Fall 12-14-2011

Non-Classical Protein Secretion and Transcriptome Studies during Stationary Phase of Bacillus Subtilis

Chun-Kai Yang

doi: https://doi.org/10.57709/2384671

NON-CLASSICAL PROTEIN SECRETION AND TRANSCRIPTOME STUDIES DURING STATIONARY PHASE OF BACILLUS SUBTILIS

by

CHUN-KAI YANG

Under the Directions of Dr. Phang C. Tai and Dr. Chung-Dar Lu

ABSTRACT

A cloned esterase and several cytoplasmic proteins which lack a classical cleavable signal-peptide were secreted during late stationary phase in *B. subtilis*. Several lines of evidence indicate that secretion of enolase, SodA, and Est55 is not due to cell lysis. The extent of possible release of these proteins mediated by membrane vesicles into the medium was also found to be minimal. We have identified a hydrophobic α–helical domain within enolase that contributes to the secretion specificity. Thus, upon the genetic deletion or replacement of a potential membrane-embedding domain, the secretion of plasmid-encoded mutant enolases is totally blocked, while that of the wild-type chromosomal enolase is not affected in the same cultures. However, mutations on the conserved basic residues flanking the hydrophobic core region show no effect.

GFP fusion experiments demonstrate that minimal length of N-terminus 140 amino acids and its tertiary structure are required to serve as a functional signal for the export of enolase.

Transcriptome analysis revealed several interesting patterns in gene expression when the cell growth switches from exponential phase into stationary phase. As expected, once cell growth enters the stationary phase, expressions of most SigA-dependent house-keeping genes (for syntheses of ATP, amino acids, nucleotides, ribosomes), and surprisingly *secY* and *yidC* homolog in the Sec-dependent general protein secretion system were significantly decreased; however, *secA* and *sipT* were found progressively induced in the stationary phase. The *sigB* gene and the SigB regulon exhibited a distinct pattern of transient induction with a peak in transition phase. A total of 62 genes were induced by three fold after cessation of SigB-dependent surge, which includes *sigW* and many of SigW-depedent genes specifically for antitoxin resistant genes, and some unknown function genes. In addition, oxidative stress response and damage repair genes also dominantly induced in stationary phase implied a high level of oxidant or thio-depleting agents in stationary phase. Besides, induction of *fruRAB* at T40 and *gap* operon at T100 suggested a sequential switch of carbon utilization from glucose to fructose. These results indicate a complex adaptation physiology as *Bacillus* cells change from the fast growing exponential phase toward the stationary phase.

INDEX WORDS: Est55, signal-less protein, enolase, secretion, EM domain, hydrophobicity, sigma factor, microarray, *Bacillus subtilis*

NON-CLASSICAL PROTEIN SECRETION AND TRANSCRIPTOME STUDIES DURING STATIONARY PHASE OF BACILLUS SUBTILIS

by

CHUN-KAI YANG

A Dissertation Submitted in Partial Fulfillment of the Requirements for the Degree of

Doctor of philosophy

in the College of Arts and Sciences

Georgia State University

2011

NON-CLASSICAL PROTEIN SECRETION AND TRANSCRIPTOME STUDIES DURING STATIONARY PHASE OF BACILLUS SUBTILIS

by

CHUN-KAI YANG

Committee Chair: Dr. Phang C Tai

Committee: Dr. Chung-Dar Lu

Dr. John Houghton

Electronic Version Approved:

Office of Graduate Studies

College of Arts and Sciences

Georgia State University

December, 2011

ACKNOWLEDGEMENTS

I would like to thank my parents and my family who support me for years. I would like thank my advisor, Dr. Phang C. Tai, for his patience on instruction and encouragement to guide me through my study. I also would like to thank Dr. Chung-Dar Lu who also advises me during my Ph. D study. In addition, I want to acknowledge Dr. John Houghton for serving on my dissertation committee and his precious advisements in my manuscript; I also would like to thank all the lab membranes in Dr. Tai's and Dr. Lu's lab for their friendship and help, especially Dr. Hsiuchin Yang for her great consultation and discussion for my research. I also want to express my special thank to all the friends who support me these years in Atlanta.

TABLE OF CONTENTS

ACKNOWLEDGEMENTS v

LIST OF TABLES viii

LIST OF FIGURES ix

INTRODUCTION 1

CHAPTER 1 9

Non-classical protein secretion of Bacillus subtilis in the stationary phase is not due to cell lysis 9

Abstract 10

Introduction 11

Materials and Methods 13

Results 18

Carboxylesterase Est55 from G. stearothermophilus is secreted 18

Secretion of Est55 from a protease-deficient strain of B. subtilis 18

The secreted Est55 is not processed 19

Other cytoplasmic proteins lacking typical signal peptide are secreted in stationary phase 20

Non-classical protein secretion is due to cell lysis? 21

Non-classical secretion is a common phenomenon 21

Lytic enzymes do not significantly alter signal-less protein secretion 22

Signal-less protein secretion is not mediated significantly by membrane vesicles 22

A hydrophobic helix domain of enolase is crucial for its secretion 23

Discussion 26

CHAPTER 2 43

A highly conserved N-terminal hydrophobic region of Bacillus subtilis enolase: A putative signal peptide in E. coli and B. subtilis 43

Abstract 44

Introduction 45

Materials and Methods 48

Results 50

A highly conserved hydrophobic α-helix domain affects secretion of enolase……50
The conserved basic residues flanking the HC region are not critical for the export……..51
The hydrophobic α-helix is unique for enolase secretion……52
The hydrophobic core dependent secretion is a general phenomenon……52
The minimal length of N-terminus of enolase that necessary for fusion protein secretion……..53
Discussion.....54

CHAPTER 3……66
Growth phase related transcriptome analysis of *Bacillus subtilis* 168 ……..…….66
Abstract.....67
Introduction 68
Materials and Methods 69
Result and discussion……...71
Clusters of time course-dependent transcriptional profiling……...…..…71
Genes that turned off in the stationary phase……...…71
Genes that are transiently induced at the transition stage……..…72
Genes that are induced in the stationary phase…….74
The expression profile of general secretion pathways…….....78

GENERAL CONCLUSION…….....86

REFERENCES……..91

APPENDIX……99
I. Genechip raw data of *Bacillus subtilis* at different growth stage**s……..……**100

LIST OF TABLES

Table 1.1. Bacterial strains and plasmids……42

Table 2.1. Bacterial strains and plasmids……64

Table 3.1. List of antitoxin resistant and membrane stress response genes from sigW regulon ……84

Table 3.2. Oxidative stress and repair genes expressed in stationary phase……..84

Table 3.3. Nutrient supply and unknown function genes that expression significantly in stationary phase……..85

Table S1. SigA regulon and genes expressed in exponential phase……101

Table S2. SigB regulon and genes expressed in transition phase……108

Table S3. SigW regulon and genes expressed in stationary phase……..110

LIST OF FIGURES

Figure 1.1. The genetic information and expression profile of Est55……31

Figure 1.2. Secretion of Est55 and other cytoplasmic proteins in B. subtilis…….33

Figure 1.3. Identification of secreted proteins in B. subtilis WB600BHM……35

Figure 1.4. Protein secretion of B. subtilis 168/pHE550 and WB600BHM/pHE550……37

Figure 1.5. A hydrophobic domain required for enolase secretion…….39

Figure 2.1. Genetic and restriction map of the expression vector pGTN-FLAG….....59

Figure 2.2. The substitution mutations in the hydrophobic core region affected the secretion of enolase……..60

Figure 2.3. Substitution mutations of positively charged residues flanking the HC region did not affect enolase secretion…….61

Figure 2.4. Hydrophobic core of enolase is unique for secretion……62

Figure 2.5. Whole N-terminus of EnoBs is capable to work as a signal for recombinant GFP……..63

Figure 3.1. The growth profile and gene expression of B. subtilis 168……..80

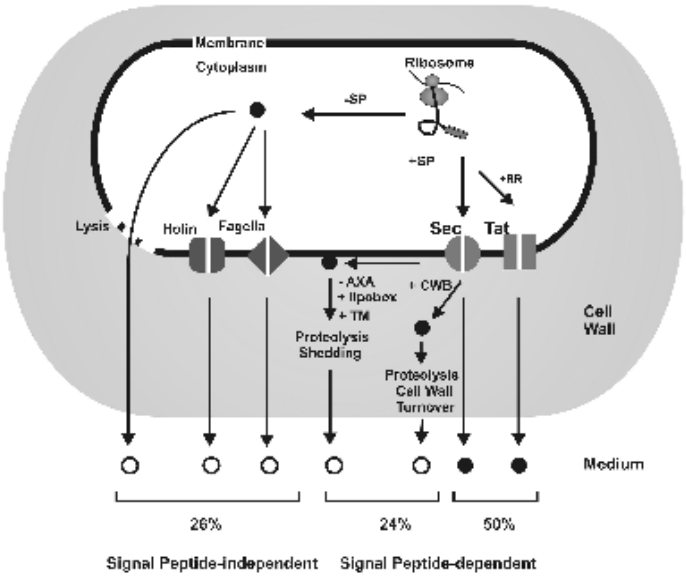
Figure 3.2. The expression profiles of different sigma factors……...81

