PROTEOME-WIDE ANALYSIS OF THE ROLE OF EXPRESSION OF BACILYSIN OPERON ON IDIOPHASE PHYSIOLOGY OF *B. SUBTILIS*

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ABSTRACT

PROTEOME-WIDE ANALYSIS OF THE ROLE OF EXPRESSION OF BACILYSIN OPERON ON IDIOPHASE PHYSIOLOGY OF *B. SUBTILIS*

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The members of the genus *Bacillus* produce a wide variety of secondary metabolites with antimetabolic and pharmacological activities. These metabolites are mostly small peptides and have unusual components and chemical bonds. These metabolites are synthesized nonribosomally by multifunctional enzyme complexes called peptide synthetases. One of those small peptides, bacilysin, is a dipeptide antibiotic composed of L-alanine and L-anticapsin which is produced and excreted by certain strains of *Bacillus subtilis.* Proteins that are responsible to synthesize bacilysin are encoded by *bac* operon. It has been shown that the biosynthesis of bacilysin is under the control of quorum sensing global regulatory pathway through the action of ComQ/ComX, PhrC (CSF), ComP/ComA in a Spo0K (Opp)-dependent manner. The objective of the study is to identify the functional roles of bacilysin biosynthesis in the regulatory cascade and idiophase cell physiology operating in *B. subtilis* by using gel-based and gel-free proteomics techniques. For this, we employed 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. Identification via GeLC analysis of 76 differentially expressed proteins from total soluble proteome of wild-type PY79 and bacilysin minus OGU1 strain indicated the direct or indirect multiple effects of bacilysin on metabolic pathways, global regulatory systems and sporulation.

Keywords: *B. subtilis*, Quorum-sensing, sporulation, bacilysin, proteomics

ÖZ

BASİLİSİN OPERONU EKSPRESYONUNUN *B. SUBTİLİS*'İN İDYOFAZ FİZYOLOJİSİ ÜZERİNDEKİ GÖREVİNİN PROTEOM ÖLÇEKLİ ANALİZİ

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Bacillus türleri, antimetabolik ve farmakolojik aktiviteye sahip çok çeşitli ikincil metabolitler üretirler. Bu metabolitler genellikle farklı türde bileşenler ve kimyasal bağlar içeren küçük peptitlerdir. Bu metabolitler peptit sentetaz adı verilen multifonksiyonel enzim kompleksleri tarafından ribozoma ihtiyaç duyulmadan sentezlenir. Bu küçük peptidlerden biri olan basilisin bazı *B. subtilis* suşları tarafından sentezlenip hücre dışına salgılanan, L-alanine ve L-anticapsin' den oluşan bir dipeptide antibiyotiktir. Basilisin üretiminde görev alan proteinler *bac* operonundan sentezlenir. Önceki çalışmalarda, basilisin biyosentezinin, ComQ/ComX, PhrC (CSF), ComP/ComA nın Spo0K (Opp) ya bağlı aktivasyonu ile, hücre yoğunluğu sinyali düzenleyici yolaklarıyla kontrol altında olduğu gösterilmişti. Bu çalışmanın amacı basilisin üreten *B. subtilis* PY79 suşuyla grubumuz tarafından oluşturulmuş basilisin üretemeyen *bacA::lacz::erm* OGU1 suşu arasındaki proteomik karşılaştırmayı jel esaslı ve jel esaslı olmayan proteomik tekniklerle gerçekleştirmek ve basilisinin düzenleyici basamaklardaki ve idyofaz hücre fizyolojisindeki fonksiyonel rolünü ortaya çıkarmaktır. GelC analiz yöntemi kullanıldığında, PY79 suşu ve basilisin üretmeyen mutant OGU1 suşu arasında toplam çözünebilir proteinler aresında 76 farklı ifade edilmiş protein görülmüştür.Bu proteinlerin farklı ifadesi basilisinin çeşitli metabolik yolaklarda, global regulator sistemlerde ve sporülasyondaki birçok direk veya indirek etkisini göstermiştir.

Anahtar kelimeler: *B. subtilis*, hücre yoğunluğu sinyali, sporülasyon, basilisin, proteomiks

To my endless curiosity

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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
LC	: Liquied Chromotography

CHAPTER 1

INTRODUCTION

1.1 Model organism *Bacillus subtilis*

A member of the genus *Bacillus*, *B. subtilis* is gram-positive, rod-shaped, and has the ability to form a tough, protective endospore, allowing the organism to tolerate extreme environmental conditions. *Bacillus subtilis* is one of the best understood prokaryotes in terms of molecular biology and cell biology. Its various genetic amenability and relatively large size have provided the powerful tools required to investigate a bacterium with all possible aspects (Zweers *et al*., 2008). Recent improvements in fluorescence microscopy techniques have provided novel and amazing insight into the dynamic structure of a single celled organism. Research on *B. subtilis* has been at the forefront of bacterial molecular biology and cytology, and the organism is a model for differentiation, gene/protein regulation, and cell cycle events in bacteria. Moreover, *B. subtilis* is one of the best fermenter prokaryote in fact it is as good as *E. coli* biotechnologically. *B. subtilis* is even more advantageous than *E.coli* in some angles for fermentation; *B. subtilis* secrete many of its secondary metabolites to culture media. However, *E. coli* does not secrete produced secondary metabolites to culture. The hydrophilic products generally aggregate and form inclusion bodies in cytoplasm of *E. coli*, thus purification of this kind of products is very hard from *E. coli* (Zweers *et al*., 2008). This unique property of *B. subtilis* makes the organism a perfect fermenter. Nonetheless, it is much more difficult to transform the *B. subtilis* than *E. coli*. *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, *B*. *subtilis* is mesophilic and may undergo growth and production of normal-sized colonies within a day when placed at 37^{o}C, just like many other members of its genus. In addition, *B. subtilis* is a facultative anaerobe, thus it requires sufficient aeration during growth (Nakano and Zuber, 1998).

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 endospores that are resistantant to chemical, irradiation and desiccation when the responses fail to recover the 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 (Frangeul *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 (Ogura *et al,*. 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 *et al.*, 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 (Spizizen, 1958; Harwood and Cutting, 1990). *B. subtilis* PY79 has also found its place as a wild type strain, being a prototrophic derivative of *Bacillus subtilis* 168 reported by Youngman *et al.,* 1984.

1.2. Primary Metabolism in *B. subtilis*

As all living organisms, *B. subtilis* needs energy to sustain its life cycle. It had been believed that *B. subtilis* provide its energy from aerobic respiration. However, Nakano and Zuber (1998) stated that although gram-positive soil bacterium *Bacillus subtilis* is thought to be a strict aerobe, studies have shown that it can grow anaerobically by using nitrite or nitrate as terminal electron acceptor, or by fermentation to produce energy. *B. subtilis* alters its metabolic activity according to the availability of oxygen and alternative electron acceptors by two-component signal transduction system ResDE. It is composed of a sensor kinase, ResE, and a response regulator, ResD, occupies an early stage in the regulatory pathway governing anaerobic respiration. Induction of *fnr* transcription is one of the essential roles of ResDE in anaerobic gene regulation under oxygen limitation. FNR is a transcriptional activator for anaerobically induced genes, including those for respiratory nitrate reductase, *narGHJI*. *B. subtilis* has two different nitrate reductases, one for the assimilation of nitrate nitrogen and the other for nitrate respiration. Unlike many anaerobes, which use pyruvate formate lyase, *B. subtilis* can maintain fermentation without external electron acceptors, since pyruvate dehydrogenase is utilized to metabolize pyruvate (Nakano and Zuber, 1998).

In response to induction to nitrate or anaerobic fermentation metabolism protein pattern of *B. subtilis* is changed when compared to aerobic respiration. If we look at the protein pattern between nitrate respiration vs. aerobic respiration and anaerobic fermentation vs. aerobic respiration, we can see that many metabolic proteins upregulated in response different environmental conditions. Proteins encoded *by feuA, hmp*, and *ytkD* were induced by nitrate respiration. Proteins encoded by *pyrR, sucD, trpC*, and *ywjH* were induced by fermentation. Proteins encoded by *acuB, pdhC, ydjL*, and *yvyD* were induced by nitrate respiration and fermentation (Clements *et al.*, 2002).

Metabolic shift of energy production is very important from the pointview of industrial applications. The organism can efficiently synthesize a wide variety of proteinases and secrete them out of the cell using secretion systems under different conditions. Many enzymes like proteases and amylases obtained from *B. subtilis* are used for various industrial applications (Schallmey, Singh, and Ward, 2004). It is an attractive host that can easily produce and secrete in large amount various industrial enzymes and biopharmaceuticals (Westers *et al*., 2004). By changing the environmental conditions, the metabolic products of *B. subtilis* might be changed and this provides secretion of multifarious compounds that can be used in industry. 2,3-butanediol is one of those compounds. The pathway for production of 2,3-butanediol via acetoin reduction is present in *B. subtilis*. Biosynthesis of 2,3-butanediol from pyruvate occur in three steps: first pyruvate is converted to α-acetolactate by α-acetolactate synthase. Then α-acetolactate decarboxylase converts α-acetolactate to acetoin. α-acetolactate synthase and decarboxylase are encoded by *alsSD* operon (Renna *et al.*, 1993). In final acetoin is converted to 2,3-butanediol by acetoin reductase, a *bdhA* gene product (Nicholson, 2008). Induction of this pathway in *B. subtilis* is done via anaerobic fermentation, which does not support the growth of the cells.

1.3. Secondary Metabolite Production in *B. subtilis* and Its Regulation

Bacillus subtilis, the model system for Gram-positive organisms, has an amazing metabolism that can produce wide range of antibiotics with a great variety of structures. It can produce more than two dozens of antibiotics with different functions. The produced antimicrobially active compounds 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 such as polyketides, an aminosugar, and a phospholipid. Summary of well-studied antibiotic regulation pathways is shown in Figure 1.1. Moreover, findings indicate that *B. subtilis* antibiotics show distinct roles beyond the 'pure' antimicrobial action: Non-ribosomally produced lipopeptides (Table 1.1) are involved in biofilm and swarming development, lantibiotics (Table 1.2) function as pheromones in quorum-sensing, and a 'killing factor' effectuates programmed cell death in sister cells (Stein, 2005).

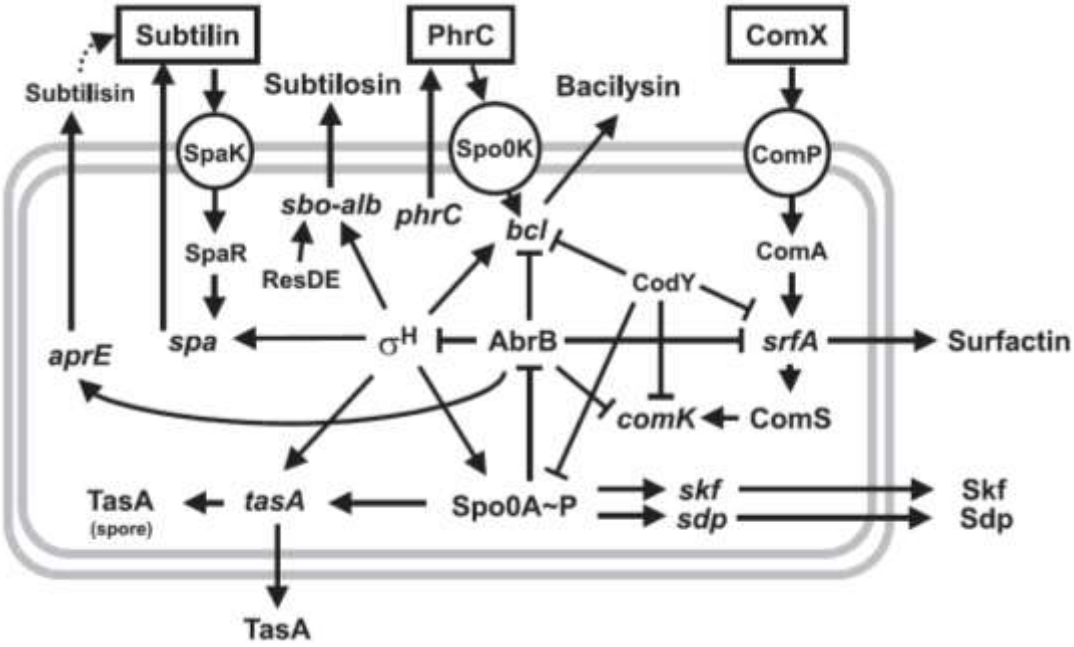

Figure 1.1. Regulatory pathways of antibiotic biosynthesis in *B. subtilis*. Survey of the regulatory pathways for the biosynthesis of the *B. subtilis* antibiotics subtilin, subtilosin, bacilysin, surfactin, the killing factor Skf and the spore-associated antimicrobial polypeptide TasA (Stein, 2005).

Table 1.1. Non-ribosomally synthesized antibiotics in *Bacillus* species (Mannanov and Sattarova, 2001)

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

Table 1.2. Lantibiotics produced in *B. subtilis* (Mannanov and Sattarova, 2001)

Lantibiotic	MW (Da)	# of aminoacids	Total Charges	Properties
Pep 5	3488	34	7	Elongated, helical
Nisin	3353	34	3	cationic, amphiphilic
Subtilin	3317	32	2	energy-dependent
Epidermin	2164	32	3	membrane
Gallidermin	2164	22	3	pore formers
Mersacidin	1825	20	0	Amphiphilic
Astagardin	1890	19	-1	hydrophobic

1.3.1. Biofilm Formation and Swarming Development

In nature, bacteria can be found as free-living organisms, however the vast majority of microorganisms form highly organized and very complex communities (Shapiro, 1998; Nadell *et al.,* 2009). For example, *Bacillus subtilis*, in non-agitated liquid culture usually forms a floating biofilm called a pellicle (Branda *et al.*, 2001). Many bacteria also show swarming, which is thought to be required before biofilm development. Biofilms are densely packed microbial cell communities that grow on surfaces and surround themselves by secreting polymers. Many species of bacteria form biofilms, and it is revealed that mechanisms are complex and diverse. The structural and physiological complexity of biofilms has bring the idea that bacteria may live as coordinated and cooperative groups, similar to multicellular organisms (Nadell *et al.*, 2009). Swarming which is a form of migration facilitates rapid colonization of bacterial populations on surfaces. Swarming development and biofilm formation is a pretty important subject to study, since these actions have been clearly shown to lead to antibiotic resistance and virulence factor production (Connelly *et al.*, 2004). Biofilms and swarming are considered as surface-associated, multicellular communities, sharing some common features. Within both types of communities, bacteria appear to undergo differentiation into different subpopulations of cells, presumably to carry out various functions. Additionally, surfactants are produced during both pellicle formation and swarming of *B. subtilis.* Surfactin has shown to be essential for coordinated movement (Nagorska *et al.*, 2010). Moreover, the study conducted by Connelly *et al.*, 2004, has also indicated that extracellular protease Epr is also essential for swarming motility (Connelly *et al*., 2004).

1.3.2. Lantibiotics

Lantibiotics are a class of antimicrobial peptides produced ribosomally. They contain the characteristic polycyclicthioether amino acids lanthionine or methyllanthionine with their antibacterial action (*lantibiotic*: ***lant***athionine containing ant***ibiotic***) (Sahl and Bierbaum, 1998). They act as both pheromone and killing factor. Many lantibiotics are only expressed during late exponential or early stationary phase and regulatory genes are present in several gene clusters. For instance, the gene clusters of subtilin (Klein *et al.*, 1993), nisin (Engelke *et al*., 1994), and mersacidin (Altena *et al.*, 2000) contain a pair of genes encoding two-component regulatory systems. The first component, a membrane-bound sensor kinase (LanK) of 380–480 residues has been autophosphorylate a His residue in its intracellular domain in response to an extracellular signal. This phosphate residue has transferred to a conserved Asp of an intracellular response regulator protein (LanR) and cause a conformational change and response regulator activate the transcription. Nisin autoregulates its own biosynthesis (Kuipers *et al.*, 1995; Dodd *et al*., 1996). With the exception of *nisRK*, which is transcribed independently of the presence of nisin A, only a very low transcription of the nisin A biosynthetic gene cluster takes place during exponential growth (Kuipers *et al*., 1995). This transcription leads to a slow accumulation of nisin A in the culture supernatant, which after reaching a critical concentration level, induce transcription of the nisin biosynthetic genes and activate the promoters of *nisABTCIP* and *nisFEG* for immunity system (Kuipers *et al*., 1995). After transcription of lantibiotics mRNA's translation occurs.

Lantibiotics, generally serve as antibiotics via pore formation (mercasidin, nisin). Inhibitions of phospholipase and peptidoglycan biosynthesis (cinnamycin) and also with various secondary effects have been recorded. For instance, nisin and Pep5 revealed massive cell wall degradation, especially in the septum area, and it was noticed that incubation of cells with these lantibiotics released autolytic enzymes (Bierbaum and Sahl, 1987). Another secondary effect of lantibiotics is the inhibition of spore outgrowth by subtilin and nisin (Liu and Hansen, 1993; Chan *et al*., 1996). In this case, the activity can clearly be attributed to the didehydroalanine residue in position 5 of both peptides; hence it was assumed that the double bond provides a reactive group for an interaction with a spore-associated factor that is essential for outgrowth. In addition to its antibiotic action,

lantibiotics can contribute to cell-cell communication and serve as signal molecules for cell density, a phenomenon called “quorum sensing” (Sahl and Bierbaum, 1998). The cells, which are not induced in this way, produce low-level of lantibiotics. Then lantibiotics accumulates to a threshold concentration (equivalent to a certain cell density); this serves as an input signal that, in the case of nisin, results in strong upregulation of its own biosynthesis through the action of a nisin-binding sensor kinase (NisK) and a corresponding transcription activator (NisR). In principle, a similar pheromone-like function can be suggested for those lantibiotics that contain a two-component regulatory system in their own gene cluster (Sahl and Bierbaum, 1998).

1.4. Dipeptide Antibiotic Bacilysin

Bacilysin is molecule that contains an L-alanine residue at the N terminus and a non-proteinogenic amino acid, L-anticapsin, at the C terminus (Yang *et al.*, 2009). 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 (Ozcengiz *et al.*, 1990; Ozcengiz and Alaeddinoglu[a], 1991). Its synthesis seemed to be under the stringent response (Inaoka, 2002) and feedback regulation (Ozcengiz and Alaeddinoglu[b], 1991). Bacilysin-negative(bac-) strain NG79 was found to be oligosporogenous. When compared with the parental strain, it was 200-300 times less resistant to heat, chloroform, and lysozyme treatments, and the spores contained considerably less dipicolinate. When NG79 was transduced, the oligosporogenous phenotype was found to be cured in all the transductants tested. External addition of bacilysin to the cultures of this strain markedly improved each measure of spore quality. The time of its addition determined the extent of acquired resistance, the optimum being 4-7 h after inoculation. This suggested that bacilysin might influence sporulation prior to stage I. Bacilysin activity completely disappeared from the extracellular fluid of aged cultures. It was demonstrated that the dipeptide can be cleaved by the alkaline serine protease that is produced by *Bacillus subtilis* who showed that basilysin is a component of the global quorum-sensing control system (Ozcengiz and Alaeddinoglu[b], 1991). This was later confirmed at a molecular level after by the studies of Yazgan et al (2001). Transposon mutagenesis (Tn10) was employed to *Bacillus subtilis* PY79 in order to isolate the genes related with the biosynthesis of bacilysin. Four different bacilysin non-producer mutants were isolated from the transposon library.It was found that biosynthesis of basilysin takes place 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). 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 (Yazgan Karataş *et al.*, 2003). According to Köroğlu *et al.,* 2011, synthesis of bacilysin reaches its maxima in the entry of stationary phase in wild-type PY79 but in bacilysin-negative mutant OGU1 bacilysin synthesis does not take place in any phase of growth (Figure 1.2).

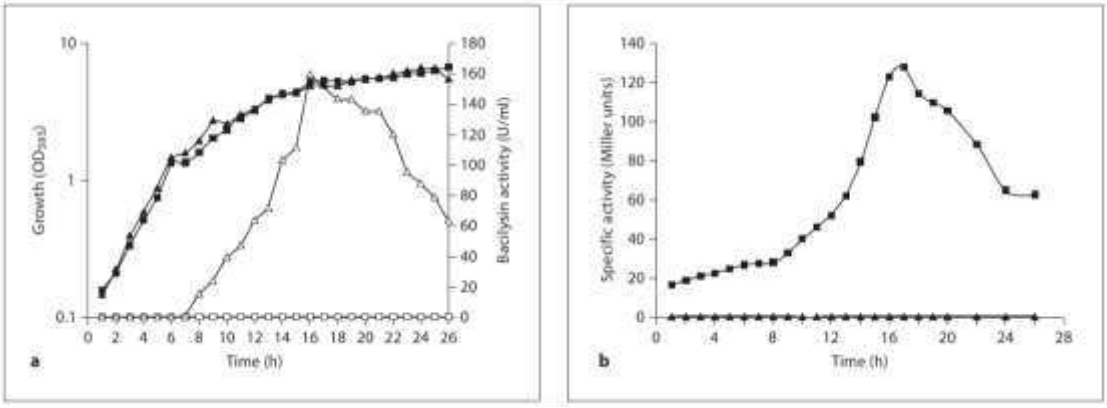

Figure 1.2. Growth, bacilysin activity and transcriptional activity of β-galactosidase a. Growth (closed symbols) and bacilysin activity (open symbols) of *B. subtilis* PY79 (triangles) and OGU1(bacA :: lacZ :: erm) (squares) in PA medium. b. Expression of transcriptional bacA-lacZ fusion in PA medium.Specific activity of B. subtilis PY79 (black triangles) and OGU1 (black squares) (from Köroğlu *et al.* 2011)

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 (Yazgan Karataş *et al.*, 2003). The *ywfBCDEF* genes of *B. subtilis* 168 were shown to carry the biosynthetic core functions and were renamed *bacABCDE* (Steinborn *et al.*, 2005) (Figure 1.3). 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, apparently in three enzymatic steps. *bacDE* (*ywfEF*) have been shown to encode the functions of amino acid ligation and bacilysin immunity respectively (Steinborn *et al*., 2005). It is shown 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, 2002). Briefly, bacilysin production is regulated on different levels, negatively by GTP via the transcriptional regulator CodY (Inaoka, 2002) and AbrB (Yazgan Karataş *et al.*, 2003). Moreover, it is obviously shown that transition phase regulators CodY and AbrB represses the expression of *bac* operon by directly interacting with its promoter. CodY and AbrB suppress the expression of *bac* operon starting with exponential phase and their suppression severety increases during early stationary and stationary phase (Köroğlu *et al.,* 2011). Positive regulation occurs by guanosine 5‟-diphosphate 3‟-diphosphate (ppGpp) (Inaoka, 2002) and quorum-sensing signal transduction pathway, namely *comP* , *comA* , *comQ,spo0H, oppA, phrK* and *phrF* have positive effect on bacilysin biosynthesis (Yazgan Karataş *et al.*, 2003; Köroğlu *et al.,* 2011).

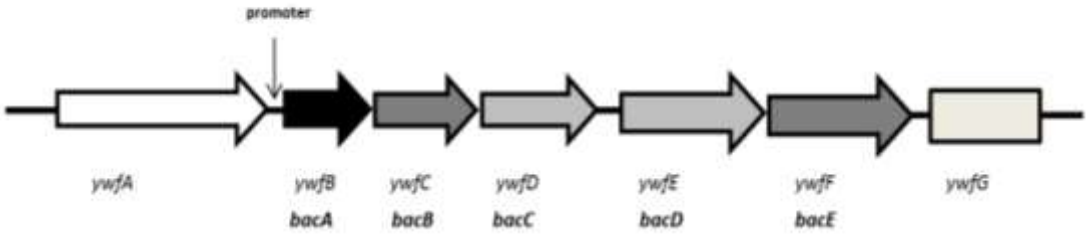

Figure 1.3. Organization of the bacilysin gene cluster bacABCDE relative to open reading frames ywfABCDEFG of *B. subtilis 168* (from Steinborn *et al*., 2005)

After a series of regulation taking place to enhance bacilysin operon, multiple biochemical steps involved in bacilysin formation from prephenate precursor are shown in Figure 1.4 (Parker and Walsh, 2013).

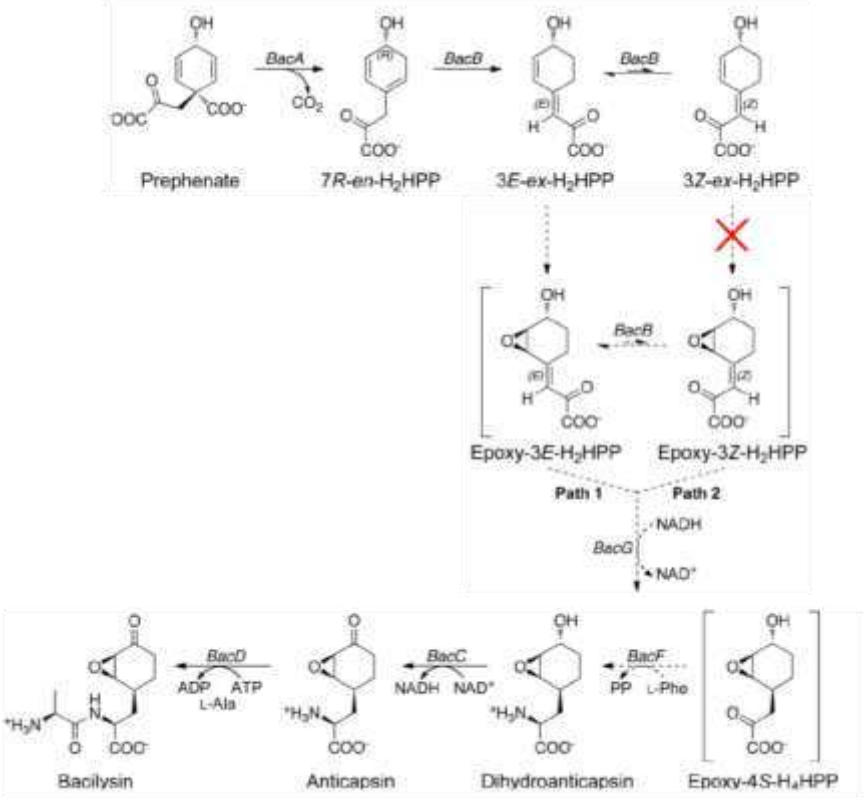

Figure 1.4. Detailed schematic presentation of the recently hypothesized biosynthetic pathway for bacilysin production from prephenate (from Parker and Walsh, 2013)

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

Cell to cell signaling is used to regulate gene expression and development by many types of cells. A form of cell to cell signaling; cell density signaling involves a regulatory response to cell density signals, called quorum sensing (Fuqua *et al.*, 1994). It is typically characterized by regulatory events that are induced when reaching high cell density. A variety of chemicals, including acyl homoserine lactones, peptides, and amino acids, are used for bacterial cell-cell signaling to regulate many biological processes, including gene transfer, development, virulence, bioluminescence, and production of antibiotics (Solomon and Grossman, 1996). Although many types of quorum-sensing systems exist, they can be grouped into two established paradigms that regulate the intraspecific interaction in many bacteria. 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 is one type.The other is oligopeptide/two component-type quorum-sensing circuits in Gram-positive bacteria responsible for the production of autoinducer (Lazazzera, 2001). Use of quorum sensing mechanisms can be exemplified in gram-negative bacteria; *Pseudomonas aeroginosa* for virulence and biofilm formation, *Rhizobium leguminosarum* for root nodule formation*, Vibrio fischeri* and *V. harveyi* for bioluminescence, *Argobacterium tumefaciens* for plasmid conjugation, *Erwinia carotovora* for antibiotic production. For Gram-positive bacteria, via quorum sensing phenomenon, *Staphylococcus aureus* and *Enterococcus faecalis* develop virulence, *Streptococcus pneumoniae* and *B. subtilis* show competence and *B. subtilis* is also induced for sporulation and antibiotic biosynthesis (Sturme *et al.*, 2002).

