

DYNAMIC ANALYSIS OF SECRETOME ALTERATIONS IN A  
BACILYSIN-KNOCK OUT MUTANT OF *BACILLUS SUBTILIS*

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## ABSTRACT

### **DYNAMIC ANALYSIS OF SECRETOME ALTERATIONS IN A BACILYSIN-KNOCK OUT MUTANT OF *BACILLUS SUBTILIS***

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The members of the genus *Bacillus* produce a wide variety of secondary metabolites with antimetabolic and pharmacological activities. Bacilysin, being produced and excreted by certain strains of *B. subtilis*, is a dipeptide antibiotic composed of L-alanine and L-anticapsin which is synthesized enzymatically. As shown earlier by our research group, the expression of bacilysin biosynthetic operon (*bacA*) is relatively constant during the exponential growth, but increases during the transition between exponential and stationary phases and reaches to its maximal level upon entry into stationary phase (16<sup>th</sup> hour). We also reported for the first time that the expression of *bacA* operon is under the control of several elements of quorum sensing global regulatory pathway of sporulation initiation and competence development as well as those of some essential two-component pleiotropic regulator proteins that play the critical role in a variety of cellular functions, including the adaptation to nutrient-limiting conditions, motility, chemotaxis and biofilm formation. More recently, we performed comparative cytosolic proteome analysis in the

bacilysin producer *B. subtilis* PY79 and its bacilysin-knock out derivative, OGU1, by using a combination of 2DE MALDI-TOF/MS and GeLC-MS/MS approaches with the aim of identifying functional roles of bacilysin biosynthesis in its producer. Yet, these studies did not cover extracellular proteins of the organisms which could point to some other important physiological alterations in response to the bacilysin knock out mutation.

The secretome of *B. subtilis* is known to change at the beginning of the sporulation phase to include new membrane- and wall-binding proteins as well as those secreted outside, like new degradative enzymes that may provide the cells with new nutrients and those engaged in detoxification, communication, defense and many more. As a Gram-positive organism, the absence of an outer membrane in *Bacillus subtilis* simplifies protein secretion pathways and allow the organism to secrete high levels of extracellular proteins. The present study aims at an investigation of dynamic changes in the secretome of *B. subtilis* in the absence of bacilysin production by employing both 2 DE gel-based and gel-free approaches, to improve protein coverage as a function of time and space. At different time points of 12, 16 and 24 hours of cultivation in glucose-based synthetic medium, the secretome components were analyzed, identified and compared in the parental strain PY79 and its and bacilysin knock out mutant OGU1. LC-MS/MS analyses identified a total of 2075 distinct proteins. Of these, a total of 166 proteins were found to be differentially expressed between two strains. As expected, the numbers of total and differentially expressed proteins identified were much lower in 2DE MALDI-TOF/MS approach, still its results well supported the findings from LC-MS/MS analyses. Overall, the differentially expressed proteins mainly belonged the functional categories of transport and metabolism, genetic processes, lifestyle, coping with stress, sporulation and germination and those unknown. The alterations should mainly be regarded to represent a defense or stress-coping mechanism assuming that the absence of bacilysin is sensed by the mutant as a type of stress condition.

The present study greatly aided in a comprehensive understanding of whole proteomic alterations in the bacilysin knock-out mutant that remains to be supported by further functional analyses.

Keywords: *Bacillus subtilis*, bacilysin, gene expression, proteome, secretome, LC-MS/MS, 2DE MALDI-TOF MS



## ÖZ

# BASİLSİN BİYOSENTEZİNİN SUSTURULDUĞU BİR *BACILLUS SUBTILIS* MUTANTINDA DİNAMİK SEKRETOM DEĞİŞİMLERİNİN ANALİZİ

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*Bacillus* cinsi bakteriler, antimetabolik ve farmakolojik aktiviteye sahip çok çeşitli ikincil metabolitler üretmektedirler. *B. subtilis*'in belirli suşları tarafından üretilen basilisin, L-alanin and L-antikapsin'den oluşan dipeptid yapısında bir antibiyotik olup enzimatik olarak sentezlenmektedir. Basilisin biyosentetik operonunda (*bacA*) gen ifadesinin, logaritmik büyüme fazı boyunca nispeten sabit olduğu, logaritmik fazdan durağan faza geçiş sürecinde arttığı ve durağan faza girildiği dönemde (16. saatte) maksimum seviyeye ulaştığı grubumuz tarafından daha önce gösterilmişti. *bacA* operonunun hücrelerde sporulasyonun başlamasını ve kompetans gelişimini kontrol eden "hücre yoğunluğu sinyali global düzenleyici mekanizma"nın çeşitli elemanları ve ayrıca besin miktarındaki azalmaya uyum, motilite, kemotaksis ve biyofilm oluşumu gibi çeşitli hücrel fonksiyonlarda kritik roller oynayan bazı esasiyel iki-bileşenli pleiotrofik regülatörler tarafından kontrol edildiği de yine grubumuzca rapor edilmişti. Daha sonra, basilisin biyosentezinin üretici

organizmadaki fonksiyonlarını aydınlatmak amacıyla, *B. subtilis* PY79 ve onun basilisin biyosentezinin bloke edildiği bir mutanlığı olan OGU1 suşunda GeLC-MS/MS ve 2DE MALDI-TOF/MS yaklaşımları bir arada kullanılarak karşılaştırmalı sitozolik proteom analizleri gerçekleştirildi. Ancak, bacilysin blokajı sonucu ortaya çıkabilecek diğere önemli fizyolojik değışimlere işaret edebilecek önemli ekstrasellüler protein analizleri bu çalışmalarda yer almadı.

*B. subtilis* sekretomunun, sporlanma fazının başlangıcında, yeni membran- ve hücre duvarı-bağlanma proteinleri ve makromolekül parçalayıcı enzimler gibi yeni besinler temin eden ve dışarıya yeni salgılanan enzimleri ve detoksifikasyon, iletişim, stres, savunma ile ilgili proteinleri içerecek biçimde değışime uğradığı bilinmektedir. Gram- pozitif bir organizma olarak, diğere membranın bulunmayışı *B. subtilis*'de protein salgı yollarını basitleştirerek ekstrasellüler proteinlerin yüksek seviyede salgılanmasına olanak tanımaktadır. Bu çalışmada, jelle dayalı ve jelden bağımsız iki ayrı yaklaşım kullanılarak ulaşılabilen proteinlerin sayısının artırılmasıyla *B. subtilis*'de basilisin üretimi yokluğunda ortaya çıkan sekretom değışimlerinin zaman ve mekan olarak dinamik bir analizi amaçlanmıştır. Glukoz içeren sentetik besi yerinde yapılan kültürasyonun 12., 16. ve 24. saatlerinde sekretom bileşenleri analiz edilmiş, tanımlanmış ve ana suş PY79 ve basilisin üretemeyen türeği OGU1 arasında farklılık gösteren proteinler ve bunların seviyeleri nicel olarak belirlenmiştir. LC-MS/MS analizlerinde toplam 2075 protein tanımlanabilmiştir. Tanımlanabilen proteinlerin 166'sının iki suş arasında farklı ifade edilen proteinler olduğu gösterilmiştir. Beklenildiği üzere, 2DE MALDI-TOF/MS yaklaşımı ile elde edilen toplam ve farklı ifade edilen protein sayıları çok daha düşük olmuş, yine de bu yöntemle elde edilen sonuçlar LC-MS/MS analiz bulgularını desteklemiştir. Bütünüyle ele alındığında, OGU1'de farklı ifade edilen proteinlerin transport ve metabolizma, genetik prosesler, yaşam biçimi, strese uyum, sporulasyon ve jermineasyon ve henüz tanımlanamayan

fonksiyonel kategorilere ait olduđu gösterilmiřtir. Basilisin biyosentezi yokluđunun hücresel tarafından bir çeřit stres kořulu olarak algılandıđı varsayıldıđında, mevcut farklılıklar genel bir savunma ve stresle bařa çıkma göstergesi olarak kabul edilmelidir. řimdiki alıřma, basilisin üretemeyen mutant organizmada tam hücre proteomu seviyesindeki deđiřimlerin kapsamlı biçimde anlaşılmasına önemli bir katkı sađlamıř olup mevcut bulguların daha ileri fonksiyonel alıřmalarla desteklenmesi hedeflenmektedir.

Anahtar kelimeler: *Bacillus subtilis*, basilisin, gen ifadesi, proteom, sekretom, LC-MS/MS, 2DE MALDI-TOF MS



**To my family...**

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## LIST OF ABBREVIATIONS

bp	: Base pair
<i>B. subtilis</i>	: <i>Bacillus subtilis</i>
kDa	: Kilodalton
OD	: Optical density
SDS-PAGE	: Sodium dodecyl sulfate polyacrylamide gel electrophoresis
pI	: Isoelectric Point
PMF	: Peptide Mass Fingerprint
PTM	: Posttranslational Modification
ROS	: Reactive Oxygen Species
RP	: Reverse Phase
SCX	: Strong Cation Exchange
ORF	: Open Reading Frame
1DE	: One Dimensional Electrophoresis
2DE	: Two Dimensional Electrophoresis
ESI	: Electrospray Ionization
IEF	: Isoelectric Focusing
LC	: Liquid Chromatography
MALDI-TOF	: Matrix-assisted Laser Desorption Ionization Time of Flight
MS	: Mass Spectrometry
KO	: Knock-out



## CHAPTER 1

### INTRODUCTION

#### 1.1. *Bacillus subtilis*

The genus *Bacillus* is composed of taxonomically and metabolically diverse group of microorganisms which share common characteristic features like being Gram-positive, rod shaped, motile and endospore-forming. Among *Bacillus* species, *Bacillus subtilis* is the most famous and extensively studied one and is a model organism (Wipat and Harwood 1999; Sonenshein et al., 2002; Lippolis et al., 2013). Although some species of *Bacillus* are pathogenic like *B. anthracis*, most of the members including *B. subtilis* are nonpathogenic. The natural habitats of *B. subtilis* are mainly the upper layer of the soil and water sources. The spore forming ability and diverse metabolic activity of *B. subtilis* provide survival under adverse conditions as well as colonization of diverse habitats. The chemoorganotroph nature of the *B. subtilis* enables the organism to oxidize organic compounds and provide the necessary growth conditions (Harwood and Cutting, 1990). The *B. subtilis* genome has evolved under unfavorable environmental conditions since the survival mechanisms of the organism include increased expression of hydrolases mainly proteases, amylases and antibiotics that are the sources of medical, chemical and industrial processes (Sonenshein et al., 2002). Genome sequence of *B. subtilis* being the first representative of soil dwelling microorganism was first published in 1997 (Kunst et al., 1997) and then updated (Barbe et al., 2009) as 4.2 Mb genome with more than 4100 genes (Ando, 2002; Kobayashi and Osagura, 2002). The standard strain *B. subtilis* 168 and its prototrophic

derivative PY79 are the commonly used strains for genetic and biochemical studies (Spizen, 1958; Harwood et al., 1990).

## **1.2. Antibiotics and secondary metabolites of *B. subtilis***

Most of the members of the genus *Bacillus* can produce a variety of peptide antibiotics which are synthesized by ribosomal or nonribosomal mechanisms and ribosomally-synthesized ones are subjected to post-translational modifications (lantibiotics and lantibiotic-like peptides). The nonribosomal mechanisms include the synthesis of peptides by large megaenzymes called nonribosomal peptide synthetases (NRPSs). Although, there are structural differences between the nonribosomal peptide antibiotics most of them share a common mode of synthesis, the multicarrier thiotemplate mechanism with NRPSs. The organization of NRPSs is as iterative functional units which form the modules catalyzing the different reactions of peptide transformations. By this way, the growing chains as a series of elongating acyl-S-proteins, covalently tied to the terminal thiol of phosphopantetheinyl arms, with themselves hitched to side chains of serine residues of carrier protein domains in the assembly line. Briefly, each elongation cycle three main domains are necessary namely adenylation, peptidyl carrier domain and condensation domain. In addition to those main domains, substrate epimerization, hydroxylation, methylation and heterocyclic ring formation domains can be included. The arrangement of domains generally correlated with gene level but in some cases depending on the complexity this can be changed (Döhren et al., 1999; Finking and Marahiel, 2004; Hamdache et al., 2013).

Among the nonribosomally-synthesized antibiotics gramicidin, tyrocidine, and bacitracin are produced by a multienzyme thiotemplate mechanism whereas surfactin and mycobacillin are produced by a distinct mechanism (Zuber et al.,

1993; Tamehiro et al., 2002). On the other hand, antibiotics like subtilin and sublancin are the examples to ribosomally-synthesized ones (Nakano and Zuber 1990). Some of the antibiotics produced by *B. subtilis* are categorized and listed in Figure 1.1 (Stein, 2005). Surfactin is a lipopeptide which is composed of a heptapeptide moiety bonded to the carboxyl and hydroxyl groups of a  $\beta$ -hydroxy fatty acid. Its synthesis is encoded by the *srfAA-AB-AC-AD* operon and the targets on cellular membranes and disrupts its integrity. Surfactin has different isoforms in *B. subtilis*, *B. pumilus*, *licheniformis* and *amyloliquefaciens*. Iturine, mycosubtilin and bacillomycin are composed of related cyclic lipopeptides belongs to iturin family. They have a strong antifungal and haemolytic whereas their antibacterial activities are limited (Stein, Fengycin has distinct structure that is cyclic, branched and contains unusual constituents. It is specifically act on filamentous fungi by inhibiting phospholipase A2 (Hamdache et al., 2013). Corneyabactin (also known as bacillibactin) is a siderophore, not an antibiotic but synthesized via nonribosomally (Stein, 2005). 3, 3- neotrehalosdiamine (NTD) synthesized by the *ntdABC* (*yhjLKJ*) operon and it is an aminosugar antibiotic which is synthesized upon induction by rifampicin. NTD can induce its own biosynthesis and inhibits growth of *Staphylococcus aureus* and *Klebsiella pneumoniae* with an unknown mechanism (Inaoka et al., 2004). Difficin is a polyketide which inhibits protein biosynthesis and it has broad spectrum antibacterial activity (Sansinenea and Ortiz, 2011). Bacilysoicin is a phospholipid antibiotic and probably acting on in membrane synthesis, repair, or recycling (Eiamphungporn and Helmann, 2008). Amicoumacins have antibacterial and anti-inflammatory action and active against *Helicobacter pylori*. Mycobacillin are active against molds and yeasts. Rhizocticin are phosphonate containing oligopeptide antibiotics with antifungal activity (Hamdache et al., 2013).

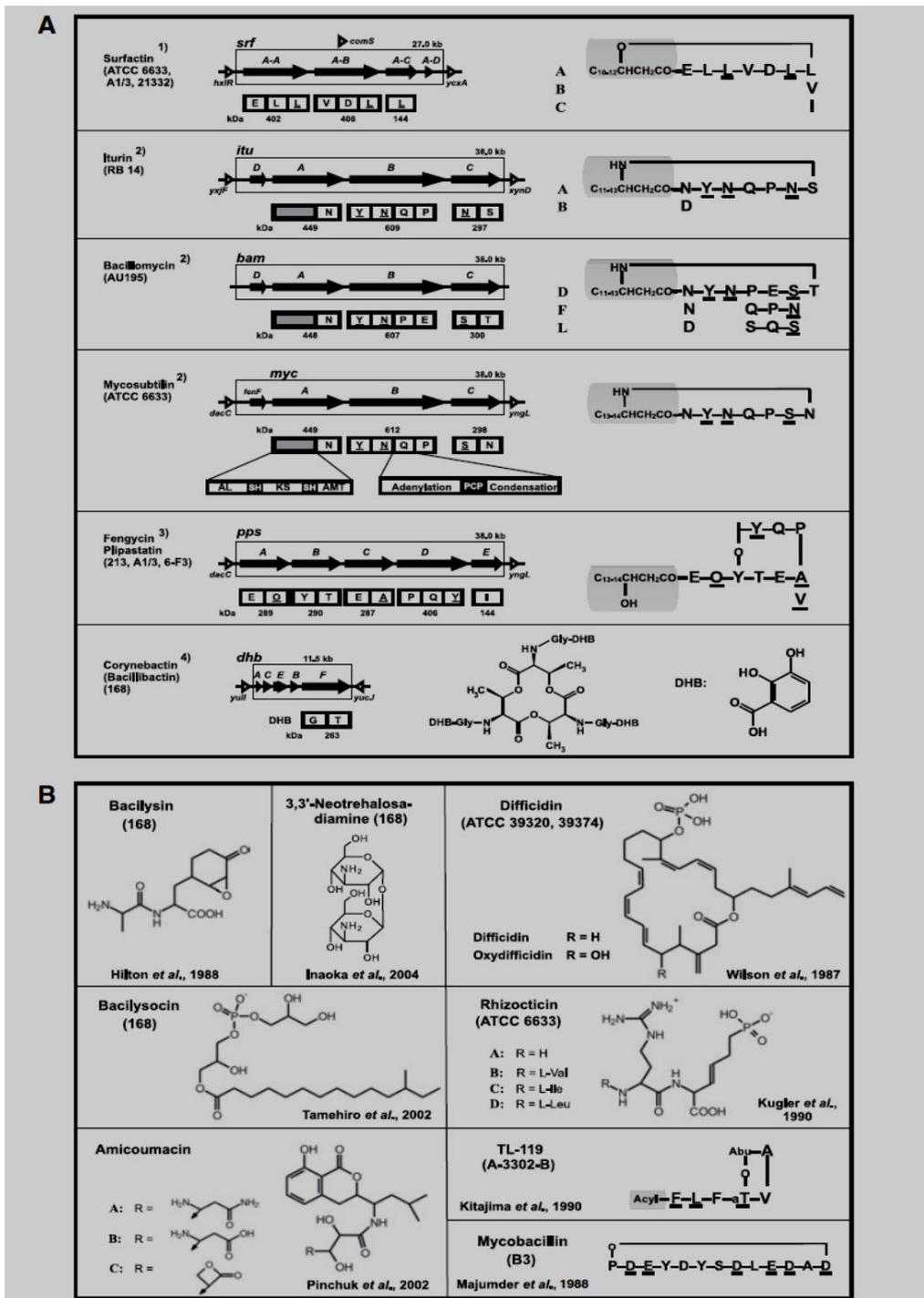


Figure 1.1. List of *B. subtilis* antibiotics. (a) Ribosomally and nonribosomally synthesized and (b) miscellaneous antibiotics (Stein, 2005).

Besides their antimicrobial actions, some of these secondary metabolites have important roles as signaling molecules. For instance, surfactin has also an important role in swarming motility and biofilm formation in *B. subtilis*, inhibits toxin production and colonization of *Staphylococcus aureus* and inhibits the aerial hyphae formation in *Streptomyces* species. In addition, the polyketide antibiotic bacillaene inhibits translation in bacteria, and besides this antimicrobial action, it inhibits the synthesis of several secondary metabolites like prodigionine in *S. coelicolor* (Gonzalez et al., 2011, Lamsa et al., 2012).

According to molecular studies, biosynthetic genes of antibiotics are found as polycistronic transcription units which are controlled by the global regulatory mechanisms and the expression of relevant genes are induced upon the entry into stationary phase of growth (Nakano and Zuber 1990).

### **1.3. General overview of Quorum sensing and two-component systems in the regulation of gene expression of *B. subtilis***

Regulation of gene expression is regulated depending on the level of cell density via small diffusible signal molecules and this process is known as Quorum sensing (QS) (Lazazzera, 2000; Li and Tian, 2012). There are mainly three QS mechanisms; first one is the acylhomoserine lactone (AHL) which found in Gram-negative bacteria which is produced by a LuxI-type synthase and is perceived by a DNA-binding LuxR-type transcriptional activator. Secondly, the autoinducing peptides (AIP) found in Gram-positive bacteria that are sensed by a two-component regulatory system which is composed of a membrane-bound sensor and an intracellular response regulator. The third one, the autoinducer-2 (AI-2) system that is common in both Gram-negative and -positive bacteria (Brackman and Coeny, 2015).

QS is an important regulatory mechanism in *B. subtilis* and takes role in many physiological processes like competence development, sporulation, production of extracellular enzymes and antibiotics (Comella and Grossman, 2005). As in the case of other prokaryotes, the signal transduction is mediated via two-component system which is composed of a histidine protein kinase and a response regulator. QS signals (AIPs) are sensed by an membrane-bound sensor histidine kinase and information is channeled to target genes through a phosphorelay to a response regulator protein (Zhang and Dong, 2004). In some instances, QS signals are transported back into the cell cytoplasm in order to interact with transcription factors and therefore, activity of the transcription factor is modulated to regulate gene expression (Rutherford and Bassler, 2012). Both routes were schematized in Figure 1.2.

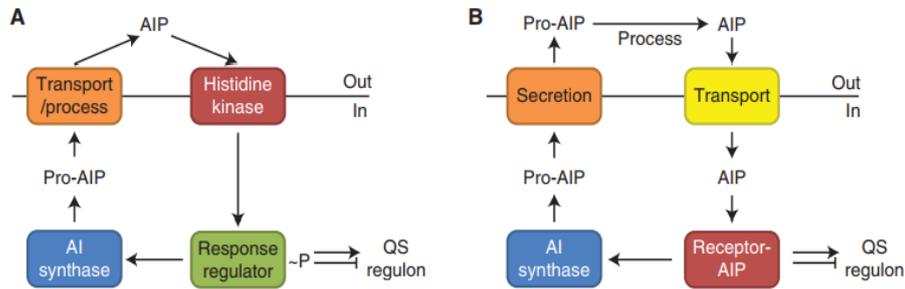


Figure 1.2. Canonical bacterial quorum-sensing (QS) circuits. Autoinducing peptide (AIP) QS in Gram-positive bacteria by (a) two-component signaling, or (b) an AIP-binding transcription factor (Rutherford and Bassler, 2012).

Rap proteins, which are phosphatases, belong to bacterial quorum sensing receptor family. In *B. subtilis*, there are 11 Rap proteins (RapA-K) and in addition to these chromosomal encoded ones there are five additional plasmid encoded proteins. Inhibitory oligopeptides of Rap proteins are called Phr

peptides and each Phr is encoded in an operon with its related Rap protein (Parashar et al., 2013). Three response regulators have been characterized as targets of Rap proteins in *B. subtilis* namely Spo0F, ComA, and DegU. The pairs of Rap and Phr proteins take role in the regulation of important processes takes place during transition to stationary phase of the growth. For instance, the regulation of sporulation by RapA/PhrA, extracellular protease production by RapG/PhrG and transfer of conjugative DNA element By RapI and PhrI (Lanigan-Gerdes et al, 2007; Bendori et al., 2015). The role of the Rap protein and Phr peptides in the process of sporulation is schematized in Figure 1.3.



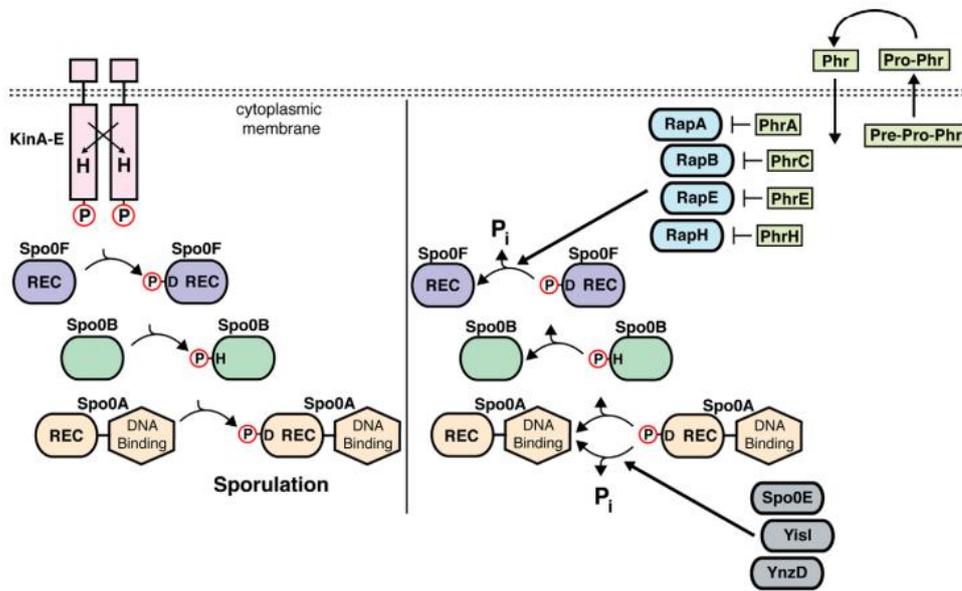


Figure 1.3. Rap proteins and Phr peptides in the regulation of *B. subtilis* sporulation signal transduction. The sporulation kinases (KinA-E) are depicted as membranous proteins for the sake of simplicity; however, KinA and KinE are actually soluble cytoplasmic receptors. Phosphoryl groups are transferred to Spo0F, Spo0B, and ultimately to Spo0A. When the level of Spo0A, P reaches a critical level, sporulation is triggered (left panel). Rap proteins dephosphorylate Spo0F, P causing phosphoryl groups to flow away from Spo0A, inhibiting sporulation (right panel). The Spo0K permease (not pictured) imports Phr peptides into the cytoplasm where they positively regulate sporulation by binding to Rap proteins and inhibiting Spo0F dephosphorylation. The Spo0E family member phosphatases, Spo0E, YisI, and YnzD, dephosphorylate *B. subtilis* Spo0A. REC, Receiver Domain; H, histidine; D, aspartic acid; P, phosphoryl group; Pi, inorganic phosphate (Parashar et al., 2011).

More than 10% of the genome is regulated by transcription factor ComA which is the main component of the ComQXPA pathway (Comella and Grossman, 2005). The pathway is activated depending on the accumulation of two pepheromones ComX (a cell driven extracellular peptide) and CSF (Competence

Sporulation Factor) as schematized at Figure 1.4. Briefly, ComX activates the ComP (membrane bound receptor protein kinase) which autophosphorylates and donated the phoshoate to ComA (response regulator). As a result, ComA dependent promoters are activated. In the case of CSF which is imported via Opp (oligopeptide permease; Spo0K) inhibits RapC which negatively regulates ComA (Comella and Grossman, 2005). In addition, at high concentrations, CSF inhibits the ComA controlled gene expression with a mechanism not completely identified (Pottathil et al., 2008).

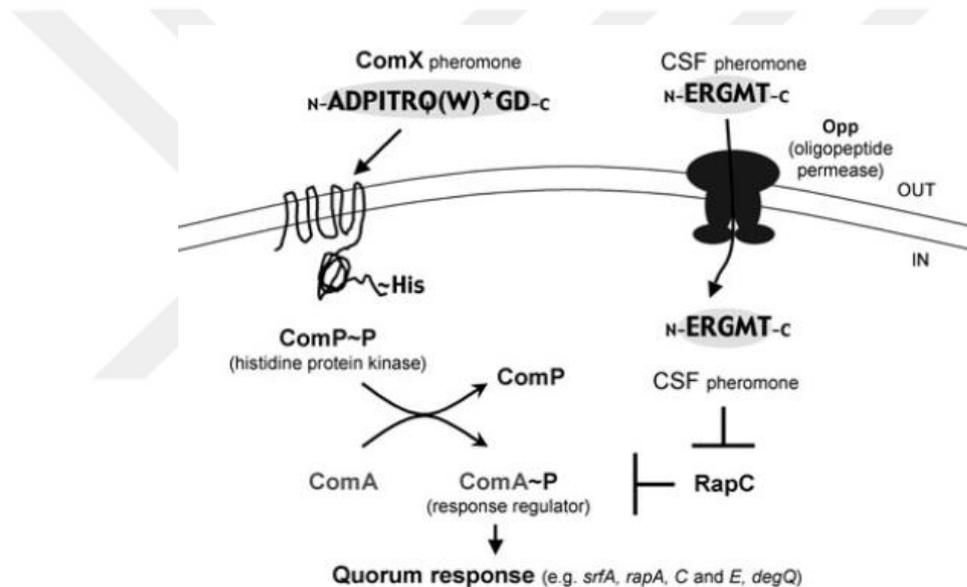


Figure 1.4. Convergent pathways regulate a quorum response in *Bacillus subtilis*.

Beside ComX and CSF, other pheromones PhrF, PhrK and most probably PhrH also increase the activity of ComA. These pheromones are transported via an oligopeptide permease and block the inhibitory activity of their cognate Rap proteins on ComA (Pottathil et al., 2008). ComA is the QS response regulator and it was repressed by RapC, RapD, RapF, RapH, and RapK. ComA

is an activator of many of the proteins including Rap proteins and surfactin (Ogura et al., 2003). Overall, the more than 20 genes directly and more than 150 genes indirectly are regulated by ComX-ComP-ComA quorum-sensing pathway (Claverys et al., 2006).

#### **1.4. Dipeptide antibiotic bacilysin**

Bacilysin [L-alanyl-(2.3-epoxycyclohexanone-4)-L-alanine] is the simplest peptide antibiotic known (Figure 1.5 b). It is composed of an N-terminal L-alanine and a C terminal non-proteinogenic amino acid, L-anticapsin (Walker and Abraham, 1970).

Bacilysin is active against some bacteria and fungi and its antibiotic activity is conferred by anticapsin. Upon the uptake of bacilysin by the target cells, anticapsin moiety blocks the glucosamine synthetase which is responsible for the bacterial peptidoglycan or fungal mannoprotein biosynthesis, leading to partial lysis of cells. Although it is synthesized nonribosomally, the mechanism of biosynthesis of bacilysin is quite distinct and has no relation with multifunctional NRPS model. It was shown by the cloning of a polycistronic operon *ywfBCDEF* and a monocistronic gene *ywfG* that these genes encode the bacilysin biosynthetic machinery (Inoaka et al., 2003). The *ywfBCDEF* genes of *B. subtilis* 168 were shown to carry the biosynthetic core functions of bacilysin synthesis and then renamed as *bacABCDE* (Steinborn et al., 2005). The *bacABC* genes are responsible from the conversion of prephenate to anticapsin and *bacDE* genes have roles in amino acid ligation and bacilysin immunity, respectively (Steinborn et al., 2005). The *ywfH* gene is divergently located and transcribed on the opposite DNA strand from *bacABCDE*. Later on, for convenience, *ywfG* and *ywfH* genes were renamed as *bacF* and *bacG*,

respectively (Parker et al., 2012). The organization of *bac* operon is shown in Figure 1.5 (a).

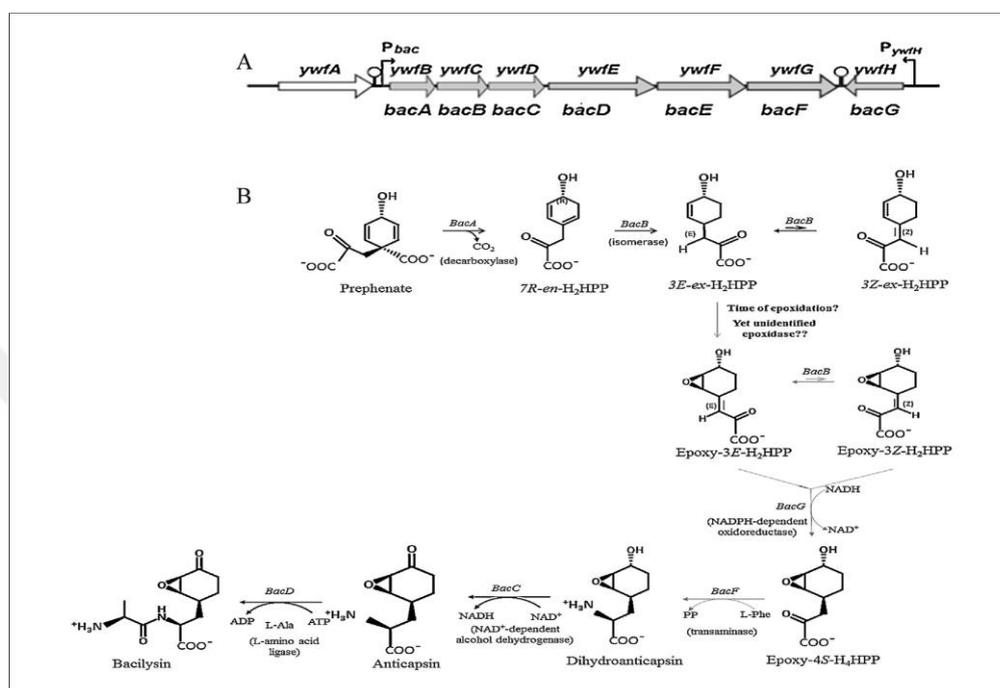


Figure 1.5. (a) Organization of the bacilysin biosynthetic gene cluster and (b) the biosynthetic pathway from prephenate to bacilysin (Özcengiz and Ögülür, 2015).