Figure 3.3. Expression of sec dependent apparatus at different stages during growth……..83

INTRODUCTION

Protein secretion is an essential function of many kinds of cells for various purposes. Recent biochemical and genetic work in bacteria has revealed, in addition to the general sec-dependent pathway, several diverse pathways, through which proteins are secreted across Gram-positive and Gram-negative bacterial membranes. Much of genetic and biochemical work has been focused in *Escherichia coli* because of the ease of genetic manipulation (Oliver and Beckwith 1981; Danese and Silhavy 1998), and the early development of in vitro translocation systems (Tai, Tian et al. 1991; Pugsley 1993; Manting and Driessen 2000). Though the secretory pathways in bacteria have been remarkably similar, there are also differences. Gram-positive bacteria are capable in producing various proteins and then secret them into growth medium. The single membrane of gram-positive bacteria, in particular *Bacillus subtilis* which secretes large amounts of various enzymes, offers unique opportunities and attracts much early attention (Tjalsma 2004). These eubacterial species are particularly attractive for this purpose because they have a high capacity to export proteins into the growth medium and because of their non-pathogenicity. For these reasons, *Bacillus subtilis* is a good model on investigating protein export mechanism. Moreover, *B. subtilis* has been the prototype model system for studying the unique features of Gram-positive bacteria, including the development towards sporulation (Abraham L. Sonenshein 2001). Nevertheless, the literatures on protein secretion and related events in *B. subtilis* are vast.

B. subtilis and protein secretion. The genome of *B. subtilis* 168 is 4,215 kb in length and contains about 4,100 genes that are predicted to encode over 250 extracellular proteins; the majority of them are secreted through the aforementioned pathways (Hirose 2000; Antelmann 2001). Protein secretion is essential for bacteria to survival in a natural environment. Bacteria can produce a large amount of proteins to maintain their physiological function (Christine Eymann 2002). Four protein secretion pathways in *Bacillus subtilis* have been well studied. The majority of secretory proteins are exported from the cytoplasm by the Sec pathway with a signal peptide that is cleaved during translocation across membrane (Simonen 1993). Some proteins are secreted to the medium via the Tat pathway (twin-arginine translocation) (Tjalsma 2000). Small numbers of proteins are exported through the dedicated pseudopilin export pathway (Com) which is also responsible for competence development. The fourth pathway, the ATP-binding cassette (ABC) transporters are only for few dedicated proteins (Tjalsma 2000).



Tjalsma et. al., 2004 Microbiol Mol Biol Rev 68(2): 207-233

Lysis or secretion? Proteomic studies revealed more secreted proteins than previous prediction. The genome-based predictions reflect only 50% of the actual composition of the extracellular proteome. This significant discrepancy is mainly due to the difficulties in the prediction of extracellular proteins lacking signal peptides (including cytoplasmic proteins) and lipoproteins (Hirose 2000; Antelmann 2001). It is not quite certain whether some of these proteins identified by proteomic analysis were released due to cell lysis (Stephenson 1999; Vitikainen 2004) or even significant since the distribution of these proteins in the cytoplasm and medium was not quantified (Hirose 2000; Antelmann 2001). These findings suggest that in addition to well known secretion pathways, *B. subtilis* can utilize alternative mechanisms to release such signal-less proteins to their environment (Tjalsma 2004). Recently, several publications have indicated many proteins are secreted via shedding of membrane vesicles in Gram-positive bacteria (Tjalsma 2004; Mashburn-Warren 2006; Lee 2009). It is also not clear how significant is the extent of such release by membrane vesicles since no quantification has been attempted.

Most of the signal-less proteins are major cytoplasmic proteins and the functions of the proteins have been identified, hence, several proteomic publications have suggested that the presence of the signal-less proteins are due to cell lysis (Simonen 1993; Tjalsma 2000; Petersohn, Brigulla et al. 2001). On the other hand, Bendsten *et al.* predicted that signal-less proteins could be secreted into growth medium and these proteins may follow other unknown mechanism instead of cell lysis (Bendtsen 2005).

Moonlighting cytoplasmic proteins. The protein Est55 of *Geobacillus* has been cloned in our lab and successfully expressed in *Bacillus subtilis* (Ewis 2004). This protein is neither Sec- nor Tat- dependent, and this protein does not utilizes ATP or proton motive force as energy source. It has been suggested that a novel secretion system may be involved in the signal-less protein secretion. Several signal-less proteins have been found in growth media in the stationary phase and these proteins are abundant in the cytosolic compartment. These proteins have defined cytosolic functions but the reason and the mechanism that they are secreted is not clear yet. Recent publications showed that GroEL can be localized on the membrane in *H. pylori* and interacted with the host (Gabriela E. Bergonzelli 2006). Nevertheless, the secretion pathway of GroEL and its extracellular function are still unknown. Similarly, S-complex has been found its multiple functions including regulation of sporulation and could be an activator of Type III secretion in *P. aeruginosa* (Denis Dacheux 2002; Haichun Gao 2002). SodA also has been predicted that will be secreted into growth medium (Tjalsma. 2006). These proteins have been named "moonlighting" proteins and have been suggested to have other functions in the extracellular compartment (Bendtsen 2005). Due to its nature which is without apparent signal peptide but could be secreted, therefore, we named those proteins as "Non-classical proteins".

Enolase and its function. Enolases (EC 4.2.1.11) are essential cytoplasmic enzymes that catalyze the reversible conversion of 2-phosphoglycerate into phosphoenolpyruvate. Enolases are considered to be evolutionarily conserved due to its universal functions in glycolysis and gluconeogenesis (Hosaka 2003). Many reports showed that eno-

lases can be exported to the cell surface or released to the culture medium in eukaryotic and prokaryotic organisms (Dudani 1993; Pancholi 1998; Pancholi 2001; Lamonica 2005; Lopez-Villar 2006; Antikainen 2007; Knaust 2007; Wang 2011) and play important roles in diseases which in turn facilitates bacterial persistence, colonization and the invasion of host tissues (Bergmann 2001; Jin 2005).

E. coli enolase was found to be exported into the medium and the export may depends on covalent binding of its substrate 2-phosphoglycerate (Boel 2004). A recent study showed that the N-terminal fragment of 169 amino acids of yeast enolase can target a cytoplasmic invertase to the cell surface (Lopez-Villar 2006). It was reported that *Bacillus anthracis* enolase was detected in the right-side out membrane vesicles (Agarwal 2008). Besides, Ferrari *et al*. found that enolase of *Neisseria meningitidis* can be found in the spontaneously released outer membrane vesicles (Ferrari 2006). Recent study on *Clonorchis sinensis* demonstrated that enolase is an excretory/secretory protein and is also a multifunctional metabolic enzyme (Wang 2011) and Yu *et al*. showed that the enolase secretes via exosomes in the eukaryotic cells (Yu 2006). On the other hand, it has also been proposed that the release of enolase in the medium could be attributed to the cell lysis (Guiral 2005; Antelmann 2006). In addition, *B. subtilis* enolase was found in the extracellular compartment by many research groups (Tjalsma 2004; Vitikainen 2004; Antelmann 2006; Zanen 2006), but how and why the enolase of this non-pathogenic strain is secreted remains unknown. Nevertheless, the molecular mechanism of enolase transport through the membrane is mostly unknown.

Hydrophbic helix and enolase. Using the crystal structure of *Enterococcus hirae* enolase (Hosaka 2003) as the template, a predicted molecular structure of enolase was modeled by Swiss-Model (Arnold 2006). *Bacillus* enolase is composed of one small N-terminal domain (P_2-N_{138}) and one large C-terminal domain (S_{139}-K_{430}). According to Swiss-Prot database (Entry No. **37869**), the C-terminal barrel domain contains four phosphorylation sites, substrate and Mg^{2+} binding sites and two catalytic motifs. A long unbent α-helix (A_{108}-L_{126}) of enolase resides in the N-terminal domain, and in which a hydrophobic core region (A_{110}-C_{118}) is predicted as the membrane-embedded (EM) domain by the PSSM_SVM scheme (Hu 2007). This predicted domain is also conserved in several non-classical proteins except SodA. This domain has been suggested on promoting secretion on those proteins. An identified EM domain shred a light to demonstrate an unknown secretion pathway that involves in the transportation of signal-less protein(s).