Signal transduction in prokaryotes is mainly carried out by two-component systems that consist 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 transferred to its response regulator pair afterwards, activating its function, allowing the cells to respond and adapt to the specific signal (Stock *et al.*, 2000).

In *B. subtilis*, several processes are known to be regulated by extracellular peptide signaling, including the genetic competence initiation, sporulation, degradative enzyme and exopolysaccharide production, antibiotic synthesis (Solomon and Grossman, 1996; Schneider *et al.,* 2002), adaptation to environmental stress (Darmon *et al.*, 2002), the production of secondary metabolites, 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, which are known as

Eight Phr peptides (PhrA, PhrC, PhrE, PhrF, PhrG, PhrH, PhrI, and PhrK) and a family of 11 Rap proteins (RapA to RapK) are encoded by *B. subtilis* species are shown in Table 1.3 (Lazazzera, 2001; Stein, 2005).

Table 1.3. 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		Unknown	Unkown	
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
RapI	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

Phr peptides are encoded in an operon with a Rap protein, and each characterized Phr blocks the activity of its cotranscribed Rap (Solomon and Grossman, 1996; Kunst *et al.*, 1997; Ogura *et al.*, 2002). Unpaired Rap protein, RapB, activity can also be inhitbited by PhrC (Perego, 1997). It might be possible that other unpaired Rap proteins are also inhibited by noncognate Phr peptides (Auchtung *et al.*, 2006). In addition to the upstream *rap* promoter expression, most *phr* genes are also expressed from a promoter upstream of *phr* that is recognized by σH alternative sigma factor mediated RNA polymerase. This regulation by σH causes increase level of each *phr* gene expression when transition of cells from exponential growth to stationary phase (Lazazzera, 2001). pre-Phr peptides that are the primary *phr* gene products are 38 to 57 amino acids in length. Export and cleavage of Pre-Phr peptides are needed to form the mature Phr pentapeptides (Lazazzera, 2001).

The oligopeptide permease (Opp), an ATP-binding cassette (ABC) transporter is needed to export those peptides (Perego *et al.*, 1991).

In order to stimulate the development of genetic competence and expression of several genes involved in other processes, two peptide pheromones, ComX and Competence and sporulation stimulating factor CSF (PhrC), accumulate during exponential growth (Mootz and Marahiel, 1997). ComX pheromone is a 10-amino-acid peptide and two genes, *comQ* and *comX*, are responsible for ComX production (Magnuson *et al.*, 1994). Production of ComX pheromone requires processing of the 55-amino-acid precursor to 10 amino acids (Weinrauch *et al.*, 1991), modification of the tryptophan residue, and export from the cell. ComQ is thought to be involved in the processing and/or modification step (Lazazzera, 2001). Membrane bound receptor histidine kinase ComP activity is stimulated by ComX. ComP has eight putative membrane-spanning helices and appears to be the direct receptor for ComX pheromone. The hydrophobic modification on the pheromone is obligatory for functioning and may help to increase the local concentration of ComX pheromone at the membrane in order to 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, 2001).

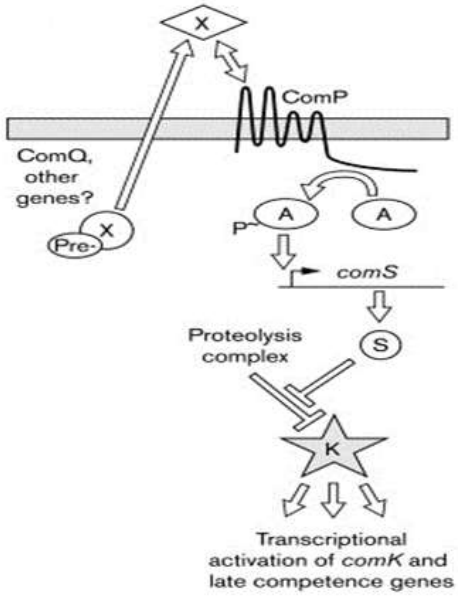

Figure 1.5. Pathways of competence regulation in *B. subtilis*, a precursor pheromone (pre-X) encoded by *comX* is secreted, processed and modified with the participation of ComQ. ComP, a membrane-localized histidine kinase, detects extracellular ComX and activates the transcription factor ComA by phosphorylation. ComA~P activates the promoter that drives transcription of *comS*. ComS binds to a proteolytic apparatus causing the release of ComK, protecting the latter from degradation. ComK initiates the transcription of its own gene as well as that of the late competence genes (from Tortosa and Dubnau, 1999).

CSF is a 5-amino-acid peptide that activates ComA by inhibiting the activity of the regulator RapC (Solomon and Grossman, 1996). It is shown that also PhrF and PhrK stimulate ComA by directly inhibiting the activity of their cognate proteins RapF and RapK. Moreover it is also shown that PhrC, PhrF, and PhrK can stimulate ComA-dependent gene in a different level and those three peptides are reguired for full expression of ComA-dependent genes (Auchtung *et al*., 2006).

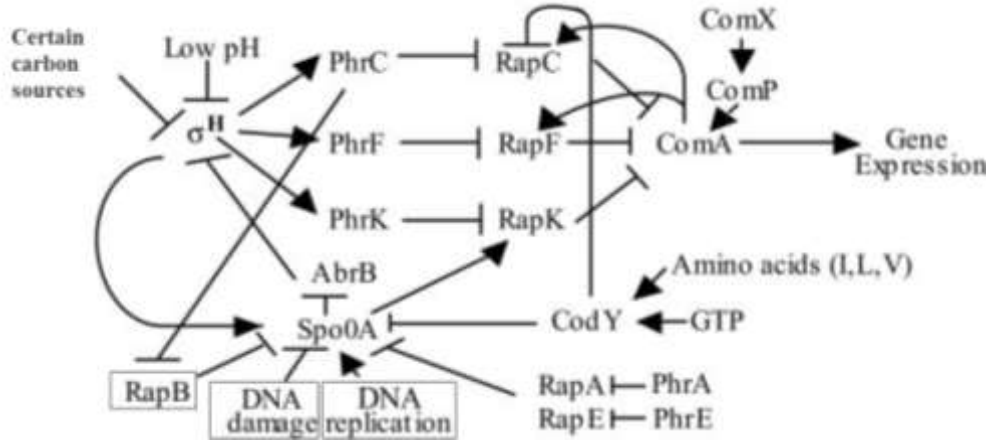

Figure 1.6. Regulation of ComA-dependent gene expression (from Auchtung *et al*, 2006).

The function of CSF in regulating ComA activity is more complicated than ComX pheromone working principle. CSF is a secreted, diffusible peptide (Solomon and Grossman, 1996). When CSF reaches a critical concentration, it is transported back into the cell by an oligopeptide permease Opp (also known as Spo0K). It binds to two different intracellular receptors to regulate the activity of the ComA transcription factor (Perego, 1997; Lazazzera, 2001). At low concentrations CSF stimulates the activity of ComA by blocking the activity of an aspartylphosphate phosphatase, RapC (Solomon and Grossman, 1996). At higher concentrations, CSF binds to an unidentified receptor, possibly the histidine-protein kinase ComP, to prevent expression of ComA-controlled genes (Lazazzera *et al.*, 1997). In addition to those functions, CSF, at high concentrations, also activates sporulation by inhibiting the activity of an alternative aspartyl-phosphate phosphatase, RapB (Solomon and Grossman, 1996; Lazazzera *et al.*, 1997; Perego, 1997).

Production of mature CSF involves several steps, starting with tanscription and translation of *phrC*, the gene encoding the precursor of CSF (Solomon and Grossman, 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 and Hoch, 1996). It is 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 (Lazazzera *et al.*, 1999). RapC, which is negative regulator of ComA~P, is a part of this homeostatic autoregulatory loop. PhrC (CSF) activates ComA and positively regulates its own expression. Moreover, it is shown that CSF reaches certain concentration that is need for stimulation of competence gene expression and cells enter stationary phase afterwards. Extracellular concentration of CSF reaches certain level to induce sporulation and block early competence gene expression (Lazazzera, 2001).

CSF stimulates sporulation in a similar mechanism by which it stimulates competence (Figure 1.9). It requires the phosphatase RapB. RapB dephosphorylates Spo0F~P (Perego *et al*., 1994) and it is part

of the phospho-transfer pathway which donates its phosphate to the transcription factor Spo0A, key element for the initiation of sporulation (Grossman, 1995). The phosphatase activity of RapB is inhibited *in vitro* by CSF, which might indicate that CSF stimulates sporulation *in vivo* by directly inhibiting RapB (Perego, 1997).

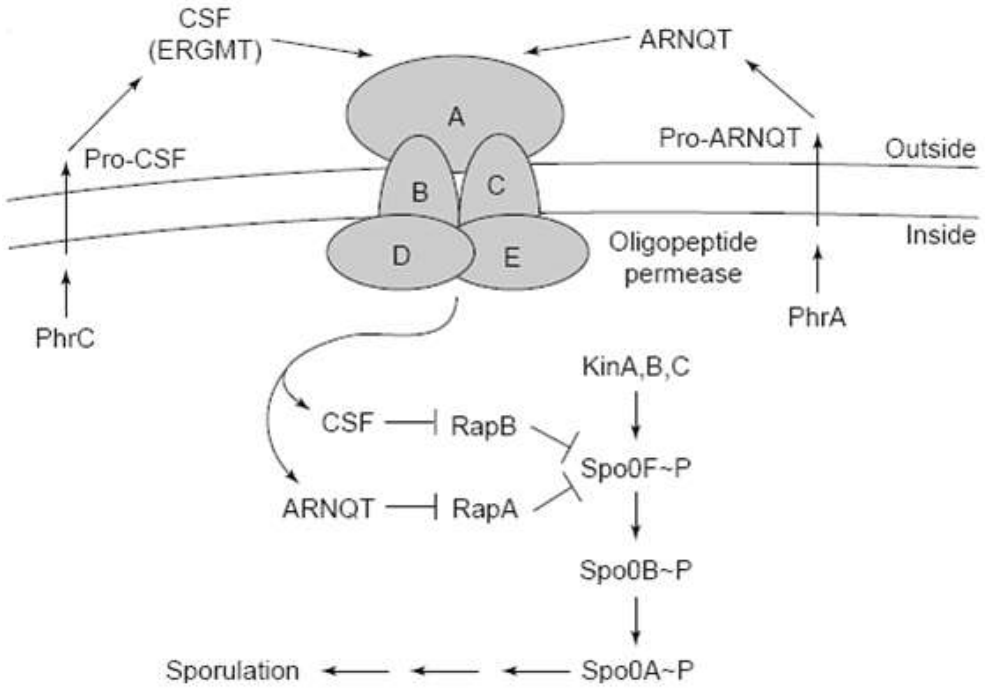

Figure 1.7. The regulation of sporulation by extracellular peptides. CSF and a peptide, ARNQT, encoded by phrA, are transported into the cell by Opp. Each peptide inhibits the activity of a phosphatase (RapB or RapA) that dephosphorylates Spo0F~P. Spo0F receives phosphate from one of three kinases (KinA,B,C) and donates phosphate to Spo0A through Spo0B (from Lazazzera and Grossman, 1988).

It is a fact that CSF accumulates in culture medium during cell growth to provide cell to cell signaling. It is also clear that PhrA pentapeptide can function in cell–cell signaling. Nonetheless, 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; Perego and Hoch, 1996; Solomon and Grossman, 1996).

The products of *spo0A* and *spo0H* genes, play a key role in the initiation of sporulation by coding response regulator of the multi-compenent signal transduction system: phosphorelay and alternative sigma factor, σH, respectively (Burbulys *et al.*, 1991; Chibazakura *et al.*, 1995). A multicomponent phosphorelay contains five histidine kinases (KinA, KinB, KinC, KinD and KinE) and two phosphorelay proteins (Spo0F and Spo0B) (Perego and Hoch, 1996). 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). Activity of Spo0A is subject to several auto-stimulatory loops which involve in transcription of *spo0A* and phosphorylation of Spo0A (Sonenshein, 2000). Transcription of *spo0A* is directly activated by Spo0A~P and indirectly activated by induced expression of σ^H. The σ^H encodes an alternative sigma factor (σH) 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 transcriptionally activate the *spoIIA* operon, which contains the sporulation specific sigma factor, sigma-F (Burbulys *et al*., 1991).

Once activated by phosphorylation, Spo0A binds to a DNA sequence containing a so-called '0A-box' (Strauch *et al.*, 1992), where it play its role by transcriptionally activating or repressing it. 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). Among 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 (Castilla-Llorente *et al.*, 2006; Molle *et al*., 2003). The levels of Spo0A protein and activity increase gradually during the early stages of sporulation (Fujita *et al.* , 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, decrease in 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 thought to be the primary kinase in the phosphorelay and is necessary for the phosphorylation of Spo0A (Burbulys *et al*., 1991). It has been shown that the fraction of cells that initiate sporulation is decreased in a *kinA* mutant background (Chung *et al.*, 1994). This result supports the idea that a certain threshold concentration of Spo0A~P is necessary to initiate sporulation and that the activity of the phosphorelay decide 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 being RapA and PhrA (Veening *et al.*, 2005). KinC and, to a minor extent, KinD, are responsible for heterogeneous expression of *spo0A* during logarithmical growth (Castilla-Llorente *et al*., 2006). 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). Submaximal levels of *abrB* expression during exponential growth has 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 (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 (Castilla-Llorente *et al*., 2006). To date, more than 35 two-component regulatory systems have been identified in *B. subtilis* by genome sequencing (Kunst *et al*., 1997).

1.6. 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). In rich medium, *B. subtilis* cells divide by binary fission approximately every 30 minutes. By contrast, deterioration of environmental conditions induces sporulation, a developmental process that takes about 8 to 10 hours. Thus, endospore formation represents a challenging way of survival, because *B. subtilis* cells only decide to sporulate after they failed to deal with starvation in other ways, like cannibalism or establishment of a genetically competent state (Veening *et al.*, 2006). Sporulation begins with an asymmetric cell division and results in the generation of two cell types, a forespore (the smaller compartment, also called the prespore) and a mother cell. The two cells go through two different fates, because the mother cell ultimately lyses by a programmed cell death mechanism, yet the forespore matures as a spore. Shortly after asymmetric division, two parallel programs of gene expression are established in each compartment under the control of transcription factors that are activated in a cell-specific manner. In addition to regulatory interactions within the forespore and mother cell, precise inter-compartmental signaling is also required to control the spatial and temporal progression of the developmental process (De Hoon *et al.*, 2010).

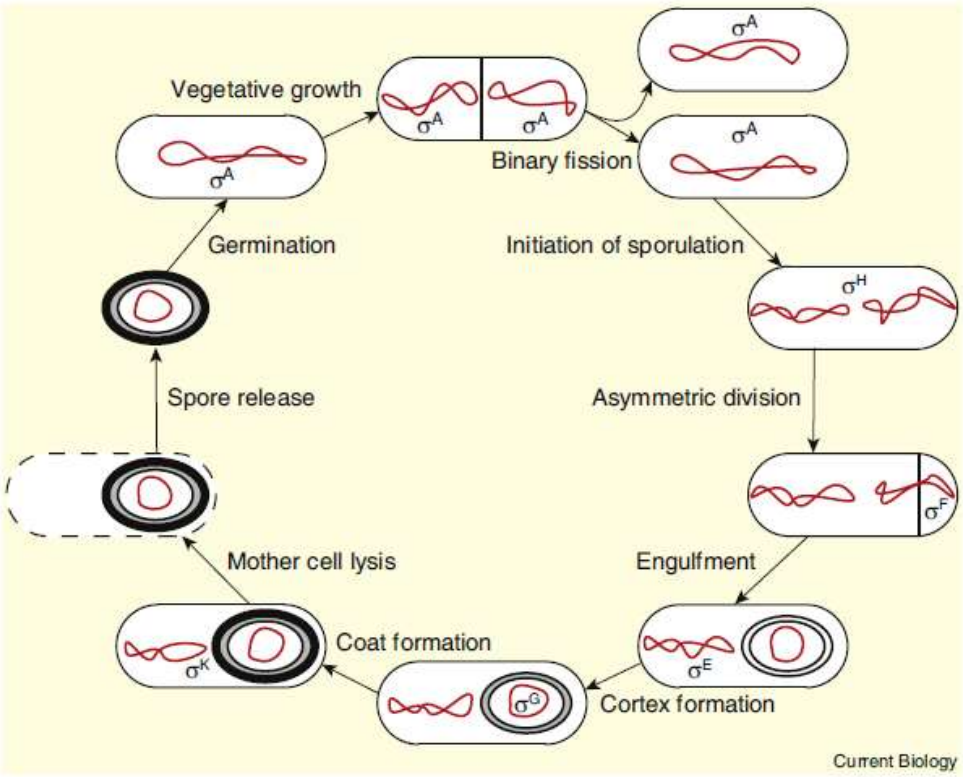

Figure 1.8. Morphological stages of *B. subtilis* life cycle (from De Hoon *et al*., 2010)

Sporulation commences only after a round of DNA replication has been completed, in order to ensure that two chromosome copies are available in the predivisional cell (Veening *et al.*, 2009). The two chromosomes are oriented with their origin of replication anchored at one cell pole and their origin-distal region at mid-cell (Teleman *et al.*, 1998). After asymmetric division, only about onethird of the forespore chromosome (i.e. the origin-proximal region) is captured in the small chamber of the dividing cell. A DNA translocase, SpoIIIE, located at the center of the polar septum, is necessary to pull the rest of this chromosome into the forespore (Ptacin *et al.*, 2010; Wu and Errington, 1994). The other chromosome is localized entirely inside the mother cell. Following asymmetric division, the next morphological stage of sporulation is the engulfment of the forespore by the mother cell. This process is analogous to phagocytosis and is driven by mother cell proteins that facilitate membrane migration around the forespore by enzymatic removal of the peptidoglycan (Morlot *et al.*, 2010; Pogliano *et al*., 2010). After completion of engulfment, the forespore, now entirely surrounded by its inner and outer membranes, is a free protoplast in the mother cell cytoplasm. Next, a series of protective structures is assembledaroundthe spore core. The cortex, a modified peptidoglycan, is synthesized between the two forespore membranes. Simultaneously, at least 70 individual coat proteins are synthesized in the mother cell to encase the spore in a multi-layered structure, with the crust as the outermost layer (De Hoon *et al*., 2010). Finally, the mother cell lyses to release the mature spore. Fully formed spores, recognized as the most resistant form of life on the planet (Nicholson *et al.,* 2000), protect the bacterial genome against heat, desiccation, radiation, and oxidation. In addition, spore formation might be an efficient way to escape predation from higher organisms (De Hoon *et al*., 2010). As soon as environmental conditions become favorable for vegetative growth, however, it is critical that *B. subtilis* quickly exits from the dormant state. This process is referred to as spore germination (De Hoon *et al*., 2010) and is triggered by the presence of nutrients in the environment. The nutrients are sensed by specific spore membrane receptors and, within minutes, the spore core rehydrates, the cortex is hydrolyzed, and the coat is shed. Ultimately, DNA replication is initiated and the first cell division follows soon.

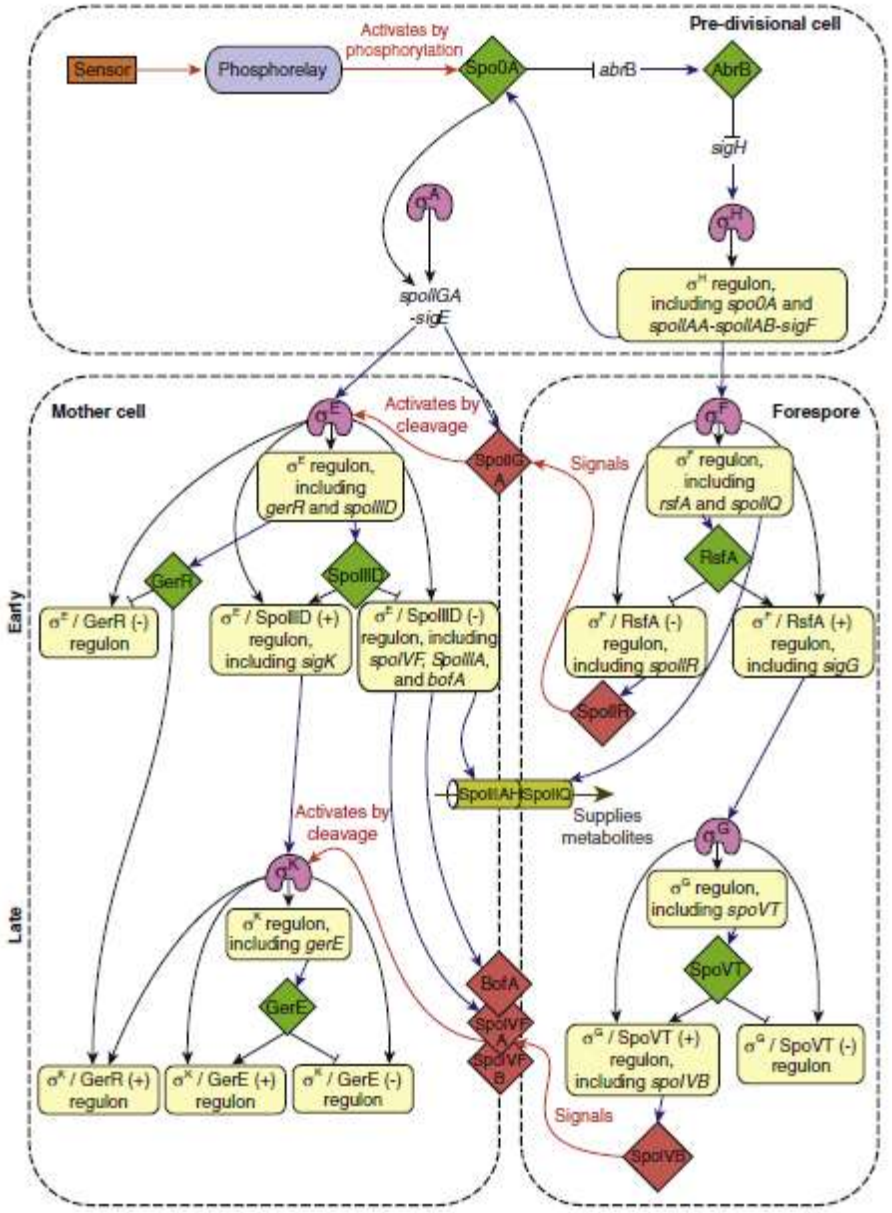

Figure 1.9. Sporulation transcriptional regulatory network in *B. subtilis* (from De Hoon *et al.*, 2010).

The *B. subtilis* sporulation transcriptional regulatory network is shown schematically in Figure 1.10. Many sigma factors whose activity is limited to specific compartments play a crucial role in this network. The sporulation regulatory network is organized spatially and temporally into modules, with the sporulation-specific sigma factors σ^F, σ^E, σ^G, and σ^K controlling the gene expression of the early forespore, early mother cell, late forespore, and late mother cell, respectively (Losick and Stragier, 1992). The sigma factor σ^H regulates transcription in the pre-divisional cell (Carter and Moran, 1986). Importantly, σ^A which is the primary sigma factor of *B. subtilis*, stay active even during the late stages of sporulation (Li and Piggot, 2001). The master regulator of sporulation, Spo0A~P, is not a sigma factor but it is a response regulator which is activated by a phosphorelay. Before initiation of sporulation, spo0A is transcribed at a low level by RNA polymerase containing σ^A.

Sporulation is initiated by changes in environmental conditions that are sensed by a group of histidine kinases which are capable of auto-phosphorylation. The phosphate group is then sequentially transferred from the histidine kinases to the Spo0B and Spo0F proteins of the phosphorelay and finally to Spo0A (Burbulys *et al*., 1991). Phosphorylation of Spo0A affects the transcription of more than 500 genes, including at least 120 as a direct target (Molle *et al*., 2003). In the pre-divisional cell, σH increases transcription level of spo0A by using a sporulation specific promoter. As mentioned in a previous section, Spo0A~P acts as an indirect positive regulator of the σ^H-encoding gene, *sigH*, by repressing transcription of its repressor AbrB. The Spo0A~P regulon is composed of low and high threshold genes (Fujita et al., 2005), implying that Spo0A~P-dependent genes are activated with different kinetics. Following asymmetric division, Spo0A~P activity is predominantly observed in the mother cell (Fujita and Losick, 2005). The sigma factor σ^H also controls transcription of the *spoIIAA–spoIIAB–sigF* operon, by encoding the early forespore- specific sigma factor σ^F, the anti-sigma factor SpoIIAB, and the anti-anti-sigma factor SpoIIAA. σ^F is the first compartment-specific regulator to be activated and directs transcription of about 50 genes (Steil *et al.*, 2003; Wang *et al*., 2006), including *sigG*. The other transcription factor to be produced under the control of σ^F is RsfA, which activates some σ^F-transcribed genes and represses others, forming with σ^F compatible and incompatible feed-forward loops. Feed-forward loops are frequent topological motifs in cellular networks in which one gene regulates another gene, and together they also regulate a downstream gene (Alon, 2006). Feed-forward loops play various functional roles, including noise filtering, fine-adjustment of expression timing, response induction and acceleration, and signal generation. Individual feed-forward loops in biological networks usually aggregate into larger motif generalizations (De Hoon *et al*., 2010), in which a feed-forward loop regulates many downstream genes. While, σ^F and RsfA form a coherent feed-forward loop, RsfA acts as an activator, in which results delayed and/or lengthened gene expression kinetics. Conversly, when RsfA acts a repressor, σ^F and RsfA form an incoherent feed-forward loop, producing a short pulse of gene expression. The late-forespore-specific sigma factor, σ^G, regulates about 100 genes (Steil *et al*., 2003; Wang *et al*., 2006) and constitutes coherent and incoherent feed-forward loops with SpoVT, which is the final forespore-specific transcription factor in the cascade. A similar way of gene expression regulation in the mother cell happens. σ^E controls transcription of the genes specific to the early mother cell Synthesis of σ^E depends on a σ^A-specific promoter that is activated by Spo0A~P and located upstream of the *spoIIGA– sigE* operon. σ^E controls the transcription of about 270 genes (Steil *et al*., 2003), including those encoding the transcription factors SpoIIID and GerR. Both SpoIIID and GerR constitute feed-forward loops with σ^E. Nonetheless, while SpoIIID activates some σ^E-dependent genes and represses others, GerR only seems to repressf σ^E-dependent genes and activates σ^K-dependent genes (Kuwana *et al.*, 2005). Expression of *sigK*, is dependent on both σE and SpoIIID (Kroos *et al.*, 1989). σ^K regulates about 150 genes (Steil *et al*., 2003), including GerE, the final mother cell-specific transcription factor in the cascade. GerE can act either as an activator or as a repressor of transcription depending on the promoter regulatory logic, like SpoIIID.