Bacilysin is produced in parallel to active growth by *B. subtilis* PY79 in synthetic medium and the production is suppressed by certain nutrients like glucose, ammonium and casamino acids as well as at certain temperatures and culture pH (Özcengiz et al., 1990; Özcengiz and Alaeddinoglu, 1991). It was well demonstrated that the bacilysin biosynthesis is under the stringent response and feedback regulation as well as quorum sensing global regulation (Figure 1.6.) (Özcengiz and Alaeddinoglu, 1991; Yazgan et al., 2001; Inaoka et al., 2003). In the study performed by Karataş et al. (2003), surfactin (*srfA*)

operon was disrupted and in this strain the bacilysin production is blocked. It reveals that surfactin biosynthetic operon (*surfA*) has a direct effect on the bacilysin biosynthesis.

The expression of *bac* operon (*bacA*) is under the regulation of four transcriptional regulator proteins namely ComA, Spo0A, AbrB and CodY. Among these, negative regulation occurs via AbrB and CodY which bind to *bac* operon promoter region simultaneously whereas positive regulation occurs via positive transcriptional regulators ComA and Spo0A, as shown by electrophoretic mobility shift analyses (EMSA) (Köroğlu et al., 2011). For the basal level *bac* operon transcription, ComA activity is sufficient while both ComA and Spo0A activity is necessary for the transition state induction. Besides, among Phr peptides PhrC, PhrF and PhrK are needed for full level expression of ComA-dependent *bac* operon expression (Karataş et al. 2003; Köroğlu et al., 2011).

Spo0A is the master transcriptional regulator which is activated under nutritional stress and high cell density conditions. The activation of Spo0A is possible via phosphorelay system including KinA, KinB, and KinC, a phosphorylation pathway starting from Spo0F phosphorylation and the phosphoryl group transfer to Spo0A at the end. Upon activation, more than 120 genes are regulated by Spo0A by binding to the promoter regions of these responsive genes and by this way the expression of more than 500 genes were changed (Burbulys et al., 1991; Hoch et al., 1993; Sonenshein, 2003). The most important results of Spo0A phosphorylation are activation of *degQ*, *sfrA*, *rapA*, *rapC* and repression of AbrB. AbrB is a transition state regulator and its depletion via Spo0A repression, the genes which are under the negative control of AbrB are activated (Strauch et al., 1990). Among those genes, *spo0H* which is a sporulation regulatory gene and involved in expression of vegetative and early stationary stage genes including mature PhrC peptide production and the

stationary phase overtranscription of *phr* genes (Lazazzera et al., 1999; Jaacks et al., 2001).

ppGpp (guanosine 50-diphosphate 30-diphosphate) has an essential role in transcription of the *bac* operon which mainly depends on the GTP level in the cell. The level of intracellular GTP is regulated through a CodY-mediated repression system (Inoaka et al., 2003).

In addition, it was shown that in *B. amyloliquefaciens* DegS/DegU, a two component signal transduction system, has a positive regulatory effect on the *bac* operon and monocistronic *bacG* at the transcriptional level (Mariappan et al., 2012). ScoC (Hpr) which is a transition state regulator and also shown to be a negative regulator of the sporulation and protease production. Similarly, the ScoC is negatively regulates bacilysin production by directly binding to the promoter region of *bacABCDEF* (Inoaka et al., 2009). A GntR type transcription factor which is encoded by *lutR* gene and takes role in broad range of physiological activities is found to be necessary for bacilysin production in *B. subtilis* (Köroğlu et al., 2008; İrigül Sönmez et al., 2014).

Major controls over bacilysin biosynthesis and its relation with sporulation initiation, competence development and surfactin production are shown in Figure 1.6.

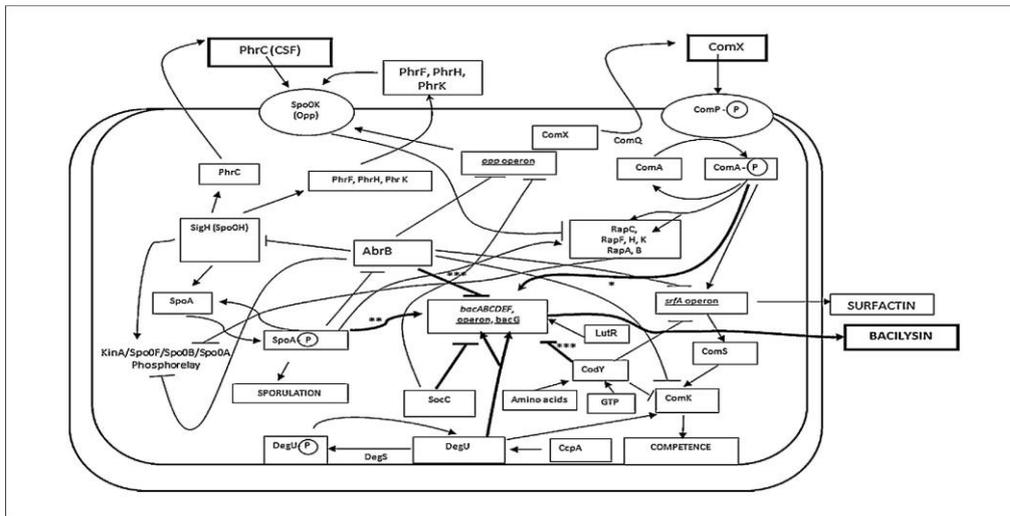


Figure 1.6. Major controls over bacilysin biosynthetic operon and their interactions with sporulation initiation, competence development and surfactin production. PhrC (CSF) and ComX pheromones are bold boxed. Positive and negative regulations are shown by arrows and T-bars, respectively, bold lines referring to direct interaction with P<sub>bac</sub>\*: basal-level transcription and transient-state induction, \*\*: transient-state induction, \*\*\*: both bind to P<sub>bac</sub> simultaneously. (Özcengiz and Ögüür 2015).

For monitoring of *bac* operon expression and find out the the regulatory mechanisms act on this operon, OGU1 mutant strain was constructed. For this purpose, the *bacA* which is the first gene of the *bac* operon was amplified and by the use of pMUTIN T3 plasmid DNA, the recombinant plasmid containing *bacA-lacZ* fusion was constructed. After transformation of recombinant plasmid into *B. subtilis* PY79 and a single cross-over event at *bacA* locus (Campbell-like insertion) whole recombinant plasmid was integrated into chromosomal DNA and as a result OGU1 (*bacA::lacZ::erm*) strain was established. The insertion of pMUTIN plasmid leads to first of all inactivation of the target gene, in our case *bacA* and therefore the expression from whole operon is inactivated. Secondly, the  $\beta$ -galactosidase which is the reporter gene

is now under the expression of the natural promoter of the target gene. Finally, the downstream genes are placed under control of a promoter (P<sub>spac</sub>) and the expression of which is regulated by the IPTG-dependent LacI repressor by this way the possible polar effects due to insertion of plasmid is eliminated (Ogasawara, 2000). After insertion of recombinant plasmid and construction of OGU1 strain, bacilysin production was blocked and instead the  $\beta$ -galactosidase synthesis occurs under the *bac* promoter. Therefore,  $\beta$ -galactosidase activity is correlated with bacilysin production. The transcriptional expression pattern of *bac* operon was quite similar to the bacilysin production pattern which was formerly determined by bioassay technique in culture fluids in that the expression was constant during the exponential phase and followed by an increment in expression during the transition phase. Finally, it reached its maximal level upon entry into stationary phase (Figure 1.7.) (Ögülür, 2008; Köroğlu et al., 2013).

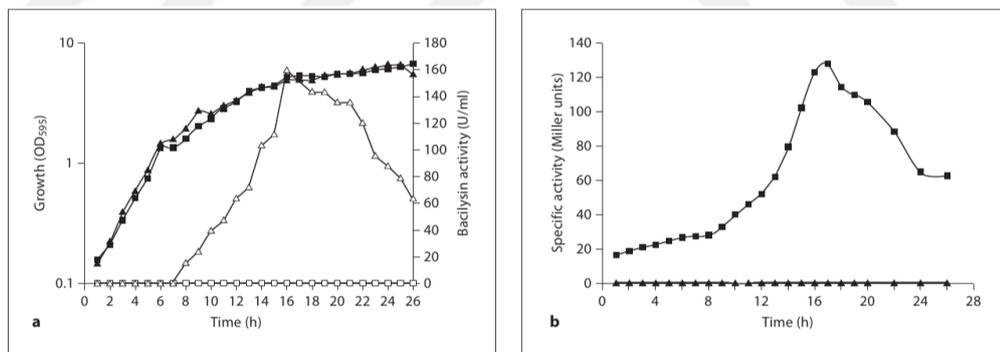


Figure 1.7. (a) Growth (closed symbols) and bacilysin activity (open symbols) of *B. subtilis* PY79 (triangles) and OGU1 (*bacA::lacZ::erm*) (squares) in PA medium. (b) Expression of transcriptional *bacA-lacZ* fusion in PA medium. Specific activity of *B. subtilis* PY79 (black triangles) and OGU1 (black squares). (Köroğlu et al. 2011).

## 1.5. Proteomics: Techniques and instruments

Mass spectrometry (MS) analyses together with the protein separation techniques have a crucial role in proteomics. Mass spectrometers are generally used for determination of mass or posttranslational modifications of the target polypeptide or protein as well as quantification purposes. Mass spectrometer has three major components namely; an ion source, an analyzer and a detector. Briefly, the mass spectrometric analysis takes place in the gas phase on ionized analytes by ionization source and then the mass to charge ratio ( $m/z$ ) of these analytes is measured by analyzer and the number of ions at each  $m/z$  value is detected by detector (Zhu et al., 2010; Aebersold and Mann, 2003; Domon and Aebersold, 2006). The different configurations are shown in Figure 1.8.

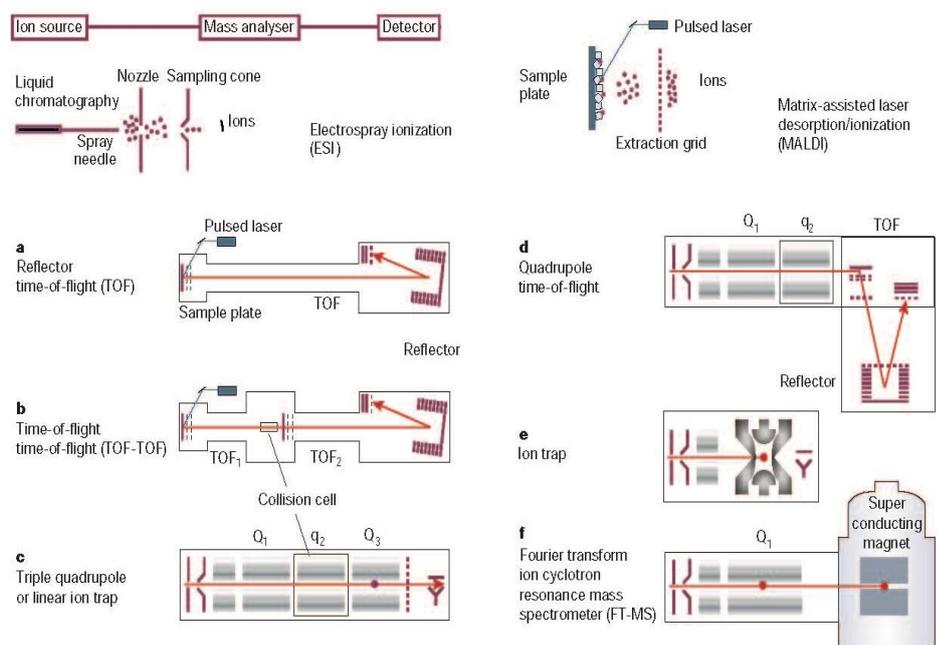


Figure 1.8. Mass spectrometer with different ionization sources and instrumental configurations with different analyzers (a-f) (Aebersold and Mann, 2003)

The two commonly used ionization techniques in proteome studies namely; matrix-assisted laser desorption/ionization (MALDI) and Electro spray ionization (ESI) are shown in Figure 1.8. MALDI ionizes samples via crystalline matrix and coupled to gel based methods (2DE) while ESI ionizes samples which are dispersed in solution. Relatively simple peptides are generally analyzed via MALDI-MS/MS whereas for complex samples ESI-MS/MS (LC-MS/MS) is chosen. The gel based methods which are coupled with MALDI are very crucial since they provide valuable information like posttranslational modifications including mass and charge modifications, protein stability and protein-protein interactions but the limitations of this approach include difficulties with the identification of poorly soluble hydrophobic proteins, membrane proteins in particular, or very acidic or basic, also small and large proteins. On the other hand, the LC coupled MS/MS

analyses provide a better protein coverage when compared to gel based methods. As a result, the combinations of different techniques are used in comparative proteome studies since each technique has its own advantages and disadvantages (Marvin et al., 2003; Aebersold and Mann, 2003).

Since the coverage of proteome by the 2DE is nearly 25%, the studies mainly focus on different protein extraction methods together with different protein prefractionation methods in order to increase the proteome coverage (Becher et al., 2011). Among the 2816 predicted cytosolic proteins, nearly 860 proteins were detected in vegetative proteome of *B. subtilis* via GeLC-MS/MS. To sum up, 1156 cytosolic proteins were detected which constitutes 40% of the predicted proteome (Becher et al., 2011).

The workflow of a proteome analysis after sample collection continues with protein extraction. There are different protein extraction techniques like Trichloroacetic acid (TCA), methanol or phenol methods. After extraction, these proteins can be separated via 1DE or 2DE approach. In 2DE approach, the proteins are separated according to their isoelectric points (pI) at first dimension with isoelectric focusing (IEF) and then according to their molecular weight (Mw) with polyacrylamide gel electrophoresis (PAGE) at second dimension. This method couples with MALDI ionization and followed by MS analysis. In 1DE approach, proteins are separated according to their sizes and then further separation is done with liquid chromatography coupled to ESI ionization and MS analysis (Aebersold and Mann, 2003; Neilson et al., 2011). In 1DE approach, SDS-PAGE is generally preferred since SDS (sodium dodecyl sulphate), a strong anionic detergent, effectively solubilize proteins. Another PAGE method available especially for isolation of membrane proteins is Blue Native (BN) PAGE (Schägger and von Jagow, 1991; Westermeier, 2006). Briefly, in this technique proteins are separated by the help of mild nonionic detergents like Triton X-100 and the three dimensional structure of the protein is maintained. (Abdallah et al., 2012; Wöhlbrand et al., 2013). BN-

PAGE method is generally used for hydrophobic membrane proteins in mitochondria, plant membranes and chloroplasts. BN is also give information about the subunit composition, molecular weight and native state of the proteins (Schägger, 1994; Abdallah et al., 2012).

In gel free methods proteins are digested and resulting peptide fragments are subsequently analyzed via MS. There are different strategies for gel-free methods and categorized under mainly label-free and label-based MS techniques (Wöhlbrand et al., 2013).

The quantification with MS analyses can be possible by either using label free peptides or isotopically labeled peptides. For labeling, modifying peptides with isobaric tags for relative and absolute quantitation (iTRAQ), labelling proteins with isotope-coded affinity tags (ICAT), or metabolically labelling proteins by incorporation of stable isotope labels with amino acids in cell culture (SILAC) are generally preferred. Besides their accuracy and efficiency, labeling methods are expensive; require specific equipment as well as software programs and experience. On the other hand, the label free methods are cheaper and do not require additional equipment for detection of labeling products. There are two main approaches for label free quantification. First one is based on spectral counting and in this approach the number of peptides of a protein is used for quantification. In the second approach which is called area under the curve (AUC), the intensity of the signal is measured based on ion spectra of a protein in MS/MS experiment (Beissbarth et al., 2004; Neilson et al., 2011).

### **1.6. Proteome of the model organism *B. subtilis***

By the availability of the complete genome sequence of *B. subtilis* 168, the proteome studies become more and more powerful. The technical improvements in proteomics area with parallel developments in *B. subtilis*

proteomics are shown in Figure 1.9 (Becher et al., 2011). The proteome of growing cells (i.e. vegetative proteome) and the proteome of non-growing cells suffering from stress or starvation has been extensively compared since a significant part of the genome is considered silent unless a certain stress stimulus is present (Wolff *et al.*, 2007). On the other hand, the total *B. subtilis* proteome has been divided into subproteomes as follows; the cytoplasmic proteome; the cell membrane proteome; the cell wall proteome and the extracellular proteome (secretome) (Antelmann et al., 2002).

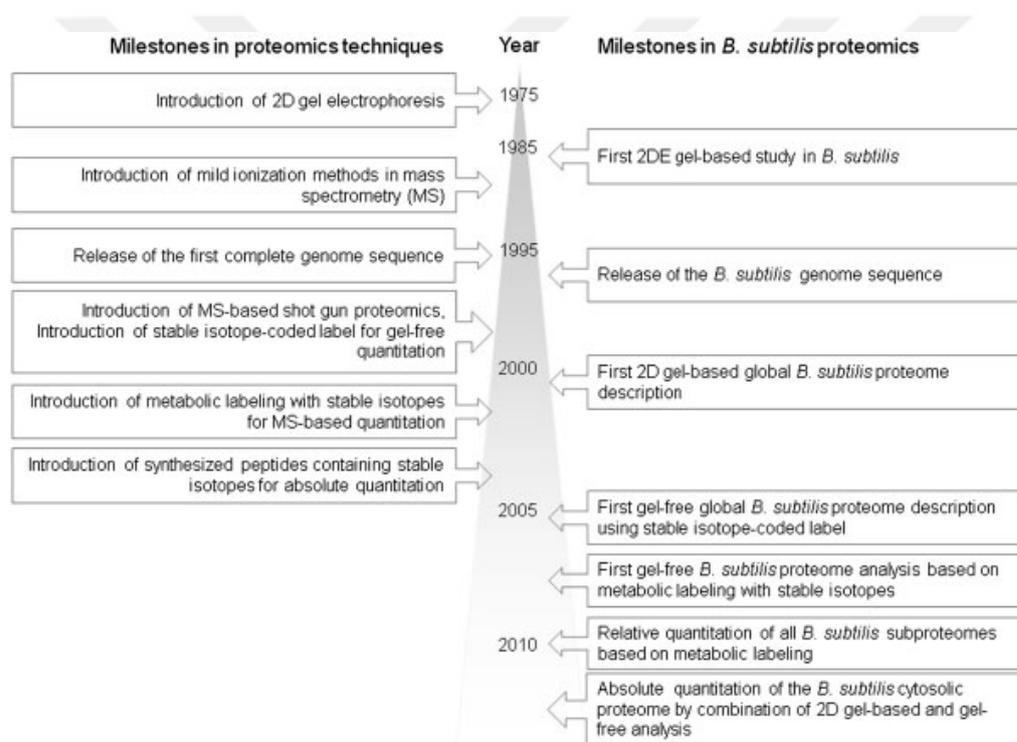


Figure 1.9. Milestones in the development of proteomics techniques and their application in *B. subtilis* (Becher et al., 2011).

### 1.6.1. Cytoplasmic (cytosolic) proteome

The first comprehensive proteome map of vegetative *B. subtilis* revealed more than 300 cytosolic proteins by the two-dimensional gel electrophoresis (2DE-PAGE) in pI 4-7 range (Büttner et al., 2001). In the same pI interval, cytosolic proteome of *B. subtilis* was expanded to a total of 693 proteins (Eymann et al., 2004). One third of these reside above pI 7 ranges which cannot be identified by 2DE-PAGE. For this reason, gel-free approach is applied for the detection of 814 cytosolic proteins, 140 of which were identified (Wolff et al., 2006).

The vegetative cytosolic proteome studies were followed by stress- or starvation-induced proteome analyses by which more than 200 stress- or starvation-induced proteins were detected. Eighty three of these were absent from the vegetative proteome map and 117 were found with a basal expression level in the vegetative cells as well (Antelmann et al., 2000; Bernhardt et al., 2003; Mostertz et al. 2004; Tam et al., 2006; Höper et al., 2006; Wolff et al., 2007). Most of the phosphate starvation-induced proteins are under the control of sigB was identified via proteomic approach (Antelmann et al., 2000). Later on, in an osmotic shock response proteomics work conducted by Hahne et al. (2010), up to 1710 proteins were identified by gel-free technique and the results were quite consistent with another osmotic shock response study performed by 2DE approach, as reported by Höper et al., (2006).

In another study, the effect of thiol oxidant diamide that leads to disulfide stress in *B. subtilis* was investigated by combining DNA macroarray and proteome analyses. As a results of disulfide stress, drastical changes in the proteome pattern was observed and the differentially expressed proteins were identical to those that were found earlier to be responsive in other stress conditions like heat, oxidative stress as well as stress due to exposure to toxic compounds (Leichert et al., 2002).

The changes occurring in response to calcium treatment was investigated via cytoplasmic proteome analysis in *B. subtilis*. As a result, significant global changes in protein abundance were found in the *B. subtilis* proteome and mainly six proteins calcium binding proteins (CaBP) were identified namely, adenylate kinase, fructose-bisphosphate aldolase, the heat-shock protein GrpE, 3-oxoacyl-acylcarrier-protein synthase (ACP), elongation factor-Tu (EF-Tu), and a thiol peroxidase (Domniguez et al., 2011).

In order to understand the response of *B. subtilis* to synthetic peptide antibiotic MP196 which targets membrane, cytoplasmic proteome was analyzed via 2DE and LC-MS based approaches. In this study, upregulations of proteins related with cell envelope, cell wall and cell membrane stress as well as energy starvation were detected. Gramicidin S belongs to a small cationic antimicrobial peptide class and it is integrated in to the lipid bilayer in order to disrupt the function of membrane. Valinomycin is a peptide antibiotic with potassium carrier ionophore function and leads to cell lysis. MP196 and the selected antibiotics valinomycin and gramicidin S are comparators to each other. Upon exposure to these three antibiotics, proteins which are the markers of stress conditions related with cell-envelope, membrane, energy limitation, and cell wall stress were induced strongly (Wenzel et al., 2014).

In another study, Reddy et al. (2015) examined the alterations in *B. subtilis* proteome after exposing the cells to super antioxidant totarol which can restrain the growth by perturbing the cell division, leads to alteration of 139 protein's expression patterns. The metabolic shutdown due to repression of major central dehydrogenases is the obvious result of totarol exposure. Besides, the important protein groups like chaperone proteins, heme biosynthesis, and ribosomal proteins were found to be differentially expressed and these leads to filamentation of the bacteria.

### 1.6.2. Cell Membrane proteome

The membrane proteins are important components of the total proteins since they have diverse functions like maintaining cell integrity, signaling, transport, energy conservation as well as taking part in pathogenicity. The membrane proteins also contain signal peptides which are cleaved upon their anchorage to the membrane and also some of them contain membrane spanning domains (Wolff *et al.*, 2007). The proteome of membrane proteins are difficult and challenging when compared to proteome cytoplasmic (cytosolic) proteins. It is mainly because of relatively insoluble nature of membrane proteins in nonionic or zwitterionic detergents with low ionic strength. The solubility of proteins is not sufficient since after solubilization, these proteins can precipitate at the pH values that closer to their pI values during the IEF process (Bunai *et al.*, 2004). Therefore, a special protocol for membrane proteome analysis is described by Hartinger *et al.* (1996) which is a discontinuous acidic PAGE system with cationic detergents in the first dimension and followed by Tris/glycine SDS in the second dimension. Another protocol is applied by Schägger and von Jagow (1987) that is a kind of 2D system with a native PAGE is used; in the first dimension (blue native-PAGE) in which Coomassie Brilliant Blue G induces a charge shift on membrane protein complexes. The combined and modified forms of these protocols are used in order to perform membrane proteome studies as in the case of the first membrane proteome of *B. subtilis* in which the ABC transporter solute binding proteins were investigated. Briefly, cytoplasmic membrane is washed in high-salt and alkaline buffers before the membrane proteins are solubilized with the detergent n-dodecyl- $\beta$ -D-maltoside. As a result of 2DE MALDI-TOF MS analysis, a total of 637 proteins were detected and among these 256 of them predicted to be membrane proteins. Among these proteins, 30 of 38 ABC transporter solute binding proteins

(SBPs) were identified and potential substrates were predicted (Bunai *et al.*, 2004).

Another comparative membrane proteome analysis of *B. subtilis* cells (stationary phase of growth) was performed via stable isotope labeling with amino acids (SILAC) and <sup>14</sup>N/<sup>15</sup>N-labeling. The efficiencies of these two labeling techniques in the identification of membrane protein were compared. SILAC technique is based on the integration of iso-topologous amino acids into proteins whereas the <sup>14</sup>N/<sup>15</sup>N-metabolic labeling uses different N-isotopes integration of amino acids into proteins. In SILAC method a total of 456 proteins were identified and 46 of them were cytoplasmic membrane proteins. In <sup>14</sup>N/<sup>15</sup>N-labeling method, 488 proteins were identified and 31 of them were cytoplasmic membrane protein. As a result, both labelling methods provide comparable and important data for membrane protein quantification (Dreisbach *et al.*, 2008).

Membrane proteome of *B. subtilis* cells were conducted with PrsA (a membrane-anchored peptidyl- prolyl cis-trans isomerase)-depleted and non-depleted cells with <sup>14</sup>N/<sup>15</sup>N-metabolic labeling method and the main aim is to identify the PrsA dependent membrane proteins. The important finding is that Penicillin binding proteins (PBPs) were shown to be among the PrsA dependent proteins and the probable reason for growth arrest in the absence of PrsA is related folding of PBPS (Hyyryläinen *et al.*, 2010).

### **1.7. Protein export systems of *B. subtilis***

There are mainly four routes for protein secretion namely; the Sec pathway, the twin-arginine translocation pathway, the ATP-binding cassette transporters and a pseudopilin export pathway that are characterized in *B. subtilis* (Harwood and

Cranenburg, 2008; Du Plessis et al 2011). The secretory proteins are identified by the presence of a N-terminal hydrophobic extension which is called the signal peptide. Both in Sec-dependent and Tat pathways, the primary sequences of signal peptides are not homologous but share a common features like a N-terminus (net positively charged), a hydrophobic core region (H-region), and a polar C-terminal end containing the signal peptidase recognition site as shown in Figure 1.10 (Harwood and Cranenburgh, 2007; Anné et al., 2016). To be more specific, type I (classical) signal sequence, the twin arginine translocon (Tat) signal sequence, the type II (lipoprotein) signal sequence, and type IV (prepilin-like) signal sequence are the different types of signal sequences found in the secretory proteins (Jankovic et al.,2007). Sec pathway is the main secretory mechanism and there are five chromosomally encoded Type I SPases (SipS, SipT, SipU, SipV, and SipW) and Type II SPases are exclusive for lipoproteins in *B. subtilis* (Pohl and Harwood, 2010).

The SPs has mainly three functions as known and first one is the block the folding of the nascent preproteins intracellularly to retain translocation competence and inhibit the activation of secretory enzymes. By the interaction of the secretion machinery components, SPs direct the translocation process and lastly, SPs serve as a topological determinant for preproteins in the membrane (Park and Schumann, 2015).

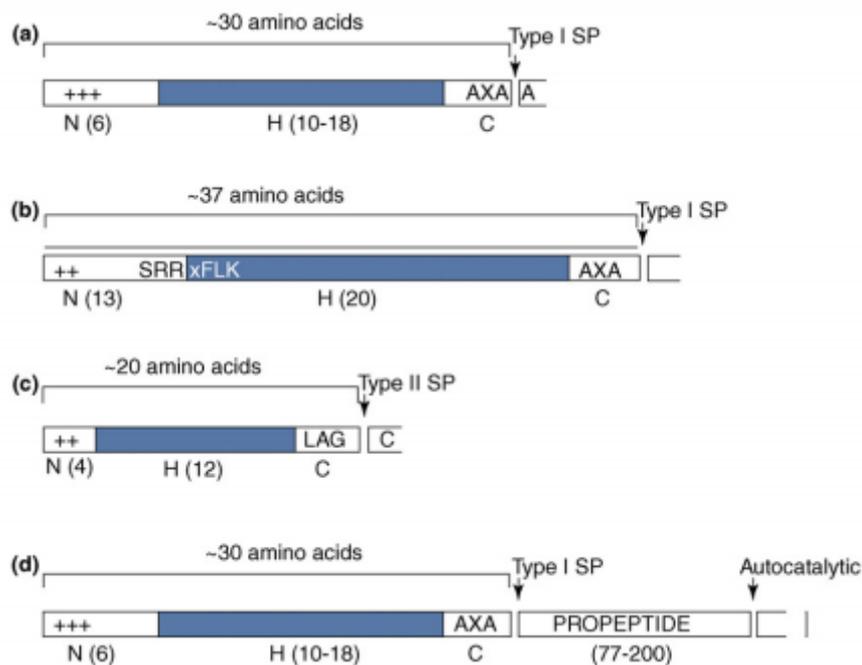


Figure 1.10. General features of the signal peptides and propeptides of *Bacillus* secretory proteins. The N-terminal (N), hydrophobic (H) and cleavage (C) regions are identified by contrasting shading and their lengths (amino acid residues) are indicated in brackets. Cleavage sites are indicated by arrows. (a) Sec-dependent signal peptide cleaved by a type I signal peptidase (SP) at the AXA cleavage site. (b) Tat-dependent signal peptide with twin arginine motif (SRRxFLK), also cleaved by a type I SP. (c) Lipoprotein signal peptide cleaved by the type II SP. (d) The signal peptide and propeptide (prepropeptide) at the N-terminal end of a secretory protein requiring the propeptide for folding on the trans side of the cytoplasmic membrane (Harwood and Cranenburgh, 2007).

The main pathway for protein secretion in *B. subtilis* is the Sec pathway (Figure 1.11.) which translocates the protein in an unfolded state. Sec pathway is composed of the SecA motor protein, the SecYEG translocon and the SecDF-YrbF heterotrimeric complex. The CsaA which is a potential chaperon

protein and found to interact with SecA and binds preproteins (Park and Schumann, 2015).

In contrast to Gram-negative organisms, Gram-positive ones including *B. subtilis* contains two SecA homologues namely SecA1 and SecA2. The essential one is SecA1 protein and maintains secretion via canonical Sec pathway. On the other hand, SecA2 is used for the secretion of less number of proteins and generally these proteins are required under certain stress conditions. The SecA2 is similar to SecA1 with smaller size due to some deletions which are effective on ATP hydrolysis rate, interactions with the Sec apparatus and its location. Probably, secretion via SecA2 may be regulated differently when compared to secretion by SecA (Green and Meccas, 2016).

The main importance of the Tat system (Figure 1.11.) is the translocation of the proteins in their folded state. Tat SPs are generally longer and less hydrophobic when compared to Sec pathway SPs. The presence of basic residues at the C terminal of the Tat SPs is probably functioning as the Sec avoidance signals (Harwood and Cranenburgh, 2007). The Tat signal sequence contains a pair of “twin” arginines in the motif S-R-R at the N-terminus of the folded protein which are released extracellularly (Tjalsma et al., 2004; Green and Meccas, 2016). In general, Tat pathway translocon is composed of a single complex with three subunits namely, TatA, TatB and TatC. In *B. subtilis*, TatB is absent and active Tat complexes are merely composed of TatA and TatC subunits (Goosens et al., 2013; Anne et al, 2016). The presence of RR or KR signals is not sufficient for a protein secreted via Tat pathway. Although, there are at least 14 peptides including either RR or KR signal, there are only four known substrates of Tat pathway namely, phosphodiesterase (PhoD), the newly identified YwbN protein, the wall-bound metallo-phosphoesterase (YkuE) and LipA (secreted esterase) are Tat-dependent in *B. subtilis* (Jongbloed et al., 2004; Goosens et al., 2013).