Transcriptome and stationary phase. Generally, in *Bacillus*, the non-classical proteins we found in the medium are presence in stationary phase. Taking Est55 as an example, this protein starts to accumulate in the cell after induction in exponential phase but it is secreted in stationary phase. Besides, protein secretion in *E. coli* also shows a similar fashion as in *B. subtilis* which is more dominated in stationary phase. This result suggests there could be a retention signal or something could trigger the secretion phenomenon in stationary phase but it is currently unclear. In addition, although the overall cell numbers remain static in stationary phase, this period of time is considered the most dynamic period in response to ever-changing growth environments. However, previous researches focused on stationary phase were mostly interested in sporulation, and no re-

search has been conducted on how *Bacilli* adapt changes caused by growth phase shift from exponential phase to stationary phase. Depending on the specific physical and chemical growth conditions, the genetic circuits of the cells are designed to make prioritized decisions for adjustments when facing multiple stress factors arising from growth. Accordingly, the nature of this design makes it a very challenging task to predict the status of the cells at the genetic level in the past. Equipped with a complete and annotated genome sequence as well as modern genomic tools, it is now feasible to conduct systematic analysis of this complicated phase of bacterial growth.

Therefore, to better understand the physiological status of *B. subtilis* during growth, DNA microarrays (GeneChip® by Affymetrix) were employed to conduct transcriptome analysis. Although many publications report investigation of bacterial responses to specific stresses or genetic mutations by this approach, however, none of them focused on how *B. subilis* adapts to growth phase changes in a temporal manner. Recently, the gene expression profile of *B. subtilis* from exponential phase to stationary phase with additional amino acids has been studied (Ye, Zhang et al. 2009), however, the enriched medium may delay *Bacilli* to enter into stationary phase. Besides, no research has studied protein secretion onto a transcription level, and no direct correlation was found between protein secretion and stationary phase. To understand how *Bacilli* manage different growth stages, mRNAs were collected from exponential phase, transition phase and stationary phase to monitor gene expression profile. The major aim of this part of work was to study transcriptional profiling when the cells shift from the exponential phase to stationary phase by a series of snapshots in this specific time window.

In this study, we provided several lines of evidence to rule out lysis is the mechanism for the presence of non-classical proteins in the growth medium. Furthermore, genetic manipulation of hydrophobic helix from enolase demonstrated a possible mechanism of non-classical protein secretion. The transcriptome study is elucidated a sophisticated system of *Bacilli* when encounter physical stress or nutritional depletion.

CHAPTER 1

Non-classical protein secretion of *Bacillus subtilis* in the stationary phase is not due to cell lysis

This work has been published by

Chun-Kai Yang, Hosam E. Ewis, XiaoZhou Zhang, Chung-Dar Lu, Hae-Jin Hu, Yi Pan, Ahmed T. Abdelal, and Phang C. Tai. Nonclassical Protein Secretion by Bacillus subtilis in the Stationary Phase Is Not Due to Cell Lysis. (2011) *J. Bacteriol*. 193: 5607-5615

ABSTRACT

The carboxylesterase Est55 has been cloned and expressed in *Bacillus subtilis* strains. Est55, which lacks a classical, cleavable N-terminal signal sequence, was found to be secreted during the stationary phase of growth such that there is more Est55 in the medium than inside the cells. Several cytoplasmic proteins were also secreted in large amounts during late stationary phase, indicating the secretion in *B. subtilis* is not unique to Est55. These proteins, which all have defined cytoplasmic functions, include GroEL, DnaK, enolase, pyruvate dehydrogenase subunits PdhB and PdhD, and SodA. The release of Est55 and those proteins into the growth medium is not due to gross cell lysis, a conclusion that is supported by several lines of evidence: constant cell density and secretion in the presence of chloramphenicol, constant viability count, the absence of EF-Tu and SecA in the culture medium, and the lack of effect of autolysins deficient mutants. The shedding of these proteins by membrane vesicles into the medium is minimal. More importantly, we have identified a hydrophobic α-helical domain within enolase that contributes to its secretion. Thus, upon the genetic deletion or replacement of a potential membrane-embedding domain, the secretion of plasmid-encoded mutant enolase is totally blocked, while the wild-type chromosomal enolase is secreted normally in the same cultures during the stationary phase, indicating the differential specificity. We conclude that the secretion of Est55 and several cytoplasmic proteins without signal peptides in *B. subtilis* is a general phenomenon and is not a consequence of cell lysis or membrane shedding; rather their secretion is through a process(es) in which protein domain structure plays a contributing factor.

INTRODUCTION

Bacillus subtilis secretes large amount of proteins into the growth medium (van Wely, Swaving et al. 2001). Of the known secretory pathways in *B. subtilis*, the majority of proteins are exported from the cytoplasm by the Sec-dependent pathway, through which secretory proteins are synthesized as precursors with typical cleavable N-terminal signal peptides (Simonen and Palva 1993; Antelmann 2001). Relatively fewer proteins are released to the medium via the cleavable twin-arginine translocation (TAT) system (Tjalsma, Bolhuis et al. 2000). Still some other proteins are exported into the medium via ATP-binding cassette transporters, a dedicated pseudopilin export pathway, a competence development system or an ESAT-6 like system (Pallen 2002).

The genome of *B. subtilis* 168 is 4,215 kbp in length and encodes about 4,100 genes that are predicted to include over 250 extracellular proteins; the majority of which are secreted through the aforementioned pathways (Hirose, Sano et al. 2000; Antelmann 2001). However, proteomic studies have revealed that genome-based predictions reflect only 50% of the actual composition of the extracellular proteome. This significant discrepancy is mainly due to the difficulties in the prediction of extracellular proteins lacking signal peptides (including cytoplasmic proteins) and lipoproteins (Hirose, Sano et al. 2000; Antelmann 2001). These findings suggest that, in addition to the well-known secretion pathways, *B. subtilis* can utilize alternative mechanisms to release such signal-less proteins into their environment (Tjalsma 2004). However, it is not quite certain whether some of these extracellular proteins, identified by proteomic analysis, were released due

to cell lysis (Stephenson and Harwood 1999; Vitikainen 2004) or were even secreted in significant amounts since the distribution of these proteins in the cytoplasm and medium was not quantified (Hirose, Sano et al. 2000; Antelmann 2001). Recently, several publications have indicated many proteins are secreted by Gram-positive bacteria via the shedding of membrane vesicles (Tjalsma 2004; Mashburn-Warren 2006; Lee 2009). Even so, it is not at all clear as to how significant this shedding of membrane vesicles is, since no quantification of this phenomenon has been attempted.

In this work, a carboxylesterase, Est55 (monomer size of 55 kDa) from *Geobacillus stearothermophilus* (Ewis, Abdelal et al. 2004) has been characterized in *B. subtilis* WB600BHM, in which six protease genes have been deleted (Wu, Lee et al. 1991). The expressed Est55, which lacks a typical export signal, was found to accumulate in the cells during exponential phase. The intracellular Est55, however, begins to be secreted in early stationary phase, and decreases into the late stationary phase to the extent that more Est55 is found in the medium than inside the cells. This secretion of Est55 in the growth medium is accompanied by the concomitant appearance of several other cytoplasmic proteins that also lack a classical signal peptide. A similar secretion pattern was also found in both *B. subtilis* 168 and an autolysins-deficient mutant of *B. subtilis* (Wu, Lee et al. 1991; Blackman, Smith et al. 1998). Negligible amounts of Est55, SodA and enolase were found in the membrane vesicles recovered from the spent medium, indicating that the membrane-shedding is not a significant factor for the secretion. Moreover, we have identified a hydrophobic membrane-embedded domain of enolase (Hu, Pan et al. 2004; Hu, Holley et al. 2007) that is necessary for its secretion. Taken together, these

findings indicate that several cytoplasmic proteins which lack a typical signal peptide are secreted into the medium in the absence of cell lysis during the stationary phase of growth, and that the secretion is a general phenomenon in *B. subtilis.*

MATERIALS AND METHODS

Bacterial strains, plasmids, culture conditions and growth. Bacterial strains and plasmids are listed in Table 1.1. All *Bacillus subtilis* strains were grown in Luria-Bertani (LB) broth or agar plates containing 0.2% glucose at 37 ^{o}C. *Geobacillus stearothermophilus* ATCC 7954 was grown in Bacto-antibiotic medium No.3 (Difco) supplemented with 0.2% glucose, 272 µM of $CaCl_2$ and 143 µM of $FeCl_2$ at 60 ^{o}C. The following antibiotics were used as required: ampicillin (100 µg/ml), chloramphenicol (100 µg/ml), and kanamycin (10 µg/ml).