1.7. Proteomics

The definition of proteome is 'the entire PROTEin complement expressed by a genOME' (Wilkins *et al.*, 1996). Proteome is the exact protein profile of certain organism in an exact time period. Multiple steps are required for proper proteome analysis; stringent control of sample preparation, two-dimensional polyacrylamide gel electrophoresis (2-D PAGE or 2-DE), image detection and analysis, spot identification and database search. As to its application, many different arrays of study are now grouped under rubric of 'proteomics'. Proteomics started in 1975 with publication of 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 cm2 in size (Klose, 1975; O'Farrell, 1975). Each single protein is separated according to its molecular weight and pI. First initiators of physiological proteomics used this powerful technique to address crucial issues of *E. coli* cell physiology such as heat shock or starvation responses (Neidhardt and VanBogelen, 2000). Gel-based

proteomics technique is successfully improved by the introduction of IPGs for IEF (Görg, 1993). This was the basis for high-throughput comparative gel-based proteomics and for the development of gel-based proteomics as a new field in cell physiology. Typical gel-based proteomics workflow is shown in Figure 1.12. Because of their low complexity, bacteria are extremely suitable model organisms for transferring the "blue-print of life to real life", and therefore becoming to a new quality in understanding life processes (Zweers *et al*., 2008). This property of microorganisms makes the bacteria to reasonable model systems for addressing the crucial and elementary issues of life processes by using proteomic approaches. On the other hand gel free approaches are as important as gel-based method. Whenever, gel-based method is not sufficient to work with; gel-free proteomics is used as a savior. In gel-free methods, extraction and solubilization of sample proteins must be in high level, in order not to miss any protein (Baggerman *et al.*, 2005).

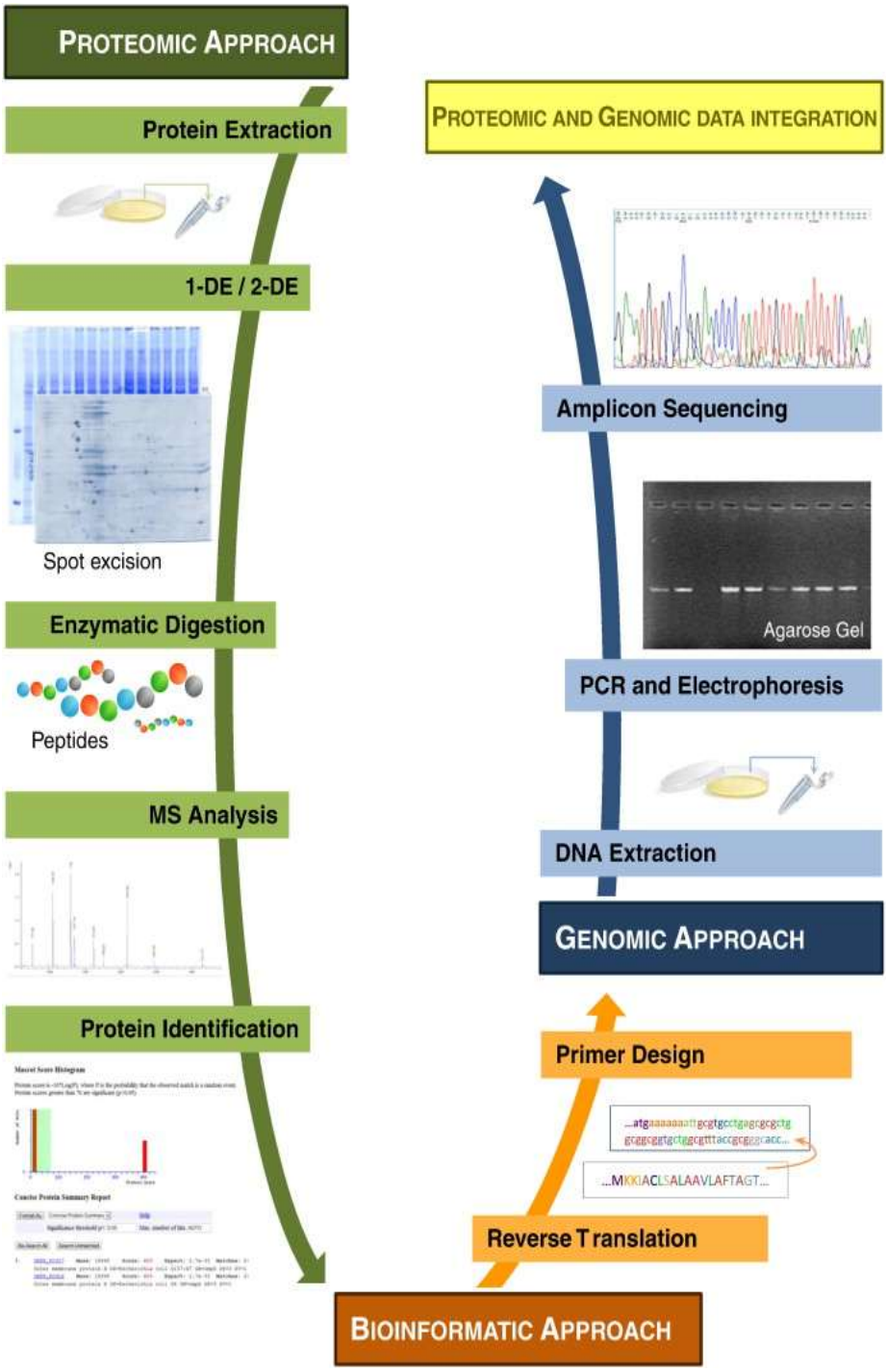

Figure 1.10. Typical proteomic workflow representing the classical gel-based approach to protein identification (from Radhouani *et al.,* 2012).

1.8 Why Proteomics?

Proteomics is the large-scale screening of the proteins in a cell or biological fluids. After the foundation of DNA double helix, whole biology leant to genetic studies, however, not all types of information can be obtained from genes alone. Genomic analysis can provide very limited information with respect to description of how organisms adapt to a constantly changing environment. Since organism contains same genetic material in all conditions, adaptations can only be revealed by investigating the protein profile of this organism in certain condition. 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. Analysis of mRNA is much more informational than DNA analysis; however it is not a direct reflection of protein content of organism in a given timepoint. It has already shown that there is a poor correlation between mRNA and protein expression level (Gygi *et al.*, 1999). There are many reasons behind this poor correlation. Firstly, splicing, polyadenylation and mRNA editing takes place after synthesis of mRNA. Secondly, in eukarya, mRNA might be subjected to regulation just before translation. Moreover, after being formed, proteins are also subjected to posttranslational modifications, proteolysis and compartmentalization. Therefore, it can be said that average number of proteins formed per gene is predicted to be one or two in bacteria, three in yeast and three or more for human (Wilkins *et al.*, 1996). Thus physiological map of an organism in certain conditions cannot be completely analyzed by gene analysis or mRNA analysis. Here, proteome take shows its strength to illuminate characteristics of organisms by the power of synthesized proteins. For example, to reveal the stress related proteomes, such as starvation physiology of different organisms, several proteome projects have been performed (Yu *et al.*, 2011). Moreover, different tissues of the same organism have the same genome, but different proteomes in the same organism certainly make the proteome amazingly more 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 at identifying 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. The challenge is to provide sufficient amount of information in experimental datasets to match the underlying complexity.

1.9. Comparison of Gel Based and Gel Free Proteomics

Though 2D PAGE is quite an old concept and has been around since the seventies, it is still convenient and chosen technique to analyze (separate and visualize) the proteins in many studies (Baggerman *et al*., 2005). Still, 2D-PAGE has important limitations; it is time-consuming, costly, insensitive to low abundance proteins and is incapable of separating all proteins present in a cell. Most of these limitations are addressed by gel-free proteomics techniques. Nonetheless, these shotgun proteomic approaches also have many limitations. These limitations are a result of the underlying key assumption that a protein can be identified based on the sequence of a single (or a few) tryptic peptide(s) derived from this protein. Many proteins undergo post-translational modifications such as truncation, phosphorylation, glycosylation etc. which are often important in the regulation of protein function and thus will show differences from one physiological condition to the other. Also a given gene may result in a large number of different protein products. It is not possible to discriminate between all of these protein isoforms by analyzing a single tryptic peptide derived from this protein. Instead, 2D-PAGE delivers a map of intact proteins, which reflects changes in protein expression level, isoforms or post-translational modifications. Post-translational

modifications will result in a shift of pI (in the case of phosphorylations) or relative mass (e.g. glycosylation or truncation) and show a different mobility on a 2DE gel. Different isoforms of the same protein will therefore be visible as different spots on the 2DE gel. In the hemolymph of the fruit fly, for instance, several protein spots with very different pI and molecular weight could be attributed to a single protein, ferritine (Vierstraete *et al*., 2004). In gel-free based methods, performing analysis on peptides, Mr and pI information is lost (Baggerman *et al*., 2005).

1.10. Why GeLC-MS/MS?

Normally, comparative proteomics between different samples are done with 2DE method (Bernhardt *et al*., 1996). However, as mentioned above, it is restricted to first dimension pI and borders of polyacrylamide gel. Also, low abundant proteins cannot be observed by low resolution techniques. On the other hand, 1D coupled LC-MS/MS technique uses the power of liquid chromatography to analyze whole proteins of an organism. It is very beneficial to use in identifying total proteome of certain organisms (Old *et al*., 2005). Thus, this method can also be used for comparative proteomics as a mean of low abundant protein comparison (Kuwana *et al*., 2002).

1.11 Proteome of the Model Bacterium *B. subtilis*

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. The first comprehensive map of the vegetative *B. subtilis* soluble 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 a pI region of 4–7. In 2007, 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).

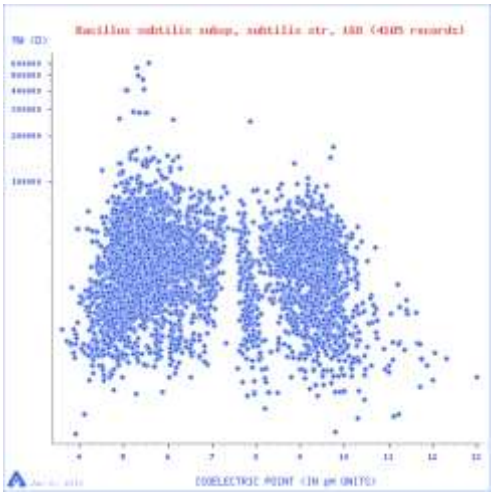

Figure 1.11. A theoretical map of *B. subtilis* 168 strain proteome; predicted 2-D gel using isoelectric point (in pH units) vs. molecular weight (in Dalton)
(http://gelbank.anl.gov/cgi-bin/2dgels/gel_insilico.pl)

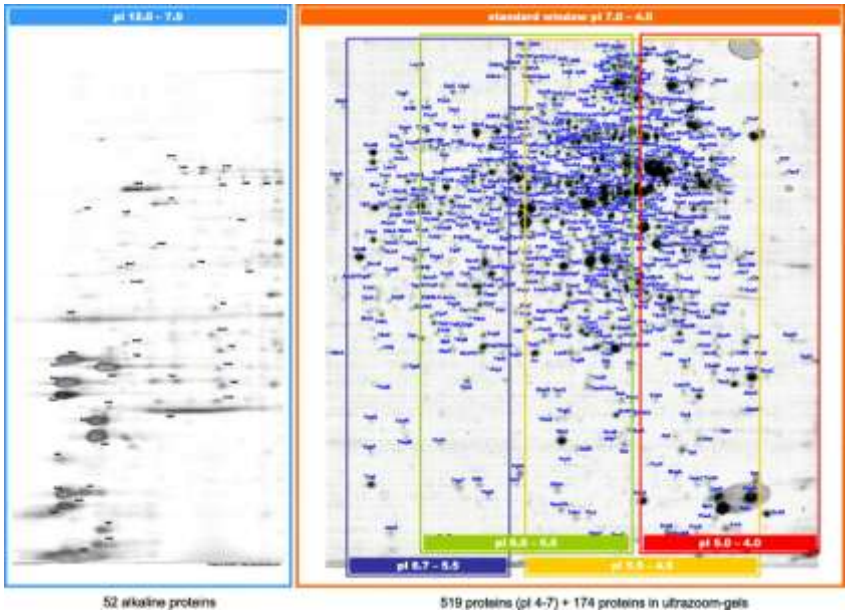

Figure 1.12. The cytoplasmic vegetative proteome map of *B. subtilis* in the standard pH range (4–7), in the narrow pH ranges (4.0–5.0, 4.5–5.5, 5.0–6.0, 5.5–6.7) and in the alkaline pH range (7–12) (Wolff *et al*., 2007)

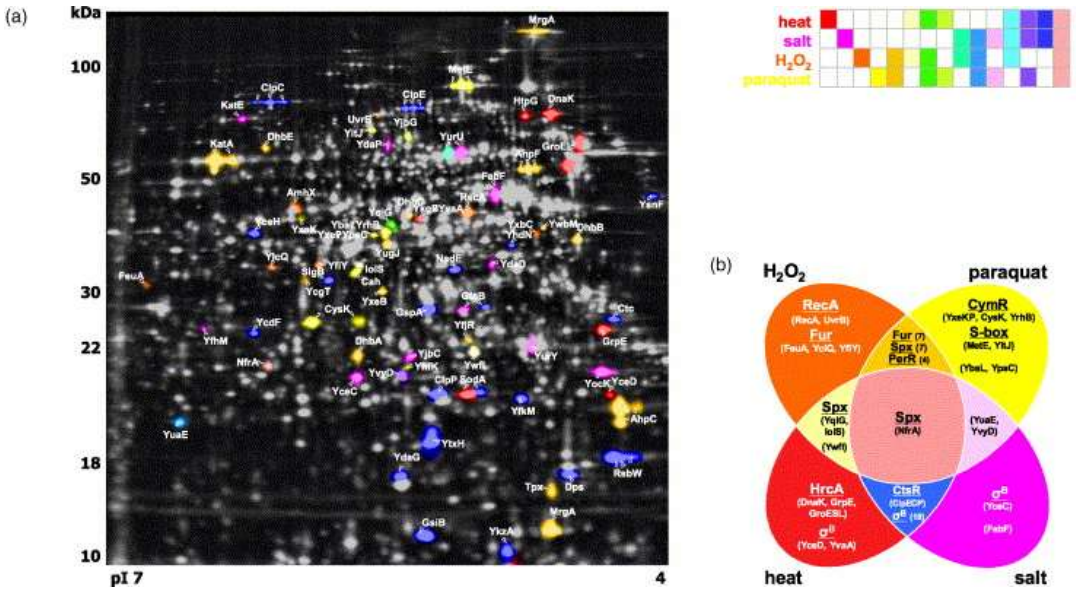

Figure 1.13. (a) Cytoplasmic proteome map of *B. subtilis.* The realistically looking 2D-pattern is a composite image from five positionally corrected 2D-autoradiographs derived from exponentially grown as well as heat, salt, hydrogen peroxide and paraquat stressed *B. subtilis* cells. (b) The specific and general stress regulons in *B. subtilis* (Wolff *et al*., 2007)

As a gram-positive model organism, not only the genome sequence of *B. subtilis* is available but also mRNA profiling of gene expression. For about 25 years of proteome research in *B. subtilis*, still one-third of *B. subtilis* 4100 genes are not assigned to a defined function yet, another one-third of genes are assigned a defined function but its corresponding protein could not be demonstrated 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 stays more or less silent under standard growth conditions and will only be expressed in response to specific conditions. Therefore, two major classes of proteomes are defined: the proteome of growing cells (vegetative proteome) (Figure 1.14) and the proteome of non-growing cells suffering from stress or starvation (Figure 1.15) (Wolff *et al*., 2007).

The study, conducted by our former lab member Aslı Aras Taşkın revealed that bacilysin biosynthesis possesses a great impact on the total soluble proteome of *B. subtilis*. It was revealed that bacilysin – minus mutant strain OGU1 and wild-type PY79 strain has 192 differentially expressed proteins in a pI range of 4-7 by using 2DE approach coupled to MALDI-ToF MS analysis of total soluble proteome of *B. subtilis*. 128 of these 192 proteins were chosen, since they were 2.5 fold or more differentially expressed. Proteins that are differentially expressed under the effect of bacilysin biosynthesis were visualized in three pH ranges. 58 differentially expressed proteins shown with 2-DE gels with a pH range of 4-7 which would cover 2/3 of all *B. subtilis* proteins (Eymann *et al.*, 2004). In zoomed pH ranges of 4.5-5.5 and 5.5-6.7, 28 and 53 differentially expressed proteins are revealed respectively (some proteins were common to both pH ranges) (Taşkın, 2010). Those differentially expressed proteins comprised many important metabolic pathways, sporulation, general stress response and two-component response regulators. Those findings which were obtained via 2-DE proteomics approach showed the important role of bacilysin biosynthesis on general physiology of *B. subtilis*.

1.11 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 implementing 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 helped us to improve our understanding not only about interactions between bacilysin biosynthesis and the regulatory pathways but also the effects of antibiotic production on expression of the genes with unknown functions.

CHAPTER 2

MATERIALS AND METHODS

2.1. Bacterial 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 (Köroğlu *et al.*, 2011).

In our study we use bacilysin minus strain OGU1 which was created in our lab before. For construction of strain OGU1, integral DNA fragment of *bacA* gene was amplified by PCR with the following gene 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 *HindIII* or *BamHI* restriction enzyme cute sites as indicated by boldface letters. This fragment was ligated into the corresponding restriction sites in pMutinT3.

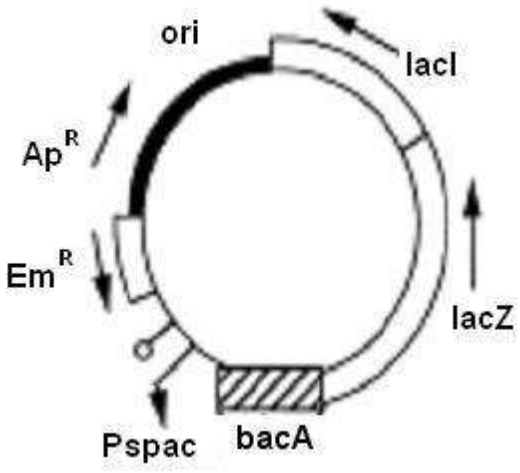

Figure 2.1. bacA::lacZ fusion construct (Köroğlu *et al.*, 2011)

The resulting recombinant plasmid (Fig. 2.1) was used to transform *B. subtilis* PY79 to Macrolide-lincosamide-streptogramin B-resistance (MLSR), thus generating strain 59 OGU1. Recombinant plasmid was driven into the chromosomal DNA of *B. subtilis* PY79 by a single cross over event (Campbell like insertion). Thus, insertional mutation of *bacA* results in no expression of bacilysin operon in the mutant strain OGU1 (Köroğlu *et al*., 2011). Due to polycistronic transcription of operon, no product in *bac* operon was synthesized, there was stop codon at the end of recombination site.

2.2. Maintenance and Growth Conditions of Bacterial Strains

B. *subtilis* strains were grown in LB liquid medium and kept on LA 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. For experimental procedures, *B. subtilis* PY79 and OGU1 strains were grown overnight at 37°C on LA plates. Inoculation with a single colony of 10 mL PA medium was made in 50 mL falcon tubes. Cells were grown at 37 ^{o}C (200 rpm) for 16 h. These cultures were then used to inoculate 50 mL of PA medium (Appendix A) to an initial optical density of about 0.1 at 595 nm (OD595). After inoculation, the cultures were incubated at 37°C (200 rpm) for 16 h. OGU1 strain was grown in lincomycin and erythromycin containing LB and PA media in all steps.

2.3. Extraction of Total Soluble Proteins

For the extraction of soluble proteome, 15 mL of culture was centrifuged at 6000 rpm for 5 min. The cells were washed with TE buffer twice and were centrifuged at 10000 rpm for 5 min. Pellet was taken, supernatant discarded. 500 µl of 40 mg/ml lysozyme solution added to pellet and 40 min. incubation was done at 37 ^{o}C. Lysozyme treated cells were centrifuged at 13000 rpm for 10 min at room temperature. Supernatant discarded and 8 M urea was added onto the pellet. Cells were vortexed at 4˚C until it was dissolved completely. Urea treated cells centrifuged at 13000 rpm for 20 min. Supernatant was taken gently and pellet was discarded. In supernatant, there are our total proteome samples. Protein samples were stored at -20˚C.

For TCA-Acetone precipitation, 15 mL of culture was centrifuged at 6000 rpm for 5 min. The cells were washed with TE buffer twice and were centrifuged at 10000 rpm for 5 min. Supernatant discarded and pellet was scrapped, and freezed with liquid nitrogen. Frozen samples were taken to mortars immediately and 3-4 times grinding was performed till cells appear like a dust. Then 4 mL of solution A (%10 TCA-Acetone, %0.007(v/v) β-mercaptoethanol) was added. Dissolved cells were taken to eppendorf tubes and 2-24 h -20 ˚C was done with 15-30 min. periodic vortexing. Samples were centrifuged at 15000xg for 15 min at +4 ˚C. After removal of supernatant, solution B (Acetone, %0.007(v/v) β-mercaptoethanol) was added and pellet was resuspended for 1h incubation at -20 ˚C. Then again samples are centrifuged in same conditions before, after centrifugation supernatant is discarded and pellet was resuspended with solution C (Acetone, 10mM DTT) for 1h incubation at -20 ˚C. Centrifugation at 15000xg was done for 15 min at +4 ˚C and supernatant was discarded. In order to get rid of acetone 2-4 h vacuuming was done. Protein dust that was obtained after vacuuming was dissolved in rehydration buffer and incubated for 30 min. at room temperature with 2-3 times vortexing. Samples were centrifuged afterwards for 5 min at 15000xg, supernatant was taken and stored at -20˚C at most for 10 days till usage.

2.4. Protein Estimation

Protein concentration was determined by the modified Bradford assay as described by Ramagli and Rodrigez (1985). 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 dH_2O) 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 duplicates. Blank was prepared with solubilization buffer of total solube proteome (8M Urea) and absorbance was measured at 595 nm. A calibration curve of absorbance versus protein concentration in micrograms was constructed. 1 mg/mL of Bovine Serum Albumin (BSA) fraction number V was used as a standard for the construction of calibration curves.

2.5. Proteome Study

2.5.1. 1D Gel Electrophoresis

For 1-D gel separation, an aliquot of 50 µg protein was solubilized with one volume of SDS–PAGE sample buffer and separated in 12% acrylamide/bis-acrylamide gel with a Bio-Rad Cell system (Bio-Rad, USA), applying approximately 16mA per gel. To visualize the separated proteins, gels were stained with Coomassie Brilliant Blue (CBB) dye.

2.5.2. Isoelectric Focusing and Two Dimensional SDS-PAGE

IPG strips (17 cm, pH 3-10, Bio-Rad) were passively rehydrated by applying 400 µg protein sample dissolved in equal volume of rehydration buffer (Appendix B) 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: 2.5h 50V, 2.5h 100V; 1 h 300 V; 1h 500V; 2h 1000 V; 2h 3000 V; 2h 5000V followed by a linear increase to 8000 V. The final phase of 8000V was terminated after 55,000 Vh. 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, 60mg 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, 150mg iodoacetamide) (Görg *et al*., 2004). The isolated proteins were separated in 12% SDS-PAGE 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).

2.5.3 Sample Preparation, LC-MS/MS Analysis and Database Search

10 equidistant pieces were prepared from one lane of 1D SDS-gel. Tryptic digestion of gel was done. Then all pepties that were coming from one 1D lane load into LC and peptide elution for LC–MS/MS was performed for each of the gel pieces as described by Eymann *et al*., 2004. The nano-LC–MS/MS analysis of peptides derived from tryptic in-gel digestion was performed on a linear trap quadrupole (LTQ) Oribtrap (Thermo Fisher Scientific, Waltham, MA) equipped with a nanoACQUITY UPLC (Waters, Milford, MA). Peptides were loaded onto a trapping column (nanoAcquity Symmetry UPLC column, C18, 5_m, 180_m by 20mm; Waters) at a flow rate of 10_l/min and washed for 3min with 99% buffer A. Peptides were eluted and separated via an analytical column (nanoAcquity BEH130 UPLC column, C18, 1.7_m, 100_m by 100mm; Waters) with a 80min gradient (from buffer A (0.1% acetic acid) to buffer B (0.1% acetic acid, acetonitrile). The mass spectrometric analysis started with a full survey scan in the Orbitrap (m/z 300–2000, resolution of 60,000) followed by collision-induced dissociation and acquisition of MS/MS spectra of the five most abundant precursor ions in the LTQ. Precursors were dynamically excluded for 30 s, and unassigned charge states as well as singly charged ions were rejected. Proteins were identified via an automated database search using the SEQUEST software (Bioworks v.3.2, Thermo Electron). The search results were imported to Scaffold 2.02.01 (Proteome Software) and used to validate MS/MS-based peptide and protein identifications. Peptide and protein identifications were accepted if they could be established at a greater than 99.9% probablility and contained at least two identified peptides. For each differentially expressed proteins name, predicted activity, subcellular localization are searched in SubtiList (http://genolist.pasteur.fr/SubtiList/genome.cgi) and SubtiWiki (http://subtiwiki.uni-goettingen.de) databases by entering the obtained protein's abbreviation to search engine.

2.5.4 Relative Abundances of Proteins

Relative spectal counts (RSC) were calculated for quantitation of abundance differences of the proteins identified by LC–MS/MS from two strains (Beissbarth *et al*., 2004; Old *et al*., 2005). For each protein, $\log_2$ ratio of abundance between Sample 1 and Sample 2 constituted an R_{SC} value (Eq. (1)). n1 and n2 designate spectral counts for the protein in Sample 1 and Sample 2, t1 and t2 are total spectral count (sampling depth) for Samples 1 and 2; and f is the correction factor set to 1.25 instead of 0.5, as proposed by Old *et al*., 2005.

$$R_{SC} = log_2[\frac{(n_2+f)}{(n_1+f)}] + log_2[\frac{(t_1-n_1+f)}{(t_2-n_2+f)}] \quad (1)$$

After calculations, the changes in R_{SC} values greater than 2-fold were accepted as significant.