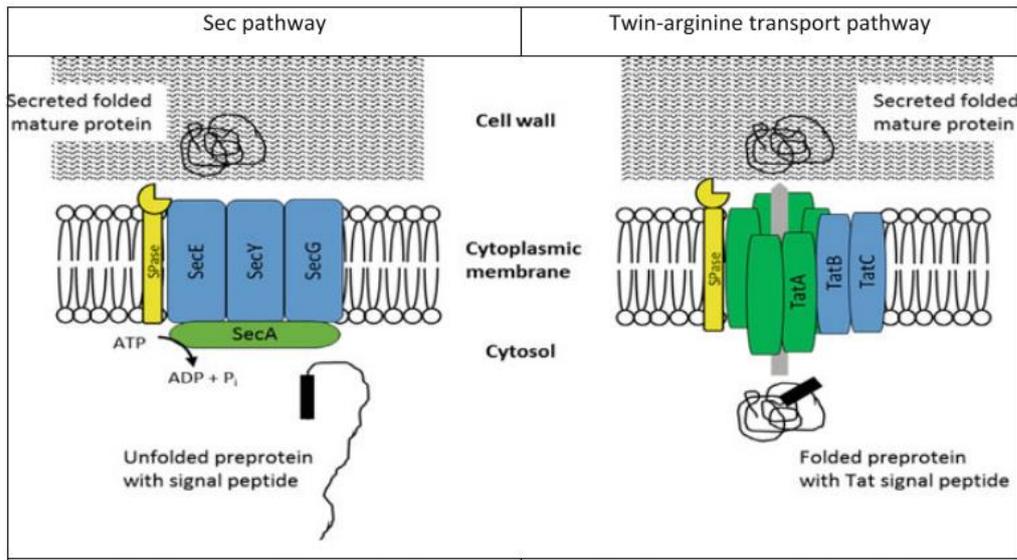


Figure 1.11. Two main secretion systems in *B. subtilis* Sec and Tat pathway (Anné et al., 2016).

ATP-binding cassette transporters composed of two hydrophobic domains with membrane spanning properties and two hydrophilic domains called nucleotide binding domain. ATP hydrolysis provides the source of energy that is necessary for export of proteins. SpaT and SunT are the two ATP transporter used for the export of lantibiotics subtilin and sublancin 168, respectively (Fu et al., 2007).

The pseudopilin export pathway recognizes the pseudopilin like signal which is a 33 residue in length. Competence related proteins ComGC, ComGD, ComGE, and ComGG are the substrates of this pathway. Pseudopilin precursors bypass the Tat and Sec pathways (Tjalsma et al., 2004).

Flagella Export Apparatus (FEA) is a specific protein export system for secretion of the flagella hook, filament, and cap forming proteins. This apparatus is thought to be ancestor of all T3SS system which takes role in the

export of flagellum across the cytoplasmic membrane into the channel of the flagellum for assembly (Anne et al., 2016).

Another transport system is found in T4SSs which transports wide range of substrates like DNA, nucleoprotein complexes and effector proteins. T4SSs are membrane spanning and multisubunit that translocate proteins that contains specific leader peptide which is cleaved by type 4 prepilin peptidase upon secretion (Anne et al., 2016). In addition, a novel export system which is called Esat-6 secretion system (ESX or Ess) is also found in the *B. subtilis*. This system was first found in *Mycobacterium tuberculosis* for the export of small proteins ESAT-6 (EsxA) and CFP-10 (EsxB). EsxA is a small protein composed of 100 aminoacid in a helix turn helix structure and does not include a signal sequence at N terminal (Huppert et al., 2104).

### **1.8. Secretome of *B. subtilis***

The secretome, a subset of proteome, is composed of secreted proteins together with the secretory protein components of a cell (Greenbaum et al., 2001; Tjalsma et al., 2004). The secretome studies are valuable for providing a better understanding in microbiology since many microbes have the ability to secrete considerable amounts of valuable substances with special properties related with survival under changing environment (Greenbaum et al., 2001). Thus secretome is highly dynamic and responsive to various environmental stimuli and pathologies (Caccia et al., 2013).

Secretome analysis was conducted as a comparative study with *B. subtilis* 168, a *secA*-temperature sensitive mutant and an *ffh* conditional mutant in minimal medium with different carbon sources (glucose, cellobiose, maltose and soluble starch). The 2D analysis of these secreted proteins revealed nearly 110 protein

spots visualized in the parental strain 168 and most these disappeared in *secA* and *ffh* mutants. Depending on the results, 23 different proteins were found and most of them were found to be secreted via SRP/Sec protein secretion system. Among these, three of them namely Gap, SodA and KatA were cytoplasmic proteins. The secretion of YfIE and YfnI (predicted membrane proteins) to the culture medium was dependent of presence of SecA and Ffh (Hirose *et al.*, 2000).

Proteome analysis of secreted proteins of *B. subtilis* under phosphate starvation was performed via 2D approach. Comparison of wild type strain (168) and two mutants *sigB* and *phoR* was performed in order to identify the phosphate starvation induced proteins and corresponding regulons. As a result, two new members of Pho regulon namely glycerophosphoryl diester phosphodiesterase (GlpQ) and a lipoprotein (YdhF) were identified (Antelmann *et al.*, 2000). Later, Antelman and coworkers reported that the maximum secretion from *B. subtilis* is maintained in the rich medium at the stationary growth phase of the organism. In the same study, previously identified 23 extracellular proteins (Hirose *et al.*, 2000) and newly identified 62 extracellular proteins of *B. subtilis* 168 were reported. It was shown by this study that the secretome changes can also occur in response to a mutation (Antelmann *et al.*, 2001).

Secretome study on the secretion stress response of *B. subtilis* was conducted by Antelmann *et al.*, (2003). For the induction of secretion stress, two methods were chosen. First one is to mutate HtrA or HtrB which are membrane-anchored protein quality control proteases and they are important players for dealing with stress. Another way is to secrete high levels of  $\alpha$ -amylase AmyQ of *Bacillus amyloliquefaciens*. The results show that two methods of inducing stress were revealed similar results. In addition, HtrA and YqxI levels show major variations in parallel. Based on the 2D and Northern blot analysis, HtrA regulation is at the transcriptional whereas the YqxI level is dependent on HtrA and regulated posttranscriptionally (Antelmann *et al.*, 2003).

Other than identification purposes, secretome can be used to attribute functions to extracellular proteins. For instance, 2D coupled with zymogram assay is performed in order to detect fibrinolytic enzymes in the medium of *B. subtilis* 168. As a result, mainly four spots having fibrinolytic activity were detected and identified as serine proteases WprA and Vpr and their processed forms (Park et al., 2002).

When the *B. subtilis* cells grown in phosphate starvation medium was compared to cells grown in complete medium (Figure 1.12.), a total of 113 different proteins were identified. In the same study, it was found that specific proteins of phosphate starvation proteins constituted 30% of the extracellular proteome (Figure 1.12 (a)). After a detailed examination, 54 proteins were found to be among the predicted extracellular proteins. Also, 17 cytoplasmic proteins, 6 phage-related proteins and 7 flagella-related proteins without signal peptide were identified in the secretome samples (Wolf et al., 2007).



mechanism that MscL channel protein is probably a candidate component (Kouwen et al., 2009).

Several *tat* mutants were investigated via secretome analysis with LC-MS/MS in order to understand the *tat* pathway and explore its potential substrates. The results of extracellular proteome part of the study showed that *tat* mutations under investigation have no major impact on the composition of the *B. subtilis* proteome. On the other hand, the differentially expressed proteins (TasA, YoaW, Epr, LytF, LytA/B) partially explained the minor phenotypic changes in *tat* mutants like delayed biofilm formation in several *tat* mutants. The more important result is the identification of QcrA protein as being a *tat* substrate in *B. subtilis* via secretome analysis (Goosens et al., 2013).

In *B. subtilis*, comparative secretome analysis of two putative sortase mutant (*yhcS-ywpE*) and parental strain (168) was investigated. Sortases are the enzymes that catalyze the anchorage of proteins to the cell wall via covalent bonding. The analysis revealed that under phosphate starvation, mutant released increased levels of YfkN into the culture medium which is a potential substrate of those sortases. From the comparison of extracellular proteins, it was found that the proteases Bpr, Vpr and PhoB, PhoD did not show any difference meaning that sortase activity does not affect either proteases or phosphate induced proteins as shown in Figure 1.13. Briefly, together with the extracellular proteome and reporter protein analysis, it was found that YhcS is involved in the cell retention of the YfkN protein, and that YhcS can anchor a secreted reporter protein to the cell wall of *B. subtilis* when this reporter is fused to the sortase recognition motif of YhcR (Fasehee et al., 2011).

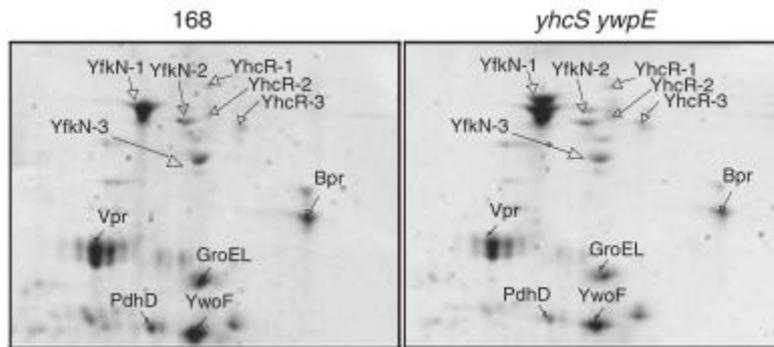


Figure 1.13. Extracellular levels of YfkN and YhcR upon phosphate starvation. *B. subtilis* 168 or mutant of *B. subtilis* *yhcS ywpE* were grown under conditions of phosphate starvation (Voigt et al., 2008).

In another study, the extracellular proteomes of *B. subtilis* and *B. licheniformis* have been compared under different growth conditions in order to find clues about their similarities and differences in terms of protein secretion. Extracellular proteins were collected 1 hour after entry into the stationary phase and protein separation performed with TCA precipitation as shown in Figure 1.14. More than 200 visible spots detected in both strains. The overall composition of the secretomes are similar in both strains but there are differences in terms of the relative amounts of the proteins with different functional groups (Figure 1.14 a,b). The main difference was seen in phosphate starvation condition. Alkaline phosphatases (PhoA and PhoB) and the phosphodiesterase (PhoD) were abundant in *B. subtilis* secretome whereas these proteins are found in small amounts in *B. licheniformis* (Figure 1.14 c, d) (Voigt et al., 2008).

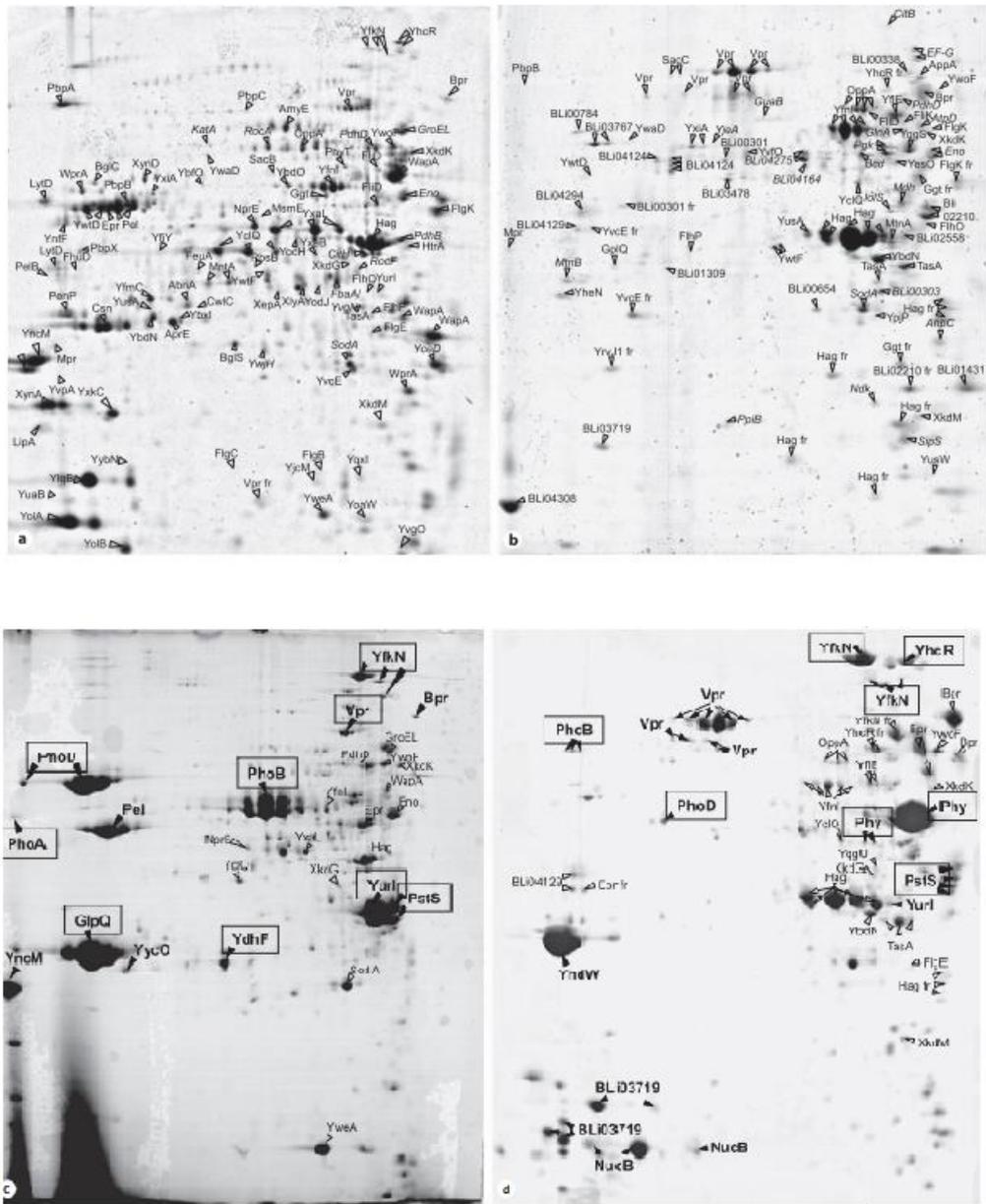


Figure 1.14. The extracellular proteomes of (a) *B. subtilis* and (b) *B. licheniformis* grown in complete medium (LB). (c) *B. subtilis* and (d) *B. licheniformis* grown in phosphate starvation medium. pH gradient 3-10 (right to left) and stained with Sypro ruby. Spots labeled in italics are presumably intracellular proteins (Voigt et al., 2008).

In addition to secretome, bacterial extracellular vesicle (EV) proteome is also popular since EV is considered as a novel secretion system which is important for communication, survival and virulence of the bacteria. Comparative proteome of EV during sporulation and vegetative growth of *B. subtilis* is investigated (Galperin et al., 2012). As a result, significant differences in terms of both quantity and quality of the vesicle contents are observed. In sporulating EV, proteins related with the ribonucleoprotein complex were abundant as well as higher hydrolase activity, nucleic acid binding and structural activity is observed. On the other hand, in vegetative EV oxidoreductase and nucleotide binding proteins are in considerable amount (Kim et al., 2016). During sporulation phase, higher than 12 % of the genes of *B. subtilis* are expressed meaning that the sporulation has also significant effects on the other cellular processes (Galperin et al., 2012).

The lipoproteome and cell wall proteome are considered as secretome or subtopics of secretome. Several extracellular proteins of *B. subtilis* are modified with the addition of lipid components to obtain lipoproteins which are found at the membrane surface. The secretion of lipoproteins in *B. subtilis* occur via Sec pathway and the lipid part is modified by Lgt (diacyl glyceryl transferase) and cleaved by LspA (type II signal peptidase) (Wolf et al., 2007). Although, lipoproteins should remain in the membrane portion, some of them are found in the earlier extracellular proteome studies. It was reported that among 10 lipoproteins detected by 2D-PAGE approach, 9 of them were already found in the extracellular proteome (secretome). The possible reason is the proteolytic shaving upon the processing of SPaseII (Tjalsma et al., 2004; Humperry-Smith et al., 2006). The cell wall proteins are involved in diverse processes including cell wall turnover during cell growth, cell division, sporulation and germination. The enzymes like penicillin binding proteins, cell wall hydrolases as well as many proteases, RNases, Dnases are found in the cell wall portion. The cell wall proteins contain both specific domains and N-

terminal signal peptides. The cell wall proteome of *B. subtilis* revealed 12 abundantly expressed cell wall proteins (Antelmann et al., 2002).

### **1.9. Findings from recent *B. subtilis* proteomics studies by our group**

Comparison of standard strain PY79 and bacilysin knock-out mutant strain OGU1 in terms of cytoplasmic proteomes revealed more than 1900 proteins separated via 2DE approach within different pH ranges (4-7, 4.5- 5.5, 5.5-6.7) (Taşkın et al., manuscript submitted). As a result, 192 differentially expressed that corresponded to 128 distinct proteins were listed. Among these, 63 proteins were found to be downregulated in mutant strain. 19 protein spots were absent and 2 protein spots were newly- induced in mutant strain. In another proteome study, 1282 proteins were identified via GeLC-MS/MS approach, 76 proteins were detected as differentially expressed. Both studies found out the strong effect of *bacA* deletion on the abundance of various proteins. One of the major functional group affected was sporulation proteins. A total of 18 proteins that are directly or indirectly involved in sporulation were determined as differentially expressed (Demir, 2013). Proteins that were found to be differentially expressed in both studies are presented in Table 1.1.

Table 1.1. Comparison of proteins that were found in 2DE and GeLC-MS/MS studies. Highlighted proteins were referring to those with similar expression in these independent studies. LC-MS/MS the Rsc values between -2 and 2 were considered significant. In 2DE MALDI-TOF MS bacilyisn knock-out mutant over PY79 control ratio was given (Adopted from Demir, 2013).

Genes names	LC-MS/MS (Rsc value)	MALDI-TOF MS/MS mutant/control ratio
ahpC	0.06755	2.557
ald	0.74636	N.D.**
atpC	-0.414235	N.D.**
atpD	-0.096144	0.343
bacC	-2.947499	0.337
bacD	-4.994989	N.D.**
citB	-0.388263	0.345
cotA	-3.216815	0.088
degU	0.54458	2.68
dppA	-0.227276	0.26
glmS	-2.06461	N.D.**
ilvC	0.14455	N.D.**
ilvD	0.28741	N.D.**
malS	-2.823487	0.386
nadE	0.80922	0.246
oppD	0.41255	0.177
sdhA	0.12699	3.225
serA	0.4181	3,215; 2,941
serC	0.3129	0.115
spoVFA		0.16
spoVFB	-2.099759	
ureC	-0.211643	0.178
yaaN	0.58232	4.366
yceD	0.33079	3.125
yceE	0.18465	N.D.**
yhcQ	-1.903889	0.326
ytfJ	-2.305726	0.109
ytkL	0.67734	3.378
yuaE	-1.141273	2.557

### 1.10. Aim of the present study

The biosynthesis of bacilysin, the dipeptide antibiotic, is under the control of quorum sensing global regulatory pathway. Its production is relatively constant during the exponential growth, but increase at the transition between exponential and stationary phases and reaches to maximum upon entry into stationary phase (16<sup>th</sup> hour) (Köroğlu et al., 2011). Similarly, in *B. subtilis*, changes in the secretome occur at the beginning of the sporulation phase, and new degradative enzymes are secreted that may provide the cell with new nutrients. The cytoplasmic proteomes of standard strain (PY79) and its bacilysin blocked mutant (OGU1) were extensively analyzed in our laboratory to shed light into the functions of bacilysin in its producer. The results pointed to the impact of bacilysin biosynthesis on cellular levels of certain proteins of sporulation and morphogenesis; the members of mother cell compartment-specific regulons in particular, quorum sensing and two component-regulatory systems, peptide transport and stress response (manuscript submitted). On the other hand, cytosolic proteomes miss extracellular proteins of the organisms which would otherwise point to important physiological alterations in response to the knock out mutations. Thus, the aim of the present study is the investigation of dynamic changes in the secretome of *B. subtilis* in the absence of bacilysin production by employing both gel-based and gel-free approaches.



## CHAPTER 2

### MATERIALS AND METHODS

#### 2.1. Bacterial strains, maintenance and culture conditions

The strains of *B. subtilis* used in this study were PY79 (Youngman *et al.*, 1984) and its *bacA::lacZ::erm* derivative OGU1. In OGU1 strain, *bacA-lacZ* fusion is a single crossover integrant of a pMutin derived plasmid and therefore *bacA* gene, thus whole *bac* operon was inactivated (Köroğlu *et al.*, 2011). Bacterial cultures were grown at 37 °C on Luria-Bertani agar (LA) plates and long term stocks were prepared by adding 20% (V/V) glycerol and kept at -80 °C.

After growing overnight at 37°C on LB plates, the strains were inoculated to 10 mL of PA medium (Perry and Abraham, 1979) (pH 7.4) contained in 14 mL Falcon tubes and the cultures were grown at 37°C (200 rpm) for 16 h. These cultures were then used to inoculate 100 mL of PA medium to an initial optical density of about 0.1 at 595 nm (OD<sub>595</sub>). After inoculation, the cultures were incubated at 37°C (200 rpm) and culture supernatants were collected at 12<sup>th</sup>, 16<sup>th</sup> and 24<sup>th</sup> hour time points, respectively, for dynamic secretome analysis.

#### 2.2. Culture media

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

### **2.3. Buffer and solutions**

Composition and preparation of buffers and solutions 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. Preparation of secreted proteins**

For the preparation of secreted proteins, the phenol extraction method of Kaffarnik et al. (2009) was used with slight modifications. The cells were centrifuged at 16,000 g for 15 min and the pellet was discarded. The supernatant was passed through a 0.45 µm filter to eliminate the remaining vegetative cells. Half volume of Tris-buffered phenol (pH 8.0) was then added and mixed vigorously. After incubation of the suspension on ice for 30 min, back extraction was performed twice with one volume of extraction buffer (0.1 M Tris-HCl, pH 8.0, 20 mM KCl, 10 mM EDTA, 0.4% (v/v) β-mercaptoethanol). Then, 5 volume of 0.1 M NH<sub>4</sub>OAc in methanol was added and incubated overnight at -20 °C. Protein was recovered by centrifugation and the pellet was washed twice with 0.1 M NH<sub>4</sub>OAc in methanol and twice with 80% acetone.

### **2.6. Protein estimation**

To determine total protein concentration, the modified Bradford assay described by Ramagli and Rodrigez (1985) was used (Appendix B). Standards

and samples were prepared in duplicates. A blank was included and absorbance was measured at 595 nm.

## **2.7. 2D gel electrophoresis**

For 2D gel electrophoresis, after 14h passive rehydration of IPG strips (11 cm, pH 3-10, BioRad) with 200 µl of rehydration buffer (Appendix B) at room temperature, IEF step was carried out Protean IEF Cell System (Bio-Rad USA). Focused strips were equilibrated with equilibration solution (Appendix B) and SDS-PAGE (Criterion TGX Stain Free Precast Gels, BIO-RAD, USA) was run for separation at second dimension (Görg *et al.*, 2004). The gel was pre-scanned (ChemiDoc™ MP, BIO-RAD, USA). The visualization of separated protein spots was accomplished by Lava Purple (Fluorotechnics, Sydney, Australia) according to the instructions of the manufacturer. LavaPurple is a fluorescence stain based on epicocconone and is also known as Deep Purple total protein staining (GE Healthcare). Epicocconone has low fluorescence in the green until it reacts with basic amino acids to form an internal charge transfer (ICT) complex that is highly fluorescent in the orange/red (Ball and Karuso, 2007). Briefly, gels stained with LavaPurple were fixed overnight in 15% ethanol, 1% citric acid; stained for 1 h in a 1:200 dilution of LavaPurple concentrate in 100 mM sodium borate buffer pH 10.8; destained for 30 min in 15% ethanol; and acidified for 30 min in 15% ethanol, 1% citric acid before being imaged (Ball and Karuso, 2007).

For analyses, fluorescence labeled and stained gels were scanned with a Typhoon 9400 multi-mode imager (GE Healthcare) by excitation with the green laser (532 nm) and detection with a 610 nm narrow band pass filter (Griebel *et al.*, 2013). 2D image analysis software Delta 2D version 4.3 was used for image analyses (Decodon, Germany).

## **2.8. LC-MS analysis**

### **2.8.1. Filter-aided sample preparation (FASP)**

A new approach of gel-free method is uses Filter-Aided Sample Preparation (FASP) method which skips the tedious and difficult gel preparation steps. FASP method is applied after extraction of proteins and enables the purification and digestion with trypsin on filter before MS analysis. This method also provides purification of detergents and chaotropic agents like SDS, CHAPS and salts and also high protein coverage is possible with this technique (Aebersold and Mann, 2003; Wisniewski et al., 2009; Nel et al., 2015). Briefly, the phenol extracted proteins from secretome samples were dissolved; protein concentrations were estimated and used for FASP protocol. 30 µg of protein extract was used as a starting material and treated with FASP™ Protein Digestion Kit (Expedeon) with minor modifications including digestion with combination of Lys-C and trypsin enzymes (Wiśniewski and Mann, 2012; Ünsaldı, 2016; Ünsaldı et al., 2017).

### **2.8.2. Zip-tip cleaning**

Cleaning of extracted proteins was performed by using Zip-tip® Millipore™ cleaning tips according to the manufacturer's instructions.

### **2.8.3. Nano-LC MS/MS analysis**

For LC MS/MS analyses, AB SCIEX TripleTOF 5600+ instrument (AB SCIEX, Redwood City, CA, USA) joined with Eksigent expert nano-LC 400 systems (AB

SCIEX) was used. Trap column (180 $\mu$ m x 20mm column, 300, nanoACQUITY UPLC® 2G-VM Trap 5 $\mu$ m Symetry® C18, Waters, UK) and separation column (75 $\mu$ m, x 150mm column, nanoACQUITY UPLC® 1.8 $\mu$ m 120 Å HSS T3, C18, Waters, UK) were used. Analyst® TF v.1.6 (AB SCIEX) software was used for MS and MS/MS data acquisition and ProteinPilot 4.5 Beta (AB SCIEX) for the peptide identification was used. The precursor tolerance was set to 10 ppm, and tolerance for fragment ions was set to 1 atomic mass unit (amu). Depending on the decoy database search, false discovery rates (FDRs) were accepted as 1%. After all, the identification of the proteins was accepted if there were at least two matching unique peptides.

#### **2.8.4. LC-MS/MS data analysis**

For the analysis of data, Perseus software supplied with MaxQuant platform together with R statistical program was used as previously described (Kulak et al., 2014). Volcano plots of mutant vs. standard strain were constructed in order to define differentially expressed proteins with at least 2-fold change with a p value < 0.05.

#### **2.9. 2DE MALDI-TOF MS data analysis**

Protein spots were cut from the gel and analyzed with 2DE MALDI-TOF MS in Greifswald University (Germany) based on the protocols described by Eymann et al. (2004). AB SCIEX TOF / TOFTM 5800 Analyzer (AB Sciex / MDS Analytical Technologies) was used for analysis. For MS/MS experiment, three most abundant peptides were taken from each MS spectrum. To increase the reliability of protein identification, the resulting sequence data was also included. For the mass accuracy, the usual range was between 10 and 30 ppm.

Proteins with at least 2-fold change with a p value < 0.05 were considered as differentially expressed proteins.

## **2.10. Protein identification and database search**

MASCOT software (Matrix Science Inc., Boston, MA, USA) together with Uniprot Reference Proteome of *B. subtilis* specific database search results a protein score and if that score was higher than 49, protein was considered as identified. For functional assignments, Subtiwiki Web Server (<http://subtiwiki.uni-goettingen.de/>) and Protein Knowledgebase (UniProtKB) (<http://www.uniprot.org/>) databases were used. PSORT (<http://wolfpsort.seq.cbrc.jp/>) and SignalP version 3.0 (<http://www.cbs.dtu.dk/services/SignalP/>) databases were used for subcellular localization and signal peptide properties of the identified proteins.

## CHAPTER 3

### RESULTS AND DISCUSSION

#### 3.1. Theoretical proteome map of extracellular proteins (secretome)

Computational prediction of bacterial protein subcellular localization (SCL) provides a quick and inexpensive means for gaining insight into protein function, verifying experimental results, annotating newly sequenced bacterial genomes, detecting potential cell surface/secreted drug targets, as well as identifying biomarkers for microbes (Yu et al., 2010).

To determine the optimal range for standard 2D gel-based secretome analysis of *B. subtilis*, the theoretical 2D gel map of *B. subtilis* has been constituted using genomic data from GELBANK database ([http://gelbank.anl.gov/cgi-bin/2dgels/gel\\_insilico.pl](http://gelbank.anl.gov/cgi-bin/2dgels/gel_insilico.pl)). Localization of each protein was determined by computational prediction program PSORTb version 3.0. Corresponding pI/Mw of these extracellular proteins were determined via ExPASy ([http://web.expasy.org/cgi-bin/compute\\_pi/pi\\_tool](http://web.expasy.org/cgi-bin/compute_pi/pi_tool)). Based on these, theoretical map revealed the presence of 73 proteins in a pI range of 3 to 12 (Figure 3.1).

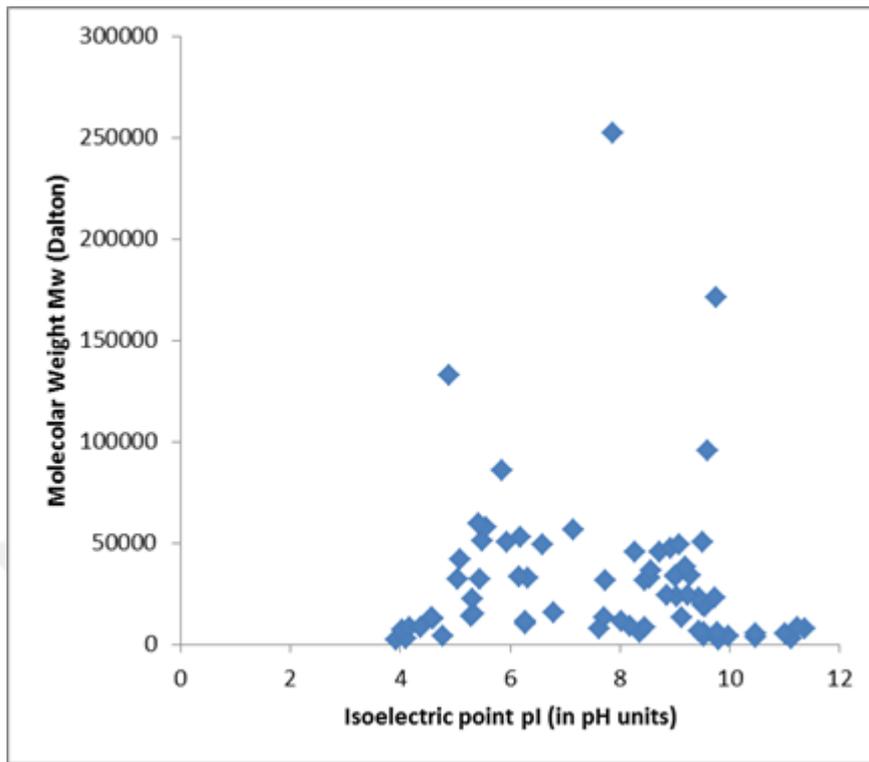


Figure 3.1. A theoretical 2D map of *B. subtilis* 168 extracellular proteome.

### **3.2. Optimization of protein extraction for 2DE PAGE of *B. subtilis* secretome**

The protein extraction part is the most challenging and critical step in proteomic studies in order to obtain reproducible and informative results. In addition, the extraction and recovery of secreted proteins is more difficult since such proteins are much lower in their concentration in the culture fluids. Moreover, the culture media is the starting point of extraction which is composed of interfering compounds like salts and the remains of dead cells, making the recovery of the proteins of interest more difficult.

