PROTEOME-WIDE ANALYSIS OF THE FUNCTIONAL ROLES OF BACILYSIN BIOSYNTHESIS IN *BACILLUS SUBTILIS*

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ABSTRACT

PROTEOME-WIDE ANALYSIS OF THE FUNCTIONAL ROLES OF BACILYSIN BIOSYNTHESIS IN *BACILLUS SUBTILIS*

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The members of the genus Bacillus produce a wide variety of secondary metabolites with antimetabolic and pharmacological activities. Most of these metabolites are small peptides that have unusual components and chemical bonds and synthesized nonribosomally by multifunctional enzyme complexes called peptide synthetases. Bacilysin, being produced and excreted by certain strains of *Bacillus subtilis*, is one of the simplest peptide antibiotics known. It is a dipeptide with an N-terminal L-alanine and an unusual amino acid, L-anticapsin, at its C-terminal. Recently, *ywfBCDEF* operon of *B. subtilis* 168 was shown to carry bacilysin biosynthesis function, the genes of this operon were renamed as *bacABCDE*. The first member of *bac* operon, *bacA* gene was proved to encode the function of L-alanine –L-anticapsin amino acid ligation. Bacilysin production is regulated at different levels, negatively by GTP via the transcriptional regulator CodY and AbrB while positive regulation occurs by guanosine 5'-diphosphate 3'-diphosphate (ppGpp) as well as a quorum-sensing mechanism through the peptide pheromone PhrC. This study aims to identify the functional role of bacilysin biosynthesis in the regulatory cascade operating in *B.*

subtilis by employing proteome-wide analysis of the bacilysin producer *B. subtilis* PY79 and its bacilysin nonproducer derivative PY79 *ΔbacA::lacZ::erm* which was recently constructed by our group. The identification of a total of 128 proteins which are differentially expressed in wild type PY79 and *bacA* inactive strains provided us with the knowledge of interaction of bacilysin biosynthesis with global regulatory pathways and the understanding of the effects of antibiotic production on the expression of the genes with unknown functions in *B. subtilis*.

Keywords: *Bacillus subtilis*; Quorum-sensing; Bacilysin; Comparative Proteomics

ÖZ

BACİLLUS SUBTİLİS' DE BASİLİSİN BİYOSENTEZİNİN FONKSİYONEL ROLÜNÜN PROTEOM ÖLÇEKLİ ANALİZİ

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Bacillus türleri, antimetabolik ve farmakolojik aktiviteye sahip çok çeşitli ikincil metabolitler üretirler. Bu metabolitlerin çoğu, küçük peptidler olup peptid sentetazlar adı verilen multifonksiyonel enzim kompleksleri tarafından ribozomal olmayan bir biçimde sentezlenirler. *Bacillus subtilis*' in bazı suşları tarafından sentezlenen basilisin L-alanin ve L-antikapsinden oluşmuş bir dipeptiddir. *B. subtilis* 168'in *ywfBCDEF* operonunun basilisin biyosentetik fonksiyonlar taşıdığı gösterilmiş ve ilgili operon *bacABCDE* olarak yeniden adlandırılmıştır. Bu operon içerisinde yer alan bacA geninin ise amino asid ligasyonundan sorumlu basilisin sentetaz olduğu doğrulanmıştır. Basilisin biyosentezi, farklı düzeylerde regüle edilir. Negatif regülasyon GTP tarafından transkripsiyonel regülatör CodY ve AbrB üzerinden gerçekleştirilir. Pozitif regülasyon ise hem ppGpp (guanozin 5' difosfat 3'-difosfat) ile, hem de peptid feromon PhrC yolu ile hücre yoğunluğu sinyali mekanizması aracılığıyla gerçekleştirilir. Bu çalışmada basilisin üretici *B. subtilis* PY79 suşu ve onun basilisin üretemeyen ve araştırma grubumuzca oluşturulmuş *B. subtilis* PY79 *ΔbacA::lacZ::erm* isimli türevi kullanılarak bu suşlarda karşılaştırmalı proteom

analizleri yapılması ve basilisinin *B. subtilis*' deki fonksiyonel rolünün ortaya çıkarılması amaçlanmıştır. Bu iki suşta farklı ifade edilen toplam 128 proteinin tanımlanması, basilisin biyosentezinin global regülatör yollarla olan etkileşimi hakkında ipuçları vermiş ve dipeptid antibiyotik basilisinin fonksiyonu bilinmeyen genlerin ekspresyonu üzerindeki etkilerinin anlaşılmasına olanak sağlamıştır.

Anahtar kelimeler: *Bacillus subtilis*; Hücre yoğunluğu sinyali; Basilisin; Proteom

To My Grandfather

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LIST OF ABBREVIATIONS

aa	: Amino acid
bp(s)	: Base pair(s)
CBB	: Coomassie Brilliant Blue
EDTA	: Ethylenediaminetetraacetic Acid
IEF	: Isoelectric Focusing
IPG	: Immobilized pH Gradients
kb	: Kilobase
lacZ	: β-galactosidase
MALDI	: Matrix-Assisted Laser Desorption/Ionization
MS	: Mass Spectrometry
ORF	: Open Reading Frame
E. coli	: *Escherichia coli*
OD	: Optical Density
2D-PAGE	: Two-Dimensional Polyacrylamide Gel Electrophoresis
TOF	: Time of Flight

CHAPTER 1

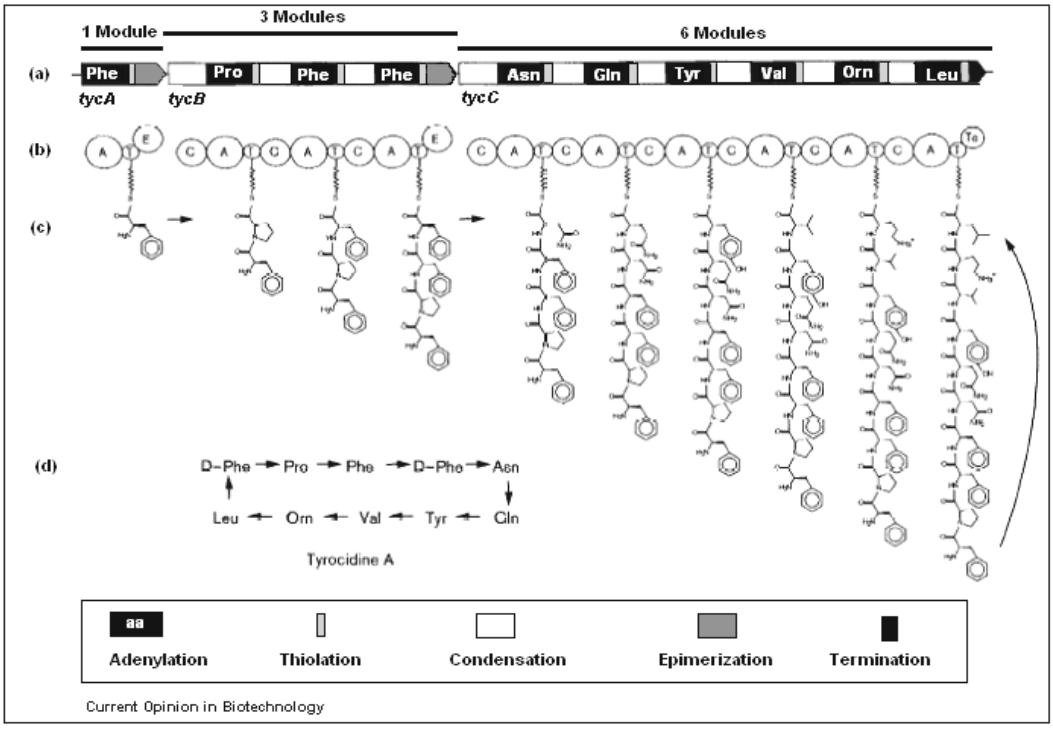
INTRODUCTION

1.1. Nonribosomal Peptide Synthesis

Microbially-produced peptides are among the most biologically active groups of compounds known. Among the microbial peptides, peptide antibiotics are currently the most important ones as they are used for antimicrobial and antitumor therapy, against plant pathogens, as immunosuppressive and cytostatic drugs, for promotion of animal growth and seed germination (Katz and Demain, 1977; Demain, 1980; Nakano and Zuber, 1990; Demain, 1992; Devine, 1995; Boman, 1995; Pichardal *et al.*, 1995; Boman, 1996; Gill *et al.*, 1996).

Two mechanisms have been identified as biosynthetic pathways for the bioactive peptides. The multicyclic lantibiotics, which contain the thioether amino acid lantihionine, for example, are synthesized ribosomally from gene-encoded peptide precursors, which are then modified by complex posttranslational processing (Schnell *et al*., 1988; Zuber *et al*., 1992). On the other hand, a large number of therapeutically useful cyclic and linear peptides synthesized via a template-directed, nucleic-acid-independent nonribosomal mechanism (Weber and Marahiel, 2001). These pharmaceutically important peptides are synthesized as secondary metabolites on large multifunctional enzymes in *Actinomycetes*, *Bacilli* and filamentous fungi, including antibiotics like penicillin and vancomycin, immunosuppressive agents like cyclosporin A, cytostatic agents like epothilone and antiviral, antitumor, biosurfactant compounds (Mootz and Marahiel, 1997; Mootz and Marahiel, 1999; Schwarzer and Marahiel, 2001; Schwarzer *et al*., 2002; Sieber *et al*., 2002).

These secondary metabolite peptides may be composed of linear, cyclic, or branched peptide chains and contain unique structural features, such as D-amino acids, N-terminally attached fatty acid chains, N- and C-methylated residues, N-formylated residues, heterocyclic elements, and glycosylated amino acids, as well as phosphorylated residues. Moreover, in contrast to proteins produced by ribosomal synthesis, small peptide products contain not only the common 20 amino acids but also hundreds of different building blocks, suggesting a nonribosomal origin of biosynthesis (Marahiel *et al*., 1997; Sieber and Marahiel, 2005; Grünewald and Marahiel, 2006). Also in contrast to the structural diversity of the products, these secondary metabolites share a common mode of synthesis, the so-called "multiple carrier thio-template mechanism". According to this model (Fig. 1.1), peptide bond formation takes place on multienzymes designated as peptide synthetases, on which amino acid substrates are first activated by ATP hydrolysis to the corresponding adenylate. This unstable intermediate is subsequently transferred to another site of the multienzyme where it is bound as a thioester to the cysteamine group of an enzyme-bound 4'-phosphopantetheinyl (4'-PP) cofactor (Marahiel *et al.*, 1997; Mootz *et al.*, 2002; Sieber and Marahiel, 2005; Grünewald and Marahiel, 2006). According to the present multiple carrier model of nonribosomal peptide synthesis, nonribosomal peptide synthetases (NRPSs) are composed of repetitive units called as modules, each about 1.000 – 1.500 amino acids in length, which are capable of incorporating one amino acid constituent at a time into peptide chain (Mootz and Marahiel, 1997; Schwarzer and Marahiel, 2001; Kallow *et al*., 2002). The number and the order of modules within a NRPS match the number and sequence of amino acids incorporated into the peptide that's why these enzymes have also been called "protein templates" (von Döhren *et al*., 1999; Schwarzer and Marahiel, 2001; Weber and Marahiel, 2001).

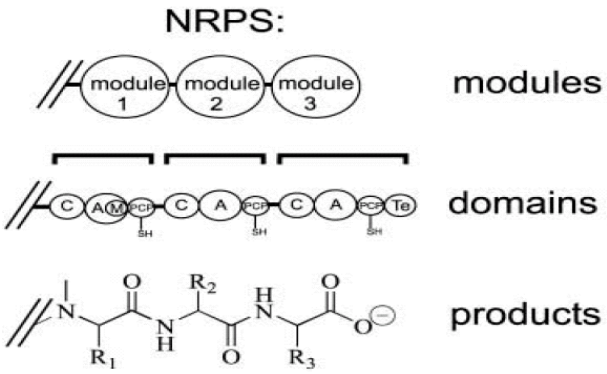


(Taken from Mootz and Marahiel, 1999).

Figure 1.1. The multiple carrier thiotemplate mechanism illustrated with the example of tyrocidine A synthesis (Mootz and Marahiel, 1997). Three peptide synthetases; (a) encoded by the genes tycA, tycB, and tycC, act in concert for the stepwise assembly of the cyclic decapeptide. (b) The substrates are recognized and adenylated with the consumption of ATP by the action of the A-domains and subsequently transferred to a thioester linkage on the cofactor 4'-phosphopantetheine (shown as a zigzagged line) of the T-domain, (c) C-domains then catalyze the condensation with the aminoacyl- or peptidyl-moieties on the neighbouring modules. At positions one and four, an epimerization domain converts L-Phe into its stereochemical isomer. (d) A thioesterase-like domain is believed to act as a cyclase to give the final product (Mootz and Marahiel, 1999).

The modules can be subdivided into domains, each responsible for catalyzing the three basic reactions: substrate recognition, activation as acyl adenylate, and covalent binding as thioester. These enzymatic activities are embedded in distinct catalytic domains with highly conserved core motifs within the module (Figure 1.2) (Linne *et al.*, 2001; Schwarzer and Marahiel, 2001). Three domains are necessary as the basic equipment of

a NRPS elongation module: an adenylation (A) - domain that selects the substrate amino acid and activates it as amino acyl adenylate; a peptidyl carrier protein (PCP)-domain that binds the co-factor 4'-phosphopantetheine (4'-PP) to which the activated amino acid is covalently attached; and a condensation (C)-domain that catalyzes peptide bond formation. These domains can therefore be described as the "toolbox" of NRPS (Konz and Marahiel, 1999; Schwarzer and Marahiel, 2001).



(Taken from: Schwarzer and Marahiel, 2001)

Figure 1.2. From modules to products: the modules of NRPS can be subdivided into domains that catalyze the single enzymatic reactions. The composition of the products is determined by the assembly of active domains found in the corresponding modules (Schwarzer and Marahiel, 2001).

1.2. *Bacillus subtilis*

Bacillus subtilis is one of the best-studied organisms in the nature and stands after *Escherichia coli* as the second among prokaryotes, in the level of detail at which it is understood. The level of understanding has made the bacterium the central source of physiological Gram-positive bacteria analysis, and of bacterial differentiation. *Bacillus*

spp were intensively studied by the mid-20th century because of its role in human, animal and insect infections and the production of many important products such as antibiotics, it also presents an important source of industrial enzymes, e.g. amylases and proteases, and other useful products (Sonenshein *et al*., 2002).

B. subtilis is commonly found in soil, water sources and in association with plants. One of the main characteristics of life in the soil and important implications for the organism's physiology is the tendency to a 'fast or feast' existence (Harwood and Cutting, 1990).

Additionally, *B. subtilis* is a chemoorganotroph, so that it is able to maintain a suitable environment containing factors it demands for its growth by simply oxidizing organic compounds belonging to a broad range of family. Moreover, just like many other members of its genus, *B. subtilis* is mesophilic and may undergo growth and production of normal-sized colonies within a day when placed at 37^{o}C. Another important characteristic of this organism is its being anaerobe and therefore it requires sufficient aeration during growth (Harwood *et al*., 1990).

B. subtilis stops growing under nutritional starvation and starts responses to restore grow by increasing metabolic diversity. The responses include the induction of motility and chemotaxis, and the production of hydrolases (proteases and carbohydrases) and antibiotics. The cells are induced to form chemical-, irradiation- and desiccation-resistant endospores when the responses fail to re-establish growth. The first morphological indication of sporulation is division of the cell into a smaller forespore and a larger mother cell, each with an entire copy of the chromosome. The former is engulfed by the latter and differential expression of their respective genomes, coupled to a complex network of interconnected regulatory pathways and developmental checkpoints, culminates in the programmed death and lysis of the mother cell and release of the mature spore (Stragier and Losick, 1996). *B. subtilis* can also differentiate

into a physiological state, the competent state, which allows it to undergo genetic transformation in an alternative developmental process (Solomon and Grossman, 1996).

B. subtilis has a genome of 4.2 Mb in size (Franguel *et al*., 1999). Its genome sequence was completed in 1997 by an international collaboration (Kunst *et al*., 1997). It is now known that *B. subtilis* uses 275 genes, 25 of which are unknown, in order to grow in a rich medium at moderate temperatures and in an aerated environment (Kobayashi and Osagura, 2002). Its genome also consists of 17 sigma factors and approximately 250 DNA binding transcriptional regulators. In addition to these, 4106 protein-coding, 86 tRNA, 30 rRNA and 3 small stable RNA genes are harbored (Ando, 2002; Kobayashi and Osagura, 2002).

In 1947, Burkholder and Giles reported that they isolated many auxotrophic mutants of *B. subtilis*, one of which is a tryptophan requiring strain called BGSC1A1, or *B. subtilis* 168. Subsequently, in 1958, transformable characteristic of this strain was reported and upon this information, *B. subtilis* 168 has become the most useful and a commonly used strain for genetic researches based on this organism (Spizen, 1958; Harwood *et al*., 1990). *B. subtilis* PY79 has also found its place as a wild type strain as being a prototrophic derivative of *Bacillus subtilis* 168.

1.3. Microbially Synthesized Bioactive Peptides by the Genus *Bacillus*

Members of genus *Bacillus* produce a large number of antibiotics as listed in Table 1.1. *B. subtilis* is able to produce more than two dozen antibiotics with an amazing variety of chemical structures. They exhibit highly rigid, hydrophobic and/or cyclic structures with unusual constituents like D-amino acids and are generally resistant to hydrolysis by peptidases and proteases. Additionally, cysteine residues are either oxidized to disulphides and/or are modified to characteristic intramolecular C–S (thioether) linkages, and as a result the peptide antibiotics are insensitive to oxidation. The anti-

microbially active compounds produced include predominantly peptides that are either ribosomally synthesized and post-translationally modified (lantibiotics and lantibiotic-like peptides) or non- ribosomally generated, as well as a couple of non-peptidic compounds. Recent findings assigned extra roles for distinct *B. subtilis* antibiotics beyond the 'pure' anti-microbial action: Non-ribosomally produced lipopeptides are involved in biofilm and swarming development, lantibiotics function as pheromones in quorum-sensing, and a 'killing factor' effectuates programmed cell death in sister cells (Stein, 2005).

Table 1.1. Bioactive peptides synthesized by *Bacillus* species

Peptide	**Organism**	**Structure**
Bacilysin	*B. subtilis*	Linear
Edeine	*B. brevis* Vm4	Linear
Gramicidin	*B. brevis*	Linear
Iturin	*B. subtilis*	Cyclopeptide
Gramicidin S	*B. braves* ATCC 9999	Cyclopeptide
Tyrocidine	*B. brevis* ATCC 8185	Cyclopeptide
Mycobacillin	*B. subtilis*	Cyclopeptide
Surfactin	*B. subtilis*	Lacton
Polymyxin	*B. polymyxa*	Polypeptide
Bacitracin	*B. licheniformis*	Polypeptide
Bacilysocin	*B. polymyxa*	Phospholipid

1.4. Global Regulation of Gene Expression by Quorum- Sensing and Two-Component Systems in *B. subtilis*

Cell-cell signaling is utilized by many types of cells to regulate gene expression and development. One form of cell-cell signaling involves a regulatory response to cell density signals. This process, sometimes called quorum sensing (Fuqua *et al*., 1994), is typically characterized by regulatory events that are induced as cells grow to high cell density. A variety of chemicals, including acyl homoserine lactones, peptides, and amino acids, are used for microbial cell-cell signaling to regulate many biological processes, including genetic exchange, development, virulence, bioluminescence, and production of antibiotics (Solomon *et al*., 1996). Even though a vast number of diverse quorum-sensing systems exist, they can be divided into two established paradigms that regulate the intraspecific behavior in many bacteria: (i) LuxI/LuxR-type quorum-sensing systems in Gram-negative bacteria responsible for the production of *N*-acyl-L-homoserine lactone autoinducers or type I autoinducers and (ii) oligopeptide/two component-type quorum-sensing circuits in Gram-positive bacteria responsible for the production of autoinducer peptides (Lazazzera *et al*., 1997; Perego, 1997; Bacon Shneider *et al*., 2002; Fuqua and Greenberg, 2002; Jian *et al*., 2002). As examples in Gram-negative bacteria; *Pseudomonas aeroginosa* use quorum sensing for biofilm formation and virulence, *Vibrio fischeri* and *V. harveyi* for bioluminescence, *Erwinia carotovora* for antibiotic production, *Argobacterium tumefaciens* for plasmid conjugation, *Rhizobium leguminosarum* for root nodule formation, and so on. In case of Gram-positive bacteria, via quorum sensing phenomenon, *Staphylococcus aureus* and *Enterococcus faecalis* develop virulence, *Streptococcus pneumoniae* and *B. subtilis* show competence and additionally *B. subtilis* gets induced for sporulation and antibiotic biosynthesis (Sturme *et al*., 2002; Taga *et al*., 2003).

Signal transduction in prokaryotes is mainly carried out by so-called two-component systems consisting of a histidine protein kinase and a response regulator. The kinase acts

as a sensor of a specific signal and upon binding, activates itself by autophosphorylation on a histidine residue. The phosphoryl group is subsequently transferred to a paired response regulator, thus activating its function, generally of transcription a regulation, allowing the cells to respond and adapt to the specific signal (Hoch and Silhavy, 1995).

In *B. subtilis*, several processes are known to be regulated by extracellular peptide signaling, including the initiation of genetic competence, sporulation, production of degradative enzymes and exopolysaccharides and antibiotic synthesis (Magnuson *et al*., 1994; Solomon *et al*., 1996; Comella and Grossman, 2005), adaptation to environmental stress (Darmon *et al*., 2002), the production of secondary metabolites (Martin, 2004), and cell division (Fukuchi *et al*., 2000). Three types of secreted peptide signaling molecules have been identified: a modified 5- to 10-amino-acid peptide, ComX, that interacts extracellularly with its receptor; lantibiotic peptides (Magnuson *et al*., 1994; Piazza *et al*., 1999; Tortosa *et al*., 2001), such as subtilin, which interact extracellularly with their receptors; and unmodified pentapeptides, known as Phr peptides, that are internalized to inhibit the activity of their target proteins, known as Rap proteins (Lazazzera, 2001; Perego and Brannigan, 2001; Stein, 2005).

B. subtilis encodes a family of 8 Phr peptides (PhrA, PhrC, PhrE, PhrF, PhrG, PhrH, PhrI, and PhrK) and a family of 11 Rap proteins (RapA to RapK) (Table 1.1). Each Phr peptide is encoded in an operon with a Rap protein, and each characterized Phr inhibits the activity of its cotranscribed Rap (Perego and Hoch, 1996; Solomon *et al*., 1996; Kunst *et al*., 1997; Jiang *et al*., 2000; Ogura *et al*., 2003; Hayashi, 2006). The PhrC peptide [also known as competence-and sporulation-stimulating factor (CSF)] also inhibits the activity of an unpaired Rap protein, RapB (Perego, 1997). It is possible that the other unpaired Rap proteins are also inhibited by noncognate Phr peptides (Auchtung *et al*., 2006).

In addition to expression from the upstream *rap* promoter, most *phr* genes are also expressed from a promoter upstream of *phr* that is recognized by RNA polymerase containing the alternative sigma factor, σ^H. This regulation by σ^H causes the level of each *phr* gene to increase as cells transition from exponential growth to stationary phase (Lazezzera *et al*., 1999; McQuade *et al*., 2001).

Table 1. 2. Processes regulated by Rap proteins and Phr peptides in *B. subtilis*. (Auchtung *et al*., 2006).

Rap protein	**Phr peptide**	**Target(s) of Rap**	**Mechanism of Rap**	**Responses regulated by target protein(s)**
RapA	PhrA	Spo0F~P	Stimulates autodephosphorylation	Activates post exponential-phase gene exp. and sporulation indirectly through Spo0A
RapB	PhrC	Spo0F~P	Stimulates autodephosphorylation	Activates post-exponential phase gene exp. and sporulation indirectly through Spo0A
RapC	PhrC	ComA	Inhibits binding of ComA to DNA	Activates exp. of genes involved in production of degradative enzymes, antibiotics, and competence
RapD		Unkown	Unknown	
RapE	PhrE	Spo0F~P	Stimulates autodephosphorylation	Act. post exponential-phase gene exp. and sporulation indirectly through Spo0A
RapF	PhrF	ComA	Inhibits binding of ComA to DNA	Activates exp. of genes involved in production of degradative enzymes, antibiotics, and competence
RapG	PhrG	DegU, ComA	Inhibits binding of DegU to DNA, unknown	Activates expression of genes involved in competence and production of degradative enzymes, and antibiotics
RapH	PhrH	ComA, DegU	Unknown	Activates exp. of genes involved in competence and production of degradative enzymes, and antibiotics

Table 1.2. Continued

	PhrI	Unknown	Unknown	RapI stimulates gene expression, excision, and transfer of ICEBs1
RapJ		Unknown	Unknown	
RapK	PhrK	ComA	Unknown	Activates exp. of genes involved in production of degradative enzymes, antibiotics, and competence

The primary *phr* gene products are pre-Phr peptides that are 38 to 57 amino acids in length. Pre-Phr peptides are exported and cleaved to form the mature Phr pentapeptides (Lazazzera, 2001; Perego and Brannigan, 2001). The oligopeptide permease (Opp), an ATP-binding cassette (ABC) transporter that imports small peptides (Perego *et al*., 1991; Rudner *et al*., 1991), transports the Phr peptides into the cell, where they can inhibit the activities of Rap proteins (Lazazzera *et al*., 1997; Perego, 1997).

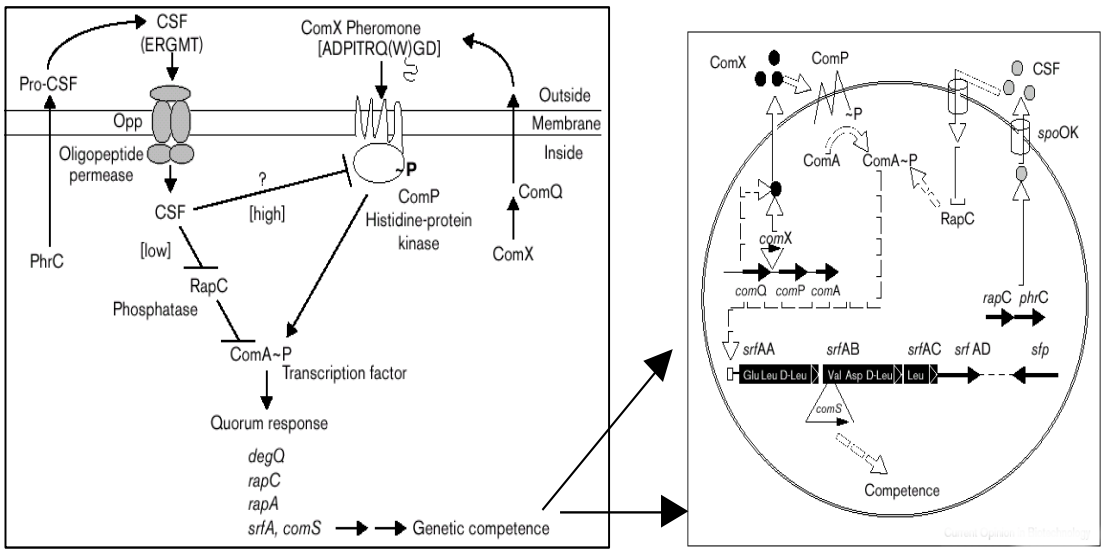
Two peptide pheromones, ComX pheromone and CSF (PhrC), accumulate during exponential growth and stimulate the development of genetic competence and expression of several genes involved in other processes (Fig. 1.3). The extracellular and intracellular mechanisms of peptide signaling are represented by ComX pheromone and CSF (PhrC), respectively (Lazazzera and Grossman, 1998).

ComX pheromone is a 10-amino-acid peptide (ADPITRQWGD) with a hydrophobic modification of unknown structure on the tryptophan residue. Two genes, *comQ* and *comX*, are known to be required for ComX pheromone production (Magnuson *et al*., 1994). *comX* encodes the 55-amino-acid precursor of ComX pheromone. *comQ* is located immediately upstream of *comX* in the chromosome (Weinrauch *et al*., 1991),

and *comQ* null mutants do not produce ComX pheromone (Magnuson *et al*., 1994). Production of ComX pheromone requires processing of the 55-amino-acid precursor to 10 amino acids, modification of the tryptophan residue, and export from the cell. ComQ may be involved in the processing and/or modification step (Lazazzera *et al*., 1999). ComX stimulates the activity of the membrane bound receptor histidine kinase ComP. ComP has eight putative membrane-spanning helices and appears to be the direct receptor for ComX pheromone. The hydrophobic modification on the pheromone is required for function and may help to increase the local concentration of ComX pheromone at the membrane where it can interact with and activate ComP. Autophosphorylated ComP donates phosphate to the response regulator ComA . The phosphorylated form of ComA activates expression of several genes, including *comS* (also known as *srfA*), which is the only ComX–ComP–ComA-controlled gene required for competence development (Lazazzera and Grossman, 1998; Core and Perego, 2003).

CSF (also known as PhrC) is a 5-amino-acid peptide (ERGMT) that contributes to the activation of ComA by inhibiting the activity of the regulator RapC (Solomon *et al*., 1996). Very recently, it was shown that also PhrF and PhrK stimulate ComA's activity by directly inhibiting the activity of their cognate proteins RapF and RapK Bongiorni *et al*., 2005; Auchtung *et al*., 2006). Auchtung and his co-workers also shown that PhrC, PhrF, and PhrK stimulate ComA-dependent gene expression to different magnitudes and that all three peptides are reguired for full expression of ComA-dependent genes.

The function of CSF in regulating ComA activity is more complicated than that of ComX pheromone. CSF is a secreted, diffusible peptide (Solomon *et al*., 1996). When CSF reaches a critical concentration, it is transported back into the cell by an oligopeptide permease Opp (also known as Spo0K). It then appears to bind to two different intracellular receptors to modulate the activity of the ComA transcription factor (Fig. 1.3) (Lazezzera and Grossman 1998; Perego, 1997).



(Taken from: Mootz and Marahiel, 1997)

Figure 1. 3. A model for two extracellular signaling peptides mediating the quorum response in *B. subtilis*.

At low concentrations (1–5 nM), competence-and sporulation-stimulating factor (CSF) stimulates the activity of ComA apparently by inhibiting the activity of an aspartylphosphate phosphatase, RapC (Fig. 1.3) (Solomon *et al*., 1996). At higher concentrations (>20 nM), CSF interacts with an as yet unidentified receptor, possibly the histidine-protein kinase ComP, to inhibit the expression of ComA-controlled genes (Fig. 1.3) (Lazezzera *et al*., 1997). In addition to these two functions, CSF, at high concentrations, also stimulates sporulation apparently by inhibiting the activity of an alternate aspartyl-phosphate phosphatase, RapB (Solomon *et al*., 1996; Lazezzera *et al*., 1997; Pereggo, 1997).

Production of mature CFS involves several steps, starting with tanscription and translation of *phrC*, the gene encoding the precursor of CSF (Solomon *et al*., 1996). The 40-amino acid primary product of *phrC* has a signal sequence and putative peptidase cleavage sites, indicating that an 11-to 25-amino acid peptide is exported (Perego *et al*, 1996). Lazezzera and coverkers (1999) found that transcription of the *rapC phrC* operon

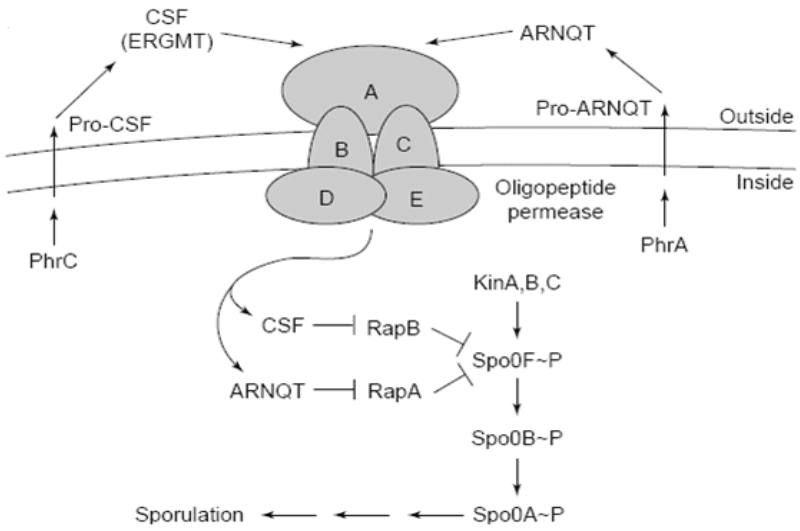
activated by high cell density through ComA~P and that *rapC* and *phrC* regulate their own expression. RapC, by negatively regulating ComA~P, is a part of this homeostatic autoregulatory loop. PhrC (CSF) stimulates ComA activity and positively regulates its own expression. Furthermore, they showed that by mid-exponential phase, CSF is at concentrations that stimulate competence gene expression and that as cells enter stationary phase, the extracellular concentration of CSF reaches levels approaching 100 nM, concentrations that are known to stimulate sporulation and inhibit early competence gene expression.

The ability of CSF to stimulate sporulation involves a similar mechanism to that by which CSF stimulates competence (Fig. 1.4). It requires the phosphatase RapB. RapB dephosphorylates Spo0F~ P (Perego *et al*., 1994) and Spo0F is part of the phospho-transfer pathway that donates phosphate to the transcription factor Spo0A, which is required for the initiation of sporulation (Grossman, 1995; Hoch, 1993). The phosphatase activity of RapB is inhibited *in vitro* by CSF, indicating that CSF stimulates sporulation *in vivo* by directly inhibiting RapB (Perego, 1997).

It is clear that CSF accumulates in culture medium during cell growth and can function in cell–cell signaling. It is also clear that PhrA pentapeptide can function in cell–cell signaling. However, it has been suggested that the active form of modified PhrA, ARNQT, may not normally accumulate to significant levels in culture medium and that PhrA may be involved in cell-autonomous signaling as part of a timing mechanism. At high internal CSF concentrations, CSF inhibits competence and promotes spore development. Specifically, CSF inhibits ComS, reducing transcription of competence genes and promoting sporulation instead (Lazazzera *et al*., 1997; Solomon, 1995; Solomon *et al*., 1996; Perego and Hoch, 1996; Mirel *et al*., 2000; Stephens, 1998).

The products of *spo0A* and *spo0H* genes, which code for response regulator of the multi-compenent signal transduction system, so called phosphorelay and alternative sigma

factor, σ^H, respectively, play a key role in the initiation of sporulation (Burbulys *et al*., 1991; Chibazakura *et al*., 1995; Dubnau *et al*., 1988; Asai *et al*., 1995).



(Taken from Lazazzera *et al*., 1998)

Figure 1. 4. The regulation of sporulation by extracellular peptides. CSF and a peptide, ARNQT, encoded by phrA, are transported into the cell by Opp. Each peptide inhibits the activity of a phosphatase (RapB or RapA) that dephosphorylates Spo0F~P. Spo0F receives phosphate from one of three kinases (KinA,B,C) and donates phosphate to Spo0A through Spo0B (Lazazzera *et al*., 1998).

A multicomponent phosphorelay consists of five histidine kinases (KinA, KinB, KinC, KinD and KinE) and two phosphorelay proteins (Spo0F and Spo0B) (Perego and Hoch, 2002). Multiple environmental and physiological signals are fed into this system, and under appropriate conditions this leads to phosphorylation of Spo0A, the key sporulation transcription factor (Grossman, 1995; Sonenshein, 2000).