CHAPTER 3

RESULTS AND DISCUSSION

3.1. 2D Gels of *B. subtilis* PY79 and OGU1

Protein profile of the organism of interest in certain timelines can be visualized and identified with gel based proteomic approaches. As mentioned earlier, 2D gels give us the chance to observe the protein pattern of organism on the basis of their molecular weight (kDa) and isoelectric point (pI), the point at which molecules have no net charge, and proteins are seen as spots. When it is coupled with MS, these protein spots could be identified. Therefore, we performed running of 2D gels of *B. subtilis* PY79 and OGU1 in duplicates in order to compare the effect of bacilysin biosynthesis on global regulatiory mechanism in cells. *B. subtilis* OGU1 strain, containing a transcriptional *bacA-lacZ* fusion at *bacA* locus, was previously constructed by our group and proven to be bacilysin inactive due to the insertional inactivation of *bac* operon (Köroğlu *et al.*, 2011). In the same study, it was shown that bacilysin biosynthetic activity of wild-type *B. subtilis* PY79 strain constantly increases during exponential growth (detected as 20-30 Miller units) and reaches its maxima (detected as 130 Miller units) at the transition between exponential and stationary phase (16^{th} hour). 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 2-DE approach; using IPG strips in a pH range 6-11 (data not shown) and 3-10 as the first dimension.Proteins that could be visualized and separated on CBB-stained 2-D gels of *B.subtilis* PY79 and OGU1 are shown in Figure 3.1 and 3.2.

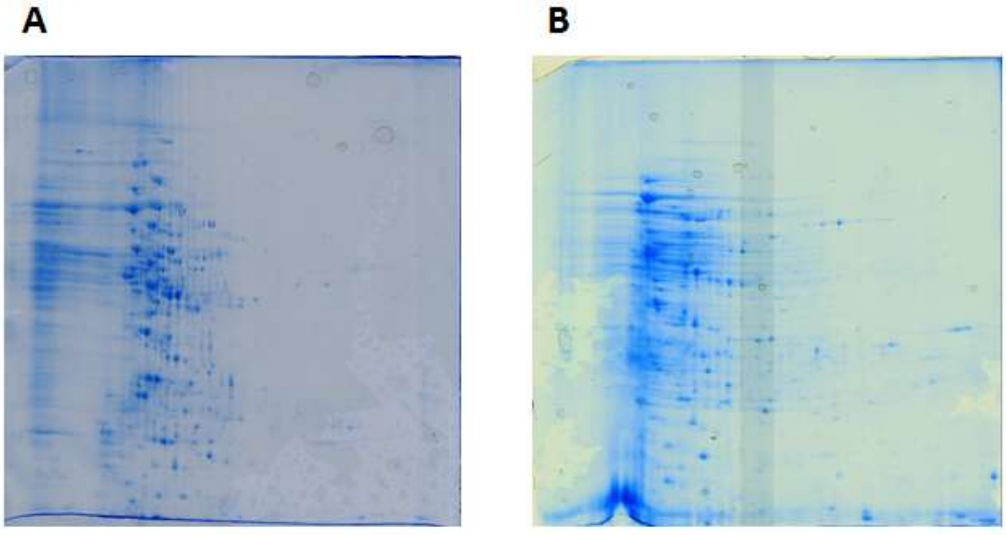

Figure 3.1. 2D gel images of total soluble proteome of *B. subtilis* in a pI range of 3-10 when the proteomes were prepared through urea extraction after 16 h growth of OGU1 (A) and PY79 (B).

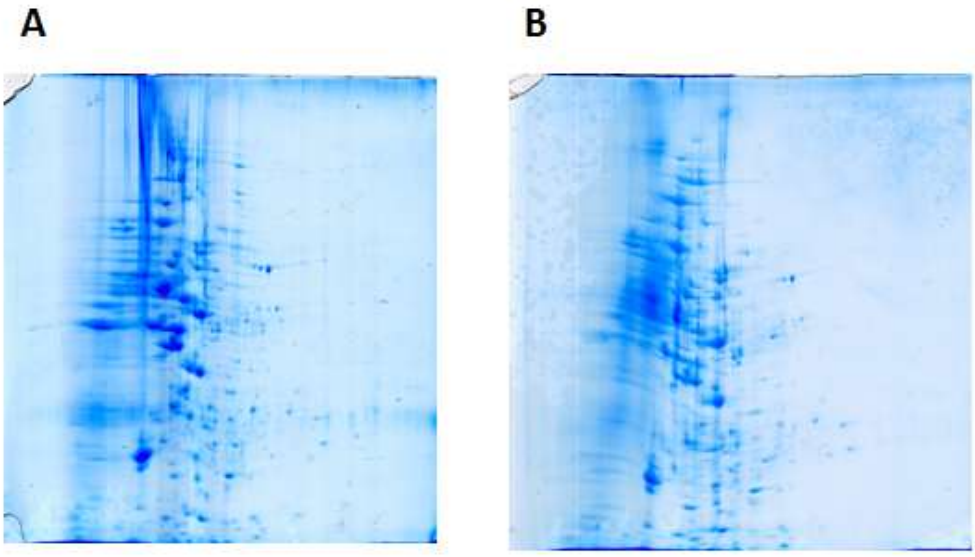

Figure 3.2. 2D gel images of total soluble proteome of *B. subtilis* in a pI range of 3-10 when the proteomes were prepared through TCA-acetone precipitation at 16th hour growth of OGU1 (A) and PY79 (B).

To compare PY79 and OGU1 strains 2DE method with an alkaline pI range 6-11 was employed initially. Spot resolution of pI 6-11 gels were extremely poor, spots were hard to differentiate and of low abundance (data not shown). General protein population of sporulating *B. subtilis* is mostly acidic or neutral (Wolff *et al.*, 2007). Thus, the pI range 6-11 was not good to study comparative proteomics of bacilysin biosynthesis effect on *B.* subtilis. Then the pI range 3-10 were chosen to analyze afterwards. However, spot resolution of the resulting gels was not sufficient for an appropriate comparison. In our experiments, relatively acidic sides of the gels were mostly occupied by horizontal streaks,which was most probably resulted from high density of acidic sporulation phase proteins. The proteins having extreme acidity tend to precipitate at their isoelectric point during isoelectric focusing (Sinha *et al.*, 1990)**.** In addition to that, basic sides of the gels had a lack of sufficient spots for a convenient comparison. It is possible that the abundance of the basic proteins was not enough to be visualized by commassie staining method. Because of these drawbacks and insufficient 2DE results, a gel free approach, called GeLC-MS, was used. Since it does not require any separation based on pI of proteins, this approach is much more applicable to acidic proteins. Moreover, low abundance proteins are more likely to be detected by the help of GeLC-MS.

3.2. 1D Gels of *B. subtilis* Strains PY79 and OGU1

Proteome samples for 1D gel analysis were extracted from *B. subtilis* cultures harvested at 16th h of incubation. 1D gels were prepared for LC-MS analysis. The gels were run at 16 mA to separate the proteins according to their molecular weight. Protein patterns in 1D gels are shown in Figure 3.3.

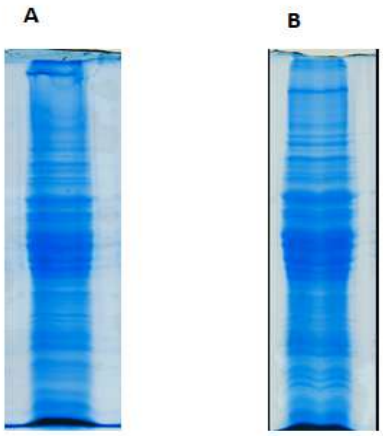

Figure 3.3. 1D gel images of total soluble proteome of *B. subtilis* OGU1 (A) and PY79 (B). In each, 50 µg of protein sample was run on gel.

3.3. LC-MS Identification of Differentially Expressed Proteins

Molecular mechanisms for the interaction of the elements of quorum sensing and stringent response systems with the genes responsible for bacilysin biosynthesis were investigated in previous studies (Yazgan *et al*., 2001; Inaoka, 2002; Yazgan Karataş *et al*., 2003; Taşkın, 2010 and Köroğlu *et al*., 2011). To further analyze the network of the existing regulatory mechanisms and bacilysin biosynthesis via differentially expressed proteins, we performed a proteomic analysis of differentially expressed proteins in *bacA* inactive OGU1 strain as compared to the wild-type PY79 strain with GeLC-MS/MS approach.

A total of 1282 proteins were identified from total soluble proteome of *B.subtilis* cells that were harvested at the stationary phase (16^{th} hour). 76 proteins were found to be differentially expressed according to the R_{SC} comparison, which are shown in Table 3.1.

Among 76 differentially expressed proteins, 18 were upregulated whereas 58 were absent or downregulated in the mutant strain. These results suggested that the protein profile of *B. subtilis* changed drastically, upon the loss of the ability to synthesize bacilysin. These proteins were next analyzed and classified according to their functions

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

Classification	Locus Name	Protein Name/Function	Gene Product	MW(kDa)	PI	Subcellular Localization[a]	Spectral Counts[b] PY79	OGU1	R_{sc}[c]
Primary Cell Metabolism	BSU11250	ornithine carbamoyltransferase/ biosynthesis of arginine	ArgF	34	4.97	C	0	6	-2.55
	BSU10790	asparagine synthase (glutamine-hydrolysing)/ biosynthesis of asparagine	AsnO	70	5.7	C	24	3	2.56
	BSU14900	cytochrome-c oxidase (subunit I)/ respiration	CtaD	68	6.99	CM	0	4	-2.09
	BSU01780	glutamine-fructose-6-phosphate transaminase/ cell wall synthesis	GlmS	65	4.8	C	279	65.5	2.06
	BSU09300	glycerol-3-phosphate dehydrogenase (menaquinone 7)/ glycerol utilization	GlpD	62	8	C	4	0	2.05
	BSU38630	catalase (major catalase in spores)/ detoxification (degradation) of hydrogen peroxide	KatX	62	5.34	C	26	5	2.08
	BSU29880	malate dehydrogenase (decarboxylating)/ malate utilization- malate dehydrogenase (decarboxylating), forms a transhydrogenation cycle with YtsJ for balancing of NADPH	MalS	61	4.9		22	2	2.82
	BSU13690	motility protein (flagellar motor rotation)/ motility and chemotaxis, H+-coupled MotA-MotB flagellar stator	MotA	29	5.01	CM	0	4	-2.09
	BSU01230	ribosomal protein L16/ translation	RplP	16	10.88		0	7	-2.74
	BSU19330	superoxide dismutase/detoxification of oxygen radicals	SodF	33	6.17	CM	5	0	2.31
	BSU38290	thiamine-phosphate pyrophosphorylase/biosynthesis of thiamine by 4-methyl-5-(beta-hydroxyethyl)thiazole phosphate to yield thiamine phosphate	ThiE	23	5.06		0	4	-2.09
	BSU11670	glycine oxidase/biosynthesis of thiamine	ThiO	40	5.9	C	0	6	-2.55
	BSU12980	L-Ala-D/L-Glu epimerase/cell wall metabolism	YkfB	39	5.1		4	0	2.05
	BSU19710	putative deacylase	YodQ	48	5.41		4	0	2.05

Table 3.1. Continued

	BSU22030	dynamin-like protein/fusion of membranes	YpbR	137	5.72	CM	6	0	2.52
	BSU24530	octanoyltransferase/ biosynthesis of lipoic acid	YqhM	32	5.93	C	0	4	-2.09
	BSU36230	UDP-glucose dehydrogenase	YwqF	47	5.03	C	4	0	2.05
	BSU30035	putative 16S pseudouridylate synthase	YtzG	27	5.62		0	4	-2.09
	BSU31150	minor undecaprenyl pyrophosphate phosphatase	YubB	30	7.9	CM	8	0	2.87
Replication	BSU10620	ATP-dependent deoxyribonuclease(subunit B)/DNA repair - recombination	AddB	134	5.39	C	4	0	2.05
	BSU17040	mismatch recognition protein/DNA repair	MutS	97	5.19	Nuc	0	4	-2.09
	BSU15940	chromosome condensation and segregation SMC protein/ structural maintenance of the chromosome	Smc	135	5.27	Nuc	4.5	0	2.19
Catabolic Proteins	BSU17260	intracellular alkaline serine protease/ protein degradation	AprX	47	4.97	C	6.5	0	2.62
	BSU32840	3-hydroxyacyl-CoA dehydrogenase (acetoacetyl-CoA)/ fatty acid degradation	FadN	89	6.53		4.5	0	2.19
	BSU13190	intracellular serine protease/ protein degradation	IspA	33	4.52	C	4.5	0	2.19
Secondary Cell Metabolism	BSU37410	antilisterial bacteriocin (subtilosin) production (processing protease)	AlbE	43	6.07	C	0	8.5	-2.98
	BSU37740	anticapsin biosynthesis protein, prephenate decarboxylase/ biosynthesis of the antibiotic bacilysin	BacA	23	7.9	C	12	0	3.33
	BSU37730	isomerase component of bacilysin synthetase/ biosynthesis of the antibiotic bacilysin(oxidase that catalyzes the synthesis of 2-oxo-3-(4-oxocyclohexa-2,5-dienyl)propanoic acid, a precursor to L-anticapsin)	BacB	26	5.19	C	57	0	5.53
	BSU37720	bacilysin biosynthesis oxidoreductase/ biosynthesis of the antibiotic bacilysin	BacC	27	4.96	C	8.5	0	2.95
	BSU37710	alanine-anticapsin ligase/ biosynthesis of the antibiotic bacilysin	BacD	52	4.68	C	39	0	4.99
	BSU37690	aminotransferase/ aminotransferase involved in bacilysin synthesis	BacF	44	5.35	C	7.5	0	2.79
Antibiotic Resistance	BSU30370	bacitracin ABC transporter (permease)/ ABC transporter (permease) for the export of bacitracin, plectasin, mersacidin and actagardine	BceB	72	9.79	CM	0	4.5	-2.22
	BSU34820	multidrug ABC transporter (ATP-binding protein)/ multiple antibiotic resistance	BmrA	64	6.47	CM	0	4	-2.09

Table 3.1. Continued

Sporulation	BSU19780	spore crust protein/ maturation of the outermost layer of the spore	CgeA	14	3.98	FS	15	0	3.68
	BSU06300	laccase, bilirubin oxidase/ resistance of the spore	CotA	58	5.89	FS	11	0	3.22
	BSU36050	spore coat protein (outer)/resistance of the spore	CotB	42	9.83		16	0	3.77
	BSU17030	spore coat morphogenetic protein/ assembly of the outer spore coat	CotE	20	4.14	FS	69	15.5	2.06
	BSU30920	spore coat protein/ spore envelope	CotI	41	4.99	SW	4	0	2.05
	BSU30910	spore coat protein/resistance of the spore	CotSA	42	7.81		5	0	2.31
	BSU17670	spore coat protein/resistance of the spore	CotU	11	6.32		10	0	3.15
	BSU17410	N-acetylmuramoyl-L-alanine amidase/ mother cell lysis	CwlC	27	5.94	sec.	11	0	3.22
	BSU25540	spore protease (degradation of SASPs)/ germination	Gpr	40	5.2		4.5	0	2.19
	BSU04110	spore coat phospholipase B/ phospholipase implicated in spore germination	LipC	23	5.84	SC	7.5	0	2.79
	BSU18670	oxalate decarboxylase, spore coat protein/ unknown	OxdD	43	5.36		4.5	0	2.19
	BSU22930	spore cortex-lytic enzyme/ degration of the spore cortex	SleB	33	9.51		4	0	2.05
	BSU16740	dipicolinate synthase (subunit B)/ dipicolic acid production	SpoVFB	21	6.15		15	2.5	2.1
	BSU09400	unknown/involved in spore cortex synthesis	SpoVR	55	5.44		13	0	3.44
	BSU37900	unknown/spore coat polysaccharide synthesis	SpsB	54	5.04		4.5	0	2.19
	BSU37830	dTDP -glucose-4,6-dehydratase/rhamnose biosynthesis, spore coat polysaccharide synthesis	SpsJ	35	6.29		7	0	2.71
	BSU37820	dTDP -4-dehydrorhamnose reductase/rhamnose biosynthesis, spore coat polysaccharide synthesis	SpsK	31	6.08		5	0	2.31
	BSU29500	sporulation protein	YtfJ	16	4.99		5	0	2.31
Regulatory Proteins	BSU35240	carboxy-terminal processing serine protease, cleaves SpoIVFA/ control of SigK activation	CtpB	52	8.64		4	0	2.05
	BSU01540	MRP family regulator/ control of alkaline protease expression, negative regulator of scoC expression	SalA	38	5.23		9	0	3.02
	BSU16790	signal peptide peptidase/protein secretion, signal peptide peptidase required for efficient processing of pre-proteins	TepA	24	5.58	C	4.5	0	2.19

Table 3.1. Continued

Membrane Transporters	BSU10220	major H+/Na+-glutamate symport protein/ glutamate and aspartate uptake	GltT	45	9.03	CM	4	0	2.05
	BSU38810	multiple sugar ABC transporter (ATP-binding protein)/ maltodextrin and melibiose (probably) uptake	MsmX	41	7.39	CM	4	0	2.05
	BSU03360	putative metallochaperone/zinc uptake	YciC	45	4.4	CM	0	9	-3.05
	BSU30595	putative ABC transporter (binding protein)	YtlA	38	4.99	CM	4.5	0	2.19
	BSU30610	ABC transporter (ATP-binding protein)	YtlC	29	5.43	CM	7	0	2.71
Proteins with unknown function	BSU02200	putative pepdidoglycan binding protein/unknown	YbfG	79	4.69	CM	6	0	2.52
	BSU06280	similar to chloroperoxydase	YdjP	30	4.62		0	4	-2.09
	BSU07650	unknown	YflK	24	7.76		0	4	-2.09
	BSU07280	similar to CDP-glucose 4,6-dehydratase/unknown	YfnG	33	5.34		8.5	0	2.95
	BSU09780	unknown	YheC	41	9.22	FS	7	0	2.71
	BSU10400	similar to alcohol dehydrogenase /unknown	YhxC	30	7.26		4	0	2.05
	BSU10950	unknown	YitD	28	4.38		4	0	2.05
	BSU12050	unknown	YjdH	15	5.11		4	0	2.05
	BSU12490	similar to manganese-containing catalase /unknown	YjqC	31	5.15		6.5	0	2.62
	BSU17400	unknown	YmaB	23	4.51		0	4	-2.09
	BSU22920	unknown	YpeB	51	6.31		11	0	3.22
	BSU22230	similar to PTS, EIIA component/unknown	YpqE	17	5.29		0	6	-2.55
	BSU26160	unknown	YqbC	25	5.16		0	4	-2.09
	BSU25860	unknown/toxin	YqcG	59	8.81		4	0	2.05
	BSU26780	unknown	YrdA	18	5.72	CM	8.5	0	2.95
	BSU28350	unknown	YsnB	19	5.33		0	4	-2.09
	BSU39870	unknown	YxbD	17	4.9		14	2	2.17

[a] Predicted protein locations from subtiwiki are C, cytoplasm; CM, cell membrane; FS, forespore; SW, spore wall; sec., secreted; SC, spore cortex; Nuc., replication-active region of the DNA.

[b] Spectral counts of proteins of strains. PY79, wild-type and OGU1, mutant.

[c] Relative spectral counts according to equation 1 (Old *et al*., 2005) to compare peptide counts between two strains. R_{SC} values equal to or greater than 2 and equal to or less than -2 were accepted as significant.

3.4. Functional Distribution of Differentially Expressed Proteins

GeLC-MS/MS results indicated that among 1282 proteins, 76 proteins were expressed differentially. Normally, in order to normalize and calculate the relative abundance, correction factor= F is used as 0.5, but, Old *et al*., 2005, suggested that correspondence between observed and expected protein ratio is poor when correction factor is 0.5. Indeed, when we did the initial normalization with $F = 0.5$ and 286 differentially expressed proteins were obtained. Percentage of differentially expressed proteins then seemed to be very high as compared to the total number of proteins (%22). Hence, we took the $F = 1.25$, as suggested by Old *et al.*, 2005, a correction factor which appeared much better to equalize observed and expected protein ratio. As a result, the number of differentially expressed proteins decreased to 76. We categorized the differentially expressed proteins according to their functions (Figure 3.4). There are 9 main different functional classes; proteins that are responsible for primary cell metabolism, replication, catabolism, secondary metabolite production, antibiotic resistance, sporulation proteins (initiation, germination and outgrowth), transcriptional regulation, metabolite transport and proteins with unknown function.

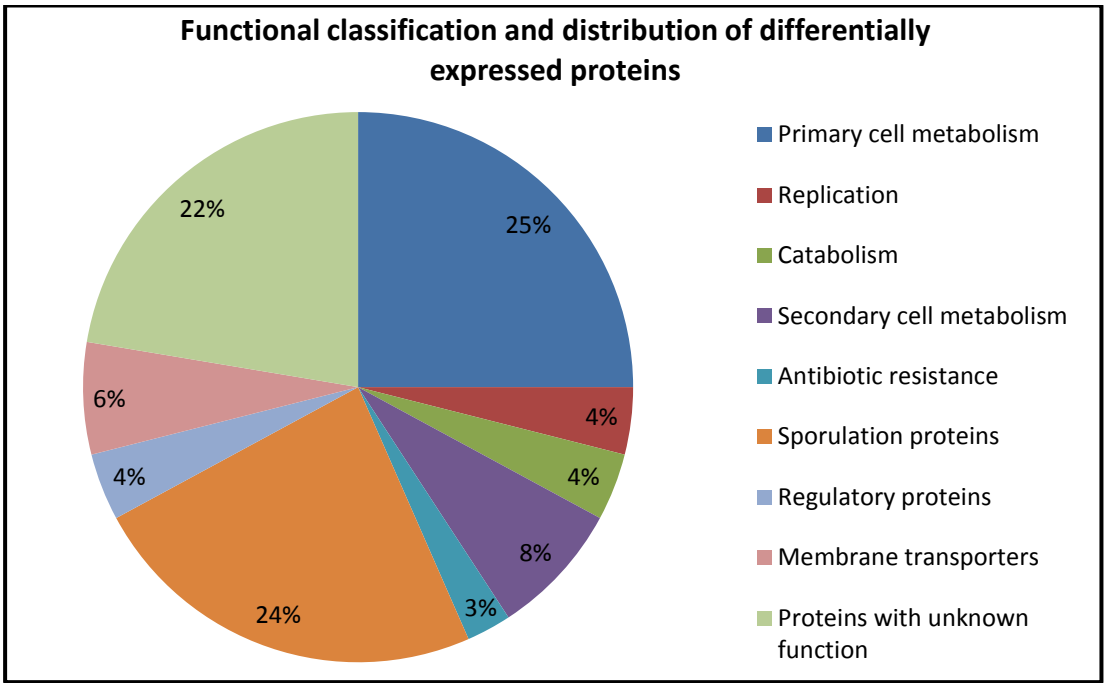

Figure 3.4. Distribution of the differentially expressed proteins of PY79 and OGU1 according to their functions.

3.5. Analysis of Differentially Expressed Proteins

3.5.1. Proteins of Primary Metabolism

A total of 19 proteins identified as being responsible for primary cell metabolism were either down- or up-regulated in the mutant strain. Those primary cell metabolism proteins cover %25 percent of total differentially expressed proteins, suggesting that bacilysin biosynthesis has some role in vegetative growth as well as in sporulation.

There are two of main interest differentially expressed proteins found in our proteome approach. First one is ArgF which is one of the proteins acting in arginine biosynthetic pathway (Ma *et al.,* 2002), catalyzing the reaction: *Carbamoyl phosphate + L-ornithine = phosphate + L-citrulline*. In our study, this protein was absent from the wild-type strain. The other proteins that are also responsible for arginine biosynthesis were expressed in both strains equally (Appendix D). Arginine biosynthetic operon *argCJDBF* is under control of the ArgR regulator protein (Larsen *et al.*, 2005). All proteins that are expressed from this operon, except ArgF, were found in GeLC-MS analysis in wild-type PY79 strain and ArgF was also found in bacilysin-minus mutant strain. Proteins that are controlled by single regulator were found to be equally expressed in both strains; however, only ArgF was found to be absent in wild-type strain. It is puzzling and needs further investigation. There was another amino acid biosynthetic protein found to be differentially expressed in our study. AsnO is a protein responsible for biosynthesis of asparagine. Unlike other proteins (AsnB, AsnH) that are involved in asparagine biosynthesis, it is expressed in a monocistronic operon expression of which depends on one of the sporulation sigma factors, sigma-E (Yoshida *et al.*, 1999). This protein was shown to be important for sporulation because *asnO* knock out strain failed to sporulate (Yoshida *et al*., 1999). Our study we supported this finding in that, AsnO was overexpressed in wild-type strain which is able to sporulate; however, in the absence of bacilysin biosynthesis its expression level was low. Nonetheless, there was an interesting observation, by Yoshida *et al*., 1999, that *asnO* expression was only seen in rich sporulation medium but not in minimal medium in sporulation stage. Our PA medium (Appendix A) was a chemically defined medium not that rich at all, and we could see the AsnO in the phase of sporulation. Thus there was a conflict between our study and some findings of Yoshida *et al*.'s (1999) study. This phenomenon might need further investigation, like comparative proteomics between samples that are grown in two different medium, one being rich sporulation the other being minimal medium.

There is a finding that flagellar motor protein complex MotAB expression was also absent from wild type, however there was expression in mutant strain which might support the idea that sporulation initiation inhibits the motility of *B.subtilis* (Nishihara and Freese, 1975). ThiE and ThiO proteins are responsible for thiamine biosynthesis (Begley *et al*., 1999). They catalyze following reactions respectively in the pathway of thiamin production:

2-methyl-4-amino-5-hydroxymethylpyrimidine diphosphate + 4-methyl-5-(2-phosphono-oxyethyl)thiazole = diphosphate + thiamine phosphate

Glycine + $H_2O + O_2 = glyoxylate + NH_3 + H_2O_2$

In our study, ThiE and ThiO were found to be absent in wild type strain. Thiamin which is synthesized by multistep reactions is a coenzyme of pyruvate dehydrogenase (Kale *et al*., 2008). Pyruvate dehydrogenase coenzyme production is deficient and pyruvate dehydrogenase is a catalyzer of the first reaction into the TCA cycle. Thus we may conclude that wild type *B. subtilis* PY79 might not need the energy comes from oxidative phosphorylation.