The classical methods of protein extraction for 2DE -PAGE studies are generally TCA-acetone, methanol or phenol based protocols (Chevallet et al., 2007; Müller et al., 2010; Bonn et al., 2014). The solid phase extraction (SPE) methods which are also preferred methods for protein extraction in terms of their efficiency at enrichment. The SPE methods are based on the affinity material used for protein extraction such as diatomaceous earth, surface-functionalized diamond nanocrystallites, gold-coated nanoparticles and commercial affinity beads. The StrataClean is a commercially available affinity bead system composed of phenol-functionalized bead system (Bonn et al., 2014). In this study, the first task was to select the most appropriate protocol to achieve a better coverage of the secretome. First of all, TCA based extraction method was performed and different rehydration buffer compositions along with different IEF procedures were experienced. TCA extracted proteins were failed to focus under different IEF conditions and the rehydration buffer compositions (data not shown). In each condition, the proteins stacked in the acidic range and formed horizontal streaks. In methanol extraction, the protein yield was very low. When the StrataClean method was tested, despite a high amount of proteins and the ease of extraction, this method is not the choice for further analysis due to its high expense and problems with provision.

Moreover, this extraction is compatible with only 1DE approach. It is known that the use of StrataClean resin is a substitute for the phenol protocol, therefore, phenol protocol was chosen for further proteome analysis. Consequently, phenol extraction was performed in order to get better results. For LC-MS analyses, FASP method was employed just after protein extraction to increase the coverage of the identified proteins.

### **3.3. Comparative secretome analyses via LC-MS/MS**

#### **3.3.1. The proteins identified from secretome samples of PY79 and OGU1**

It is known that *B. subtilis* secretes large amounts of extracellular proteins, which have important roles in nutrient trap, detoxification, communication, defense and many more. The analyses based on prediction algorithms revealed that the genome encodes nearly 300 proteins that can be categorized as extracellular (Tjalsma et al., 2000; Van Dijk et al., 2002; Yamane et al., 2004). Up to date, 113 extracellular proteins were identified by the help of proteomics (Antelmann et al., 2006). In contrast to Gram-negative bacteria, the Gram-positive bacterium *B. subtilis* is lacking an outer membrane. Thus, the thick negatively charged cell wall is thought to perform some roles of the periplasm found in Gram-negative bacteria and the lack of an outer membrane enables the secretion of large amounts of proteins directly into the extracellular medium.

In *B. subtilis*, proteins retained in the cell wall include DNAses, RNAses, proteases, enzymes involved in the synthesis of peptidoglycan (penicillin-binding proteins) and cell-wall hydrolases (Smith, et al., 2000) that are involved in cell wall turnover during cell growth, cell division, sporulation and germination (Margot et al., 1996; Babe et al., 1998). These wall-binding proteins are retained in the cell wall due to the presence of specific wall-

binding domains (CWB) in addition to *N*-terminal signal peptides (Tjalsma et al., 2000). In addition to the CWB domains the peptidoglycan hydrolases also possess catalytic domains which are not bound to the cell wall (Smith et al., 2000). In all former *B. subtilis* secretome studies, a considerable number of the proteins found in the extracellular medium (about 50%) were not predicted to be secreted, because they either lack known export signals or possess an additional retention signal. Among the proteins found in the extracellular proteome lacking known export signals are cytoplasmic proteins (Antelmann et al., 2001; Voigt et al., 2006). Among them are proteins from a range of physiological pathways and cellular functions. Other proteins found in the extracellular medium include flagellum-related and phage proteins.

It is known that cells of *B. subtilis* secrete the highest amounts of protein in the stationary growth phase when grown in a rich medium (e.g. Luria broth). On the other hand, cell wall proteins were always present to a higher extent in the extracellular proteome of cells in the exponential growth phase than of cells in the stationary growth phase (Antelmann et al., 2001 and 2002; Voigt et al., 2006). A considerable number of proteins involved in transport processes, among them several ABC transporter-binding proteins, were found to be secreted into the extracellular medium. Many of these proteins contain a conserved lipobox that can be lipid-modified by the lipoprotein diacylglycerol transferase (Lgt) prior to cleavage by SPase II. These proteins should be retained in the membrane by the lipid anchor.

LC-MS analysis of 12<sup>th</sup> hour, 16<sup>th</sup> hour and 24<sup>th</sup> hour secretome samples of *B. subtilis* standard strain PY79 and its bacilysin blocked mutant strain OGU1 provided the data illustrated in Figure 3.2. At above-indicated time intervals, a total of 414, 458 and 743 proteins were identified from PY79, respectively. These numbers varied in the case of OGU1 in that the respective number of the identified ones was 694, 489 and 687. When the abundance of proteins in two

strains was compared in terms of OGU1 to PY79 ratio, it corresponded to 1.67, 1.07 and 1.09 in 12<sup>th</sup>, 16<sup>th</sup> and 24<sup>th</sup> hour secretome samples, respectively. Of these, the proteins overlapping in two strains were determined as 386, 407 and 615, at 12<sup>th</sup>, 16<sup>th</sup> and 24<sup>th</sup> hours of cultivation, respectively. The highest number of OGU1-specific proteins was obtained at 12<sup>th</sup> hour. Conversely, the number of overlapping proteins as well as PY79-specific proteins was the lowest at this time. These indicated that bacilysin exerts a more profound impact on cellular physiology at 12<sup>th</sup> hour as compared with later stages. Considering the timing of onset of bacilysin biosynthesis,  $t_8$ , as determined both by the level of bacilysin in culture broths and basal and transient-state induction of *bacA* operon expression (Section 1.3), we can postulate that bacilysin does have crucial roles in its producer organism during active commitment to sporulation.

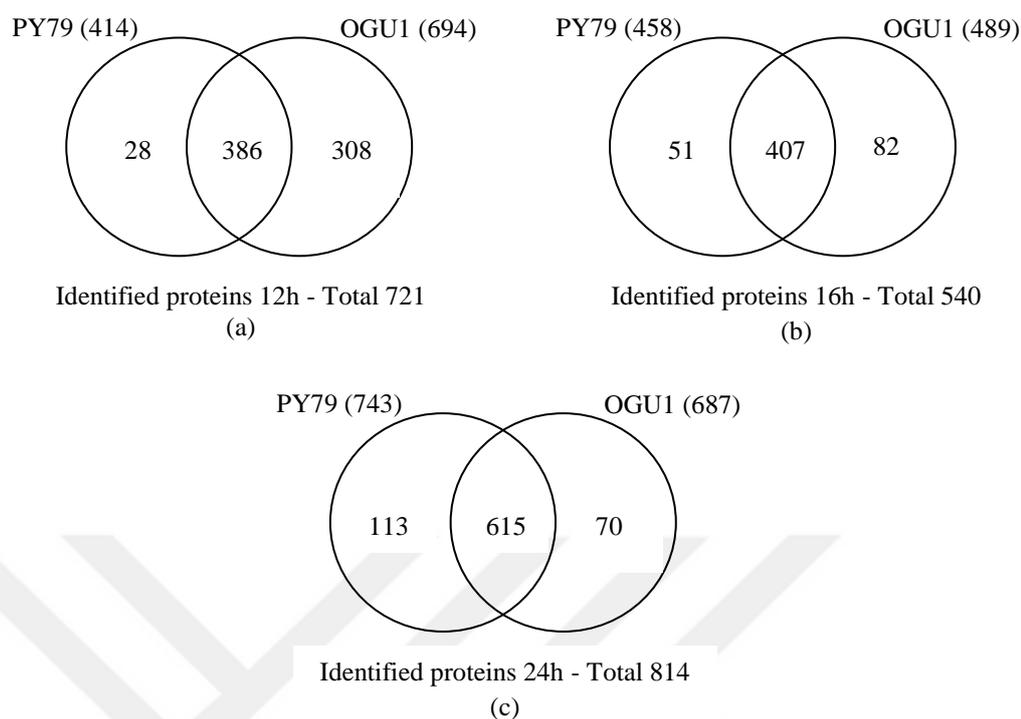


Figure 3.2. Total number of proteins identified in the secretome samples of PY79 and OGU1 at 12<sup>th</sup>, 16<sup>th</sup> and 24<sup>th</sup> hours of cultivation.

As shown in Figure 3.3, secretome samples did not only contain extracellular proteins, but also a considerable number of cytoplasmic proteins. The presence of cytoplasmic protein is common in secretome of many bacteria and eukaryotes (Ebner et al., 2015). There are several explanations for the presence of cytoplasmic proteins without signal peptide in the medium. First is cell lysis which could be supported by the secretome analysis with the conditional mutant strains of *B. subtilis* genes like *ffh*, *secA* and *prsA*. Deletion of those essential secretion genes was lead to an increase in the amount of cytoplasmic proteins probably because the cells become sensitive to lysis (Antelmann et al., 2006). Also, the detection of cytoplasmic proteins in the secretome of multiple protease deficient strains of *B. subtilis* showed that these proteins can be present in the medium due to decreased level of extracellular proteases which

in turn leads to the increased levels of autolysins in the extracellular environment (Krishnappa et al., 2013). Prophage-encoded holins which could oligomerize in the membrane and form membrane pores could be suggested as another explanation. Through these pores, the lytic phage enzymes gain access to the bacterial cell wall and lead to cytoplasmic protein leakage (Voigt et al., 2009). Even in the temperature sensitive *secA* mutant of *B. subtilis* in which Sec translocon is absent, the cytoplasmic proteins were present in the medium (Hirose et al. 2000). It was also suggested that apart from classical secretion pathways of *B. subtilis*, there might be alternative mechanisms for the release of cytoplasmic proteins without any signal peptide (Yang et al., 2011). One possible mechanism could be the secretion of proteins in Gram-positive bacteria via shedding of membrane vesicles (Tjalsma et al., 2004; Mashburn-Warren et al., 2006; Lee et al., 2009). *B. subtilis* also produce similar extracellular vesicles in terms of size and morphology with other Gram-positive bacteria. The extracellular vesicles are found to be constituents of the biofilms and show strain specific differences. In addition, the lipopeptide surfactin produced by wild-type strain of *B. subtilis* has vesicle-disrupting role (Brown et al., 2014; Kim et al., 2015). Final explanation is related with some cytoplasmic proteins called moonlighting proteins which can be found in different subcellular locations in order to perform multiple unrelated functions. One example is enolase protein which is an essential cytoplasmic enzyme for the conversion of 2- phosphoglycerate into phosphoenolpyruvate in a reversible way. Although, enolases do not contain any known signal peptide sequence, it was shown that various enolases can be exported to the cell surface or released to the culture medium in eukaryotic and prokaryotic organisms (Yang et al., 2014). It was strongly suggested that various cytoplasmic proteins without a known signal peptide are secreted into the medium in the absence of cell lysis during the stationary phase of growth and that secretion is a general phenomenon in *B. subtilis*. In addition this phenomenon is not restricted to *B. subtilis*, in *Staphylococcus aureus*, the similar scenario is valid for enolase

secretion in to the extracellular medium and more over the 25% of *S. aureus* secretome is constituted by cytoplasmic proteins without a known signal peptide (Ebner et al., 2015).

When the abundance of cytoplasmic proteins in two strains was compared in terms of OGU1 to PY79 ratio, it corresponded to 1.74, 0.94 and 0.93 in 12<sup>th</sup>, 16<sup>th</sup> and 24<sup>th</sup> hour secretome samples, respectively. When the abundance of extracellular proteins in two strains was compared in terms of OGU1 to PY79 ratio, it corresponded to 1.56, 0.92 and 0.95 in 12<sup>th</sup>, 16<sup>th</sup> and 24<sup>th</sup> hour secretome samples, respectively. These were as expected in view of the general (total) protein abundance in OGU1 compared to PY79 at 12<sup>th</sup> hour of cultivation.

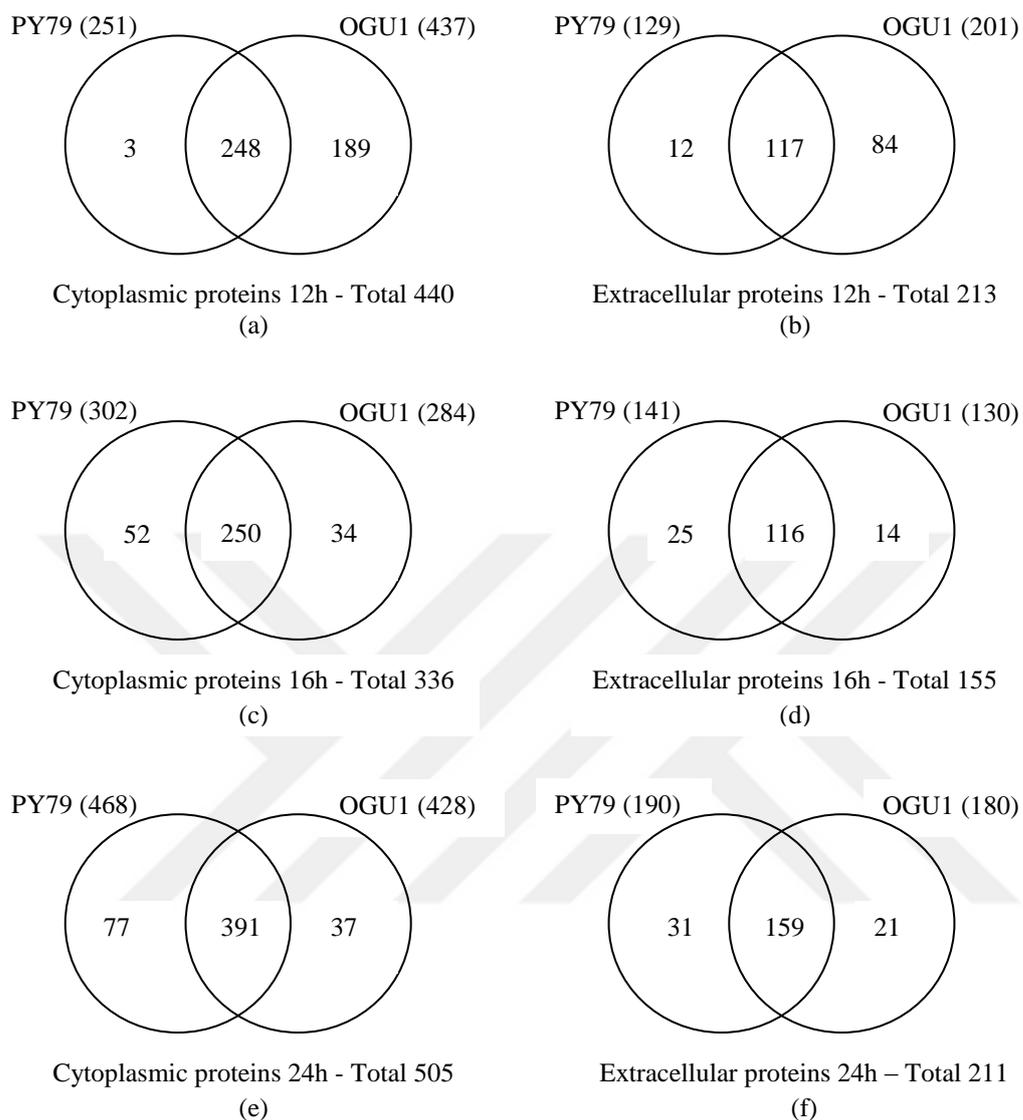


Figure 3.3. Total number of (a, c, e) extracellular (b, d, f) cytoplasmic proteins identified in the secretome samples of PY79 and OGU1 at 12<sup>th</sup>, 16<sup>th</sup> and 24<sup>th</sup> hours of cultivation. For the determination of the subcellular localizations pSORT, Subtiwiki and signalP databases were used.

The number of proteins that could be identified only in PY79 or OGU1, but not in the other; i.e. strain-specific are shown in Figure 3.2 and 3.3. However, these do not reflect the actual numbers of differentially expressed proteins. Of these, only 108, 46 and 12 were found as differentially expressed between PY79 and OGU1 in 12<sup>th</sup>, 16<sup>th</sup> and 24<sup>th</sup> hour samples, respectively, when 2-fold increase or decrease in protein abundance is taken as the criterion of significance.

#### **3.3.1.1. Differentially expressed extracellular proteins of *B. subtilis* at 12<sup>th</sup> hour secretome samples**

In secretome analysis of 12<sup>th</sup> hour samples, a total of 108 proteins were found to be differentially expressed in OGU1. 60 of them were cytoplasmic proteins and overrepresented when compared to the parental strain. Their distribution is shown in a volcano plot for 12<sup>th</sup> hour in Figure 3.4.

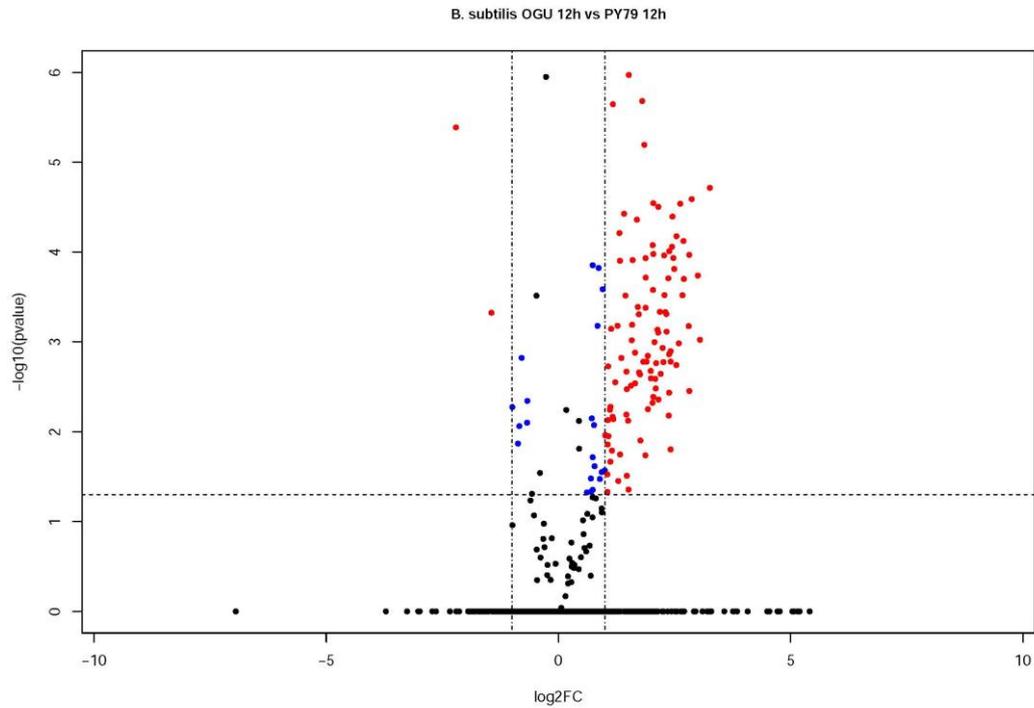


Figure 3.4. The volcano plot showing the distribution of differentially expressed proteins with at least 2-fold change in their levels with a p value < 0.05 in 12<sup>th</sup> hour secretome samples of *B. subtilis* PY79 and OGU1. The x and y axis correspond to p values (in  $-\log_{10}$ ) and fold change (FC) ( $\log_2$ ) respectively. The dashed horizontal line shows where  $p = 0.05$  ( $-\log 0.05=1.3$ ) with points above the line having  $p < 0.05$  and points below the line having  $p > 0.05$ . The dashed vertical lines correspond to  $\log_2=1$  which means no change and shown in black dots. The blue dots have p values less than 0.05 but have fold change values close to 2. Red dots are the ones that are accepted as differentially expressed both having p values equal to or less than 0.05 and fold change values are greater than 2 and less than -2. Red dots at the negative values are decreasing while on the positive values of fold change is increasing in the OGU1 when compared to parental strain PY79.

Differentially expressed proteins from 12<sup>th</sup> hour secretome samples were analyzed according to their abundance, functional category and the properties like pI, MW, signal peptide presence and their subcellular localization which were all tabulated in Table 3.1. Overall, all of the differentially expressed proteins identified at 12<sup>th</sup> hour were overrepresented in OGU1 except for FabZ and FlgK.



Table 3.1. Differentially expressed proteins in the secretome of 12<sup>th</sup> hour cultures as identified by LC-MS/MS

Locus Name	Protein	Gene	log2FC	Fold Change	P Value	Functional Category Codes	Functional Category	Function	SSL	Mass (Da)	pI	SP
BSU09310	Phosphoglucomutase	<i>pgcA</i>	1.107	2.15	0.0057	SW 1.1	Cellular processes	Interconversion of glucose 6-phosphate and alpha-glucose 1-phosphate	C	64663	4	
BSU35770	Putative major teichoic acid biosynthesis protein C	<i>tagC</i>	1.173	2.25	0.0000	SW 1.1	Cellular processes	Biosynthesis of teichoic acid	C	50826	7.34	
BSU00500	Bifunctional protein GlmU	<i>glmU</i>	1.688	3.22	0.0000	SW 1.1	Cellular processes	Cell wall metabolism	C	49610	5.65	
BSU33930	Phosphoglycerate kinase	<i>pgk</i>	2.036	4.10	0.0003	SW 2.1	Metabolism	Enzyme in glycolysis/ gluconeogenesis	C	42190	4	
BSU18570	Probable oxidoreductase YoaE	<i>yoaE</i>	1.153	2.22	0.0162	SW 2.1	Metabolism	Unknown	C	76167	7.33	
BSU33910	2,3-bisphosphoglycerate-independent phosphoglycerate mutase	<i>gpmI</i>	2.700	6.50	0.0002	SW 2.2	Metabolism	Enzyme in glycolysis / gluconeogenesis	C	56309	5.21	
BSU19370	2-oxoglutarate dehydrogenase E1 component	<i>odhA</i>	1.922	3.79	0.0014	SW 2.2	Metabolism	TCA cycle	C	106278	5.87	
BSU18360	Aldose 1-epimerase	<i>galM</i>	1.119	2.17	0.0053	SW 2.2	Metabolism	Unknown	C	37228	5.6	
BSU33900	Enolase	<i>eno</i>	1.879	3.68	0.0002	SW 2.2	Metabolism	Enzyme in glycolysis/ gluconeogenesis	C	46581	4	
BSU32610	Fructosamine deglycase FrlB	<i>friB</i>	2.541	5.82	0.0001	SW 2.2	Metabolism	Metabolism of aminoacylated fructose	C	36876	5.44	
BSU33040	Fumarate hydratase class II	<i>fumC</i>	1.802	3.49	0.0000	SW 2.2	Metabolism	TCA cycle	C	50532	5.48	
BSU23850	Glucose-6-phosphate 1-dehydrogenase	<i>zwf</i>	1.355	2.56	0.0015	SW 2.2	Metabolism	Initiation of the pentose phosphate pathway	C	55632	5.28	
BSU37660	Phosphate acetyltransferase	<i>pta</i>	1.288	2.44	0.0353	SW 2.2	Metabolism	Overflow metabolism	C	34791	4.65	

Table 3.1. (Cont'd)

Locus Name	Protein	Gene	log2FC	Fold Change	P Value	Functional Category Codes	Functional Category	Function	SSL	Mass (Da)	pI	SP
BSU37120	Probable fructose-bisphosphate aldolase	<i>fbxA</i>	1.461	2.75	0.0064	SW 2.2	Metabolism	Enzyme in glycolysis/ gluconeogenesis	C	30401	5.03	
BSU29220	Probable NAD-dependent malic enzyme 4	<i>yskJ</i>	1.165	2.24	0.0068	SW 2.2	Metabolism	Malate utilization	C	43667	5.05	
BSU14860	Pyruvate carboxylase	<i>pyc</i>	2.154	4.45	0.0044	SW 2.2	Metabolism	Replenishment of the oxaloacetate pool	C	127937	5	
BSU14580	Pyruvate dehydrogenase E1 component subunit alpha	<i>pdhA</i>	2.999	8	0.0002	SW 2.2	Metabolism	Links glycolysis and TCA cycle	C	41548	5	
BSU37110	Transaldolase	<i>tal</i>	1.514	2.86	0	SW 2.2	Metabolism	Pentose phosphate pathway	C	22971	5.88	
BSU13180	5-methyltetrahydropteroyl triglutamate--homocysteine methyltransferase	<i>metE</i>	2.132	4.38	0.0007	SW 2.3	Metabolism	Biosynthesis of methionine	C	86806	4	
BSU31930	Alanine dehydrogenase	<i>ald</i>	2.591	6.03	0.001	SW 2.3	Metabolism	Alanine utilization	C	39684	5	
BSU28290	Ketol-acid reductoisomerase	<i>ilvC</i>	3.047	8.26	0.0009	SW 2.3	Metabolism	Biosynthesis of branched-chain amino acids	C	37458	5	
BSU02690	L-asparaginase 2	<i>ansZ</i>	2.491	5.62	0.0002	SW 2.3	Metabolism	Asparagine utilization	C	40103	6	+
BSU24080	Leucine dehydrogenase	<i>yqiT</i>	2.671	6.37	0.0003	SW 2.3	Metabolism	Utilization of branched-chain keto acids	C	39992	4.94	
BSU38920	Peptidase T	<i>pepT</i>	2.183	4.54	0.0005	SW 2.3	Metabolism	Peptide degradation	C	45509	4.54	
BSU10020	Phosphoserine aminotransferase	<i>serC</i>	1.499	2.83	0.0075	SW 2.3	Metabolism	Biosynthesis of serine	C	40136	5	
BSU36900	Serine hydroxymethyltransferase	<i>glyA</i>	1.272	2.41	0.0007	SW 2.3	Metabolism	Biosynthesis of glycine	C	45490	5.48	
BSU32250	Threonine synthase	<i>thrC</i>	1.443	2.72	0.0003	SW 2.3	Metabolism	Biosynthesis of threonine	C	37464	5.19	
BSU38960	Uncharacterized protein YxjG	<i>yxjG</i>	1.596	3.02	0.0001	SW 2.3	Metabolism	Unknown	C	43165	5	

Table 3.1. (Cont'd)

Locus Name	Protein	Gene	log <sub>2</sub> FC	Fold Change	P Value	Functional Category Codes	Functional Category	Function	SSL	Mass (Da)	pI	SP
BSU36640	Urease subunit alpha	<i>ureC</i>	1.986	3.96	0.0021	SW 2.3	Metabolism	Utilization of urea as alternative nitrogen source	C	61187	5.09	
BSU11250	Ornithine carbamoyltransferase	<i>argF</i>	1.877	3.67	0.0004	SW 2.3	Metabolism	Biosynthesis of arginine	C	34663	4.97	
BSU36370	3-hydroxyacyl-[acyl-carrier-protein] dehydratase FabZ	<i>fabZ</i>	-1.443	0.37	0.0005	SW 2.4	Metabolism	Fatty acid biosynthesis	C	15740	9	
BSU15500	Dihydroorotase	<i>pyrC</i>	1.465	2.76	0.0021	SW 2.5	Metabolism	Pyrimidine biosynthesis	C	46533	5.43	
BSU37670	Putative heme-dependent peroxidase YwfH	<i>ywfH</i>	1.99	3.97	0.0025	SW 2.6	Metabolism	Biosynthesis of heme	C	29505	4	
BSU00110	Pyridoxal 5'-phosphate synthase subunit PdxS	<i>pdxS</i>	2.384	5.22	0.0001	SW 2.6	Metabolism	Pyridoxal-5-phosphate biosynthesis	C	31612	5	
BSU22790	DNA-binding protein HU 1	<i>hupA</i>	2.301	4.93	0.0005	SW 3.1	Genetics	DNA packaging, function of the signal recognition complex	C	9884	9.5	
BSU16120	DNA topoisomerase 1	<i>topA</i>	1.841	3.58	0	SW 3.1	Genetics	DNA replication	C	79078	9	
BSU01280	50S ribosomal protein L5	<i>rplE</i>	2.104	4.3	0.0017	SW 3.3	Genetics	Translation	C	20148	9	
BSU22080	Carboxypeptidase 1	<i>ypwA</i>	2.691	6.46	0.0001	SW 3.3	Genetics	Unknown	C	58175	4.77	
BSU01130	Elongation factor Tu	<i>tuf</i>	1.927	3.8	0.0056	SW 3.3	Genetics	Translation	C	43593	4	
BSU15430	Isoleucine--tRNA ligase	<i>ileS</i>	1.18	2.27	0.0072	SW 3.3	Genetics	Translation	C	104845	5.19	
BSU00380	Methionine--tRNA ligase	<i>metG</i>	1.059	2.08	0.0139	SW 3.3	Genetics	Translation	C	76188	4.96	
BSU28090	Valine--tRNA ligase	<i>valS</i>	2.365	5.15	0.0002	SW 3.3	Genetics	Translation	C	101745	4.87	
BSU30320	Leucine--tRNA ligase	<i>leuS</i>	2.044	4.12	0.0001	SW 3.3	Genetics	Translation	C	91543	4.86	

Table 3.1. (Cont'd)

Locus Name	Protein	Gene	log2FC	Fold Change	P Value	Functional Category Codes	Functional Category	Function	SSL	Mass (Da)	pI	SP
BSU16160	ATP-dependent protease ATPase subunit ClpY	<i>clpY</i>	1.827	3.55	0.0017	SW 3.3	Genetics	Protein degradation	C	52586	5	
BSU18000	Aconitate hydratase A	<i>citB</i>	1.223	2.33	0.0028	SW 3.4	Genetics	TCA cycle	C	99334	4	
BSU17460	Glutamine synthetase	<i>glnA</i>	1.07	2.1	0.0019	SW 3.4	Genetics	Glutamine biosynthesis, control of trna and glnr activity	C	50278	4	
BSU16690	Polyribonucleotide nucleotidyltransferase	<i>ppp</i>	1.764	3.4	0.0125	SW 4.1	Lifestyles	DNA repair, competence development, RNA degradation	C	77464	4	
BSU16770	dihydrodipicolinate synthase	<i>dapA</i>	2.242	4.73	0.0012	SW 4.2	Sporulation and Germination	Biosynthesis of lysine and peptidoglycan	C	31042	4	
BSU16750	Aspartate-semialdehyde dehydrogenase	<i>asd</i>	1.897	3.72	0.0017	SW 4.2	Sporulation and Germination	Biosynthesis of threonine, lysine, dipicolic acid, peptidoglycan	C	37847	4.97	
BSU11540	Oligoendopeptidase F homolog	<i>yjbG</i>	2.454	5.48	0	SW 4.2	Sporulation and Germination	Protein degradation	C	77073	5.13	
BSU08760	Sporulation-control protein spo0M	<i>spo0M</i>	1.578	2.99	0.001	SW 4.2	Sporulation and Germination	Sporulation	C	29733	4	
BSU06030	60 kDa chaperonin	<i>groL</i>	2.474	5.56	0.0001	SW 4.3	Coping with stress	Protein folding and re-folding	C	57425	4	
BSU30650	General stress protein 20U	<i>dps</i>	1.063	2.09	0.0074	SW 4.3	Coping with stress	Iron storage, survival of stress conditions	C	16594	4.44	

Table 3.1. (Cont'd)

Locus Name	Protein	Gene	log <sub>2</sub> FC	Fold Change	P Value	Functional Category Codes	Functional Category	Function	SSL	Mass (Da)	pI	SP
BSU33400	Glyoxal reductase	<i>yvgN</i>	2.444	5.44	0.0001	SW 4.3	Coping with stress	Unknown	C	31663	5.07	
BSU03130	NH(3)-dependent NAD(+) synthetase	<i>nadE</i>	1.735	3.33	0.0022	SW 4.3	Coping with stress	NAD biosynthesis	C	30395	4.89	
BSU33240	Oxalate decarboxylase OxdC	<i>oxdC</i>	1.871	3.66	0.0183	SW 4.3	Coping with stress	Unknown	C	43566	5	
BSU25020	Superoxide dismutase [Mn]	<i>sodA</i>	1.412	2.66	0	SW 4.3	Coping with stress	Detoxification of oxygen radicals	C	22490	5	
BSU28500	Thioredoxin	<i>trxA</i>	3.259	9.57	0	SW 4.3	Coping with stress	Protection of proteins against oxidative damage	C	11393	4	
BSU25880	Uncharacterized protein YqxJ	<i>yqxJ</i>	1.007	2.01	0.011	SW 5.1	Prophages and mobile genetic elements	Unknown	C	14341	4.82	
BSU12410	Uncharacterized protein YjoA	<i>yjoA</i>	1.322	2.5	0.0001	SW 6.4	Proteins with unknown functions	Unknown	C	17793	5.81	
BSU23910	Uncharacterized protein YqjE	<i>yqjE</i>	2.149	4.44	0	SW 6.6	Proteins with unknown functions	Unknown	C	39647	4.76	
BSU13010	6-phosphogluconolactonase	<i>pgl</i>	1.134	2.2	0.0007	SW 4.3	Coping with stress	Pentose phosphate pathway	C	38411	5	
BSU29120	Malate dehydrogenase	<i>mdh</i>	2.043	4.12	0	SW 2.2	Metabolism	TCA cycle	C	33644	4.73	