Activity of Spo0A is subject to several auto-stimulatory loops (Strauch *et al*., 1992; Strauch *et al*., 1993; Fujita and Sadaie, 1998). These loops involve transcription of *spo0A* and phosphorylation of Spo0A. Transcription of *spo0A* is directly activated by Spo0A~P and indirectly activated by induced expression of *sigH*. The *sigH* gene encodes an RNA polymerase sigma factor (σ^H) that recognizes an alternative promoter located upstream of *spo0A* (Predich *et al*., 1992) and activates transcription of genes involved in the phosphorylation of Spo0A such as *kinA* and *spo0F*. Furthermore, sigma-H activates transcription of the *spoIIA* operon, which contains the sporulation specific sigma factor, sigma-F (Hoch, 1991).

Once activated by phosphorylation, Spo0A binds to a DNA sequence containing a so-called '0A-box' (Strauch *et al*, 1990), where it exerts its role by acting as a transcriptional activator or repressor. Besides being required for the onset of sporulation, Spo0A is also involved in the transcriptional regulation of various other stationary phase processes. Spo0A influences the expression of 520 *B. subtilis* genes showing that it has indeed a profound effect on the global gene expression pattern of *B. subtilis* (Fawcett *et al*, 2000; Liu *et al*, 2003). Of these 520 genes, 121 are under the direct control of Spo0A. Several of these encode proteins that themselves are directly or indirectly involved in transcriptional regulation, explaining the global effect of Spo0A on transcription (Molle *et al*., 2003; Castilla-Llorente *et al*., 2006). The levels of Spo0A protein and activity increase gradually during the early stages of sporulation (Fujita and Losick, 2005) and the progressive increase of activated Spo0A explains the temporal fashion by which the low- and high-threshold Spo0A-regulated genes are activated or repressed (Fujita *et al*., 2005).

A major role of phosphorylated Spo0A is to repress the expression of *abrB*, a gene encoding a transcriptional regulator that represses various stationary phase processes (Robertson *et al*., 1989). During exponential growth, AbrB represses expression of *sigH*, *kinA* and *abrB* itself (Strauch, 1995). Thus, alleviation of AbrB repression by Spo0A~P

at the beginning of the stationary growth phase, stimulates *sigH* and *kinA* expression and therefore *spo0A* transcription and indirectly phosphorylation of Spo0A. In conclusion, the complex autostimulation of *spo0A* could be the basis of the bistable sporulation gene expression (Veening *et al*., 2005).

KinA is the primary kinase in the phosphorelay and is necessary for the phosphorylation of Spo0A (Burbulys *et al*., 1991). It has been demonstrated that the fraction of cells that initiate sporulation is decreased in a *kinA* mutant background (Chung *et al*., 1994). This result suggests that a certain threshold concentration of Spo0A~P is necessary to initiate sporulation and that the activity of the phosphorelay determines the threshold level for autostimulation of Spo0A. This implies that influences on the phosphorelay by external phosphatases could alter the heterogeneous sporulation gene expression, examples are RapA and PhrA (Veening *et al*., 2005). Recently, Castillo-Llorente *et al*. (2008) showed that KinC and, to a minor extent, KinD, are responsible for heterogeneous expression of *spo0A* during logarithmical growth. The low-threshold Spo0A-regulated gene *abrB* has been reported to be expressed at submaximal levels during logarithmical growth of wild-type cells, compared with the levels reached in strains with an inactivated phosphorelay (Perego *et al*., 1988; Trach and Hoch, 1993). Submaximal levels of *abrB* expression during logarithmical growth were also observed in *kinA*, *kinB* or *kinE* mutant strains, but the expression levels were higher in *kinC* and *kinD* mutant strains, and maximum *abrB* expression levels were observed in the absence of both KinC and KinD (Trach and Hoch, 1993; LeDeaux *et al*., 1995; Jiang *et al*., 2000). From these results, it was inferred that KinC and KinD would be responsible for generating Spo0A~P to levels causing partial repression of *abrB* transcription during logarithmical growth (Castillo-Llorente *et al*., 2008).

To date, more than 35 two-component regulatory systems have been identified in *B. subtilis* by genome sequencing (Guo *et al*., 2010). The histidine kinases and response regulators of *B. subtilis* are shown in Figure 1.5.

Class[a]	Family[b]	Kinase	Response regulator	Organization[c]	Adaptive role
I	Others-B	LytS	LytT	HR	Rate of autolysis
		YesM	YesN	HR	Unknown and nonessential
		YwpD		Orphan[d]	Unknown and nonessential
II	NarL	DegS	DegU	HR	Degradative enzyme and competence
		YhcY	YhcZ	HR	Unknown and nonessential
		YvqE	YvqC	HR	Unknown and nonessential
		YvfT	YvfU	HR	Unknown and nonessential
		YocF	YocG	HR	Unknown and nonessential
		YdfH	YdfI	HR	Unknown and nonessential
		YxjM	YxjL	HR	Unknown and nonessential
		YfiJ	YfiK	HR	Unknown and nonessential
		ComP	ComA	HR	Early competence
IIIA	OmpR	YcbM	YcbL	RH	Unknown and nonessential
		YxdK	YxdJ	RH	Unknown and nonessential
		YtsB	YtsA	RH	Unknown and nonessential
		YvcQ	YvcP	RH	Unknown and nonessential
		YbdK	YbdJ	RH	Unknown and nonessential
		YvqB	YvqA	RH	Unknown and nonessential
		YkoH	YkoG	RH	Unknown and nonessential
		YrkQ	YrkP	RH	Unknown and nonessential
		YclK	YclJ	RH	Unknown and nonessential
		YvrG	YvrH	RH	Unknown and nonessential
		YycG	YycF	RH	Essential functions
		ResE	ResD	RH	Aerobic and anaerobic respiration
		PhoR	PhoP	RH	Alkaline phosphatase and phosphodiesterase
		YccG	YccH	RH	Unknown and nonessential
IIIB	NtrB	KinA	Spo0F	Orphan	Initiation of sporulation
		KinB	Spo0F	Orphan	Initiation of sporulation
		KinC		Orphan	
		YkvD		Orphan	
		YkrQ		Orphan	
IV	Others-A	YufL	YufM	HR	Unknown and nonessential
		YdbF	YdbG	HR	Unknown and nonessential
		CitS	CitT	HR	Mg^{2+}/citrate transport
		YcbA	YcbB	HR	Unknown and nonessential
V	CheY	CheA	CheY	RH	Chemotaxis

[a] Classes defined by comparing the *B. subtilis* kinases around the histidine.
[b] Families of two-component systems defined in *E. coli* by comparing the response regulator C-terminal domains.
[c] Organization of each kinase-regulator pair on the *B. subtilis* chromosome (HR, 5′ histidine kinase-3′ response regulator; RH, 5′ response regulator-3′ histidine kinase).
[d] "Orphan" designates an histidine kinase gene not directly associated with a response regulator gene in an operon on the chromosome.

Figure 1. 5. Two- component systems in *B. subtilis* (Sonenshein *et al.*, 2002)

1.5. Sporulation in *B. subtilis*

Sporulation by the bacterium *B. subtilis* is a multistage, developmental process that is responsible for the conversion of a growing cell into a dormant cell type known as the spore or endospore (Stragier and Losick, 1996; Piggot and Losick, 2002). Soon after a cell commits to sporulation, it builds an asymmetrically positioned septum that divides the sporulating cell, called a sporangium, into two unequally sized cells with distinct developmental fates. The smaller chamber, called the forespore, ultimately becomes the spore. The larger compartment, called the mother cell, nurtures the developing spore during its formation. In the next stage of sporulation, the edge of the septum migrates toward the forespore pole of the cell and engulfs the smaller forespore compartment, resulting in a protoplast with two membrane layers that is entirely surrounded by the mother cell. The space between this double layer of membrane becomes the site of assembly of two layers of specialized peptidoglycan called the germ cell wall and the cortex. The last structure to be formed is a proteinacious shell, called the coat, that encircles and protects the spore. In its final act, the mother cell lyses, releasing the now mature spore into the environment. In *B. subtilis*, the coat is composed of a heterogeneous group of over 20 polypeptides, ranging in size from about 6 to 69 kDa, which are arranged in three main structural layers: a diffuse undercoat, a laminated lightly staining inner layer, and a thick and electron-dense outer coat. A large number of genes have been directly implicated in coat assembly. These include genes for 18 coat structural proteins (*cot* genes), as well as genes encoding morphogenetic proteins that act by guiding the assembly of the structural components but need not be part of the final structure (Serrano *et al*., 1999). TasA is one of the structural components that is associated with the assembly of the spore coat. The *tasA* gene product is secreted into the medium (Figure 1.6) and is the major protein component of the biofilm extracellular matrix (Branda *et al*., 2006). In addition, TasA has a broad-spectrum antibacterial activity which inhibit the growth of competitor bacteria in nature, a trait that could be useful early in

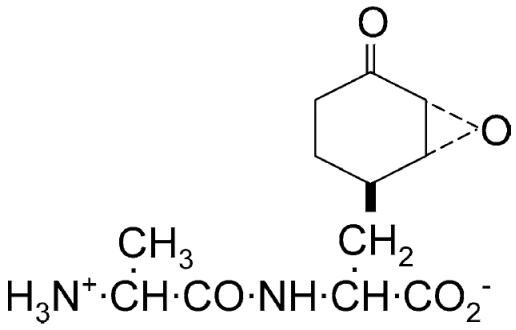
sporulation, as well as immediately upon germination (Katz and Demain, 1977; Stöver and Dricks, 1999).

A series of sequentially activated transcription factors ensures that sporulation genes are activated at the proper times and in the correct compartments (Stöver and Dricks, 1999). The master regulator for entry into sporulation is the DNA-binding protein Spo0A (Hoch, 1993), which is a member of the response regulator family of transcription factors (Perego and Hoch, 2002), many of its effects on the global pattern of gene transcription are likely to be mediated indirectly by regulatory genes under its control. 121 genes, which are organized as 30 single-gene units and 24 operons, were found to be under the direct control of Spo0A. Forty of these genes are under the positive control of Spo0A, and 81 are under its negative control. Among identified members of the regulon with transcription that was stimulated by Spo0A are genes for metabolic enzymes and genes for efflux pumps. Among members with transcription that was inhibited by Spo0A are genes encoding components of the DNA replication machinery and genes that govern flagellum biosynthesis and chemotaxis. Also included in the regulon are many genes with products that are direct or indirect regulators of gene transcription (Molle *et al*., 2003)

Fawcett *et al*. (2000) and Molle and colleagues (2003) have shown that Spo0A regulates the *skf* operon, which encodes the sporulation killing factor (SkfA) (Figure 1.8). SkfA induces the lysis of sibling *B. subtilis* cells that have not entered the sporulation pathway (i.e., Spo0A inactive), providing a source of nutrients to support this key differentiation process.

1.6. Dipeptide Antibiotic Bacilysin

The dipeptide bacilysin is one of the simplest peptide antibiotics known (Fig. 1.6) which displays activity against some bacteria and fungi (Kenig and Abraham, 1976; Tschen, 1990). Its proposed amino acid ligase mode of biosynthesis which has been understood in 2000s might offer strategies to engineer new derivatives with improved properties (Steinborn *et al*., 2005).



(Taken from Rogers *et al*., 1965)

Figure 1. 6. Structure of bacilysin

Bacilysin [L-alanyl-(2.3-epoxycyclohexanone-4)-L-alanine] molecule contains an L-alanine residue at the N terminus and a non-proteinogenic amino acid, L-anticapsin, at the C terminus (Walker and Abraham, 1970). Its antibiotic activity depends on the anticapsin moiety, which becomes released by peptidases (Kenig *et al*., 1976; Chmara *et al*., 1982) after bacilysin uptake into susceptible cells by a distinct peptide permease system (Perry and Abraham, 1979; Chmara *et al*., 1981). The intracellular anticapsin then blocks the glucosamine synthetase, and hence, bacterial peptidoglycan or fungal mannoprotein biosynthesis. This leads to cell protoplasting and lysis (Whitnney and Funderburk, 1970; Kenig *et al*., 1976; Chmara *et al*., 1982; Chmara, 1985; Milewski, 1993). Based on its metabolic target, the antibiotic activity

of anticapsin becomes specifically antagonized by glucosamine or N-acetylglucosamine (Walton and Rickes, 1962; Kenig and Abraham, 1976).

Biosynthesis of anticapsin branches from prephenate (Roscoe and Abraham, 1966; Hilton *et al*., 1988). The experimental evidence suggested that the peptide formation with L-alanine occurs in a non-ribosomal mode catalysed by an enzyme, namely bacilysin synthetase (Sakajoh *et al*., 1987).

Bacilysin production by *B. subtilis* is active when the cells are grown in synthetic medium and becomes repressed and/or inhibited by certain nutrients, like glucose and casamino acid, and temperatures above 30°C (Özcengiz *et al*., 1990; Özcengiz and Alaeddinoglu, 1991; Basalp *et al*., 1992). Its synthesis seemed to be under the stringent response (Inaoka *et al*., 2003) as well as under feedback regulation (Özcengiz and Alaeddinoğlu, 1991), and as shown more recently, it was discovered to be a component of the global quorum-sensing control system (Yazgan *et al*., 2001; Karatas *et al*., 2003). It was definitely shown that the biosynthesis of basilysin is under quorum sensing global regulation through the action of ComQ/ComX, PhrC (CSF), ComP/ComA and in a Spo0K (Opp)-dependent manner in *B. subtilis* (Yazgan *et al.*, 2001; Karatas *et al.,* 2003). The disruption of lipopeptide antibiotic surfactin biosynthetic (*srfA*) operon in the bacilysin producer resulted in a bacilysin-negative phenotype, thus the study verified that the *srfA* operon functions directly in the production of bacilysin (Karatas *et al*., 2003). The loss of bacilysin production in *spo0H* and/or *spo0A*-blocked mutants as well as an increase in the production of bacilysin in *abrB*-disrupted mutants and the suppression of bacilysin-negative phenotype by an *abrB* mutation in *spo0A*-blocked mutants revealed that the transcription of some gene(s) involved in bacilysin formation is under the negative control of *abrB* gene product which is relieved by Spo0A protein (Karatas *et al*., 2003).

The *ywfBCDEF* genes of *B. subtilis* 168 were shown to carry the biosynthetic core functions and were renamed *bacABCDE* (Fig. 1.7) (Inaoka *et al*., 2003; Steinborn *et al*., 2005). In accordance with the similarity features of the genes *bacABC*, the deduced proteins were good candidates to catalyse the proposed conversion of prephenate to anticapsin (Roscoe and Abraham, 1966; Hilton *et al*., 1988), apparently in three enzymatic steps. *bacDE* (*ywfEF*) have been shown to encode the functions of amino acid ligation and bacilysin immunity respectively (Inaoka *et al*., 2003; Steinborn *et al*., 2005).

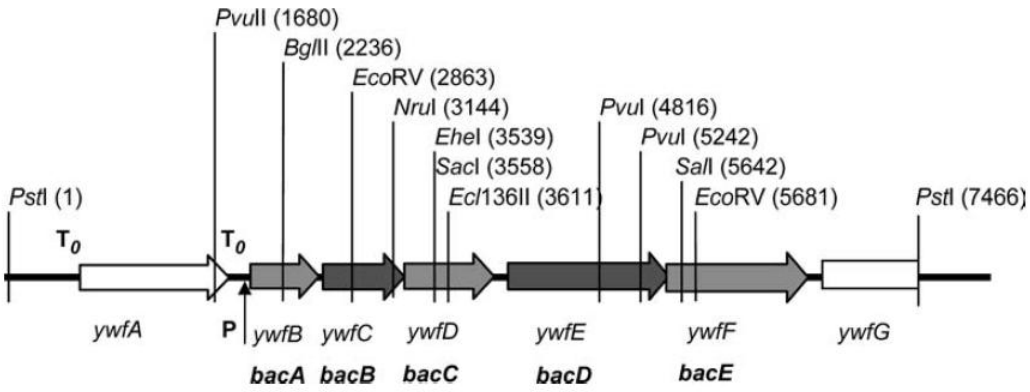
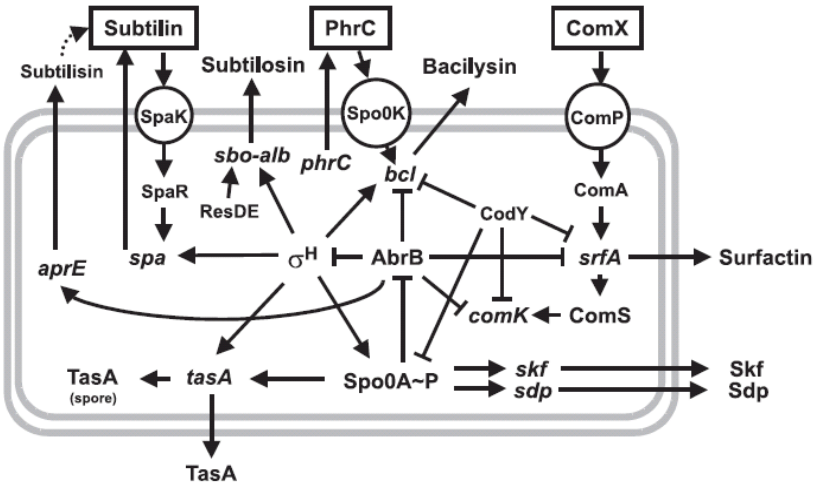


Figure 1. 7. Organization of the bacilysin gene cluster *bacABCDE* relative to open reading frames *ywfABCDEFG* of *B. subtilis* 168 (Steinborn *et al*., 2005).

The study of Inaoka *et al*. (2003) was also showed that guanosine 5'-diphosphate 3'-diphosphate (ppGpp) plays a crucial role in transcription of the *bacABCDE* operon and that the transcription of these genes is dependent upon the level of intracellular GTP which is transmitted as a signal via the CodY-mediated repression system. It was proposed that bacilysin production in *B. subtilis* is controlled by a dual regulation system composed of the guanine nucleotides ppGpp and GTP (Inaoka *et al*., 2003).

Briefly, bacilysin production is regulated on different levels (Fig. 1.8), negatively by GTP via the transcriptional regulator CodY (Inaoka *et al*., 2003) and AbrB (Karatas *et al*., 2003). Positive regulation occurs by guanosine 5'-diphosphate 3'-diphosphate (ppGpp) (Inaoka *et al*., 2003) and a quorum-sensing mechanism through the peptide pheromone PhrC (Karatas *et al*., 2003).



(Taken from: Stein, 2005)

Figure 1. 8. Regulatory pathways of antibiotic biosynthesis in *B. subtilis.* Survey of the regulatory pathways for the biosynthesis of the *B. subtilis* antibiotics subtilin, subtilosin, bacilysin, surfactin, the killing factor Skf and the spore-associated anti-microbial polypeptide TasA (Stein, 2005).

1.7. Proteomics

The release of the first genome sequence of a living organism, the bacterium *Haemophilus influenzae,* opened a new era in life sciences, called –omics (structural, comparative, functional and so on) (Fleischmann *et al*., 1995**)**. The year 1995, however, should also be kept in our mind because the term 'proteomics' was coined (Wasinger *et al*., 1995**)** describing the complete set of proteins expressed under defined physiological conditions. Whereas the genome sequence only provides the "blue-print" of life, the proteomics is required to bring the genes to "real life of the proteins" (Hecker *et al*., 2008)

Proteomics started in 1975 as O'Farrell and Klose (O'Farrell, 1975; Klose, 1975). published the now famous and most frequently cited two-dimensional gel electrophoresis technique (2-DE) that allows the separation of thousands of proteins in an area of 20x20 cm^2 in size. Each single protein is separated according to its molecular weight and pI. Only a few years later Fred Neidhardt and Ruth Van Bogelen, the pioneers of physiological proteomics used this powerful technique to address crucial issues of *E. coli* cell physiology such as heat shock or starvation responses (Neidhardt *et al*., 2000). In the 1980s and 1990s amongst others Angelika Görg (1993) successfully improved the gel-based proteomics technique by the introduction of IPGs for IEF. This was the basis for high-throughput comparative gel-based proteomics studies (Görg *et al*., 2000; Görg *et al*., 2004) and for the development of gel-based proteomics as a new field in cell physiology.

In the post-genomic era, the field of proteome analysis has acquired importance very rapidly (Pandey *et al*., 2000; Aebersold *et al*., 2003; Huber *et al*., 2003). Proteomic studies now includes protein expression profiling of biological samples in a given physiological state to afford a large-scale characterization of all detectable proteins including their post-translational modifications (structural proteomics) (Wasinger *et*

al., 1995), comparison of protein expression level in two or more physiological states (i.e. normal versus altered/pathological conditions) (comparative proteomics or quantitative proteomics) (Lasonder *et al*., 2002; Florens *et al*., 2002), protein-protein interaction analysis (Uetz *et al*., 2000; Gavin *et al*., 2002), sub-cellular protein localization analysis (Huh *et al*., 2003) and definition of a biological role for open reading frames with poorly known function (functional proteomics) (Minshull *et al*., 2005).

Monteoliva and Albar (2004) stated that classical proteomics work involves: (1) a separation step in which proteins of interest are separated by 2-DE [isoelectric focusing (IEF) followed by separation as a function of molecular mass]; (2) protein visualization and image analysis; (3) excising the spots to be analyzed; (4) in gel digestion of proteins and pooling of the released peptides; (5) analysis of the peptide mass fingerprint (PMF) for every digested protein through matrix-assisted laser desorption/ionisation TOF-MS (MALDI-TOF MS); (6) matching peptide masses against protein databases to obtain candidate proteins; (Andersen and Mann, 2000), and (7) validating identification by acquisition of MS/MS spectra of selected peptides to confirm their sequences (Figure 1.9) (Corthals *et al*., 1999; Molloy *et al*., 2002).

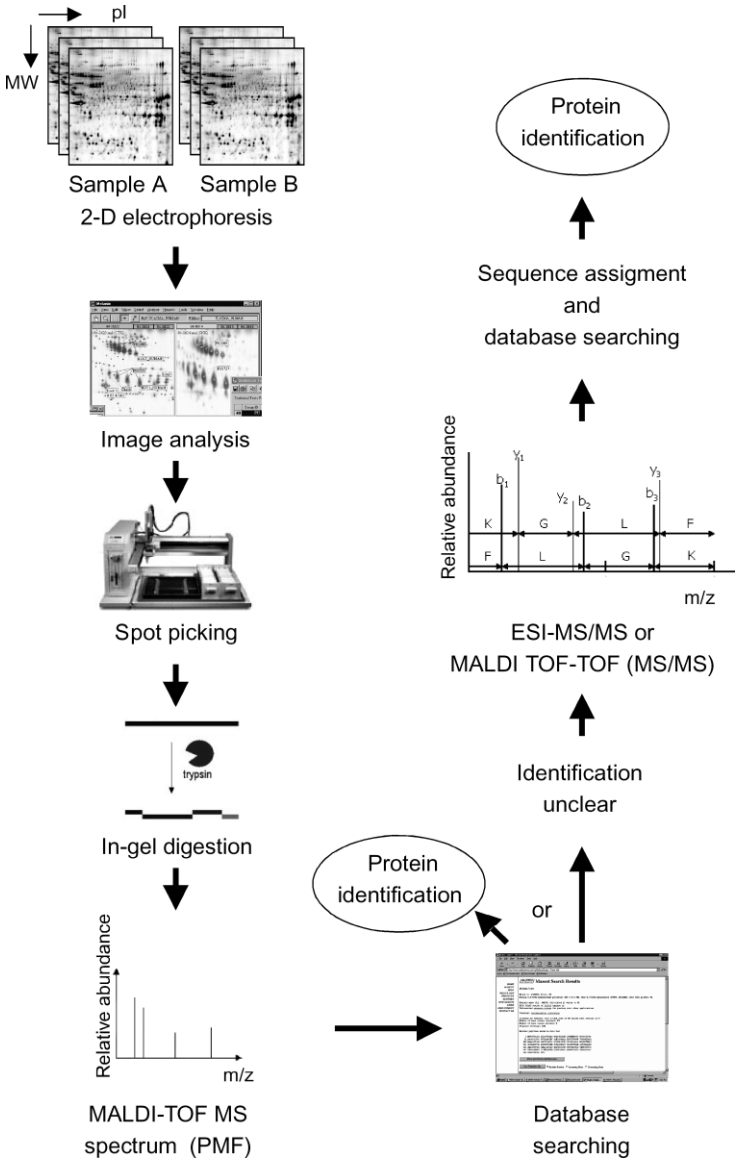


Figure 1.9. Workflow of a classical 2DE based proteome study: Samples A and B are resolved by 2- DE in replicate gels and silver stained. 2-D images are analysed by specific software and differentially expressed protein spots are proteolytically digested and analysed by MALDI-TOF MS. The peptide mass fingerprint (PMF) is matched against genomic or protein databases to obtain candidate proteins. Tandem mass spectrometry (MS/MS) and peptide sequencing by analysis of fragmentation spectra can assist in identifying peptides when ambiguity remains after MALDI-TOF analysis (Monteoliva and Albar, 2004).

Because of their low complexity, bacteria are extremely suitable model organisms for transferring the "blue-print of life to real life", and thereby becoming to a new quality in understanding life processes (Hecker *et al*., 2008). These low complexity organisms are reasonable model systems to address crucial and elementary issues of life processes by using proteomic approaches (Regula *et al*., 2000; Jaffe *et al*., 2004; Hermann *et al*., 2006).

B. subtilis, the best analyzed representative of the Gram-positive bacteria, has been established as a model system for functional genomics of bacteria. The extensive knowledge on genetics, molecular biology and physiology of this model organism was the main reason to be chosen as the primary model for physiological proteomics. A first comprehensive map of the vegetative *B. subtilis* proteome was published by Büttner *et al*. (2001), three years later Eymann *et al*. (2004) expanded the cytosolic proteome map of growing cells to a total number of 693 proteins in the region pI 4–7. In 2007 in their proteome study, Wolff *et al*. published that 1395 *B. subtilis* proteins were identified most of which are vegetative proteins synthesized in growing cells to put on house-keeping functions (950 cytosolic proteins, 268 membrane proteins, 12 cell wall-bound proteins).

E.coli is another most extensively studied organism in bacteria and its 2-DE map was constructed by Pasquali *et al*. in 1996 (updated in 1998), the 2-DE map having published in SWISS-2DPAGE format in the expasy website (http://www.expasy.org/ch2d/publi/ecoli.html).

1.8. Why Proteomics?

Genome sequencing provides a genetic 'map' of potential protein products and predicts functional pathways. While this information is a necessity for understanding a biological system, the genome itself is 'static' in terms of its linear progression when challenged by outside stimuli. Thereby, many types of information can not be obtained from the study of genes alone. Genomic analyses can provide very limited information with respect to describing how microorganisms adapt to a constantly changing environment. While the presence of certain genes characterized by their sequence identity to a database homology suggests an organism may have the ability to adapt to certain ecological niches and to utilize various substrates, the knowledge of protein expression under these conditions is essential for fully understanding how the organism responds to a given challenge (Cordwell *et al*., 2001).

In recent years, DNA microarray technology and serial analysis of gene expression have become increasingly popular for the analysis of mRNA expression. Monitoring gene expression at the mRNA level has advantages in that it has been easily scalable to cover near-to-total prokaryotic and simple eukaryotic genomes, is achievable using high-throughput technologies thus aiding in faster lead discovery times, and is readily roboticised thus reducing sample handling and error (Zweiger *et al*., 1999). However, biological influences including the stability, half-life, post-transcriptional, co-translational and degradative modification of proteins (Turner *et al*., 2000) combine to suggest that mRNA abundance and response to environmental stimuli may not completely represent the expression of a corresponding protein in vivo (Gygi *et al*., 1999). While this effect is still not fully understood in microbes, a sensible technical approach is to combine the determination of mRNA levels with a concurrent analysis of protein expression levels. The global analysis of protein expression from a genome under a given set of conditions is much better summarized by proteomics (Humphery-Smith *et al*., 1997; Pandey *et al*., 2000). Indeed, Gygi *et al*. (1999) has

shown that there is a poor correlation between mRNA and protein expression level. The first reason is that mRNA is subject to post-transcriptional control in the form of alternative splicing, polyadenylation and mRNA editing. Secondly, in eukaryotes, mRNA then can be subjected to regulation at the level of translation. After being formed, proteins are also subjected to post-translational modification, proteolysis and compartmentalization. Hence, the average number of proteins formed per gene is predicted to be one or two in bacteria, three in yeast and more for humans (Wilkins *et al*., 1996). To our experience, there can be over 5 proteins encoded bu the same gene in the basidiomycete P. Chrysosporium (Özcan *et al*., 2007; Yıldırım *et al*., submitted). The fact that different tissues of the same organism have the same genome, but different proteomes in the same organism certainly makes the proteome bewilderingly complex than genome. The most significant attempt and example for investigation of the proteome of different tissues has been initiated by the Human Proteome Project (HUPO). HUPO aims to identify tissue and organ specific proteomes (www.hupo.org). There are 100 000 different protein sequences estimated in the human organism, and perhaps 10-100 times as many different protein forms. Analysis of the human proteome is a much more challenging task than that of the human genome (Zubarev, 2006). The challenge is to provide sufficient amount of information in experimental datasets to match the underlying complexity.

1.9. Why 2-DE?

2-DE allows routine separation of thousands of proteins, and thus represents the dominant technique in the field of proteomics (Wildgruber *et al*., 2000; Nyman, 2001), although it was developed 35 years ago by O'Farrell and Klose (O'Farrell, 1975; Klose, 1975). The first dimension of 2-DE is isoelectric focusing (IEF). During IEF, proteins are separated in a pH gradient until they reach a stationary position where their net charge is zero. In the second dimension, the proteins separated by

isoelectric focusing are separated orthogonally by electrophoresis according to their molecular weight.

2-DE has its advantages in proteome analysis, as well as its limitations. The main strength of 2-DE is its extremely high resolution power compared to other separation techniques and a good visualization of obtained results. Resolution, reproducibility and protein load capacity have been greatly enhanced with the introduction of immobilized pH gradients (IPG) (Gorg *et al*., 1988). 2-DE can provide more than 10 000 detectable protein spots in a single gel run (Klose and Kobalz, 1995). Thus, proteins with posttranslational modifications, such as processing, phosphorylation and glycosylation, can be easily detected as separate spots on a 2-DE gel. Furthermore, 2-DE instruments and reagents now commercially available allow a good reproducibility with respect to other techniques. Massive 2-DE comparison can be easily performed in parallel by using commercial instruments, allowing the increase in the number of experiments to be set up with the aim to detect subtle protein changes associable to various physiological states (Renzone *et al*., 2005).

In spite to its advantages, it is now accepted that 2-DE is far to be a perfect methodology for proteome analysis (Rabilloud, 2002). Primary weakness is the difficulty in detecting low abundant proteins. Furthermore, certain classes of proteins are known to be absent or underrepresented in a 2-DE map, as in the case of very acid/basic proteins, or very large/hydrophobic proteins associated to membranes. In general, only proteins with a molecular weight of 10-100 kDa and a pI of 4- 8 migrate well within 2-DE gels. Several attempts to overcome these shortcomings have been done (Renzone *et al*., 2005). For instance, sample pre-fractionation can be used to increase the amount of low abundant proteins; similarly, modification in experimental conditions for protein solubilization can give a better resolution and detection of hydrophobic components (Molloy *et al*., 1998 and Herbert, 1999).

Proteomics area is branching out everyday with the new developing technologies, which holds the promise of new and more precise methods. To precisely compare protein expression profile of two samples, the fluorescent two-dimensional difference gel electrophoresis (2D-DIGE), liquid phase separations combined with mass spectrometry (LC-MS), protein microarrays, several combinations of stable isotope labeling and shotgun peptide sequencing protocols have been introduced (Goshe and Smith, 2003; Tao and Aebersold, 2003). It is evident that each method has its own strengths and weaknesses and no single method will be optimal in all applications. However, the continuing development of innovative strategies for protein separation and analysis are providing a wealth of new tools for multi-dimensional protein profiling (Steel *et al*. 2005).

1.10. Steps in Proteome Work

1.10.1. Isoelectric Focusing and Immobilized pH Gradient

Isoelectric focusing (IEF) is an electrophoretic method which separates proteins according to their isoelectric points (pI) (O' Farrell, 1975). In nature, proteins are amphoteric molecules. They carry either negative, positive or zero net charge, depending on their surrounding pH level. The net charge of a protein is the sum of all the positive and negative charges of R groups (amino acid side chains) and amino- and carboxyl-termini. pI is the specific pH where the net charge of a protein is zero. Proteins are positively-charged with pH values below their pI and negatively-charged with pH values above their pI. If the net charge of a protein is plotted versus the pH of its environment, the resulting curve intersects the x-axis at its pI.

The presence of the pH gradient is essential to the IEF method. In the pH gradient, under the influence of an electrical field, a protein will migrate to a location in the gradient where its net charge is zero. Proteins with positive net charges will migrate

toward the cathode, becoming progressively less positively charged as it moves through the pH gradient until it reaches its pI. On the contrary, proteins with negative net charges will migrate toward anode, becoming less negatively charged as it moves through the pH gradient until it also reaches a zero net charge. When a protein diffuses away from its pI, it suddenly gains charge and moves back to its pI position. This is called "focusing" effect of IEF, which concentrates proteins at their pIs and permits proteins to be separated on the basis of very small charge differences (Righetti *et al*., 1989).

IEF is performed under denaturing conditions. Complete solubilization and denaturation is achieved by using a mixture of detergent, urea and ampholines. Employing this mixture ensures that each protein is present in only one configuration, aggregation and intramolecular interaction.

Immobilized pH gradients (IPG) have been introduced for the generation of the pH gradient (Bjellqvist *et al*., 1982). The pH gradient in IPG is prepared by covalently incorporating a gradient of carboxylic and tertiary amino groups as buffering agents at the time of casting. Due to the preformed buffering groups, the gradient can not drift and is not influenced by the sample composition. As commercial products, pre-manufactured gel strips and dedicated IPG instruments are available; a reproducible and more reliable data generation is also possible. Another advantage of the IPG over the traditional carrier ampholyte- mediated IEF is the amount of protein samples which could be increased more than 10 fold.

After the first dimension is completed by either carrier-ampholyte-generated system or automated IPG systems, the second dimension is achieved on the base of protein size using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

1.10.2. Visualization of Protein Spots and Image Analysis

There are a number of visualizing methods for the protein spots resolved by the 2-DE. The highest sensitivity is achieved with radioactivity/fluorography detection. A number of non-radioactive methods (Silver staining, Coomassie Brilliant Blue staining) are applied, with big difference in sensitivity, linearity and dynamic range. Sensitive protein stainings, like the ammoniacal silver staining, permits the detection of proteins at or below nanogram quantities. Hoving *et al*. (2000) reported the calculation down to 100 copies of a protein per cell with sensitive silver staining.

Following staining, evaluation and comparison of the complex 2-DE patterns seems to be very difficult and nearly impossible. Therefore, the gel images have to be converted into digital data with a scanner, camera or more sophisticated gel image documentation systems. This high resolution image (preferably as much as 16 bit or greater) is then evaluated with the aid of the image analysis softwares (e.g. PDQuest, Biorad; Delta2D, Decodon; Phoretix, Nonlinear; Melanie, GeneBio).