$pyruvate + CoA + NAD^+ = acetylCoA + CO_2 + NADH + H^+$

There might be a supportive data found in our study also, about change in energy demand of *B. subtilis* just before sporulation. It was shown that CtaD protein, which is one of the required protein for oxidative phosphorylation (Van der Oost *et al.*, 1991), was overexpressed in mutant strain. By an indirect or a direct effect of bacilysin biosynthesis, energy need of *B. subtilis* might have changed in the phase of sporulation. In the wild-type PY79 there are two important deficiencies in the path of oxidative phosphorylation. In fact, lack of those proteins might indicate that energy need of transition from vegetative state to spore form supplied with glycolysis. Thus, we can speculate that bacilysin synthesis might have an effect on oxidative phosphorylation mechanisms in *B. subtilis*. Moreover, in wild-type strain there was overexpression in a protein called NAD independent glyceraldehyde-3-dehydrogenase (GlpD)(Nilsson *et al.*, 1994). Differential expression of those 4 proteins ThiE,ThiO, CtaD and GlpD might help us to connect the scenario. NAD cycle is important for oxidative phosphorylation. NAD is reduced when taking H^+ in glycolysis then molecules that are responsible for oxidation in the bacteria, take the H^+ from NAD and NAD cycle continues. However, in an organism, if oxidation machinery is defected NADH to NAD conversion cannot be maintained to carry on glycolysis. Thus, in order to stabilize glycolysis, cells need a NAD independent enzyme which is GlpD. For synthesis of pyruvate, because of energy demand, *B. subtilis* needs to synthesize GlpD protein. Presence of bacilysin biosynthesis might provide a kind of protein expression pattern, which would turn off the high-energy supply machinery and continue to sporulation with lower energy in order to become more stable and more oxidative stress free. In bacteria SodA provides detoxification of free radicals and we found that SodA expression level in both strains is equal (Appendix D). However, the organism *B. subtilis* might need to get rid of oxidative stress, in sporulation process. According to our findings, another superoxide dismutase enzyme (SodF) was found to be overexpressed in PY79 strain. This idea also supports the finding, Bosak *et al.*, 2008, that expression timing of SodF which is during sporulation of mother cell. There is also overexpression of KatX protein in wild type strain, which is one of the major catalase protein to detoxify hydrogen peroxide from germinating spores (Bagyan *et al.*, 1998). These findings suggesting the idea that bacilysin biosynthesis has direct or indirect effect on stress response of *B. subtilis.*

In our study, dynamin like protein (YpbR) was found to be absent from the bacilysin-minus mutant. YpbR provides fusion of membranes, and foci formation (Capuano *et al.*, 1996; Bürmann *et al.*, 2012). Its loss from the bacilysin minus strain indicates that absence of bacilysin biosynthesis might have effect on YpbR dependent mechanisms.

Changes in the expression profiles of the proteins mentioned above suggest that bacilysin biosynthesis have tremendous effects on cell metabolism. Further studies and analyses are needed to reveal the relevant mechanisms.

3.5.2 Catabolic Proteins

Differential expression of the three catabolic proteins was found in our study. Intracellular alkaline serine protease (AprX), 3-hydroxyacyl-CoA dehydrogenase (FadN) and intracellular serine protease (IspA) were found to be absent in OGU1. AprX is a putative intracellular enzyme that is synthesized in stationary phase. Its expression is dependent on various transition state regulatory genes (degU, degQ, hpr, abrB, sinR), however its presence is not essential for growth or sporulation (Valbuzzi *et al.*, 1999). For the other two proteins, there is nearly no information in the literature. Though, it can be speculated that there may be protein and fatty acid catabolism occurring in order to provide aminoacid and lipid precursors to spore specific components. Thus, direct or indirect induction signals related with bacilysin biosynthesis seem to be needed for expression of those proteins.

3.5.3. Secondary Metabolite Synthesis Proteins

In our study, as expected *bac* operon proteins were absent from OGU1 strain. Thus, anticapsin biosynthesis protein, prephenate decarboxylase (BacA), oxidase (BacB), bacilysin biosynthesis oxidoreductase (BacC), alanine-anticapsin ligase (BacD), aminotransferase (BacF), are proteins responsible for biosynthesis of bacilysin and were not seen in OGU1 strain, proving the loss of biosynthetic ability in OGU1 strain. In *B. subtilis,* one major regulatory switch in the adaptation to low oxygen tension is the two component regulatory system ResDE (Nakano and Zuber, 1990; Nakano *et al.*, 2000). The activated ResD -via phosphorylation by ResE- binds to the promoter regions of genes that function in nitrate respiration. These genes are *fnr* (encoding the anaerobic gene regulator Fnr), *nasDEF* (encoding the nitrite reductase operon), and *hmp* (encoding the flavohemoglobin). Response regulator ResDE was found to be equal (Appendix D) in both strains in our study. There was an interesting finding that the *alb* operon, which was proved to be induced by ResDE two component regulatory system (Nakano et al., 2000), was more active in OGU1 strain. AlbACEF proteins that are synthesized from *alb* operon have more peptide count in OGU1 strain, in fact AlbE was absent from wild type but overexpressed in bacilysin minus strain OGU1 (Appendix D). So it can be speculated that beside ResDE system, there may be more complex mechanisms since our finding points out that Alb proteins were differentially expressed despite same level of protein expression of ResDE proteins. Alb proteins are responsible for synthesis and secretion of antilisterial bacteriocin subtilosin (Zheng *et al.*, 1999). Especially AlbE is responsible signal peptide peptidase that provide export of subtilosin via cutting its signal peptide (Tjalsma *et al.,* 2000). It was an interesting result that in the bacilysin-minus mutant OGU1 strain, all proteins were expressed, responsible for subtilosin biosynthesis and export; however, in the bacilysin producer wild type PY79 strain AlbAC had less peptide count and AlbEF were missing. This might mean that in OGU1 there is a mechanism trigerred by absence of bacilysin biosynthesis, in order to produce subtilosin and secrete it to outside. In *B. subtilis,* bacilysin is a responsible dipeptide antibiotic that resides in cascades for competence and quorum sensing; however, in the absence of bacilysin, *B. subtilis* might use subtilosin as a competence agent by releasing it to environment.

3.5.4. Antibiotic Resistance Proteins

Interestingly, in bacilysin minus strain, there appear new antibiotic resistance systems emerging. In normal conditions, upon induction with the bacitracin, in producer organism *B. subtilis*, BceRS two component regulatory system is activated and it ends up with the synthesis of BceAB proteins which provides bacitracin resistance (Ohki *et al*., 2003). In our study, bacitracin ABC transporter (ATP-binding protein) BceA protein expression level was found to be same in both strains; however, permease BceB was only expressed in OGU1 but not in the wild type. Moreover, multidrug ABC transporter protein BmrA, which provides multiple antibiotic resistance (Steinfels *et al*., 2004), was newly expressed in bacilysin minus strain but absent from the wild type. Those new resistance systems may emerge because of two antibiotics lincomycin erythromycin used in the culture media to cultivate OGU1. There may be other mechanisms in *B. subtilis* for synthesis of those antibiotic resistance mechanisms. Absence of bacilysin biosynthesis seems to have effect on those mechanisms. It should be further investigated in order to understand the role of bacilysin biosynthesis on these antibiotic resistance mechanisms.

3.5.5. Sporulation Proteins

In our study, a total of 18 sporulation proteins were detected as differentially expressed. Two of those proteins which are SpoVFB (dipicolinate synthase subunit B) and spore coat morphogenic protein (CotE), were found to be expressed in mutant strain at a low abundance. Other 16 proteins spore crust protein (CgeA), laccase, bilirubin oxidase (CotA), outer spore coat protein (CotB), spore coat protein (CotI), spore coat protein (CotSA), spore coat protein (CotU), N-acetylmuramoyl-L-

alanine amidase (CwlC), spore protease (Gpr), spore coat phospholipase B (LipC), oxalate decarboxylase (OxdD),spore cortex-lytic enzyme (SleB), spore cortex synthesis protein (SpoVR), spore coat polysaccharide synthesis protein (SpsB), dTDP-glucose-4,6-dehydratase (SpsJ), dTDP-4-dehydrorhamnose reductase (SpsK), sporulation protein (YtfJ) were expressed exclusively in PY79 but not in OGU1. On the other hand, not just spore coat protein but also spore coat synthesis proteins (SpoVR, SpsB, SpsJ, SpsK) were absent from OGU1, indicating a general downregulation of spore coat synthesis proteins. With regard to spore coat protein defects in OGU1 strain, it is assumed that spore formation is highly decreased in the absence of bacilysin, as proved by our group much earlier (Ozcengiz and Alaeddinoglu[b], 1991; Yazgan *et al.*, 2001). In the absence of CwlC coupled with CwlH or CwlB, mother cell is disabled to lyse, thus CwlC is shown to be essential in mother cell lysis (Lewis, 2000). Not just spore formation proteins but also mother cell lysis protein, (CwlC), is absent from OGU1. Moreover, spore protease (Gpr) is crucial for germination. Thus when SASP degrading protein (Gpr) is absent from mutant strain, Gpr mutant *B. subtilis* cells are constrained to return to vegetative state (Sanchez-Salas *et al.*, 1992). In our study this protein was absent from OGU1 strain which is another proof of defective germination and outhgrowth. Another protein which is spore coat phospholipase B (LipC) is not expressed in bacilysin mutant strain. This protein is one of the key elements in germination as its absence causes defective L-alanine stimulated germination (Masayama *et al*., 2007). The idea that absence of bacilysin cause sporulation defective phenotype (Köroğlu *et al*., 2011) was supported by our findings. Nonetheless, according to differentially expressed proteins not only the proteins that are responsible for entry into sporulation but also germination and outgrowth proteins were defective in bacilysin minus strain. Our proteomics study indicates that bacilysin biosynthesis might have a strong effect on sporulation process of *B. subtilis*.

3.5.6. Regulatory Proteins

According to GeLC-MS/MS results, three regulatory proteins were found to be differentially expressed in our study. Carboxy-terminal processing serine protease (CtpB) was missing in bacilysin minus strain. Absence of CtpB, which is one of the key signal in forespore stage to cause delayed and defective sporulation (Campo , 2007) and is directly correlated with our earlier findings (Yazgan *et al.*, 2001; Köroğlu *et al.*, 2011;). Moreover, MRP family regulator (SalA) was deficient in OGU1 strain. This protein is a repressor of ScoC (Ogura *et al.*, 2004) which is the negative regulator of sporulation, thus the absence of SalA is expected to cause an increase in the level of active ScoC which leads to defective sporulation. Signal peptide peptidase (TepA) is a protein responsible for protein translocation accross membrane and its presence or absence does not affect the sporulation process (Bolhuis *et al*., 1999). This protein was absent from OGU1 strain. It is interesting that a protein which is not responsible for sporulation is expressed in a spore forming strain but not in sporulation deficient strain. In order to clarify other possible roles of this protein, further studies are needed.

3.5.7. Transporter Proteins

YciC is a putative metallochaperone responsible for metal uptake, mainly zinc (Gaballa and Helmann, 1998). This protein was found to be absent from the wild type strain, which might mean that metal uptake in mutant strain is more favorable than in sporulating wild type one. On the other hand, major H^+/Na^+ glutamate symport protein (GltT), multiple sugar ABC transporter (MsmX), putative ABC transporter (YtlA) and ABC transporter (YtlC) were the transporter proteins that were not expressed in OGU1.

3.5.8. Replication Proteins

In our study, it is found that three proteins involved in replication processes were differentially expressed. In OGU1, ATP-dependent deoxyribonuclease (subunit B) (AddB) and chromosome condensation and segregation protein (Smc) were no longer present while the presence of, mismatch recognition protein (MutS) only in this bacilysin minus strain, suggested the activation of this type of repair.

3.5.9. Proteins with Unknown Functions

Our GeLC-MS/MS results indicated 17 different proteins with unknown functions were differentially expressed either in PY79 or OGU1. The amount of differentially expressed proteins, whose functions are not known, cover %22 percentage of total differentially expressed proteins. Although *B. subtilis* is a model organism, there is still a long way to identify all proteins in this organism.

3.6. Comparison of Results with Those Obtained from 2DE-MALDI-TOF MS Approach

Proteomic comparison of bacilysin-minus mutant strain OGU1 and wild-type PY79 was done by our former lab member Aslı Aras Taşkın (2010) in different pI ranges by 2DE gel electrophoresis coupled to MALDI-TOF/MS analysis. Proteins that were found to be differentially expressed in both studies are presented in Table 3.2. According to her findings, sporulation proteins were less abundantly expressed in OGU1 strain. Less abundant expression or absence of 18 sporulation proteins in OGU1 strain is well correlated with this previous study in that OGU1 shows sporulation defective phenotype in the absence of bacilysin biosynthesis. Especially one of the sporulation control protein YtfJ (Caldwell *et al.* 2001) was found to be less abundantly expressed in mutant strain in both studies. To support this phenomena, not only the YtfJ protein but also CtpB and SalA which are the positive regulators of sporulation (Campo and Rudner, 2007; Ogura et al., 2004) were found to be absent in bacilysin-minus OGU1 strain via GeLC-MS study. Those findings might support the idea that absence of bacilysin biosynthesis cause defective sporulation. Thus, it might be said that bacilysin biosynthesis has direct or indirect effects on sporulation regulation.

In Aslı Aras Taşkın's study, 2010, it was suggested that bacilysin-minus OGU1 strain was more sensitive to oxidative stress because of decrease level of oxygen-radical neutralizing proteins (YgaF, MsrA) as compared to wild-type (Taşkın, 2010). In the present study, two important oxygen radical detoxification proteins (KatX, SodF) were also found to be absent from bacilysin-minus OGU1 strain. These results might indicate that biosynthesis of bacilysin biosynthesis has a positive effect on relieving from oxidative stress.

All above mentioned findings, when compiled with those of an extensive 2D-MALDI-TOF/MS analysis of comparative total soluble proteome of PY79 and OGU1 (Taşkın *et al.,* manuscript in preparation), the work of our group clearly indicates crucial roles of bacilysin formation in cell physiology, particularly in sporulation. The exact network remains to be defined; still it is quite possible that dipeptide antibiotic bacilysin acts by itself as a quorum-sensing peptide.

Table 3.2. Comparison of proteins that were found in 2DE and GeLC-MS/MS studies. Highlighted proteins were found to be differentially expressed in both studies by the comparison of PY79 and OGU1 strains.

OGU1	S.C.	PY79	S.C	Rsc	mutant/control(2DE)
ahpC	56.5	ahpC	54.5	-0.06755	2.557
ald	29	ald	17	-0.74636	N.D.**
atpC	4.5	atpC	6.5	0.414235	N.D.**
atpD	122.5	atpD	132.5	0.096144	0.343
bacC	0	bacC	8.5	2.947499	0.337
bacD	0	bacD	39	4.994989	N.D.**
citB	120	citB	159	0.388263	0.345
cotA	0	cotA	10.5	3.216815	0.088
degU	62.5	degU	43	-0.54458	2.68
dppA	20.5	dppA	24.5	0.227276	0.26
glmS	65.5	glmS	278.5	2.06461	N.D.**
ilvC	81.5	ilvC	74.5	-0.14455	N.D.**
ilvD	48.5	ilvD	40	-0.28741	N.D.**
malS	2	malS	22	2.823487	0.386
nadE	16.5	nadE	9	-0.80922	0.246
oppD	5	oppD	3.5	-0.41255	0.177
sdhA	101	sdhA	93.5	-0.12699	3.225
serA	129	serA	97.5	-0.4181	3,215; 2,941
serC	36.5	serC	29.5	-0.3129	0.115
spoVFA					0.16
spoVFB	2.5	spoVFB	15	2.099759	
ureC	220	ureC	257.5	0.211643	0.178
yaaN	8	yaaN	5	-0.58232	4.366
yceD	32	yceD	25.5	-0.33079	3.125
yceD	32	yceD	25.5	-0.33079	3.125
yceD	32	yceD	25.5	-0.33079	3.125
yceE	21.5	yceE	19	-0.18465	N.D.**
yhcQ	4.5	yhcQ	20.5	1.903889	0.326
ytfJ	0	ytfJ	5	2.305726	0.109
ytkL	11	ytkL	6.5	-0.67734	3.378
yuaE	2	yuaE	6	1.141273	2.557

**N.D. : proteins that were found in OGU1 strain but expressed in PY79 strain for 2DE 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, 1282 proteins were identified in GeLC-MS/MS analysis, 76 of those are differentially expressed.

- Our comparative proteome analysis proved that the inactivation of *bacA* locus led to the complete loss of *bac* operon-encoded proteins BacABCDEF in the mutant strain.

- The study revealed the strong impact of *bacA* deletion on the abundance of numerous proteins. One of the major functional group affected by the deletion of bacilysin production consisted of sporulation proteins. A total of 18 proteins that are directly or indirectly involved in sporulation were determined as differentially expressed. It might be claimed that bacilysin biosynthesis has an important role in sporulation.

- In our study, it was observed that certain new antibiotic resistance systems are activated in the bacilysin minus strain. It was shown that in mutant OGU1 strain, expression of antibiotic resistance proteins (BceB and BmrA) were activated. This might be an indication of activation of survival as a response to the loss of bacilysin biosynthesis as a weapon for competition.

- Also AlbE, which is the protein responsible for synthesis and secretion of subtilosin, was expressed in mutant OGU1 strain while not in the wild-type PY79. This might be an indication of adaption to competetion via secreting a new antibiotic to environment, upon the loss of bacilysin biosynthesis.

- Our results have shown that the absence of bacilysin create changes in the expression level of energy metabolism proteins, particularly those of ThiEO, CtaD and GlpD. These findings suggest that bacilysin might have direct or indirect effect on energy metabolism of *B. subtilis*, in a manner not yet exactly known.

- Absence of catabolic proteins in bacilysin-minus OGU1 strain can be speculated such that *B. subtilis* might need protein and lipid precursors to get prepared for sporulation

- According to our study, the expression of the sporulation regulators in OGU1 was impaired. Absence of the carboxy-terminal processing serine protease (CtpB), MRP family regulator (SalA) in bacilysin-minus strain might reflect a strong role of bacilysin biosynthesis on sporulation regulation.

- Our study revealed 17 differentially expressed proteins with unknown functions emphasizing the significance of these proteins, whose functions are not defined, in *B.subtilis* physiology as linked to bacilysin biosynthetic function. The functions of these proteins remain to be investigated via constructing knock-out strains as well as overexpression trials.

- Absence of some ABC type membrane transporters (MsmX, YtlA and YtlC) in bacilysin minus strain is an interesting finding that should require further investigation in order to determine the role of bacilysin biosynthesis on the regulation of metabolite transport. These membrane transporters might be related with the sporulation initiation as in in the case of Opp (oligopeptide permease) which exports the cell density signal proteins for the onset of sporulation (Perego et al., 1991).

- Absence of bacilysin biosytnthesis led to many changes in the global regulation systems, two component regulatory systems and adaptation to competition for food and space in nature; along with a sporulation phenotype defective in many structural features. Thus, in order to illuminate further roles of bacilysin formation in the physiology of its producer, further studies with particular reference to differentially expressed proteins will be in progress in our laboratory in near future.

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APPENDIX A

Culture Media

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

KH2PO4	1 g/L
KCl	0.2 g/L
MgSO4.7H2O*	0.5 g/L
Glutamate.Na.H2O	4 g/L
Sucrose*	10 g/L
Ferric citrate**	0.15 g/L
Trace elements**	1 mL
CoCl2.6H2O	0.0001 g/L
Ammonium molybdate	0.0001 g/L
MnCl2.4H2O	0.001 g/L
ZnSO4.7H2O	0.0001 g/L
CuSO4.5H2O	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	5 g/L
Agar	15 g/L

Distilled H_2O was added up to 1000ml and then autoclaved for 15 minutes.

APPENDIX B

Buffers and 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

Acrylamide/Bisacrylamide Solution:

Acrylamide	146 g
N.N‟-Methylene-bis Acrylamide	4 g

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

Tris HCl (1.5 M):

Tris base	54.45 g
dH_2O	150 mL

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

Tris HCl (0.5 M):

Tris base	6 g
dH_2O	60 mL

pH is adjusted to 6.8 with HCl, distilled wate

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

Add distilled water to 1 L and store 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
dH_2O	800 mL

Rehydration Buffer:

Urea	8 M
Thiourea	2 M
Ampholite	0,2 M
DTT	28 mM
CHAPS	2% m/v

dH_2O up to 10 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
HCl	Merck
IPG strips	BioRad
KH_2PO_4	Merck
Methanol	Merck
$MgSO_4$ $7H_2O$	Merck
Molecular Weight Standard	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- HCl	Sigma
Urea	Fluka

APPENDIX D

Raw Data of Identified Proteins

Table D.1. Spectral count comparison of identified proteins

ogu1	SC	py79	SC	*	**	result
aadK	5	aadK	5	1	0.988608	-0.01653
		aag	2	2.6	0.9887	1.362116
accA	18.5	accA	17	0.924051	0.988535	-0.13059
accB	12	accB	9.5	0.811321	0.988494	-0.31835
accC	59.5	accC	49.5	0.835391	0.988136	-0.27669
accD	24	accD	26.5	1.09901	0.98871	0.119823
ackA	31	ackA	24.5	0.79845	0.988307	-0.3417
acpA	17	acpA	16	0.945205	0.988558	-0.0979
acsA	5.5	acsA	7.5	1.296296	0.988697	0.357996
addA	4	addA	4	1	0.988609	-0.01653
		addB	4	4.2	0.988788	2.054123
adeC	5.5	adeC	6.5	1.148148	0.988653	0.182844
		adhB	2	2.6	0.9887	1.362116
adk	19	adk	34	1.740741	0.989267	0.784134
ahpC	56.5	ahpC	54.5	0.965368	0.988493	-0.06755
ahpF	17.5	ahpF	15	0.866667	0.988491	-0.22315
alaR	9.5	alaR	7.5	0.813953	0.988517	-0.31364
alaS	30	alaS	27	0.904	0.988463	-0.16235
alaT	3	alaT	4	1.235294	0.988654	0.288392
albA	45	albA	21	0.481081	0.987523	-1.07376
albC	11	albC	6	0.591837	0.988384	-0.77359
albE	8.5			0.128205	0.98823	-2.98056
albF	2			0.384615	0.988521	-1.39517
ald	29	ald	17	0.603306	0.988064	-0.74636
alsD	45	alsD	42	0.935135	0.988455	-0.11351
alsS	120.5	alsS	99	0.823409	0.987592	-0.29833
amhX	11.5	amhX	11.5	1	0.988605	-0.01653
ampS	5.5	ampS	3	0.62963	0.988497	-0.68412
ansZ	8.5	ansZ	5.5	0.692308	0.988474	-0.54724

appB	6.5	appB	7	1.064516	0.98863	0.0737
		appF	3	3.4	0.988744	1.749204
		aprX	6.5	6.2	0.988899	2.616164
apt	5.5	apt	5	0.925926	0.988586	-0.12759
argB	2	argB	4.5	1.769231	0.988721	0.806757
argC	4	argC	4	1	0.988609	-0.01653
argD	7	argD	7	1	0.988607	-0.01653
argF	6			0.172414	0.988342	-2.55297
argG	15	argG	12	0.815385	0.98847	-0.31118
argH	14	argH	14	1	0.988604	-0.01654
argJ	19	argJ	19	1	0.988601	-0.01654
argS	32	argS	31.5	0.984962	0.988572	-0.03844
aroA	67.5	aroA	91.5	1.349091	0.989645	0.41697
aroB	19.5	aroB	19	0.975904	0.988579	-0.05176
aroE	35.5	aroE	33.5	0.945578	0.988504	-0.09741
aroF	34.5	aroF	40	1.153846	0.988838	0.190256
aroK	2.5	aroK	4	1.4	0.988676	0.468997
artP	39.5	artP	41	1.03681	0.988657	0.035694
artQ	5.5	artQ	3	0.62963	0.988497	-0.68412
artR	10.5	artR	13	1.212766	0.988717	0.26193
asd	55.5	asd	45	0.814978	0.988116	-0.31241
asnB	17	asnB	10	0.616438	0.988292	-0.71496
asnH	18	asnH	24.5	1.337662	0.98889	0.403596
asnO	3	asnO	24	5.941176	0.989541	2.555581
asnS	36	asnS	38.5	1.067114	0.988704	0.077324
aspB	59.5	aspB	47	0.794239	0.988025	-0.34974
aspS	61.5	aspS	58	0.944223	0.988424	-0.0996
atpA	138	atpA	127	0.921005	0.98805	-0.13606
atpB	2.5	atpB	3.5	1.266667	0.988654	0.324575
atpC	4.5	atpC	6.5	1.347826	0.988697	0.414235
atpD	122.5	atpD	132.5	1.080808	0.988994	0.096144
atpF	21.5	atpF	13	0.626374	0.988223	-0.692
atpG	35.5	atpG	29.5	0.836735	0.988327	-0.2741
atpH	15	atpH	12.5	0.846154	0.988492	-0.25771
azoR1	9	azoR1	9.5	1.04878	0.988629	0.052213
azoR2	6	azoR2	11	1.689655	0.98883	0.740523
		bacA	11.5	10.2	0.989121	3.334716
		bacB	57	46.6	0.991144	5.529425

		bacC	8.5	7.8	0.988988	2.947499
		bacD	39	32.2	0.990343	4.994989
		bacF	7.5	7	0.988944	2.791315
bcd	22	bcd	19	0.870968	0.988467	-0.21604
bceA	14	bceA	12.5	0.901639	0.988537	-0.16601
bceB	4.5			0.217391	0.988409	-2.21845
bceR	2	bceR	2	1	0.98861	-0.01653
bdbD	21	bdbD	19	0.910112	0.988512	-0.15255
bdhA	96	bdhA	63.5	0.66581	0.987117	-0.60552
bioA	6.5	bioA	6	0.935484	0.988586	-0.11278
bioB	9.5	bioB	7.5	0.813953	0.988517	-0.31364
bioD	4	bioD	2.5	0.714286	0.988542	-0.50205
bkdAA	2	bkdAA	4	1.615385	0.988699	0.67548
bkdAB	9.5	bkdAB	11.5	1.186047	0.988695	0.229758
bkdB	12	bkdB	14.5	1.188679	0.988716	0.232987
bmrA	4			0.238095	0.988432	-2.08718
		bpr	2	2.6	0.9887	1.362116
bsaA	2.5	bsaA	2.5	1	0.98861	-0.01653
buk	11	buk	13.5	1.204082	0.988716	0.251562
cah	2	cah	2	1	0.98861	-0.01653
carB	10.5	carB	12.5	1.170213	0.988694	0.210367
cccA	10	cccA	9.5	0.955556	0.988584	-0.08215
		cccB	3	3.4	0.988744	1.749204
ccpA	7	ccpA	6.5	0.939394	0.988585	-0.10676
ccpC	3	ccpC	2.5	0.882353	0.988587	-0.19713
ccpN	4.5	ccpN	2.5	0.652174	0.98852	-0.63333
		cgeA	15	13	0.989276	3.684885
cggR	2	cggR	3	1.307692	0.988654	0.370561
cheA	3	cheA	4.5	1.352941	0.988676	0.419669
cheR	2.5			0.333333	0.988499	-1.60165
de	9	cheV	6	0.707317	0.988473	-0.5163
cheW	2.5	cheW	2	0.866667	0.988588	-0.22301
cinA	5	cinA	5	1	0.988608	-0.01653
citB	120	citB	159	1.321649	0.99029	0.388263
citG	47	citG	26.5	0.57513	0.987677	-0.81593
citZ	34.5	citZ	35.5	1.027972	0.988638	0.023315
clpC	131	clpC	137	1.045369	0.988811	0.047779
clpP	68	clpP	53.5	0.790614	0.987932	-0.35647