Construction, cloning and expression of Est55 in *B. subtilis* strains. The *est55* gene was PCR-amplified from pCOS4 clone (Ewis, Abdelal et al. 2004) using the appropriate primer pair. PCR products were subcloned into the *E. coli*/*B. subtilis* shuttle vector pDG148 in which the ATG initiation codon of *est55* was fused to a ribosomal binding site 7 bp upstream provided by the vector (Yansura and Henner 1984). The resulting plasmid pHE550 carrying kanamycin resistance as a selective marker allows the IPTG-induced expression of Est55 in *Bacillus subtilis* from the P_{spac} promoter (Yansura and Henner 1984). Plasmid transformation of *B. subtilis* was performed by natural competence protocol (Spizizen 1958; Anagnostopoulos 1961), and occasionally by the protoplast method (Chang and Cohen 1979).

Cloning of *B. subtilis* enolase and its mutants. Appropriate primer pairs were used for PCR amplification of the *eno* gene using *B. subtilis* 168 chromosomal DNA, while the restriction enzyme sites *Xba*I and *Sal*I were introduced into the forward and reverse primer sequences, respectively. The PCR product was digested by *Xba*I/*Sal*I and subcloned into the corresponding sites of the shuttle vector pDG148, in which the cloned gene was placed under the control of P_{spac} promoter. To construct the *B. subtilis* 168 enolase mutant deleted of α-helix EnoBsΔH (**^{102}NKGKLGANAILGVSMACARAAADFL^{126}**), a series of over-lap extension PCR (Ho 1989) were carried out. The PCR-amplified DNA fragments were inserted into pDG148 and the resulting pEnoBsΔH plasmids were used to transform into *E. co*li DH5α. An enolase EM (18, 19) domain replacement, with a short sequence of a α-helix from β-galactosidase (EMR, ^{110}**AILGVSMAC**^{118} to ^{110}**EQTMVQDIL**^{118}), was similarly constructed, resulting in the plasmid pEnoBsEMR. The sequences of the entire inserts in the pDG148-derived plasmids were confirmed by DNA sequence analysis.

Construction of shuttle expression vector pGTN-FLAG. The strong constitutive promoter region of *B. subtilis groESL* operon (Phan, Nguyen et al. 2006) was amplified by the appropriate primer pair using *B. subtilis* 168 genomic DNA as the template, and the *B. subtilis gsiB* SD sequence (Jurgen 1998) that stabilizes the transcribed mRNA molecules was introduced downstream of the P_{groE} promoter. The strong tandem terminators t_1t_2 and t_0 were amplified from pMUTIN4 (Vagner 1998), and the DNA fragment, containing the P_{groE}/SD_{gsiB} and t_1t_2/t_0, was generated through over-lap extension PCR (Ho

1989). The *Bam*HI, *Kpn*I and *Pst*I sites between SD_{gsiB} and the terminators, as well as the flanking *Hin*dIII and *Eco*RI sites, were introduced into the final DNA fragment by appropriately modified PCR primers. The final DNA product was digested with *Hin*dIII and *Eco*RI, and cloned into the corresponding sites of the *E. coli-B. subtilis* shuttle vector pNW33N to generate vector pGT. A short DNA fragment, containing 5'-*Bam*HI-FLAG (DYKDDDK)-*Sac*I/*Sac*II/*Sal*I/*Pst*I-3', was generated by annealing a pair of complementary oligonucleotide primers and cloning them into the *Bam*HI/*Pst*I sites of pGT to generate pGTN-FLAG. To express FLAG-tagged enolase, the *eno* gene was PCR amplified and cloned into the *Sac*I/*Pst*I sites of pGTN-FLAG.

Analysis of Est55 and other proteins in *G. stearothermophilus* and *B. subtilis*. Cell growth was monitored by OD_{600}. The protease inhibitor phenylmethylsulfonyl fluoride (PMSF) was added to the culture (final conc. 1 mM) in early-log phase (OD_{600} ~ 0.2) in most cases, except for the protease deficient strain WB600BHM and *G. stearothermophilus* when the esterase activity was measured. Samples (10 ml) were collected at various growth phases, as indicated, and were centrifuged at 13,400 x *g* for 10 min. Culture supernatants were filtered (0.22 μm Millex, Millipore) to remove residual cells. For gel electrophoresis and subsequent Western blot analysis, samples of the medium were loaded directly or (where indicated) were first concentrated by 10 % tricholoroacetic acid (TCA) on ice overnight. After centrifugation the protein precipitate was washed with 70% cold acetone three times and re-suspended in 0.5 ml of SDS-electrophoresis buffer. Cell pellets were washed once and re-suspended in 1 ml of 10 mM Tris-HCl (pH 8.0), containing 1 mM EDTA and 1 mM PMSF. Cells were ruptured in an Aminco French

Press at 10,000 psi. For analysis of soluble fractions, the cell extracts were centrifuged at 16,000 x *g* for 5 min to remove large debris, or inclusion body if any.

Measurement of carboxylesterase activity. The esterase activity of Est55 was measured with *p*-nitrophenyl caproate (Sigma-Aldrich) as substrate, and the presence of *p*-nitrophenol was monitored by spectrophotometry, as described previously (Wood, Fernandez-Lafuente et al. 1995). The protein concentration was measured by the Bradford method, using bovine serum albumin as standard (Bradford 1976).

Gel electrophoresis and immunoblotting. Protein samples were analyzed by standard Laemmli sodium dodecyl sulfate (SDS) electrophoresis in 12.5 % polyacrylamide gels (Laemmli 1970). For comparison of protein profiles, using Coomassie Blue staining, equal volumes of secreted protein fraction and cell extracts (corresponding to 50 µl culture volumes) were applied to the gel for electrophoresis. For immunoblot analysis, protein samples were diluted 10 times, and (following electrophoresis) proteins on the gel were transferred to polyvinylidene difluoride (PVDF) membrane (Problot, Applied Biosystems) (Towbin, Staehelin et al. 1979) for further analysis. Membranes were developed in alkaline phosphatase substrates, according to manufacturer protocols. The immunoblots were first scanned by densitometer GS-800 (BioRad, Calif.) and then quantitated by Quality One® computational software (BioRad, Calif.). The percent secretion of individual protein was presented as ratio, which is calculated based on the amount of extracellular portion vs. total individual protein (intracellular + extracellular).

Antibodies. Antisera against purified Est55, SecA, EF-Tu, SodA (Liu 2007) and enolase were from lab stocks. Rabbit antibodies were developed in rabbit (New Zealand) by subcutaneous injections of 5 mg of purified enzyme mixed with an equal volume of Freund's complete adjuvant (Cooper 1995). The primary antibody against FLAG-tag was purchased from Sigma-Aldrich (Sigma-Aldrich, St. Louis, MO). The alkaline phosphatase-linked goat anti-rabbit antibodies (Bio-Rad) were used as secondary antibodies for all reactions, except for EF-Tu, for which alkaline phosphatase-linked donkey anti-goat antibodies (Promega) were employed.

Est55 purification. For intracellular Est55, a one-liter culture of *B. subtilis* WB600BHM harboring pHE550 was induced by 1 mM IPTG (as above) for 4 hours. The cell pellet was harvested, washed once and re-suspended in 20 ml of 10 mM Tris-HCl buffer (pH 8.0), after which the sample was ruptured through an Aminco French Press at 16,000 psi. The cell debris was removed by centrifugation at 7,500 x *g* for 15 min at 4 ^{o}C. The supernatant was heated at 60 ^{o}C for 20 min, and the precipitated proteins removed by centrifugation at 18,000 x *g* for 20 min at 4 ^{o}C.

To purify the extracellular Est55, a one-liter culture of *B. subtilis* WB600BHM harboring pHE550 was induced by 1 mM IPTG at OD_{600} ~ 0.2 for 10 hours. The culture was centrifuged for 15 min at 7,500 x *g* to collect the supernatant that contained the secreted Est55. The purification protocol was as described (10).

N-terminal amino acid analysis. Proteins from the secreted fraction after filtration were precipitated by 10% TCA, washed with cold 70% acetone, separated by SDS-

PAGE, and then electrophoretically transferred to PVDF membrane (Towbin, Staehelin et al. 1979). Protein bands were excised and their amino terminal sequences were determined by ABI Procise Sequencer 493A in the Molecular Biology Core Facility of Georgia State University.

RESULTS

Carboxylesterase Est55 from *G. stearothermophilus* is secreted. We have previously characterized a carboxylesterase, Est55, of *G. stearothermophilus* ATCC7954 that was cloned and expressed in *E. coli* (Ewis, Abdelal et al. 2004). The protein contains no typical cleavable signal sequence (Fig.1.1A). To investigate whether this hydrolytic enzyme can be secreted by its parent strain *G. stearothermophilus* ATCC7954. Enzymatic activity measurements (Fig. 1.1B) and immunoblot analysis (Fig. 1.1C) were used to detect the presence of chromosome-encoded Est55 in the intracellular and extracellular fractions of bacterial culture. Expression of the chromosome-encoded Est55 was induced when cell growth reached early stationary phase, followed by the presence of Est55 in the extracellular fractions that continued into late stationary phase. These results indicate that Est55 is expressed and secreted from its parent strain in the stationary phase.