1.10.3. Mass Spectrometry Analysis

An array of approaches has been developed for addressing proteome analysis and protein identification. Methods of protein identification have included immunoblotting, peptide sequencing, amino acid composition and more the use of mass spectrometry (Celis *et al*., 2000). One of the earliest methods used for protein identification was microsequencing by Edman chemistry to obtain N-terminal amino acid sequences. Since the invention, little has changed in this technique, but improvements in sequencing technology have increased in the sensitivity and ease of it. One of the biggest hamper that has limited the use of the Edman sequencing in the past was N-terminal modification of proteins. In order to bypass this problem, mixed peptide sequencing is employed (Graves and Haystead, 2002).

Mass spectrometry (MS) enables protein structural information (i. e., peptide masses or amino acid sequences) to be obtained. Hence, this information can be employed to identify the protein by searching nucleotide and protein databases. Identification of proteins by mass spectrometry requires three major stages: (i) sample preparation, (ii) sample ionization and (iii) mass analysis. Extraction of a whole protein or its constituent peptide from the gel is commonly very difficult and mostly inefficient. On the other hand, if a protein is "in gel" digested with proteases (e.g. with trypsin) many of the peptides can be extracted from the gel (Andersen and Mann, 2000). Therefore *in situ* digestion is more efficient at sample recovery than other common methods such as electroblotting. Additionally, the conversion of a protein into its parts provides more information than that can be obtained from a whole protein itself.

First requisite for biological sample to be analyzed by MS is that the molecule must be charged and dry (Graves and Hystead, 2002). Conversion of the samples into desolvated ions is the most commonly used application. Although there are different techniques available, the most preferable methods are Electrospray Ionization (ESI) and Matrix-assisted Laser Desorption/Ionization (MALDI). In both methods, peptides are converted into ions by the addition or loss of one or more protons. Both methods allow the formation of ions without major loss of sample integrity. This is important since mass information about proteins and peptides is obtained in their native state.

Mass analysis follows the conversion of protein or peptides into molecular ions. This is performed by the mass analyzer in a mass spectrometer which resolves the molecular ions on the bases of their mass and charge in a vacuum. Although there are few available techniques, a time-of-flight (TOF) is one of the simplest mass analyzer. It measures the m/z (mass to charge) ratio of an ion by determining the time required for it to transverse the length of a flight tube. From the MS scan, after subtracting the backgrounds, amino acid sequence information for the peptide is

obtained with the aid of software (Graves and Haystead, 2002). The information obtained is then used to search DNA and protein databases (SWISS-PROT, FASTA; MOWSE, ProFound, PepFrag, PepSea and so on).

1.10.4. Proteome and Bioinformatics

Over the last three to four decades, 2-DE has established itself as the *de facto* approach to separating proteins from cell and tissue samples (Anderson and Anderson, 2002). Due to the sheer volume of data and its experimental geometric and expression uncertainties, quantitative analysis of these data with image processing and modelling has become an actively pursued research topic. The results of these analyses include accurate protein quantification, isoelectric point and relative molecular mass estimation, and the detection of differential expression between samples run on different gels. Systematic errors such as current leakage and regional expression inhomogeneities are corrected for, which is followed by each protein spot in the gel being segmented and modelled for quantification (Dowsey *et al*., 2003).

Computer algorithms identify proteins based on PMF and fragmentation (MS/MS) information to search protein databases. Currently, there are different softwares (e.g. MASCOT, SEQUEST) using different algorithms to identify proteins from PMF and MS/MS information. Several papers have been published using both methodologies (Lewis *et al*., 2000; Predic *et al*., 2002; Kanamoto *et al*., 2002).

Bioinformatic softwares give additional and very useful information by analysing the amino acid sequences of proteins. General approach is to establish a theoretical (*in silico*) proteome map by using the available genome sequence and comparing it with the experimental data (Rosen *et al*., 2004; Gade *et al*., 2005). By analyzing the amino acid sequence, bioinformatic softwares can predict several aspects of a protein such as epitope prediction (Hopp and Woods, 1981; Kolaskar and Tongaonkar, 1990; Alix,

1999), pI/M_W values (Bjellqvist *et al*., 1993), existence of signal peptide (Nielsen *et al*., 1997), subcellular localization (Dönnes and Höglund, 2004) and function (Watson *et al*., 2005).

1.11. Proteome of the Model Bacterium *B. subtilis*

For *B. subtilis*, with the whole genome sequence available not only mRNA profiling of gene expression could be performed, but also the gate towards high throughput protein identification was pushed open. Though we can look back on 25 years of proteome research in *B. subtilis* (Streips *et al*., 1985; Richter *et al*., 1986; Wolff *et al*., 2007), still one-third of *B. subtilis* 4100 genes are not assigned to a defined function yet (Sonenshein *et al*., 2001), and only for about one-third of all genes the corresponding protein could be demonstrated so far (Wolff *et al*., 2007).

A considerable part of the genome remains more or less silent under standard growth conditions and will be expressed only in response to specific stimuli. Therefore, two major classes of proteomes have to be defined: the proteome of growing cells (vegetative proteome) and the proteome of non-growing cells suffering from stress or starvation (Wolff *et al*., 2007)

1.11.1. Cytosolic Proteome Map of Growing cells

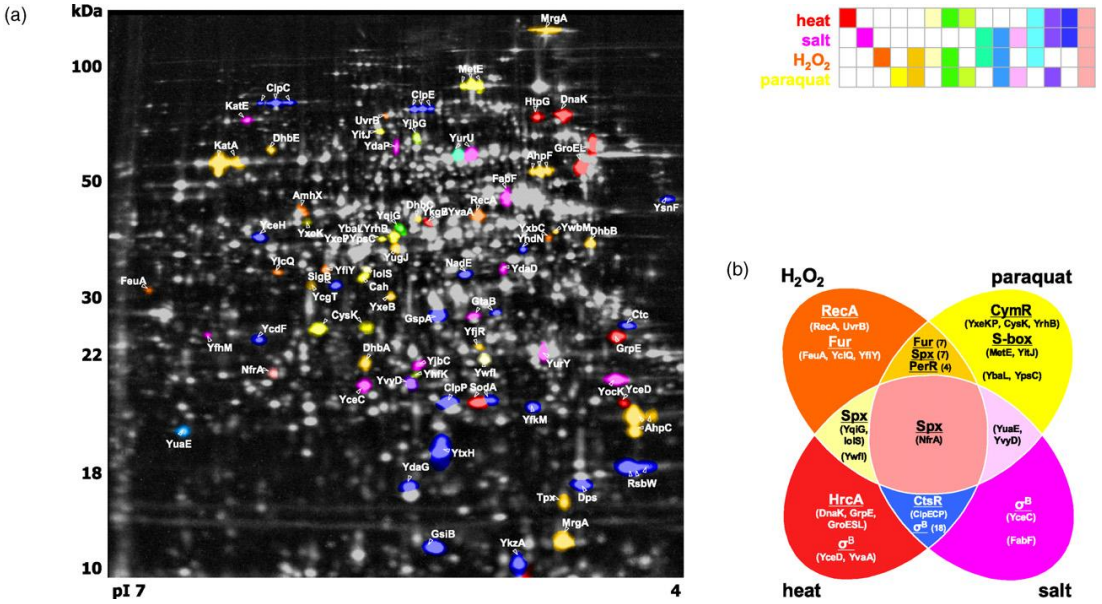
Macroarray analyses revealed that approximately 2500 a total of 4107 genes of *B. subtilis* are actively transcribed under exponential growth conditions (Eymann *et al*., 2004). A first comprehensive map of the vegetative *B. subtilis* proteome was published by Büttner *et al*. (2001), who were able to identify more than 300 cytosolic proteins by standard 2D-PAGE located in an analytical window of pI 4–7. Three years later Eymann *et al*. (2004) expanded the cytosolic proteome map of growing cells to a total number of 693 proteins in the region pI 4–7.

Proteins having a pI beyond 7, which theoretically applies to one-third of the *B. subtilis* proteome, are not covered by conventional 2D-PAGE (pI 4–7) (Wolff *et al*., 2007). A gel-free approach, in which the cytosolic proteome fraction of growing *B. subtilis* cells was dissected via two-dimensional liquid chromatography, yielded a total of 814 identified proteins of which 140 possessed a pI beyond 7 (Wolff *et al*., 2006). Surprisingly 129 of the 814 proteins are characterized by a pI between 4 and 7 and have no transmembrane domains but still they were not detected via standard 2D-PAGE. The incapability to detect these proteins on the gel could be caused by their low abundance, as 10 of the 129 proteins are transcriptional regulators that are most likely difficult to visualize by current staining methods. There are also proteins smaller than 10 kDa or larger than 100 kDa which will probably bottom out the gel or will not migrate into the 2D-gel, respectively. Despite the high-resolution power of 2D-PAGE one can not rule out that some proteins could not be identified from the gel due to protein comigration, which results in masking of small protein spots by higher abundant proteins (Wolff *et al*., 2007).

Combining the results from 2D-PAGE analyses with those obtained by a peptide fractionation via 2D-LC followed by MS/MS acquisition, Wolff *et al*. (2007) was able to identify 1014 cytosolic proteins from exponentially growing cells. In their study, most of the proteins of the central carbohydrate metabolism (glycolysis, pentose phosphate shunt and citric acid cycle), almost all amino acid synthesis pathways, purine and pyrimidine metabolism, fatty acid metabolism, and the main cellular functions like replication, transcription, translation and cell wall synthesis have been identified. However, according to Wolff *et al.* 2007, gel-free approaches allowed the identification of additional proteins, as alkaline, hydrophobic and low-abundant ones.

1.11.2. Cytosolic Proteome Map of Non-Growing cells

Tam *et al*. (2006) complemented the vegetative proteome map of *B. subtilis* by constructive, another one in response to stress and starvation using the 2D-gel-based approach. In total more than 200 stress or starvation induced proteins have been identified (Antelmann *et al*., 2000; Bernhardt *et al*., 2003; Mostertz *et al*. 2004; Tam *et al*., 2006; Höper *et al*., 2006). Eighty-three of them were absent in the vegetative proteome map, whereas the remaining stress and starvation induced proteins exhibit a basal expression level already in vegetative growing cells, indicating a basic physiological role in growing and non-growing cells (Wolff *et al*., 2007).



(Taken from Wolff *et al*., 2007)

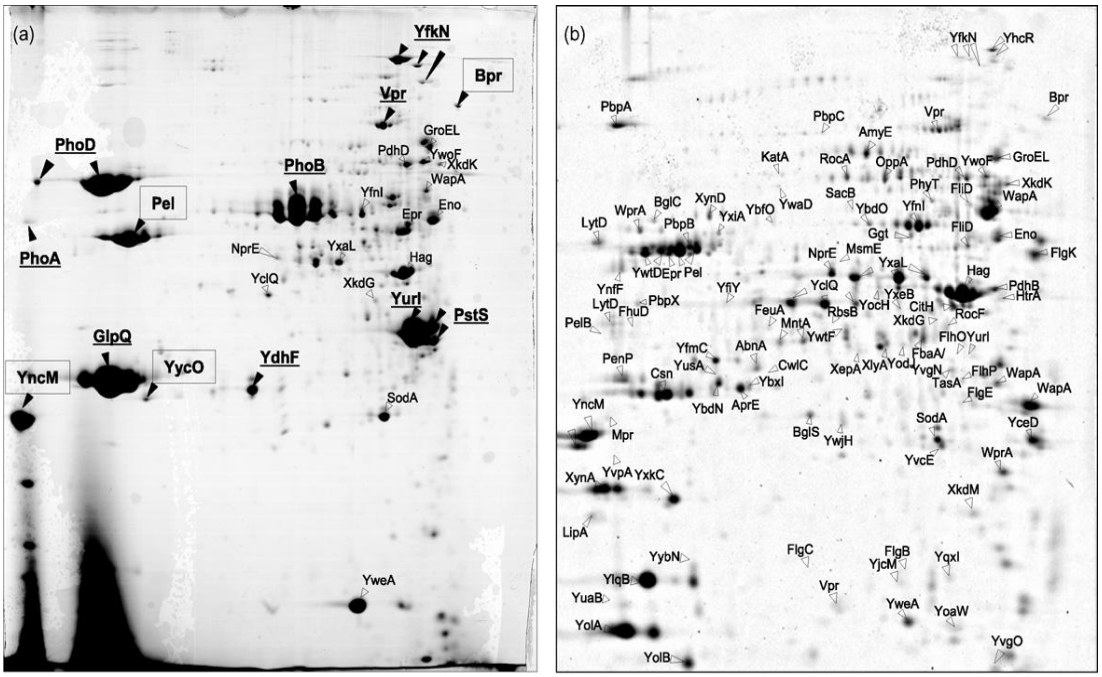
Figure 1.10. a) Cytoplasmic proteome map of *B. subtilis*. b) The specific and general stress regulons in *B. subtilis*. Commonly shared (generally induced) and unique (specifically induced) stress regulons and proteins in the proteome of *B. subtilis* exposed to heat, salt, hydrogen peroxide and paraquat stress according to the fused stress proteome map in (a). The specific and general stress regulons are underlined and the encoded specific or general stress proteins are listed in parentheses (Tam *et al*., 2006).

1.11.3. The Extracytoplasmic Proteome of *B. subtilis* Dissected by 2D-PAGE

As a Gram-positive bacterium *B. subtilis* lacks an outer membrane. It is therefore able to secrete large amounts of extracellular proteins directly into the environment. These proteins perform several very important functions, such as trapping of nutrients, environmental detoxification, cell-to-cell communication, combating competitors. The way a protein is translocated and its final destination are determined by the presence of particular signal peptides and retention signals. According to previous studies there are at least four distinct secretion pathways for approximately 300 proteins predicted to be located extracellularly (Tjalsma *et al*., 2000; Dijl *et al*., 2002). *B. subtilis* extracytoplasmic proteome can be divided into three subproteomes: the true extracellular proteome, the lipoproteome and the cell wall associated proteome.

1.11.4. The Extracellular Proteome of *B. subtilis*

The first proteome analyses on secreted proteins in *B. subtilis* were performed in minimal medium with different carbon sources (Hirose *et al*., 2000) as well as under phosphate starvation conditions (Antelmann *et al*., 2000). However, the highest level of protein secretion in *B. subtilis* is observed when the cells have entered stationary growth phase in rich medium (Antelmann *et al*., 2001).



PhoPR-dependent phosphate starvation-induced proteins (underlined)
PhoPR-independent phosphate starvation-induced proteins (framed)

Figure 1. 11. The extracellular proteome of *B. subtilis* 168 under conditions of phosphate starvation (a) and in complete medium (b) (Wolff *et al*., 2007).

A master gel for the secreted proteome of *B. subtilis* was defined in Luria Broth (LB) medium during the stationary phase (Tjalsma *et al*., 2000). In total, 113 different proteins of the *B. subtilis* secretome could be identified. Fifty-four of them were predicted to be secreted because of an N-terminal signal peptide with a SPase I cleavage site and the lack of retention signals (Tjalsma *et al*., 2000; Tjalsma *et al*., 2004). The remaining proteins were unpredicted secretory proteins which either possess signal peptides and retention signals (18 lipoproteins, 6 cell wall-binding proteins and 6 membrane proteins) or lack signal peptides (17 cytoplasmic proteins, 6 phage-related proteins and 7 flagella-related proteins). The identified extracellular proteins were involved in the uptake, transport or utilization of carbohydrates, proteins, nucleotides, lipids and phosphate, in the cell wall metabolism, in environmental detoxification or they show flagella- and phage-related functions (Antelmann *et al*., 2001; Tjalsma *et al*., 2004).

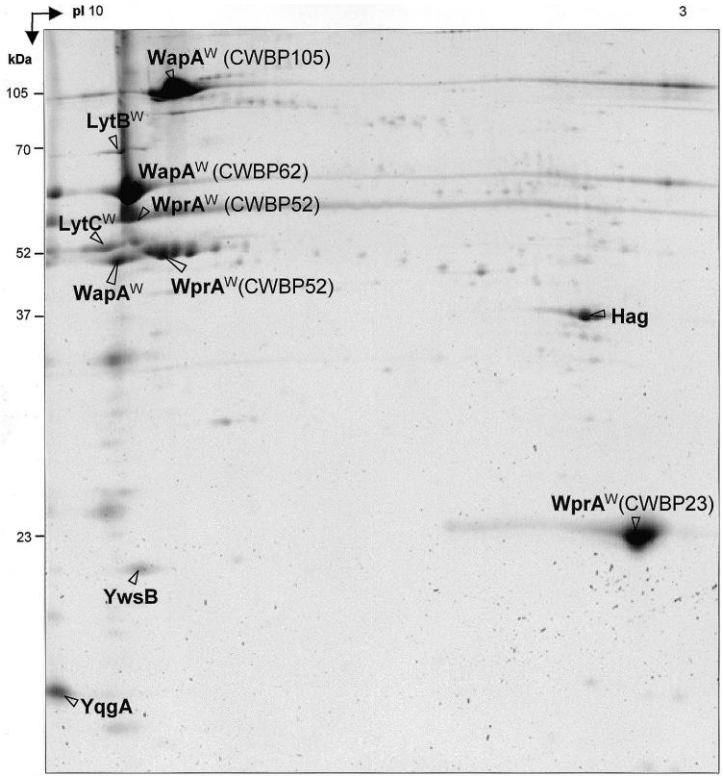
1.11.4.1. The Lipoproteome of *B. subtilis*

Humphery-Smith *et al*. (2006) reported that a separation of lipoprotein containing fraction by 2D-PAGE yielded 10 lipoproteins which have not been identified in the cytoplasmic proteome. Interestingly, nine of these lipoproteins were also components of the extracellular proteome. These proteins were most likely released into the growth medium by proteolytic shaving (Tjalsma *et al*., 2005).

1.11.4.2. The Cell Wall Proteome of *B. subtilis*

The cell envelope of the Gram-positive bacterium *B. subtilis* is composed of a single cytoplasmic membrane surrounded by a relatively thick cell wall (30–40 nm) consisting of peptidoglycan and covalently attached anionic polymers such as teichoic or teichuronic acids. Peptidoglycan, the substrate of autolysins, is a polymer of amino sugars cross-linked by short peptides which constitutes the major skeletal component of the cell wall (Archibald *et al*., 1993). 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 (Pooley *et al*., 1996). In *B. subtilis*, proteins retained in the cell wall include DNAses, RNAses (Merchante *et al*., 1995), proteases (Margot and Karamata, 1996; Babe and Schmidt, 1998), enzymes involved in the synthesis of peptidoglycan (penicillin-binding proteins) and cell-wall hydrolases (Foster, 1993; Blackman *et al*., 1998; Smith *et al*., 2000) that are involved in cell wall turnover during cell growth, cell division, sporulation and germination (Yanouri *et al*., 1993; Murray *et al*., 1996; Popham *et al*., 1996; Murray *et al*., 1997). These wall-binding proteins are retained in the cell wall due to the presence of specific wall-binding domains in addition to N-terminal signal peptides (Lazarevic *et al*., 1992; Kuroda *et al*., 1993; Ghuysen *et al*., 1994; Margot *et al*., 1994).

The global cell wall proteome of the wildtype strain *B. subtilis* 168 was studied by Antelmann *et al*. (2002). Using MALDI-TOF MS as well as *N*-terminal sequencing, they identified 12 abundantly expressed cell wall proteins shown in Figure 1.12.



(Taken from Antelmann *et al*., 2002)

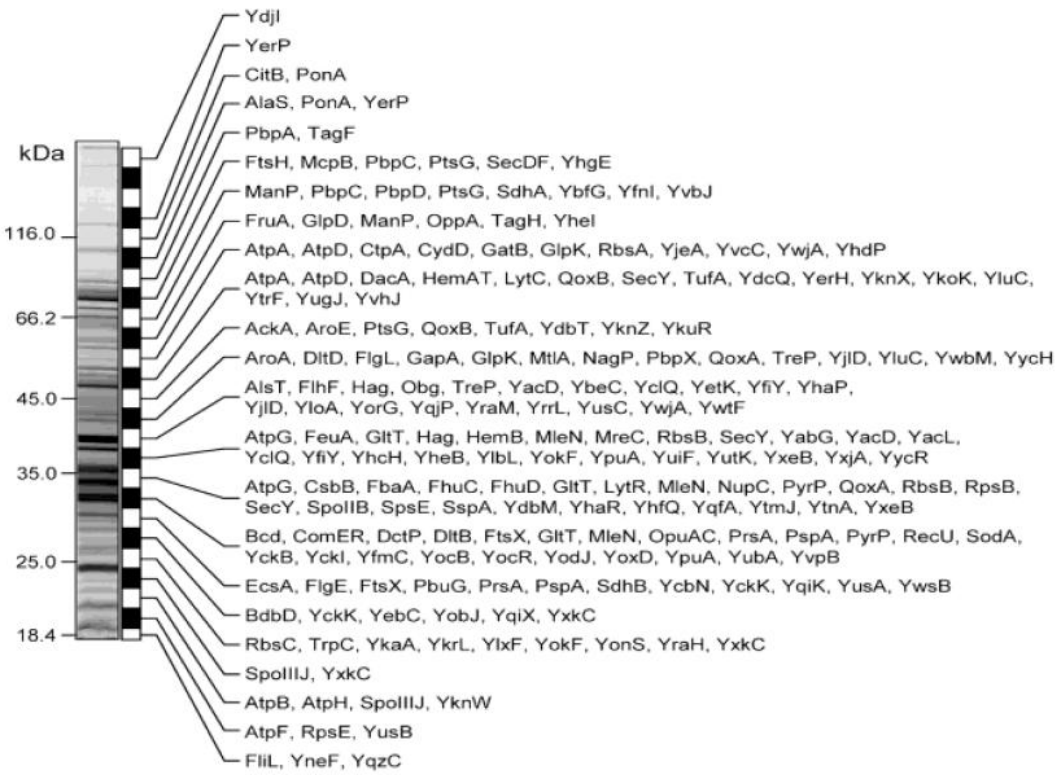
Figure 1.12. The cell wall proteome of the *B. subtilis* wild type 168.

Overall, the comprehensive analyses of the *B. subtilis* extracytoplasmic proteome by a 2D-gel-based approach led to the identification of 119 additional proteins which were either secreted, lipid anchored or associated to the cell wall.

1.11.5. The Membrane Proteome of Vegetative Cells

Membrane proteins constitute an important facet in physiological proteomics due to their importance in maintaining cell integrity, signal sensing, transport processes, energy conservation and their role in virulence regarding pathogenic bacteria. Similar to the extracellular proteins, membrane proteins possess signal peptides marking them for cellular export. However, in most cases these signal peptides are not removed by a signal peptidase and, consequently, they serve in the anchoring of proteins in the membrane. Furthermore, membrane proteins may contain additional membrane-spanning domains, which determine the topology of these proteins in the cytoplasmic membrane (Wolff *et al*., 2007).

With a profiling of ABC-transporter solute-binding proteins, Bunai *et al*. (2004) initiated the work on the *B. subtilis* membrane protein fraction. Eymann and coworkers (2004) established a technique in which the cytoplasmic membrane is washed in high-salt and alkaline buffers before the membrane proteins are solubilized with the detergent n-dodecyl-β-D-maltoside. As a result, 268 identified proteins which contain membrane-spanning domains identified with this technique was successfully reported to start the dissection of the *B. subtilis* membrane proteome of exponentially growing cells (Eymann *et al*., 2004; Wolff *et al*., 2006). Most of the detected membrane proteins are still of unknown function. The functionally defined proteins can be assigned to several essential categories, such as permeases and transporters, components of respiratory chains and ATP-synthase, redox active enzymes (e.g. oxidoreductases and dehydrogenases), two component systems, proteins involved in cell motility, cell division, autolysis, chemotaxis, osmoregulation, penicillin-binding proteins, parts of translocation systems and signal peptidases, as well as extracellularly acting proteases. Of the 268 membrane proteins, 16 were predicted lipoproteins according to Tjalsma *et al*., (2000) 11 of those have not been detected in 2D-gel-based studies.



(Taken from Wolff *et al*., 2007)

Figure 1.13. Analysis of membrane proteins of *B. subtilis*. Identification of proteins by a combined SDS-PAGE LC-MS/MS approach. Proteins of the membrane protein fraction were separated by SDS-PAGE, the gel was divided into sections as indicated by the black-white bar adjacent to the gel lane, and identification was accomplished by ESI-MS/MS after tryptic in-gel digestion of the proteins and separation of the resulting peptides by reverse-phase chromatography. The proteins identified and the molecular weight of standard proteins are indicated (Wolff *et al*., 2007).

In conclusion, the genome of *B. subtilis* contains more than 4100 genes. In proteome study of Wolff *et al*. (2007) 1395 proteins were identified. Most of them were vegetative proteins which are synthesized in growing cells to display house-keeping functions (950 cytosolic proteins, 268 membrane proteins, 12 cell wall-bound proteins). In response to nutrient starvation 155 proteins were determined to be

induced and can be used as proteomic signatures for carbon, nitrogen or phosphate starvation. Finally, their work resulted in the detection of 113 extracellular and 21 lipid-anchored proteins, mostly expressed in non-growing cells. The total proteome information they obtained covered more than one-third of *B. subtilis* theoretical proteome. Most proteins of the main metabolic pathways, predominantly located in the proteomic window of pI 4–7, were covered by Wolff and coworkers' study (2007).

1.12. Comparative Proteomics

In comparative proteomic studies, proteins from different biological states are quantitatively compared to obtain a thorough understanding of the biological processes affecting their expression and/or in which they are involved (Cordwell *et al*., 2001). In general, comparative proteomics approach can be utilized to monitor the effects of genetic modification, to compare protein profiles of strains, different stages (phases) of growth and species of microorganisms as well as to characterizing bacterial stress responses.

Proteomics can be used to monitor the effects of gene disruption or insertion on the overall pattern of gene expression. Proteins with altered cellular levels may therefore be those that interact with the given target gene product or be a constituent of a pathway within which the gene-products plays a key role. The effects of gene knock-out on protein expression provides evidence for the localization of an unknown gene within a pathway, and as such several proposals have been suggested that involve the schematic knock-out of every gene within a given genome to provide information on the function of so-called 'hypothetical' or 'FUN' ('function unknown') genes and their corresponding gene-products (Hinton, 1997; Dujon, 1998). Elsewhere such an approach has been adopted to create a minimal gene set capable of self-replication by systematic removal of genes from *M. genitalium* genome (Hutchison *et al*., 1999).

The analysis of global protein expression in response to genetic manipulation can provide additional information with respect to protein function in association with altered cellular phenotypes (Cordwell *et al*., 2001). These types of analyses have been used to explore the mechanisms behind several pathways in bacteria, including, exopolysaccharide biosynthesis (Guerreiro *et al*., 2000), response to nutrient limitation (Eberl *et al*., 2000), inactivation of sigma-factors (Elias *et al*., 2000) and quorum-sensing (Callahan *et al*., 2000).

In *B. subtilis*, several studies have successfully used comparative proteomic methods to analyze the physiological responses of this bacterium to starvation, stress conditions and mutation. The extensive knowledge on genetics, molecular biology and physiology of this model organism was the main reason to be chosen as primary model for physiological proteomics (Hecker *et al*., 2008).

In order to identify new phosphate starvation-inducible (Psi) proteins , Antelmann *et al*., (2000) analyzed the phosphate starvation response in *B. subtilis* by using the 2-DE technique for comparison of the cytoplasmic and secreted proteins from wild-type *B. subtilis*, the *phoR* mutant, and the *sigB* mutant. In this study, two alkaline phosphatases (APases) (PhoA and PhoB), an APase-alkaline phosphodiesterase (PhoD), a glycerophosphoryl diester phosphodiesterase (GlpQ), and the lipoprotein YdhF were identified as very strongly induced PhoPR-dependent proteins secreted into the extracellular medium. In another comparative proteome study of Antelmann *et al*., (2002), the extracellular and surface proteome of the wild type *B. subtilis* 168 was compared with that of mutants lacking different extracellular and wall binding proteases as well as the alternative sigma factor SigmaD and it was found that the cell wall proteins were specific targets for the extracellular proteases and were stabilized also in a *sigD* mutant (Antelmann *et al*., 2000).

A comparative proteome study was performed by Hirose *et al*. (2000) to analyze the proteome of *B. subtilis* extracellular proteins, by comparing expression profiles of parental *B. subtilis* 168, a *secA*-temperature sensitive mutant and an *ffh* conditional mutant. They reported that approximately 100 to 110 spots were visualized in a gel of *B. subtilis* 168 extracellular proteins and over 90% and 80% of these disappeared in the absence of SecA and Ffh, respectively.

Mader *et al*. (2002) studied the gene expression profile of *B. subtilis* exponentially grown in minimal medium with and without 0.2% Casamino Acids (CAA) to provide an insight into the response of *B. subtilis* to different amino acid availabilities. In this study, it was reported that 120 genes were predominantly involved in amino acid biosynthesis, sporulation, and competence, which were downregulated in CAA-containing medium.

To characterize the pleiotropic DegS-DegU regulon, by combining proteomic and transcriptomic approaches, a comparative analysis of wild-type *B. subtilis* and the *degU32*(Hy) mutant grown in complex medium was performed by Mader *et al*., (2002). Besides genes already known to be under the control of DegU-P, novel putative members of this regulon were identified in this study.

A comparative investigation of protein expression by 2D-gel electrophoresis was undertaken using *B. subtilis* cultures grown in defined medium under aerobic, anaerobic nitrate respiration, or fermentation condition as reported by Clements *et al*. (2002). Their differential protein profiling analysis between aerobic and anaerobic conditions showed that anaerobic fermentation induced at least 44 proteins and nitrate respiration induced at least 19 proteins as compared to aerobic controls.

Comparative proteome and transcriptome analysis was combined by Eymann *et al*. (2002) in order to define the “RelA regulon” of *B. subtilis*. Comparison of protein

synthesis patterns of wild-type and *relA* mutant cells which were cultivated under conditions which provoke the stringent response revealed significant differences. Wild-type and *relA* mutant proteome and transcriptome patterns showed that 20 proteins and ca. 40 transcriptional units were negatively controlled by the stringent response, whereas 13 proteins and 50 transcriptional units seemed to be positively controlled (Eymann e*t al*., 2002).

Mostertz *et al*. (2004) analyzed the global gene expression of *B. subtilis* in response to oxidative stress in order to characterize the peroxide and the superoxide stress stimulons of *B. subtilis*. By monitoring variations in gene expression after treatment with H_2 O_2 or the O_2^- at the level of transcription using DNA macroarray hybridization and at the level of protein synthesis by 2-D gel electrophoresis. This study indicated that both stimuli strongly induced the PerR-dependent stress response and the SOS response was activated primarily by H_2 O_2, which also induced a slight Sigma B-dependent stress response (Mostertz *et al*., 2004)

For understanding of the adaptational response of *B. subtilis* to continued growth at low temperature, Budde *et al*. (2006) combined transcriptomic and proteomic approach to evaluate the changes that occur in *B. subtilis* cells cultivated at low temperature. Propagation of *B. subtilis* in minimal medium at 15 ºC triggered the induction of 279 genes and the repression of 301 genes in comparison to cells grown at 37 ºC. This study revealed that post-transcriptional regulation phenomena had a major contribution in the adaptation reaction of *B. subtilis* to growth at low temperature.

The global response of the soil bacterium *B. subtilis* to salicylic acid was analyzed by Duy *et al*. (2007) by using comparative proteomics and transcriptomics. The results demonstrated that salicylic acid caused predominantly the induction of the SigmaB-dependent general stress response in *B. subtilis* which was not related to the acidic

conditions. In another study, Duy *et al*. (2007) investigated the response of *B. subtilis* to the fungal-related antimicrobials 6-brom-2-vinyl-chromanon (chromanon) and 2-methylhydroquinone (2-MHQ) using proteome and transcriptome analyses. Authors reported that chromanon, a derivative of aposphaerins from *Aposphaeria* species caused predominant protein damage in *B. subtilis* as indicated by the induction of the HrcA, CtsR, and Spx regulons. The expression profile of the ganomycin-related substance 2-MHQ was similar to that of catechol as reflected by the common induction of the thiol-specific oxidative stress response. Several putative ring-cleavage dioxygenases and oxidoreductases were differentially up-regulated by 2-MHQ, catechol, and chromanon including *yfiDE*, *ydfNOP*, *yodED*, *ycnDE*, *yodC*, and *ykcA*.

In their study, Mostertz *et al*. (2008) showed that a comparative proteomics approach was a useful tool to gain new information on the role of TrxA (thioredoxin A) protein and its integration into the metabolism. The depletion of TrxA during growth on sulfate as the sole sulfur source was shown to cause a significant decrease of growth, the massive induction of genes involved in sulfur utilization and minor up-regulation of the PerR-dependent oxidative stress response at the proteome and at the transcriptome level. In addition, this study provided experimental evidence that TrxA was dispensable for growth with alternative sulfur sources. Mostertz *et al*. (2008) suggested that the lack of TrxA in cells grown on sulfate leads to a limitation of the building blocks for protein synthesis, i.e., cysteine and methionine, which caused a distinct decrease of growth followed by the activation of sporulation and the induction of the stringent response.

Kouwen *et al*. (2009) investigated the impact of a membrane bound protein MscL, on *B. subtilis* secretome biogenesis, by comparing the extracellular proteomes of MscL deficient and proficient strains during normal growth and under hypo-osmotic shock conditions. It has been shown that the MscL protein did not contribute directly to the

biogenesis of the *B. subtilis* exoproteome. Instead, MscL prevented the excessive release of particular proteins under conditions of hypoosmotic shock in an unprecedented manner. This showed that *B. subtilis* contained a system for the release of cytoplasmic content, including proteins, under conditions of fluctuating osmolarity.

1.13. The Aim of the Present Study

Under the light of above-mentioned findings related with bacilysin biosynthesis and *B. subtilis* proteomics, the present research aimed to identify the functional role of bacilysin biosynthesis in the regulatory cascade operating in *B. subtilis* by employing comparative proteome-wide analysis of the bacilysin producer *B. subtilis* PY79 and its bacilysin non-producer derivative *bacA::lacz::erm* OGU1 strain which was recently constructed by our group. This study provided better understanding of interactions between bacilysin biosynthesis and the regulatory pathways and the effects of antibiotic production on expression of the genes with unknown functions.

CHAPTER 2

MATERIALS AND METHODS

2.1. Bacteral Strains

The strains of *B. subtilis* used in this study were the prototrophic wild-type strain PY79 (Youngman *et al*., 1984) and *bacA::lacz::erm* strain OGU1 the latter being bacilysin non-producer strain which was constructed by our group (İ. Öğülür, unpubl. results).

2.2. Culture Media

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

2.3. Buffers and Solutions

Composition and preparation of buffers and solutions are given in Appendix B.

2.4. Chemicals and Enzymes

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

2.5. Maintenance of Bacterial Strains

B. subtilis strains were grown in Luria-Bertani (LB) liquid medium and kept on Luria-Bertani (LB) agar plates. All cultures were stored at 4°C and sub-cultured monthly. 20 % glycerol stock was prepared for each strain and kept at -80°C.

2.6. Growth Conditions and Protein Extraction

B. subtilis PY79 and OGU1 strains were grown overnight at 37°C on Luria-Bertani (LB) agar plates (Appendix A). Then, they were used to inoculate 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_{595}). After inoculation, the cultures were incubated at 37°C (200 rpm) for 16 h.