clpQ	3.5	clpQ	3	0.894737	0.988587	-0.17702
clpX	46	clpX	47	1.021164	0.988632	0.01372
clpY	42.5	clpY	46	1.08	0.988745	0.094701
cmk	16.5	cmk	13	0.802817	0.988447	-0.33362
codY	28.5	codY	32.5	1.134454	0.988774	0.165711
comA	4.5	comA	3	0.73913	0.988542	-0.45272
		comEA	3	3.4	0.988744	1.749204
comEB	7.5	comEB	11.5	1.457143	0.988785	0.52687
comER	7.5	comER	22.5	2.714286	0.989273	1.425013
comGA	7.5	comGA	12	1.514286	0.988807	0.582398
comK	5	comK	7.5	1.4	0.988719	0.46906
		cotA	10.5	9.4	0.989077	3.216815
		cotB	16	13.8	0.989321	3.771107
cotE	15.5	cotE	69	4.19403	0.990982	2.055268
cotF	5.5	cotF	15.5	2.481481	0.989052	1.29532
		cotH	3	3.4	0.988744	1.749204
		cotI	4	4.2	0.988788	2.054123
cotJC	2	cotJC	4	1.615385	0.988699	0.67548
cotO	2	cotO	9	3.153846	0.98892	1.641039
		cotQ	2	2.6	0.9887	1.362116
		cotS	2.5	3	0.988722	1.568599
		cotSA	5	5	0.988833	2.305726
		cotU	10	9	0.989054	3.154047
		cotY	2	2.6	0.9887	1.362116
coxA	4	coxA	12.5	2.619048	0.988986	1.373064
		cpgA	2	2.6	0.9887	1.362116
csd	19	csd	18	0.950617	0.988557	-0.08967
cshA	8	cshA	18	2.081081	0.989051	1.041449
cshB	5	cshB	8.5	1.56	0.988764	0.625244
csn	15	csn	9.5	0.661538	0.988359	-0.613
cspD	2			0.384615	0.988521	-1.39517
cspR	3.5	cspR	2	0.684211	0.988543	-0.56411
cssR	5	cssR	3	0.68	0.98852	-0.57305
ctaC	18	ctaC	18	1	0.988602	-0.01654
ctaD	4			0.238095	0.988432	-2.08718
ctc	4	ctc	8	1.761905	0.988786	0.800867
ctpA	9	ctpA	12	1.292683	0.988739	0.354031
		ctpB	4	4.2	0.988788	2.054123

		cwlC	10.5	9.4	0.989077	3.216815
		cwlJ	3	3.4	0.988744	1.749204
cwlO	3			0.294118	0.988476	-1.78226
cymR	3	cymR	2	0.764706	0.988565	-0.40362
cypX	16	cypX	5.5	0.391304	0.988137	-1.37085
cysC	8	cysC	5.5	0.72973	0.988496	-0.47126
cysE	2			0.384615	0.988521	-1.39517
cysH	3.5	cysH	2	0.684211	0.988543	-0.56411
cysI	24	cysI	25	1.039604	0.988643	0.039556
cysJ	35.5	cysJ	31.5	0.891156	0.988415	-0.18306
cysK	51	cysK	50.5	0.990431	0.988563	-0.03047
cysS	21.5	cysS	23	1.065934	0.988667	0.075674
dacA	9	dacA	11	1.195122	0.988695	0.240755
		dacB	2	2.6	0.9887	1.362116
dacF	4	dacF	12.5	2.619048	0.988986	1.373064
dapA	23	dapA	25.5	1.103093	0.98871	0.125174
dapB	5	dapB	7	1.32	0.988697	0.384138
dapF	4.5	dapF	4	0.913043	0.988587	-0.14781
dapG	15	dapG	17.5	1.153846	0.988714	0.190076
dat	40	dat	41	1.024242	0.988635	0.018067
dck	2	dck	2.5	1.153846	0.988632	0.189957
ddl	15.5	ddl	11.5	0.761194	0.988426	-0.41046
def	2			0.384615	0.988521	-1.39517
degS	19.5	degS	12.5	0.662651	0.988291	-0.61067
degU	62.5	degU	43	0.694118	0.987713	-0.54458
degV	3			0.294118	0.988476	-1.78226
deoD	9.5	deoD	6	0.674419	0.988451	-0.58504
dhaS	65	dhaS	52	0.803774	0.988	-0.33256
dinB	7	dinB	7	1	0.988607	-0.01653
		dinG	2	2.6	0.9887	1.362116
disA	9	disA	5.5	0.658537	0.988451	-0.61942
divIB	2.5			0.333333	0.988499	-1.60165
divIVA	26.5	divIVA	23.5	0.891892	0.988464	-0.1818
dltA	4.5	dltA	4	0.913043	0.988587	-0.14781
dnaA	5	dnaA	3	0.68	0.98852	-0.57305
dnaI	3.5	dnaI	2	0.684211	0.988543	-0.56411
dnaK	140.5	dnaK	157.5	1.119929	0.989298	0.147884
dnaN	10.5	dnaN	9.5	0.914894	0.988561	-0.14492

dppA	20.5	dppA	24.5	1.183908	0.988778	0.227276
dppB	2	dppB	5	1.923077	0.988743	0.927084
dppE	22	dppE	31.5	1.408602	0.989021	0.478338
dprA	2	dprA	3	1.307692	0.988654	0.370561
dps	18	dps	12.5	0.714286	0.988358	-0.50232
dra	3	dra	3.5	1.117647	0.988632	0.14397
drm	4	drm	6	1.380952	0.988698	0.449265
		dsdA	2	2.6	0.9887	1.362116
efp	20	efp	17.5	0.882353	0.98849	-0.19727
eno	203.5	eno	195.5	0.960928	0.988148	-0.0747
epsB	4.5	epsB	5.5	1.173913	0.988653	0.214862
epsM	3	epsM	2.5	0.882353	0.988587	-0.19713
era	2.5	era	2.5	1	0.98861	-0.01653
etfA	3	etfA	3.5	1.117647	0.988632	0.14397
exuR	2			0.384615	0.988521	-1.39517
ezrA	11.5	ezrA	10.5	0.921569	0.988561	-0.13443
fabD	8	fabD	6.5	0.837838	0.98854	-0.27189
fabF	34	fabF	40	1.170213	0.98886	0.210609
fabG	41.5	fabG	35.5	0.859649	0.988323	-0.23513
fabHA	19.5	fabHA	16	0.831325	0.988446	-0.28328
fabHB	23	fabHB	22	0.958763	0.988555	-0.07736
fabI	36	fabI	23	0.651007	0.988016	-0.63665
fabL	17	fabL	17	1	0.988602	-0.01654
fadB	2.5	fadB	3.5	1.266667	0.988654	0.324575
fadE	5	fadE	18	3.08	0.989185	1.607243
fadH	2	fadH	6.5	2.384615	0.98881	1.237521
		fadN	4.5	4.6	0.988811	2.1854
fapR	5.5	fapR	4	0.777778	0.988542	-0.3792
fbaA	49.5	fbaA	42.5	0.862069	0.988275	-0.23114
ffh	9	ffh	10.5	1.146341	0.988673	0.180602
fhuD	4.5	fhuD	4.5	1	0.988609	-0.01653
flgE	7	flgE	5.5	0.818182	0.988541	-0.30613
flgK	7	flgK	7	1	0.988607	-0.01653
flgL	4.5	flgL	4.5	1	0.988609	-0.01653
fliF	4.5	fliF	3.5	0.826087	0.988564	-0.29223
fliG	4.5	fliG	7	1.434783	0.98872	0.504465
fliW	2	fliW	2	1	0.98861	-0.01653
fliY	2.5	fliY	3	1.133333	0.988632	0.164078

fmt	4.5	fmt	4	0.913043	0.988587	-0.14781
folD	27.5	folD	23	0.843478	0.988397	-0.26241
folK	3	folK	3.5	1.117647	0.988632	0.14397
frr	17.5	frr	9.5	0.573333	0.988247	-0.81961
fruA	29.5	fruA	23	0.788618	0.988308	-0.35957
fruC	13.5	fruC	7	0.559322	0.988316	-0.8552
fruK	9.5	fruK	7	0.767442	0.988495	-0.39856
fruR	2	fruR	2	1	0.98861	-0.01653
ftsA	10	ftsA	6.5	0.688889	0.988451	-0.55442
ftsE	13	ftsE	6	0.508772	0.988294	-0.9919
ftsH	61	ftsH	63.5	1.040161	0.988691	0.040398
ftsX	8	ftsX	6.5	0.837838	0.98854	-0.27189
ftsY	5.5	ftsY	3	0.62963	0.988497	-0.68412
ftsZ	39.5	ftsZ	32.5	0.828221	0.98828	-0.28892
fur	3	fur	2	0.764706	0.988565	-0.40362
fusA	268	fusA	290.5	1.083565	0.989483	0.100533
gabD	7.5	gabD	6.5	0.885714	0.988563	-0.19168
gabP	3.5	gabP	5	1.315789	0.988676	0.379498
galE	20	galE	14	0.717647	0.988335	-0.49558
galK	4.5	galK	3	0.73913	0.988542	-0.45272
galT	3			0.294118	0.988476	-1.78226
gapA	297	gapA	268.5	0.904443	0.987179	-0.16352
gatA	45.5	gatA	39	0.860963	0.988299	-0.23296
gatB	45.5	gatB	46.5	1.02139	0.988632	0.01404
gcaD	12.5	gcaD	9	0.745455	0.988449	-0.44057
gcp	2	gcp	2	1	0.98861	-0.01653
gcvH	8	gcvH	5	0.675676	0.988474	-0.58232
		gcvPA	3	3.4	0.988744	1.749204
gcvPB	6.5	gcvPB	5.5	0.870968	0.988563	-0.2159
gcvT	8.5	gcvT	9	1.051282	0.988629	0.055651
gdh	4	gdh	14.5	3	0.989075	1.569114
		gerD	3	3.4	0.988744	1.749204
gerM	25.5	gerM	22.5	0.88785	0.988465	-0.18835
		gerPC	3	3.4	0.988744	1.749204
gerQ	5.5	gerQ	13	2.111111	0.988941	1.061959
gerR	17	gerR	32.5	1.849315	0.98929	0.871457
		gerT	2	2.6	0.9887	1.362116
		ggaB	2	2.6	0.9887	1.362116

gidA	16.5	gidA	19	1.140845	0.988713	0.173727
glcK	2	glcK	2	1	0.98861	-0.01653
		glgD	2.5	3	0.988722	1.568599
		glgP	2	2.6	0.9887	1.362116
glmM	13	glmM	10	0.789474	0.988471	-0.35777
glmS	65.5	glmS	278.5	4.191011	0.998138	2.06461
glnA	936	glnA	864	0.92318	0.984794	-0.13742
glnH	5.5	glnH	18.5	2.925926	0.989185	1.533205
		glnQ	2.5	3	0.988722	1.568599
		glpD	4	4.2	0.988788	2.054123
		glpK	2	2.6	0.9887	1.362116
glpP	3	glpP	3.5	1.117647	0.988632	0.14397
glpX	16.5	glpX	13.5	0.830986	0.98847	-0.28384
gltA	53	gltA	68	1.276498	0.989251	0.336599
gltB	5	gltB	4	0.84	0.988564	-0.26813
gltC	3.5	gltC	2	0.684211	0.988543	-0.56411
		gltT	4	4.2	0.988788	2.054123
gltX	32.5	gltX	24.5	0.762963	0.988239	-0.40738
glyA	146.5	glyA	139.5	0.952623	0.988223	-0.08711
glyQ	6	glyQ	6.5	1.068966	0.98863	0.079718
glyS	35	glyS	37	1.055172	0.988682	0.061057
gmk	5	gmk	7	1.32	0.988697	0.384138
gndA	122.5	gndA	94.5	0.773737	0.987302	-0.38852
		gpr	4.5	4.6	0.988811	2.1854
gpsA	3.5	gpsA	3	0.894737	0.988587	-0.17702
greA	6.5	greA	4	0.677419	0.988497	-0.57857
groEL	412.5	groEL	421	1.020544	0.98878	0.01306
grpE	8.5	grpE	6	0.74359	0.988496	-0.44411
gsaB	5.5	gsaB	7.5	1.296296	0.988697	0.357996
gtaB	37.5	gtaB	28.5	0.767742	0.988192	-0.39844
guaA	55	guaA	66.5	1.204444	0.989094	0.252548
guaB	109	guaB	90.5	0.8322	0.987732	-0.28281
guaC	9.5	guaC	7.5	0.813953	0.988517	-0.31364
gudB	16.5	gudB	15	0.915493	0.988536	-0.14401
gyrA	2	gyrA	3	1.307692	0.988654	0.370561
gyrB	9.5	gyrB	9.5	1	0.988606	-0.01653
hag	47	hag	44.5	0.948187	0.988476	-0.09348
hbs	2			0.384615	0.988521	-1.39517

hemAT	8.5	hemAT	10	1.153846	0.988673	0.190016
hemB	12.5	hemB	10	0.818182	0.988494	-0.3062
hemE	4.5	hemE	6	1.26087	0.988675	0.317988
hemH	9	hemH	8.5	0.95122	0.988584	-0.08871
hemL	33	hemL	21	0.649635	0.988062	-0.63963
hemY	5	hemY	4	0.84	0.988564	-0.26813
		hfq	3	3.4	0.988744	1.749204
hisA	13	hisA	11	0.859649	0.988516	-0.23484
hisB	6	hisB	5.5	0.931034	0.988586	-0.11966
hisC	13	hisC	9	0.719298	0.988427	-0.49213
hisD	10.5	hisD	7.5	0.744681	0.988473	-0.44203
hisF	13	hisF	9.5	0.754386	0.988449	-0.42339
hisG	9	hisG	6.5	0.756098	0.988496	-0.42005
hisH	5.5	hisH	4	0.777778	0.988542	-0.3792
hisI	34.5	hisI	42	1.20979	0.988926	0.258692
hisS	35	hisS	30	0.862069	0.988371	-0.231
hisZ	14	hisZ	12	0.868852	0.988515	-0.21948
hit	2	hit	2	1	0.98861	-0.01653
hmp	6	hmp	7	1.137931	0.988652	0.169948
hom	82	hom	88	1.072072	0.988836	0.084205
hprK	6.5	hprK	8.5	1.258065	0.988696	0.314805
hprT	11	hprT	9.5	0.877551	0.988539	-0.20508
htpG	56.5	htpG	53.5	0.948052	0.988449	-0.09372
htrA	10	htrA	9	0.911111	0.988562	-0.1509
htrB	2.5	htrB	2	0.866667	0.988588	-0.22301
		hutP	2	2.6	0.9887	1.362116
icd	248.5	icd	254.5	1.024024	0.988752	0.01793
ileS	38.5	ileS	49	1.264151	0.989058	0.322295
ilvA	11	ilvA	10.5	0.959184	0.988583	-0.07669
ilvB	12	ilvB	6.5	0.584906	0.988361	-0.79061
ilvC	81.5	ilvC	74.5	0.915408	0.988258	-0.14455
ilvD	48.5	ilvD	40	0.829146	0.988209	-0.28741
ilvH	3	ilvH	3	1	0.988609	-0.01653
infB	53	infB	64.5	1.211982	0.989095	0.261549
infC	11.5	infC	9	0.803922	0.988494	-0.33157
iolR	2			0.384615	0.988521	-1.39517
iolS	27.5	iolS	30	1.086957	0.988708	0.103911
		iscU	2.5	3	0.988722	1.568599

iseA	4	iseA	2.5	0.714286	0.988542	-0.50205
		ispA	4.5	4.6	0.988811	2.1854
ispC	2			0.384615	0.988521	-1.39517
ispE	3			0.294118	0.988476	-1.78226
ispF	2			0.384615	0.988521	-1.39517
ispG	7	ispG	9.5	1.30303	0.988718	0.365502
ispH	8	ispH	6	0.783784	0.988518	-0.36813
jag	4	jag	3	0.809524	0.988565	-0.32145
K1C10_HUMAN	94.5	K1C10_HUMAN	100.5	1.062663	0.98883	0.071478
K1C9_HUMAN	79	K1C9_HUMAN	71	0.900312	0.988215	-0.16861
K22E_HUMAN	42	K22E_HUMAN	30.5	0.734104	0.988079	-0.46325
K2C1_HUMAN	128	K2C1_HUMAN	144.5	1.12766	0.989281	0.157784
		kamA	3	3.4	0.988744	1.749204
katA	2	katA	6	2.230769	0.988787	1.141273
katX	5	katX	25.5	4.28	0.989518	2.082409
kduD	4	kduD	4	1	0.988609	-0.01653
kduI	15.5	kduI	14.5	0.940299	0.988559	-0.10541
kinE	6	kinE	6	1	0.988608	-0.01653
ksgA	3	ksgA	3.5	1.117647	0.988632	0.14397
ktrC	8.5	ktrC	8	0.948718	0.988585	-0.09251
ldh	26.5	ldh	24	0.90991	0.988487	-0.15291
lepA	6.5	lepA	10	1.451613	0.988763	0.521353
leuA	19	leuA	22	1.148148	0.988734	0.182964
leuB	25	leuB	18.5	0.752381	0.98831	-0.42743
leuC	8.5	leuC	8	0.948718	0.988585	-0.09251
leuD	3.5	leuD	2.5	0.789474	0.988565	-0.35763
leuS	36	leuS	35	0.973154	0.988548	-0.05588
levD	28.5	levD	21	0.747899	0.988264	-0.43612
levE	53.5	levE	40	0.753425	0.987984	-0.42591
levG	29	levG	22	0.768595	0.988286	-0.3967
lgt	3	lgt	2	0.764706	0.988565	-0.40362
liaH	5	liaH	5	1	0.988608	-0.01653
ligA	8.5	ligA	8	0.948718	0.988585	-0.09251
lip	3.5	lip	4.5	1.210526	0.988654	0.259171
lipA	4	lipA	6	1.380952	0.988698	0.449265
		lipC	7.5	7	0.988944	2.791315
lonA	5	lonA	5	1	0.988608	-0.01653
lpdV	11	lpdV	11.5	1.040816	0.988628	0.041215

lutA	8.5	lutA	10	1.153846	0.988673	0.190016
lutB	60.5	lutB	66	1.089069	0.988825	0.106882
lutC	43.5	lutC	38.5	0.888268	0.988367	-0.18781
luxS	7	luxS	6.5	0.939394	0.988585	-0.10676
lysA	4	lysA	4	1	0.988609	-0.01653
lysC	19.5	lysC	11.5	0.614458	0.988246	-0.71967
lysS	22	lysS	27.5	1.236559	0.988844	0.290146
lytB	7.5	lytB	10.5	1.342857	0.98874	0.408969
lytC	5	lytC	9	1.64	0.988786	0.697426
lytE	2	lytE	4	1.615385	0.988699	0.67548
lytR	12	lytR	12	1	0.988605	-0.01653
maf	9	maf	9.5	1.04878	0.988629	0.052213
malR	4.5	malR	4.5	1	0.988609	-0.01653
malS	2	malS	22	7.153846	0.989497	2.823487
map	5	map	4	0.84	0.988564	-0.26813
mbl	21	mbl	14	0.685393	0.98829	-0.56199
mccB	3	mccB	2.5	0.882353	0.988587	-0.19713
mcpA	3	mcpA	5	1.470588	0.988698	0.539995
mcpB	10	mcpB	11	1.088889	0.98865	0.106389
mcpC	7.5	mcpC	5.5	0.771429	0.988519	-0.39106
mcsA	2			0.384615	0.988521	-1.39517
mcsB	5.5	mcsB	6	1.074074	0.98863	0.086597
mdh	120.5	mdh	134.5	1.11499	0.989173	0.141326
mecA	2			0.384615	0.988521	-1.39517
menB	17.5	menB	13	0.76	0.988402	-0.41276
metA	3	metA	3	1	0.988609	-0.01653
metC	4.5	metC	3	0.73913	0.988542	-0.45272
metE	140.5	metE	151.5	1.077601	0.98903	0.091909
metI	10	metI	6.5	0.688889	0.988451	-0.55442
metK	38.5	metK	35	0.91195	0.988436	-0.14975
metQ	21.5	metQ	19	0.89011	0.988489	-0.18465
metS	58	metS	55.5	0.957806	0.98847	-0.07893
mfd	10.5	mfd	14	1.297872	0.988761	0.359842
mhqA	2	mhqA	2	1	0.98861	-0.01653
		mhqD	2	2.6	0.9887	1.362116
minC	5	minC	5.5	1.08	0.988631	0.094535
minD	18	minD	14.5	0.818182	0.988447	-0.30627
mleA	6	mleA	6	1	0.988608	-0.01653

mlpA	3	mlpA	4	1.235294	0.988654	0.288392
		mmgD	2	2.6	0.9887	1.362116
		mmgE	3	3.4	0.988744	1.749204
mnaA	4	mnaA	3	0.809524	0.988565	-0.32145
mntA	15	mntA	15.5	1.030769	0.988626	0.027217
mntB	14	mntB	11	0.803279	0.988471	-0.33276
		mntR	2.5	3	0.988722	1.568599
moaE	2	moaE	2	1	0.98861	-0.01653
moeA	4	moeA	3.5	0.904762	0.988587	-0.16095
motA	4			0.238095	0.988432	-2.08718
motB	3			0.294118	0.988476	-1.78226
mpr	4	mpr	2.5	0.714286	0.988542	-0.50205
mraW	2	mraW	3	1.307692	0.988654	0.370561
mreB	45	mreB	42	0.935135	0.988455	-0.11351
mreC	8	mreC	5.5	0.72973	0.988496	-0.47126
mrgA	2.5	mrgA	4	1.4	0.988676	0.468997
		msmX	4	4.2	0.988788	2.054123
mtnA	2.5	mtnA	2	0.866667	0.988588	-0.22301
mtnB	3			0.294118	0.988476	-1.78226
mtnD	14.5	mtnD	16	1.095238	0.98867	0.114806
mtnK	2	mtnK	2	1	0.98861	-0.01653
mtnN	11	mtnN	9	0.836735	0.988517	-0.27382
mtnW	2			0.384615	0.988521	-1.39517
mtnX	3	mtnX	4	1.235294	0.988654	0.288392
mtrA	6	mtrA	6.5	1.068966	0.98863	0.079718
murAA	4	murAA	4.5	1.095238	0.988631	0.114749
murAB	4	murAB	4	1	0.988609	-0.01653
murB	9	murB	3.5	0.463415	0.988363	-1.12651
murC	7.5	murC	8	1.057143	0.988629	0.063672
murD	7	murD	10.5	1.424242	0.988763	0.493891
murE	6	murE	9	1.413793	0.988741	0.483236
murF	9	murF	7.5	0.853659	0.98854	-0.2449
		murG	2	2.6	0.9887	1.362116
mutS	4			0.238095	0.988432	-2.08718
mutSB	3	mutSB	8	2.176471	0.988831	1.105787
mutTA	2			0.384615	0.988521	-1.39517
nadA	15.5	nadA	19.5	1.238806	0.988781	0.292673
nadC	8.5	nadC	5.5	0.692308	0.988474	-0.54724

		nadD	2	2.6	0.9887	1.362116
nadE	16.5	nadE	9	0.577465	0.98827	-0.80922
nadF	3	nadF	3	1	0.988609	-0.01653
nadR	2			0.384615	0.988521	-1.39517
nagA	2.5	nagA	3	1.133333	0.988632	0.164078
		nagP	3	3.4	0.988744	1.749204
		nap	2	2.6	0.9887	1.362116
narG	15.5	narG	32	1.985075	0.989336	0.973725
narH	3.5	narH	4.5	1.210526	0.988654	0.259171
narJ	3.5	narJ	3	0.894737	0.988587	-0.17702
nasB	8.5	nasB	11	1.25641	0.988718	0.312938
nasC	6.5	nasC	16.5	2.290323	0.989051	1.179668
nasD	45	nasD	30.5	0.686486	0.987944	-0.5602
nasE	2	nasE	2	1	0.98861	-0.01653
nasF	9.5	nasF	9	0.953488	0.988584	-0.08528
ndh	31	ndh	27.5	0.891473	0.98844	-0.18251
ndk	6	ndk	3.5	0.655172	0.988497	-0.62674
nfrA	5	nfrA	6	1.16	0.988653	0.197661
nin	27	nin	27.5	1.017699	0.988619	0.008798
noc	9	noc	7	0.804878	0.988518	-0.32982
nos	3	nos	3	1	0.988609	-0.01653
nrdE	4	nrdE	4.5	1.095238	0.988631	0.114749
nrdF	4.5	nrdF	2.5	0.652174	0.98852	-0.63333
nrgB	19.5	nrgB	17.5	0.903614	0.988512	-0.16289
nrnA	4.5	nrnA	4	0.913043	0.988587	-0.14781
nsrR	4	nsrR	3	0.809524	0.988565	-0.32145
ntdA	4.5	ntdA	3.5	0.826087	0.988564	-0.29223
ntdB	2			0.384615	0.988521	-1.39517
nudF	11	nudF	7.5	0.714286	0.98845	-0.50219
nusA	20	nusA	16	0.811765	0.988423	-0.31767
nusG	12.5	nusG	8.5	0.709091	0.988427	-0.51275
obg	10	obg	10.5	1.044444	0.988628	0.046236
odhA	131	odhA	134	1.022684	0.988678	0.015933
odhB	93	odhB	97.5	1.047745	0.988764	0.050986
ogt	3			0.294118	0.988476	-1.78226
oppA	46	oppA	41.5	0.904762	0.988388	-0.16124
		oppB	2	2.6	0.9887	1.362116
oppD	5	oppD	3.5	0.76	0.988542	-0.41255