Table 3.1. (Cont'd)

Locus Name	Protein	Gene	log <sub>2</sub> FC	Fold Change	P Value	Functional Category Codes	Functional Category	Function	SSL	Mass (Da)	pI	SP
BSU04140	Penicillin-binding protein 3	<i>pbpC</i>	1.849	3.6	0	SW 1.1	Cellular processes	Unknown	CM	74406	6	
BSU23980	Arginine-binding extracellular protein ArtP	<i>artP</i>	2.537	5.81	0.0018	SW 1.2	Transporters	Arginine uptake	CM	28312	5.09	+
BSU27040	Fructose permease IID component	<i>levG</i>	2.067	4.19	0.001	SW 1.2	Transporters	Fructose uptake and phosphorylation	CM	30087	9.94	
BSU30770	Manganese-binding lipoprotein MntA	<i>mntA</i>	2.624	6.16	0	SW 1.2	Transporters	Manganese uptake	CM	33418	6.16	+
BSU36830	ATP synthase subunit alpha	<i>atpA</i>	1.562	2.95	0.0031	SW 2.1	Metabolism	ATP synthesis	CM	54598	5	
BSU14590	Pyruvate dehydrogenase E1 component subunit beta	<i>pdhB</i>	2.814	7.03	0.0001	SW 2.2	Metabolism	Links glycolysis and TCA cycle	CM	35474	4	
BSU28440	Succinate dehydrogenase flavoprotein subunit	<i>sdhA</i>	1.328	2.51	0.0179	SW 2.2	Metabolism	TCA cycle	CM	65152	5.71	
BSU09950	Foldase protein PrsA	<i>prsA</i>	2.085	4.24	0.0026	SW 3.3	Genetics	Protein folding	CM	32510	9.12	+
BSU31080	Uncharacterized protein YuaB	<i>yuaB</i>	1.01	2.01	0.0109	SW 4.1	Lifestyles	Biofilm formation, control of entry into sporulation via the phosphorelay	CM	19257	9	+
BSU31010	Uncharacterized protein YuaG	<i>yuaG</i>	1.508	2.84	0.044	SW 4.1	Lifestyles	Involved in the control of membrane fluidity	CM	55994	5.14	
BSU11430	Oligopeptide-binding protein OppA	<i>oppA</i>	2.869	7.31	0	SW 4.1	Lifestyles	Initiation of sporulation, competence development	CM	61525	5	+
BSU27440	ABC transporter glutamine-binding protein GlnH	<i>glnH</i>	2.328	5.02	0.0008	SW 4.2	Sporulation and Germination	Glutamine uptake	CM	29756	8	+

Table 3.1. (Cont'd)

Locus Name	Protein	Gene	log <sub>2</sub> FC	Fold Change	P Value	Functional Category Codes	Functional Category	Function	SSL	Mass (Da)	pI	SP
BSU12110	Uncharacterized protein YjfA	<i>yjfA</i>	1.709	3.27	0.0004	SW 4.2	Sporulation and Germination	Unknown	CM	16880	9.5	+
BSU30610	Uncharacterized ABC transporter ATP-binding protein YtlC	<i>ytlC</i>	1.312	2.48	0.0001	SW 4.2	Sporulation and Germination	Unknown	CM	29303	5.43	
BSU03000	Glycine betaine-binding protein OpuAC	<i>opuAC</i>	2.028	4.08	0.0001	SW 4.3	Coping with stress	Compatible solute transport	CM	32215	8.02	+
BSU07260	Lipoteichoic acid synthase 1	<i>ltaSI</i>	2.413	5.32	0.0157	SW 4.3	Coping with stress	Biosynthesis of lipoteichoic acid	CM	73315	5	
BSU01935	Sporulation-killing factor biosynthesis protein SkfC	<i>skfC</i>	1.117	2.17	0.0215	SW 4.3	Coping with stress	May be involved in spore killing	CM	56292	8.15	
BSU25380	UPF0365 protein YqfA	<i>yqfA</i>	2.027	4.07	0.0048	SW 4.3	Coping with stress	Resistance against sublancin	CM	35641	4.92	
BSU26820	Uncharacterized protein YrpD	<i>yrpD</i>	1.053	2.08	0.0298	SW 6.7	Proteins with unknown functions	Unknown	CM	24876	9.96	+
BSU26890	Chitosanase	<i>csn</i>	2.323	5.01	0.0005	SW 2.2	Metabolism	Chitin degradation	S	31497	8.89	+
BSU39330	Extracellular endo- $\alpha$ -(1 $\rightarrow$ 5)-L-arabinanase 2	<i>abn2</i>	1.585	3	0.0006	SW 2.2	Metabolism	Arabinan degradation	S	52607	7.37	+
BSU27030	Levanase	<i>sacC</i>	2.202	4.6	0.0023	SW 2.2	Metabolism	Degradation of levan to fructose	S	75951	6.79	+
BSU02700	Lipase EstA	<i>estA</i>	1.876	3.67	0.0001	SW 2.4	Metabolism	Lipid degradation	S	22791	10	+

Table 3.1. (Cont'd)

Locus Name	Protein	Gene	log <sub>2</sub> FC	Fold Change	P Value	Functional Category Codes	Functional Category	Function	SSL	Mass (Da)	pI	SP
BSU19800	3-phytiase	<i>phy</i>	1.054	2.08	0.0467	SW 2.6	Metabolism	Utilization of inositol hexakisphosphate (phytate)	S	41946	4.94	+
BSU09190	Endonuclease YhcR	<i>yhcR</i>	2.381	5.21	0.0014	SW 3.2	Genetics	Utilization of nucleic acids	S	132686	4.69	+
BSU15300	Bacillopeptidase F	<i>bpr</i>	2.042	4.12	0.0041	SW 3.3	Genetics	Protein degradation	S	154578	4.98	+
BSU38090	Minor extracellular protease vpr	<i>vpr</i>	1.648	3.13	0.0013	SW 3.3	Genetics	Protein degradation	S	85608	5.77	+
BSU39940	Uncharacterized protein YxaL	<i>yxaL</i>	1.65	3.14	0.0029	SW 3.3	Genetics	Unknown	S	43877	6.38	+
BSU35410	Flagellar hook-associated protein I	<i>flgK</i>	-2.208	0.22	0	SW 4.1	Lifestyles	Motility and chemotaxis	S	54355	4.38	
BSU35360	Flagellin	<i>hag</i>	2.148	4.43	0.0008	SW 4.1	Lifestyles	Motility and chemotaxis	S	32626	4	
BSU24620	Spore coat-associated protein N	<i>tasA</i>	1.472	2.77	0.0034	SW 4.1	Lifestyles	Biofilm formation	S	28305	5	+
BSU18800	Beta-lactamase	<i>penP</i>	2.374	5.18	0.0066	SW 4.3	Coping with stress	Resistance to beta-lactam antibiotics	S	33446	9.21	+
BSU12610	Phage-like element PBSX protein XkdG	<i>xkdG</i>	2.415	5.33	0.0017	SW 5.1	Prophages and mobile genetic elements	Unknown	S	34614	5.19	+
BSU25890	Uncharacterized protein YqxI	<i>yqxI</i>	2.282	4.86	0.0003	SW 5.1	Prophages and mobile genetic elements	Unknown	S	16000	5.4	
BSU36460	Uncharacterized protein YwoF	<i>ywoF</i>	2.094	4.27	0.0033	SW 6.7	Proteins with unknown functions	Unknown	S	51452	4.79	+

Table 3.1. (Cont'd)

Locus Name	Protein	Gene	log <sub>2</sub> FC	Fold Change	P Value	Functional Category Codes	Functional Category	Function	SSL	Mass (Da)	pI	SP
BSU39230	tRNA nuclease WapA	<i>wapA</i>	1.471	2.77	0.0309	SW 1.1	Cellular processes	Intercellular competition	S, CW	258162	6	+
BSU07840	Trifunctional nucleotide phosphoesterase protein YrKN	<i>yfKN</i>	2.803	6.98	0.0007	SW 2.6	Metabolism	Probably phosphate acquisition	S, CW	159706	5	+
BSU34520	Uncharacterized FAD-linked oxidoreductase YvdP	<i>yvdP</i>	1.755	3.38	0.0023	SW 4.2	Sporulation and Germination	Protection of the spore	SW	50085	6.1	
BSU40530	Spore coat protein F	<i>cotF</i>	1.73	3.32	0.0005	SW 4.2	Sporulation and Germination	Resistance of the spore	SW	18725	7.37	
BSU02310	Putative hydrolase YbfO	<i>ybfO</i>	2.415	5.33	0.0013	SW 4.3	Coping with stress	Unknown	S	51788	6.68	+
BSU02040	Uncharacterized protein YbdN	<i>ybdN</i>	2.26	4.79	0.0017	SW 6.7	Proteins with unknown functions	Unknown	S	31503	8.8	+
BSU18780	Uncharacterized protein YoaW	<i>yoaW</i>	2.818	7.05	0.0035	SW 4.2	Sporulation and Germination	Unknown	S	16609	5.16	+
BSU07270	Probable glucose-1-phosphate cytidylyltransferase	<i>yfhH</i>	2.383	5.22	0.0037	SW 4.2	Sporulation and Germination	Unknown	U	28671	5	
BSU12280	Uncharacterized protein YjIC	<i>yjIC</i>	1.509	2.85	0	SW 6.7	Proteins with unknown functions	Unknown	U	15583	4.26	

Table 3.1. (Cont'd)

Locus Name	Protein	Gene	log <sub>2</sub> FC	Fold Change	P Value	Functional Category Codes	Functional Category	Function	SSL	Mass (Da)	pI	SP
BSU33540	FMN-dependent NADH-azoreductase 2	<i>azoR2</i>	2.273	4.83	0.0001	SW 4.2	Coping with stress	Resistance to 2-methylhydroquinone	U	23272	5.11	

SSL: Subcellular localization predicted via PSORTb and Uniprot. C: Cytoplasm, CM: Cytoplasmic Membrane, CW: Cell Wall, S: Secreted,S and U:Unknown.

SP: Signal Peptide which is predicted by SignalP prediction program.

In secretome analysis of 12<sup>th</sup> hour samples, 108 differentially expressed proteins were classified into functional groups as based on their functions provided in the Subtiwiki database. As shown in Figure 3.5, most of the differentially expressed proteins were categorized under “metabolism” (35 %) and “information pathways” (14 %). Regarding the rest of functional categories related, “cell envelope and cell division” and “exponential and early exponential lifestyles” were together make up the 10 % of the whole. “Transporters” and “electron transport synthesis and ATP synthesis” categories contained 6 % of differentially expressed proteins identified.

Some of the proteins were categorized under ‘proteins with unknown functions’ in which the functions of the proteins are unknown or have not previously been determined. The “poorly characterized or putative enzymes” category was containing a protein or an enzyme that is assigned to a specific superfamily based on sequence homology. This assignment leads to prediction of the structural fold, active site residues, a range of its potential catalytic activities, and even the likely catalytic mechanism. These two categories together constituted 5 % of the differentially expressed proteins.

Among all categories, “sporulation and germination” (11 %) and “coping with stress” (13 %) were particular interest since these two categories were quite prominent in our former comparative cytosolic proteome analysis.

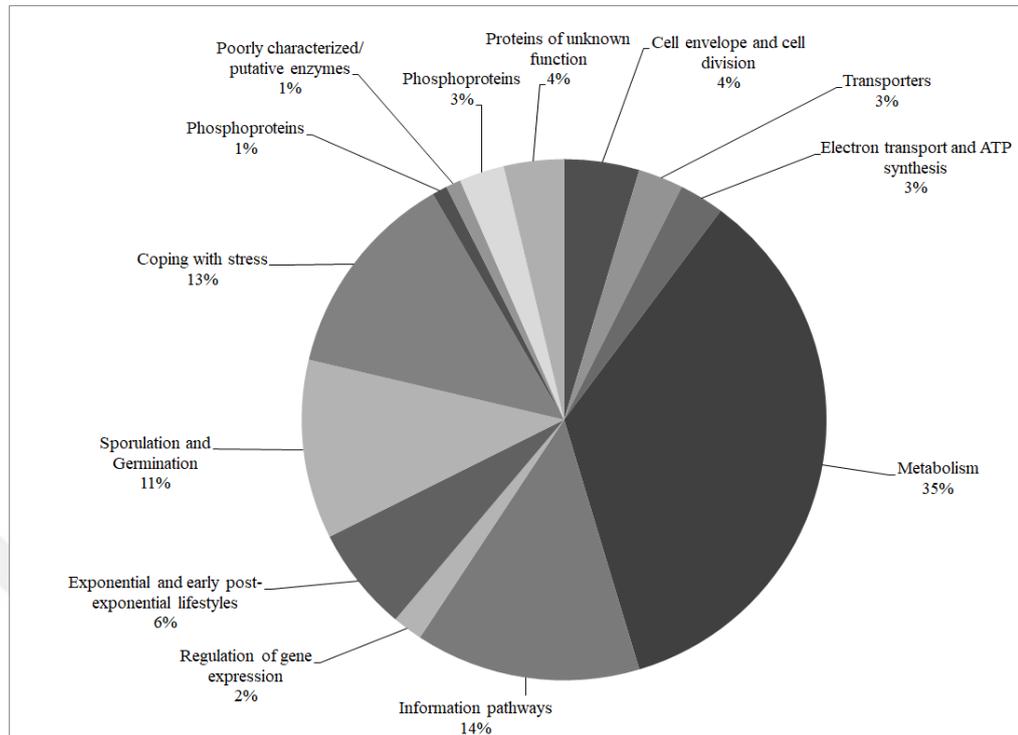


Figure 3.5. Functional distribution of differentially expressed proteins of 12<sup>th</sup> hour

### 3.3.1.1.1. Differentially expressed extracellular proteins in 12<sup>th</sup> hour secretome samples

Among differentially expressed proteins with extracellular localization, 18 were secreted proteins and except for *flgK*, all of them were more abundant in the mutant strain.

Of these 18 overrepresented proteins, 8 were upregulated catabolic enzymes, namely chitosanase, extracellular endo-1,5-alpha-L-arabinosidase, levanase, extracellular lipase, phytase, endonuclease YhcR, bacillopeptidase F, minor extracellular serine protease. It was found that the Chitosanase which degrades chitosan by hydrolyzing the glycosidic bonds was major component of the secretome of *B. subtilis* (Voigt et al., 2009). This carbohydrate degrading

enzyme is important for energy metabolism. Extracellular degradation of arabinose, which is found in plant tissues, is achieved by mainly two arabinase enzymes AbnA and Abn2. Control of gene expression of *abn2* is different than *abnA* in terms of both being subjected to catabolite repression and having sigma  $\sigma^A$  and  $\sigma^H$  dependent promoter, respectively (Inacio et al., 2008).

Levanase encoded by *sacC* and degrades two unusual polysaccharide mainly levan and inulin in addition to sucrose. This enzyme is induced by low concentrations of fructose (Marvasi et al., 2010). *sacC* was found to be among  $\sigma^B$ -dependent stress genes (heat, ethanol and salt stress) (Petersohn et al., 2001). Intriguingly, the above-mentioned enzymes of polysaccharide catabolism displayed 5, 3 and 4.6 fold increments in OGU1 in the absence of their substrate inducers.

There are two lipolytic extracellular enzymes of *B. subtilis* lipase A and lipase B, and these two enzymes are under the control of different regulatory mechanisms so expressed under different conditions. For instance, only lipase A is responsible of lipolytic activity when glucose is the only carbon source during cell growth in minimal medium (Eggert et al., 2001). It was found that LipA was among the upregulated proteins under the salt stress (Steil et al., 2003).

Phytate is one of the organic phosphorous sources found in soil and utilized by *B. subtilis* by the action of phytase which is encoded by *phy* gene. By the help of this action, both phosphate and Zn, Fe and Ca ions become available to cells (Maogual et al., 2014).

*yhcR* gene encodes a high-molecular weight, sugar-nonspecific 5' nucleotidase that is activated by calcium ions. This nuclease activity, located principally in the cell wall, renders the extracellular nucleotides available for the organism and it is important for adaptation to distinct environmental niches (Oussenko et

al., 2004). It is coexpressed with the YhcS which is a sortase that is responsible from the anchorage of proteins to the cell wall. YhcR is a cell wall bound protein by the help of YhcS (Fasehee et al., 2011). *yhcR* expression was found to be increased in *sigB* mutant under the phosphate starvation (Allenby et al., 2005). The level of this protein was found to be 5.2 fold elevated in OGU1.

*bpr* (bacillopeptidase F) and *vpr* (minor extracellular serine protease) genes encode extracellular proteolytic enzymes in order to degrade proteins from the environment (Pohl et al., 2013; Veening et al., 2008). These proteins were among the upregulated extracellular proteins with 5.2 and 4.1 fold increases in their levels, respectively. Bpr is one of the major exoprotease of *B. subtilis* known to be regulated positively by DegU-P since the regulatory part of *bpr* includes three direct repeats of a DegU-binding consensus sequence (Verhamme et al., 2007; Tsukahara and Ogura, 2008; Marlow et al., 2014;). It was reported that *bpr* is highly expressed and secreted by a group of vegetative cells in a heterogeneous population for the benefit of the whole population (Veening et al., 2008). In another study, it was reported that transcription from *bpr* promoter region increases during biofilm development (Marlow et al., 2014). The expression of *vpr* is positively regulated by PhoP under the phosphate starvation conditions (Allenby et al., 2005). The *vpr* promoter is  $\sigma^H$ -dependent and is also regulated by CodY which represents one of the rare cases for  $\sigma^H$ -dependent genes. In addition, this is a strong regulation since *vpr* became more abundant in the secretome of *B. subtilis* in the *CodY* deleted mutant (Barbieri et al 2015). In *B. subtilis*, minor extracellular proteases (such as Epr, Bpr, Vpr, NprB, WprA and Mpr) are not essential for growth or sporulation. However; their transcription is tightly controlled by different regulators, like AbrB, DegU, ScoC, SinR, and SpoIIID. CodY was also added to this list due to its control on the expression of two extracellular proteases (Vpr and Mpr). Therefore, the expression of extracellular proteases is induced during nutrient exhaustion in stationary phase as well as other starvation

conditions (Barbieri et al 2015; Allenby et al., 2005). In the cytoplasmic proteome of 16 h , DegU level was found to be 2.7 fold increased in the OGU1 strain (Aras Taşkın, 2011; Taşkın et al, submitted), quite consistent with overrepresentation of Bpr in our work which is known to be activated by DegU. The two-component response regulator DegU is involved in the regulation of synthesis of degradative enzymes as well as competence in *B. subtilis* (Dahl et al., 1992). Certain mutations in the *degS* or *degU* genes, causing a more stable phosphorylation of DegU, result in the hyperproduction of degradative enzymes (Antelmann et al., 2001). Therefore, the upregulation of these extracellular proteases could be regarded as the absence of bacilysin is sensed as a type of stress condition that the organism increases its extracellular enzymes in order to utilize all possible sources from the environment as a coping mechanism.

The SigB general stress regulon contains 100 genes (Petersohn et al., 2001). These genes provide a nonspecific response to stress by encoding proteins that protect the DNA, membranes, and proteins from the damaging effects of stress. Proteins induced by SigB help the cell to survive potentially harmful environmental conditions, such as heat, osmotic, acid, or alkaline shock. Interestingly, in 12<sup>th</sup> hour secretome, none of the overexpressed stress proteins is a member of SigB regulon, but are rather the members of SigA (vegetative sigma factor), AbrB, SigH, DegU, CcpA, PhoP, LevA, CodY and WalR regulons. During exponential growth sigB is kept in an inactive complex by binding to its anti-sigma factor, RsbW (Benson and Haldenwang, 1993). It follows that taken together, the general increment in the synthesis of macromolecule-degrading enzymes in OGU1 might be regarded as a defense or stress-coping mechanism if the absence of bacilysin is sensed by the mutant as a type of stress condition.

WapA is a cell wall-associated protein precursor which is also known as contact-dependent growth inhibition protein. It contains a C-terminal toxin domain with RNase activity (cleaves tRNA). The function of WapA is to bind to the cell surface receptors on the target cells and deliver tRNA toxin to inhibit their growth (interspecies competition) (Koskiniemi et al., 2013). WapA displayed a 2.8 fold increase in OGU1, most possibly to provide an alternative competition mechanism in the absence of bacilysin.

As stated in Section 3.3.1, flagellum-related and phage proteins are also secretome components in *B. subtilis*. *flgK* encoding flagellar hook-filament junction proteins and *hag* encoding the flagellin monomer were differentially expressed in OGU1. FlgK was strongly downregulated while Hag 4.4 times upregulated. About 20,000 subunits of Hag make up one flagellum (Haiko and Westerlund-Wikström, 2013). Both proteins are related with motility and chemotaxis, i.e. lifestyle but they face with different regulations: *flgK* is positively regulated by DegU and ComK; whereas both *flgK* and *hag* are negatively regulated by *ScoC* (Mirel et al., 1994; Kodgire and Rao, 2001). In addition, *hag* is regulated negatively by CodY and, CsrA regulates translation initiation of *hag* by binding to its mRNA thereby blocking ribosome binding (Yakhnin et al., 2007). The expression of *hag* is known to increase during the transition from logarithmic growth phase to stationary phase and to decline afterwards. As known, flagellar gene expression is tightly regulated in order to coordinate flagellar assembly as a function of time and space (Hsueh et al., 2011). It was found in our laboratory that many cytoplasmic proteins under *ScoC* and *CodY* regulation are in OGU1 at 16<sup>th</sup> hour. (Aras Taşkın., manuscript submitted), in this respect, Hag protein of secretome represents a controversion.

TasA, found 2.8 times overrepresented in our 12<sup>th</sup> hour secretome, is a major component of biofilm matrix that forms amyloid fibers to bind cells together in

the biofilm (Dragoš et al., 2017). It also takes role in the assembly of the spore coat as a spore coat- associated protein (Stöver et al., 1999). Besides, TasA has an antimicrobial action which inhibits the growth of competitor bacteria. The expression of this protein is under the negative control of SinR and AbrB and positive control of LutR and RemA. The *tapA-sipW-tasA* genes belong to one of the two operons, *tapA* (other operon is *epsA-O*) whose expression is necessary for biofilm development under the control of SinR (Strauch et al., 2007; Chu et al., 2008; Diethmaier et al., 2011). The secretion of TasA and TapA depends on the SipW protein, the latter was being at the same level in the cytoplasmic proteomes of both PY79 and OGU1 (Demir, 2013). The upregulation of TasA is thought to be beneficial for OGU1 based on its antimicrobial activity in order to compensate the absence of bacilysin.

*penP* encodes a secreted protein beta-lactamase. *B. subtilis* displays a significant level of intrinsic resistance against a variety of  $\beta$ -lactam antibiotics, but the underlying mechanisms are poorly understood. Although there are three putative  $\beta$ -lactamase genes *penP*, *ybbE* and *yblX* in the genome, no  $\beta$ -lactamase activity could be detected in the growing cells or supernatants (Colombo al., 2004). PenP was found as much as 5-fold upregulated in OGU1 when compared to PY79.

*xkdG* encodes a phage like element, PBSX (a defective *B. subtilis* prophage) protein that was 5-fold upregulated OGU in the present study. It was reported that PBSX phage-like particles binds to cell wall and disrupt nonlysogenic *Bacillus* strains, leading to death (McDonnell et al., 1994). The *xkdG* gene belongs to *xpf* regulon which is downregulated in *B. subtilis* under high salinity conditions (Nagler et al., 2016). Besides, it was proposed that the expression of genes from the defective PBSX phage leads to excretion of chromosomal DNA fragments with different sizes to the media in a growth-phase specific manner (Shingaki et al., 2003).

The putative erythromycin esterase, 5.3-fold upregulated in the OGU1 is encoded by *ybfO*. The run-off transcription followed by macroarray analysis (ROMA) showed that this gene is a member of  $\sigma$ W regulon. Among SigB-independent stress induction phenomena, the salt shock induction of the SigW regulon was certainly the most interesting (Petersohn et al., 2001). SigW is one of seven extracellular (ECF) type sigma factors of *B. subtilis*, the functions of all of which are still not well understood. In general, this class of sigma factors controls uptake or secretion of specific molecules and ions or responses to a variety of stresses. SigW in particular has been implicated in detoxification responses and the production of antimicrobial compounds (Turner and Helmann, 2000). Like the other members of  $\sigma$ W regulon, YbfO is probably among the proteins that have functions related with detoxification of antibiotics, many of which target cell wall biosynthesis (Cao et al., 2002). Besides, *ybfO* is among the AbrB regulated genes repressed during logarithmic growth phase (Banse et al., 2008). *ybfO* has a signal peptide and categorized within the group of extracellular proteins.

Overall, the extracellular proteome of 12<sup>th</sup> hour cultures were composed of increased catabolic enzymes, proteins with antimicrobial activity the flagellum-related and phage proteins and those related with coping with stress in OGU1 most probably caused by missing bacilysin activity when compared to PY79. Such a pattern very likely indicates that the loss of bacilysin is interpreted by the producer organism as a kind of stress which then initiates significant alterations in global gene expression patterns, including those of extracellular proteins.

### 3.3.1.1.2. Differentially expressed cytoplasmic membrane proteins in 12<sup>th</sup> hour secretome samples

The secretome samples of 12<sup>th</sup> hour contained 108 differentially expressed cytoplasmic membrane proteins which were shown in Table 3.1.

It was known that the *yqiXYZ* operon encoding an amino acid ABC transport system shows significantly different levels of expression in response to amino acid availability (Mader et al., 2002). ArtP (YqiX) is high affinity arginine ABC transporter protein and found to be upregulated in the secretome of mutant strain in our study. LevG, fructose permease IID component is involved in the phosphoenolpyruvate-dependent sugar phosphotransferase system (PTS) which is responsible for the phosphorylation of incoming sugar substrates concomitant with their translocation across the cell membrane. *levD*, *levE*, *levF* and *levG* and *sacC* share the same operon that is involved in the regulation of levanase expression (Reizer et al., 1999; Pereira et al., 2001). LevG and SacC were found to be overrepresented in the mutant strain for unpredictable reasons. *MntA* encodes manganese-binding lipoprotein A and takes important role in the transport of manganese ions. It is known that the Mn (II) uptake in *B. subtilis*, at the level of transcription, is active in the exponential growth and is tightly regulated (Que and Helmann, 2000). Mn (II) uptake regulation also occurs during initiation of sporulation. The mutation of *mntA* leads to defective sporulation in *B. subtilis* (Que and Helmann, 2000). *mntA* was also found among upregulated genes under cold stress (Kaan et al., 2002). The MntA protein levels in OGU1 did not show any alteration in the 16<sup>th</sup> hour cytoplasmic proteome when compared to PY79 (Demir, 2013). Many sporulation proteins as well as spore coat proteins were either downregulated or absent in OGU1 strain in cytoplasmic proteome of 16<sup>th</sup> hour. Yet, the sporulation frequency of OGU1 was the same with the PY79 (manuscript submitted). The 6-fold increase in the level of *mntA* might have provided a

benefit for sporulation initiation in OGU1 at 12<sup>th</sup> hour and probably contributed to comparable sporulation frequency with the standard strain.

*skfC* encodes sporulation-killing factor biosynthesis protein (SkfA) and is induced in a Spo0A dependent manner during sporulation. SkfA causes lysis of Spo0A inactive cells (not enter the sporulation pathway) in order to provide source of nutrients for the sporulating Spo0A active cells. SkfA is induced in phosphate starvation conditions (Molle et al., 2003). Besides, it was shown to protect extracellular lipase (*lipA*) against proteolysis by an unknown mechanism (Westers et al., 2005). In the present secretome study, the SkfC was found to be 2-fold and *lipA* was 3.7-fold upregulated in OGU1 strain.

*prsA* encodes a peptidylprolyl cis-trans isomerase lipoprotein, which facilitates the folding and quality control of the secreted proteins. A higher portion of secreted proteins of *B. subtilis* is exported via Sec pathway in an unfolded state and therefore the PrsA has an important role in their folding together with HtrA and HtrB (other major foldases). The overexpression of *prsA* leads to an increase in the yields of heterologous protein secretion one example is subtilisin in *B. subtilis* (Kontinen et al., 1993; Krishnappa et al., 2014). PrsA is necessary for the stable and proper folding of extracellular proteins at the membrane and cell wall interface during posttranslocational phase. (Wahlström et al., 2003). Besides, when the cellular levels of PrsA decrease below a certain threshold (200 molecules/cell), cell lysis occurs (Vitikainen et al., 2004). Penicillin binding proteins were not stable and peptidoglycan cross-linkage was decreased in *prsA* mutant (Hyyryläinen et al., 2010). Overexpression of PrsA also promotes the recombinant proteins of biotechnological importance (Jakob et al., 2015). In the 16<sup>th</sup> hour comparative cytoplasmic proteome of OGU1, the PrsA levels were found to be the same in both standard strain and the mutant strain in cytoplasmic proteome at 16<sup>th</sup> hour (Demir, 2013). In the

present secretome study, PrsA was 4.2 fold upregulated in the mutant which is in correlated with the high amount of secreted proteins in the mutant.

Three membrane associated proteins which are all related with energy metabolism namely; AtpA (ATP synthase), PdhB (pyruvate dehydrogenase) and SdhA (succinate dehydrogenase) were found to be among overrepresented proteins. PdhB, in particular, was as much as 7 fold upregulated.

There are four ABC transporter binding proteins upregulated in the mutant strain; OppA (oligopeptide binding), OpuAC (glycine betaine binding), GlnH (glutamine binding) and YltC (an uncharacterized one). The OppA (oligopeptide ABC transporter binding protein) which transports cell density signal peptides, is necessary for sporulation and competence in *B. subtilis*. In *lgt* (lipoprotein diacylglyceryl transferase) mutant of *B. subtilis*, *oppA* and several lytic enzymes were upregulated in extracellular proteome (Antelmann et al., 2001). It was reported that Tn 917 transposition mutation of *oppA* gene resulted in loss of in the bacilysin expression. It was also shown in the same study that biosynthesis of dipeptide antibiotic bacilysin is linked to ComA and PhrC (CSF) in a Spo0K (Opp)-dependent manner by constructing *phrC*-, *comA*- and *oppA*-disrupted mutants of *B. subtilis*. The results indicated that the biosynthesis of bacilysin did not take place when the oligopeptide permease function was impaired (Yazgan et al., 2001). Since the *oppA* gene is under the control of CodY, the increase in the level of OppA at the beginning of stationary phase was an unexpected result (Gilois et al., 2007). In the cytoplasmic proteome at 16<sup>th</sup> hour, the CodY regulated genes including oligopeptide ABC transporter ATP-binding protein (OppD) were found to be downregulated (Aras Taşkın, 2011; Demir, 2013). In our secretome result, the levels of OppA were found to be upregulated 7-fold in 12<sup>th</sup> hour cultures and which then decreased at 16<sup>th</sup> hour time point.

Four fold increased OpuAC is a substrate binding domain of OpuA protein that takes role in glycine betaine transport in *B. subtilis* (Horn et al., 2006). Glycine betaine is one of the important osmoprotectants. This compatible solute is important for adaptation of *B. subtilis* to high salinity conditions as well as high and low temperature stress (Hoffmann and Bremen, 2011). During the changes in the external salinity especially the sudden and dramatic ones, the *opuA* expression is upregulated (Hoffmann et al., 2013).