For the extraction of total soluble proteins, 5 mL of culture was centrifuged at 4000 rpm for 15 min. The supernatant was decanted, and the pellet was washed twice with cold TE buffer (0.2 mM EDTA, 2 mM Tris-HCl) and centrifuged at 10000 rpm for 5 min at room temperature (Eppendorf, Germany). The supernatant was then discarded, the remaining pellet was re-suspended in lysozyme (in 0.04 M TE buffer) and incubated at 37˚C for 60 min. After centrifugation at 13000 rpm for 10 min at room temperature, 8 M urea was added onto the pellet, vortexed at 4˚C until it was dissolved completely and centrifuged at 13000 rpm for 10 min after which the pellet was discarded. Protein samples were stored at -20˚C at most for 10 days till usage. The proteins obtained by using this procedure represented total cellular proteins since the debris contained whole soluble protein complements of the cells.

2.7. Protein Estimation

To determine total protein concentration, the modified Bradford assay described by Ramagli and Rodrigez (1985) was used. (Appendix B) The Bradford assay, as a colorimetric protein assay, is based on an absorbance shift in the dye Coomassie when the previously red form of Coomassie reagent changes and stabilizes into Coomassie blue by the binding of protein. 5X Bradford Reagent (containing 500 mg Coomassie Brillant Blue G-250, 250 ml of 96% ethanol and 500 ml of 85% ortho-phosphoric acid; completed to a 1 L with dH2O) was diluted 1:4 with dH_2O and filtered at least three times using Whatman No. 1 filter paper. Standards and samples were prepared as shown in Table 2.1 in duplicate.

Blank was prepared by using the buffer in which the proteins solubilize (8M Urea) and absorbance was measured at 595 nm. Calibration curve of absorbance versus protein concentration in microgram was drawn. 1 mg/ ml Bovine Serum Albumin (BSA) fraction number V was used as a standard for the construction of calibration curves.

2.8. Proteome Study

2.8.1. Isoelectric Focusing

IPG strips (17 cm, pH 4–7, Bio-Rad; 18 cm, GE Healthcare Immobiline DryStrip pH 4.5-5.5 and 18 cm, GE Healthcare Immobiline DryStrip pH 5.5-6.7) were passively rehydrated by applying 350 µl of rehydration buffer (8M urea, 2M thiourea, 2% w/v CHAPS, 28mM DTT and 0.5% v/v ampholyte 4-7) containing 400 µg protein sample for 14 h at room temperature (20-25˚C) with mineral oil coverage. Isoelectric focusing (IEF) was performed on the Protean IEF Cell (Bio-Rad USA). Rehydrated strips were taken to the IEF process by applying the following voltage profile: 1 h

100V; 1 h 300 V; 1 h 600V; 1 h 1000 V; 2 h 3000 V; 2 h 5000V followed by a linear increase to 8000 V. The final phase of 8000V was terminated after 50,000 Vh.

2.8.2. SDS-PAGE

The IPG strips were equilibrated for 15 min each in 3 mL of solution 1 (6M urea, 50mM Tris–HCl (pH 8.8), 30% v/v glycerin, 2% w/v SDS, 50mg DTT) and then in 3 mL of solution 2 (6M urea, 50mM Tris–HCl (pH 8.8), 30% v/v glycerin, 2% w/v SDS, 225mg
iodoacetamide) (Görg *et al*., 2004). The isolated proteins were separated in 12% acrylamide/bis-acrylamide gels with a Bio-Rad Cell system (Bio-Rad, USA), applying approximately 25mA per gel.

To visualize the separated proteins, each gel was stained with colloidal Coomassie blue according to Neuhof *et al*. (1988) (Appendix B).

2.8.3. Evaluation of 2-DE Data

Coomassie stained gels were scanned by using a scanner (HP Scanjet 4070 Photosmart scanner, USA). CCB-stained gels were stored in a little amount of water in dark at 4˚C for protein analysis. Spot pattern analyses and labeling of the spots to be cut were accomplished by using the 2D image analysis software Delta 2D version 3.3 (Decodon, Germany).

2.8.4. Sample Preparation and Analysis by Mass Spectroscopy

The identification of protein spots were accomplished by using mass spectrometry according to the established protocols. Proteins were cut from the gel and sent to the Greifswald University (Germany) for MALDI-TOF/MS analysis. Briefly, protein spots were excised from CBB-stained 2-D gels, destained and digested with trypsin (Promega, Madison, WI, USA) and for extraction of peptides, the gel pieces were covered with 60 µl 0.1% trifluoroacetic acid in 50% CH3CN and incubated for 30 min at 40˚C. Peptide solutions were mixed with an equal volume of saturated α-cyano-3-hydroxycinnamic acid solution in 50% acetonitrile-0.1% trifluoroacetic acid (v/v) and applied to a sample plate for MALDI-TOF-MS. Mass analyses were carried out on the Proteome-Analyzer 4700 (Applied Biosystems, Foster City, CA, USA). The three most abundant peptides in each MS spectrum were chosen for MS/MS experiment. The resulting sequence data were included for the database search to increase the reliability of protein identification. Mass accuracy was usually in a range between 10 and 30 ppm.

2.8.5. Protein Identification

B. subtilis whole genomic DNA sequence data which are available in the organism's genome project web address (http://genolist.pasteur.fr/SubtiList/) have already been translated to amino acid sequences in FASTA format and has been published on November 20, 1997 in the study of Kunst *et al*. (1997). The peak lists of each protein spot (peptide mass fingerprint and MS/MS data) obtained from MALDI-TOF MS measurement were analyzed with the aid of "Peptide Mass Fingerprint" and "MS/MS Ion Search" engines of MASCOT software (Matrix Science Inc., Boston, MA, USA) against the *B. subtilis* PMF database. The searches considered oxidation of methionine and modification of cysteine by carbamidomethylation as well as partial cleavage leaving one internal cleavage site. Of the results given by the

MASCOT software, those having a protein score value higher than 98 were considered for protein identification. To find out putative functions, protein accession numbers of the identified spots which were included in the MASCOT output were searched in the website of the *Bacillus subtilis* genome sequencing project (Subtilist Web Server) and Protein Knowledgebase (UniProtKB) (http://www.uniprot.org/). UniProtKB is a curated protein sequence database which strives to provide a high level of annotation (such as the description of the function of a protein, protein families, its domains structure, post-translational modifications, variants, etc.), a minimal level of redundancy and high level of integration with other databases. For the proteins which were certainly assigned to the particular genes, i.e. the identified ones, the functional classification was made by consulting to the functional categories list contained in the website of the *B. subtilis* genome sequencing project (Subtilist Web Server).

2.8.6. Bioinformatic analyses

For each identified protein, subcellular localization predictions were obtained from PSORT (http://wolfpsort.seq.cbrc.jp/) by submitting amino acid sequences of the identified proteins to this server. The existence of signal peptide sequences was checked in the public web server of signal peptide prediction program SignalP version 3.0 (http://www.cbs.dtu.dk/services/SignalP/).

CHAPTER 3

RESULTS AND DISCUSSION

3.1. Construction of *B. subtilis* OGU1 strain

For construction of strain OGU1, integral DNA fragment of *bacA* gene was amplified by PCR with the following specific primers: *bacA*-F (5'-GCC **AAG CTT** ATG ATT ATA TTG GAT AAT- 3') and *bacA*-R (5'- GC**G GAT CC**G GAT AAA TAT TTT ATT AAA- 3'). These primers had the *HindIII* or *BamHI* site as indicated by boldface letters. This fragment was ligated into the corresponding restriction sites in pMutinT3, resulting in *bacA::lacZ* fusion into the vector.

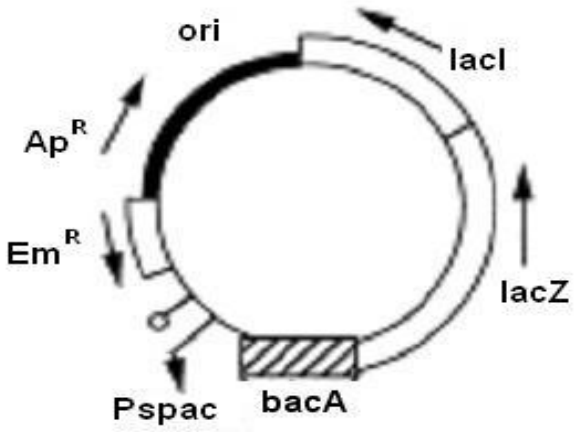


Figure 3.1. The construct of *bacA::lacZ* fusion.

Resulting recombinant plasmid (Fig. 3.1) was used to transform *B. subtilis* PY79 to Macrolide-lincosamide-streptogramin B-resistance (MLSR), thus generating strain

OGU1. Recombinant plasmid was driven into the chromosomal DNA of *B. subtilis* PY79 by a single cross over event (Campbell like insertion).

3.2. Theoretical Protein Map

Based on theoretical calculations, separation of proteins in one single 2-DE gel with a pH range of 4–7 would cover 2/3 of all *B. subtilis* proteins (Eymann *et al*., 2004). To determine the optimal range for standard 2-D gel-based proteome analysis of *B. subtilis*, the theoretical 2-D gel map of *B. subtilis* has been constituted using genomic data from GELBANK database (http://gelbank.anl.gov/cgi-bin/2dgels/gel_insilico.pl) (Figure 3.2). This theoretical map revealed the presence of 4105 records of proteins in a pI range of 3 to 13 for 2-D gel based proteome analysis of *B. subtilis*.

The pI range for *B. subtilis* proteins is between pH 3.6 (for hypothetical protein BSU27380) and 12.9 (for ribosomal protein L34). Within this p*I* range, the molecular weights of proteins vary from 2,297 Da (for tetracycline resistance leader peptide) to 609,594 Da (for polyketide synthase of type I).

When the theoretical map was drawn for pH 4-7 range, it was revealed that the map covered 65 % of the whole theoretical proteome (Figure 3.2 and Figure 3.3). Since the 65 % of coverage is sufficent for a proteome research, it was preferentially chosen in the present study. However, the protein profile displayed in 3.4, clearly contains regions of spot crowding in which separation and thus unequivocal spot identification is compromised. Therefore, in addition to pI range between 4 and 7, two different narrow pI ranges were used (pH 5.5–6.7 and 4.5–5.5) to increase resolution and spot identification in these overcrowded regions.

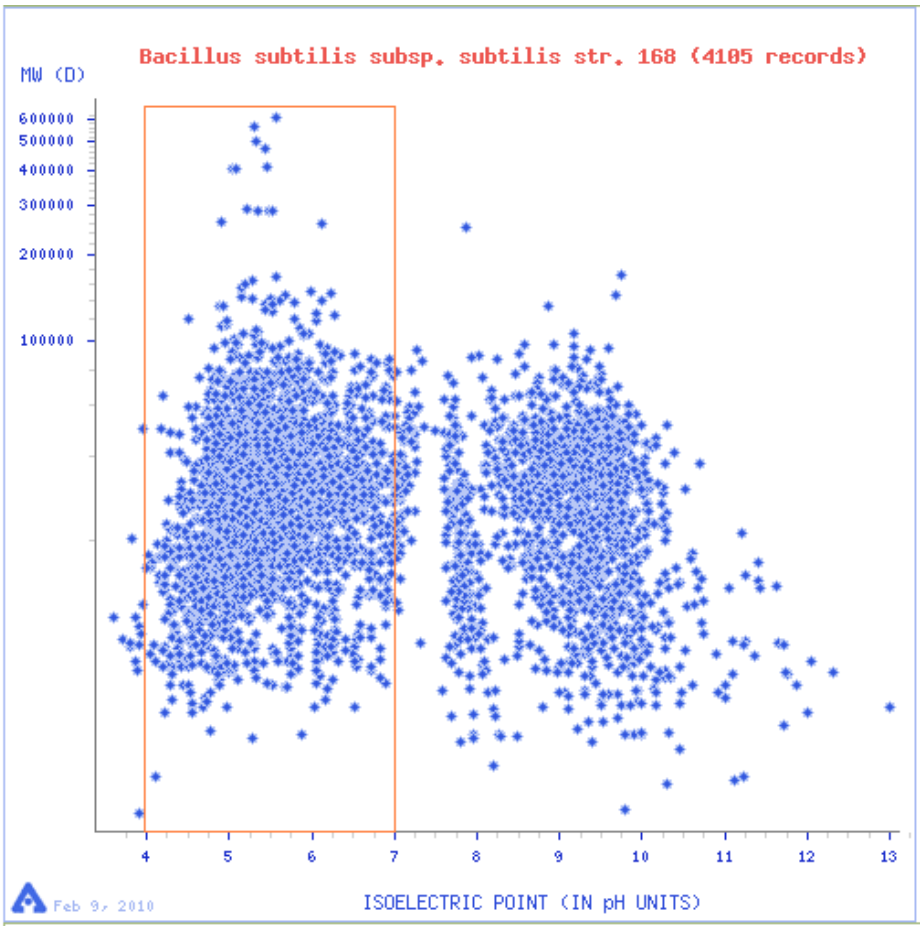


Figure 3.2. A theoretical map of *B. subtilis* 168 strain proteome; predicted a 2-D gel using isoelectric point (in pH units) vs. molecular weight (in Dalton). The orange colored rectangle indicates the margins of the master gels that were used in this study (http://gelbank.anl.gov/cgi-bin/2dgels/gel_insilico.pl).

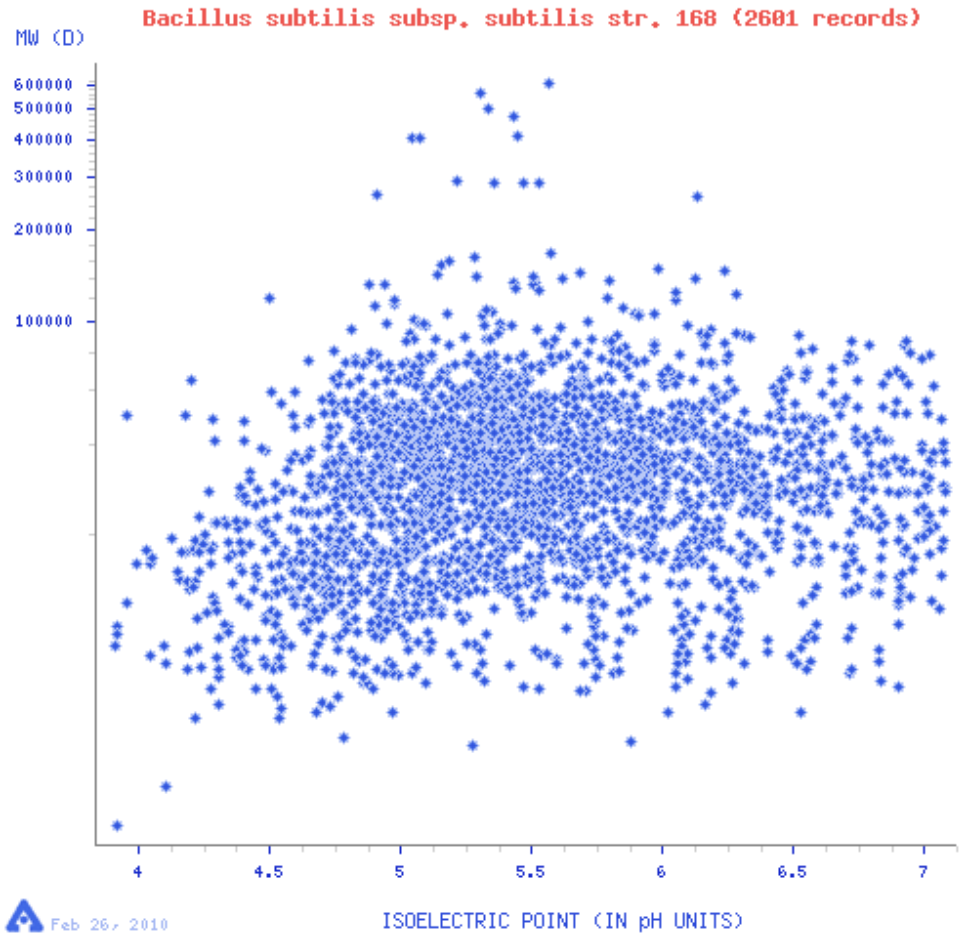


Figure 3.3. A theoretical map of *B. subtilis* 168 strain proteome in pI 4-7 range (http://gelbank.anl.gov/cgi-bin/2dgels/gel_insilico.pl).

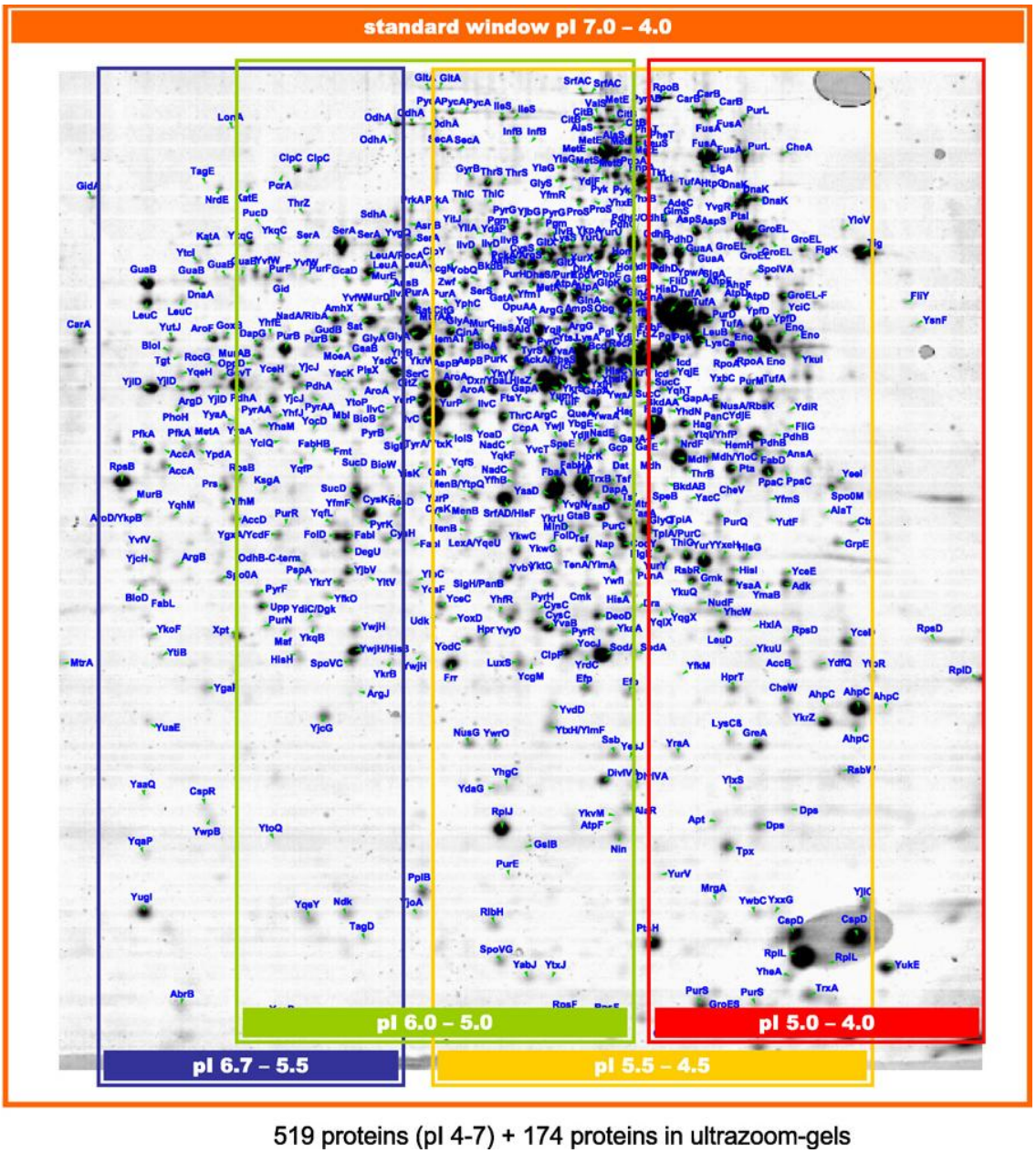


Figure 3.4. The cytoplasmic vegetative proteome map of *B. subtilis* in the standard pH range (4–7) and in the narrow pH ranges (4.0–5.0, 4.5–5.5, 5.0–6.0, 5.5–6.7) (Wolff *et al*., 2007).

3.3. Master Gels of *B. subtilis* Strains

The use of proteomics as a tool for understanding the physiology of bacterial cells under different conditions requires the development of master gels (Voigt *et al*., 2004). Therefore, we created master gels for proteins with isoelectric points between 4-7, 4.5-5.5 and 5.6-6.7 for wild-type *B. subtilis* PY79 and bacilysin bisynthesis-blocked *B. subtilis* strain OGU1, respectively. The master gels were then used to compare protein profiles of wild-type and bacilysin inactive strains.

B. subtilis OGU1 strain containing a transcriptional *bacA-lacZ* fusion at *bacA* locus was previously constructed by our group and proved to be bacilysin inactive due to the insertional inactivation of *bac* operon with bacilysin activity assays (İ. Öğülür, unpubl. results). In the same study, it was shown that bacilysin biosynthetic activity of wild-type *B. subtilis* PY79 strain was constantly increasing during exponential growth (detected as 20-30 Miller units) but maximally increased at the transition between exponential and stationary phase and reached to its maximal level (detected as 130 Miller units) at 16^{th} hour (I.Öğülür, unpubl. results). In this study, our aim was to understand the interactions between bacilysin biosynthesis and the regulatory pathways operating in *B. subtilis*, therefore, the cells were collected for protein extraction at 16^{th} h of incubation. The soluble protein fraction of *B. subtilis* was then visualized by using 2-DE approach by using IPG strips in a pH range 4.5-5.5, 4-7 and 5.5-6.7 as the first dimension.

A total of 1986 protein spots could be separated and detected on Coomassie-stained 2-D gels of *B. subtilis* in three pI ranges (1100 for pI 4-7, 243 for pI 4.5-5.5 and 643 for pI 5.5-6.7) by the help of Delta2D (Decodon, Germany) image analysis software. However, since we were only interested in differentially expressed proteins (2.5 fold or more) between two strains, a total of 192 spots were selected and cut from master

gels. Figures from 3.5 to 3.10 show the colloidal CBB-stained master gels for *B. subtilis* PY79 and OGU1 strains.

Figure 3.5. 2-D master gel of the total soluble proteome of *B. subtilis* PY79 in a pI range of 4-7.

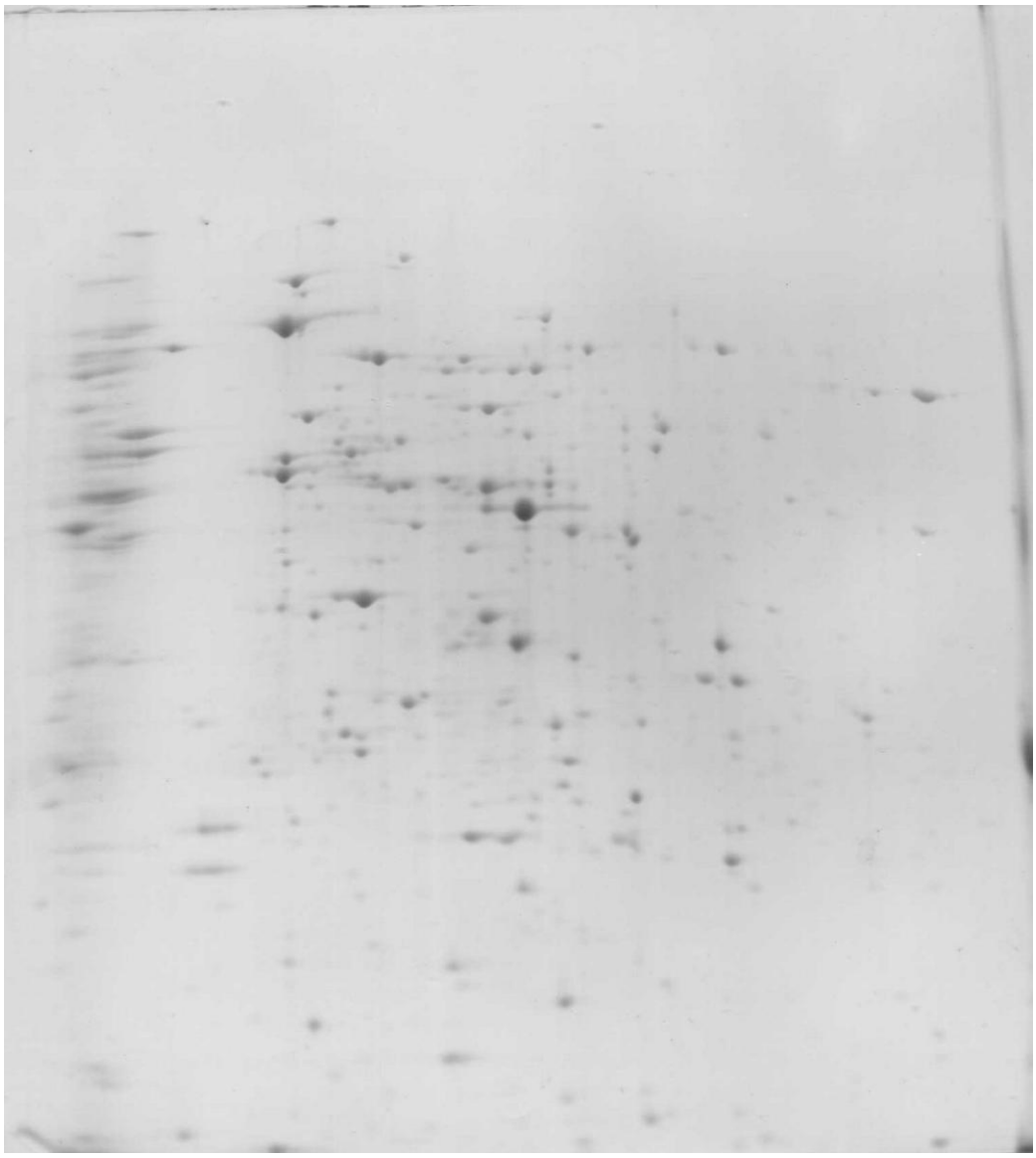


Figure 3.6. 2-D master gel of the total soluble proteome of *B. subtilis* OGU1 in a pI range of 4-7.

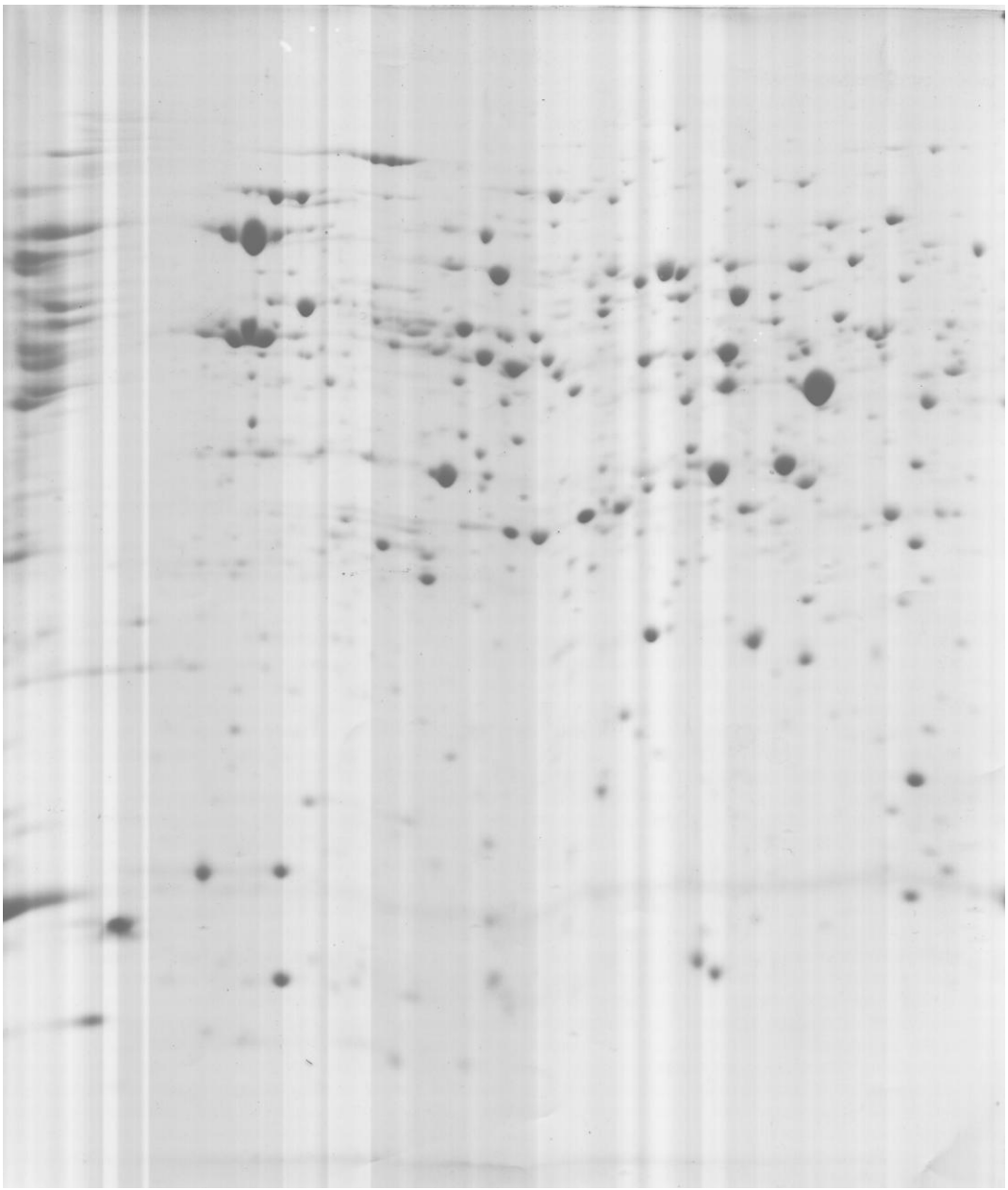


Figure 3.7. 2-D master gel of the total soluble proteome of *B. subtilis* PY79 in a pI range of 4.5-5.5.

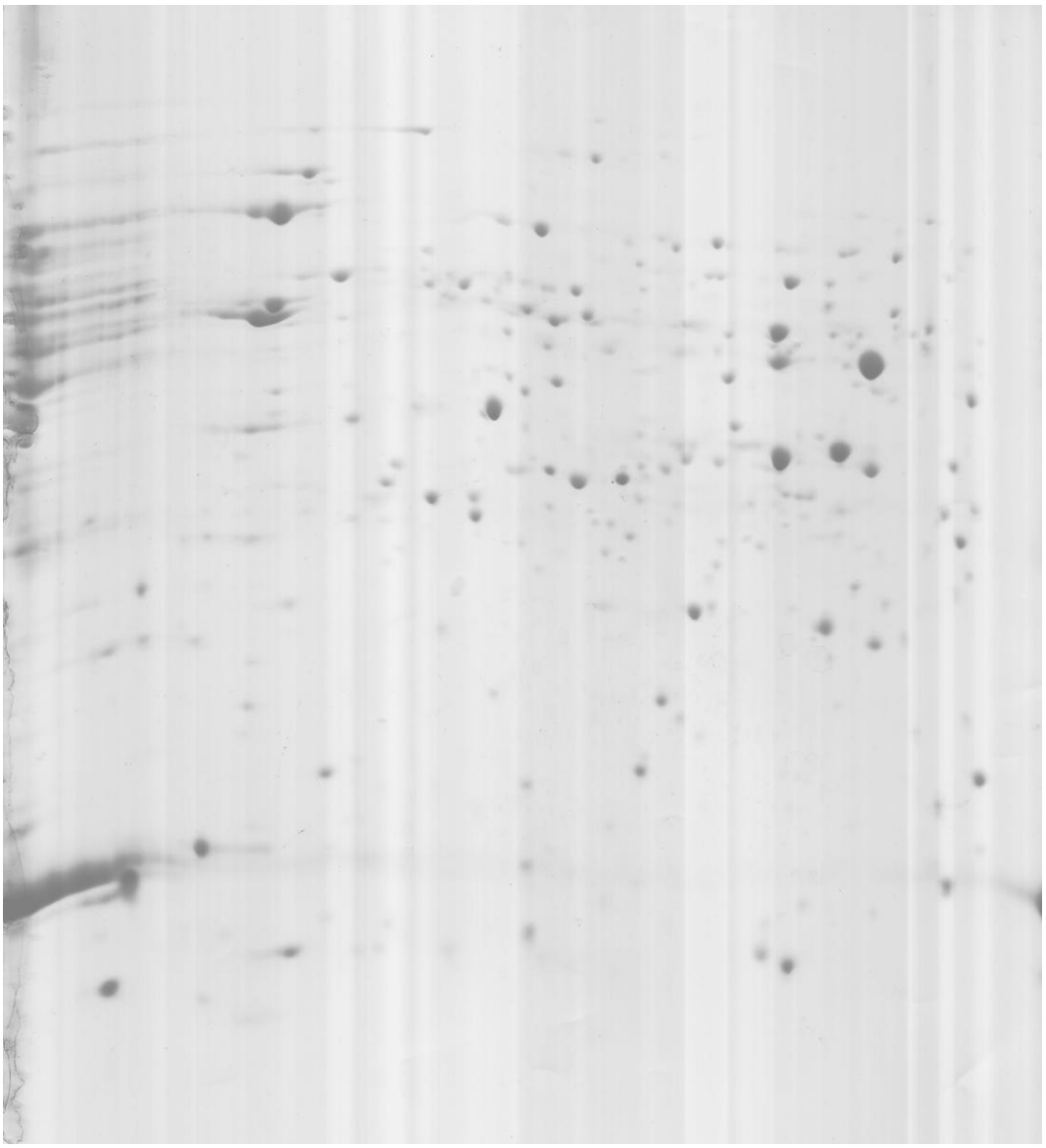


Figure 3.8. 2-D master gel of the total soluble proteome of *B. subtilis* OGU1 in a pI range of 4.5-5.5.

