filtered and stored at 4 °C. Protected from the light.

**SDS-PAGE Loading Buffer**

| | |
|---|---|
| $dH_2O$ | 3 mL |
| Tris-Cl (0.5 M) | 1 mL |
| Glycerol | 1.6 mL |
| SDS (10%) | 0.4 mL |
| β-mercaptoethanol | 0.4 mL |
| Bromophenol blue (0.5%, w/v) | 0.4 mL |

**SDS-PAGE Running Buffer (5x)**

| | |
|---|---|
| Tris base | 15 g |
| Glycine | 72 g |
| SDS | 5 g |

Distilled water to 1 L. Stored at 4 °C.

**Coomassie Blue R-250 Stain**

| | |
|---|---|
| Coomassie blue R-250 | 0.25 g |
| Methanol | 125 mL |
| Glacial acetic acid | 25 mL |
| $dH_2O$ | 100 mL |

**Destaining Solution**

| | |
|---|---|
| Methanol | 100 mL |
| Glacial acetic acid | 100 mL |
| $dH_2O$ | 800 mL |

**Washing or Decolorization Solution**

| | |
|---|---|
| Ammonium bicarbonate | 0.8 g |
| Acetonitrile | 15 mL |
| $dH_2O$ | 35 mL |

**Trypsin solution (1x)**

10x trypsin solution available was diluted with dH20.

## C. Chemicals and Enzymes

| Chemicals | Supplier |
|---|---|
| Acetone | Sigma |
| Acetonitrile | Sigma |
| Acrylamide | Sigma |
| Agar | Merck |
| Agarose | Prona |
| Ammonium Bicarbonate | Sigma |
| Ammonium Molybdate | Sigma |
| β – mercaptoethanol | Sigma |
| Bromophenol Blue | Sigma |
| $Ca(NO_3)_4$ | Merck |
| $CaCl_2$ | Merck |
| Chloroform | Merck |
| $CoCl_2.6H_2O$ | Merck |
| Coomassie Blue R-250 | Sigma |
| $CuCl_2.6H_2O$ | Merck |
| $CuSO_4.5H_2O$ | Merck |
| EDTA | AppliChem |
| Erythromycin | Sigma |
| Ethanol | Sigma |
| Ethidium Bromide | Sigma |
| $FeCl_{3.}6H_2O$ | Merck |
| Ferric citrate | Merck |
| $FeSO_4.7H_2O$ | Merck |
| Glacial Acetic Acid | Merck |
| Glucose | Merck |
| Glutamate.Na.$H_2O$ | Merck |

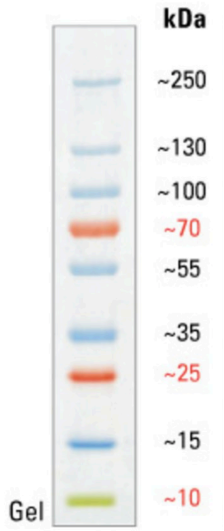
| | |
|---|---|
| Glutamine | Sigma |
| Glycerol | Merck |
| IPTG | Sigma |
| $K_2HPO_4$ | Merck |
| KCl | Merck |
| $KH_2PO_4$ | Merck |
| L-Alanine | Sigma |
| L-Arginine | Sigma |
| L-Cystidine | Sigma |
| L-Glycine | Sigma |
| L-Histidine | Sigma |
| L-Leucine | Sigma |
| L-Methionine | Sigma |
| L-Phenylalanine | Sigma |
| L-Proline | Sigma |
| L-Threonine | Sigma |
| L-Tyrosine | Sigma |
| L-Valine | Sigma |
| Lincomycin | Sigma |
| Methanol | Merck |
| $MgSO_4.7H_2O$ | Merck |
| $MgSO_4.Na.H_2O$ | Merck |
| $MnCl_2.4H_2O$ | Merck |
| N,N'- Methylenebisacrylamide | Sigma |
| $Na_2.MoO_4.2H_2O$ | Merck |
| $Na_2HPO_4.7H_2O$ | Merck |
| $Na_3.Citrate.2H_2O$ | Merck |
| NaCl | Merck |
| $NaH_2PO_4.7H_2O$ | Merck |
| $(NH_4)_2SO_4$ | Merck |

| | |
|---|---|
| Nutrient Broth | Merck |
| PMSF | Sigma |
| SDS | Merck |
| Sucrose | Merck |
| Tris-Base | Merck |
| Tryptone | Difco |
| Yeast Extract | Oxoid |
| $ZnCl_2$ | Sigma |
| $ZnSO_4.7H_2O$ | Sigma |

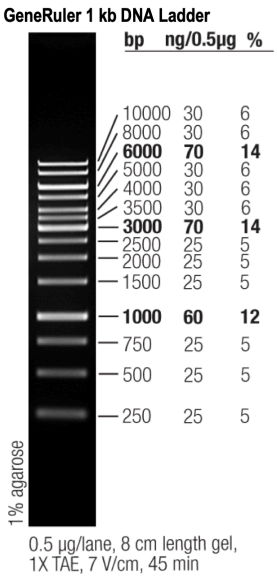
**Enzymes**

| | |
|---|---|
| Lysozyme | Sigma |
| Proteinase K | Sigma |
| RNAse I | ThermoFisher |

## D. Markers




PageRuler Plus Prestained Protein Ladder (ThermoFisher Scientifics, MA, USA)




GeneRuler 1 kb DNA ladder (ThermoFisher Scientifics, MA, USA)