CotF is synthesized as a precursor which is processed to a mature form during spore coat assembly. After processing CotF is found in two mature forms in *B. subtilis* spores, 5 and 8 kDa polypeptides which are spore coat components and their synthesis depends on  $\sigma K$  (Lai et al., 2003; Abhyankar et al., 2011). The increased level of CotF or its precursor was detected in the absence of a protease encoded by *yabG* (Takamatsu et al., 2000). In cytoplasmic proteome of OGU1, although no significant change was found in CotF, other spore coat proteins CotE, CotA, CotB, CotI, CotSA, CotU and proteins related with spore coat synthesis (SpoVR, SpsB, SpsJ, SpsK) were found to be significantly downregulated in OGU1 (Demir, 2013). The loss of these proteins, CotA in particular, led to the disappearance of brownish pigmentation of spores with negative effects on spore resistance. Contrarily, in the present study, CotF was found to be 3.3 times upregulated in OGU1 at 12<sup>th</sup> hour. It is possible that the increase in some of the coat proteins could compensate the decrease in the other coat proteins with overlapping functions.

OxdC (oxalate decarboxylase) is one of the most abundant bacterial cell wall proteins expressed under acidic stress conditions (Mäkelä et al., 2010). TrxB and OxdC expression level was found to be equal cytoplasmic proteome in both OGU1 and PY79 (Demir, 2013).

### 3.3.1.1.3. Differentially expressed cytoplasmic proteins of 12<sup>th</sup> hour secretome samples

A total of 63 cytoplasmic proteins were found to be differentially expressed in 12<sup>th</sup> hour secretome samples and the highest number of differentially expressed cytoplasmic proteins was found at 12<sup>th</sup> hour as compared to 16<sup>th</sup> hour and 24<sup>th</sup> hour secretome samples. Among these, there were 31 proteins belonged to the category of metabolism, 12 engaged in genetic processes such as replication, translation, genetic regulation, protein degradation and DNA packaging, 4 sporulation and germination proteins, 8 proteins for coping with stress, 3 proteins of cellular processes, cell wall metabolism in particular, along with those of unknown functions.

Among differentially expressed cytoplasmic proteins, DapA (dihydrodipicolinate synthase) and Asd (aspartate-semialdehyde dehydrogenase) of aspartate pathway take role in DPA (dipicolinic acid) synthesis. PepF (YjbG) oligoendopeptidase and Spo0M (sporulation-control protein) are related with sporulation. All these were upregulated in 12<sup>th</sup> hour secretome of OGU1. In *B. subtilis*, DAP (*dap*) operon is composed of *spoVFA* and *spoVFB* (forming the two subunits of dipicolinate synthase), *asd* (forming aspartate semialdehyde dehydrogenase), *dapG*, and *dapA* (forming dihydrodipicolinate synthase). These enzymes are indeed the aspartate biosynthetic pathway components (Jakobsen et al., 2009). DapA is expressed both during growth and sporulation phases and its absence results in DPA deficiency in spores (Chen et al., 1993; Orsburn et al., 2010). In the cytoplasmic proteome at 16<sup>th</sup> hour, SpoVFA and SpoVFB as well as DapG were downregulated in OGU1 (Aras Taşkın, 2011; Demir, 2013). Levels of these proteins could be higher at 12<sup>th</sup> hour which probably decreased at 16<sup>th</sup> hour.

Spo0M is sporulation control protein acting at the stage 0 of sporulation. Its expression is  $\sigma^H$ - and  $\sigma^W$ - dependent. Spo0M is a substrate of FtsH (ATP-dependent metalloprotease) (Cao et al., 2002). *spo0M* KO mutant is sensitive to lysis at the growth phase and the sporulation process is blocked at the stage 0 while overproduction of Spo0M results in a decrease in frequency of sporulation. These effects on sporulation frequency depend on the amount of Spo0M protein in the individual cells (Nguyen and Schumann, 2012). A considerable group of proteases, chaperones and late sporulation related proteins do interact with Spo0M, Spo0M is also believed to be involved in various processes during the vegetative growth (Vega-Cabrera et al. 2017). PepF has probably some functions in protein turnover and sporulation. When overexpressed, the sporulation is inhibited and it most probably hydrolyzes peptides of the Phr family important for phosphorelay taking the cells into spore formation and takes role in competence as well (Kanamaru et al., 2002). Both Spo0M and PepF were overrepresented by 5.5- and 3-fold, respectively in OGU1 at 12<sup>th</sup> hour.

Acotinate hydratase (CitB) which is encoded by *citB* gene is a member of TCA cycle. Besides, expression level of CitB is increases during sporulation since it is necessary for normal sporulation process (Fouet and Sonenshein, 1990). CitB was also found to in the transcriptional units that CodY directly interacts (Molle et al., 2003). CitB was found 2.3-fold overrepresented in OGU1. Consistently, it was also found to 2.9-fold decrease in OGU1 in the cytoplasmic proteome (Aras Taşkın, 20011).

Eight proteins were grouped in the category of coping with stress and they were upregulated in OGU1. *groEL* (chaperonin) increases the fitness of the cell by aiding the assembly, translocation and folding of the proteins. Under the short term ethanol stress, the chaperons DnaK and GroEL are recruited to the cell membrane and important for coping with stressful conditions by aiding

protein folding and assembly (Seydlova et al., 2012). The Dps (general stress protein 20U) encodes for miniferritin which is a member of  $\sigma^B$  regulon and important for iron detoxification as well as having a DNA binding and protection ability. *dps* mutants are defective in coping with stress conditions like severe ethanol, low temperature and paraquat stress (Chiancone et al., 2004; Höper et al., 2005; Reder et al., 2012). GroEL, DnaK and *dps* levels were no different in OGU1 and PY79 as revealed by 16<sup>th</sup> hour cytoplasmic proteome analysis (Demir, 2013). NadE (NH<sub>3</sub>-dependent NAD<sup>+</sup> synthetase) is among the  $\sigma^B$ -dependent genes and catalyzes the amidation of deamido-NAD to form NAD in an ATP-dependent manner. NadE is induced upon ethanol and salt stress as shown by macroarray studies (Petershon et al., 2001). Besides, *nadE* is induced by variety of antibiotics (nisin, gallidermin, gramicidin A/S, valinomycin) that are effective on the bacterial membrane. For this reason, NadE has been considered as a marker protein for membrane stress. (Wenzel et al., 2012). SodA (Superoxide dismutase) functions for detoxification of free radicals. The mutants of *katX* (catalase) or the *sodA* were all defective to cope with harsh ethanol stress (Casillas-Martinez and Setlow; 1997). These proteins are upregulated in paraquat stress and at low temperature stress (Höper et al., 2005). SodA expression level in both PY79 and OGU1 strains was equal in 16<sup>th</sup> hour cytoplasmic proteome, in addition, another superoxide dismutase SodF and catalase KatX were downregulated in OGU1 (Demir, 2013). However, it was 2.66-fold overexpressed by 12<sup>th</sup> hour cultures in the present work. TrxA (thioredoxin) is a thiol-disulfide oxidoreductase and functions as a reducing agent in the cytoplasm (Scharf et al., 1998). The absence of TrxA leads to deoxyribonucleoside and cysteine or methionine auxotrophy. In addition, the absence of *trxA* leads to defects in endospore formation and cytochrome C synthesis. *TrxA* is known to be induced by heat, salt and ethanol stress (Möller and Hederstedt, 2008). According to mRNA profiling experiments, *trxA* was found to be related with sulfur utilization in exponential

and stationary phase cells. Besides, the growth was adversely affected due to the disrupted sulfur metabolism (Mostertz et al., 2008).

#### **3.3.1.1.4. Differentially expressed proteins with unknown function or location of 12<sup>th</sup> hour secretome samples**

*ybdN* is among the AbrB regulated genes (Banse et al., 2008). The signal peptide of *ybdN* was used for increased secretion of subtilisin from *B. amyloliquefaciens* (Degering et al., 2010). The uncharacterized protein YbdN was nearly 5-fold upregulated in secretome of OGU1 strain.

YoaW, as a secreted protein with unknown function, was found necessary for normal biofilm formation, *yoaW* expression is under the regulation of AbrB (Hamon et al., 2004; Chagneau and Saier, 2004). It was proposed that YoaW is taking role in formation of extracellular matrix required for maturation in late stage of biofilm formation. YoaW is among the proteins affecting the depth of the biofilm (Hamon et al., 2004). Intriguingly, YoaW was 7-fold upregulated in 12<sup>th</sup> hour secretome of OGU1 secretome.

*azoR2* which was formerly known as *yvaB* encodes for azoreductase which is under the regulation of MarR-type repressor *YkvE* (*MhqR*) Töwe et al., 2007). *AzoR2* transcription increases under thiol stress caused by catechol and 2-methylhydroquinone (2-MHQ) and together with *AzoR1*, they confer resistance to thiol stress (Montra et al., 2008). Azoreductases contain flavin and catalyze NADH-dependent quinone reductions, thereby (protecting the cells from free radicals and reactive oxygen species (Töwe et al., 2007; Montra et al., 2008). *AzoR2* was found to be 5-fold upregulated in the OGU1 strain. The *yfnH* encodes for probable glucose-1-phosphate cytidyltransferase. *yfnHGF* operon is a member of  $\sigma^K$  and GerE operons (Eichenberger et al., 2004). YnfH was

another upregulated protein in OGU1 secretome. In the absence of pyruvate, *yjlCD* operon is downregulated in the period of anaerobic conditions, nitrate and nitrite reduction and fermentative growth (Ye et al., 2000; Chumsakul et al., 2017). It was reported that *yjlC* and *ndh* are transcribed as a single transcriptional unit and Yj1C is induced by high NADH/NAD<sup>+</sup> ratio (Gyan et al., 2006). In the present study, Yj1C was a 2-fold upregulated protein in OGU1.

### **3.3.1.2. Differentially expressed proteins of *B. subtilis* at 16<sup>th</sup> hour secretome samples**

According to the secretome results from 16<sup>th</sup> hour cultures, at which bacilysin level peaks in the culture fluid, a total of 46 proteins were found to be differentially expressed in OGU1 (Figure 3.6 and Table 3.2). Among these proteins, 27 were cytoplasmic proteins and half of them were found to be overrepresented with respect to parental strain. Additionally, 4 of 5 cytoplasmic membrane proteins were overexpressed in bacilysin KO mutant. When secreted proteins are considered, 9 were found to be differentially expressed. Remaining, 5 proteins were categorized as unknowns.

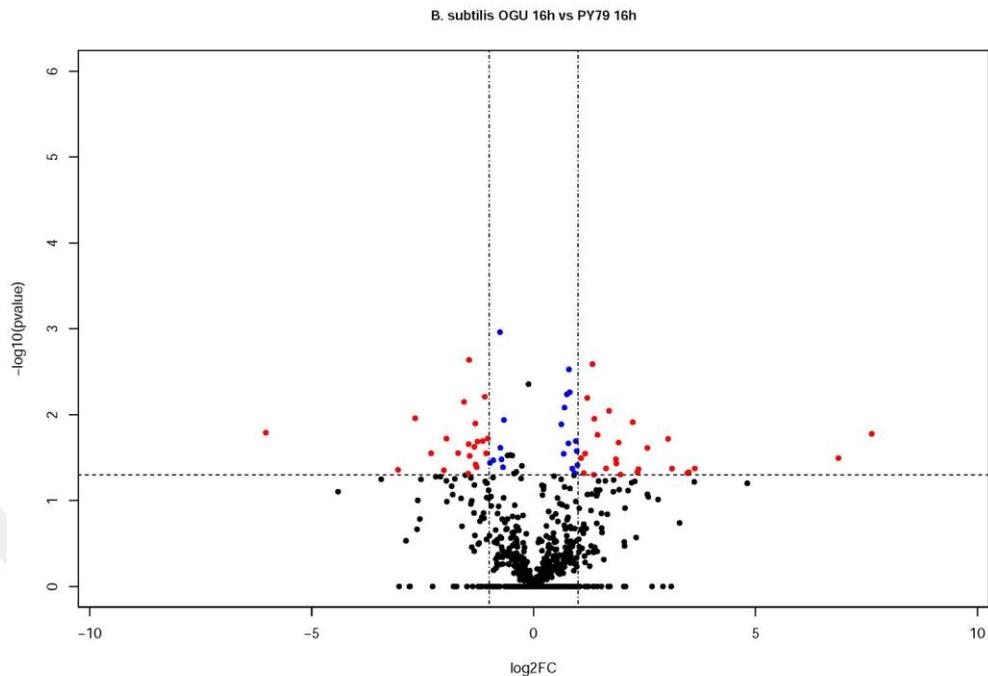


Figure 3.6. The volcano plot showing the distribution of differentially expressed proteins with at least 2-fold change in their levels with a p value < 0.05 in 16<sup>th</sup> hour secretome samples of *B. subtilis* PY79 and OGU1. The x and y axis correspond to p values (in  $-\log_{10}$ ) and fold change (FC) ( $\log_2$ ) respectively. The dashed horizontal line shows where  $p = 0.05$  ( $-\log_{10} 0.05 = 1.3$ ) with points above the line having  $p < 0.05$  and points below the line having  $p > 0.05$ . The dashed vertical lines correspond to  $\log_2 = 1$  which means no change and shown in black dots. The blue dots have p values less than 0.05 but have fold change values close to 2. Red dots are the ones that are accepted as differentially expressed both having p values equal to or less than 0.05 and fold change values are greater than 2 and less than -2. Red dots at the negative values are decreasing while on the positive values of fold change is increasing in the OGU1 when compared to parental strain PY79.

Table 3.2. Differentially expressed proteins in the secretome of 16<sup>th</sup> hour cultures as identified by LC-MS/MS

Locus Name	Protein	Gene	log <sub>2</sub> FC	Fold Change	P Value	Functional Category Codes	Functional Category	Function	SSL	Mass (Da)	pI	SP
BSU01150	30S ribosomal protein S10	<i>rpsJ</i>	-2.310	0.20	0.0282	SW 3.3	Genetics	Translation	C	11666	10	
BSU01420	30S ribosomal protein S11	<i>rpsK</i>	-1.031	0.49	0.0189	SW 3.3	Genetics	Translation	C	13925	12	
BSU01220	30S ribosomal protein S3	<i>rpsC</i>	-1.567	0.34	0.0071	SW 3.3	Genetics	Translation	C	24323	10	
BSU01440	50S ribosomal protein L17	<i>rpQ</i>	-1.438	0.37	0.0302	SW 3.3	Genetics	Translation	C	13751	10	
BSU01320	50S ribosomal protein L18	<i>rpR</i>	-1.311	0.40	0.0127	SW 3.3	Genetics	Translation	C	12969	10	
BSU01210	50S ribosomal protein L22	<i>rpV</i>	-1.701	0.31	0.0280	SW 3.3	Genetics	Translation	C	12460	11	
BSU27940	50S ribosomal protein L27	<i>rpmA</i>	-1.068	0.48	0.0282	SW 3.3	Genetics	Translation	C	10372	10	
BSU13180	5-methyltetrahydropteroyl triglutamate--homocysteine methyltransferase	<i>metE</i>	-1.263	0.42	0.0205	SW 2.3	Metabolism	Biosynthesis of methionine	C	86806	4	
BSU23250	6,7-dimethyl-8-ribitylumazine synthase	<i>ribH</i>	1.367	2.58	0.0112	SW 2.6	Metabolism	Riboflavin biosynthesis	C	16287	5	
BSU23070	D-3-phosphoglycerate dehydrogenase	<i>serA</i>	-2.023	0.25	0.0444	SW 2.3	Metabolism	Biosynthesis of serine	C	57129	5.62	
BSU14600	Dihydrolypoyllysine-residue acetyltransferase component of 2- pyruvate dehydrogenase complex	<i>pdhC</i>	-1.146	0.45	0.0203	SW 2.2	Metabolism	Links glycolysis and TCA cycle	C	47539	4	
BSU19360	Dihydrolypoyllysine-residue succinyltransferase component of 2- oxoglutarate dehydrogenase complex	<i>odhB</i>	-2.669	0.16	0.0110	SW 2.2	Metabolism	TCA cycle	C	46003	4.86	
BSU00780	Dihydroneopterin aldolase	<i>folB</i>	1.864	3.64	0.0370	SW 2.6	Metabolism	Biosynthesis of folate	C	13517	5.23	

Table 3.2. (Cont'd)

Locus Name	Protein	Gene	log <sub>2</sub> FC	Fold Change	P Value	Functional Category Codes	Functional Category	Function	SSL	Mass (Da)	pI	SP
BSU16740	Dipicolinate synthase subunit B	<i>dpaB</i>	2.338	5.06	0.0467	SW 4.2	Sporulation and Germination	Dipicolic acid production	C	21869	6.15	
BSU01780	Glutamine--fructose-6-phosphate aminotransferase [isomerizing]	<i>glmS</i>	-3.054	0.12	0.0439	SW 1.1	Cellular processes	Cell wall synthesis	C	65338	4	
BSU00090	Inosine-5'-monophosphate dehydrogenase	<i>guaB</i>	1.070	2.10	0.0320	SW 2.5	Metabolism	Biosynthesis of GMP	C	52991	6	
BSU13190	Major intracellular serine protease	<i>isp</i>	2.235	4.71	0.0122	SW 3.3	Genetics	Protein degradation	C	33849	4.52	
BSU35770	Putative major teichoic acid biosynthesis protein C	<i>tagC</i>	1.702	3.25	0.0090	SW 1.1	Cellular processes	Biosynthesis of teichoic acid	C	50826	7.34	
BSU16090	Succinyl-CoA ligase [ADP-forming] subunit beta	<i>sucC</i>	-1.306	0.40	0.0380	SW 2.1	Metabolism	TCA cycle	C	41372	4	
BSU12410	Uncharacterized protein YjoA	<i>yjoA</i>	1.633	3.10	0.0423	SW 6.4	Proteins with unknown functions	Unknown	C	17793	5.81	
BSU36660	Urease subunit gamma	<i>ureA</i>	1.914	3.77	0.0211	SW 2.3	Metabolism	Utilization of urea as alternative nitrogen source	C	11455	4.98	
BSU25550	30S ribosomal protein S20	<i>rpsT</i>	-1.335	0.40	0.0237	SW 3.3	Genetics	Translation	C	9599	11	
BSU16160	ATP-dependent protease ATPase subunit ClpY	<i>clpY</i>	1.439	2.71	0.0172	SW 3.3	Genetics	Protein degradation	C	52586	5	
BSU03050	L-lactate dehydrogenase	<i>ldh</i>	3.030	8.17	0.0191	SW 2.2	Metabolism	Pyruvate fermentation to lactate	C	34802	5.39	
BSU01490	50S ribosomal protein L13	<i>rplM</i>	-1.473	0.36	0.0486	SW 3.3	Genetics	Translation	C	16375	10	

Table 3.2. (Cont'd)

Locus Name	Protein	Gene	log <sub>2</sub> FC	Fold Change	P Value	Functional Category Codes	Functional Category	Function	SSL	Mass (Da)	pI	SP
BSU01170	50S ribosomal protein L4	<i>rplD</i>	-1.281	0.41	0.0408	SW 3.3	Genetics	Translation	C	22391	10	
BSU23370	Uncharacterized protein YpuA	<i>ypuA</i>	1.162	2.24	0.0283	SW 4.3	Coping with stress	Unknown	C	31295	4.52	
BSU27440	ABC transporter glutamine-binding protein GlnH	<i>glnH</i>	1.957	3.88	0.0498	SW 4.2	Sporulation and Germination	Glutamine uptake	CM	29756	8	+
BSU03420	DNA-entry nuclease inhibitor	<i>nin</i>	1.327	2.51	0.0026	SW 4.1	Lifestyles	Genetic transformation, DNA uptake	CM	14997	4.92	
BSU11430	Oligopeptide-binding protein OppA	<i>oppA</i>	1.210	2.31	0.0064	SW 4.1	Lifestyles	Initiation of sporulation, competence development	CM	61525	5	+
BSU24990	Phosphate-binding protein PstS	<i>pstS</i>	3.495	11.28	0.0467	SW 2.6	Metabolism	High-affinity phosphate uptake	CM	31684	4.85	+
BSU03610	L-cystine-binding protein TcyA	<i>tcyA</i>	-1.464	0.36	0.0220	SW 1.2	Transporters	Cystine uptake	CM	29514	9.12	+
BSU14700	Bacillolysin	<i>nprE</i>	-6.033	0.02	0.0162	SW 2.3	Metabolism	Degradation of proteins	S	56522	7	+
BSU35860	Gamma-DL-glutamyl hydrolase	<i>pgdS</i>	2.343	5.07	0.0462	SW 1.1	Cellular processes	Polyglutamic acid degradation	S	45247	8.52	+
BSU25750	Sporulation-specific extracellular nuclease	<i>nucB</i>	2.362	5.14	0.0430	SW 2.5	Metabolism	DNA degradation after mother cell lysis	S	14968	5.17	+
BSU03830	Uncharacterized ABC transporter solute-binding protein YclQ	<i>yclQ</i>	1.851	3.61	0.0329	SW 1.2	Transporters	Acquisition of iron	S	34792	6.63	+
BSU18630	Expansin-YoaJ	<i>yoaJ</i>	3.467	11.06	0.0478	SW 4.4	Lifestyles	Interaction with plant roots	S, CW	25638	9	+
BSU09420	Probable peptidoglycan endopeptidase LytE	<i>lytE</i>	-1.098	0.47	0.0062	SW 1.1	Cellular processes	Major autolysin, cell elongation and separation	S, CW	35455	10	+

Table 3.2. (Cont'd)

Locus Name	Protein	Gene	log2FC	Fold Change	P Value	Functional Category Codes	Functional Category	Function	SSL	Mass (Da)	pI	SP
BSU27580	Putative N-acetylmuramoyl-L-alanine amidase YrvJ	<i>yrvJ</i>	-1.965	0.26	0.0190	SW 1.1	Cellular processes	Cell wall metabolism	S, CW	55501	5.19	+
BSU39230	tRNA nuclease WapA	<i>wapA</i>	-1.451	0.37	0.0023	SW 1.1	Cellular processes	Intercellular competition	S, CW	258162	6	+
BSU05730	Uncharacterized protein YdhF	<i>ydhF</i>	1.132	2.19	0.0479	SW 4.2	Sporulation and Germination	Unknown	S	26525	6.16	+
BSU01650	Uncharacterized protein YbbC	<i>ybbC</i>	3.118	8.68	0.0424	SW 4.2	Sporulation and Germination	Unknown	U	46036	7.37	+
BSU12280	Uncharacterized protein YjC	<i>yjC</i>	2.562	5.90	0.0243	SW 6.7	Proteins with unknown functions	Unknown	U	15583	4.26	
BSU14750	Uncharacterized protein YlaE	<i>ylaE</i>	6.867	116.71	0.0321	SW 6.7	Proteins with unknown functions	Unknown	U	22454	4.13	+
BSU36730	Uncharacterized protein YwmD	<i>ywmD</i>	7.618	196.40	0.0167	SW 6.7	Proteins with unknown functions	Unknown	U	23965	4.68	+

Table 3.2. (Cont'd)

Locus Name	Protein	Gene	log <sub>2</sub> FC	Fold Change	P Value	Functional Category Codes	Functional Category	Function	SSL	Mass (Da)	pI	SP
BSU18280	UPF0748 protein YngK	<i>yngK</i>	3.632	12.40	0.0424	SW 4.2	Sporulation and Germination	Unknown	U	58868	8.35	+

SSL: Subcellular localization predicted via PSORTb and Uniprot. C: Cytoplasm, CM: Cytoplasmic Membrane, CW: Cell Wall, S: Secreted, S and U: Unknown.

SP: Signal Peptide which is predicted by SignalP prediction program.

When 16<sup>th</sup> hour secretome samples were analyzed, there were 46 differentially expressed proteins most of which represented by “metabolism” (26%) and “information pathways” (26 %) followed by “cell envelope and cell division” (13 %). “Sporulation and germination” category had 11 % of the differentially expressed proteins while the “coping with stress” category had only 2% while (Figure 3.7).

The proteins of unknown functions constituted 7% of the differentially expressed proteins. The two proteins encoded by *ylaE* and *ywmD* showed highest level of overrepresentation in the mutant strain.

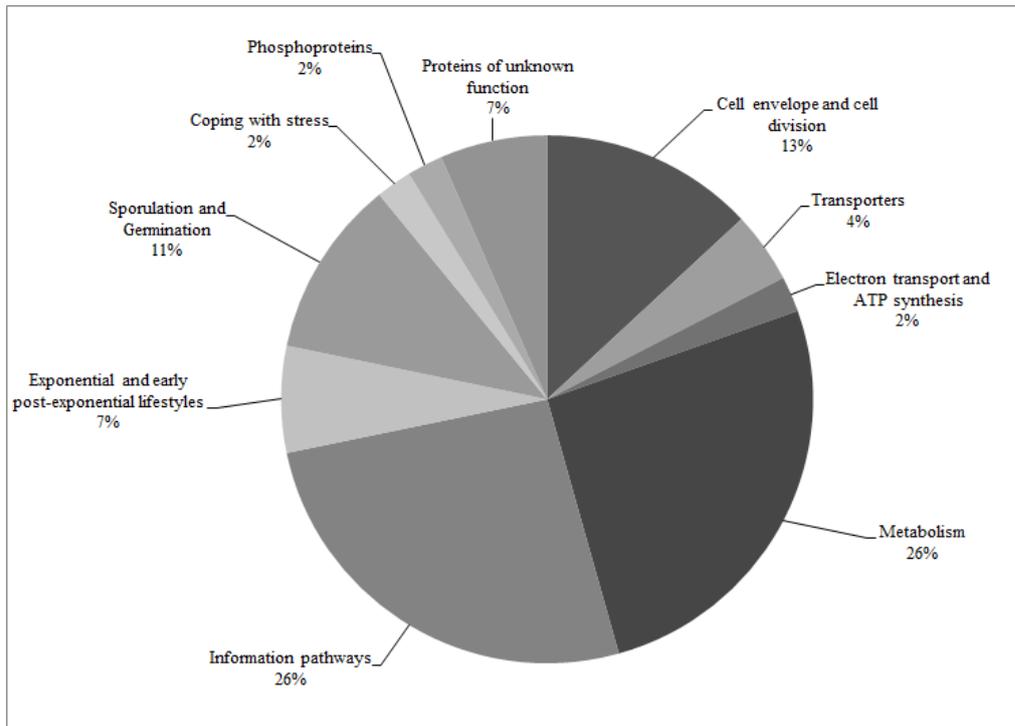


Figure 3.7. Functional distribution of differentially expressed proteins of 16<sup>th</sup> hour

### 3.3.1.2.1.1. Differentially expressed extracellular proteins of 16<sup>th</sup> hour secretome samples

*nprE* encodes an extracellular neutral protease B (bacillolysin) that is among the major extracellular enzymes of *B. subtilis*. Its regulation is under the control of CodY, AbrB and ScoC and repressed during exponential growth (Chumsakul et al., 2011; Barbieri et al., 2016). With another extracellular enzyme AprE, NprE, takes role in preventing autolysis at the stationary phase (Barbieri et al., 2016). *lytE* encodes the major autolysin in vegetative cells of *B. subtilis*. The interactions of LytE with MreBH (an actin-like protein), FtsZ and PBP2b (penicillin binding protein 2b) suggest its close relation to cell wall

turnover. It was also proposed that its inactivation could lead to increased  $\beta$ -lactam resistance because of the delay in the cell lysis process (Lou and Helman, 2012; Dominguez-Cuevas et al., 2013). *lytE* belongs to Spo0A, SigA, SigI (heat shock), WalR and SigH regulons. Its expression is also under the regulation of Spo0A (negative regulation) and WalR (positive regulation) (Schirner and Errington, 2009). WapA was found as a secreted, cell wall-associated protein overexpressed in 12<sup>th</sup> hour OGU cultures, as already mentioned (Section 3.3.1.1.1). The three extracellular proteins NprE (~7-fold), LytE (2-fold) and WapA (2.7-fold) were downregulated in the 16<sup>th</sup> hour secretome samples of OGU1. The levels of WapA and LytE are found to be the same in both OGU1 and PY79 in the cytoplasmic proteome at 16<sup>th</sup> hour (Demir, 2013). The downregulation in LytE is important in terms of prevention of the cell lysis and increase in cellular fitness.

The major sporulation-specific extracellular nuclease NucB is regulated by mother cell specific  $\sigma^E$ . The studies done with *nucB* mutants revealed that NucB is released during the late sporulation phase in order to digest the released mother cell DNA. The induction of *nucB* occurs in the mother cell and is secreted right before the mother cell lysis and probably digested nucleotides by NucB may be utilized for growth for the nonsporulated cells in the culture (Hosoya et al., 2007). It was demonstrated in *B. licheniformis* that NucB could lead to biofilm dispersal. By this way, the spores can disperse through the environment easily (Nijland et al., 2010). The secretion of nucleic acid degrading enzymes was found to be upregulated under different starvation and stress conditions. For instance, NucB is upregulated in the secretome of *B. licheniformis* under phosphate starvation (Voigt et al., 2006). The NucB was as much as 5.14 times upregulated in the secretome of the OGU1 in 16<sup>th</sup> hour culture fluids in the present study. The upregulation of this nuclease probably could provide nucleotides for yet nonsporulating cells.

PgdS protein is an enzyme called gamma-DL-glutamyl hydrolase which degrades poly-g-glutamate (PGA, a natural polymer). In *B. subtilis* cells, depending on cultural and physiological conditions, PGA can be used for different purposes like sequestration of toxic metal ions to increase resistance to changing environmental conditions or can be used as a glutamate source under a starvation during late stationary phase (Candela and Fouet, 2006). This enzyme was 5.1-fold upregulated in OGU1. This upregulation could contribute to adaptation of OGU1 to the absence of bacilysin.

YclQ, previously known as FpiA, is a petrobactin (3,4-catecholate siderophore) ABC transporter solute-binding protein which takes role in iron transport as being an exogenous siderophore (Zawadzka et al., 2009). It is known that the cellular fitness is strongly enhanced by non-endogenous siderophores (Miethke et al., 2013). The YclQ was found to be 3.6-fold upregulated in OGU1, probably reflecting an elevated need of the mutant to iron sources.

#### **3.3.1.2.2. Differentially expressed cytoplasmic membrane proteins of 16<sup>th</sup> hour secretome samples**

There are three ABC transporter binding proteins found to be upregulated in the mutant strain; OppA (oligopeptide binding), GlnH (glutamine binding), PstS (phosphate binding) and one of them TcyA (cysteine binding) was found to be downregulated in the mutant OGU1 strain in the present study. OppA and GlnH were already found to be upregulated at 12<sup>th</sup> hour secretome samples of OGU1 strain.

*pstS* (phosphate binding) is the lipoprotein component of the high-affinity phosphate transport system and it is the first gene of the *pst* operon. It was found that this operon is under the PhoPR-dependent regulation. The *pst* operon was found to be induced under phosphate starvation condition and the PstS protein was found in the extracellular fraction. Besides, *pst* operon can be induced by alkine stress and this induction state is prevented when the phosphate concentrations increased (Antelmann et al., 2000; Atalla and Schumann, 2003). In order to get higher concentrations of PstS than other components of the transporter the primary transcript of the *pst* operon is processed differentially. The *pstS*-specific mRNA with a significantly longer half-life than the mRNA for rest of genes belongs to *pst* operon (Allenby et al., 2004). PstS was found to be 11-fold upregulated in 16h secretome of OGU1 strain.

The *nucA* encodes a membrane associated nuclease and *nin* encodes inhibitor of NucA and both of these proteins are regulated by ComK. It was also stated that the DNase activity of NucA was stimulated by manganese. The NucA being a membrane associated DNase, cleaves the bound DNA during transformation and therefore it was found that the transformation efficiency decreased in *nucA* mutant (Provvedi et al., 2001). The Nin was found to be induced under nitrogen starvation conditions (Jarmer et al., 2002). In the present study, Nin was found to be 2.5 fold upregulated in 16<sup>th</sup> hour secretome of OGU1 strain. The inhibitory activity of Nin on the NucA probably affects the transformation efficiency of OGU1 strain.