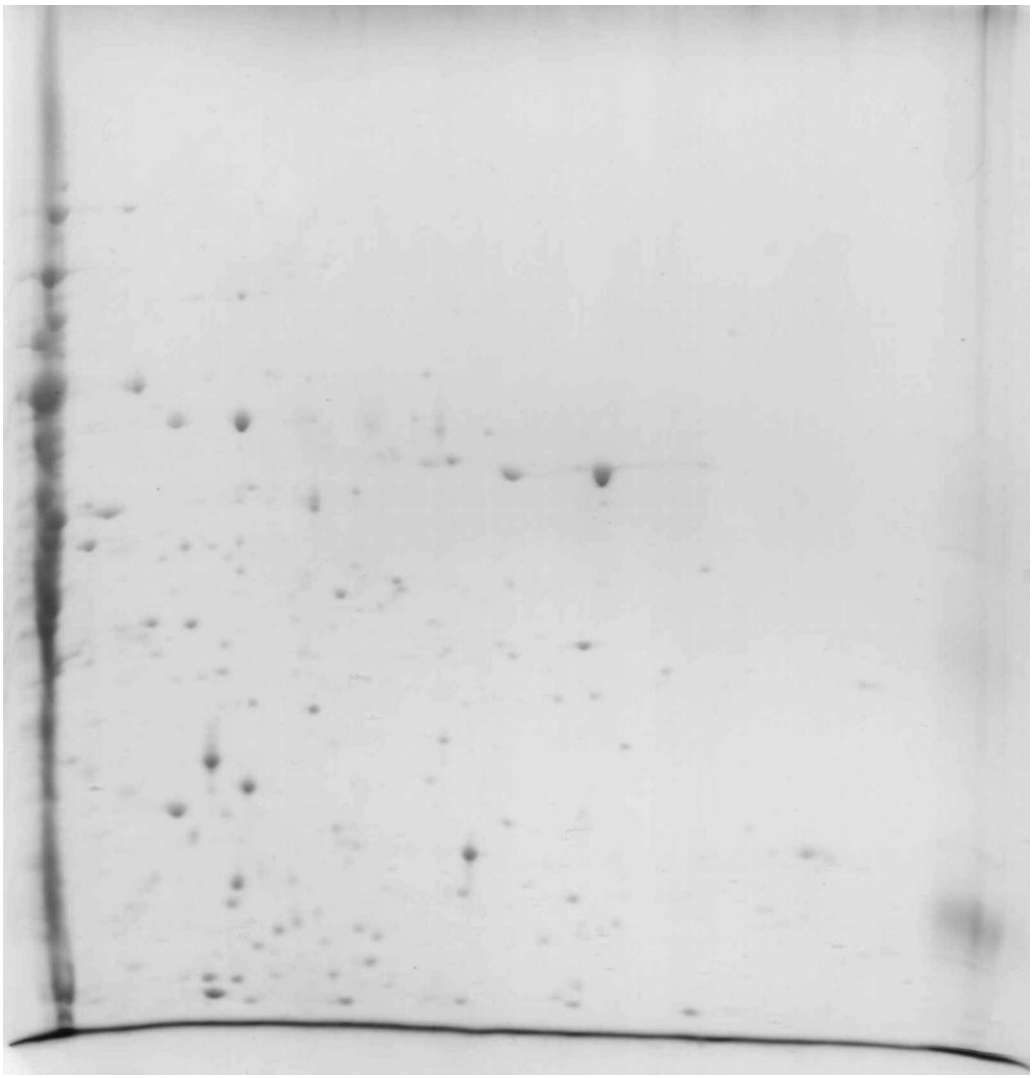


Figure 3.9. 2-D master gel of the total soluble proteome of *B. subtilis* PY79 in a pI range of 5.5-6.7.

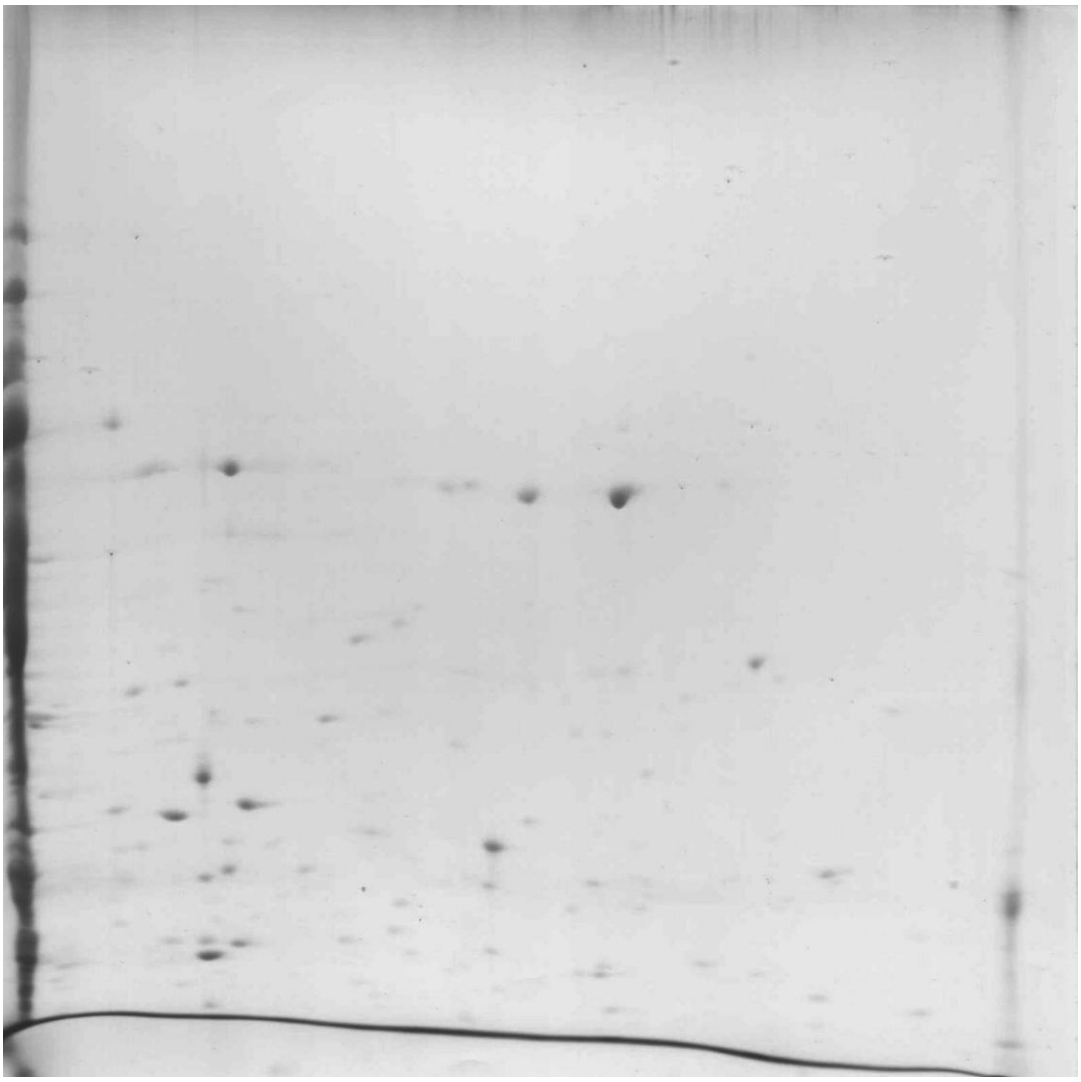


Figure 3.10. 2-D master gel of the total soluble proteome of *B. subtilis* OGU1 in a pI range of 5.5-6.7.

3.4. Identification of Differentially Expressed Proteins by MALDI-TOF MS

Molecular mechanisms for the interaction of the elements of quorum sensing and stringent response systems with the genes responsible for bacilysin biosynthesis were investigated by previous studies (Yazgan *et al*., 2001; Karatas *et al*., 2003; Inaoka *et al*., 2003). To explore such interactions and to further investigate interactions between the existing the regulatory mechanisms and bacilysin biosynthesis, we performed a proteomic analysis of differentially expressed proteins in *bacA* inactive OGU1 strain as compared to the wild-type PY79 strain.

For the analysis of different three pI ranges, 2D gels of the harvested cells were run in duplicates for control and each strain. Function of Delta2D software (Decodon, Germany) was used for the comparison of the protein spots from the mothere and mutant cells. The proteins with levels (relative spot intensities) increased or decreased by at least 2.5-fold as compared to the control cells were selected as differentially expressed ones. To correct the quantitative variability, the spot volumes were normalized as a percentage of the total volume in all of the spots in the gel. SDs of the spot intensities from the two replicates were in the range of 20%. For pI ranges 4-7, 4.5-5.5 and 5.5-6.7, selected spots for MS analysis are shown in Figures 3.11, 3.12, 3.13 respectively.

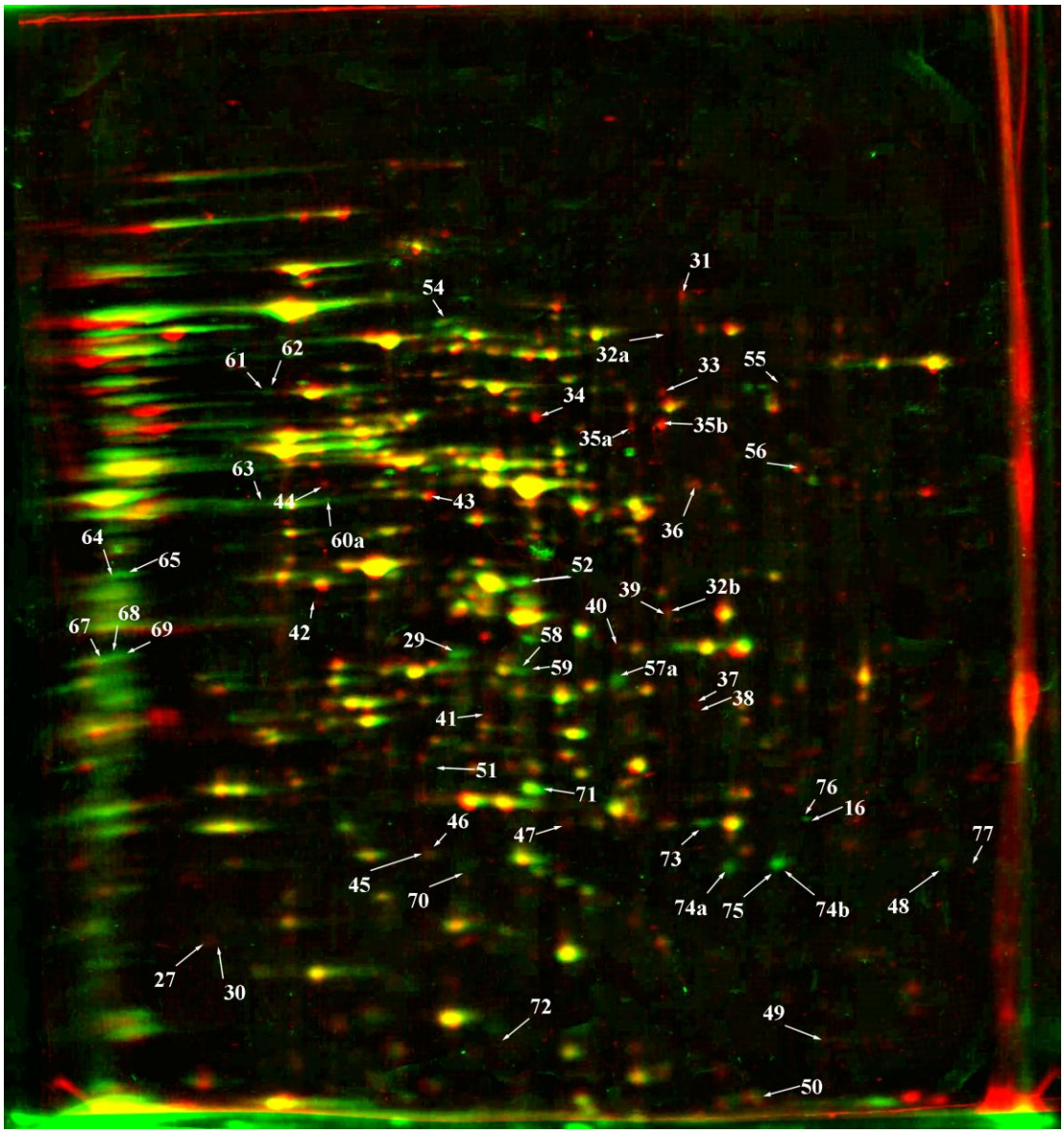


Figure 3.11. Dual channel 2-D imaging of the differentially expressed proteins in a pI range of 4-7.

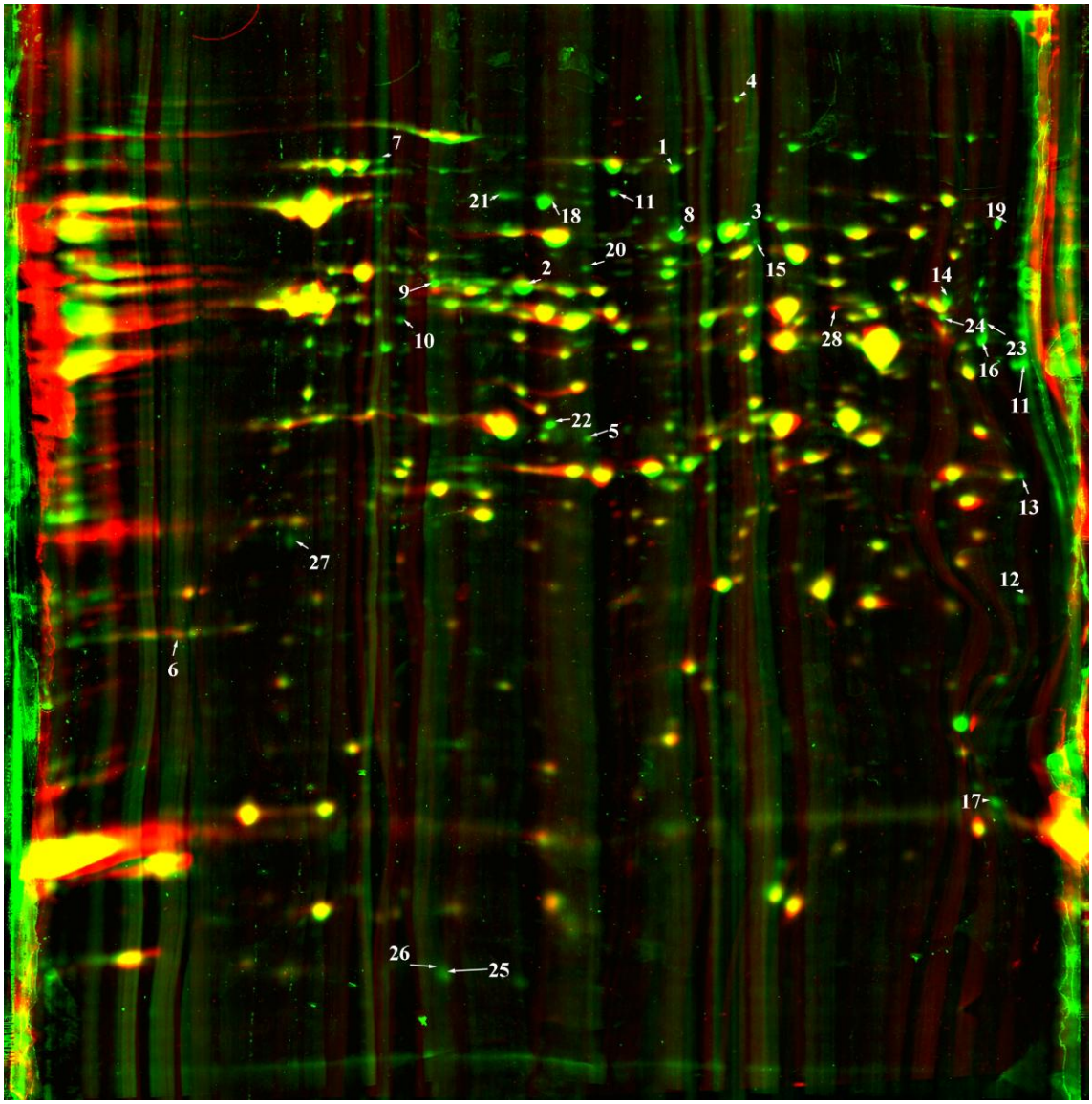


Figure 3.12. Dual channel 2-D imaging of the differentially expressed proteins in a pI range of 4.5-5.5.

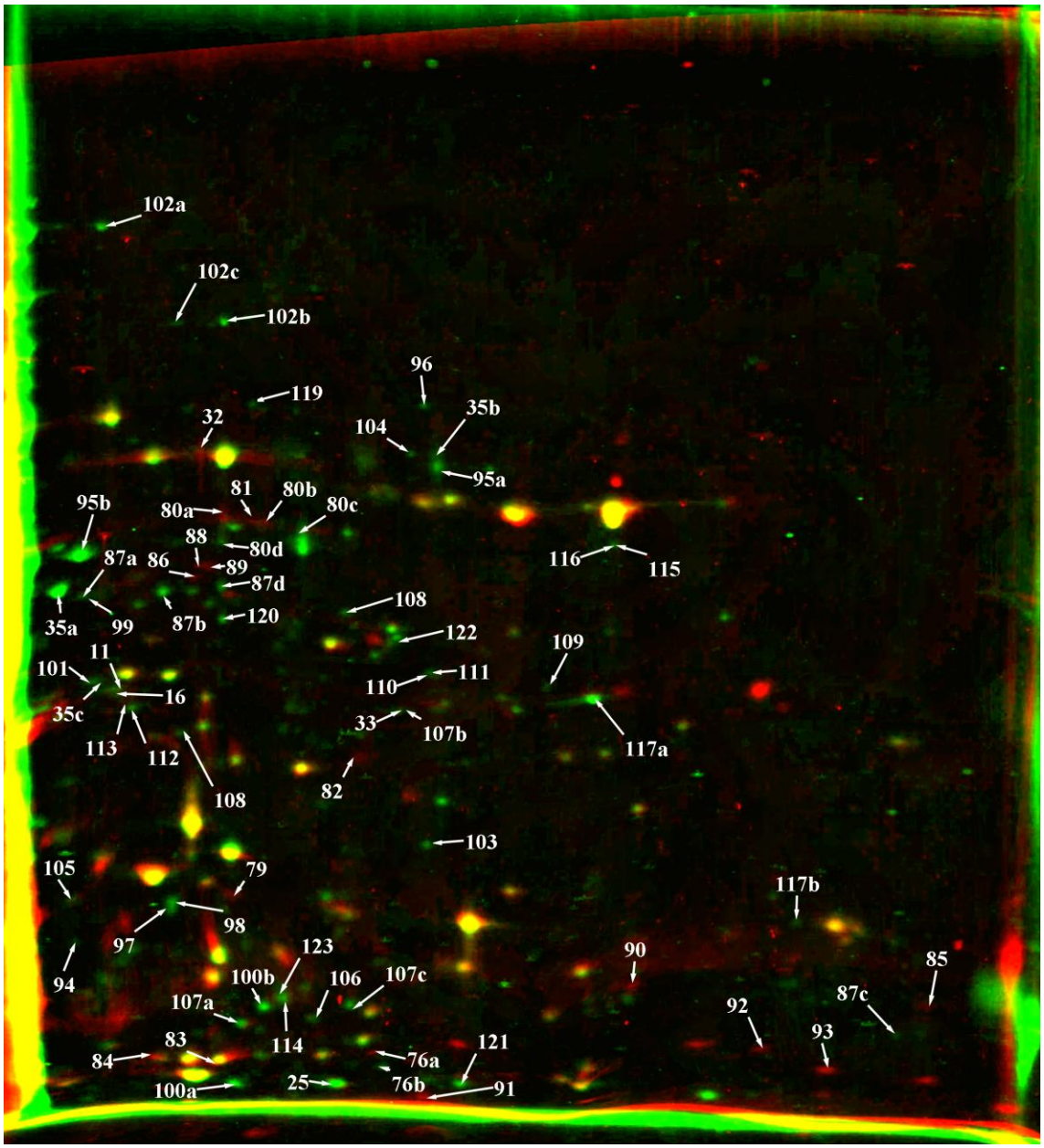


Figure 3.13. Dual channel 2-D imaging of the differentially expressed proteins in a pI range of 5.5-6.7.

The separation of the pI range 4.5-5.5 allowed detection of 243 protein spots in each gel. Among these, the quantitative spot intensity analysis revealed 45 differentially expressed protein spots between the wild-type PY79 and *bacA* inactive OGU1. Out of these, 29 spots could be identified by MALDI-TOF MS which corresponded to 28 different gene products and their identities along with the quantitative data are shown in Table 3.1.

In pI range 4-7, a total of 1100 protein spots were visualized in each gel. Among these, quantitative spot intensity analysis revealed 57 differentially expressed protein spots between the wild-type PY79 and *bacA* inactive OGU1. Out of these, 42 protein spots could be identified by MALDI-TOF MS which corresponded to 58 proteins and 51 different gene products. Their identities as well as quantitative data are shown in Table 3.1.

Ultrazoom gels covering the pI range 5.5-6.7 facilitated the visualization of 643 protein spots. Of these spots, 69 were found to be differentially expressed. After MALDI-TOF/MS analysis, 57 of 69 protein spots were identified which corresponded to 72 proteins and 53 different gene products. The identities and quantitative data of identified spots are shown in Table 3.1.

In this study, another experiment was conducted with different protein concentrations, the quantitative spot intensity analysis of differentially expressed proteins in the mutant strain in pI range 4-7 allowed detection of 21 differentially expressed protein spots between the wild-type PY79 and *bacA* inactive OGU1. After MALDI-TOF/MS analysis, 10 of 21 protein spots were identified which corresponded to 9 different gene products (data not shown). In addition to the differentially expressed proteins tabulated in Table 3.1, DNA repair protein (RecA), cell wall biosynthesis protein UDP-N-acetylmuramoylalanyl-D-glutamyl-2,6-diaminopimelate-D-alanyl-D-alanine ligase (MurF), amino acid metabolism protein

aspartokinase I (DapG) were found to be down-regulated in the mutant strain while peptidyl-prolyl isomerase (PpiB) and ornithine acetyltransferase (ArgJ) were found to be up-regulated and/or newly induced in bacilysin-minus strain, respectively. (data not shown)

As a result, out of a total of 192 selected spots, 166 proteins could be identified. Further analysis of the data indicated that the number of distinct ORFs was 128, after excluding the ones occurring as two or more spots. In other words, 128 different gene products (distinct ORFs) could be characterized from 166 protein spots that gave statistically significant scores. Of 128 ORFs, as many as 14 proteins yielded multiple spots on the master gel. Multiple spots can be attributed either to post-translational modifications or to artificial chemical modifications occurring during protein preparation (Büttner *et al*., 2001).

Our findings on 192 differentially expressed proteins out of a totally visualized 1986 spots might not be found to reflect whole picture. However, we selected the most accurate proteins that were 2.5-fold or more differentially expressed in each gel. Nevertheless, our master gel was stained with CBB, which is less sensitive than silver, so that the total number of differentially expressed protein spots in the master gels might have been underestimated. Also there may be other possibilities that differentially expressed proteins may be in low abundance and/or smaller than 10 kDa or larger than 100 kDa which will probably bottom out the gel or will not migrate into the 2D-gel, respectively. In addition, some proteins could not be identified from the gel due to protein comigration, which results in masking of small protein spots by higher abundant proteins (Wolff *et al*., 2007).

The proteins detected to be differentially expressed in PY79 and its *bac*$^{-}$ constructed strain and their abundance in each of the pI ranges are shown in the venn diagram presented in Fig. 3.14.

It is known that under the IEF/SDS-PAGE conditions, proteins are separated according to two parameters, isoelectric point and molecular weight, that can be deduced from their amino acid composition. Migration of a known protein can be predicted from its sequence. Generally predictability of protein migration is ± 0.15 pH units for migration on the IEF gel and ± 5000 daltons for migration on the SDS gel (Boucherie *et al*., 1995; Garrels *et al*., 1997; Link *et al*., 1997). The difference between the expected migration of an identified protein and its observed 2-D gel location may be used for investigating post-translational modifications (Rabilloud, 2000). In the present study, the analysis of theoretical versus experimental pI values indicated the existence of 14 protein spots whose horizontal migration differed from the expected migration according to the calculated pI values. The deviation from the theoretical pI values of these protein spots can be the result of post-translational charge modifications in *B. subtilis* cells. The proteins subjected to charge modifications are shown in Table 3.1.

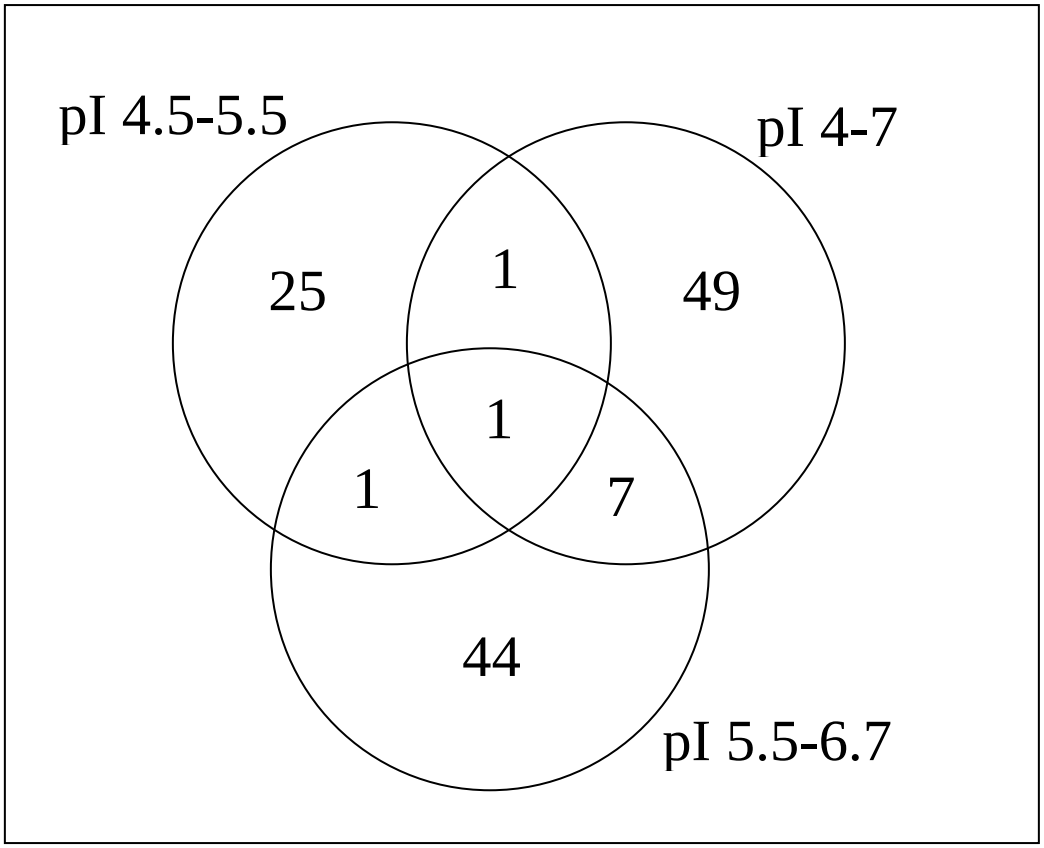


Figure 3.14. The proportions of number of proteins identified in each of the pI ranges

When the theoretical Mr values were compared with those experimentally observed, the data revealed the existence of 11 spots with an Mr that was different from the theoretical value, suggesting a major change in the protein mass due to post-translational modifications or to artificial chemical modifications occurring during protein preparation (Büttner *et al*., 2001). The proteins subjected to mass modifications are shown in Table 3.1.

Table 3.1. Differentially expressed proteins of the total proteome of *B. subtilis* PY79 compared to OGU1.

No	Locus Name	Protein Name/ Function	Gene Product	Mass (kDa)	pI	Subcellular[a] Localization	Signal Peptide[b]	Multiple Spots	Modification*	pI Range of Detection	Mutant/Control Ratio
1	BSU16690	Polynucleotide phosphorylase	PnpA(ComR)	77,415	5,08	C	-	-	-	4,5-5,5	0,182
2	BSU01130	Elongation factor Tu	TufA	43,565	4,92	C	-	-	-	4,5-5,5	0,270
3	BSU38500	D-alanyl –D-alanine carrier protein ligase	DltA	55,773	5,10	C	-	-	-	4,5-5,5	0,411
4	BSU18000	Aconitate hydratase	CitB	99,271	5,09	C	-	-	-	4,5-5,5	0,345
5	BSU19390	Unknown similar to nitric-oxide reductase/probable D-alanine amino transferase	YojN/ Dat	33,459	5,16	CM	-	-	-	4,5-5,5	0,232
6	BSU40090	Alkyl hydroperoxide reductase (small subunit)(General stress protein 22)	AhpC	20,614	4,48	C	-	-	-	4,5-5,5	2,557
7	BSU25470	Class I heat-shock protein (molecular chaperone)	DnaK	65,961	4,76	C	-	-	-	4,5-5,5	N.D.**
8	BG14010	Unknown similar to unknown proteins	YurX	48,263	5,12	U	-	-	-	4,5-5,5	N.D.**
9	BSU29980	Unknown similar to Xaa-His dipeptidase	YtjP	51,020	4,85	C	-	-	-	4,5-5,5	N.D.**
10	BSU01430	RNA polymerase (alpha subunit)	RpoA	34,778	4,8	C	-	-	-	4,5-5,5	N.D.**
11	BSU28290	Ketol-acid reductoisomerase	IlvC	37,434	5,49	C	-	2	C-M	4,5-5,5	N.D.**
								-	-	5,5-6,7	0,322
12	BSU22230	Unknown similar to phosphotransferase system enzyme II	YpqE	17,924	5,43	C	-	-	-	4,5-5,5	N.D.**

Table 3.1 Continued

13	BSU34870	HisF cyclase-like protein	HisF	27,277	5,39	C	-	-	-	4,5-5,5	N.D.**
14	BSU31930	L-alanine dehydrogenase	Ald (SpoVN)	39,658	5,28	C	-	-	-	4,5-5,5	N.D.**
15	BSU24060	Probable branched-chain alpha-keto acid dehydrogenase E3 subunit (dihydrolipoamide dehydrogenase)	LpdV (BfmBC)	48,644	5,07	C	-	-	-	4,5-5,5	N.D.**
16	BSU29750	3-deoxy-D-arabino-heptulosonate 7-phosphate synthase / chorismate mutase-isozyme 3	AroA	39,514	5,48	C	-	-	-	4,5-5,5	N.D.**
								-	-	5,5-6,7	0,322
								-	-	4-7	0,074
17	BSU36800	ATP synthase (subunit epsilon)	AtpC	14,200	5,41	CM	-	-	-	4,5-5,5	N.D.**
18	BSU01780	L-glutamine-D-fructose-6-phosphate amidotransferase	GlmS (GcaA, YbxD)	65,296	4,99	C	-	-	-	4,5-5,5	N.D.**
19	BSU21870	Dihydroxy-acid dehydratase	IlvD	59,510	5,44	C	-	-	-	4,5-5,5	N.D.**
20	BSU34910	Histidinol dehydrogenase	HisD	46,227	4,96	C	-	-	-	4,5-5,5	N.D.**
21	BSU37710	Alanine-anticapsin ligase bacD	YwfE	52,234	4,87	C	-	-	-	4,5-5,5	N.D.**
22	BSU01120	Elongation factor G	FusA	76,496	4,82	C	-	-	-	4,5-5,5	N.D.**
23	BSU01540	Unknown similar to ATP-binding Mrp-like protein	YbaL (SalA)	38,614	5,39	CM	-	-	-	4,5-5,5	N.D.**

Table 3.1. Continued

24	BSU29470	Acetate kinase	AckA	43,110	5,34	C	-	-	-	4,5-5,5	N.D.**
25	BSU29570	Small acid-soluble spore protein (major alpha-type SASP) (SspA)	SspA	7,066	4,94	E	-	-	-	4,5-5,5	N.D.**
26	BSU09750	Small acid-soluble spore protein (major beta-type SASP) (SspB)	SspB	6,975	4,99	E	-	-	-	4.5-5.5	N.D.**
27	BSU02910	Unknown similar to tellurium resistance protein	YceE	20,935	4,58	C	-	-	-	4,5-5,5	N.D.**
								-	-	4-7	3,125
28	BSU15500	Dihydroorotase	PyrC	46,579	5,53	C	-	-	-	4,5-5,5	N.D.***
29	BSU37720	Unknown similar to glucose 1-dehydrogenase	YwfD	27,306	5,12	C	-	-	-	4-7	0,337
30	BSU02900	Unknown similar to tellurium resistance protein	YceD	20,681	4,49	C	-	-	-	4-7	3,125
31	BSU28440	Succinate dehydrogenase	SdhA (CitF)	65,308	5,77	CM	+	-	-	4-7	3,225
32	BSU23070	D-3-phosphoglycerate dehydrogenase	SerA	57,079	5,69	C	-	2	M	4-7	3,215; 2,941
								-	-	5,5-6,7	3,058
33	BSU40420	Adenylosuccinate synthetase	PurA	47,642	5,58	C	-	-	-	4-7	3,921
								-	-	5,5-6,7	0,293
34	BSU23860	Unknown similar to 6-phosphogluconate dehydrogenase	YqjI	51,742	5,25	C	-	2	M	4-7	3,154; 2,680
35	BSU36900	Serine hydroxymethyltransferase	GlyA (GlyC)	45,461	5,56	C	-	2	C	4-7	4,048; 5,780
								3	C-M	5,5-6,7	0,017; 0,072; 0,115
36	BSU28820	Putative aminopeptidase	YsdC	39,192	5,63	C	-	-	-	4-7	3,058

Table 3.1. Continued

37	BSU15380	Similar to unknown proteins from other organisms	YlmE	25,687	5,65	C	-	-	-	4-7	2,680
38	BSU35490	Two-component response regulator DegU involved in degradative enzyme and competence regulation	DegU	25,850	5,66	C	-	-	-	4-7	2,680
39	BSU16100	Succinate-CoA synthetase (ADP-forming) subunit alpha	SucD	31,362	5,66	C	-	-	-	4-7	2,941
40	BSU30800	Dihydroxynaphthoic acid synthetase (Naphthoate synthase)	MenB	29,880	5,47	C	-	-	-	4-7	2,724
41	BSU29410	Similar to unknown proteins from other organisms	YtkL	24,815	5,14	U	-	-	-	4-7	3,378
42	BSU14010	Two-component response regulator CheV	CheV	34,611	4,82	C	-	-	-	4-7	4,329
43	BSU16090	Succinyl-CoA synthetase (beta subunit)	SucC	41,345	5,04	C	-	2	C-M	4-7	3,236 ; 0,178
44	BSU39880	Similar to unknown proteins from *B. subtilis* (Iron ion binding protein)	YxbC (YxaQ)	37,434	4,78	C	-	-	-	4-7	7,751
45	BSU00120	Unknown similar to amidotransferase	YaaE (PdxT)	21,433	5,14	C	-	-	-	4-7	2,617
46	BSU06520	Bifunctional purine biosynthesis protein PurH	PurH (PurHJ)	55,704	5,28	C	-	-	-	4-7	2,617
47	BSU30670	Autoinducer-2 production protein LuxS	LuxS (YtjB)	17,702	5,29	C	-	-	-	4-7	2,680
48	BSU31030	Uncharacterized protein YuaE	YuaE	19,098	6,19	U	-	-	-	4-7	2,557
49	BSU29850	Uncharacterized protein YtoQ	YtoQ	16,780	5,84	C	-	-	-	4-7	3,472

Table 3.1. Continued

50	BSU12410	Uncharacterized protein YjoA	YjoA	17,781	5,83	C	-	-	-	4-7	2,531
51	BSU28420	Uncharacterized protein YsmA	YsmA	17,062	5,63	U	-	-	-	4-7	2,531
52	BSU31390	Unknown similar to polyribonucleotide nucleotidyltransferase (General stress protein 13)	YugI	14,274	5,95	C	-	-	-	4-7	8,474
53	BSU39420	Deoxyribose-phosphate aldolase	Dra (DeoC)	22,197	5,11	C	-	-	-	4-7	2,500
54	BSU29880	Probable NAD-dependent malic enzyme	MalS	62,109	5,08	U	-	-	-	4-7	0,386
55	BSU00160	Unknown similar to cortical fragment-lytic enzyme	YaaH	48,606	5,72	E	-	-	-	4-7	0,374
56	BSU02940	Unknown similar to toxic anion resistance protein	YceH	41,646	5,90	C	-	-	-	4-7	0,266
57	BSU37120	Probable fructose-1,6-bisphosphate aldolase	FbaA	30,381	5,19	U	-	2	C	4-7	0,348; 0,337
58	BSU27990	Septum site-determining protein MinD	MinD (DivIVB)	29,388	5,16	C	-	-	-	4-7	0,260
59	BSU12920	D-aminopeptidase	DppA (DciAA)	30,223	5,34	U	-	-	-	4-7	0,260
60	BSU33930	Phosphoglycerate kinase	Pgk	42,163	4,96	C	-	2	C	4-7	0,160; 0,178
61	BSU36810	ATP synthase (subunit beta)	AtpD	51,388	4,80	CM	-	-	-	4-7	0,343
62	BSU17460	Glutamine synthetase	GlnA	50,246	5,05	C	-	-	-	4-7	0,343