Secretion of Est55 from a protease-deficient strain of *B. subtilis*. To facilitate the study of Est55 secretion in *B. subtilis*, plasmid pHE550 (harboring the *est55* gene) was constructed. Est55 was induced from the P_{spac} promoter and expressed in *B. subtilis* WB600BHM, in which six protease genes have been inactivated (Wu, Lee et al. 1991).

Induction of Est55 from the P_{spac} promoter was initiated by the addition of 1 mM IPTG in early log phase (Fig. 1.2A) and monitored over time by immunoblots. Virtually all of Est55 remained soluble inside the cells. Under the conditions used, the intracellular levels of Est55 reached a plateau at about 9 hrs after inoculation and, thereafter, gradually decreased through the late stationary phase. Concomitantly, the extracellular Est55 in the medium also started to appear at 9 hrs, reaching a plateau at 12 hr (Fig. 1.2B). At this time point, there was almost twice as much Est55 in the medium as there was inside the cells (Fig. 1.2B). These results indicate that Est55 is secreted by *B. subtilis* during stationary phases, and that this secretion is substantial: there was more Est55 present in the medium than inside the cells.

The secreted Est55 is not processed. To investigate further the possibility that the Est55 was subjected to N-terminal cleavage during secretion, both the intracellular and extracellular Est55 that were expressed from *B. subtilis* WB600BHM harboring pHE550 were purified to homogeneity. The first 10 amino acids of Est55 from both protein sources were determined as being M-E-R-T-V-V-E-T-R-Y, which are identical to the derived protein sequences from the published nucleotide sequences, as well as the sequences of Est55 protein expressed and purified from *E. coli* (Ewis, Abdelal et al. 2004). In addition, the sizes of intracellular and extracellular Est55 were identical on SDS-PAGE (data not shown). These results indicate that there is no cleavage of the secreted Est55 on neither N- nor C-termini during the secretion process. Moreover, the secretion is not affected by a proton motive force inhibitor or by azide, and in the presence of chloramphenicol the intracellular Est55 is secreted into the medium (data not shown), indi-

cating that neither Sec nor TAT system is involved in the secretion of Est55 even though it contains twin-arginine residues in the N-terminus (Fig.1.1A).

Other cytoplasmic proteins lacking typical signal peptide are secreted in stationary phase. To determine whether the secretion of Est55 is unique or a general phenomenon in *B subtilis*, we analyzed the protein profiles of extracellular fractions during growth on SDS-PAGE, as shown in Fig. 1.3A. Surprising, the extracellular proteins are abundant in late stationary phase. Secretion of several major proteins lasted for several hours into the late stationary phase; these proteins were subjected to N-terminal amino acid sequencing. The identified proteins included: DnaK (band a), GroEL (band b), homoserine dehydrogenase (band d), enolase (band e), YdjL of unknown function (band f), flagellin Hag (band g), pyruvate dehydrogenase subunit PdhB (band h), and superoxide dismutase SodA, (band j) (Fig. 1.3A). Remarkably, most of these proteins are normally considered to be intracellular cytoplasmic proteins. Similar to Est55, no N-terminal cleavage can be detected for any of these proteins, as judged from sequence comparison, except for chitosanase (band i) which had lost its signal peptide; secretion of chitosanase is SecA-dependent (Rivas, Parro et al. 2000).

The secretion patterns for two of these proteins, enolase and SodA, were shown by immunoblot analysis to be similar to that of Est55 (Fig. 1.3B). Thus, the presence of these proteins, which lack any of the classical signal peptide sequences, in the growth medium of cells in late stationary phase represents a non-classical secretion of proteins by *B. subtilis*.

Non-classical protein secretion is due to cell lysis? The presence of these intracellular proteins in the growth medium of cells in stationary phase is most likely not due to cell lysis as evidenced by the constant OD_{600} (1. 2A) and cell viability counts throughout the growth phases at about $3x10^9$/mL (data not shown). It is possible; however, that the constant cell density or viability in stationary phase could be the result of concomitant cell growth and cell lysis at a comparable rate. To test this possibility, chloramphenicol was added to the culture of WB600BHM harboring pHE550 at the time point when signal-less proteins start to be secreted to the medium. The cell density remained constant for three hours after the addition of chloramphenicol (Fig. 1.2A, filled diamond). Under these conditions, the secretion of enolase and SodA continued, as shown by immunoblots (Fig. 1.3B). These results strongly suggest that the secretion of these proteins is not due to cell lysis, as these proteins were secreted from existing pools in the cells, and new protein synthesis is not required for their presence in the medium. To further rule out the possibility of general cell lysis under the conditions used, immunoblots were performed with specific antibodies against two major (and abundant) intracellular proteins functioning in protein secretion and translation: SecA, and EF-Tu. As shown in Fig. 1.3C, SecA was undetectable, and only very low levels of EF-Tu (less than 5% of the intracellular fractions) could be detected in the medium.

Non-classical secretion is a common phenomenon. To examine the possibility that the observation of non-classical protein secretion is due to the unique genetic background of the *Bacillus* strain WB600BHM, we also performed similar experiments in *B. subtilis* 168 harboring pHE550 in the presence of the protease inhibitor PMSF. The re-

sults were similar to those for the protease-deficient *B. subtilis* WB600BHM harboring pHE550. The quantities of secreted Est55, SodA, GroEL, and enolase at 12 hr samples were estimated to be 30-50% of the total amounts of each individual protein (Fig. 1.4A). The extracellular protein profiles and the signal-less proteins, as identified by N-terminal peptide sequencing in strain 168 (data not shown) were similar to those in strain WB600BHM, as described in Fig. 1.3A. These findings strongly suggest that the observed appearance of a specific set of cytoplasmic proteins (without signal peptides) in the medium appears to be a general phenomenon in *B. subtilis* during late stationary phase.

Lytic enzymes do not significantly alter signal-less protein secretion. Cell lysis has been intensively studied and its mechanism has been correlated to autolysins (Kodamaa 2007). We examined a *B. subtilis lytC lytD* double mutant SH128 lacking two major autolysin genes and its parental strain 1A304. The cells were grown at 30^{o}C because of a growth defect of SH128 at 37^{o}C. The distribution of intra- and extra-cellular GroEL, enolase, and SodA were analyzed by immunoblots. Quantitative analysis revealed that the ratios of secreted proteins in strain 1A304 were comparable to those in SH128 (Fig. 1.4B). These findings support the notion that the observed secretion of a specific set of cytoplasmic proteins is a general phenomenon in *B. subtilis* during the stationary phase and is not due to cell autolysis.

Signal-less protein secretion is not mediated significantly by membrane vesicles. It was reported that enolases from *Bacillus anthracis* and *Neisseria meningitidis*

enolases can be detected in the membrane vesicles in the media (Agarwal 2008). However, the amounts have not been reported. To determine the extent of membrane vesicle-mediated secretion in our studies, medium fractions were separated after 11 hrs incubation, and the membrane fraction was collected by ultracentrifugation. Distribution of signal-less proteins in the pellet and soluble fraction was determined by immunoblot analysis. Negligible amounts of Est55 and SodA, and less than 15% of enolase were detected in the membrane pellet fraction (Fig. 1.4C). (The pellet fraction by ultracentrifugation may also contain large macromolecule complexes, such as enolase with a molecular mass of about 400,000 daltons). These results indicate that the secretion mediated by the shedding of membrane vesicles is not significant.

A hydrophobic helix domain of enolase is crucial for its secretion. To provide further evidence for the secretion of cytoplasmic proteins in the absence of cell lysis, we examined the possible structural properties required for these proteins to be secreted. We used *B. subtilis* enolase (EnoBs) as the model system. Enolase is a key glycolytic enzyme and has been reported as a plasminogen-binding protein that is displayed on the bacterial cell surface (Agarwal 2008; Feng 2009). Using the crystal structure of *Enterococcus hirae* enolase as a template, a predicted 3D molecular structure was generated by Swiss-Model (Swiss-Prot database entry No. 37869). A long, unbent α-helix (from A108 to L126) and a membrane-embedded domain (EM domain) (Hu, Holley et al. 2007) residing in the N-terminal region of enolase (Fig. 1.5A, 1.5B) were identified as potential targets. A plasmid containing the *eno* gene in which the α-helix had been deleted (EnoBsΔH) was used for the expression of the mutated enolase in *B. subtilis*. The expressed EnoBsΔH

protein remained soluble and could be identified with a slightly smaller size than the wild-type enolase (EnoBs) as monitored by immunoblots. The results showed that the deletion of this α-helix domain completely abolished secretion of plasmid-coded EnoBsΔH, while the chromosomal wild-type enolase was secreted from the same cell cultures under identical conditions (Fig. 1.5C).