The *yoaJ* gene encodes a protein structurally similar to plant expansins which have functions like extension and stress relaxation of plant cell walls under acidic pHs (Georgelis et al., 2011). Bacterial YoaJ (EXLX1) has two domains; one catalytic domain and one cellulose binding domain. *B. subtilis* YoaJ

promotes plant root colonization with a yet unknown mechanism (Kim et al., 2013). A comparison of the wild type and *yoaJ* mutant of *B. subtilis* showed that *yoaJ* could have a synergic action with bacterial hydrolases and leads to an efficient autolysis in wild type cells (Kerff et al., 2008). *YoaJ* could also take role in formation or modification of bacterial biofilms formed during colonization of plant roots. With as high as 11.1 fold elevated levels, it was a significantly upregulated cell-wall associated, secreted protein in OGU1.

### **3.3.1.2.3. Differentially expressed cytoplasmic proteins of 16<sup>th</sup> hour secretome samples**

27 cytoplasmic proteins were found to be differentially expressed in 16<sup>th</sup> hour secretome samples. Among these, 9 of them are 30S ribosomal protein subunits S10, S11, S20, S3 and 50S ribosomal protein subunits L13, L17, L18, L22, L27. These proteins and amino acid biosynthetic enzymes, MetE of methionine (5-methyltetrahydropteroyl triglutamate--homocysteine methyltransferase) and SerA of serine (D-3-phosphoglycerate dehydrogenase) are all belong to the the  $\sigma$ A regulon and downregulated in the OGU1 secretome of 16<sup>th</sup> hour.

In the 16<sup>th</sup> hour cytoplasmic proteome of OGU1 identified via LC-MS/MS approach, some ribosomal proteins including S3, S11, L13, L17 and L18 were found remained unchanged (Demir, 2013).

*pdhC* encodes the E<sub>2</sub> subunit of pyruvate dehydrogenase that forms a link between TCA cycle and glycolysis. It belongs to the *pdhABCD* operon. This operon has two promoters, first one is for the entire operon and the other is for the *pdhC*. The mutants of *pdhC* had a reduced sporulation phenotype (Gao et al., 2002). This protein did have no significant changes in the cytoplasmic proteome of OGU1 at 16<sup>th</sup> hour (Demir, 2013) while it was PdhC 2-fold downregulated in the secretome of OGU1, in the present work. It is to be

remembered that a secretome study can only identify the cytoplasmic proteins leaking out of the cells, thus the relative abundance of such proteins cannot provide reliable information about their expression levels.

SucC, succinyl-CoA synthetase (beta subunit), OdhB, the E2 subunit of 2-oxoglutarate dehydrogenase, MalD, and PdhC, a component of pyruvate dehydrogenase are the components of TCA cycle. Ldh, the enzyme L-lactate dehydrogenase, on the other hand converts pyruvate to lactate and is crucial in *B. subtilis* fermentation for reoxidation of the NADH formed by glycolysis. It was proposed that the ratio of NADH/NAD<sup>+</sup> has a regulatory role and during the transition to oxygen-limited growth, NADH is less effectively oxidized due to a decrease in respiratory activity. Therefore, there is a need for LDH to reoxidize NAD<sup>+</sup> (Larsson et al., 2005; Blencke et al., 2003). In the present secretome study, SucC, OdhB and PdhC were all downregulated while Ldh was strongly (8.2-fold) upregulated in OGU1.

UreA the urease enzyme which utilizes urea as an alternative energy source was found 3.8-fold overrepresented in our study. The *ureABC* operon is under the negative regulation of CodY and GlnR while it is positively regulated by PucR and TnrA. *ureC* was found earlier to be upregulated in 16<sup>th</sup> hour cultures of OGU1 as shown by 2DE MALDI-TOF MS approach (Aras Taşkın, 2011) while the levels of *ureA* and *ureC* did not change when compared to PY79 at the same hour in LC-MS/MS approach (Demir, 2013) used to identify differentially expressed cytoplasmic proteins.

The levels of 3 enzymes for riboflavin (RibH), folate (FolB) and GMP (GuaB) biosynthesis were elevated 2.6, 3.6 and 2.1 times, respectively, in 16<sup>th</sup> hour secretome samples of OGU1.

IspA is the major intracellular serine protease of *B.subtilis* and is under the CodY repression in the presence of branched chain amino acids (Belitsky and

Sonenshein, 2013). IspA were found to be absent from cytoplasmic proteome of OGU1 at 16 h (Demir, 2013), but to the contrary and intriguingly, it was 4.7 times more abundant in our 16<sup>th</sup> hour samples. ClpY is a subunit of two component ATP dependent protease having chaperone-like activity and ClpYQ complex functions for elimination of abnormal proteins and the rapid degradation of regulatory proteins (Kang et al., 2003). ClpY level was 2.7-times elevated in 16<sup>th</sup> hour OGU1 secretome, but was strongly underrepresented in 12<sup>th</sup> hour secretome samples in the present work.

*tagC* codes for putative major teichoic acid biosynthetic protein C. It was induced upon DNA damage (Au et al., 2005). TagC was a 2.25- and 3.25-fold upregulated protein at 12<sup>th</sup> hour and 16<sup>th</sup> hour, respectively in OGU1 strain in the present secretome study while its levels did not change in OGU1 when compared to PY79 in 16<sup>th</sup> hour cytoplasmic proteome (Demir, 2013). The DltA (D-alanine-D-alanyl carrier protein ligase) which is also important for cell wall synthesis downregulated in 16<sup>th</sup> hour cytoplasmic proteome of OGU1 strain (Aras Taşkın, 2011), thus the increment obtained for TagC in the present work is not much trustable.

Bacterial endospore is composed of a core DNA which is surrounded by several layers of protein, peptidoglycan, dipicolinic acid (DPA) and calcium in high amounts. SpoVFA and SpoVFB subunits form DPA synthase which catalyzes the final step in the synthesis of DPA during spore maturation (Errington, 1993; Jamroskovic et al., 2014). DPA has crucial functions in endospores such as lowering the water content and protecting the DNA and proteins from damage. The final step in DPA synthesis is catalyzed by DPA synthase SpoVFB and SpoVFA was demonstrated as downregulated proteins in cytoplasmic proteome of OGU1 when compared to PY79 (Aras Taşkın, 2011; Demir, 2103). On the contrary, SpoVFB was found to be 5-fold upregulated in OGU1 strain in 16<sup>th</sup> hour in the present secretome study.

Besides, when the DPA levels in the spores of OGU1 and PY79 were compared, no changes have been observed (manuscript submitted). It was reported that the DPA and calcium levels may show differences in individual spores in a single spore population (Jamroskovic et al., 2016).

The *glmS* gene encodes glutamine-fructose-6-phosphate amidotransferase and this enzyme catalyzes the reaction that generates glucosamine-6-phosphate from fructose-6-phosphate and glutamine. This product is the raw material for UDP-N-acetylglucosamine (GlcNAc) production which is used for cell wall biosynthesis (Winkler et al., 2004). GlmS is subject to Clp-dependent proteolysis under glucose starvation conditions (Gerth et al., 2008). Besides, *glmS* gene harbors the unique ribozyme which regulates *glmS* expression via ligand-induced (GlcNAc) self-cleavage. As a result of this ribozyme activity, intracellular degradation of *glmS* transcript via RNase J1 and consequent downregulation is achieved (Roth et al., 2006). Regarding GlmS, our former and current proteomic studies gave consistent results in that this cytoplasmic protein is a downregulated one in 16<sup>th</sup> hour cultures.

YpuA, one of the members of  $\sigma^M$  regulon, is responsive to cell wall stress (Jervis et al., 2007). Upon exposure to synthetic peptide antibiotic MP196 which targets bacterial cell wall synthesis, *ypuA* gene expression is strongly activated (Wenzel et al., 2014). This protein's abundance was ca. 2-fold increased in OGU1's secretome in 16<sup>th</sup> hour.

#### **3.3.1.2.4. Differentially expressed proteins with unknown localization and/or function of 16<sup>th</sup> hour secretome samples**

All of the 6 proteins with unknown function and localization were overrepresented in OGU1. There were 3 differentially expressed proteins belonging to the functional category of sporulation and germination category namely, YdhF, YbbC and YngK with unknown particular functions. Of these, YbbD which shares the same cluster with YbbC is an ortholog of the cell wall-recycling NagZ (N-acetylglucosaminidase) of *E. coli* and *ybbC* was found to be expressed in late exponential and early stationary phase of growth cycle (Litzinger et al., 2010; Nicolas et al., 2012). YngK is categorized as a newly identified sporulation and germination related protein as based on former transcriptional profiling studies (Nicolas et al., 2012). As stated earlier, although, the loss or downregulation of many sporulation proteins, the sporulation frequency of OGU1 is no different than that PY79 (manuscript submitted).

YdhF is a lipoprotein which is a member of Pho regulon and was found to be secreted into the medium. *ydhF* was found to be strongly induced under phosphate starvation conditions, but the function of this protein has not yet been determined (Antelmann et al., 2000). YjlC was another protein with unknown function. It was 2-fold and up to 6-fold overexpressed in 12<sup>th</sup> hour and 16<sup>th</sup> hour secretomes of OGU1. These proteins are thought to be candidate components of stress-coping in OGU1.

Two unknown proteins were found in anomalously high levels from 16<sup>th</sup> hour secretome samples of OGU1: The one was YwmD, claimed to contain a signal peptide (not verified in this work) and was found as an overproduced protein in a *degU32* hypersecretion mutant by Antelmann et al. (2004). The *ywmD* gene is located in *licR-gerBC* region (Presecan et al., 1997) though its function has

not yet been characterized. Another overproduced protein was YlaE known to be negatively regulated by AbrB and Abh (Chumsakul et al., 2011). The expression levels of these proteins were exceptionally high, corresponding to ca 117- and 196-fold elevated levels when compared to the parental strain.

### **3.3.1.3. Differentially expressed proteins of *B. subtilis* of 24<sup>th</sup> hour secretome samples**

According to the 24<sup>th</sup> hour secretome results, only a total of 12 proteins were found to be differentially expressed between PY79 and OGU1 (Figure 3.8). The differentially expressed proteins, their levels, properties and functions are tabulated in Table 3.3.

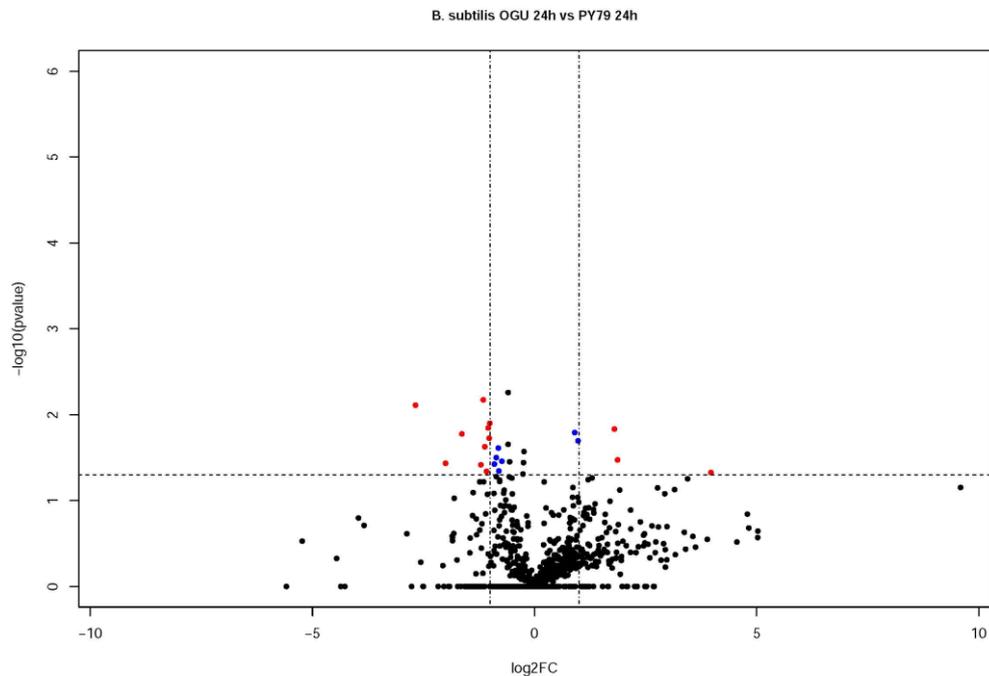


Figure 3.8. The volcano plot showing the distribution of differentially expressed proteins with at least 2-fold change in their levels with a p value < 0.05 in 24<sup>th</sup> hour secretome samples of *B. subtilis* PY79 and OGU1. The x and y axis correspond to p values (in  $-\log_{10}$ ) and fold change (FC) ( $\log_2$ ) respectively. The dashed horizontal line shows where  $p = 0.05$  ( $-\log_{10} 0.05 = 1.3$ ) with points above the line having  $p < 0.05$  and points below the line having  $p > 0.05$ . The dashed vertical lines correspond to  $\log_2 = 1$  which means no change and shown in black dots. The blue dots have p values less than 0.05 but have fold change values close to 2. Red dots are the ones that are accepted as differentially expressed both having p values equal to or less than 0.05 and fold change values are greater than 2 and less than -2. Red dots at the negative values are decreasing while on the positive values of fold change are increasing in the OGU1 when compared to parental strain PY79.

Table 3.3. Differentially expressed proteins in the secretome of 24<sup>th</sup> hour cultures as identified by LC-MS/MS

Locus Name	Protein	Gene	log <sub>2</sub> FC	Fold Change	P Value	Functional Category Codes	Functional Category	Function	SSL	Mass (Da)	pI	SP
BSU02910	Uncharacterized protein YceE	<i>yceE</i>	-2.678	0.16	0.0077	SW 4.3	Coping with stress	Required for survival of ethanol stress and at low temperatures	C	20948	4.39	
BSU18440	Glutamate synthase [NADPH] small chain	<i>gltB</i>	-1.208	0.43	0.0383	SW 2.3	Metabolism	Glutamate biosynthesis	C	54862	7	
BSU01370	Adenylate kinase	<i>adk</i>	-1.043	0.49	0.0143	SW 2.5	Metabolism	ADP formation	C	24119	4.45	
BSU22600	3-phosphoshikimate 1-carboxyvinyltransferase	<i>aroA</i>	-2.004	0.25	0.0368	SW 2.3	Metabolism	Biosynthesis of aromatic amino acids	C	45240	6	
BSU23070	D-3-phosphoglycerate dehydrogenase	<i>serA</i>	-1.155	0.45	0.0067	SW 2.3	Metabolism	Biosynthesis of serine	C	57129	5.62	
BSU29750	Protein AroA(G)	<i>aroA</i>	-1.121	0.46	0.0236	SW 2.3	Metabolism	Biosynthesis of aromatic amino acids	C	39539	5.34	
BSU03510	Surfactin synthase subunit 3	<i>srfAC</i>	-1.635	0.32	0.0167	SW 4.3	Coping with stress	Antibiotic synthesis	C	143872	4.97	
BSU33410	Stress response protein YvgO	<i>yvgO</i>	-1.080	0.47	0.0457	SW 4.3	Coping with stress	Survival of ethanol stress	CM	17602	4.85	+
BSU03420	DNA-entry nuclease inhibitor	<i>nin</i>	-1.006	0.50	0.0126	SW 4.1	Lifestyles	Genetic transformation, DNA uptake	CM	14997	4.92	
BSU37280	Nitrate reductase alpha chain	<i>narG</i>	3.970	15.67	0.0471	SW 2.1	Metabolism	Nitrate respiration, nitrogen assimilation	CM	139099	6.06	
BSU33770	Sporulation delaying protein C	<i>sdpC</i>	1.799	3.48	0.0147	SW 4.3	Coping with stress	Killing of non-sporulating sister cells	S	22221	9	+
BSU38260	Probable deferriochelate/ferroxidase EfeN	<i>eFeN</i>	1.869	3.65	0.0336	SW 4.3	Coping with stress	Ferrous iron conversion	S	45693	8	+

SSL: Subcellular localization predicted via PSORTb and Uniprot. C: Cytoplasm, CM: Cytoplasmic Membrane, CW: Cell Wall, S: Secreted, S and U: Unknown.

SP: Signal Peptide which is predicted by SignalP prediction program.

The number of differentially expressed proteins of 24<sup>th</sup> hour was only 12 and half of these belonged to “metabolism” and “exponential and early post-exponential life styles”. 5 proteins were categorized under “coping with stress” and the remaining one protein belonged to “electron transport and ATP synthesis” (Figure 3.9).

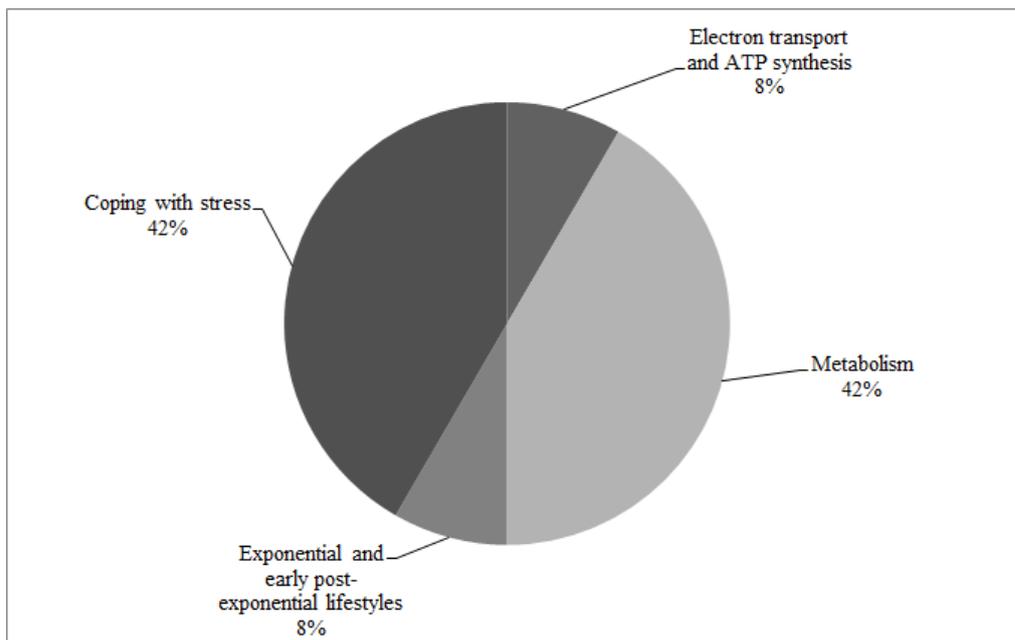


Figure 3.9. Functional distribution of differentially expressed proteins of 24<sup>th</sup> hour

### 3.3.1.3.1. Differentially expressed extracellular proteins of 24<sup>th</sup> hour secretome samples

*efeB* (*ywbN*) encodes a heme peroxidase which is a member of EfeUOB transporter and is secreted via Tat pathway (Jongbloed et al., 2004). EfeB protein belongs to Dyp-type peroxidase family and contains an iron-heme cofactor. Under low salinity conditions, EfeB (YwbN) deprived cells become

iron starved and some cells undergo induced cell lysis instead of entering into the stationary phase (Ploeg et al., 2011). Among the components of EfeUOB transporter, only EfeB has an additional promoter region which is under the control of cell envelope stress  $\sigma$  factors (w, m and x) indicating that elevated high EfeB synthesis is indicative of stressful conditions (Miethke et al., 2013). Accordingly, EfeB was nearly 4-fold upregulated in 24<sup>th</sup> hour secretome of OGU1.

*B. subtilis* produces a mixed population of cells in response to starvation or nutrient deprivation depending on the phosphorylation status of Spo0A which is the master regulatory factor of entry into sporulation. The cells with active Spo0A produce and export a peptide-antibiotic-like killing factor (Skf) (discussed in Section 3.3.1.1.2) and a protein toxin (SdpC) in order to kill Spo0A inactive cells. By this process, Spo0A active cells gather nutrient from the dead cells which enables Spo0A active cells to slow down or delay the sporulation process till a point where the sporulation is irreversible. The *sdpC* gene encodes the toxin SdpC which is composed of 63 amino acid protein derived from the C-terminal portion. It collapses the proton motive force and induces autolysis in the target cells. The *sdpC* is under the regulation of Spo0A (positive) and AbrB (negative) (Ellermeier et al., 2006; Strauch et al., 2007). Besides its cannibalistic action, SdpC is also found to be effective on other Gram positive bacteria (Firmicutes phylum) and *E. coli* with damaged outer membrane (Lamsa et al., 2012). SdpC was a 3.5-fold upregulated protein in 24<sup>th</sup> hour secretome of OGU1..

### **3.3.1.3.2. Differentially expressed cytoplasmic membrane proteins of 24<sup>th</sup> hour secretome samples**

*nin* encodes an inhibitor of NucA. NucA is induced under nitrogen starvation and it has also an effect on the transformation efficiency (Provvedi et al., 2001). The 2.5-fold Nin upregulation was recorded at 16 h (Section 3.3.1.2.2), but the protein became 2-fold downregulated thereafter.

*narG* encodes a nitrate reductase and transcription of its operon *narGHIIJ* is positively controlled by the transcriptional regulator Fnr which is a member of a regulatory cascade acting for adaptation to low oxygen conditions (Reents et al., 2006). Besides, the nitrates which function as the final electron acceptor during anaerobic respiration are the inducers of the *narGHIIJ* operon. The TnrA is a transcription factor which binds DNA to activate transcription of the *nar* operon and several nitrogen-controlled genes. Being a global regulator TnrA activates and represses many genes during nitrogen-limited growth (Nakano et al., 1998; Yoshida et al., 2003). One such nitrogen regulated gene is *narG* which is activated when nitrogen is limited (Richardson et al., 2001; Yoshida et al., 2003; Reents et al., 2006). In the present study, NarG was found to be as much as 15-fold upregulated in OGU1.

*srfAC* encodes surfactin synthase subunit 3 which takes role in the surfactin biosynthesis pathway (Tanovic et al., 2008). In *B. subtilis*, surfactin is a lipopeptide and it is involved in the extracellular matrix production for biofilm formation via paracrine signaling pathway in the neighboring cells (Marvasi et al., 2010), although *B. subtilis* 168 (from which PY79 has been derived) has never shown to produce this lipopeptide (Nakano et al., 1992; Kearns and Losick, 2003). The surfactin biosynthetic operon has some role in bacilysin production since bacilysin biosynthesis is also blocked in a *srfA*-blocked

mutant. (Yazgan Karataş et al., 2003). The SrfAC was found to be 3-fold downregulated in OGU1 mutant, but not in PY79 for the reasons that are yet obscure.

YvgO was found to be among the proteins which are important for the survival under the ethanol stress (Höper et al., 2005; Chumsakul et al., 2011). Its synthesis is dependent on alternative sigma factor  $\sigma^B$  and repressed by AbrB. The mechanism of its exact function is unknown. YvgO is located in cytoplasmic membrane and was downregulated in OGU1 6-fold.

In the proteomics study of Antelmann et al (2002), the wall binding proteins disappeared from the extracellular proteome during the stationary phase and are subjected to proteolysis. Quite consistently, no cell wall-binding protein could be detected in 24<sup>th</sup> hour secretome samples in the present study.

#### **3.3.1.3.3. Differentially expressed cytoplasmic proteins of 24<sup>th</sup> hour secretome samples**

All of the 7 cytoplasmic proteins identified in 24<sup>th</sup> hour secretome samples were downregulated in OGU1 (Table 3.3). Cytoplasmic proteins functions in aromatic amino acid biosynthesis were SerA, AroA and AroE. These proteins contain S-bacillithiolation sites in their cystein residues and are oxidized in response to hypochlorite stress in *B. subtilis* (Chi et al., 2013).

YceE is also important for survival under ethanol as well as low temperature stress and its synthesis is also dependent on alternative sigma factor  $\sigma^B$  (Höper et al., 2005; Chumsakul et al., 2011). YceE could not be detected in the 16<sup>th</sup> hour cytoplasmic proteome of PY79 with 2DE approach while there were no

significant change with respect to this protein between two strains when LC-MS/MS was the approach used (Aras Taşkın, 2011; Demir, 2013). In the present secretome study, YceE was downregulated in OGU1 by 2-fold, respectively.

Most of the cytoplasmic other than NarG, SdpC and EfeB were downregulated in OGU1 at 24<sup>th</sup> hour at which bacilysin itself disappears from the culture fluids. As expected, the number of differentially expressed proteins was very low when compared to those obtained in 12<sup>th</sup> and 16<sup>th</sup> hours.

#### **3.3.1.4. LC-MS/MS-identified differentially expressed proteins commonly detected at time intervals**

Since differentially expressed proteins were analyzed in a time dependent manner (12<sup>th</sup>, 16<sup>th</sup> and 24<sup>th</sup> hour, respectively) in this study, the proteins commonly identified at selected times of cultivation were also tabulated (Table 3.4). Eight overrepresented proteins were common to the time points of 12<sup>th</sup> hour and 16<sup>th</sup> hour while only two proteins, SerA that functions in aromatic amino acid biosynthesis (underrepresented) and Nin that acts as a DNA-entry nuclease inhibitor (overrepresented) were common to the 16<sup>th</sup> hour and 24<sup>th</sup> hour. There were no proteins common in all three time intervals. Briefly, teichoic acid biosynthesis protein TagC, two uncharacterized proteins Yj1C and YjoA showed an increment in their expression from 12<sup>th</sup> hour to 16<sup>th</sup> hour. The level of remaining five upregulated proteins decreased during the same time period. Among these MetE, ClpY and WapA were related to cellular metabolism and GlnH and OppA were related to sporulation.

Table 3.4. Differentially expressed proteins common to the selected time intervals

Locus Number	Fold Change at 12 <sup>th</sup> h	Fold Change at 16 <sup>th</sup> h	Fold Change at 24 <sup>th</sup> h	Protein	Gene	Functional Category Codes	Functional Category	Function	SSL	Mass (Da)	pI	SP
BSU35770	2.25	3.25	-	Putative major teichoic acid biosynthesis protein C	<i>tagC</i>	SW 1.1	Cellular processes	Biosynthesis of teichoic acid	C	50826	7.3	
BSU13180	4.38	0.42	-	5-methyltetrahydropteroyl triglutamate--homocysteine methyltransferase	<i>metE</i>	SW 2.3	Metabolism	Biosynthesis of methionine	C	86806	4	
BSU16160	3.55	2.71	-	ATP-dependent protease ATPase subunit ClpY	<i>clpY</i>	SW 3.3	Genetics	Protein degradation	C	52586	5	
BSU12410	2.50	3.10	-	Uncharacterized protein YjoA	<i>yjoA</i>	SW 6.4	Proteins with unknown functions	Unknown	C	17793	5.8	
BSU11430	7.31	2.31	-	Oligopeptide-binding protein OppA	<i>oppA</i>	SW 4.1	Lifestyles	Initiation of sporulation, competence development	CM	61525	5	+
BSU27440	5.02	3.88	-	ABC transporter glutamine-binding protein GlnH	<i>glnH</i>	SW 4.2	Sporulation and Germination	Glutamine uptake	CM	29756	8	+
BSU39230	2.77	0.37	-	tRNA nuclease WapA	<i>wapA</i>	SW 1.1	Cellular processes	Intercellular competition	S, CW	258162	6	+
BSU12280	2.85	5.90	-	Uncharacterized protein YjIC	<i>yjIC</i>	SW 6.7	Proteins with unknown functions	Unknown	U	15583	4.3	
BSU23070	-	0.25	0.45	D-3-phosphoglycerate dehydrogenase	<i>serA</i>	SW 2.3	Metabolism	Biosynthesis of serine	C	57129	5.6	
BSU03420	-	2.51	0.50	DNA-entry nuclease inhibitor	<i>nin</i>	SW 4.1	Lifestyles	Genetic transformation, DNA uptake	CM	14997	4.9	

SSL: Subcellular localization predicted via PSORTb and Uniprot. C: Cytoplasm, CM: Cytoplasmic Membrane, CW: Cell Wall, S: Secreted, U: Unknown.

SP: Signal Peptide which is predicted by SignalP prediction program.

### 3.4. Comparative secretome analyses via 2DE MALDI-TOF MS

#### 3.4.1. Differentially expressed proteins of 12<sup>th</sup> hour secretome samples

In 12<sup>th</sup> hour secretome samples, 6 proteins were identified as differentially expressed with 2DE MALDI-TOF MS (Figure 3.10). All were secreted proteins, one half of them containing a signal peptide (Table 3.5).

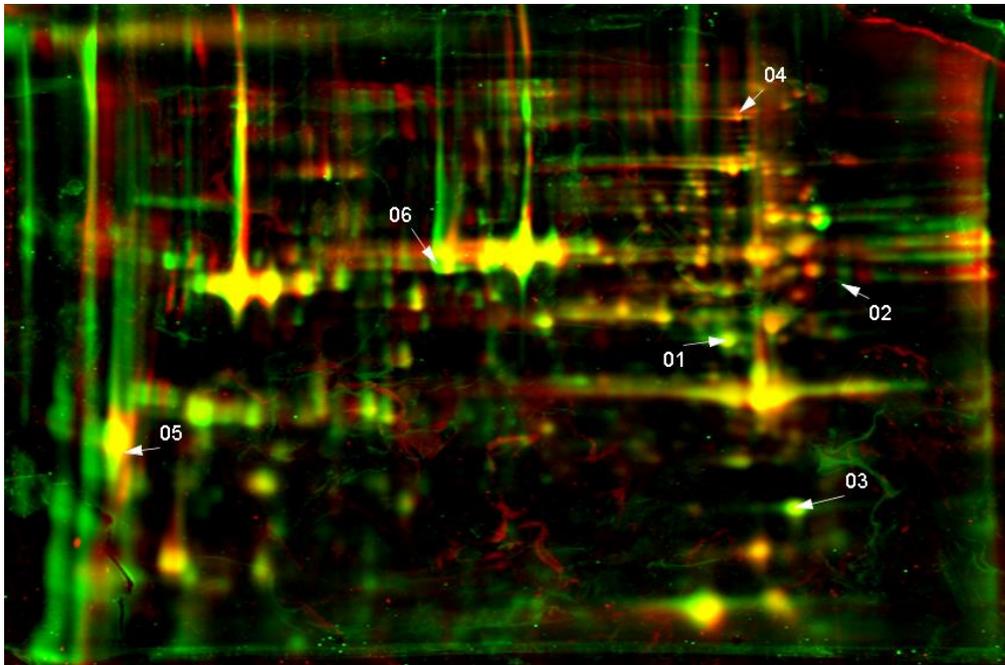


Figure 3.10. Dual channel 2DE imaging of the differentially expressed protein spots at 12<sup>th</sup> hour cultures of *B. subtilis* strains PY79 (red) and OGU1 (green) in a pI range of 3-10. Overlapping spots are shown yellow.

Table 3.5. Differentially expressed proteins in the secretome of 12<sup>th</sup> hour cultures as identified by 2DE MALDI-TOF MS

Locus Number	Mass (KDa)	PI	OGU/PY79 Ratio 12h	P Value	Spot Label	Protein	Gene	Functional Category Codes	Functional Category	Function	SSL	SP
BSU12610	34.59	5.19	2.64	0.01	1	putative capsid protein of PBSX prophage	<i>xtdG</i>	SW 5.1	Prophages and mobile genetic elements	Unknown	S	+
BSU35410	54.32	4.38	0.52	0.05	2	flagellar hook-filament junction	<i>flgK</i>	SW 4.1	Lifestyles	Motility and chemotaxis	S	
BSU12660	16.35	4.58	4.29	0.05	3	conserved hypothetical protein; PBSX phage	<i>xtdM</i>	SW 5.1	Prophages and mobile genetic elements	Unknown	S	
BSU18000	99.27	4.90	3.30	0.02	4	aconitate hydratase (aconitase)	<i>citB</i>	SW 3.4	Genetics	TCA cycle	C	
BSU17690	26.58	9.97	0.39	0.03	5	conserved hypothetical protein	<i>yncM</i>	SW 6.7	Proteins with unknown functions	Proteins with unknown functions	S	+
BSU02310	51.76	6.68	2.31	0.03	6	putative exported hydrolase	<i>ybfO</i>	SW 4.3	Coping with stress	Unknown	S	+

SSL: Subcellular localization predicted via PSORTb and Uniprot. C: Cytoplasm, CM: Cytoplasmic Membrane, CW: Cell Wall, S: Secreted, S and U: Unknown.

SP: Signal Peptide which is predicted by SignalP prediction program.