Table 3.1. Continued

63	BSU36640	Urease (alpha subunit)	UreC	61,147	5,24	C	-	-	-	4-7	0,178
64	BSU29120	Malate dehydrogenase	Mdh (CitH)	33,622	4,92	E	+	-	-	4-7	0,246
65	BSU03130	NH(3)-dependent NAD(+) synthetase (Sporulation protein outB)	NadE	30,376	5,07	C	-	-	-	4-7	0,246
66	BSU40550	Manganese-dependent inorganic pyrophosphatase	PpaC	33,967	4,73	C	-	-	-	4-7	0,246
67	BSU24310	Bifunctional protein folD Methylenetetrahydrofolate dehydrogenase / Methenyltetrahydrofolate cyclohydrolase	FolD (YqiA)	30,666	5,67	C	-	-	-	4-7	0,384
68	BSU00730	Cysteine synthetase A	CysK	32,799	5,64	C	-	-	-	4-7	0,384
69	BSU29210	Acetyl-CoA carboxylase (beta subunit)	AccD (YttI)	28,859	5,48	C	-	-	-	4-7	0,384
70	BSU16500	Elongation factor Ts	Tsf	32,333	5,17	C	-	-	-	4-7	0,333
71	BSU33540	Unknown similar to NAD(P)H dehydrogenase	YvaB	23,257	5,26	U	-	-	-	4-7	0,320
72	BSU29500	Uncharacterized protein YtfJ	YtfJ	16,336	5,16	U	-	-	-	4-7	0,109
73	BSU37110	Unknown similar to transaldolase (pentose phosphate)	YwjH (Tal**)**	22,899	5,89	C	-	-	-	4-7	0,230
74	BSU37920	Unknown similar to spore coat protein	YwdL (GerQ**)**	20,262	6,58	E	-	2	C	4-7	0,243; 0,121
								-	-	5,5-6,7	0,351

Table 3.1 Continued

75	BSU21690	Peptidyl methionine sulfoxide reductase	MsrA (YppP)	20,169	5,75	C	-	-	-	4-7	0,121
76	BSU27890	Unknown similar to transcriptional regulator	YrxA	19,707	5,79	C	-	-	-	4-7	0,074
								-	-	5,5-6,7	3,745 ; 0,111
77	BSU11720	Enoyl-acyl carrier protein reductase	FabI (YjbW)	27,856	5,67	C	-	-	-	4-7	0,375
78	BSU00510	Phosphoribosylpyrophosphate synthetase	Prs	34,846	5,94	C	-	-	-	5,5-6,7	0,180
79	BSU17250	Uncharacterized protein YmaE	YmaE	26,559	9,11	U	-	-	C	5,5-6,7	2,100
80	BSU22600	5-enolpyruvoylshikimate-3-phosphate synthase	AroE	45,211	6,33	C	-	-	-	5,5-6,7	2,304; 2,525; 0,152; 0,238
81	BSU16130	Glucose-inhibited division protein	Gid (YlyC/ TrmFO)	48,032	5,83	C	-	-	-	5,5-6,7	2,525
82	BSU36950	Unknown similar to unknown proteins	YwlC	36,984	5,87	C	-	-	-	5,5-6,7	2,793
83	BSU22070	Xanthine phosphoribosyltransferase	Xpt	21,025	5,86	C	-	-	-	5,5-6,7	2,808
84	BSU31760	Unknown similar to pyrazinamidase/nicotinamidase	YueJ (PncA)	20,510	5,66	C	-	-	-	5,5-6,7	3,257
85	BSU15910	Beta-ketoacyl-acyl carrier protein reductase	FabG (YlpF)	26,265	7,77	C	-	-	C	5,5-6,7	3,257
86	BSU09260	Unknown similar to adenosylmethionine-8-amino-7-oxononanoate aminotransferase	YhxA	49,840	5,71	C	-	-	-	5,5-6,7	4,016

Table 3.1 Continued

87	BSU03010	Amidohydrolase (Aminoacylase)	AmhX	42,503	6,11	C	-	4	C-M	5,5-6,7	0,095; 0,195; 0,221; 0,368
88	BSU00260	Unknown similar to toxic cation resistance	YaaN	43,803	5,71	C	-	-	-	5,5-6,7	4,366
89	BSU35120	Unknown similar to unknown proteins (Uncharacterized protein YvlB)	YvlB	41,055	5,5	C	-	-	-	5,5-6,7	4,366
90	BSU28000	Cell-division inhibitor (septum placement)	MinC	24,982	6,24	C	-	-	-	5,5-6,7	4,366
91	BSU21680	Unknown similar to peptide methionine sulfoxide reductase	YppQ (MsrB)	16,591	5,87	C	-	-	-	5,5-6,7	7,633
92	BSU36910	Uncharacterized protein YwlG	YwlG	19,364	6,43	C	-	-	-	5,5-6,7	7,633
93	BSU37520	Unknown similar to unknown proteins (Uncharacterized protein YwhD)	YwhD	19,444	6,45	C	-	-	-	5,5-6,7	8,333
94	BSU25180	Unknown similar to unknown proteins (Phosphoprotein YqfN)	YqfN	23,690	5,39	C	-	-	C	5,5-6,7	N.D.**
95	BSU33040	Fumarate hydratase	CitG (FumC)	50,499	5,57	C	-	2	C-M	5,5-6,7	0,023; 0,072
96	BSU06300	Spore coat protein (outer)	CotA (Pig)	58,462	5,91	C	-	-	-	5,5-6,7	0,088
97	BSU07690	Unknown similar to methionine aminopeptidase	YflG (MapB)	27,193	5,65	C	-	-	-	5,5-6,7	0,093
98	BSU15530	Dihydroorotate dehydrogenase (electron transfer subunit)	PyrK	28,081	5,68	C	-	-	-	5,5-6,7	0,093
99	BSU10070	Unknown similar to aminoacylase	YhaA	43,241	5,87	C	-	-	-	5,5-6,7	0,095

Table 3.1 Continued

100	BSU11850	Unknown similar to unknown proteins	YjcG	19,649	5,65	C	-	2	M	5,5-6,7	0,113; 0,157
101	BSU10020	Phosphoserine aminotransferase	SerC (YhaF)	40,110	5,64	C	-	-	-	5,5-6,7	0,115
102	BSU19370	2-oxoglutarate dehydrogenase (E1 subunit)	OdhA (CitK)	105,674	5,91	C	-	3	M	5,5-6,7	0.157;0.187;0.390
103	BSU16730	Dipicolinate synthase subunit A	SpoVFA (DpaA)	31,927	5,91	C	-	-	-	5,5-6,7	0,160
104	BSU14530	Unknown similar to unknown proteins (Ribonuclease J 1 YkqC)	YkqC (RnjA)	61,477	5,93	C	-	-	-	5,5-6,7	0,192
105	BSU23120	Two-component response regulator involved in aerobic and anaerobic respiration	ResD (YpxD)	27,468	5,75	C	-	-	-	5,5-6,7	0,251
106	BSU19360	2-oxoglutarate dehydrogenase (dihydrolipoamide trans-succinylase, E2 subunit)	OdhB (CitM)	45,959	5,05	C	-	-	C	5,5-6,7	0,252
107	BSU07830	Unknown similar to NAD(P)H-flavin oxidoreductase	YfkO	25,612	5,73	C	-	3	C-M	5,5-6,7	0,262; 0,293; 0,300
108	BSU40250	Unknown similar to formaldehyde dehydrogenase	YycR	42,919	5,82	C	-	-	-	5,5-6,7	0,289
109	BSU23280	Riboflavin-specific deaminase	RibD (RibG)	39,280	6,1	C	-	-	-	5,5-6,7	0,300
110	BSU12980	Unknown similar to chloromuconate cycloisomerase (L-Ala-D/L-Glu Epimerase)	YkfB	39,447	5,95	C	-	-	-	5,5-6,7	0,313
111	BSU24570	Probable aminomethyltransferase	GcvT (YqhI)	39,754	5,81	C	-	-	-	5,5-6,7	0,313

Table 3.1 Continued

112	BSU18360	Unknown similar to unknown proteins (Aldose 1-epimerase)	YoxA(GalM)	37,204	5,64	C	-	-	-	5,5-6,7	0,325
113	BSU03050	L-lactate dehydrogenase	Ldh (LctE)	34,895	5,49	C	+	-	-	5,5-6,7	0,325
114	BSU09180	Unknown similar to unknown proteins (Spore coat protein F-like protein YhcQ)	YhcQ	24,774	5,77	E	-	-	-	5,5-6,7	0,326
115	BSU28260	3-isopropylmalate dehydratase (large subunit)	LeuC	52,358	6,14	C	-	-	-	5,5-6,7	0,330
116	BSU00090	Inosine-monophosphate dehydrogenase	GuaB (GnaB)	52,957	6,18	C	-	-	-	5,5-6,7	0,330
117	BSU12290	Unknown similar to NADH dehydrogenase	YjlD	41,927	6,28	CM	-	2	C-M	5,5-6,7	0,332; 0,335
118	BSU11700	Hydroxyethylthiazole phosphate biosynthesis	ThiF (YjbU)	36,376	5,72	C	-	-	-	5,5-6,7	0,343
119	BSU30820	2-succinyl-6-hydroxy-2,4-cyclohexadiene-1-carboxylate synthase / 2-oxoglutarate decarboxylase	MenD	64,051	5,71	C	-	-	-	5,5-6,7	0,354
120	BSU14280	Molybdopterin biosynthesis protein	MoeA	46,590	5,69	C	-	-	-	5,5-6,7	0,366
121	BSU08720	Unknown similar to bacterioferritin comigratory protein	YgaF (Bcp)	18,097	5,89	C	-	-	-	5,5-6,7	0,380
122	BSU11460	Oligopeptide ABC transporter (ATP-binding protein) (initiation of sporulation, competence development	OppD	39,738	6,06	CM	-	-	-	5,5-6,7	0,177

Table 3.1 Continued

123	BSU35020	N-acetylglucosamine-6-phosphate isomerase	NagB	26,974	5,76	C	-	-	-	5,5-6,7	0,326

[a] Predicted location of proteins by PSORTb version 2.0.4. C: Cytoplasm, CM: Cytoplasmic Membrane, E: Extracellular and U:Unknown

[b] Signal Peptides predicted by SignalP prediction program.

[c] The calculated expression level of mutant strain compared to wild-type

[*] C, charge difference; M, mass difference; C-M, both charge and mass difference

[**] Proteins not detected in OGU1 strain.

[***] Proteins not detected in wild-type strain

3.5. Functional Classification

The identified 128 distinct proteins were classified into functional groups based on their functions provided in the Uni-prot protein database and SubtiList web server (http://genolist.pasteur.fr/SubtiList/). Among these 128 proteins, 116 proteins could be classified according to three main headings for functions of proteins in the SubtiList web server which are cell envelope and cellular processes, intermediary metabolism and information pathways. 4 of these 116 proteins were found to have more than one functional class. These proteins are YvaB (unknown similar to NADPH dehydrogenase), NadE (NH(3)-dependent NAD(+) synthetase), FbaA (probable fructose-1,6-bisphosphate aldolase) and Ald (L-alanine dehydrogenase). Tables 3.2, 3.3, 3.4 and 3.5 show functional classes of identified proteins.

Some of the identified proteins were 'hypothetical proteins' of which the functions are unknown or have not previously been determined. Sequences of some of these hypothetical proteins were identical or almost identical to the known proteins, of which functions have been clearly defined. Therefore, these hypothetical proteins were functionally classified based on their respective homologues. Nevertheless, 16 hypothetical proteins remained unclassified and could not be matched any functional group of proteins in SubtiList web server, as seen in Table 3.6.

Regarding main functional headings , the percentage values among the ORFs identified in this study were 47.8 for intermediary metabolism, 21.2 for cell envelope and cellular processes, 12.1 for information pathways and 12.1 for stress response. 6.8 % of proteins were remained unclassified since their functions are uncharacterized. Figure 3.15. shows the relative distribution of ORFs to the functional classes of cellular physiology listed under the main headings of SubtiList web server.

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Table 3.2. Intermediary metabolism proteins.

Spot No	Locus Name	Protein Name	Gene Product	Functional Class	Mutant/Control Ratio[a]	pI Range of Detection
1	BSU16690	Polynucleotide phosphorylase (PNPase)	PnpA(ComR)	Metabolism of nucleotides and nucleic acids	0,182	4,5-5,5
28	BSU15500	Dihydroorotase	PyrC	Metabolism of nucleotides and nucleic acids	N.D.***	4,5-5,5
33	BSU40420	Adenylosuccinate synthetase	PurA	Metabolism of nucleotides and nucleic acids (Purine metabolism); AMP biosynthesis via de novo pathway.	3,921	4-7
					0,293	5,5-6,7
46	BSU06520	Bifunctional purine biosynthesis protein PurH	PurH (PurHJ)	Metabolism of nucleotides and nucleic acids	2,617	4-7
53	BSU39420	Deoxyribose-phosphate aldolase	Dra (DeoC)	Metabolism of nucleotides and nucleic acids	2,500	4-7
78	BSU00510	Phosphoribosylpyrophosphate synthetase	Prs	Metabolism of nucleotides and nucleic acids	0,180	5,5-6,7
83	BSU22070	Xanthine phosphoribosyltransferase	Xpt	Metabolism of nucleotides and nucleic acids (purine biosynthesis)	2,808	5,5-6,7
98	BSU15530	Dihydroorotate dehydrogenase (electron transfer subunit)	PyrK	Metabolism of nucleotides and nucleic acids (Pyrimidine biosynthesis)	0,093	5,5-6,7
116	BSU00090	Inosine-monophosphate dehydrogenase	GuaB (GnaB)	Metabolism of nucleotides and nucleic acids (Purine metabolism)	0,330	5,5-6,7
4	BSU18000	Aconitate hydratase	CitB	Metabolism of carbohydrates and related molecules	0,345	4,5-5,5
24	BSU29470	Acetate kinase	AckA	Metabolism of carbohydrates and related molecules	N.D.**	4,5-5,5
123	BSU35020	N-acetylglucosamine-6-phosphate isomerase	NagB	Metabolism of carbohydrates and related molecules (N-acetylneuraminate degradation)	0,326	5,5-6,7

Table 3.2. Continued

29	BSU37720	Unknown similar to glucose 1-dehydrogenase	YwfD	Metabolism of carbohydrates and related molecules (Antibiotic biosynthesis)	0,337	4-7
31	BSU28440	Succinate dehydrogenase	SdhA (CitF)	Metabolism of carbohydrates and related molecules (Tricarboxylic acid cycle)	3,225	4-7
34	BSU23860	Unknown similar to 6-phosphogluconate dehydrogenase	YqjI	Metabolism of carbohydrates and related molecules (Carbohydrate degradation; pentose phosphate pathway)	2,680; 3,154	4-7
36	BSU28820	Putative aminopeptidase	YsdC	Metabolism of carbohydrates and related molecules	3,058	4-7
39	BSU16100	Succinate-CoA synthetase (ADP-forming) subunit alpha	SucD	Metabolism of carbohydrates and related molecules (Tricarboxylic acid cycle)	2,941	4-7
43	BSU16090	Succinyl-CoA synthetase (beta subunit)	SucC	Metabolism of carbohydrates and related molecules (Tricarboxylic acid cycle)	3,236 ; 0,178	4-7
54	BSU29880	Probable NAD-dependent malic enzyme	MalS	Metabolism of carbohydrates and related molecules (Tricarboxylic acid cycle)	0,386	4-7
57	BSU37120	Probable fructose-1,6-bisphosphate aldolase	FbaA*	Metabolism of carbohydrates and related molecules (Carbohydrate degradation; glycolysis; sporulation)	0,337; 0,348	4-7
60	BSU33930	Phosphoglycerate kinase	Pgk	Metabolism of carbohydrates and related molecules	0,160; 0,178	4-7

Table 3.2. Continued

64	BSU29120	Malate dehydrogenase	Mdh (CitH)	Metabolism of carbohydrates and related molecules (Tricarboxylic acid cycle)	0,246	4-7
73	BSU37110	Unknown similar to transaldolase (Pentose phosphate)	YwjH (Tal**)**	Metabolism of carbohydrates and related molecules (Carbohydrate degradation; pentose phosphate pathway)	0,230	4-7
95	BSU33040	Fumarate hydratase	CitG (FumC)	Metabolism of carbohydrates and related molecules (Tricarboxylic acid cycle)	0,023; 0,072	5,5-6,7
102	BSU19370	2-oxoglutarate dehydrogenase (E1 subunit)	OdhA (CitK)	Metabolism of carbohydrates and related molecules (Tricarboxylic acid cycle)	0.157;0.187;0.390	5,5-6,7
106	BSU19360	2-oxoglutarate dehydrogenase (dihydrolipoamide trans-succinylase, E2 subunit)	OdhB (CitM)	Metabolism of carbohydrates and related molecules (Tricarboxylic acid cycle)	0,252	5,5-6,7
108	BSU40250	Unknown similar to formaldehyde dehydrogenase	YycR	Metabolism of carbohydrates and related molecules	0,289	5,5-6,7
112	BSU18360	Unknown similar to unknown proteins (Aldose 1-epimerase)	YoxA(GalM)	Metabolism of carbohydrates and related molecules	0,325	5,5-6,7
11	BSU28290	Ketol-acid reductoisomerase	IlvC	Metabolism of amino acids and related molecules (L-valine/L-isoleucine biosynthesis)	N.D.**	4,5-5,5
					0,322	5,5-6,7
13	BSU34870	HisF cyclase-like protein	HisF	Metabolism of amino acids and related molecules (L-histidine biosynthesis)	N.D**	4,5-5,5
14	BSU31930	L-alanine dehydrogenase	Ald* (SpoVN)	Metabolism of amino acids and related molecules	N.D**	4,5-5,5

Table 3.2. Continued

16	BSU29750	3-deoxy-D-arabino-heptulosonate 7-phosphate synthase / chorismate mutase-isozyme 3	AroA	Metabolism of amino acids and related molecules	N.D**	4,5-5,5
					0,322	5,5-6,7
					0,074	4-7
18	BSU01780	L-glutamine-D-fructose-6-phosphate amidotransferase	GlmS (GcaA, YbxD)	Metabolism of amino acids and related molecules	N.D**	4,5-5,5
19	BSU21870	Dihydroxy-acid dehydratase	IlvD	Metabolism of amino acids and related molecules(L-valine/L-isoleucine biosynthesis)	N.D**	4,5-5,5
20	BSU34910	Histidinol dehydrogenase	HisD	Metabolism of amino acids and related molecules (Histidine biosynthesis)	N.D**	4,5-5,5
21	BSU37710	Alanine-anticapsin ligase bacD (L-amino acid ligase)	YwfE (BacD)	Antibiotic biosynthesis; bacilysin biosynthesis.	N.D**	4,5-5,5
32	BSU23070	D-3-phosphoglycerate dehydrogenase	SerA	Metabolism of amino acids and related molecules (L-serine biosynthesis)	2,941; 3,215	4-7
					3,058	5,5-6,7
35	BSU36900	Serine hydroxymethyltransferase	GlyA (GlyC)	Metabolism of amino acids and related molecules (L-serine biosynthesis)	4,048; 5,780	4-7
					0,017; 0,072; 0,115	5,5-6,7
45	BSU00120	Unknown similar to amidotransferase	YaaE (PdxT)	Metabolism of amino acids and related molecules	2,617	4-7
62	BSU17460	Glutamine synthetase	GlnA	Metabolism of amino acids and related molecules	0,343	4-7
63	BSU36640	Urease (alpha subunit)	UreC	Metabolism of amino acids and related molecules	0,178	4-7
68	BSU00730	Cysteine synthetase A	CysK	Metabolism of amino acids and related molecules	0,384	4-7

Table 3.2. Continued

80	BSU22600	5-enolpyruvoylshikimate-3-phosphate synthase	AroE	Metabolism of amino acids and related molecules	2,304; 2,525; 0,152; 0,238	5,5-6,7
99	BSU10070	Unknown similar to aminoacylase	YhaA	Metabolism of amino acids and related molecules	0,095	5,5-6,7
101	BSU10020	Phosphoserine aminotransferase	SerC (YhaF)	Metabolism of amino acids and related molecules/L-serine biosynthesis	0,115	5,5-6,7
111	BSU24570	Probable aminomethyltransferase	GcvT (YqhI)	Metabolism of amino acids and related molecules	0,313	5,5-6,7
115	BSU28260	3-isopropylmalate dehydratase (large subunit)	LeuC	Metabolism of amino acids and related molecules (Leucine biosynthesis)	0,330	5,5-6,7
15	BSU24060	Probable branched-chain alpha-keto acid dehydrogenase E3 subunit (Dihydrolipoamide dehydrogenase)	LpdV (BfmBC)	Metabolism of lipids(branched-chain fatty acid biosynthesis)	N.D**	4,5-5,5
69	BSU29210	Acetyl-CoA carboxylase (beta subunit)	AccD (YttI)	Metabolism of lipids	0,384	4-7
77	BSU11720	Enoyl-acyl carrier protein reductase	FabI (YjbW)	Metabolism of lipids	0,375	4-7
85	BSU15910	Beta-ketoacyl-acyl carrier protein reductase	FabG (YlpF)	Metabolism of lipids (fatty acid biosynthesis)	3,257	5,5-6,7
40	BSU30800	Dihydroxynaphthoic acid synthetase (Naphthoate synthase)	MenB	Metabolism of coenzymes and prosthetic groups	2,724	4-7
65	BSU03130	NH(3)-dependent NAD(+) synthetase (Sporulation protein outB)	NadE*	Metabolism of coenzymes and prosthetic groups	0,246	4-7

Table 3.2. Continued

67	BSU24310	Bifunctional protein folD Methylenetetrahydrofolate dehydrogenase / Methenyltetrahydrofolate cyclohydrolase	FolD (YqiA)	Metabolism of coenzymes and prosthetic groups	0,384	4-7
84	BSU31760	Unknown similar to pyrazinamidase/nicotinamidase	YueJ (PncA)	Metabolism of coenzymes and prosthetic groups	3,257	5,5-6,7
86	BSU09260	Unknown similar to adenosylmethionine-8-amino-7-oxononanoate aminotransferase	YhxA	Metabolism of coenzymes and prosthetic groups	4,016	5,5-6,7
109	BSU23280	Riboflavin-specific deaminase	RibD (RibG)	Metabolism of coenzymes and prosthetic groups	0,300	5,5-6,7
118	BSU11700	Hydroxyethylthiazole phosphate biosynthesis	ThiF (YjbU)	Metabolism of coenzymes and prosthetic groups (Thiamine biosynthesis)	0,343	5,5-6,7
119	BSU30820	2-succinyl-6-hydroxy-2,4-cyclohexadiene-1-carboxylate synthase / 2-oxoglutarate decarboxylase	MenD	Metabolism of coenzymes and prosthetic groups (Cofactor biosynthesis; menaquinone biosynthesis; menaquinone-2 from chorismate: step 2/8)	0,354	5,5-6,7
120	BSU14280	Molybdopterin biosynthesis protein	MoeA	Metabolism of coenzymes and prosthetic groups (Molybdopterin biosynthesis protein)	0,366	5,5-6,7
66	BSU40550	Manganese-dependent inorganic pyrophosphatase	PpaC	Metabolism of phosphate	0,246	4-7

* Proteins with more than one functinal class
** Proteins not detected in OGU1 strain.
*** Proteins not detected in wild-type strain

Table 3.3. Proteins involved in cell envelope and cellular processes.

Spot No	Locus Name	Protein Name	Gene Product	Functional Class	Mutant/Control Ratio	pI Range of Detection
5	BSU19390	Unknown similar to nitric-oxide reductase/probable D-alanine amino transferase	YojN/ Dat	Membrane bioenergetics (electron transport chain and ATP synthase)	0,232	4,5-5,5
17	BSU36800	ATP synthase (subunit epsilon)	AtpC	Membrane bioenergetics (electron transport chain and ATP synthase)	N.D.**	4,5-5,5
61	BSU36810	ATP synthase (subunit beta)	AtpD	Membrane bioenergetics (electron transport chain and ATP synthase)	0,343	4-7
71	BSU33540	Unknown similar to NAD(P)H dehydrogenase	YvaB*	Membrane bioenergetics (electron transport chain and ATP synthase) (Aromatic hydrocarbons catabolism; Detoxification)	0,320	4-7
107	BSU07830	Unknown similar to NAD(P)H-flavin oxidoreductase	YfkO	Membrane bioenergetics (electron transport chain and ATP synthase)	0,262; 0,293; 0,300	5,5-6,7
113	BSU03050	L-lactate dehydrogenase	Ldh (LctE)	Membrane bioenergetics (electron transport chain and ATP synthase	0,325	5,5-6,7
117	BSU12290	Unknown similar to NADH dehydrogenase	YjlD	Membrane bioenergetics (electron transport chain and ATP synthase)	0,332; 0,335	5,5-6,7
3	BSU38500	D-alanyl –D-alanine carrier protein ligase	DltA	Cell wall biogenesis; lipoteichoic acid biosynthesis.	0,411	4,5-5,5
110	BSU12980	Unknown similar to chloromuconate cycloisomerase (L-Ala-D/L-Glu Epimerase)	YkfB	Cell wall degradation; peptidoglycan degradation	0,313	5,5-6,7
58	BSU27990	Septum site-determining protein MinD	MinD (DivIV)	Cell Division	0,260	4-7

Table 3.3. Continued

81	BSU16130	Glucose-inhibited division protein	Gid (YlyC/ TrmFO)	Cell division	2,525	5,5-6,7
90	BSU28000	Cell-division inhibitor (septum placement)	MinC	Cell division	4,366	5,5-6,7
12	BSU22230	Unknown similar to phosphotransferase system enzyme II	YpqE	Transport/binding proteins and lipoproteins	N.D.**	4,5-5,5
42	BSU14010	Two-component response regulator CheV	CheV	Motility and Chemotaxis	4,329	4-7
47	BSU30670	Autoinducer-2 production protein LuxS	LuxS (YtjB)	Sensors (signal transduction)	2,680	4-7
14	BSU31930	L-alanine dehydrogenase	Ald* (SpoVN)	Stage V sporulation	N.D.**	4,5-5,5
25	BSU29570	Small acid-soluble spore protein (major alpha-type SASP) (SspA)	SspA	Sporulation	N.D**	4,5-5,5
26	BSU09750	Small acid-soluble spore protein (major beta-type SASP) (SspB)	SspB	Sporulation	N.D**	4,5-5,5
122	BSU11460	Oligopeptide ABC transporter (ATP-binding protein) (initiation of sporulation, competence development	OppD	Transport/binding proteins and lipoproteins	0,177	5,5-6,7
55	BSU00160	Unknown similar to cortical fragment-lytic enzyme	YaaH	Sporulation	0,374	4-7
57	BSU37120	Probable fructose-1,6-bisphosphate aldolase	FbaA*	Metabolism of carbohydrates and related molecules (Carbohydrate degradation; glycolysis, sporulation)	0,348; 0,337	4-7
65	BSU03130	NH(3)-dependent NAD(+) synthetase (Sporulation protein outB)	NadE*	Metabolism of coenzymes and prosthetic groups (Sporulation)	0,246	4-7
72	BSU29500	Uncharacterized protein YtfJ	YtfJ	Sporulation	0,109	4-7
74	BSU37920	Unknown similar to spore coat protein	YwdL (GerQ**)**	Germination Sporulation	0,243; 0,121	4-7
					0,351	5,5-6,7

Table 3.3. Continued

96	BSU06300	Spore coat protein (outer)	CotA (Pig)	Sporulation	0,088	5,5-6,7
104	BSU16730	Dipicolinate synthase subunit A	SpoVFA (DpaA)	Sporulation	0,192	5,5-6,7
114	BSU09180	Unknown similar to unknown proteins (Spore coat protein F-like protein YhcQ)	YhcQ	Sporulation	0,326	5,5-6,7

* Proteins with more than one functinal class.
** Proteins not detected in OGU1 strain.
*** Proteins not detected in wild-type strain.

Table 3.4. Information pathway proteins.

Spot No	Locus Name	Protein Name	Gene Product	Functional Class	Mutant/Control Ratio	pI Range of Detection
2	BSU01130	Elongation factor Tu	TufA	Translation elongation	0,270	4,5-5,5
7	BSU25470	Class I heat-shock protein	DnaK	Protein folding	N.D.**	4,5-5,5
9	BSU29980	Unknown similar to Xaa-His dipeptidase	YtjP	Protein modification	N.D.**	4,5-5,5
10	BSU01430	RNA polymerase (alpha subunit)	RpoA	Transcription elongation	N.D**	4,5-5,5
22	BSU01120	Elongation factor G	FusA	Translation elongation (Protein biosynthesis)	N.D**	4,5-5,5

Table 3.4. Continued

38	BSU35490	Two-component response regulator involved in degradative enzyme and competence regulation	DegU	Transcription; Transcription regulation RNA Synthesis	2,680	4-7
59	BSU12920	D-aminopeptidase	DppA (DciAA)	Protein modification	0,260	4-7
70	BSU16500	Elongation factor Ts	Tsf	Translation elongation	0,333	4-7
76	BSU27890	Unknown similar to transcriptional regulator	YrxA	Transcription regulation	0,074	4-7
					3,745; 0,111	5,5-6,7
87	BSU03010	Amidohydrolase (Aminoacylase)	AmhX	Protein modification	0,095; 0,195; 0,221; 0,368	5,5-6,7
91	BSU21680	Unknown similar to peptide methionine sulfoxide reductase	YppQ (MsrB)	Protein modification	7,633	5,5-6,7
97	BSU07690	Unknown similar to methionine aminopeptidase	YflG (MapB)	Protein modification	0,093	5,5-6,7
104	BSU14530	Unknown similar to unknown proteins (Ribonuclease J 1 YkqC)	YkqC (RnjA)	mRNA processing rRNA processing	0,192	5,5-6,7
105	BSU23120	Two-component response regulator involved in aerobic and anaerobic respiration	ResD (YpxD)	Transcription regulation	0,251	5,5-6,7

* Proteins with more than one functinal class.
** Proteins not detected in OGU1 strain.
*** Proteins not detected in wild-type strain.

Table 3.5. Stress response proteins

Spot No	Locus Name	Protein Name	Gene Product	Functional Class	Mutant/Control Ratio[a]	pI Range of Detection
6	BSU40090	Alkyl hydroperoxide reductase (small subunit)(General stress protein 22)	AhpC	Detoxification	2,550	4,5-5,5
27	BSU02910	Unknown similar to tellurium resistance protein	YceE	Detoxification (Stress response)	N.D.**	4,5-5,5
					3,125	4-7
30	BSU02900	Unknown similar to tellurium resistance protein	YceD	Detoxification (Stress response)	3,125	4-7
52	BSU31390	Unknown similar to polyribonucleotide nucleotidyltransferase (General stress protein 13)	YugI	RNA modification (Stress response)	8,474	4-7
56	BSU02940	Unknown similar to toxic anion resistance protein	YceH	Detoxification	0,266	4-7
71	BSU33540	Unknown similar to NAD(P)H dehydrogenase	YvaB*	Membrane bioenergetics (electron transport chain and ATP synthase) Aromatic hydrocarbons catabolism Detoxification	0,320	4-7
75	BSU21690	Peptidyl methionine sulfoxide reductase	MsrA (YppP)	Detoxification	0,121	4-7
88	BSU00260	Unknown similar to toxic cation resistance	YaaN	Detoxification	4,366	5,5-6,7
121	BSU08720	Unknown similar to bacterioferritin comigratory protein	YgaF (Bcp)	Detoxification	0,380	5,5-6,7

* Proteins with more than one functinal class.
** Proteins not detected in OGU1 strain.
*** Proteins not detected in wild-type strain.

Table 3.6. Proteins with unknown function.

Spot No	Locus Name	Protein Name	Gene Product	Functional Class	Mutant/Control Ratio[a]	pI Range of Detection
8	BG14010	Unknown similar to unknown proteins	YurX	Unknown	N.D.**	4,5-5,5
23	BSU01540	Unknown similar to ATP-binding Mrp-like protein	YbaL (SalA)	Unknown	N.D.**	4,5-5,5
37	BSU15380	Similar to unknown proteins from other organisms	YlmE	Unknown	2,680	4-7
41	BSU29410	Similar to unknown proteins from other organisms	YtkL	Unknown	3,378	4-7
44	BSU39880	Similar to unknown proteins from *B. subtilis*	YxbC (YxaQ)	Unknown	7,751	4-7
48	BSU31030	Uncharacterized protein	YuaE	Unknown	2,557	4-7
49	BSU29850	Uncharacterized protein	YtoQ	Unknown	3,472	4-7
50	BSU12410	Uncharacterized protein	YjoA	Unknown	2,531	4-7
51	BSU28420	Uncharacterized protein	YsmA	Unknown	2,531	4-7
79	BSU17250	Uncharacterized protein	YmaE	Unknown	2,100	5,5-6,7
82	BSU36950	Unknown similar to unknown proteins	YwlC	Unknown	2,793	5,5-6,7
89	BSU35120	Unknown similar to unknown proteins	YvlB	Unknown	4,366	5,5-6,7

Table 3.6. Continued

89	BSU35120	Unknown similar to unknown proteins	YvlB	Unknown	4,366	5,5-6,7
92	BSU36910	Uncharacterized protein	YwlG	Unknown	7,633	5,5-6,7
93	BSU37520	Unknown similar to unknown proteins	YwhD	Unknown	8,333	5,5-6,7
94	BSU25180	Unknown similar to unknown proteins (Phosphoprotein YqfN)	YqfN	Unknown	N.D.**	5,5-6,7
100	BSU11850	Unknown similar to unknown proteins	YjcG	Unknown	0,113; 0,157	5,5-6,7

* Proteins with more than one functinal class.
** Proteins not detected in OGU1 strain.
*** Proteins not detected in wild-type strain.