Within this α-helix, there is a hydrophobic EM domain (residue 110 to 118) (Fig. 1.5B) that is predicted by the PSSM-SVM scheme (Hu, Pan et al. 2004; Hu, Holley et al. 2007) to be important for embedding proteins into the membranes. To determine its importance for secretion, this EM domain was replaced with a β-galactosidase sequence (EMR), leaving the predicted α-helix structure effectively unchanged by prediction, but with reduced hydrophobicity. To facilitate the detection of enolase with similar sizes, two plasmids were constructed with an N-terminal FLAG tag to yield pEnoFLAG (FLAG-tagged EnoBs), and pEMRFLAG with the replaced EM domain.

Based on modeling by AMMP (http://www.cs.gsu.edu/~cscrwh/ammp/ammp.html), or Swiss-Model (http://swissmodel.expasy.org), the replacement shows no predicted change on conformation (Fig. 1.5A). The expressed Eno-FLAG and EMR-FLAG proteins from plasmids were soluble in the cell extracts, and displayed slightly larger sizes than the chromosomal EnoBs, as determined by immunoblots (Fig. 1.5D). The results showed that both the soluble Eno-FLAG and EnoBs can be secreted into the growth medium concomitantly, and that modification of this EM domain in the α-helix completely abolished secretion of the EMR-FLAG (Fig. 1.5D). The same result was obtained when

FLAG antibody was used to detect specific, FLAG-tagged enolase. Importantly, while the mutated enolase (encoded by the plasmid) was not detected, the wild-type chromosomal enolase was detected in the medium, indicating that the structural EM element is important for enolase secretion.

It is possible that the mutation of enolase resulted in a protein that might be more susceptible to degradation. Hence, we determined the stability of plasmid encoded EnoBs and EMR in the medium, as well as SecA which was found to be negligible in the medium (Fig. 1.3C). The soluble crude extracts of the stationary-phase cells were prepared and added into the stationary-phase culture. The stability of added EnoBs and EMR, incubated in the medium at 37 ^{o}C, were also monitored by immunoblots. As shown in Fig. 1.5E, EnoBs/EMR of the added crude extracts was stable for at least 120 min without apparent degradation, while no EnoBs/EMR was found in the control sample without the addition of crude extracts. Thus, the absence of EMR in the growth medium is a consequence of the mutation of the membrane- embedding domain. SecA was also monitored for its stability under the same condition. The results showed that the extracellularly added SecA was stable for at least 2 hr (Fig. 1.5E), thus support the absence of SecA as a valid indicator for the lack of cell lysis (Fig. 1.3C).

The absence of EMR-FLAG and EnoBsΔH in the stationary phase medium demonstrates the importance of the predicted hydrophobic α-helix structure in EnoBs secretion. The differential secretion from the same cells for the presence of enolase in the late stationary phase, together with other lines of evidences strongly indicate that cell lysis is

a negligible factor for the secretion of non-classical proteins, and that the secretion is a general phenomenon in *B. subtilis*.

DISCUSSION

In this study, carboxylesterase Est55, along with several conventional endogenous cytoplasmic proteins from *B. subtilis,* were found to be secreted during the late stationary phase. N-terminal amino acid sequence analyses of these proteins confirmed the absence of any classical, signal peptide cleavage that is known to be involved in a Sec-dependent pathway (Tjalsma, Bolhuis et al. 2000). More importantly, several lines of evidence show that secretion of these proteins without a classical, signal peptide in *B. subtilis* in the stationary phase is not due to cell lysis; rather it is a general phenomenon.

The appearance of cytoplamic proteins in the stationary phase in *B. subtilis* is controversial. The existence of non-classical protein secretion has been reported (Antelmann 2001; Tjalsma 2004), while others attributed the appearance to cell lysis (Tjalsma, Bolhuis et al. 2000; Antelmann 2006). None of these reports, however, provide conclusive, quantitative analyses as to whether cell lysis contributes significantly to the observed phenomenon. In this study, the cells were grown in LB broth with glucose to minimize the effect of sporulation and cell lysis. We have observed gradual cell lysis in the stationary phase in LB broth, but glucose supplement prevents the lysis of cells, even in the very late stationary phase. Under these conditions, we show that cell lysis cannot account for the secretion of proteins in the late stationary phase by several lines of evi-

dences: constant cell viability count, no change in cell density or secretion in the presence of chloramphenicol (Fig. 1.2A), the negligible amounts of SecA and EF-Tu in the medium, minimal effect on secretion using an autolysin mutant, and (most importantly) the defective secretion of mutated enolase enzymes that harbor mutations in a hydrophobic membrane-embedding domain. The differential secretion of mutated and wild-type enolases from the same cell culture provides the most compelling evidence that cell lysis plays little role in the secretion of these signal peptide-less proteins.

Mutation of the α-helix EM domain in enolase results in a complete abolition of secretion but a mutation of another α-helix (Q132P) has no effect (data not shown). These finding strongly indicate the importance of this hydrophobic helix in the export of this protein. However, though this domain is essential, it is not sufficient to promote the secretion of green-fluorescent protein as a marker protein (unpublished). A recent report introduced a new prediction algorithm for the identification of non-classical secretory proteins, based on the biological and chemical properties; such as threonine contents, trans-membrane helices, and protein disorder in the structure (Bendtsen 2005). It is noted that enolase, pyruvate dehydrogenase (S-complex) (Caulfield, Horiuchi et al. 1984; Caulfield, Furlong et al. 1985; Hemila, Palva et al. 1990), and GroEL are predicted to be non-secreted proteins using this prediction algorithm, while in our study they are identified as abundant, secreted proteins.

Recent publications (7, 14) indicated that several cytoplasmic proteins, present in extracellular environment, are associated with membrane vesicles in Gram-positive bac-

teria. However, the extent to which the membrane-vesicles mediate release of these proteins has not been reported. We found that such release is not significant for Est55, SodA and enolase (Fig. 1.4C). In addition to enolase, the presence of a small amount of GroEL and pyruvate dehydrogenase in the pellet fraction is likely due to the co-sedimentation of the large complexes (all over 400,000 mass) by ultra-centrifugation. Alternatively, these cytoplasmic proteins may not be secreted or remain as the large complexes in the medium.

Autolysins have been proposed to play several roles in motility, cell separation, competency, antibiotic-induced lysis, pathogenesis, cell wall synthesis and turnover, and differentiation (Blackman, Smith et al. 1998). The LytC and LytD proteins account for 95% of autolysin activity in *B. subtilis*. In this study we used a strain with *lytC lytD* double mutations, which is devoid of prophage-associated lytic enzymes (Blackman, Smith et al. 1998). We found that a similar secretion pattern in the autolysin-deficient mutant and its parent strain (Fig. 1.4B), indicating that autolysins are not a major factor. Moreover, the detection of this particular class of proteins in the extracellular environment of several species of Gram-positive bacteria, other than *B. subtilis*, supports the notion that these proteins are secreted through specialized route(s) (Sibbald, Ziebandt et al. 2006).

It has also been reported that *B. subtilis* possesses an active export system represented by the *yukABCDE* operon (Pallen 2002). This operon is homologous to the ESAT-6 secretion system, which is involved in the secretion of small virulence proteins

that lack a classical signal peptide in several pathogenic Gram-positive bacteria (Burts, Williams et al. 2005). The possible role of this system in the secretion in our study has been investigated in *B. subtilis* L16601, and its derivative mutants that contain a systemic deletion in each of the genes in *yukABCDE* operon (Sao-Jose, Baptista et al. 2004). We found similar protein profiles (data not shown), indicating that the ESAT-6 homologous transport system is not involved in the secretion of this particular class of signal-less proteins. Furthermore, this class of proteins does not possess the conserved WXG motif proposed for the ESAT-6/WXG100 superfamily of secreted proteins (Pallen 2002).