There were two phage related proteins overrepresented in OGU1: XkdG was found to be 5-fold and 2.64-fold overrepresented in OGU1 at 12<sup>th</sup> hour secretome samples via LC-MS/MS (section 3.3.1.1.1) and 2DE MALDI-TOF MS, respectively while XkdM was identified only by 2DE MALDI-TOF MS as a 4.29 times overrepresented protein. As discussed earlier (section 3.3.1.1.1), two genes (*xkdG* and *xkdM*) belong to *xpf* regulon which is downregulated in *B. subtilis* under high salinity conditions (Nagler et al., 2016). Besides, it was proposed that the expression of genes from the defective PBSX phage leads to excretion of chromosomal DNA fragments with different sizes to the media in a growth-phase specific manner (Shingaki et al., 2003).

YbfO and YncM are two unknown proteins that were found as nearly 7- and 10-fold overrepresented proteins, respectively in OGU1. The *ybfO* encodes for a putative erythromycin esterase and it was also found by LC-MS MS to be overrepresented by 5.3-fold at 12<sup>th</sup> hour. YncM, secreted via Sec pathway, is the major component of the secretome and was reported among the downregulated proteins in the extracellular proteome of *B. subtilis wapa* mutant (Antelmann et al., 2002; Voigt et al., 2009). In the absence of phytate (phosphorus source in plants), YncM was among the upregulated proteins (Antelmann et al., 2007). Besides, under another phosphate starvation condition, YncM protein was upregulated (Antelmann et al., 2000). The ResDE which is a two-component signal transduction system has a global impact on the both aerobic and anaerobic respiration. In a *resDE* mutant, mRNA levels of *yncM* were increased (Ye et al., 2000). The function of YncM has not yet been determined, but it was 2.5- fold underrepresented in OGU1, as shown by the current study.

As mentioned in section 3.3.1.1, *flgK* which encodes the flagellar hook-filament junction protein was found to be 4.5-fold underrepresented in OGU1 at 12<sup>th</sup> hour via LC-MS/MS approach. Consistently, FlgK was nearly 2-fold

underrepresented in 12<sup>th</sup> hour secretome samples as shown via 2DE MALDI-TOF MS approach.

According to 2DE MALDI-TOF MS data, acotinate hydratase (CitB) was 2.31-fold overrepresented in OGU1. Quite consistently, it was found to be overrepresented by 2.33-fold in this mutant strain at 12<sup>th</sup> hour via LC-MS/MS approach.

### **3.4.2. Differentially expressed proteins of 16<sup>th</sup> hour secretome samples**

In 16<sup>th</sup> hour secretome samples, 6 proteins were found to be differentially expressed via 2DE MALDI-TOF MS (Figure 3.11). Of these, one was cytoplasmic protein and 5 were secreted ones (Table 3.6).

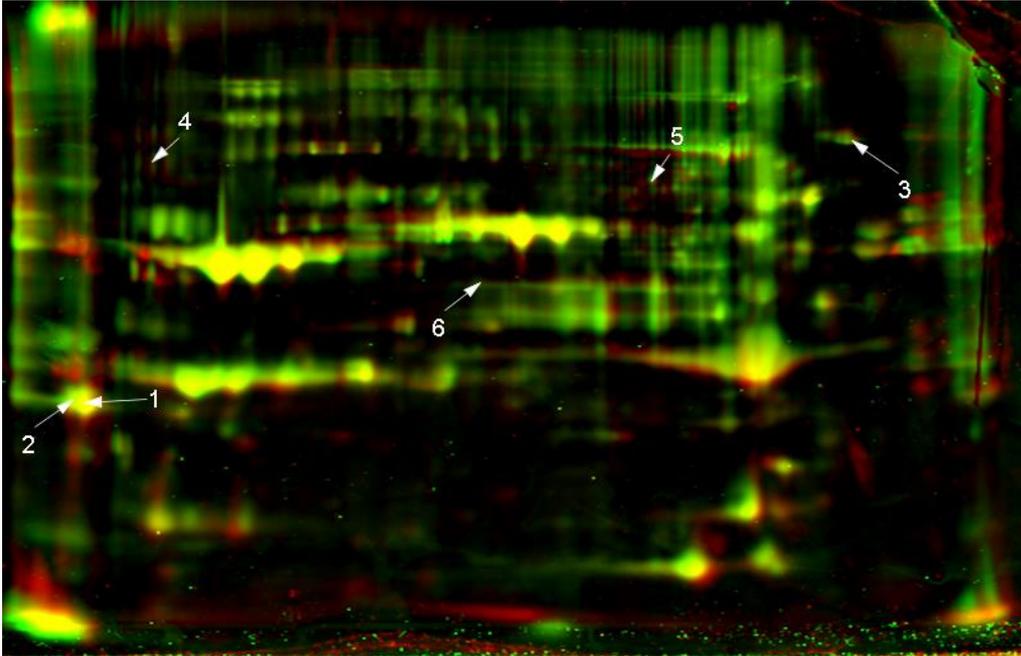


Figure 3.11. Dual channel 2DE imaging of the differentially expressed protein spots at 16<sup>th</sup> hour cultures of *B. subtilis* strains PY79 (red) and OGU1 (green) in a pI range of 3-10. Overlapping spots are shown yellow.

Table 3.6. Differentially expressed proteins in the secretome of 16<sup>th</sup> hour cultures as identified by 2DE MALDI-TOF MS

Locus Number	Mass (KDa)	PI	OGU1/PY79 Ratio 16h	P Value	Spot Label	Protein	Gene	Functional Category Codes	Functional Category	Function	SSL	SP
BSU17690	26.45	9.97	0.52	0.01	1	Uncharacterized protein YncM	<i>yncM</i>	SW 6.7	Proteins with unknown functions	Proteins with unknown functions	S	+
BSU26820	24.73	9.96	0.52	0.01	2	Uncharacterized protein YrpD	<i>yrpD</i>	SW 6.7	Proteins with unknown functions	Proteins with unknown functions	S	+
BSU15300	154.34	4.98	5.80	0.04	3	Bacillopeptidase F	<i>bpr</i>	SW 2.3	Metabolism	Protein degradation	S	+
BSU17460	50.11	4.87	0.30	0.02	4	Glutamine synthetase	<i>glnA</i>	SW 3.4	Genetics	Glutamine biosynthesis, control of trna and glnr activity	C	
BSU09190	132.47	4.69	4.09	0.05	5	Endonuclease YncR	<i>yncR</i>	SW 2.5	Metabolism	Utilization of nucleic acids	S	+
BSU39940	43.72	6.38	5.78	0.03	6	Uncharacterized protein YxaL	<i>yxaL</i>	SW 3.3	Genetics	Similar to ser/thr kinase, increases the processivity of the pcrA helicase	S	+

SSL: Subcellular localization predicted via PSORTb and Uniprot. C: Cytoplasm, CM: Cytoplasmic Membrane, CW: Cell Wall, S: Secreted, \$ and U: Unknown.

SP: Signal Peptide which is predicted by SignalP prediction program.

*glnA* encodes for glutamine synthetase (GS) which has dual roles in cell metabolism. First, ATP-dependent synthesis of glutamine is catalyzed by GS and by this reaction inorganic ammonium is incorporated. GS controls the activity of transcription factors namely TnrA and GlnR by direct protein-protein interactions. These are two transcription factors for nitrogen-regulated genes. GS is regulated by feedback inhibition via high levels of glutamine which depends on nitrogen availability (Fedorova et al., 2013). In our previous cytosolic proteome studies, GlnA was downregulated in OGU1 as revealed by 2DE MALDI-TOF MS approach (Aras Taşkın, 2011) while its level was unchanged according to LC-MS/MS data at 16<sup>th</sup> hour cytoplasmic proteome (Demir, 2013). The protein existed as a 3.3-fold lowered level in the present secretome study via 2DE MALDI-TOF MS while it was 2-fold overrepresented in OGU1 in 12<sup>th</sup> hour secretome samples via LC-MS/MS (section 3.3.1.1.2).

The *bpr* which encodes for bacillopeptidase F protein was 4-fold upregulated at 12<sup>th</sup> hour in the present secretome study via LC-MS/MS approach (section 3.3.1.1.2). Consistently, 2DE MALDI-TOF MS approach, Bpr was found to be upregulated by 5.8-fold in 16 h secretome samples.

YncM with a yet unknown function displayed a 2.5-fold decrease in OGU1 at 12<sup>th</sup> hour, as revealed by 2DE MALDI-TOF MS (section 3.4.1). The level of this protein was much lower (5-fold decreased) in 16 cultures using the same proteomic approach. YrpD is also an unknown protein which is upregulated in the absence of phytate (phosphorus source in plants) and contains a typical Sec-type signal peptide (Antelmann et al., 2007). It was found to be among the genes which are positively controlled by Spo0A during vegetative growth (Garti-Levi et al., 2013). The level of YrpD was 2-times lower in OGU1 16 h secretome samples.

YhcR (nonspecific endonuclease) enables organisms to use extracellular nucleotides (Oussenko et al., 2004). *yhcR* was induced in a *sigB* mutant under

phosphate starvation (Allenby et al., 2005). In this study, by using LC-MS/MS, YhcR was found to be 5-fold upregulated in 12 h secretome samples via (section 3.3.1.1.1). It was also 4-fold upregulated as shown in 16 secretome samples by 2DE MALDI-MS/MS.

YxaL, as an unknown protein, was demonstrated to interact and increase processivity of a helicase PcrA. It has a beta propeller conformation by which it probably provides an efficient connection between PcrA and its substrate (Noirot-Gros et al., 2002). YxaL was also reported to be strongly expressed in glucose minimal medium (Yoshida et al., 2000). This protein was 6-fold upregulated in the OGU1 strain at 16<sup>th</sup> hour.

### **3.4.3. Differentially expressed proteins of 24<sup>th</sup> hour secretome samples**

Only 4 proteins were found to be differentially expressed in OGU1 as shown in Figure 3.12 and Table 3.7. Among these, GlnA was nearly 3-fold underexpressed. NADH dehydrogenase as well as a conserved hypothetical protein (YncM) was also downregulated while the level of a putative exported protein YwmD was increased by 3.5 fold. The latter is among DegU-regulated genes as shown by Antelmann et al., 2004 and Kobayashi, 2007. As mentioned in Section 3.3.1.2.4, YwmD was found to overrepresented in mutant strain using LC-MS/MS approach. YncM was also downregulated in OGU1 at 16<sup>th</sup> hour, as mentioned in Section 3.4.2. As to NADH dehydrogenase which takes part in a regulatory loop that functions to prevent a large fluctuation in the NADH/NAD<sup>+</sup> ratio in *B. subtilis* (Gyan et al., 2006) and is known to be repressed during anaerobic conditions (Marino et al., 2000), a nearly 7-fold decrease was recorded in its level in the secretome of OGU1 at 24<sup>th</sup> hour.

Table 3.7. Differentially expressed proteins in the secretome of 24<sup>th</sup> hour cultures as identified by 2DE MALDI-TOF MS

Locus Number	Mass (kDa)	PI	OGU1/PY79 Ratio 24h	P Value	Spot Label	Protein	Gene	Functional Category Codes	Functional Category	Function	SSL	SP
BSU17690	26.45	9.97	0.22	0.04	1	conserved hypothetical protein	<i>yncM</i>	SW 6.7	Proteins with unknown function	Unknown	S	+
BSU12290	41.79	6.29	0.16	0.00	2	NADH dehydrogenase	<i>ndh</i>	SW 2.1	Metabolism	Respiration	CM	
BSU17460	50.11	4.87	0.37	0.05	3	glutamine synthetase	<i>glnA</i>	SW 3.4	Genetics	Glutamine biosynthesis, control of TnrA and GlnR activity	C	
BSU36730	23.82	4.68	3.48	0.03	4	putative exported protein	<i>ywmD</i>	SW 6.7	Proteins with unknown function	Unknown	U	

SSL: Subcellular localization predicted via PSORTb and Uniprot. C: Cytoplasm, CM: Cytoplasmic Membrane, CW: Cell Wall, S: Secreted,S and U: Unknown.

SP: Signal Peptide which is predicted by SignalP prediction program.

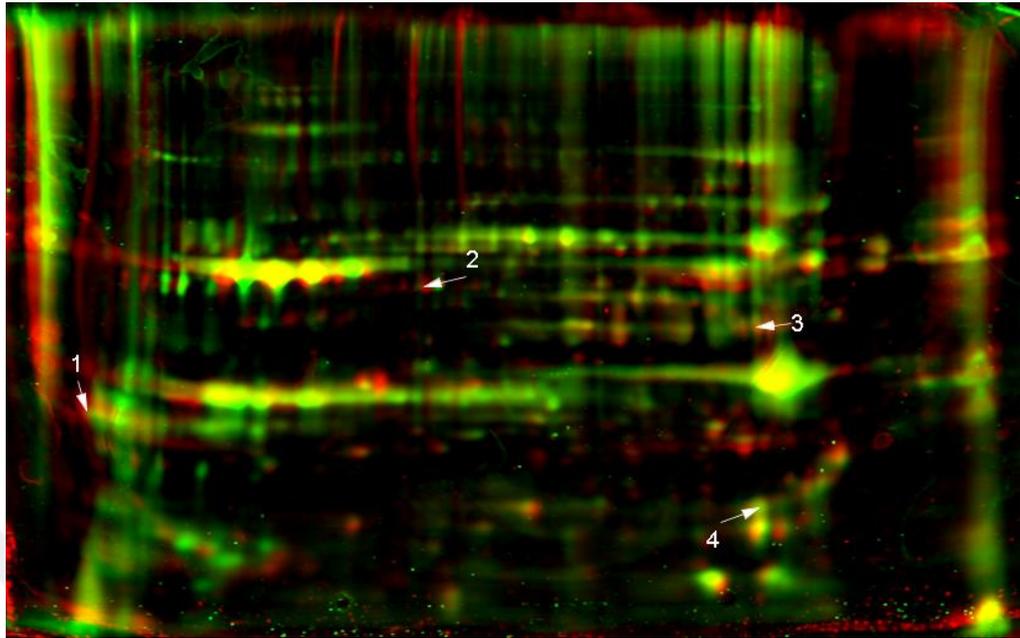


Figure 3.12. Dual channel 2DE imaging of the differentially expressed protein spots at 24<sup>th</sup> hour cultures of *B. subtilis* strains PY79 (red) and OGU1 (green) in a pI range of 3-10. Overlapping spots are shown yellow.



## CHAPTER 4

### CONCLUSION

- Even though the proteomics is a powerful tool to verify subcellular localizations, it still has limitations because not all proteins can be visualized. One reason is that several proteins are regulated in a growth-phase dependent manner and their expression levels are different depending on the physiological conditions including starvation and stress. Thus, a considerable part of the proteome remains silent under the applied physiological conditions. In the present study, such limitations were largely overcome by monitoring dynamic secretome changes in *B. subtilis* and its *bacA* KO mutant. Moreover, protein coverage was greatly improved by employing two different proteomics approaches.
- LC-MS analysis of 12<sup>th</sup>, 16<sup>th</sup> and 24<sup>th</sup> hour secretome samples of *B. subtilis* standard strain PY79 and OGU1 showed that the protein abundance was the highest at 24<sup>th</sup> hour in both strains. When the abundance of total proteins in two strains was compared in terms of OGU1 to PY79 ratio, it corresponded to 1.67, 1.07 and 1.09 in 12<sup>th</sup>, 16<sup>th</sup> and 24<sup>th</sup> hour secretome samples, respectively. Thus, the highest number of OGU1-specific proteins was obtained at 12<sup>th</sup> hour of cultivation.

- The secreted proteins such as various hydrolytic enzymes, carbohydrate-, protein and peptide- as well as nucleic acid- and lipid-degrading ones were upregulated in bacilysin KO mutant, but not in the parental strain at 12<sup>th</sup> hour. The changes in overexpression ratios of these enzymes ranged from 3 to >5. The general increment in the synthesis of macromolecule-degrading enzymes in OGU1 might be regarded as a defense or stress-coping mechanism if the absence of bacilysin is sensed by the mutant as a type of stress condition. Overall, the extracellular proteome of 12<sup>th</sup> hour cultures was not only composed of increased catabolic enzymes, but of proteins with antimicrobial activity, the flagellum-related and phage proteins and those related with coping with stress as the members of extracellular stress regulon SigW in OGU1, all most probably caused by missing bacilysin activity when compared to the parental strain. It is very likely that the loss of bacilysin is interpreted by the producer organism as a kind of stress which then initiates significant alterations in global gene expression patterns, including those of extracellular proteins.
- The secretome samples of 12<sup>th</sup> hour contained a total of 19 differentially expressed cytoplasmic membrane proteins, the majority being significantly overexpressed. Five of these proteins were related with sporulation and germination, 4 with stress, another 4 with transport and 3 with metabolism.
- The secretomes did not only contained extracellular proteins, membrane proteins, wall-associated proteins, all with secretion signals, but also a considerable number of cytoplasmic proteins, as practiced by the *B. subtilis* secretome workers of other laboratories. A total of 63 cytoplasmic proteins were found to be differentially expressed in 12<sup>th</sup>

hour secretome samples and the highest number of differentially expressed cytoplasmic proteins was found at 12<sup>th</sup> hour as compared to 16<sup>th</sup> hour and 24<sup>th</sup> hour secretome samples. Among these, there were 31 proteins belonged to the category of metabolism, 12 engaged in genetic processes such as replication, translation, genetic regulation, protein degradation and DNA packaging, 4 sporulation and germination proteins, 8 proteins for coping with stress, 3 proteins of cellular processes, cell wall metabolism in particular, along with those of unknown functions.

- In 16<sup>th</sup> hour secretomes, as much as 25 out of 46 differentially expressed proteins were overrepresented in OGU1 with respect to the parental strain. Of 46 such proteins, 27 were located in cytoplasm, 5 were in cytoplasmic membrane, 4 were in cell wall and 5 were secreted to extracellular milieu. The location of remaining 5 proteins could not be predicted.
- When differentially expressed extracellular proteins of 16<sup>th</sup> hour are dealt with, NprE, a major extracellular neutral protease B (bacillolysin) of *B. subtilis*, LytE, peptidoglycan endopeptidase involved in cell wall autolysis, and tRNA nuclease WapA were underexpressed by ca. 7-, 2- and 2.7-fold. WapA was 2.8-times overexpressed in 12<sup>th</sup> hour OGU cultures, constituting an example to dynamic alteration of protein expression. The major sporulation-specific extracellular nuclease NucB which is a component of mother cell-specific  $\sigma^E$  regulon and released before the mother cell lysis was as much as 5.14 times upregulated in the secretome of the OGU1 in 16<sup>th</sup> hour. PgdS, the gamma-DL-glutamyl hydrolase that degrades poly-g-glutamate under starvation conditions was 5.1-fold elevated. YoaJ, a protein structurally similar to

plant expansins is known have a synergic action with bacterial hydrolases in *B. subtilis* and leads to an efficient autolysis in wild type cells. With its as high as 11.1 fold elevated levels, was a significantly upregulated cell-wall associated, secreted protein in OGU1. YclQ which is a petrobactin (3,4-catecholcatecholate siderophore) ABC transporter solute-binding protein which acts as an exogenous siderophore was 3.6-fold upregulated in OGU1, probably reflecting an elevated need of the mutant to iron sources.

- Regarding differentially expressed cytosolic proteins at 16<sup>th</sup> hour, some were matching with those identified in our former cytoplasmic proteome studies. However, the present findings were generally contradictory in terms of their relative abundance, i.e. up- or down-regulation. This brought us to the conclusion that the relative abundance of such proteins in secretome samples do not generally provide reliable information about their expression levels since only those leaking out of the cells can be identified. As to 6 distinct proteins with unknown function and/or location, all were upregulated and only one (YdhF) had a secretion signal, belonged to the functional category of sporulation and germination, but its exact function is completely unknown. It was quite interesting that 3 of 6 unknowns are categorized as sporulation and germination proteins. The two unknowns, namely YlaE and YwmD, existed in unusually high abundances; with 117- and 196-fold increased levels with respect to the parental strain. These two proteins certainly deserve priority among the other important proteins and further functional studies must be promptly performed to shed light onto their relation with bacilysin production in *B. subtilis*.

- The number of differentially expressed proteins was quite low, only 12, in 24<sup>th</sup> hour stationary cultures which should have completed their sporulation process by this time. Nine of these proteins were underrepresented in OGU1. Bacilysin completely disappears from culture fluids in such late cultures, thereby accounting for the drastical decrease observed in the number of differentially expressed proteins. Those overrepresented in late OGU1 cultures included nitrate reductase, (NarG), sporulation delaying protein SdpC, a probable deferrochelataase/peroxidase (EfeN) at 11.7, 3.5 and 3.7-fold elevated levels, respectively.
- The gel-based methods which are coupled to MALDI TOF MS analysis are very crucial since they provide valuable information like posttranslational modifications including mass and charge modifications, protein stability and protein-protein interactions, but the limitations of this approach include difficulties with the identification of poorly soluble hydrophobic proteins, membrane proteins in particular, or very acidic or basic, also small and large proteins that do not enter the gel. Thus, as expected, the numbers of total and differentially expressed proteins identified were much lower in 2DE MALDI TOF MS approach, still its results well supported the findings from LC-MS/MS analyses.
- Using 2DE MALDI-TOF MS as an alternative approach, only a total of 16 proteins were found to be differentially expressed in secretome samples. Still, the results supported those obtained with LC-MS/MS analysis. Secretome analysis at 12<sup>th</sup> hour with this technique revealed 6 differentially expressed proteins and all of these except one were secreted proteins. Two of them were phage related, namely; XkdG and

XkdM which were overrepresented in mutant strain. A putative erythromycin esterase, YbfO, and an unknown protein YncM were also overrepresented by nearly 7- and 10-fold, respectively in the OGU1 at 12<sup>th</sup> hour. The cytoplasmic protein aconitase CitB was 2.3- fold overrepresented while flagellar hook-filament junction protein FlgK was underrepresented in OGU1. Another six proteins were found as differentially expressed proteins of 16<sup>th</sup> hour secretome samples. Half of these were unknown or putative proteins namely, YxaL, YwmD and YrpD. Glutamine synthetase GlnA, bacillopeptidase F Bpr and nonspecific endonuclease YhcR were the remaining differentially expressed proteins. Except for YxaL and Bpr, all the proteins were underrepresented in mutant strain. When 24<sup>th</sup> hour secretome samples were analyzed by MALDI-TOF MS, there were only 4 differentially expressed proteins. Two were secreted unknown proteins namely YwmD and YncM, the others being GlnA and Ndh. Except for YwmD, all of the identified proteins were underrepresented in late cultures.

- Our secretome data clearly support our former findings on pleiotropic effects of bacilysin on cellular physiology in *B. subtilis*. There remains a huge work to be completed by taking the parental strain PY79, the Bac- deleted mutant OGU1 and a complemented strain into focus and carrying out phenotypic analyses, transcript analyses as well as several DNA-binding experiments (EMSA). The causality relationship between bacilysin and the cellular functions reported herein can also be cross-checked by external addition of pure bacilysin to the cultures of OGU1 and re-determining the levels of at least certain key proteins.

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## APPENDIX A

### COMPOSITIONS AND PREPERATION OF CULTURE MEDIA

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

$\text{KH}_2\text{PO}_4$	1 g/L
KCl	0.2 g/L
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}^*$	0.5 g/L
Glutamate.Na.H <sub>2</sub> O	4 g/L
Sucrose*	10 g/L
Ferric citrate**	0.15 g/L
Trace elements**	1 ml
$\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$	0.0001 g/L
Ammonium molybdate	0.0001 g/L
$\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$	0.001 g/L
$\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$	0.0001 g/L
$\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$	0.00001 g/L

\*Autoclave separately

\*\*Filter sterilization

### **Luria Bertani (LB) Medium (1000ml)**

Tryptone 10 g/L

Yeast Extract 5 g/L

NaCl 5 g/L

Distilled H<sub>2</sub>O was added up to 1000ml and then autoclaved for 15 minutes.

### **Luria Bertani (LB) Agar Medium (1000 ml)**

Tryptone 10 g/L

Yeast Extract 5 g/L

NaCl<sub>2</sub> 5 g/L

Agar 15 g/L

## APPENDIX B

### BUFFERS AND STOCK SOLUTIONS

#### SDS-PAGE SOLUTIONS

Acrylamide/BisAcrylamide	146 g
N,N'-Methylene-bis Acrylamide	4 g
Distilled water to 500 mL	
Filtered and stored at 4 C. Protected form light.	

#### Tris HCl (1.5 M)

Tris base	54.45 g
dH <sub>2</sub> O	150 ml
pH is adjusted to 8.8 with HCl, distilled water to 300 mL and stored at 4°C.	

#### Tris HCl (0.5 M)

Tris base	6 g
dH <sub>2</sub> O	60 ml
pH is adjusted to 6.8 with HCl, distilled water to 100 mL and stored at 4°C.	

### **Tris-EDTA Buffer (TE)**

Tris 10 mM

EDTA 1 mM

pH is adjusted to 8.0 with HCl.

### **Running Buffer (5X)**

Tris base 15 g

Glycine 72 g

SDS 5 g

Distilled water to 1 L and stored at 4°C.

### **Sample Buffer**

dH<sub>2</sub>O 3 mL

Tris HCl (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) (in water) 0.4 mL

### **Bradford Reagent 5X**

%85 H3PO4	100 ml
Methanol	50 ml
Comassie blue G	100 mg
dH2O	up to 200 ml

### **Rehydration Buffer**

Urea	8 M
Thiourea	2 M
Ampholite	1%
DTT	50 mM
CHAPS	2% m/v
dH2O	up to 10 ml

## **Equilibration Buffers:**

### **Solution I**

Tris-HCl, pH 6.8	50 mM
Urea	6 M
Glycerol	30% (v/v)
SDS	1%
DTT (freshly added)	2%

### **Solution II**

Tris-HCl, pH 6.8	50 mM
Urea	6 M
Glycerol	30% (v/v)
SDS	1%
Iodoacetamide (IAA) (freshly added)	2.5%
Bromophenol blue (freshly added)	3.5 $\mu$ M

## APPENDIX C

### CHEMICALS AND ENZYMES

<b>Chemicals</b>	<b>Supplier</b>
Acetic acid	Merck
Acetone	Merck
Acrylamide	Sigma
Ammonium sulfate	Merck
Ampholytes pH (3-10)	Fluka
$\beta$ - mercaptoethanol	Merck
Bis-acryamide	Sigma
Bovine Serum Albumin (BSA)	Sigma
Bromophenol blue	Merck
CaCl <sub>2</sub> H <sub>2</sub> O	Merck
CHAPS	Merck
Comassie Brilliant Blue G 250	Sigma
DTT	Fluka
EDTA	Sigma
Ethanol	Merck
Glucose	Merck

Glycerol	Merck
Glycine	Merck
HCl	Merck
IPG strips	BioRad
KH <sub>2</sub> PO <sub>4</sub>	Merck
Methanol	Merck
MgSO <sub>4</sub> 7H <sub>2</sub> O	Merck
NaOH	Merck
NH <sub>4</sub> Cl	Merck
NH <sub>4</sub> HCO <sub>3</sub>	Applichem
Ortho-phosphoric acid	Merck
Phenylmethylsulfonyl fluoride (PMSF)	Merck
SDS	Sigma
TEMED	Sigma
Thiamine	Sigma
Thiourea	Fluka
Trichloroacetic acid (TCA)	Merck
Trifluoroacetic acid (TFA)	Merck
Tris- HCl	Sigma
Urea	Merck

## CURRICULUM VITAE

### PERSONAL INFORMATION

Surname, Name: Tekin İşlerel, Elif

Nationality: Turkish (TC)

Date and Place of Birth: 7 June 1985, Akçakoca/Düzce

Marital Status: Married

Phone: +90 312 210 51 90

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### EDUCATION

1996- 2003 Düzce Arsal Anatolian High School, Düzce, Türkiye

GPA 4.58/5

2003-2008 B. Sc. Degree: Biology Department, METU, Ankara, Türkiye

GPA 3.58 /4, High honor student

2008-2011 M.Sc. Degree: Biology Department, METU, Ankara, Türkiye

GPA 3.79 /4, Course Performance Award

2011- 2017                      Ph.D. Degree: Biology Department, METU, Ankara,  
Türkiye  
  
GPA 3.92/4

**M.Sc. Thesis topic:** Dynamic Expression of Three “RNA Processing and modification” Genes of *Phanerochaete chrysosporium* under Pb Stress

**PhD Thesis topic:** Dynamic Analysis of Secretome Alterations in a Bacilysin-Knockout Mutant of *Bacillus subtilis*

## **PRACTICES**

Formal Intern                      : Full-time (1 month), Department of Molecular Biology and PCR, Düzen Laboratories  
  
Voluntary Intern                    : Full-time (2 month), Molecular Microbiology Laboratory Department of Biology, METU

## **WORK EXPERIENCE**

Research Assistant    : Full-time (2009- 2017)  
  
Department of Biological Sciences, Middle East Technical University  
  
BIOL 355 Microbiology Laboratory (fall semester)  
  
BIOL 352 Genetics Laboratory (spring semester)

## **FOREIGN LANGUAGES**

English In Elementary School, High School, Univesity

Fluent, Upper-Intermediate

German

In High School, as a second foreign language

Beginner

French

University, taken as a course

Beginner

## **COMPUTER KNOWLEDGE**

MS Office (Word, Excel, Powerpoint)

MiniTAB

SPSS

## **PROJECTS**

METU-BAP-07.02.2009.00.01 (2008-2010): Functional genomics of heavy metal stress response in *Phanerochaete chrysosporium* (Özcengiz, G., Tekin, E.)

METU-BAP-07-02-2014-007-332 (2014-2015): Analysis of secretome alteration in a bacilysin blocked mutant of *Bacillus subtilis* (Özcengiz, G., Tekin, E.)

Tübitak- KBAG-116Z351- (2017-2018) Dynamic comparative secretome analysis of *Bacillus subtilis* standard strain (PY79) and its bacilysin blocked mutant (OGU1)

## **PUBLICATIONS**

### PAPERS (International, SCI):

Tekin, E., İçgen, B., Özcengiz, G. 2013. Real-Time PCR Investigation of the dynamic expression of three “RNA Processing and Modification” genes of *Phanerochaete chrysosporium* exposed to lead. *Bull Environ Contam Toxicol.* 90: 27–33.

### CONGRESS ABSTRACTS, WORKSHOPS, SEMINARS:

Tekin, E., İçgen, B., Özcengiz, G. (2009). Time-dependent effect of Lead (Pb) on the expression of genes coding for Polyadenylate binding protein, ATP-dependent RNA Helicase and Splicing factor RNPS1. XVI. National Biotechnology Congress (13-16 December 2009, Antalya) Abstract Book, page: 30.

Tekin İşlerel, E., Özcengiz G., (2017). Dynamic comparative secretome analysis of *Bacillus subtilis* PY79 and its bacilysin blocked mutant. 19th

International Conference on Bacilli & Gram-Positive Bacteria (11-15 June, Berlin)

Özcengiz G., Aras Taşkın, A., Demir M., Tekin İşlerel, E., Yılmaz, Ç., İrigül Sönmez, Ö., Yazgan Karataş, A. (2017). Bacilysin is a small, fine-tuning effector of mother cell compartment-specific  $\sigma E$  and  $\sigma K$  regulons in *Bacillus subtilis*. 19th International Conference on Bacilli & Gram-Positive Bacteria (11-15 June, Berlin)

Proteomics Bioinformatics Workshop during the X Annual Congress of the European Proteomics Association (22-25 June 2016, İstanbul, Türkiye)

## **CERTIFICATES**

Certificate of IV. Basic Cell Culture and Stem Cell Course, 2011, GMMA Center of Research and Development, Ankara, Türkiye

Certificate of Animal Use in Experimental Research Course, 2011, GMMA Center of Research and Development, Ankara, Türkiye

Certificate of IV. Basic Biostatistics Course, 2011, GMMA Center of Research and Development, Ankara, Türkiye

Certificate of Participation, Horizons in Molecular Biology and Genetics Symposium, 2009, Bilkent University, Ankara, Türkiye

Certificate of Participation, International Symposium on Biotechnology: Developments and Trends, 2009, METU, Ankara, Türkiye