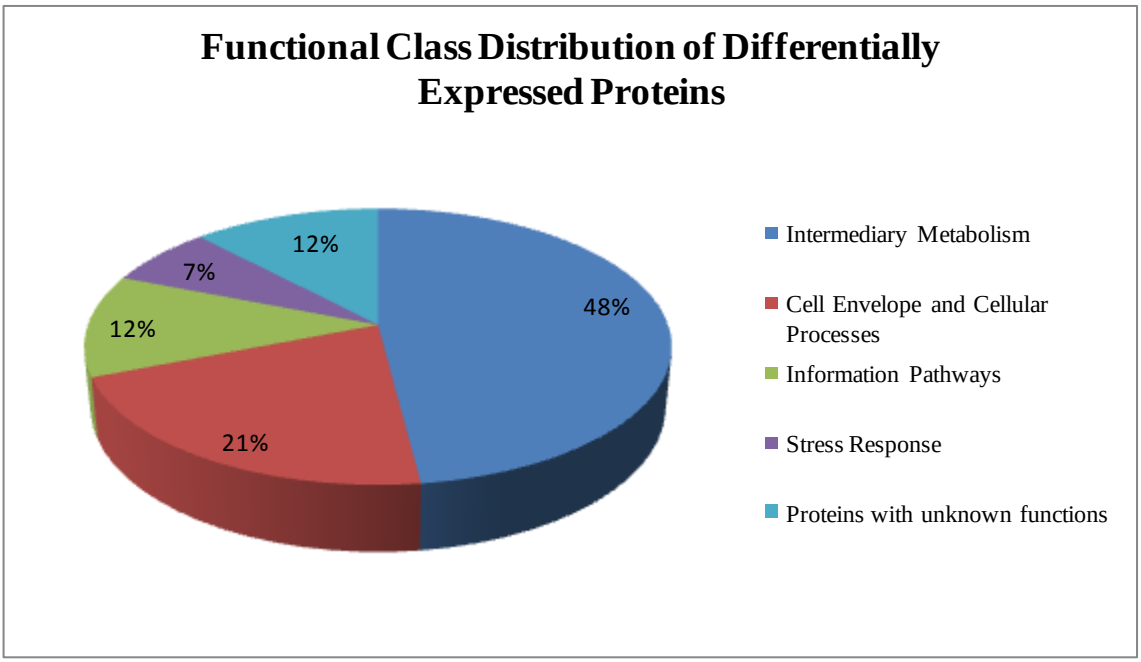

Figure 3.15. Relative quantitative distribution of 128 differentially expressed proteins to the major functional branches of cellular physiology.

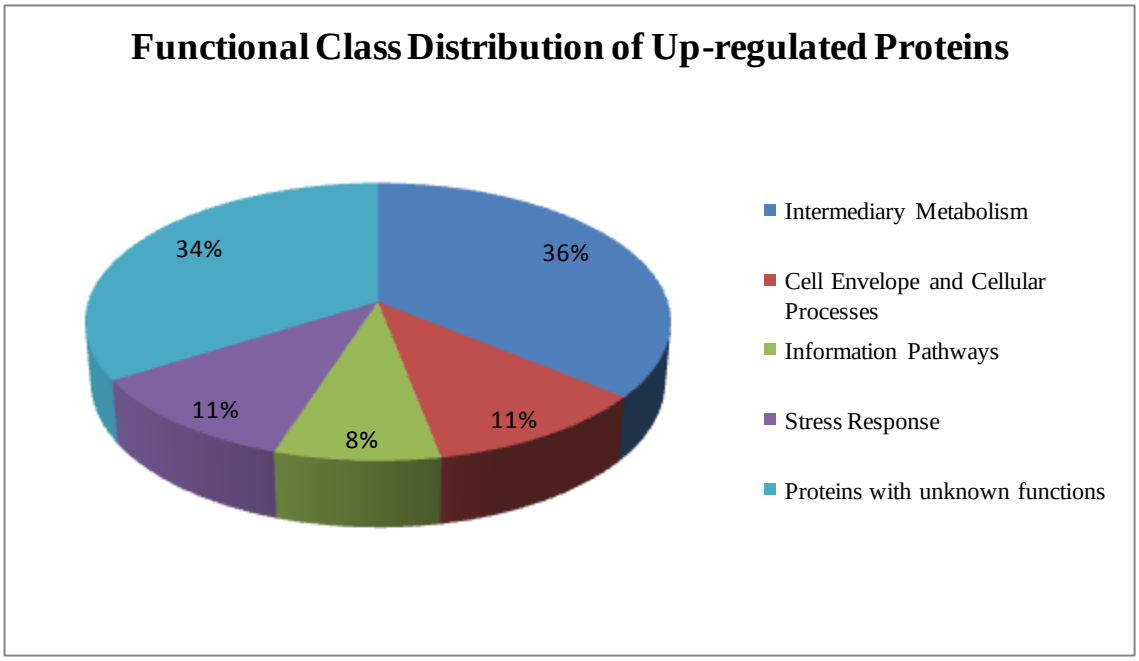

Figure 3.16. Relative quantitative distribution of up-regulated proteins in the mutant strain to the major functional branches of cellular physiology.

Further analysis of functional classifications of up-regulated proteins in the mutant strain revealed a relatively high percentage (34%) of proteins with unknown functions. This might suggest the significance of these unknown proteins in *B. subtilis* physiology in the absence of bacilysin production. Relative quantitative distribution of up-regulated proteins in the mutant strain to the major functional branches of cellular physiology is shown in Figure 3.16.

The percentage values among the down-regulated ORFs identified in this study were 51 for intermediary metabolism, 29 for cell envelope and cellular processes, 12 for information pathways and 6 for stress response. 2 % of proteins were remained unclassified since their functions are uncharacterized. Figure 3.17. shows the relative distribution of down-regulated ORFs to the functional classes of cellular physiology listed under the main headings of SubtiList web server.

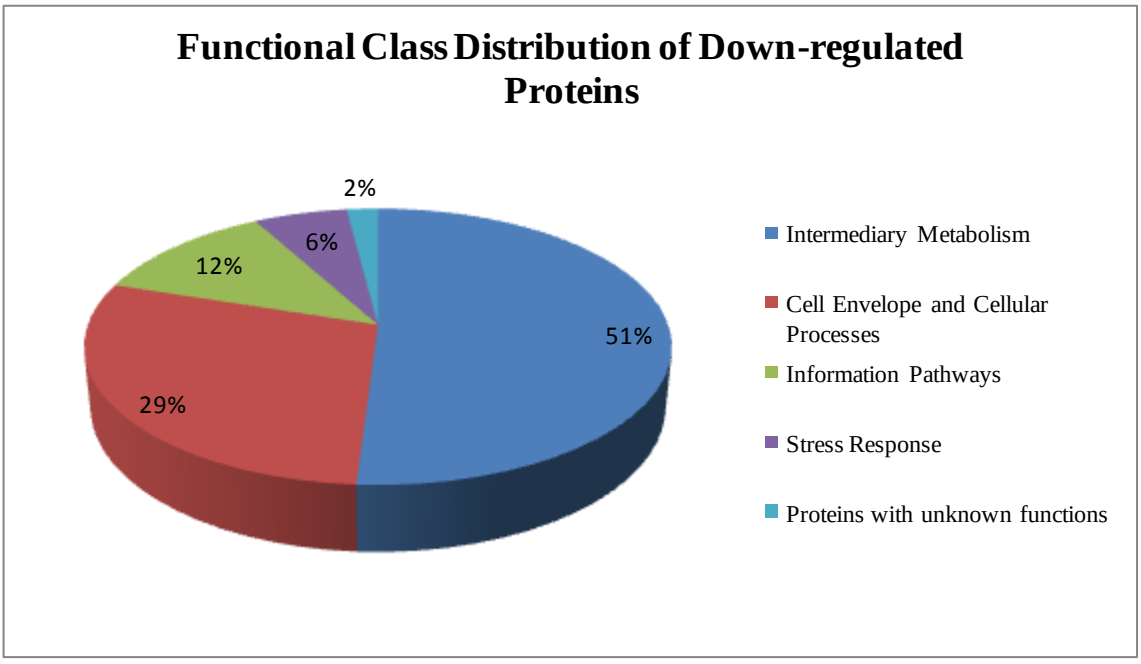

Figure 3.17. Relative quantitative distribution of down-regulated proteins in the mutant strain to the major functional branches of cellular physiology.

3.6. Subcellular Location and Signal Peptides

According the results obtained from the PSORTb search, 105 proteins were predicted to be located in the cytoplasm, 7 proteins were found to be on cytoplasmic membrane, and 6 were found to be extracellular. The localizations of 10 hypothetical proteins could not be determined. SignalP analysis predicted that 3 of the 128 identified proteins had a signal peptide sequence (Table 3.1).

3.7. Analysis of Differentially Expressed Proteins

Various studies have been performed earlier to investigate the regulation of bacilysin biosynthesis (Özcengiz *et al*., 1990; Özcengiz and Alaeddinoglu, 1991; Basalp *et al*., 1992) and molecular mechanisms of interactions of this antibiotic expression with quorum-sensing and stringent response elements in *B. subtilis* (Yazgan *et al*., 2001; Karatas *et al*., 2003; Inaoka *et al*., 2003). Recently, the effects of the products of *srfA*, *oppA*, *comA*, *phrC*, *phrF*, *phrK*, *comQ* (*comX*), *comP*, *spo0H*, *spo0A*, *abrB* and *codY* on the transcription of bacilysin biosynthetic operon *bacABCDE* was studied in our laboratory (manuscript in preparation). However, proteome-wide analysis of the functional role of bacilysin biosynthesis in regulatory cascade operating in *B. subtilis*, hence in cellular physiology has been studied for the first time in present study.

In order to obtain a comprehensive view of the changes of protein synthesis patterns in the absence of bacilysin biosynthesis and to assess the effect on global regulatory mechanisms caused by the loss of bacilysin biosynthesis in *B. subtilis*, we compared the abundance of all differentially expressed proteins in the wild type and OGU1 strain by performing comparative proteome analysis. In the present study, by using 2-DE gel electrophoresis coupled to MALDI-TOF/MS analyses, we determined the impact of *bacA* deletion on the abundance of numerous proteins that had never analysed before. This has enabled us to redefine the role of quorum-sensing and two-component systems as being two of the major regulatory mechanisms in *B. subtilis*.

Our comparative proteome analyses revealed that expression of 63 out of 128 distinct proteins decreased in bacilysin inactive OGU1 strain as compared to the wild-type. We found 36 of 128 protein spots to be expressed more abundantly in mutant strain. In addition, according to our results, 19 protein spots were found to be absent in OGU1 strain and 2 protein spots was newly- induced in mutant strain. 8 of these 128 proteins (IlvC, AroA, PurA, GlyA, SucC, YrxA, AroE and YceE) were found to be induced in one pI range and decreased in other pI range. This can be explained by the fact that some of these proteins observed as multiple spots indicating the possibility of multimerizations or fragmentations (*M*r variations) and charge modifications (pI variations) due to post-translational modifications or to artificial chemical modifications imposed during the experimental processing of the protein sample (Wolff *et al*., 2007).

On the other hand, this study indicated that expressions of a total of 48 hypothetical proteins were either up- or down-regulated in the absence of bacilysin biosynthesis and consequently have to be considered significantly related with antibiotic production in *B. subtilis*. In our study, such proteins constituted 37.5 % of the identified proteins. Out of these 48 unknown protein spots, 6 were found to be missing in mutant strain. 19 unknown proteins were found to be less abundantly and 21 found to be more abundantly expressed in mutant strain. The other two unknown proteins were stress response protein YceE and transcriptional regulator YrxA. YceE was found to be lost in pI 4.5-5.5 range and more abundantly expressed in pI range 4-7. The expression of YrxA was found to be decreased by 13.5-fold in pI range 4-7. However, in the pI range 5.5-6.7, YrxA was identified in two spots and while one of them was found to be induced the other one was decreased. This result can be attributed to the presence of multimerizations or fragmentations resulted from post-translational modifications. Table 3.7 summarizes the expression alterations of unknown proteins identified in our study.

Table 3.7. Hypothetical Proteins.

Spot No	Locus Name	Protein Name/ Function	Gene Product	Mass (kDa)	pI	Subcellular[a] Localization	Signal[b] Peptide	Multiple Spots	Mutant/Control Ratio	pI Range of Detection
5	BSU19390	Unknown similar to nitric-oxide reductase/probable D-alanine amino transferase	YojN/ Dat	33,459	5,16	CM	-	-	0,232	4,5-5,5
8	BG14010	Unknown similar to unknown proteins	YurX	48,263	5,12	U	-	-	N.D.*	4,5-5,5
9	BSU29980	Unknown similar to Xaa-His dipeptidase	YtjP	51,020	4,85	C	-	-	N.D.*	4,5-5,5
12	BSU22230	Unknown similar to phosphotransferase system enzyme II	YpqE	17,924	5,43	C	-	-	N.D.*	4,5-5,5
21	BSU37710	Alanine-anticapsin ligase bacD	YwfE	52,234	4,87	C	-	-	N.D.*	4,5-5,5
23	BSU01540	Unknown similar to ATP-binding Mrp-like protein	YbaL (SalA)	38,614	5,39	CM	-	-	N.D.*	4,5-5,5
27	BSU02910	Unknown similar to tellurium resistance protein	YceE	20,935	4,58	C	-	-	N.D.*	4,5-5,5
								-	3,125	4-7
29	BSU37720	Unknown similar to glucose 1-dehydrogenase	YwfD	27,306	5,12	C	-	-	0,337	4-7
30	BSU02900	Unknown similar to tellurium resistance protein	YceD	20,681	4,49	C	-	-	3,125	4-7

Table 3.7. Continued

34	BSU23860	Unknown similar to 6-phosphogluconate dehydrogenase	YqjI	51,742	5,25	C	-	2	3,154	4-7
									2,680	
36	BSU28820	Putative aminopeptidase	YsdC	39,192	5,63	C	-	-	3,058	4-7
37	BSU15380	Similar to unknown proteins from other organisms	YlmE	25,687	5,65	C	-	-	2,680	4-7
41	BSU29410	Similar to unknown proteins from other organisms	YtkL	24,815	5,14	U	-	-	3,378	4-7
44	BSU39880	Similar to unknown proteins from *B. subtilis*	YxbC (YxaQ)	37,434	4,78	C	-	-	7,751	4-7
45	BSU00120	Unknown similar to amidotransferase	YaaE (PdxT)	21,433	5,14	C	-	-	2,617	4-7
48	BSU31030	Uncharacterized protein YuaE	YuaE	19,098	6,19	U	-	-	2,557	4-7
49	BSU29850	Uncharacterized protein YtoQ	YtoQ	16,780	5,84	C	-	-	3,472	4-7
50	BSU12410	Uncharacterized protein YjoA	YjoA	17,781	5,83	C	-	-	2,531	4-7
51	BSU28420	Uncharacterized protein YsmA	YsmA	17,062	5,63	U	-	-	2,531	4-7
52	BSU31390	Unknown similar to polyribonucleotide nucleotidyltransferase	YugI	14,274	5,95	C	-	-	8,474	4-7
55	BSU00160	Unknown similar to cortical fragment-lytic enzyme	YaaH	48,606	5,72	E	-	-	0,374	4-7
56	BSU02940	Unknown similar to toxic anion resistance protein	YceH	41,646	5,90	C	-	-	0,266	4-7

Table 3.7. Continued

71	BSU33540	Unknown similar to NAD(P)H dehydrogenase	YvaB	23,257	5,26	U	-	-	0,320	4-7
72	BSU29500	Uncharacterized protein YtfJ	YtfJ	16,336	5,16	U	-	-	0,109	4-7
73	BSU37110	Unknown similar to transaldolase (pentose phosphate)	YwjH (Tal**)**	22,899	5,89	C	-	-	0,230	4-7
74	BSU37920	Unknown similar to spore coat protein	YwdL (GerQ**)**	20,262	6,58	E	-	+	0,243; 0,121	4-7
								-	0,351	5,5-6,7
76	BSU27890	Unknown similar to transcriptional regulator	YrxA	19,707	5,79	C	-	-	0,074	4-7
								2	3,745;0,111	5,5-6,7
79	BSU17250	Uncharacterized protein YmaE	YmaE	26,559	9,11	U	-	-	2,100	5,5-6,7
82	BSU36950	Unknown similar to unknown proteins	YwlC	36,984	5,87	C	-	-	2,793	5,5-6,7
84	BSU31760	Unknown similar to pyrazinamidase/nicotinamidase	YueJ (PncA)	20,510	5,66	C	-	-	3,257	5,5-6,7
93	BSU09260	Unknown similar to adenosylmethionine-8-amino-7-oxononanoate aminotransferase (Uncharacterized aminotransferase YhxA)	YhxA	49,840	5,71	C	-	-	4,016	5,5-6,7
88	BSU00260	Unknown similar to toxic cation resistance	YaaN	43,803	5,71	C	-	-	4,366	5,5-6,7

Table 3.7. Continued

89	BSU35120	Unknown similar to unknown proteins (Uncharacterized protein YvlB)	YvlB	41,055	5,5	C	-	-	4,366	5,5-6,7
91	BSU21680	Unknown similar to peptide methionine sulfoxide reductase	YppQ (MsrB)	16,591	5,87	C	-	-	7,633	5,5-6,7
92	BSU36910	Uncharacterized protein YwlG	YwlG	19,364	6,43	C	-	-	7,633	5,5-6,7
93	BSU37520	Unknown similar to unknown proteins (Uncharacterized protein YwhD)	YwhD	19,444	6,45	C	-	-	8,333	5,5-6,7
94	BSU25180	Unknown similar to unknown proteins (Phosphoprotein YqfN)	YqfN	23,690	5,39	C	-	-	N.D.*	5,5-6,7
97	BSU07690	Unknown similar to methionine aminopeptidase	YflG (MapB)	27,193	5,65	C	-	-	0,093	5,5-6,7
99	BSU10070	Unknown similar to aminoacylase	YhaA	43,241	5,87	C	-	-	0,095	5,5-6,7
100	BSU11850	Unknown similar to unknown proteins	YjcG	19,649	5,65	C	-	+	0,113; 0,157	5,5-6,7
104	BSU14530	Unknown similar to unknown proteins (Ribonuclease J 1 YkqC)	YkqC (RnjA)	61,477	5,93	C	-	-	0,192	5,5-6,7
107	BSU07830	Unknown similar to NAD(P)H-flavin oxidoreductase	YfkO	25,612	5,73	C	-	+	0,262; 0,293; 0,300	5,5-6,7
108	BSU40250	Unknown similar to formaldehyde dehydrogenase	YycR	42,919	5,82	C	-	-	0,289	5,5-6,7

Table 3.7. Continued

110	BSU12980	Unknown similar to chloromuconate cycloisomerase (L-Ala-D/L-Glu Epimerase)	YkfB	39,447	5,95	C	-	-	0,313	5,5-6,7
112	BSU18360	Unknown similar to unknown proteins (Aldose 1-epimerase)	YoxA	37,204	5,64	C	-	-	0,325	5,5-6,7
114	BSU09180	Unknown similar to unknown proteins (Spore coat protein F-like protein YhcQ)	YhcQ	24,774	5,77	E	-	-	0,326	5,5-6,7
117	BSU12290	Unknown similar to NADH dehydrogenase	YjlD	41,927	6,28	CM	-	+	0,332; 0,335	5,5-6,7
121	BSU08720	Unknown similar to bacterioferritin comigratory protein	YgaF (Bcp)	18,097	5,89	C	-	-	0,380	5,5-6,7

[a] Predicted location of proteins by PSORTb version 2.0.4. C: Cytoplasm, CM: Cytoplasmic Membrane, E: Extracellular and U:Unknown

[b] Signal Peptides predicted by SignalP prediction program.

[*] Proteins not detected in OGU1 strain.

Our findings about the differentially expressed proteins are discussed according to their roles in global regulatory pathways and bacilysin biosynthesis. The general information about these proteins are below;

YwfE (Alanine-anticapsin ligase BacD) and YwfD (Unknown similar to glucose-1-dehydrogenase);

The *ywfBCDEF* genes of *B. subtilis 168* were shown to carry the biosynthetic core functions and were renamed as *bacABCDE* to denote their relevance to bacilysin formation (Rajavel *et al*., 2009). The products of the first three genes are known to be critical for formation of anticapsin (Steinborn *et al*., 2005). Experimental evidence revealed that proteins of the *bacABC* cluster were sufficient to produce anticapsin and disruption of any of these genes inhibited anticapsin synthesis. The fourth gene, *bacD*, encodes an L-amino acid ligase that has been previously characterized in vitro and shown to have promiscuous activity (Tabata *et al*., 2005). It follows that the BacD enzyme catalyzes amide bond formation between Ala and anticapsin. The fifth gene, bacE, is involved in host resistance (Steinborn *et al*., 2005). Intriguingly, upon closer inspection of the gene cluster one finds a sixth ORF, *ywfG*, which generally is not annotated with the rest of the cluster. Additionally, a separate, monocistronic gene, *ywfH*, located immediately downstream of the bacilysin gene cluster, has been reported to be important in the production of bacilysin (Inaoka *et al*., 2003).

Bioinformatic analysis has led to the assignment of possible activities for each of the seven proteins BacA-E. The three proteins known genetically to be involved in formation of anticapsin have the following putative functions: BacA is homologous to prephenate dehydratases, which are involved in decarboxylation of prephenate in the aromatic amino acid biosynthesis pathway, BacB is a member of the bicupin iron enzyme family, and BacC is proposed to have nicotinamide-dependent reductase or

dehydrogenase activity. The protein encoded by the sixth gene *ywfG* is homologous to aminotransferases. YwfH has putative nicotinamide-dependent reductase or dehydrogenase activity (Mahlstedt and Walsh, 2009).

Consistent with the study conducted by Sakajoh *et al*. (1987) which indicated that the *bacD* mutation inactivates the amino acid-ligation function for the conversion of anticapsin into bacilysin, our proteomic analysis proved that alanine-anticapsin ligase BacD protein was absent from bacilysin-negative OGU1 strain. However, in the case of YwfD (unknown similar to glucose-1-dehydrogenase) protein, the expression in the mutant strain was found to be decreased by 4.6 and 2.9- fold. The biochemical characterization of BacA and BacB suggested that the production of L-anticapsin occurs from 2-*oxo*-3-(4-oxocyclohexa-2,5-dienyl) propanoic acid by a ring epoxidation and a transamination reaction in the ketone group (Rajavel *et al*., 2009). In the same study, authors proposed that other enzymes, apart from the *bac* operon members, could play a role in L-anticapsin synthesis.

Sporulation Proteins of *B. subtilis* ;

Our comparative analysis revealed that 12 of the 128 differentially expressed proteins were sporulation proteins. Among these, L-alanine dehydrogenase (SpoVN), major alpha-type small acid-soluble spore protein (SspA) and major beta-type small acid-soluble spore protein (SspB) were found to be lost and other 9 protein spots were found to be less abundantly expressed in mutant strain.

It has been known that conventional solubilization techniques and even aggressive solubilization techniques designed for separation of membrane proteins by 2-DE involving strong chaotropes and reducing agents, proved to be ineffective for solubilization of *B. subtilis* spore-coat proteins (Lai *et al*., 2003). In our study,

identification of sporulation proteins indicated the effectiveness of our solubilization technique for *B. subtilis* total proteome.

Expression of **L-alanine dehydrogenase (SpoVN)** was shown to play a role in providing energy for late sporulation events. Siranosian *et al*. (1993) reported that *ald* mutants are blocked at a late stage in sporulation. This protein was found to be absent in mutant OGU1 strain.

It has been known that spores of *B. subtilis* contain a number of small, acid-soluble spore proteins (SASP) which comprise up to 20% of total spore core protein. **Major alpha-type small acid-soluble spore protein (SspA) and major beta-type small acid-soluble spore protein (SspB)** have been shown to confer resistance to UV radiation, heat, peroxides, and other sporicidal treatments (Moeller *et al*., 2009). Indeed, spores of *B. subtilis* strains which carry deletion mutations in one gene (*sspA*) or two genes (*sspA* and *sspB*) were shown to be much more sensitive to heat and UV radiation than wild-type spores (Mason and Selow, 1987). In the present study, these two spore proteins were found to be missing in bacilysin biosynthesis-blocked OGU1 strain.

An unknown protein similar to cortical fragment-lytic enzyme (YaaH) which is synthesized in the mother cell compartment and localizes in spores of *B. subtilis* was found to be less abundantly expressed in bacilysin minus strain by 2.6-fold. Kodama *et al*. (1999) reported that the inactivation of the *yaaH* gene did not impair vegetative growth or prevent the development of resistance to heat, chloroform, and lysozyme in spores. On the other hand, in their study, germination of the *B. subtilis* spores was shown defective without the *yaaH* gene product.

In a study by Ragkousi *et al*. (2003), functional analysis of *ywdL* revealed an **unknown protein similar to spore coat protein (YwdL)** present in the spore coats, a proteinaceous layer surrounding the spore's outer membrane. CwlJ was previously shown to be essential for spore germination by Ca- dipicolinic acid (Nicholson and

Setlow, 1990). In the study of Ragkousi and coworkers (2003), YwdL was found to be essential for the localization of CwlJ in the spore coats and thus for Ca^{2+}-dipicolinic acid-induced spore germination. In our study, this protein was identified in 3 distinct spots and these spots found be less abundantly expressed in mutant strain by 4.1, 8.2 and 2.8-fold as compared to wild–type strain.

The *cotA* gene codes for a 65-kDa protein belonging to the outer spore coat of *B. subtilis*. It corresponds to the former *pig* locus (Rogolsky, 1968; Donovan *et al*., 1987) and was one of the first *cot* genes cloned (Donovan *et al*., 1987). The *cotA* gene is expressed under the control of sigma K. **Spore coat protein (CotA)** seems to be responsible for most of the protection afforded by the spore coat against UV light and hydrogen peroxide. The absence of CotA has no apparent effect on spore resistance, but it results in the loss of the usual brownish pigmentation of the colonies (Hullo *et al*., 2001). The expression of this spore coat protein was found to be decreased by 11.3-fold in bacilysin blocked strain.

One of the novel protective feature of the spore core is that it has the high levels (25% of core dry weight) of dipicolinic acid (DPA) plus its associated divalent cations, predominantly as a 1:1 chelate with Ca^{2+} (Ca-DPA) (Coleman *et al*., 2007; Gerhardt *et al*., 1989; Setlow, 2006). The high core Ca-DPA level helps to reduce core water content, an important element in spore resistance to wet heat, and Ca-DPA also plays a more direct role in protecting spore DNA against several types of damage (Gerhardt *et al*., 1989; Paidhungat *et al*., 2000; Setlow *et al*., 2006; Setlow, 2006). Setlow *et al*. (2006) showed that spores that lack both Ca-DPA and α/β-type SASP die rapidly during spore formation, most likely due to DNA damage. DPA is made only during sporulation in the mother cell compartment of the sporulating cell and is then taken up into the developing spore (Errington, 1993). The final step in DPA synthesis is catalyzed by DPA synthase, comprised of the **dipicolinate synthase subunit A (SpoVFA)** and –B

proteins (Errington, 1993). In our study, SpoVFA protein was found to be 6.2-fold less expressed in mutant strain.

Acotinate hydratase (CitB) is another protein required for sporulation. *citB* encodes aconitase of the tricarboxylic acid cycle and is required for normal sporulation, and its expression also increases during sporulation (Uratani-Wong *et al*., 1981; Dingman *et al*., 1987; Fouet and Sonenshein, 1990). In addition, Molle *et al*. (2002) reported that *citB* was one of the four transcriptional units that CodY direct interacts. The expression of this protein was also found to decrease by 2.9-fold in mutant strain.

The transition state of *B. subtilis*, which carries the cells from vegetative growth to stationary phase, is a stage in which the cell population is highly differentiated. In this stage, which is governed by the phosphorylation status of Spo0A, the cells can carry out a number of highly specialized functions (Hoch, 1998). These functions include not only those controlling the initiation of the spore-forming process but also those responsible for competence and motility as well as the production of scavenging enzymes and a host of secondary metabolites (Smith, 1993). The fate of the individual cells within the population is determined by synthesis, secretion, processing, and uptake of signaling peptides used for cell-to-cell communication (Solomon *et al*., 1996; Perego, 1999). In addition, a number of other genes called transition state regulators play an important role in this differentiation process (Perego *et al*., 1988; Strauch *et al*., 1989). Hyper-expression and/or loss of function of any of these genes profoundly affects several aspects of the cell physiology. One gene that belongs to this group of transition state regulators is *scoC*. Caldwell *et al*. (2001) reported that ScoC regulates, either directly or indirectly, the expression of at least 560 genes in the *B. subtilis* genome. It has been shown recently that *scoC* plays a direct role in the initiation of sporulation by acting as a repressor of the two major signaling peptide transport systems, *opp* and *app* (Koide *et*

al., 1999). Caldwell *et al*., (2001) found that **uncharacterized protein YtfJ** is one of the sporulation control genes and is affected by *scoC* mutation. In the same study, YtfJ was found to be regulated by Spo0A and σ^F regulon. This protein was found to be 9.1-fold less abundantly expressed in mutant strain in our study. This result is consistent with the expression patterns of other sporulation proteins in mutant strain indicating that sporulation is affected in the absence of bacilysin biosynthesis.

Wang *et al*. (2006) proposed that **spore coat protein F-like protein (YhcQ)** was one of the forespore-specific proteins and display extensive sequence similarity to CotF, which is a well characterized spore coat protein. In the present study, YhcQ was found to be expressed 3.0-fold less in the mutant strain compared to that in wild-type strain. YhcQ is yet an uncharacterized protein.

NH_3-dependent NAD(+) synthetase (Sporulation protein OutB)(NadE) is involved in spore germination and outgrowth and is also essential for growth (Nessi *et al*., 1994). In the case of *outB* mutation, temperature-sensitive outgrowth phenotype has been reported at 46 °C in rich medium (Albertini and Galizzi, 1975). However, each of the *nadE* (*outB*) mutants with reduced NAD synthetase activities was also impaired in growth at a permissive temperature of 35 °C, indicating the essential nature of this protein (Albertini *et al*., 1987). NadE was subsequently shown to belong to the family of σ^B-dependent general stress proteins (Antelmann *et al*., 1997; Malcolm *et al*., 2001). In addition, transcriptional studies showed that *nadE* is strongly induced in response to heat, ethanol and salt stress or after starvation for glucose in a σ^B-dependent manner. Sporulation protein OutB was found to be less abundantly expressed in the mutant strain (decrease 4.0-fold) in our study.

Another protein involved in spore formation was a **probable fructose-1,6-biphosohate aldolase (FbaA)** which was found to be 2.8 and 2.9-fold less abundantly expressed in the mutant strain as compared to wild-type strain. Endo and Kurusu (2007) reported that

FbaA is an essential protein in *B. subtilis* and its expression level is constant during the course of growth and sporulation. Marino and his co-workers (2000) reported that FbaA was induced under anaerobic conditions. In a study investigating the protein profile of cold-shocked sporulating *B. subtilis* cells, FbaA protein was found to be induced in vegetative *B. subtilis* cells in response to cold shock (Movahedi and Waites, 2002).

Oligopeptide ABC transporter (Oligopeptide permease) (OppD) of *B. subtilis* is an ATP binding cassette (ABC) transporter that imports small peptides (Perego *et al*., 1991; Rudner *et al*., 1991). It also functions to regulate two developmental processes, sporulation and genetic competence. In fact, *B. subtilis opp* was first identified as a sporulation locus, named *spo0K*, because null mutations caused decreased sporulation efficiency (Perego *et al*., 1991; Rudner *et al*., 1991). It contributes to the regulation of sporulation and competence, at least in part, by importing specific signaling peptides derived from *phr* gene products (Lazazzera *et al*., 1998; Lazazzera *et al*., 2001; Perego and Brannigan, 2001; Pottathil and Lazazzera, 2003). Yazgan *et al*. (2001) showed that bacilysin biosynthesis did not take place when the oligopeptide permease function was impaired. It was therefore very important to find in the present study that in the absence of bacilysin biosynthesis, the expression of OppD decreased by 5.6-fold in the mutant strain as compared to wild-type strain. More interestingly, it has been known that promoter of the *opp* oligopeptide transport operon is the target for ScoC which is a repressor of early sporulation gene expression. Recently, Inaoka *et al*. (2009) have found that ScoC negatively regulates bacilysin production in *B. subtilis*. In this respect, our finding provided further clue.

Our findings on down-regulation of sporulation proteins in thebacilysin-negative strain supports the possible relationship between bacilysin biosynthesis and sporulation which was first studied by Özcengiz and Alaaddinoğlu (1991). In their study, when compared to parental strain, bacilysin-negative strain was found to be less resistant to heat, chloroform, and lysozyme treatments, and the spores contained less dipicolinate. The

improvement of the spore quality of the mutant cells with external addition of bacilysin and the restoration of sporulation function by transduction of *bac* locus to the bacilysin-negative mutant suggested bacilysin might influence sporulation (Özcengiz and Alaaddinoğlu, 1991). In this respect, our findings provided further clue on the possible interaction of bacilysin biosynthesis and sporulation.

Enzymes that sequentially remove the NH_2-terminal amino acid from protein and peptide substrates are called aminopeptidases (Taylor, 1993). Enzymatic studies demonstrated that **D-aminopeptidase DppA** hydrolyzes D-Ala- D-Ala with high affinity (Remaut et al., 2001). The dpp transport operon has been well characterized and shows conservation in structure across both Gram-negative (Abouhamad & Manson, 1994) and Gram-positive (Guedon et al., 2001; Slack et al., 1995) bacterial species. D-aminopeptidase gene *dppA*, the first ORF of the dipeptide ABC transport (*dpp*) operon which is involved in dipeptide transport, has been shown to be maximally expressed in the stationary phase of growth and early during sporulation as reported by Mathiopoulos *et al*. (1991) with expression driven from the promoter region upstream of the first gene, dppA (Abouhamad & Manson, 1994). *dpp* expression is induced by all mechanisms known to turn on sporulation and is indirectly under Spo0A control (Slack *et al.,* 1991). However, it is not required for sporulation to take place. The dpp operon is negatively regulated by two trans-acting factors, AbrB and CodY (control of *dciA/dpp* operon) (Slack *et al*., 1991; 1993; 1995; Serror and Sonenshein, 1996). AbrB is a well-known repressor of some genes expressed early during sporulation (Robertson *et al*., 1989), and *codY* codes for a product required for repression of the *dpp* operon during growth in a medium containing rapidly metabolized sources of carbon and nitrogen (Slack *et al*., 1995). CodY competes with AbrB for the promoter region of the dpp operon, but the repressor activity of CodY requests the presence of an unidentified co-repressor. Nutritional adaptation and spore formation are usually considered as competitive and mutually exclusive processes (Mathiopoulos *et al*., 1991), but the choice between the two pathways depends on an integration of multiple signals (Cheggour *et al*., 2000). In

our study, **DppaA** protein was found to be 3.8-fold down-regulated in the mutant strain. Lower expression of DppaA in the mutant strain with respect to the wild-type suggested that either AbrB and/or CodY represses *dpp* operon more effectively in the absence of bacilysin. It follows that bacilysin somehow counteracts with the functions of these repressors.

General Stress Response in *B. subtilis*

Petersohn and coworkers (2001) found that a large group of stress proteins may have a rather nonspecific, but nevertheless very essential, protective function in response to stress or starvation, regardless of a specific stress stimulus. Therefore, the proteins were called nonspecific or general stress proteins (Richter and Hecker, 1986; Hecker *et al*., 1988; Hecker *et al*., 1990). Stress induction of this protein group was shown to be mediated by the alternative sigma factor σ^B, the general stress sigma factor of gram-positive bacteria. So far, more than 150 σ^B -dependent genes have been identified by proteome studies and transcription profiling (Antelmann *et al*., 1997; Helmann *et al*., 2001; Petersohn *et al*., 2001; Price *et al*., 2001; Yu *et al*. 2008). Many of the general stress genes display basal level transcription from vegetative σ^A-dependent promoters. However, activation of σ^B activity following metabolic or environmental stress dramatically increases the transcription of the general stress genes (from approximately 1% in growing cells to 20% or even more in starved or stressed bacteria (Bernhardt *et al*., 1997).