A recent report (Antikainen 2007) presented a pH effect on the release of enolase and glyceraldehyde-3-phosphate dehydrogenase of *Lactobacillus crispatus*. The authors showed that these non-classical secretory proteins were localized on the cell surface at pH 5 but released into the medium at pH 8. This release from the cell surface at alkaline pH was attributed to a quick response of *Lactobacilli* to changing environments (Antikainen 2007). Such pH-dependent release of non-classical secretory proteins is unlikely in our study since we did not observe any significant change of pH during late stationary phase. It is worth noting that these proteins that are secreted in *B. subtilis*, are accumulated in the cytoplasm in the early stationary phase before being secreted in the late stationary phase. It is not yet clear what signal(s) triggers the secretion. One important element may be the structural domains within the proteins. With the exception of SodA, all proteins identified in this work possess a putative short EM domain (Hu, Pan et al. 2004; Hu, Holley et al. 2007). The mutation study of the EM domain for enolase may shed light on the possible importance of such structure for the secretion mechanism. Re-

gardless of the mechanism of secretion, the presence of several cytoplasmic proteins such as GroEL, SodA, and enolase in the medium with defined physiological cytoplasmic functions raises an intriguing question: What is the significance of their presence in the medium? It is noted that many of these key proteins are involved in energy consumption. Perhaps "dumping" these enzymes provides a way to quickly reduce ATP consumption or glycolysis, and re-orientate metabolism in preparation for sporulation or for cell survival. Alternatively, it would be interesting to explore whether the secreted proteins have functions that differ from their cytoplasmic counterparts. It has been reported that many proteins have "moonlighting" functions (Jeffery 1999; Jeffery 2003), and that *Streptococcus* enolase, for example, is an important factor for plasminogen binding and adhesion during host invasion (Kolberg, Aase et al. 2006).

In conclusion, this study provides several lines of strong evidence for the non-classical secretion of large amounts of cytoplasmic proteins in stationary phase growth, and excludes cell lysis as the cause of this phenomenon *in B. subtilis*.

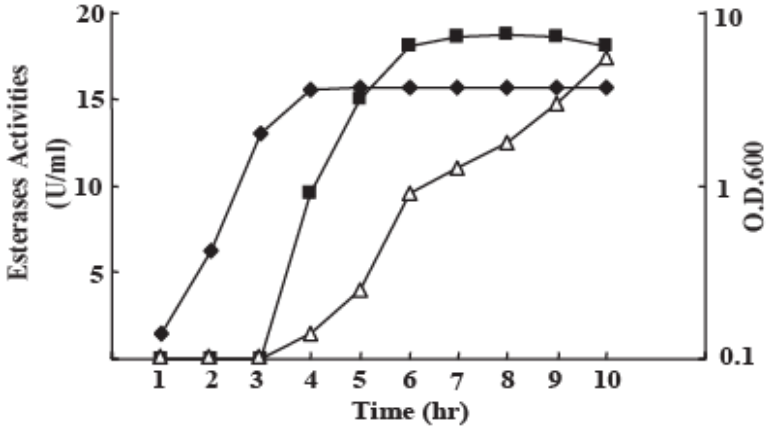
ACKNOWLEDGMENTS

The authors thank Sui-Lam Wong, Mario A. Santos and Simon J. Foster for providing strains and John E. Houghton for reviewing the manuscript. This work was supported in parts by the National Institute of Health grant GM 34766. The Biology Core Facilities are supported by Georgia Research Alliance, and Center for Biotechnology and Drug Design. CKY and HJH are fellows of the Program in Molecular Basis of Diseases at Georgia State University.

A

```
                                                    SD
GGGTGGTGGTTTATAATATTTCTTGAATCATTTGAATATTCAGGAGGTATGGGG 108

ATGGAGCGAACCGTTGTTGAAACAAGGTACGGACGGTTGCGCGGGGAAATGAAT 162
 M  E  R  T  V  V  E  T  R  Y  G  R  L  R  G  E  M  N

GAAGGCGTTTTTGTTTGGAAAGGAATTCCGTACGCGAAAGCGCCGGTCGGTGAG 216
 E  G  V  F  V  W  K  G  I  P  Y  A  K  A  P  V  G  E

CGCCGGTTTTTGCCGCCGGAGCCGCCCGATGCGTGGGATGGGGTGCGGGAGGCG 271
 R  R  F  L  P  P  E  P  P  D  A  W  D  G  V  R  E  A

ACATCGTTCGGTCCTGTCGTGATGCAGCCGTCGGATCCGATTTTCAGCGGATTG 325
 T  S  F  G  P  V  V  M  Q  P  S  D  P  I  F  S  G  L
```

B



C

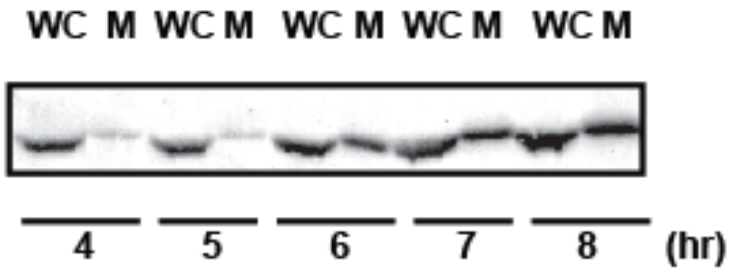
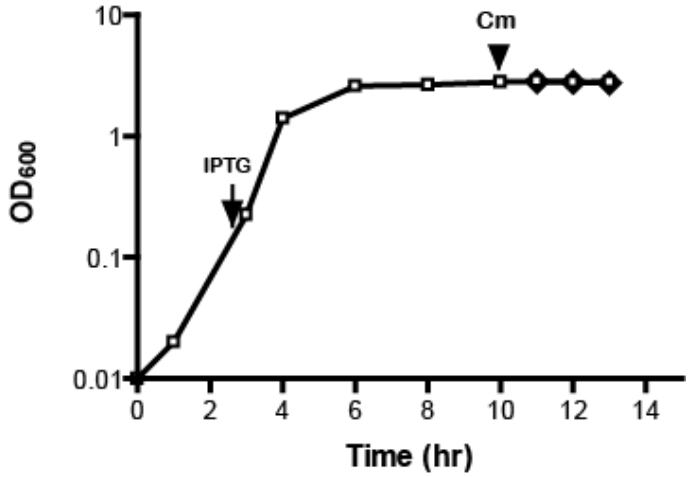
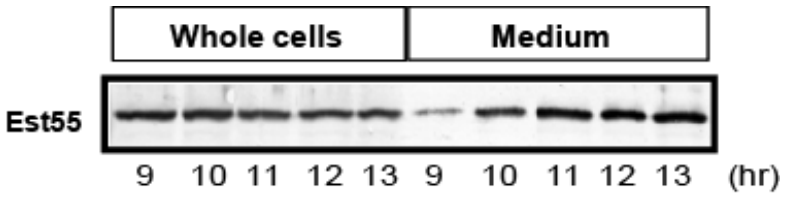


Figure 1.1. (A) Partial DNA sequence of *est55* and the deduced amino acids. The probable Shine-Dalgarno (SD) sequence is bold italicized. The determined N-terminal amino acid from purified extracellular protein is italicized, while the twin arginine residues are boxed (adapted from Dr. Ewis dissertation). (B) Est55 expression profiles in *G. stearothermophilus* by enzymatic activities. Cell growth was monitored (filled diamonds), and the intracellular (filled squares) and extracellular (open triangles) Est55 esterase activities were measured. (C) The Est55 of whole cell fraction (WC) and medium fraction (M) at the indicated time points of the growth curve were determined by immunoblots.

A



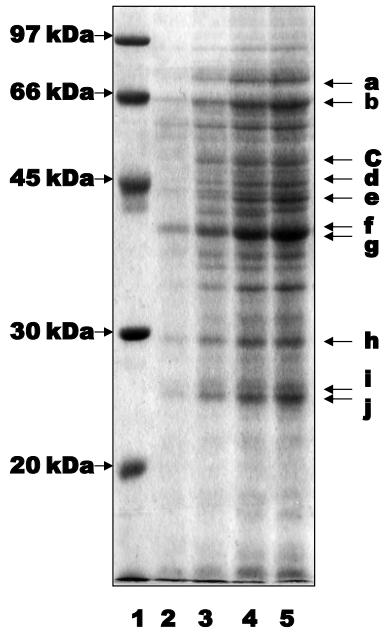
B



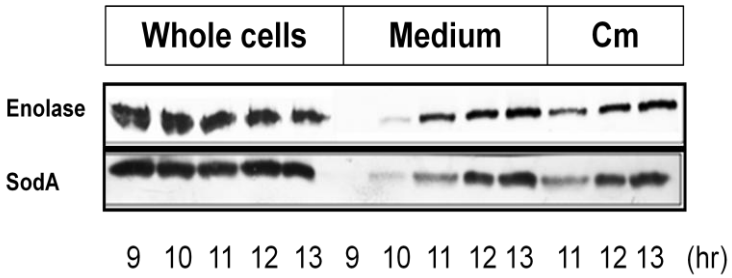
hr	9	10	11	12	13
Ratio (%)	18.5	42.0	59.1	64.8	68.0

Figure 1.2. Secretion of Est55 and other cytoplasmic proteins in *B. subtilis*. (A) Cell growth was monitored (open squares). *B. subtilis* WB600BHM carrying pHE550 were induced with 1mM IPTG to express *est55* (filled arrow). Chloramphenicol (Cm) was added 10-hours after incubation (filled diamond) indicated in the text. (B) The amounts of Est55 from whole cells and medium were detected by immunoblots. The ratios of secreted Est55 in the medium at various times were calculated.