		oppF	2	2.6	0.9887	1.362116
opuAA	17	opuAA	20.5	1.191781	0.988758	0.236808
opuAC	13	opuAC	18	1.350877	0.988826	0.417685
opuCA	5	opuCA	2	0.52	0.988475	-0.96014
opuCC	3.5	opuCC	2.5	0.789474	0.988565	-0.35763
oxdC	132.5	oxdC	120	0.906542	0.987986	-0.15899
		oxdD	4.5	4.6	0.988811	2.1854
pabA	22	pabA	18.5	0.849462	0.988444	-0.25215
pabB	2	pabB	4	1.615385	0.988699	0.67548
padC	2	padC	3	1.307692	0.988654	0.370561
		paiB	2	2.6	0.9887	1.362116
panB	2.5	panB	2	0.866667	0.988588	-0.22301
patA	15.5	patA	18	1.149254	0.988714	0.184323
		pbpB	3	3.4	0.988744	1.749204
pbpC	15.5	pbpC	15.5	1	0.988603	-0.01654
pbpD	2.5			0.333333	0.988499	-1.60165
pbpE	7	pbpE	6	0.878788	0.988563	-0.20301
pbpH	3			0.294118	0.988476	-1.78226
pbpX	3.5	pbpX	2	0.684211	0.988543	-0.56411
		pcp	3	3.4	0.988744	1.749204
pcrA	8.5	pcrA	10	1.153846	0.988673	0.190016
		pdaA	3.5	3.8	0.988766	1.909701
pdaB	3	pdaB	5	1.470588	0.988698	0.539995
pdhA	23.5	pdhA	23	0.979798	0.988577	-0.04602
pdhB	14.5	pdhB	19.5	1.31746	0.988825	0.381547
pdhC	26.5	pdhC	32	1.198198	0.988842	0.244678
pdhD	87	pdhD	76	0.875354	0.988077	-0.20937
pdp	8.5	pdp	11	1.25641	0.988718	0.312938
pdxK	9	pdxK	7	0.804878	0.988518	-0.32982
pdxS	96.5	pdxS	100.5	1.040921	0.98874	0.041523
pdxT	12.5	pdxT	14.5	1.145455	0.988693	0.179515
pel	10.5	pel	2	0.276596	0.988229	-1.87123
pepF	37	pepF	32	0.869281	0.98837	-0.21898
pepT	21	pepT	15	0.730337	0.988334	-0.47029
pfkA	36.5	pfkA	26.5	0.735099	0.988149	-0.46119
pgcA	33	pgcA	22	0.678832	0.988106	-0.57614
pgi	68	pgi	46	0.68231	0.987599	-0.5695
pgk	122	pgk	128.5	1.052738	0.988838	0.057953

pgm	39.5	pgm	38.5	0.97546	0.988546	-0.05246
pheB	2	pheB	2	1	0.98861	-0.01653
pheS	15.5	pheS	12.5	0.820896	0.98847	-0.30146
pheT	43.5	pheT	43	0.988827	0.988567	-0.0328
		phoA	2.5	3	0.988722	1.568599
phoH	11.5	phoH	11	0.960784	0.988583	-0.07428
phoP	4	phoP	4	1	0.988609	-0.01653
phoR	2	phoR	6	2.230769	0.988787	1.141273
pksC	10	pksC	7.5	0.777778	0.988495	-0.37926
pksD	4	pksD	3	0.809524	0.988565	-0.32145
pksE	9.5	pksE	9	0.953488	0.988584	-0.08528
pksF	2			0.384615	0.988521	-1.39517
pksG	5.5	pksG	6.5	1.148148	0.988653	0.182844
pksI	4	pksI	5	1.190476	0.988653	0.235075
pksJ	61	pksJ	79.5	1.297189	0.989403	0.360018
pksL	21	pksL	41	1.898876	0.989488	0.909901
pksM	11	pksM	22.5	1.938776	0.989116	0.939357
pksR	20	pksR	24.5	1.211765	0.9888	0.260861
pksS	11	pksS	6	0.591837	0.988384	-0.77359
plsC	13	plsC	9.5	0.754386	0.988449	-0.42339
plsX	9.5	plsX	11.5	1.186047	0.988695	0.229758
plsY	3			0.294118	0.988476	-1.78226
pnbA	6	pnbA	4	0.724138	0.988519	-0.48232
pncA	7	pncA	6	0.878788	0.988563	-0.20301
pncB	17	pncB	18	1.054795	0.988647	0.060489
pnpA	66	pnpA	74	1.118959	0.988933	0.146102
polA	26	polA	28	1.073394	0.988686	0.085765
		polC	2	2.6	0.9887	1.362116
polX	5	polX	6.5	1.24	0.988675	0.293908
ponA	33.5	ponA	33	0.985612	0.988572	-0.03749
ppaC	96	ppaC	97	1.010283	0.988606	-0.00177
ppiB	3	ppiB	3	1	0.988609	-0.01653
pps	3	pps	5	1.470588	0.988698	0.539995
ppsA	27.5	ppsA	30.5	1.104348	0.98873	0.126843
ppsB	21	ppsB	28	1.314607	0.988911	0.378544
ppsC	12	ppsC	13.5	1.113208	0.988671	0.138286
ppsD	31.5	ppsD	30.5	0.969466	0.988551	-0.06135
ppsE	31	ppsE	24	0.782946	0.988285	-0.37002

prfA	12.5	prfA	9.5	0.781818	0.988472	-0.37182
prfB	13	prfB	10.5	0.824561	0.988494	-0.295
prkA	72.5	prkA	180	2.457627	0.993378	1.28768
prkC	6	prkC	6.5	1.068966	0.98863	0.079718
proA	28.5	proA	33	1.151261	0.988796	0.186959
proB	2.5			0.333333	0.988499	-1.60165
proG	2			0.384615	0.988521	-1.39517
proI	2			0.384615	0.988521	-1.39517
proS	31	proS	28	0.906977	0.988462	-0.15761
		prpC	2	2.6	0.9887	1.362116
prs	50	prs	40.5	0.814634	0.988164	-0.31295
prsA	86	prsA	99.5	1.154728	0.989168	0.19184
pspA	8	pspA	4	0.567568	0.98843	-0.83393
pta	15.5	pta	8.5	0.58209	0.988293	-0.79768
ptb	2	ptb	2	1	0.98861	-0.01653
ptsG	24	ptsG	27	1.118812	0.988732	0.145619
ptsI	30	ptsI	23	0.776	0.988285	-0.38287
pucL	5.5	pucL	9.5	1.592593	0.988786	0.655107
pupG	3.5	pupG	3	0.894737	0.988587	-0.17702
purA	48.5	purA	37	0.768844	0.988076	-0.39654
purB	138	purB	132	0.956912	0.988273	-0.08056
purC	26	purC	20	0.779817	0.988332	-0.37573
purD	49	purD	45	0.920398	0.988408	-0.13649
purE	19.5	purE	16.5	0.855422	0.988468	-0.24203
purF	43.5	purF	26.5	0.620112	0.987834	-0.70706
purH	85.5	purH	64.5	0.757925	0.987634	-0.41782
purK	15.5	purK	13	0.850746	0.988492	-0.2499
purL	134	purL	114.5	0.855823	0.987673	-0.24251
purM	9.5	purM	7.5	0.813953	0.988517	-0.31364
purN	7	purN	3.5	0.575758	0.988452	-0.81322
purQ	7.5	purQ	7	0.942857	0.988585	-0.10145
purR	6.5	purR	6.5	1	0.988608	-0.01653
purS	9	purS	4	0.512195	0.988385	-0.98209
purT	6	purT	5	0.862069	0.988564	-0.23072
pycA	125	pycA	162	1.293069	0.990199	0.35659
pyk	70.5	pyk	60.5	0.860627	0.98813	-0.23377
pyrAA	15	pyrAA	14	0.938462	0.988559	-0.10823
pyrAB	68	pyrAB	76	1.115523	0.988932	0.141664

pyrB	4	pyrB	4	1	0.988609	-0.01653
pyrC	16.5	pyrC	14.5	0.887324	0.988514	-0.18913
pyrD	3.5			0.263158	0.988454	-1.94275
pyrE	11	pyrE	10	0.918367	0.988561	-0.13945
pyrF	20	pyrF	16.5	0.835294	0.988446	-0.27641
pyrG	31	pyrG	23.5	0.767442	0.988262	-0.3989
pyrH	18.5	pyrH	17.5	0.949367	0.988557	-0.09157
pyrK	2.5	pyrK	2	0.866667	0.988588	-0.22301
pyrR	21	pyrR	15.5	0.752809	0.988356	-0.42654
qcrA	8	qcrA	7	0.891892	0.988563	-0.18166
qcrB	7.5	qcrB	7.5	1	0.988607	-0.01653
qcrC	9	qcrC	11	1.195122	0.988695	0.240755
qoxA	39	qoxA	33	0.850932	0.988325	-0.24983
qoxB	8	qoxB	7	0.891892	0.988563	-0.18166
queA	3	queA	4	1.235294	0.988654	0.288392
queC	18	queC	12	0.688312	0.988336	-0.55579
queE	3.5			0.263158	0.988454	-1.94275
queF	4	queF	3	0.809524	0.988565	-0.32145
racA	2			0.384615	0.988521	-1.39517
		racX	2	2.6	0.9887	1.362116
rapA	6	rapA	10.5	1.62069	0.988808	0.680369
rapB	6.5	rapB	5	0.806452	0.988541	-0.32697
rapC	15	rapC	13.5	0.907692	0.988537	-0.15636
rapF	7.5	rapF	5.5	0.771429	0.988519	-0.39106
rapG	2			0.384615	0.988521	-1.39517
rapH	6	rapH	7.5	1.206897	0.988674	0.254869
rapK	26.5	rapK	11.5	0.459459	0.987932	-1.13951
rbgA	4.5	rbgA	5	1.086957	0.988631	0.103798
recA	141	recA	155	1.098418	0.989163	0.119708
relA	14	relA	16	1.131148	0.988693	0.161381
resA	6.5	resA	2.5	0.483871	0.98843	-1.06409
resC	3			0.294118	0.988476	-1.78226
resD	54.5	resD	52	0.955157	0.988472	-0.08292
resE	4.5	resE	5	1.086957	0.988631	0.103798
rho	3	rho	2.5	0.882353	0.988587	-0.19713
ribE	2.5	ribE	3	1.133333	0.988632	0.164078
ribH	4.5	ribH	5.5	1.173913	0.988653	0.214862
rimM	3	rimM	2.5	0.882353	0.988587	-0.19713

		rluB	2	2.6	0.9887	1.362116
rnc	3.5	rnc	4	1.105263	0.988631	0.127895
rnjA	20.5	rnjA	18.5	0.908046	0.988512	-0.15583
rnjB	12.5	rnjB	7.5	0.636364	0.988383	-0.66893
rnr	4.5	rnr	13.5	2.565217	0.989008	1.343135
rny	23.5	rny	19.5	0.838384	0.988422	-0.27112
rocA	3	rocA	2	0.764706	0.988565	-0.40362
rocD	5.5	rocD	4.5	0.851852	0.988564	-0.24792
rocF	9	rocF	8.5	0.95122	0.988584	-0.08871
rodZ	8.5	rodZ	3.5	0.487179	0.988385	-1.05433
rok	8.5	rok	3.5	0.487179	0.988385	-1.05433
rph	3	rph	4	1.235294	0.988654	0.288392
rplA	68	rplA	81	1.187726	0.989155	0.232469
rplB	8.5	rplB	14	1.564103	0.988851	0.62916
rplC	19	rplC	23	1.197531	0.988779	0.243783
rplD	18	rplD	21.5	1.181818	0.988757	0.224696
rplE	40	rplE	45	1.121212	0.988813	0.148828
rplF	26	rplF	23.5	0.908257	0.988487	-0.15553
rplI	4.5	rplI	2.5	0.652174	0.98852	-0.63333
rplJ	37.5	rplJ	30.5	0.819355	0.988281	-0.30445
rplK	35	rplK	37	1.055172	0.988682	0.061057
rplL	2			0.384615	0.988521	-1.39517
rplM	8.5	rplM	5	0.641026	0.988451	-0.6583
rplN	15	rplN	6.5	0.476923	0.988226	-1.08526
rplO	7.5	rplO	5.5	0.771429	0.988519	-0.39106
rplP	7			0.151515	0.988297	-2.73945
rplQ	3			0.294118	0.988476	-1.78226
		rplR	3.5	3.8	0.988766	1.909701
rplT	4.5	rplT	4.5	1	0.988609	-0.01653
rplU	2	rplU	4	1.615385	0.988699	0.67548
rplV	2			0.384615	0.988521	-1.39517
rplX	3.5	rplX	6	1.526316	0.98872	0.593688
rpoA	63	rpoA	45.5	0.727626	0.987801	-0.47644
rpoB	144.5	rpoB	159.5	1.102916	0.989206	0.125666
rpoC	71	rpoC	79	1.110727	0.988931	0.135445
rpoE	8.5	rpoE	8	0.948718	0.988585	-0.09251
rpsB	84.5	rpsB	72.5	0.860058	0.988034	-0.23486
rpsC	40	rpsC	34	0.854545	0.988324	-0.24371

rpsD	21.5	rpsD	17	0.802198	0.9884	-0.3348
rpsE	51.5	rpsE	42.5	0.829384	0.988185	-0.28704
rpsG	13.5	rpsG	14.5	1.067797	0.988648	0.078166
rpsH	9	rpsH	7.5	0.853659	0.98854	-0.2449
		rpsI	2.5	3	0.988722	1.568599
rpsK	4.5	rpsK	6	1.26087	0.988675	0.317988
rpsL	11.5	rpsL	12.5	1.078431	0.988649	0.092465
rpsM	4	rpsM	3	0.809524	0.988565	-0.32145
rsbR	6	rsbR	5	0.862069	0.988564	-0.23072
rsbRB	14	rsbRB	13	0.934426	0.98856	-0.11445
rsbRC	3	rsbRC	2	0.764706	0.988565	-0.40362
rsbW	8	rsbW	3	0.459459	0.988385	-1.13885
rsfA	3	rsfA	5	1.470588	0.988698	0.539995
		rsmG	2	2.6	0.9887	1.362116
salA	14	safA	12.5	0.901639	0.988537	-0.16601
		salA	9	8.2	0.98901	3.019681
sat	42.5	sat	37.5	0.885714	0.988367	-0.19197
sbcC	8	sbcC	6	0.783784	0.988518	-0.36813
sbcD	3			0.294118	0.988476	-1.78226
scoC	3	scoC	2	0.764706	0.988565	-0.40362
sdaAA	4	sdaAA	3.5	0.904762	0.988587	-0.16095
sdaAB	3			0.294118	0.988476	-1.78226
sdhA	101	sdhA	93.5	0.92665	0.988225	-0.12699
sdhB	24	sdhB	22.5	0.940594	0.988532	-0.105
sdhC	3			0.294118	0.988476	-1.78226
sdpA	4.5	sdpA	4	0.913043	0.988587	-0.14781
sdpC	9	sdpC	5	0.609756	0.988429	-0.73049
secA	50	secA	54.5	1.087805	0.988785	0.105149
secDF	30.5	secDF	31	1.015748	0.988618	0.006027
secY	2			0.384615	0.988521	-1.39517
sepF	3			0.294118	0.988476	-1.78226
serA	129	serA	97.5	0.758157	0.987142	-0.4181
serC	36.5	serC	29.5	0.81457	0.988282	-0.3129
serS	26	serS	24	0.926606	0.988509	-0.12665
sigA	3	sigA	3	1	0.988609	-0.01653
sigE	3	sigE	4	1.235294	0.988654	0.288392
sigF	7	sigF	3.5	0.575758	0.988452	-0.81322
sigH	3	sigH	3	1	0.988609	-0.01653

		sigX	2	2.6	0.9887	1.362116
sigW	2			0.384615	0.988521	-1.39517
sipS	7.5	sipS	8.5	1.114286	0.988652	0.139653
sipT	5	sipT	3	0.68	0.98852	-0.57305
sipW	7	sipW	4	0.636364	0.988474	-0.6688
skfB	11	skfB	7	0.673469	0.988428	-0.58711
skfC	22.5	skfC	11.5	0.536842	0.988112	-0.91468
skfE	3	skfE	2	0.764706	0.988565	-0.40362
skfG	3.5			0.263158	0.988454	-1.94275
		sleB	4	4.2	0.988788	2.054123
		smc	4.5	4.6	0.988811	2.1854
sodA	19.5	sodA	15.5	0.807229	0.988424	-0.32575
		sodF	5	5	0.988833	2.305726
speE	3.5	speE	2.5	0.789474	0.988565	-0.35763
spo0A	17	spo0A	15	0.890411	0.988514	-0.18412
spo0J	11	spo0J	10	0.918367	0.988561	-0.13945
spo0M	33.5	spo0M	41	1.215827	0.988927	0.265874
spoIIB	3.5	spoIIB	4	1.105263	0.988631	0.127895
spoIIIAH	16.5	spoIIIAH	21	1.253521	0.988802	0.30974
spoIIIJ	6	spoIIIJ	8	1.275862	0.988697	0.335072
spoIIQ	7	spoIIQ	3.5	0.575758	0.988452	-0.81322
spoIVA	59	spoIVA	101	1.697095	0.990451	0.749225
		spoVAD	2.5	3	0.988722	1.568599
spoVC	2	spoVC	3.5	1.461538	0.988676	0.531058
		spoVD	2	2.6	0.9887	1.362116
spoVFB	2.5	spoVFB	15	4.333333	0.989164	2.099759
spoVID	19.5	spoVID	43.5	2.156627	0.989667	1.093791
		spoVR	12.5	11	0.989165	3.443715
spoVS	6.5	spoVS	12	1.709677	0.988852	0.75755
spoVT	5	spoVT	9.5	1.72	0.988808	0.766171
sppA	4.5	sppA	2	0.565217	0.988498	-0.83981
		spsA	3	3.4	0.988744	1.749204
		spsB	4.5	4.6	0.988811	2.1854
		spsD	3.5	3.8	0.988766	1.909701
		spsJ	7	6.6	0.988921	2.706394
		spsK	5	5	0.988833	2.305726
srfAA	341.5	srfAA	391.5	1.145879	0.99069	0.182959
srfAB	735.5	srfAB	828.5	1.12623	0.992502	0.160644

srfAC	211	srfAC	222.5	1.054181	0.989017	0.06019
srfAD	13.5	srfAD	11.5	0.864407	0.988515	-0.22688
srtN	4.5	srtN	3.5	0.826087	0.988564	-0.29223
ssb	32	ssb	29	0.909774	0.988461	-0.15316
sspE	5	sspE	7.5	1.4	0.988719	0.46906
sucC	155	sucC	137	0.8848	0.987729	-0.19439
sucD	42.5	sucD	42.5	1	0.988589	-0.01656
sul	2	sul	2	1	0.98861	-0.01653
swrC	45	swrC	34.5	0.772973	0.988122	-0.38875
tagC	5	tagC	6.5	1.24	0.988675	0.293908
tagE	10	tagE	13.5	1.311111	0.988761	0.374484
tagF	2	tagF	3	1.307692	0.988654	0.370561
tagH	2			0.384615	0.988521	-1.39517
tasA	51	tasA	26.5	0.5311	0.987498	-0.93109
tcyA	9.5	tcyA	15	1.511628	0.98885	0.579927
tenA	27.5	tenA	16	0.6	0.988087	-0.75426
		tepA	4.5	4.6	0.988811	2.1854
		tgl	2	2.6	0.9887	1.362116
tgt	5.5	tgt	5	0.925926	0.988586	-0.12759
thdF	4.5	thdF	4	0.913043	0.988587	-0.14781
thiC	57.5	thiC	33.5	0.591489	0.987516	-0.7757
thiD	18	thiD	15	0.844156	0.988469	-0.26115
thiE	4			0.238095	0.988432	-2.08718
thiF	6	thiF	4	0.724138	0.988519	-0.48232
thiG	28.5	thiG	17	0.613445	0.988086	-0.72228
thiM	5.5	thiM	7.5	1.296296	0.988697	0.357996
thiO	6			0.172414	0.988342	-2.55297
thiU	3	thiU	2.5	0.882353	0.988587	-0.19713
thrB	8.5	thrB	8.5	1	0.988607	-0.01653
thrC	48.5	thrC	30	0.628141	0.987765	-0.6886
thrS	35	thrS	33.5	0.958621	0.988527	-0.07762
thyA	2	thyA	2	1	0.98861	-0.01653
thyB	3	thyB	2	0.764706	0.988565	-0.40362
tig	49.5	tig	43.5	0.881773	0.988319	-0.19847
tkmA	2	tkmA	2	1	0.98861	-0.01653
tkt	82.5	tkt	76.5	0.928358	0.988302	-0.12422
tlpA	10	tlpA	5.5	0.6	0.988406	-0.75379
tlpB	2			0.384615	0.988521	-1.39517

tmk	2	tmk	3	1.307692	0.988654	0.370561
tpi	44	tpi	27.5	0.635359	0.987856	-0.67198
tpx	32.5	tpx	18.5	0.585185	0.987973	-0.79049
trmB	3	trmB	4	1.235294	0.988654	0.288392
trmFO	6.5	trmFO	8.5	1.258065	0.988696	0.314805
trmK	2.5	trmK	2	0.866667	0.988588	-0.22301
trmU	3	trmU	3.5	1.117647	0.988632	0.14397
trpA	16.5	trpA	7.5	0.492958	0.988204	-1.03758
trpB	41	trpB	30	0.739645	0.988102	-0.45236
trpC	2.5	trpC	2	0.866667	0.988588	-0.22301
trpD	3.5	trpD	2	0.684211	0.988543	-0.56411
trpE	8.5	trpE	5.5	0.692308	0.988474	-0.54724
trpS	9.5	trpS	7.5	0.813953	0.988517	-0.31364
trxB	9.5	trxB	5	0.581395	0.988407	-0.79923
TRYP_PIG	38	TRYP_PIG	52	1.356688	0.989214	0.424443
tsf	92	tsf	59.5	0.651475	0.987119	-0.63692
tufA	343	tufA	425.5	1.239651	0.992161	0.29858
tyrA	5.5	tyrA	4.5	0.851852	0.988564	-0.24792
tyrS	14.5	tyrS	17.5	1.190476	0.988737	0.235197
udk	2	udk	2	1	0.98861	-0.01653
ung	2			0.384615	0.988521	-1.39517
upp	12	upp	19	1.528302	0.988915	0.595849
ureA	21	ureA	15	0.730337	0.988334	-0.47029
ureC	220	ureC	257.5	1.169492	0.990179	0.211643
uvrA	13	uvrA	9.5	0.754386	0.988449	-0.42339
uvrB	5	uvrB	7	1.32	0.988697	0.384138
valS	45.5	valS	47	1.032086	0.988654	0.029101
walR	7.5	walR	5	0.714286	0.988496	-0.50212
wapA	39.5	wapA	52	1.306748	0.989146	0.370237
wprA	62.5	wprA	55	0.882353	0.988246	-0.19763
xpt	21.5	xpt	21	0.978022	0.988578	-0.04863
yaaH	4	yaaH	11	2.333333	0.988919	1.206317
yaaN	8	yaaN	5	0.675676	0.988474	-0.58232
yaaQ	3	yaaQ	5	1.470588	0.988698	0.539995
yaaT	4	yaaT	4	1	0.988609	-0.01653
yabD	3	yabD	3	1	0.988609	-0.01653
		yabS	2	2.6	0.9887	1.362116
yacC	7	yacC	8.5	1.181818	0.988674	0.224575

yacD	3.5	yacD	2.5	0.789474	0.988565	-0.35763
yacL	2.5			0.333333	0.988499	-1.60165
yacO	3	yacO	4	1.235294	0.988654	0.288392
ybaE	3.5	ybaE	3	0.894737	0.988587	-0.17702
ybbI	2.5	ybbI	6	1.933333	0.988765	0.93479
ybbP	2			0.384615	0.988521	-1.39517
		ybbR	2	2.6	0.9887	1.362116
		ybfG	6	5.8	0.988877	2.519916
		ybfI	3	3.4	0.988744	1.749204
ybfQ	3.5	ybfQ	2.5	0.789474	0.988565	-0.35763
ybgE	5.5	ybgE	4.5	0.851852	0.988564	-0.24792
		ybgG	2	2.6	0.9887	1.362116
		ybxA	2	2.6	0.9887	1.362116
ybxB	3.5	ybxB	3.5	1	0.988609	-0.01653
ycbJ	18.5	ycbJ	10.5	0.594937	0.988247	-0.76625
yccK	4	yccK	3.5	0.904762	0.988587	-0.16095
ycdA	26	ycdA	32.5	1.238532	0.988886	0.292508
ycdH	7	ycdH	2	0.393939	0.988386	-1.36081
yceC	25	yceC	22	0.885714	0.988465	-0.19182
yceD	32	yceD	25.5	0.804511	0.988306	-0.33079
yceE	21.5	yceE	19	0.89011	0.988489	-0.18465
yceH	27.5	yceH	25.5	0.930435	0.988508	-0.1207
ycgN	4	ycgN	4	1	0.988609	-0.01653
yciC	9			0.121951	0.988207	-3.05274
yckB	3	yckB	4.5	1.352941	0.988676	0.419669
yclM	11.5	yclM	13	1.117647	0.988672	0.144028
yclQ	10	yclQ	8.5	0.866667	0.988539	-0.22308
ycnD	2	ycnD	2	1	0.98861	-0.01653
ycnI	9	ycnI	7.5	0.853659	0.98854	-0.2449
ycsE	4.5	ycsE	6.5	1.347826	0.988697	0.414235
ycsF	5	ycsF	4.5	0.92	0.988586	-0.13686
ycsN	3	ycsN	2.5	0.882353	0.988587	-0.19713
ydaF	8	ydaF	6.5	0.837838	0.98854	-0.27189
ydaG	2	ydaG	2	1	0.98861	-0.01653
ydbJ	2			0.384615	0.988521	-1.39517
ydbM	11.5	ydbM	7.5	0.686275	0.988428	-0.55993
ydbS	2			0.384615	0.988521	-1.39517
ydcC	28	ydcC	46	1.615385	0.989396	0.676498

ydcI	3.5	ydcI	6	1.526316	0.98872	0.593688
ydeE	2	ydeE	4	1.615385	0.988699	0.67548
		ydeI	2	2.6	0.9887	1.362116
ydeO	2			0.384615	0.988521	-1.39517
		ydeQ	2	2.6	0.9887	1.362116
ydeS	3	ydeS	3.5	1.117647	0.988632	0.14397
ydfI	2			0.384615	0.988521	-1.39517
ydfG	3			0.294118	0.988476	-1.78226
		ydgC	2	2.6	0.9887	1.362116
ydgE	2			0.384615	0.988521	-1.39517
ydgI	2	ydgI	3.5	1.461538	0.988676	0.531058
ydhD	3	ydhD	7.5	2.058824	0.988809	1.025584
ydhE	3	ydhE	4	1.235294	0.988654	0.288392
ydhK	2			0.384615	0.988521	-1.39517
ydiB	3			0.294118	0.988476	-1.78226
ydiC	2			0.384615	0.988521	-1.39517
		ydhI	2	2.6	0.9887	1.362116
ydiF	7.5	ydiF	5	0.714286	0.988496	-0.50212
ydiR	22	ydiR	20.5	0.935484	0.988533	-0.11285
ydiS	5	ydiS	3.5	0.76	0.988542	-0.41255
ydjH	4	ydjH	4	1	0.988609	-0.01653
ydjI	8	ydjI	7.5	0.945946	0.988585	-0.09673
ydjN	9	ydjN	5.5	0.658537	0.988451	-0.61942
ydjP	4			0.238095	0.988432	-2.08718
yeeB	2	yeeB	3	1.307692	0.988654	0.370561
yeeF	2	yeeF	2	1	0.98861	-0.01653
yerH	6.5	yerH	7.5	1.129032	0.988652	0.158621
yerI	5	yerI	3.5	0.76	0.988542	-0.41255
		yesJ	2	2.6	0.9887	1.362116
yezE	2.5	yezE	3	1.133333	0.988632	0.164078
yezG	9	yezG	4.5	0.560976	0.988407	-0.85081
yfhB	21	yfhB	14.5	0.707865	0.988312	-0.51542
yfhC	9.5	yfhC	8	0.860465	0.98854	-0.23344
yfhL	3	yfhL	2	0.764706	0.988565	-0.40362
yfhM	6.5	yfhM	7.5	1.129032	0.988652	0.158621
yfiK	2			0.384615	0.988521	-1.39517
yfiR	6.5	yfiR	4	0.677419	0.988497	-0.57857
yfiT	2			0.384615	0.988521	-1.39517