Comparative phenotypic studies on *sigB* mutants and wild-type bacteria have proven that high-level expression of the general stress regulon provides stressed cells with multiple, nonspecific, prospective stress resistance in anticipation of "future stress"

(Engelmann and Hecker**,** 1996; Gaidenko *et al*., 1998; Völker *et al*., 1999). This protective function is particularly important for cells that are no longer able to grow (Völker *et al*., 1999). Therefore, the general stress response might be an essential alternative for all resting *Bacillus* cells that do not sporulate efficiently either because the cell density is too low (Grossman and Losick, 1988) or because stress conditions (e.g. osmotic stress, oxygen limitation) do not allow sporulation (Hoffmann *et al*., 1995; Ruzal *et al*., 1998). In the present study, we identified 9 protein spots that are involved in detoxification and known as stress proteins (Table 3.5). 4 of these 9 protein spots (**AhpC, YceD, YugI and YaaN**) were more abundantly expressed in bacilysin-negative strain. This result is consistent with the suggestion that general stress proteins are essential alternative for cells that do not sporulate efficently, since we observed lower expression of sporulation proteins in bacilysin-negative strain. Another general stress protein **YceE**, on the other hand, was found to be missing in one pI range and to be more abundantly expressed in an other pH range. As discussed earlier, this might be resulted from biologically important post-translational modifications or to artificial chemical modifications imposed during the experimental processing of the protein sample (Wolff *et al*., 2007).

Höper *et al*. (2005) reported that **YceD** (**unknown protein similar to tellurium resistance protein) and YceE** (**unknown protein similar to tellurium resistance protein)** belong to the TerD family of proteins (Pfam Accession no. PF02342) and are important for tellurite resistance. Also these proteins could be involved in protection from reactive oxygen species. In the same study, *yceD*, *yceE* mutants were impaired in survival at 4°C, supporting the postulation that these general stress proteins are essential for viability.

The **AhpC** subunit of the *B. subtilis* **alkyl hydroperoxide reductase** was identified as a general stress protein induced in response to heat or salt stress or after entry of the organism into the stationary phase (Antelmann *et al*., 1996). Two-dimensional gel

analyses revealed an especially strong induction of AhpC and AhpF in cells subjected to oxidative stress. Transcriptional studies showed a 3- to 4-fold induction of *ahp* mRNA after heat or salt stress or starvation for glucose and a 20-fold induction by oxidative stress indicating that it was a general stress protein (Antelmann *et al*., 1996). This protein was found to be 2.6-fold more abundantly expressed in our mutant strain.

Another typical general stress protein **YugI (a putative polyribonucleotide nucleotidyltransferase or general stress protein 13)** was shown to be elevated after heat shock, salt stress, ethanol stress, glucose starvation, oxidative stress (Bernhardt *et al*. 1997; Antelmann *et al*. 1997) and cold shock (Kaan *et al*. 2002). It was also classified as a protein negatively controlled by the stringent response by Eymann *et al*. (2002). This protein was found to be 8.5-fold more abundantly expressed in the mutant strain when compared to the wild type.

The other stress proteins identified in our study were YceH, YvaB, MsrA and YgaF which were found to be less abundantly expressed in the mutant strain. **MsrA (peptidyl methionine sulfoxide reductase)** is predicted to reduce methionine sulfoxides to methionine, thus restoring protein function after oxidative stress in *B. subtilis*, analogous to the role of MsrA in *E. coli* (Moskovitz *et al*., 1995). In a study conducted by Mostertsz *et al*. (2008), expression of MsrA protein was found to be increased in response to superoxide and superoxide stress. Leichert *et al.* (2003) showed that MsrA expression was elevated when cells were exposed to thiol compounds inducing disulfide stress. It has been known that disulfide bonds play a major role in stabilizing protein structures, and extracellular proteins are particularly dependent on disulfide bonds to stabilize their structure. According to our results, MsrA which is essential for *B. subtilis* cells to stabilize protein structures 8.2 fold less abundantly expressed in bacilysin-negative cells. The mutant cells might have become more susceptible to superoxide or disulfide stress due to a lower expression of MsrA.

YgaF (unknown protein similar to bacterioferritin comigratory protein) is a member of PerR regulon members of which encodes antioxidant proteins in *B. subtilis* (Horsburgh *et al*., 2001). YgaF was found to be a peroxidase (Cummings and Connerton, 1997) which displays 40–48% identity with antioxidant proteins of the peroxiredoxin family (Chae *et al*., 1994), including bacterial bacterioferritin co-migratory protein (Bcp) homologues and mammalian thioredoxin-dependent peroxidase. In present study, this protein was found to be 2.6-fold less abundantly expressed in bacilysin-negative strain as compared to the wild type. We can propose that inactivation of bacilysin biosynthesis in *B. subtilis* PY79 led the cells to less efficient production of antioxidant proteins resulting in a more susceptible phenotype to oxidative stress.

In a global analysis of general stress response in *B. subtilis* study, Petersohn *et al*., (2001) found that **YceH (unknown protein similar to toxic anion resistance protein**) was a putative σ^B – dependent gene product and showed homology to tellurite resistance protein TelA of *R. sphaeroides*. This protein was found to be 3.7-fold less abundantly expressed in the mutant strain in our study. In the same study, Petersohn *et al*., (2001) showed that **YaaN (unknown similar to toxic cation resistance protein)** was σ^B – independent and induced by salt shock in *B. subtilis*. This protein is also an uncharacterized protein and was found to be 4.4-fold more abundantly expressed in bacilysin-negative strain according to our results. These unknown proteins are probably necessary to overcome any stress caused by the absence of bacilysin production.

It has been known that members of the σ^B-regulon are involved in the protection of DNA, membranes, and proteins against oxidative damage, which might represent an important component within the complex stress response (Petersohn *et al*., 1999). **YtkL**, which is classified as **'similar to unknown proteins from other organisms'** is a member of this regulon. Petersohn *et al*. (2001) found that this protein was induced by ethanol shock. Recently, Rukmana *et al*. (2009) found that after *B. subtilis* cells were exposed to antibiotic enduracidin, which inhibits the transglycosylation step of the

peptidoglycan biosynthesis, YtkL expression was up-regulated. In our study, this protein was found to be 3.4-fold more abundantly expressed in bacilysin-negative strain OGU1, providing further evidence that inability to form bacilysin exerts some sort of stress onto the cells. This unknown protein might be induced to provide a possible adaptation to the lack of thedipeptide antibiotic bacilysin.

Metabolic Pathway Proteins

In *B. subtilis*, the genes of the branched-chain amino acids (isoleucine, leucine and valine; BCAAs) biosynthetic pathway are organized in three genetic loci: the *ilvBHC-leuABCD* (*ilv-leu*) operon, *ilvA*, and *ilvD* (Fink, 1993). It has been known that the *ilvBHCleuABCD* (*ilv-leu*) operon included genes encoding acetolactate synthase (*ilvBH*), ketol-acid reductoisomerase (*ilvC*), 2-isopropylmalate synthase (*leuA*), 3-isopropylmalate dehydrogenase (*leuB*), and 3-isopropylmalate dehydratase (*leuCD*); the *ilvA* gene encoding threonine dehydratase; and the *ilvD* gene encoding dihydroxy-acid dehydratase (Mader *et al*., 2003). These enzymes catalyze the formation of the α-keto acid precursors of isoleucine, valine, and leucine. These genes, as well as *ybgE*, encoding a branched-chain amino acid aminotransferase, were recently demonstrated to represent direct targets of the global transcriptional regulator CodY (Mader *et al*., 2003). In our study, three BCAAs, **IlvC (ketol-acid reductoisomerase), IlvD (dihydroxy-acid dehydratase), LeuC (3-isopropylmalate dehydratase-large subunit)** were found to be differentially expressed. IlvC and IlvD were found to be absent from mutant strain in pH range of 4.5-5.5. Nevertheless, IlvC was found to be 3.1-fold down-regulated in mutant strain in an other pH range. This finding may be caused by the fact that IlvC and IlvD appeared as multiple spots on 2-D gels (Wolff *et al*., 2007). The third branched-chain amino acid gene product, LeuC, was found to be less abundantly expressed by 3-fold in mutant strain in our study. Although it has been known that these gene products were subjected to multiple mechanisms of regulation such as tRNA-mediated transcriptional anti-termination in response to leucine availability (Grandoni *et al*., 1992;

Marta *et al*., 1996) or catabolite repression (Shivers and Sonenshein, 2005), lower expression of these proteins in mutant strain might have resulted from direct negative regulation of CodY.

Expression of urease, which is encoded by the *ureABC* operon, is regulated in response to nitrogen availability in *B. subtilis* (Ramos *et al*., 1997). This metalloenzyme hydrolyze urea to form carbonic acid and two molecules of ammonia (Mobley *et al*., 1995). Degradation of urea provides ammonium for incorporation into intracellular metabolites and facilitates survival in acidic environments (Collins *et al*., 1993; Mobley *et al*., 1995). Wray *et al*. (1997) reported that the *B. subtilis ureABC* operon was transcribed from three promoters, one of which was predominant P3 promoter which is regulated by CodY, TnrA, and GlnR. Authors found that CodY was the major factor in the repression of P3 expression in the cells grown in a medium containing amino acids and GlnR and TnrA also contributed to this repression.

Since CodY is a DNA binding protein (Serror and Sonenshein, 1996), it was presumed that CodY functioned directly as a repressor to inhibit transcription from these promoters, but because of the fact that no consensus binding sequence has been identified for CodY, the possibility of CodY only indirectly regulates expression of the P2 and P3 promoters could not been ruled out. In the present study, **UreC (urease α-subunit)**, which is mainly regulated by CodY, was found to be 5.6-fold down-regulated in bacilysin-minus mutant.

The aromatic amino acids phenylalanine, tyrosine and tryptophan are formed by a sequence of reactions starting with the synthesis **of 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase (DAHP)** which occurs by the enzymatically catalysed condensation of D-erythrose 4-phosphate and phosphoenolpyruvate (Henner & Yanofsky, 1993). The synthesis is catalysed by DAHP synthase (EC 4.1, 2. IS), encoded in *B. subtilis* by the *aroA* gene. On the other hand, **AroE (5-enolpyruvoylshikimate-3-**

phosphate synthase) is a shikimate pathway enzyme catalyzes the biosynthesis of chorismate (Herrmann, 1995). Chorismate mutase catalyzes the [3,3] Claisen rearrangement of chorismate (Weiss and Edward, 1980) to prephenate (Andrews *et al*., 1973).

Previously, it was suspected that the anticapsin was derived from either tyrosine or phenylalanine. However, radioactive isotope labeling studies indicated that neither of these compounds were the anticapsin precursors (Roscoe and Abraham, 1966). *B. subtilis* cells fed radiolabeled shikimate produced labeled bacilysin (Roscoe and Abraham, 1966), indicating that the precursor must be an intermediate along the aromatic amino acid biosynthetic pathway. Several years later genetic knockout studies narrowed down the possible precursors (Hilton *et al*., 1988). Bacteria typically produce Tyr and Phe by first converting chorismate to prephenate through the action of chorismatemutase. Prephenate is then decarboxylated and aromatized to form either phenylpyruvate (prephenate dehydratase) or 4-hydroxyphenylpyruvate (prephenate dehydrogenase), which can be transaminated to produce Phe and Tyr, respectively. Mutations in prephenate dehydratase and prephenate dehydrogenase, the enzymes that catalyze decarboxylation, did not halt production of bacilysin, so it was concluded that anticapsin must be derived from prephenate (Hilton *et al*., 1988).

In our comparative proteome study, AroA (3-deoxy-D-arabino-heptulosonate-7 phosphate synthase-chorismate mutase) was found to be lost in pH 4.5-5.5 range and less abundantly expressed in mutant strain by 3.1- and 13.5-fold in other pI ranges. Similarly, AroE (5-enolpyruvoylshikimate-3-phosphate synthase) appeared in four spots in pH range 5.5-6.7, two of them less abundantly expressed and other two more abundantly expressed in the mutant strain. Wolff *et al.* (2007) also showed that AroA yielded multiple spots on the cytoplasmic vegetative proteome map of *B. subtilis*.

Two-component response regulators in *B. subtilis*;

It has been known that in bacteria, extracellular signals are transduced into the cell predominantly by two-component systems (TCSs) (Hoch 2000; Stock *et al*. 2000; Mascher *et al*. 2006; Gao *et al*. 2007). The prototypical TCS consists of a sensor kinase that responds to specific signals by modifying the phosphorylated state of a cognate response regulator (i.e., the second component). Sensor kinases are usually integral membrane proteins that autophosphorylate from ATP at a conserved histidine residue and then transfer the phosphoryl group to a conserved aspartate in the response regulator. Phosphorylation of a response regulator changes the biochemical properties of its output domain, which can participate in DNA binding and transcriptional control, perform enzymatic activities, bind RNA, or engage in protein–protein interactions (Gao *et al*. 2007; Mitrophanov and Groisman, 2008). To date, more than 35 two-component regulatory systems have been identified in *B. subtilis* by genome sequencing, this functioning in the adaptation to environmental stress (Darmon *et al.*, 2002), the production of secondary metabolites (Martin, 2004), and cell division (Fukuchi *et al.*, 2000). Our comparative proteome analysis revealed that LuxS (autoinducer-2 production protein), ResD (two-component response regulator), CheV (two-component response regulator) and DegU (two-component response regulator) were differentially expressed in OGU1 when compared to the wild-type PY79 strain. Three of these proteins were more abundantly expressed in bacilysin-negative strain while the fourth one, ResD was found to be less abundantly expressed in the mutant strain.

It has been known that cell-to-cell communication in bacteria is mediated by quorum-sensing systems that produce chemical signal molecules called autoinducers (AI). The studies of quorum- sensing had been limited to species-specific (intra-species or type 1) communication, in which acylhomoserine lactones (Gram-negative bacteria) or oligopeptides (Gram-positive bacteria) are employed as the AI-1s (Bassler *et al*., 1993). Since the interactions between AI-1s and their cognate receptors are highly specific, the

type 1 communication is restricted to bacteria within the same species, and generally does not permit any cross talk among different bacterial species (Pei and Zhu, 2004). Investigators showed that bacteria had an alternative mechanism to sense the presence of other species in a mixed population (interspecies or type 2 communication) (Bassler *et al*., 1993; Surette *et al*., 1999; Schauder *et al*., 2001). It has been shown that LuxS and its product AI-2 served as a universal language (bacterial Esperanto or universal bacterial lexicon) used by bacteria to mediate their intra- and inter-specific behaviours (Winans, 2002; Xavier and Bassler, 2003). In addition **LuxS/AI-2** have been shown to control a variety of cellular processes, such as production of pathogenicity factors, toxin production, biofilm formation, and swarming motility (Forsythand Cover, 2000; Day *et al*., 2001; Kim *et al*., 2003; Marouni and Sela, 2003; Merritt *et al*., 2003; Sperandio *et al*., 2003; Wen and Burne. 2004; Joyce *et al*., 2004; Merritt *et al*., 2005). Lombardia *et al*. (2006) reported that *B. subtilis* operates a *luxS*-dependent quorum-sensing system that regulates its morphogenesis and social behavior. In the same study, the researchers investigated whether an interrelationship does exist between sporulation and AI-2-dependent quorum-sensing and found that Spo0A and SinR had a notorious negative effect on *luxS* expression. These results indicated that the regulation of *luxS* expression in *B. subtilis* was complex and intricate, being under the control of an auto-regulatory AI-2-dependent feedback loop and under the control of the master regulatory proteins Spo0A and SinR.

In our proteome study, AI-2-dependent quorum sensing protein LuxS was found to be 2.7-fold over expressed in the mutant strain. Our study also revealed that regulation of LuxS expression and antibiotic production were intricately interconnected (Federle and Bassler, 2003) which is consistent with the former findings.

Chemotaxis is based on a two-component signal transduction network that allows bacteria to navigate within their environment and enables bacteria to move toward more favorable conditions (Karatan *et al*., 2001). In peritrichously flagellated bacteria such as

E. coli and *B. subtilis*, cells move up gradients of attractants and down gradients of repellents by modulating the duration and probability of smooth runs and reorientating tumbles (Rao *et al*., 2008). Although *E. coli* chemotaxis pathway uses a modified two-component system, *B. subtilis* has three adaptation systems: the methylation system (Goldman and Ordal, 1984), the CheC–CheD–CheYp system (Muff and Ordal, 2007) and the CheV system (Karatan *et al*., 2001). CheV has two domains, an N-terminal CheW-like coupling domain and a C-terminal response-regulator domain that is phosphorylated by CheA (Rosario *et al*. 1994; Fredrick and Helmann, 1994). In all chemotactic bacteria, CheW facilitates the coupling between the receptors and the CheA kinase (Borkovich *et al*., 1989; Simon *et al*., 1989; Hanlon *et al*., 1992). Rao *et al*. (2008) demonstrated that in *B. subtilis*, CheV is redundant to CheW in that a null mutation of one of these genes does not affect the adaptation and gradient sensing (although not as well as wild type). However, *cheW cheV* null mutants showed increased tumbling, which was consistent with the inability of the receptors to interact with and activate CheA in such strains. Our study revealed that **two-component response regulator CheV** was 4.3-fold over expressed in the mutant strain when compared to the wild-type.

Response regulator **DegU,** and the histidine protein kinase DegS, were originally identified to constitute a two-component system involved in the synthesis of degradative enzymes in *B. subtilis* (Kunst *et al*., 1988; Henner *et al*., 1988). Hamoen *et al*. (2000) reported that the response regulator DegU is involved in various late-growth developmental processes in *B. subtilis*, including the production of degradative enzymes and the development of genetic competence. In a *B. subtilis* culture, the development of competence is typically initiated toward the end of exponential growth, and is optimal in minimal medium with glucose as the carbon source. A sufficiently high cell density is another prerequisite for optimal competence. Environmental signals are interpreted by a complex signal transduction pathway, which ultimately leads to the synthesis of the competence transcription factor, encoded by *comK* (Dubnau, 1993). ComK activates the transcription of the late competence operons (*comC*,-*E*, -*F*, and -*G*), encoding the DNA

binding and uptake machinery, as well as the transcription of genes necessary for general recombination. In addition,ComK is required for the expression of its own gene (van Sinderen *et al*., 1995; Haijema *et al*., 1995; Haijema *et al*., 1996). In the same study, it was shown that full induction of ComK requires the response regulator DegU (Van Sinderen and Venema, 1994; Hahn *et al*., 1996) and DegU binds specifically to the *comK* promoter. In our study, this two component system response regulator protein was expressed in 2.7-fold higher levels in the mutant strain.

A two-component regulatory system, composed of a histidine sensor kinase (ResE) and **a response regulator (ResD),** plays an important role in the anaerobic metabolism of *B. subtilis* and probably other low-GC-content gram-positive bacteria (Nakano and Zuber**,** 2001). It has been known that *B. subtilis* has a range of adaptive responses which are induced under adverse conditions to maintain viability and, in some cases, to restore growth (Allenby *et al*., 2004; Allenby *et al*., 2005; Antelmann *et al*., 2000; Hulett, 2002). *B. subtilis* produces and exports a peptide sporulation killing factor (SkfA) that induces lysis of sibling cells that have not been induced to enter the sporulation pathway. Induction of the sporulation killing factor by cells that have entered the sporulation pathway and the subsequent lysis of the noninduced portion of the population provide cells with activated Spo0A (Spo0A_P) with a source of nutrients (including phosphorus from the cell wall, nucleic acids, membranes, and proteins) (Fawcett *et al*., 2000; Gonzalez-Pastor *et al*., 2003; Molle *et al*., 2003). Allenby *et al*. (2006) found that transcription of *skfA* is markedly induced when cells of *B. subtilis* are subjected to phosphate starvation which is a common feature of life in the soil, the natural environment of *B. subtilis*. At least three signal transduction systems have a role in phosphate deficiency response of *B. subtilis*. The interconnected pathways involve the PhoP-PhoR two component system, whose primary role is the phosphate deficiency response (Hulett, 1996; Sun *et al*., 1996; Birkey *et al*., 1998), the Spo0 phosphorelay required for the initiation of stationary phase and sporulation (Burbulys et al., 1991), and a signal transduction system ResD-ResE, which also has a role in respiratory regulation

during late growth (Azevedo *et al*., 1993; Nakano *et al*., 1997; Nakano *et al*., 2000; Sun *et al*., 1996; Zhang and Hulett, 2000).

The PhoP regulon is up-regulated in a *spo0A* null mutant that is unable to initiate sporulation (Jensen et al., 1993; Pragai et al., 2004). Maximal induction of the PhoP regulon also requires an active ResD-ResE respiration signal transduction system (Hulett, 1996). An *spo0A abrB resD* null mutant is not able to mount a specific response to phosphate starvation, showing that the induction of the PhoP regulon is dependent not only on the phosphate-specific PhoPR signal transduction system but also on this network of regulatory elements (Sun et al., 1996). If, despite these regulatory responses, phosphate starvation persists, Spo0A initiates sporulation and terminates the phosphate response by repressing the transcription of *phoPR* via AbrB and ResD-ResE (Hulett, 1994; Hulett, 1996). ResD was found to be 3.9-fold down-regulated in the mutant strain when compared to the wild-type, suggesting its possible relation with the sporulation defect of the mutant strain.

Competence in *B. subtilis*

PNPase is a non-essential multifunctional enzyme responsible for Mg^{2+}- and inorganic phosphate (P_i)-dependent 3′ → 5′ processive exoribonuclease activity (Grunberg-Manago, 1999; Condon, 2003). PNPase can convert ribonucleoside-5′-monophosphates (NMPs) into ribonucleoside-5′-diphosphates (NDPs) and can synthesize RNA polymers using NDP substrates (Ochoa, 1957; Mohanty and Kushner, 2000; Campos-Guillen *et al*., 2005; Sarkar and Fisher, 2006) or DNA polymers using dNDP substrates, in the absence of DNA template, when $MgCl_2$ is replaced by $FeC1_3$ (Beljanski, 1996). A

Δ*pnpA* strain shows a number of phenotypes including cold sensitivity, competence deficiency, tetracycline sensitivity and long multiseptate growth. All of these phenotypes are presumably linked to mRNA turnover and/or recycling of ribonucleoside-5′-diphosphates (NDPs) (Luttinger *et al*., 1996; Wang and Bechhofer, 1996; Danchin, 1997). It has been known that **PnpA/ComR (polynucleotide phosphorylase)** was necessary for the expression of late competence genes (Luttinger *et al*., 1996). We found that this late competence gene product was found to be 5.4-fold less expressed in the bacilysin minus strain.

Cell Division and Cell Wall Proteins of *B. subtilis*

In *E. coli*, the *min* locus contains three genes, *minC*, *minD*, and *minE*. Mutations of *minC* or *minD* give a minicell phenotype, whereas *minE* null mutations prevent division, giving rise to long aseptate filaments (De Boer *et al*. 1989). It is well established that **MinC** is an inhibitor of cell division, which in conjunction with **MinD**, prevents division at the cell poles (De Boer *et al*. 1990, 1992; Mulder *et al*. 1992; Marston *et al*., 1998). Homologs of *minC* and *minD* have been characterized in the Gram-positive bacterium *Bacillus subtilis* (Levin *et al*. 1992; Varley and Stewart 1992; Lee and Price 1993). In our study, septum site-determining protein MinD was found to be 3.8-fold less abundantly expressed while cell-division inhibitor protein MinC was found 4.4-fold more abundantly expressed in mutant strain.

It has been known that the cell wall of most Gram-positive bacteria is composed of a thick peptidoglycan fabric containing, in general, two types of anionic polymers: the lipoteichoic acid (LTA) and wall teichoic acid (WTA) which are in most cases modified with a D-alanyl ester or a glycosyl residue (Fischer, 1988; Hyyrylainen *et al*., 2000). In *B. subtilis* and several other Gram-positive bacteria such as *Staphylococcus aureus*, the *dlt* operon is responsible for the D-alanylation of lipoteichoic and wall teichoic acid (Perego *et al*., 1995; Peschel *et al*., 1999) and is known to be transcribed from Spo0A-

controlled promoter (Molle *et al*., 2003). Inactivation of the **DltA (D-alanine-D-alanyl carrier protein ligase)** leads in many pathogenic bacteria to a higher susceptibility to cationic antibiotics and host defensins, abolishes biofilm production and reduces pathogenicity of these bacteria (Peschel *et al*., 1999; Gross *et al*., 2001; Peschel *et al*., 2001; Collins *et al*., 2002). This significantly essential protein for cell wall biosynthesis was found to be 2.4-fold less abundantly expressed in mutant strain compared to wild-type strain PY79. It remains to be determined if a block in bacilysin biosynthesis leads to less efficient teichoic acid synthesis, of a more susceptible phenotype to certain antibiotics and defects in biofilm production.

Other proteins

Hunt *et al*., (2006) reported that *ykqC,* with other six genes of unknown function (*yacA, ydiC, ylaN, ymdA, yneS and yqjK*) was essential for viability of *B. subtilis*. YkqC has been identified as a novel endoribonuclease, **RNase J1** (Even *et al*., 2005). The enzyme is able to cleave the leader sequences of ThrS and ThrZ, (encoding threonyl-tRNA synthetase) mRNAs, and additional data suggest that RNase J1 may also contribute to global mRNA degradation (Even *et al*., 2005). Cells that were depleted for **YkqC** showed a very striking morphology, in which the cells tend to become elongated, and the distribution of the nucleoids was affected, with large regions at one or both poles of the cell devoid of DNA. Anucleated cells were frequent and there were occasional cells in which the cell division septum had bisected a nucleoid (Hunt *et al*., 2006). As part of the same study, when the response of YkqC depleted cells to certain antibiotics was tested, surprisingly, it was found that YkqC depletion resulted in cells that were much more resistant to trimethoprim which targets DNA synthesis by inhibiting dihydrofolate reductase, an enzyme in the biosynthetic pathway of the cofactor folate. It can be concluded that the primary morphological phenotype is an indirect effect resulting from a primary deficiency in RNA processing or degradation. In the present study, this

essential gene product was found to be 5.2-fold less abundantly expressed in OGU1, indicating that inactivation of bac operon have somehow resulted in a lowered RNA processing or degradation.

Hunt *et al*. (2006) also studied the functional role of **YwlC** (RNA-binding protein), a 2.8 fold upregulated protein in our study, and found that this gene was not required for viability. The crystal structure of YrdC, the *E. coli* homologue of YwlC, and nucleic acid-binding studies have shown that YrdC binds preferentially to dsRNA (Teplova *et al*., 2000). The protein binds double-stranded RNA with high specificity, providing support for a role as a translational.YrdC exhibits selective binding to RNA over DNA is consistent with a role of the protein in translation (Teplova *et al*., 2000). In addition, in *E. coli*, *yrdC* has been described as a possible ribosome maturation factor needed for correct processing of 16S ribosomal RNA, and for maintenance of normal amounts of translating ribosomes (Kaczanowska and Ryden-Aulin, 2004; Kaczanowska and Ryden-Aulin, 2005). Threonylcarbamoyladenosine (t^6A) is a universal modification found at position 37 of ANN decoding tRNAs, which imparts a unique structure to the anticodon loop enhancing its binding to ribosomes *in vitro* (Yacoubi *et al*., 2009). Using a combination of bioinformatic, genetic, structural and biochemical approaches, Yacoubi *et al*., (2009) found that the universal protein family YrdC is involved in the biosynthesis of this hypermodified base.

In order to identify additional targets of global regulator CodY in *B. subtilis*, Molle *et al.* (2003) conducted a study which showed that the *ykfABCD* operon was under CodY control. This operon was found to be controlled via the *dpp* promoter and to encode an **L-alanine-D/L-glutamate epimerase (YkfB)**, a γ-D-glutamyl-L-diamino acid peptidase (YkfC) and a transport protein (YkfD) (Schmidt *et al*., 2001). YkfA is related to a microcin-resistance protein in *E. coli* (Gonzalez-Pastor *et al*., 1995; Schmidt *et al*., 2001). These proteins may be involved in recycling of peptidoglycan degradation products (Molle *et al*., 2003). As mentioned earlier in this text, CodY directly regulates

Dpp, then the coregulation of the *ykfABCD* and *dpp* operons seems to be reasonable since the *dpp* operon encodes a D-aminopeptidase and a dipeptide uptake system and *ykf* and *dpp* operons are functionally related (Mathiopoulos *et al*., 1991; Cheggour *et al*., 2000). In our study, YkfB was found to be 3.2-fold down-regulated in the mutant strain, pointing to another gene again under the negative regulation of CodY protein.

The modified nucleosides of tRNA function usually by performing structural tasks, e.g. by blocking or reinforcing the ability of the base to form particular pairs with other bases (Helm, 2006). Addition of a single methyl group at one of the Watson–Crick positions, e.g. N1 atom of adenosine, can bring about a large conformational rearrangement, by preventing formation of a single base pair. For the model organisms *E. coli* and *Saccharomyces cerevisiae*, nearly all tRNA modification enzymes have been identified to date (Dunin-Horkawicz *et al*., 2006). However, this is not the case for other organisms, which contain modifications at different positions (Roovers *et al*., 2008). One example of a modification enzyme that remains unidentified is the one responsible for formation of m^1A at position 22 of tRNA. Roovers and coworkers (2008) demonstrated that the modification enzyme belonged to the COG2384 protein family, **YqfN** protein, **the tRNA: m1A22 methyltransferase (TrmK)** was encoded by the *yqfN* ORF in *B. subtilis*. This, recently characterized YqfN protein was found to be lost from the constructed OGU1 mutant strain in our study.

CHAPTER 4

CONCLUSION

- To identify the functional roles of bacilysin biosynthesis in regulatory cascades operating in *B. subtilis*, the comparative proteomic analysis of differentially expressed proteins in *bacA*–deleted OGU1 strain as compared to the wild-type PY79 was made for the first time, with more than 1900 proteins separated in 2-D PAGE within pH ranges 4-7, 4.5-5.5 and 5.5-6.7, respectively out of 171 differentially expressed proteins selected, 159 protein spots were identified which corresponded to 123 distinct proteins.

- Our comparative proteome analysis proved that the inactivation of BacA led to the complete loss of YwfE protein in mutant strain, namely L-alanine-L-anticapsin ligase which is required for bacilysin biosynthesis.

- The study revealed the impact of *bacA* deletion on the abundance of numerous proteins. One of the major functional group affected by the inactivation of bacilysin production consisted of sporulation proteins. A total of 12 sporulation proteins identified and all were down-regulated in the mutant strain.

- Expression of various proteins which are the targets of global regulatory protein CodY repressor were less abundantly expressed in the mutant strain. This suggested that the possibilty that bacilysin biosynthetic function and/or bacilysin itself counteracts with CodY function, hence bac operon blockage led to a further decrease in CodY- regulated gene product abundance.

- In our study, expression of general stress proteins were altered as a result of bacA inactivation. 4 of the 9 stress proteins identified (**AhpC, YceD, YugI and YaaN**) were over expressed in bacilysin blocked mutant strain. Considering the defects in sporulation in bacilysin-minus mutant, general stress proteins might have constituted essential alternative for cells that can not sporulate efficently.

- Our study demonstrated that the expression of some metabolic pathway proteins were also influenced by the inactivation of bacilysin biosynthesis in *B. subtilis*. Branched-chain aminoacid pathway proteins which are the direct targets of global transcriptional regulator CodY were found to be down-regulated in bacilysin-minus strain. The increased repression of these metabolic proteins in the absence of bacilysin biosynthetic function suggested the possibility that bacilysin biosynthesis and/or bacilysin itself might counteract with CodY function.

- Our study demonstrated that the expression of some essential two-component regulator proteins were also influenced by the inactivation of bacilysin biosynthesis in *B. subtilis*. Certain regulatory proteins of anaerobic respiration, phosphate regulation, chemotaxis, competence and sporulation were found to be differentially expressed in bacilysin-minus strain.

- The presence of differentially expressed cell division and cell wall structure proteins necessitates morphological studies in bacilysin-minus mutant.

- Our study revealed 48 unknown differentially expressed proteins emphasizing the significance of these hypothetical proteins in *B.subtilis* physiology as linked to bacilysin biosynthetic function. The functions of these proteins remain to be investigated.

- Further comparative proteomic analysis of differentially expressed proteins in *bacA*–deleted OGU1 strain and wild-type PY79 in alkaline pH range (6-11) would contribute to the identification of novel proteins involved in bacilysin biosynthesis and provide us with better understanding of interactions between bacilysin biosynthesis and the regulatory pathways and the effects of antibiotic production on expression of the genes with unknown functions.

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APPENDIX A

COMPOSITIONS AND PREPERATION OF CULTURE MEDIA

Perry and Abraham (PA) Medium (pH 7.4)

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

*Autoclave separately

**Filter sterilization

Luria Bertani (LB) Medium (1000ml)

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

Distilled H_2O 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
$NaCl_2$	5 g/L
Agar	15 g/L

APPENDIX B

BUFFERS AND STOCK SOLUTIONS

Coomassie Brilliant Blue (CBB) Stock Solution

Coomassie Brilliant Blue G-250	5g
dH2O	100 ml

Fixation Solution

40 % Ethanol	125 ml
10 % Acetic Acid	25 ml
50 % dH2O	100 ml

CCB Dye Solution

Ammonium sulfate	100g
85% phosphoric acid	12 ml
CBB stock solution	20 ml
Distilled water add to	1000 ml

CCB Staining Solution

CCB dye solution	200 ml
Methanol	50 ml

The gel is put in 200 mL CCB dye solution then 50 mL methanol is added and the gel is kept in this solution for 24 to 48 hours.

SILVER STAINING SOLUTIONS

Fixation Solution

50 % Methanol	100 ml
12% Acetic Acid	24 ml
0.05% Formaldehyde	100 µl

Pretreatment Solution

0.02% Sodium Thiosulfate

Impregnate Solution

0.2% $AgNO_3$

Development Solution

2.25% Sodium Thiosulfate
0.05% Formaldehyde
2% Pretreatment solution

Stop Solution

%50 Methanol

%12 Acetic Acid

SDS-PAGE SOLUTIONS

Acrylamide/Bis

Acrylamide	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
dH2O	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
dH2O	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 Stored at 4°C.

Sample Buffer

dH2O		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

Destaining Solution

Methanol	100 mL
Glacial Acetic acid	100 mL
dH2O	800 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	0,2 M
DTT	28 mM
CHAPS	2% m/v
dH2O	up to1 0 ml

APPENDIX C

CHEMICALS AND ENZYMES

Chemicals	Supplier
Acetic acid	Merck
Acetone	Merck
Acrylamide	Sigma
Ammonium sulfate	Merck
Ampholines pH (3-10)	Fluka
Bis-acryamide	Sigma
Bovine Serum Albumin (BSA)	Sigma
$CaCl_2$ H_2O	Merck
CH_3CN	Applichem
CHAPS	Merck
Comassie Brillant Blue G 250	Sigma
DTT	Fluka
Ethanol	Merck
Glucose	Merck
Glycerol	Merck
Glycine	Merck
H_3PO_4	Merck
HCI	Merck
IPG strips	BioRad
KH_2PO_4	Merck
Methanol	Merck
$MgSO_4$ $7H_2O$	Merck
Molecular Weight Standard (14,400-116,000)	Fermentas
NaOH	Merck
NH_4Cl	Merck
NH_4HCO_3	Applichem
SDS	Sigma
TEMED	Sigma
TFA	Applichem
Thiamine	Sigma
Thiourea	Fluka
Trichloroacetic acid (TCA)	Merck
Tris- HCI	Sigma
Urea	Fluka