yfiY	8.5	yfiY	5	0.641026	0.988451	-0.6583
yfjC	2.5	yfjC	2.5	1	0.98861	-0.01653
yfjD	2			0.384615	0.988521	-1.39517
		yfjN	2	2.6	0.9887	1.362116
yfjR	10	yfjR	4	0.466667	0.98834	-1.11646
		yfkD	3.5	3.8	0.988766	1.909701
yfkN	12	yfkN	16	1.301887	0.988782	0.364329
yfkO	5	yfkO	8.5	1.56	0.988764	0.625244
yflG	16	yflG	13.5	0.855072	0.988492	-0.24258
yflK	4			0.238095	0.988432	-2.08718
yfmC	5.5	yfmC	4.5	0.851852	0.988564	-0.24792
yfmG	111.5	yfmG	76	0.685144	0.986975	-0.56444
yfmJ	11	yfmJ	6.5	0.632653	0.988406	-0.67734
yfmK	2	yfmK	2	1	0.98861	-0.01653
		yfmM	3.5	3.8	0.988766	1.909701
yfmP	2	yfmP	2	1	0.98861	-0.01653
yfmQ	5	yfmQ	2	0.52	0.988475	-0.96014
yfmR	10.5	yfmR	8.5	0.829787	0.988517	-0.28585
yfmS	7	yfmS	5	0.757576	0.988519	-0.4172
yfmT	20.5	yfmT	15	0.747126	0.988357	-0.43747
		yfnG	8.5	7.8	0.988988	2.947499
		yfnH	3	3.4	0.988744	1.749204
ygaC	4.5	ygaC	5.5	1.173913	0.988653	0.214862
ygaF	3.5	ygaF	2.5	0.789474	0.988565	-0.35763
ygxA	3.5			0.263158	0.988454	-1.94275
yhaA	17	yhaA	19	1.109589	0.988691	0.133617
yhaM	4	yhaM	7	1.571429	0.988742	0.635743
yhaP	2.5	yhaP	2.5	1	0.98861	-0.01653
yhaQ	4.5	yhaQ	4	0.913043	0.988587	-0.14781
yhaX	3.5	yhaX	4	1.105263	0.988631	0.127895
		yhbE	2	2.6	0.9887	1.362116
yhbH	3	yhbH	10	2.647059	0.98892	1.388316
yhcB	2			0.384615	0.988521	-1.39517
yhcI	2			0.384615	0.988521	-1.39517
		yhbJ	3	3.4	0.988744	1.749204
yhcM	2	yhcM	3.5	1.461538	0.988676	0.531058
yhcN	19	yhcN	61	3.074074	0.990468	1.606334
yhcQ	4.5	yhcQ	20.5	3.782609	0.989319	1.903889

		yhcR	2	2.6	0.9887	1.362116
yhcS	2			0.384615	0.988521	-1.39517
yhcW	2			0.384615	0.988521	-1.39517
yhdA	7	yhdA	4	0.636364	0.988474	-0.6688
yhdL	2			0.384615	0.988521	-1.39517
yhdP	9.5	yhdP	6.5	0.72093	0.988473	-0.48879
yhdR	16	yhdR	8	0.536232	0.988248	-0.91613
		yheC	7	6.6	0.988921	2.706394
		yheG	2	2.6	0.9887	1.362116
yhfE	9.5	yhfE	12	1.232558	0.988717	0.285285
yhfI	2			0.384615	0.988521	-1.39517
yhfJ	4.5	yhfJ	7	1.434783	0.98872	0.504465
		yhfK	3	3.4	0.988744	1.749204
yhfM	2.5	yhfM	2.5	1	0.98861	-0.01653
		yhfO	2	2.6	0.9887	1.362116
yhfP	4	yhfP	4	1	0.988609	-0.01653
yhfR	16.5	yhfR	14	0.859155	0.988492	-0.23571
		yhfW	2.5	3	0.988722	1.568599
yhgC	5	yhgC	4	0.84	0.988564	-0.26813
yhjB	2			0.384615	0.988521	-1.39517
yhxA	4	yhxA	3	0.809524	0.988565	-0.32145
		yhxC	4	4.2	0.988788	2.054123
yisK	10	yisK	3.5	0.422222	0.988318	-1.26088
yisT	5	yisT	3.5	0.76	0.988542	-0.41255
yisY	2	yisY	3.5	1.461538	0.988676	0.531058
		yitA	3	3.4	0.988744	1.749204
		yitC	3	3.4	0.988744	1.749204
		yitD	4	4.2	0.988788	2.054123
yitJ	10.5	yitJ	6	0.617021	0.988406	-0.71343
yitK	11	yitK	9	0.836735	0.988517	-0.27382
yitL	2	yitL	3	1.307692	0.988654	0.370561
		yitS	2	2.6	0.9887	1.362116
		yitU	3	3.4	0.988744	1.749204
yitV	5	yitV	3.5	0.76	0.988542	-0.41255
yjbC	2.5	yjbC	4	1.4	0.988676	0.468997
yjbM	2.5	yjbM	3	1.133333	0.988632	0.164078
yjcG	28	yjcG	27	0.965812	0.988552	-0.0668
yjcH	9.5	yjcH	10	1.046512	0.988628	0.049089

yjcK	3	yjcK	2.5	0.882353	0.988587	-0.19713
		yjdH	4	4.2	0.988788	2.054123
yjdI	2			0.384615	0.988521	-1.39517
yjhA	5	yjhA	4	0.84	0.988564	-0.26813
yjiC	4	yjiC	2	0.619048	0.98852	-0.70854
yjjA	7	yjjA	5.5	0.818182	0.988541	-0.30613
yjlC	16.5	yjlC	18.5	1.112676	0.988691	0.137626
yjoA	11	yjoA	9.5	0.877551	0.988539	-0.20508
yjoB	2			0.384615	0.988521	-1.39517
		yjqC	6.5	6.2	0.988899	2.616164
ykaA	7	ykaA	9	1.242424	0.988696	0.296757
		ykfB	4	4.2	0.988788	2.054123
ykfD	3	ykfD	2.5	0.882353	0.988587	-0.19713
ykgB	24	ykgB	16	0.683168	0.988244	-0.56675
ykkE	3			0.294118	0.988476	-1.78226
yknT	2	yknT	2	1	0.98861	-0.01653
yknW	15.5	yknW	11.5	0.761194	0.988426	-0.41046
yknX	36	yknX	20	0.57047	0.987883	-0.82737
yknY	6.5	yknY	4.5	0.741935	0.988519	-0.44729
yknZ	5.5	yknZ	4.5	0.851852	0.988564	-0.24792
ykpA	13.5	ykpA	9.5	0.728814	0.988427	-0.47317
ykpB	4	ykpB	4	1	0.988609	-0.01653
ykqA	2			0.384615	0.988521	-1.39517
ykrA	4	ykrA	3	0.809524	0.988565	-0.32145
ykrB	8	ykrB	6.5	0.837838	0.98854	-0.27189
ykrK	3.5	ykrK	2	0.684211	0.988543	-0.56411
ykrL	7	ykrL	7	1	0.988607	-0.01653
yktB	6	yktB	5	0.862069	0.988564	-0.23072
yktC	10	yktC	6.5	0.688889	0.988451	-0.55442
ykuI	2	ykuI	2	1	0.98861	-0.01653
ykuQ	27.5	ykuQ	15.5	0.582609	0.988065	-0.79672
ykuR	8.5	ykuR	5	0.641026	0.988451	-0.6583
ykuU	22.5	ykuU	14.5	0.663158	0.988245	-0.60964
ykuV	2.5	ykuV	3	1.133333	0.988632	0.164078
ykvY	9.5	ykvY	5.5	0.627907	0.988429	-0.68817
ykwC	61	ykwC	69.5	1.136546	0.988958	0.168637
ylaG	28.5	ylaG	27	0.94958	0.98853	-0.09128
ylaJ	2.5	ylaJ	5.5	1.8	0.988743	0.831664

		ylaK	3.5	3.8	0.988766	1.909701
ylbL	2			0.384615	0.988521	-1.39517
ylbN	3	ylbN	2.5	0.882353	0.988587	-0.19713
yllA	12.5	yllA	10.5	0.854545	0.988516	-0.24343
ylmA	8.5	ylmA	5	0.641026	0.988451	-0.6583
ylmB	3	ylmB	5.5	1.588235	0.98872	0.651059
		ylmD	2	2.6	0.9887	1.362116
ylnE	5	ylnE	3	0.68	0.98852	-0.57305
yloB	6.5	yloB	11.5	1.645161	0.988829	0.702023
yloC	4.5	yloC	5.5	1.173913	0.988653	0.214862
yloI	5.5	yloI	3	0.62963	0.988497	-0.68412
yloN	2	yloN	3	1.307692	0.988654	0.370561
yloV	10.5	yloV	10	0.957447	0.988583	-0.0793
ylxF	3	ylxF	2	0.764706	0.988565	-0.40362
ylxS	2.5	ylxS	2.5	1	0.98861	-0.01653
		ylyB	2	2.6	0.9887	1.362116
ymaB	4			0.238095	0.988432	-2.08718
ymaE	5.5	ymaE	6.5	1.148148	0.988653	0.182844
ymcB	5.5	ymcB	5.5	1	0.988608	-0.01653
ymdB	5.5	ymdB	3	0.62963	0.988497	-0.68412
ymfF	2.5	ymfF	2.5	1	0.98861	-0.01653
ymfH	12	ymfH	14	1.150943	0.988694	0.186412
		ymzB	2	2.6	0.9887	1.362116
ynbA	3	ynbA	3	1	0.988609	-0.01653
		yncD	3.5	3.8	0.988766	1.909701
yncF	3	yncF	2	0.764706	0.988565	-0.40362
yncM	5.5	yncM	3	0.62963	0.988497	-0.68412
		yndB	2.5	3	0.988722	1.568599
yneF	4	yneF	3	0.809524	0.988565	-0.32145
yneJ	2			0.384615	0.988521	-1.39517
yneK	3			0.294118	0.988476	-1.78226
		yneT	3	3.4	0.988744	1.749204
yoaA	5	yoaA	3	0.68	0.98852	-0.57305
yoaD	2.5	yoaD	2	0.866667	0.988588	-0.22301
yoaE	5	yoaE	6	1.16	0.988653	0.197661
yobI	4	yobI	3	0.809524	0.988565	-0.32145
yobJ	4	yobJ	3	0.809524	0.988565	-0.32145
yobK	2	yobK	2.5	1.153846	0.988632	0.189957

		yobM (+1)	2	2.6	0.9887	1.362116
yocD	2	yocD	2	1	0.98861	-0.01653
yodC	5.5	yodC	5.5	1	0.988608	-0.01653
yodF	3.5	yodF	3	0.894737	0.988587	-0.17702
yodJ	4	yodJ	2	0.619048	0.98852	-0.70854
		yodQ	4	4.2	0.988788	2.054123
yodS	2			0.384615	0.988521	-1.39517
yoxA	6	yoxA	5.5	0.931034	0.988586	-0.11966
yoxD	17	yoxD	16.5	0.972603	0.98858	-0.05665
		ypbR	6	5.8	0.988877	2.519916
ypdA	6	ypdA	3	0.586207	0.988475	-0.78724
		ypeB	10.5	9.4	0.989077	3.216815
		ypeP	3	3.4	0.988744	1.749204
ypfD	86	ypfD	90	1.045845	0.988745	0.04834
ypgQ	3	ypgQ	2.5	0.882353	0.988587	-0.19713
ypgR	21.5	ypgR	15.5	0.736264	0.988334	-0.45864
yphC	14.5	yphC	10	0.714286	0.988404	-0.50225
yphF	2	yphF	2	1	0.98861	-0.01653
yphP	8	yphP	10	1.216216	0.988696	0.265998
ypiA	3	ypiA	5	1.470588	0.988698	0.539995
ypiB	2.5	ypiB	2	0.866667	0.988588	-0.22301
ypiF	2.5	ypiF	3.5	1.266667	0.988654	0.324575
ypjQ	8	ypjQ	6	0.783784	0.988518	-0.36813
ypmB	2			0.384615	0.988521	-1.39517
ypmQ	4.5	ypmQ	3.5	0.826087	0.988564	-0.29223
ypmR	2			0.384615	0.988521	-1.39517
ypmS	2.5	ypmS	2	0.866667	0.988588	-0.22301
ypoP	3			0.294118	0.988476	-1.78226
ypqE	6			0.172414	0.988342	-2.55297
ypuA	6	ypuA	6	1	0.988608	-0.01653
ypwA	13	ypwA	10.5	0.824561	0.988494	-0.295
yqaP	16	yqaP	9.5	0.623188	0.988315	-0.69922
yqbC	4			0.238095	0.988432	-2.08718
yqcF	10.5	yqcF	11.5	1.085106	0.98865	0.101368
		yqcG	4	4.2	0.988788	2.054123
		yqcI	2	2.6	0.9887	1.362116
		yqeC	2	2.6	0.9887	1.362116
yqeH	3	yqeH	2	0.764706	0.988565	-0.40362

yqeK	2.5	yqeK	3	1.133333	0.988632	0.164078
yqeT	2.5	yqeT	4	1.4	0.988676	0.468997
yqeY	5	yqeY	8	1.48	0.988741	0.549262
yqeZ	2	yqeZ	2.5	1.153846	0.988632	0.189957
yqfA	96	yqfA	115	1.195373	0.989408	0.242099
yqfF	6	yqfF	5	0.862069	0.988564	-0.23072
yqfG	2			0.384615	0.988521	-1.39517
yqfL	2	yqfL	3	1.307692	0.988654	0.370561
yqfO	14	yqfO	11	0.803279	0.988471	-0.33276
yqgA	3	yqgA	3.5	1.117647	0.988632	0.14397
yqhL	2	yqhL	2	1	0.98861	-0.01653
yqhM	4			0.238095	0.988432	-2.08718
yqhP	2	yqhP	2	1	0.98861	-0.01653
yqhS	3.5	yqhS	3.5	1	0.988609	-0.01653
yqhT	7.5	yqhT	3	0.485714	0.988408	-1.05864
yqhY	9	yqhY	8	0.902439	0.988562	-0.1647
yqiG	2.5	yqiG	2	0.866667	0.988588	-0.22301
yqiW	7	yqiW	8.5	1.181818	0.988674	0.224575
yqjE	14	yqjE	10	0.737705	0.988426	-0.45568
yqjM	6	yqjM	6	1	0.988608	-0.01653
yqjY	3	yqjY	3	1	0.988609	-0.01653
yqkF	12.5	yqkF	7.5	0.636364	0.988383	-0.66893
yqzC	9.5	yqzC	11	1.139535	0.988673	0.17201
yqzD	3			0.294118	0.988476	-1.78226
yrdA	6.5	yraA	2	0.419355	0.988408	-1.27058
		yrdA	8.5	7.8	0.988988	2.947499
yrdC	6	yrdC	5.5	0.931034	0.988586	-0.11966
yrhJ	5.5	yrhJ	3.5	0.703704	0.98852	-0.52362
yrkA	3	yrkA	5	1.470588	0.988698	0.539995
		yrkC	2.5	3	0.988722	1.568599
yrkL	3	yrkL	2	0.764706	0.988565	-0.40362
yrpD	12.5	yrpD	9	0.745455	0.988449	-0.44057
yrrM	2.5			0.333333	0.988499	-1.60165
yrrN	4.5	yrrN	4	0.913043	0.988587	-0.14781
yrrS	9.5	yrrS	9.5	1	0.988606	-0.01653
yrvD	2.5			0.333333	0.988499	-1.60165
yrvM	2	yrvM	2	1	0.98861	-0.01653
yrvO	4	yrvO	3	0.809524	0.988565	-0.32145

ysaA	12	ysaA	12.5	1.037736	0.988627	0.036938
ysdB	2			0.384615	0.988521	-1.39517
ysdC	6.5	ysdC	8	1.193548	0.988674	0.238824
ysmA	2	ysmA	2.5	1.153846	0.988632	0.189957
ysnA	3	ysnA	2	0.764706	0.988565	-0.40362
ysnB	4			0.238095	0.988432	-2.08718
ysxC	3.5	ysxC	4.5	1.210526	0.988654	0.259171
ytaG	10.5	ytaG	15.5	1.425532	0.988827	0.495291
		ytcG	2	2.6	0.9887	1.362116
ytcI	6.5	ytcI	10.5	1.516129	0.988785	0.584121
ytdI	6.5	ytdI	11.5	1.645161	0.988829	0.702023
		ytfJ	5	5	0.988833	2.305726
ytiB	4	ytiB	4.5	1.095238	0.988631	0.114749
ytjP	6.5	ytjP	7.5	1.129032	0.988652	0.158621
ytkA	3			0.294118	0.988476	-1.78226
ytkL	11	ytkL	6.5	0.632653	0.988406	-0.67734
ytkP	2	ytkP	3	1.307692	0.988654	0.370561
		ytlA	4.5	4.6	0.988811	2.1854
		ytlC	7	6.6	0.988921	2.706394
		ytoI	2	2.6	0.9887	1.362116
ytoP	4.5	ytoP	5	1.086957	0.988631	0.103798
ytoQ	10	ytoQ	7	0.733333	0.988473	-0.46419
ytpA	3	ytpA	2.5	0.882353	0.988587	-0.19713
ytpQ	2.5	ytpQ	3.5	1.266667	0.988654	0.324575
ytpR	2	ytpR	3	1.307692	0.988654	0.370561
ytqB	2	ytqB	2	1	0.98861	-0.01653
ytrF	2			0.384615	0.988521	-1.39517
ytsJ	26.5	ytsJ	20	0.765766	0.988309	-0.40199
ytsP	2	ytsP	2	1	0.98861	-0.01653
yttA	6	yttA	2	0.448276	0.988431	-1.17433
ytvA	2			0.384615	0.988521	-1.39517
ytxG	3	ytxG	3	1	0.988609	-0.01653
ytxH	32.5	ytxH	23	0.718519	0.988173	-0.49407
ytzB	2	ytzB	3	1.307692	0.988654	0.370561
ytzG	4			0.238095	0.988432	-2.08718
yuaC	2	yuaC	2	1	0.98861	-0.01653
yuaE	2	yuaE	6	2.230769	0.988787	1.141273
yuaG	67.5	yuaG	81	1.196364	0.989177	0.242957

yuaI	4.5	yuaI	5	1.086957	0.988631	0.103798
yubA	2			0.384615	0.988521	-1.39517
		yubB	8	7.4	0.988966	2.871518
yueB	11	yueB	6.5	0.632653	0.988406	-0.67734
yueD	5.5	yueD	4.5	0.851852	0.988564	-0.24792
yufN	46.5	yufN	47.5	1.020942	0.988632	0.013406
yufO	3	yufO	4.5	1.352941	0.988676	0.419669
		yugI	2	2.6	0.9887	1.362116
yugJ	16	yugJ	13.5	0.855072	0.988492	-0.24258
yugK	2	yugK	2	1	0.98861	-0.01653
		yugP	2	2.6	0.9887	1.362116
		yugT	2	2.6	0.9887	1.362116
yuiE	33	yuiE	39.5	1.189781	0.988883	0.234567
yukB	4	yukB	3	0.809524	0.988565	-0.32145
yukC	10	yukC	8	0.822222	0.988517	-0.29906
yukJ	5	yukJ	3	0.68	0.98852	-0.57305
yulF	3.5	yulF	3	0.894737	0.988587	-0.17702
yumB	2	yumB	10	3.461538	0.988965	1.775404
yumC	27	yumC	24	0.893805	0.988464	-0.17871
yurL	6.5	yurL	14	1.967742	0.98894	0.960496
yurO	10.5	yurO	19.5	1.765957	0.989005	0.8045
yurP	70.5	yurP	85.5	1.209059	0.989242	0.258281
		yurT	2	2.6	0.9887	1.362116
yurU	27	yurU	23.5	0.876106	0.988442	-0.20759
yurX	31	yurX	26.5	0.860465	0.988395	-0.23365
yurY	64.5	yurY	55	0.855513	0.988156	-0.24233
yutE	3	yutE	3	1	0.988609	-0.01653
yutF	2	yutF	2	1	0.98861	-0.01653
yutJ	2.5	yutJ	4	1.4	0.988676	0.468997
yuxL	3	yuxL	4	1.235294	0.988654	0.288392
yvaA	13	yvaA	8	0.649123	0.988383	-0.6403
yvaG	4	yvaG	3.5	0.904762	0.988587	-0.16095
yvaK	9	yvaK	9.5	1.04878	0.988629	0.052213
yvbF	3			0.294118	0.988476	-1.78226
yvbH	5.5	yvbH	3.5	0.703704	0.98852	-0.52362
yvbJ	11.5	yvbJ	13	1.117647	0.988672	0.144028
yvbK	5.5	yvbK	5.5	1	0.988608	-0.01653
yvcA	7.5	yvcA	7.5	1	0.988607	-0.01653

yvcI	2			0.384615	0.988521	-1.39517
yvcJ	7	yvcJ	7	1	0.988607	-0.01653
yvcK	2	yvcK	2.5	1.153846	0.988632	0.189957
		yvcP	3	3.4	0.988744	1.749204
yvcR	4.5	yvcR	3	0.73913	0.988542	-0.45272
yvcT	8	yvcT	6.5	0.837838	0.98854	-0.27189
yvdD	7	yvdD	3	0.515152	0.98843	-0.97372
yvdT	2			0.384615	0.988521	-1.39517
yvgL	2	yvgL	3	1.307692	0.988654	0.370561
yvgN	19.5	yvgN	13.5	0.710843	0.988335	-0.50932
yvhJ	4	yvhJ	2.5	0.714286	0.988542	-0.50205
yvkC	6	yvkC	8	1.275862	0.988697	0.335072
yvlB	25	yvlB	27	1.07619	0.988687	0.089519
yvmB	2.5	yvmB	2	0.866667	0.988588	-0.22301
yvoE	5.5	yvoE	4.5	0.851852	0.988564	-0.24792
yvqK	2	yvqK	3	1.307692	0.988654	0.370561
yvrC	12	yvrC	4	0.396226	0.98825	-1.35265
yvrD	6.5	yvrD	5	0.806452	0.988541	-0.32697
yvrHb	3	yvrHb	3	1	0.988609	-0.01653
yvrN	3			0.294118	0.988476	-1.78226
yvrO	3	yvrO	6	1.705882	0.988743	0.754185
yvrP	47	yvrP	41	0.875648	0.988321	-0.20853
yvyD	25.5	yvyD	16.5	0.663551	0.988199	-0.60885
yvyG	3			0.294118	0.988476	-1.78226
ywaA	59	ywaA	55	0.93361	0.988403	-0.11594
ywfH	18	ywfH	13.5	0.766234	0.988402	-0.40097
ywfI	25.5	ywfI	19	0.757009	0.98831	-0.41858
		ywhD	2	2.6	0.9887	1.362116
ywiB	3			0.294118	0.988476	-1.78226
ywjG	2	ywjG	2	1	0.98861	-0.01653
ywjH	64.5	ywjH	57.5	0.893536	0.988267	-0.17943
ywlC	4	ywlC	5	1.190476	0.988653	0.235075
ywlF	4	ywlF	3.5	0.904762	0.988587	-0.16095
ywlG	4.5	ywlG	3.5	0.826087	0.988564	-0.29223
		ywnB	2	2.6	0.9887	1.362116
ywnH	2.5	ywnH	2	0.866667	0.988588	-0.22301
		ywoC	2	2.6	0.9887	1.362116
ywpB	4	ywpB	3	0.809524	0.988565	-0.32145

ywpJ	9	ywpJ	9	1	0.988606	-0.01653
		ywqF	4	4.2	0.988788	2.054123
ywqH	3	ywqH	3	1	0.988609	-0.01653
ywqJ	2.5	ywqJ	5	1.666667	0.988721	0.7206
ywqK	5	ywqK	5	1	0.988608	-0.01653
ywrF	2	ywrF	3	1.307692	0.988654	0.370561
		ywrJ	2	2.6	0.9887	1.362116
ywrO	7.5	ywrO	8.5	1.114286	0.988652	0.139653
ywtE	3	ywtE	2	0.764706	0.988565	-0.40362
ywtF	3			0.294118	0.988476	-1.78226
yxaA	3.5	yxaA	2	0.684211	0.988543	-0.56411
yxbB	25	yxbB	16.5	0.67619	0.988221	-0.58159
yxbC	29.5	yxbC	23.5	0.804878	0.98833	-0.33009
yxbD	2	yxbD	13.5	4.538462	0.98912	2.166421
yxeK	2			0.384615	0.988521	-1.39517
yxeM	7	yxeM	4.5	0.69697	0.988497	-0.53752
yxeP	2.5	yxeP	2.5	1	0.98861	-0.01653
yxiE	8	yxiE	12.5	1.486486	0.988807	0.555667
yxjG	45	yxjG	31.5	0.708108	0.987989	-0.51539
yxjH	13	yxjH	13.5	1.035088	0.988627	0.033251
		yxjI	2	2.6	0.9887	1.362116
yxkC	16.5	yxkC	18	1.084507	0.988669	0.100599
yxnB	14.5	yxnB	7	0.52381	0.988271	-0.94991
yxxD	12	yxxD	7	0.622642	0.988383	-0.70038
yyaF	72.5	yyaF	99	1.359322	0.989754	0.428029
		yybA	2	2.6	0.9887	1.362116
		yybI	3.5	3.8	0.988766	1.909701
yybJ	10	yybJ	7	0.733333	0.988473	-0.46419
yycH	2	yycH	2	1	0.98861	-0.01653
		yycN	2	2.6	0.9887	1.362116
yydA	3	yydA	4	1.235294	0.988654	0.288392
yydB	3.5	yydB	3.5	1	0.988609	-0.01653
yydD	10.5	yydD	8	0.787234	0.988495	-0.36183
yydG	4.5	yydG	3.5	0.826087	0.988564	-0.29223
yydI	10	yydI	9.5	0.955556	0.988584	-0.08215
zosA	3	zosA	3	1	0.988609	-0.01653
zwf	43.5	zwf	29.5	0.687151	0.987967	-0.55877

* n2+f/n1+f, ** t1-n1+f/t2-n2+f