A


97 kDa
66 kDa
45 kDa
30 kDa
20 kDa
a
b
c
d
e
f
g
h
i
j
1 2 3 4 5

B


Whole cells
Medium
Cm
Enolase
SodA
9 10 11 12 13 9 10 11 12 13 11 12 13 (hr)

C

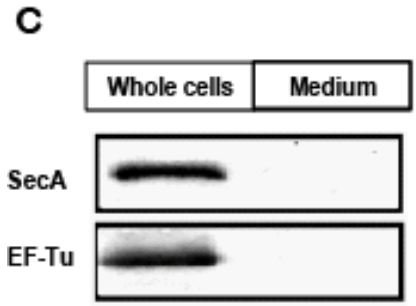
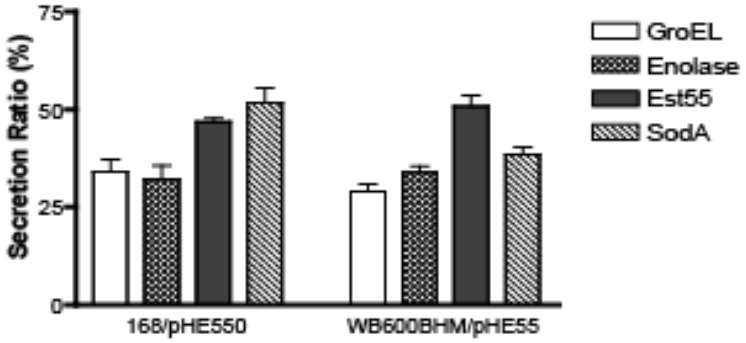
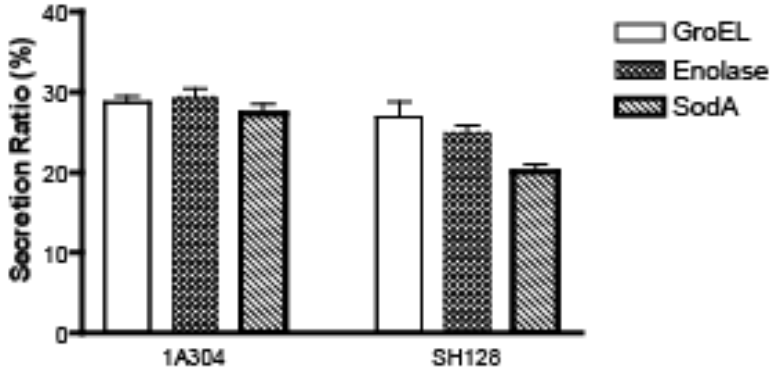

C
Whole cells
Medium
SecA
EF-Tu

Figure 1.3. Identification of secreted proteins in *B. subtilis* WB600BHM. (A) The protein profile of *B. subtilis* WB600BHM carrying pHE550 during growth. The profile was shown by Coomassie Blue staining after gel electrophoresis of ~~the~~ extracelluar samples at various times of cell growth. Lane 1, molecular weight markers. Lanes 2-5, 4hr, 6hr, 8hr, and 10hr samples respectively. Protein bands from extracellular samples were identified by N-terminal sequences: a-DnaK, b-GroEL, c-Est55, d-homoserine dehydrogenase, e-enolase, f-YdjL of unknown function, g-flagellin Hag, h-PdhB subunit of pyruvate dehydrogenase, i-chitosanase, and j-superoxide dismutase SodA. (B) Immunoblots of intracellular and extracelluar enolase and SodA. Whole cells and growth medium fractions were taken from the 9^{th} hour to the 13^{th} hour time points corresponding to the control samples of Figure 2A, The effect of chloramphenicol (Cm) on the secretion of enolase and SodA were examined by immunoblots from the extracellular samples from Figure 2A. (C) Absence of cytoplasmic markers EF-Tu and SecA in the growth medium. Protein samples from whole cell and growth medium were taken from the 10^{th} hrs culture.

A


75
50
25
0
Secretion Ratio (%)
GroEL
Enolase
Est55
SodA
168/pHE550
WB600BHM/pHE55

B


40
30
20
10
0
Secretion Ratio (%)
GroEL
Enolase
SodA
1A304
SH128

C

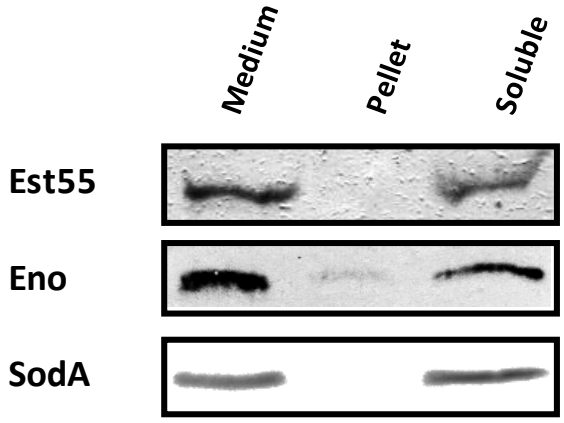
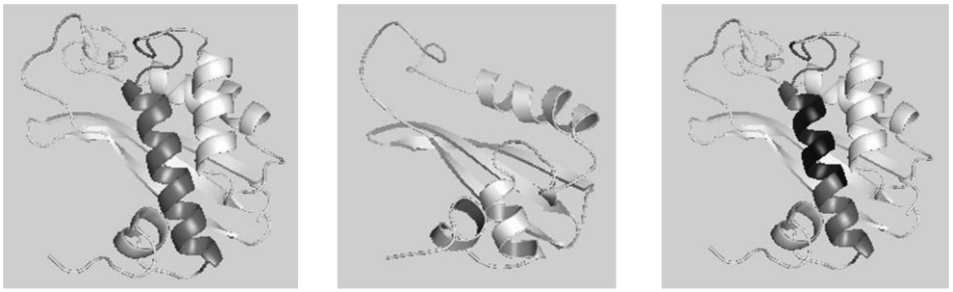
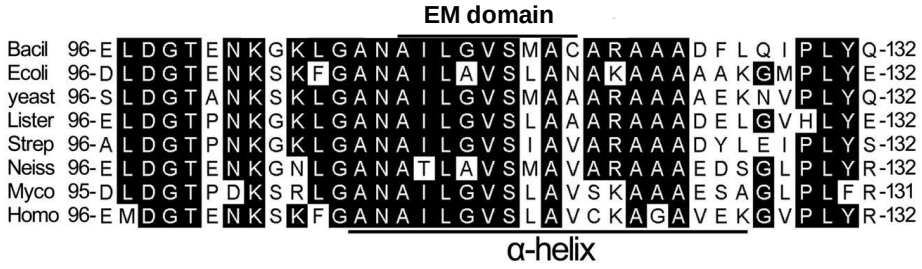

Medium
Pellet
Soluble
Est55
Eno
SodA

Figure 1.4. Protein secretion of *B. subtilis* 168/pHE550 and WB600BHM/pHE550 (A) the *lytClytD* double mutant SH128 and its parental strain 1A304 (B) Extracellular samples were collected after 12 hrs incubation. GroEL, enolase, Est55, and SodA were detected by immunoblots and quantified. The secretion ratios were presented as the percentages of extracellular proteins to the total amounts. The values are averages of at least three independent experiments. (C) Lack of significant presence of signal-less proteins in the extracellular membrane vesicle. The soluble fraction was the supernatant of ultracentrifugation of the collected medium after 11 hrs incubation. The membrane vesicle fraction (MV) was the pellet of ultracentrifugation resuspended to the original volume. Equal volumes of samples were used for immunoblotts.

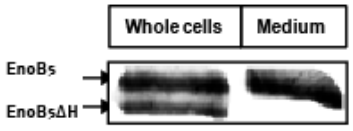
A


Wild type
α-Helix deletion
EM replacement

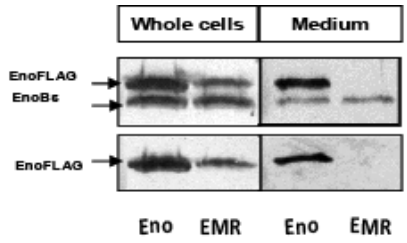
B


EM domain
Bacil 96-E L D G T E N K G K L G A N A I L G V S M A C A R A A A D F L Q I P L Y Q-132
Ecoli 96-D L D G T E N K S K F G A N A I L A V S L A N A K A A A A A K G M P L Y E-132
yeast 96-S L D G T A N K S K L G A N A I L G V S M A A A R A A A A E K N V P L Y Q-132
Lister 96-E L D G T P N K G K L G A N A I L G V S L A A A R A A A D E L G V H L Y E-132
Strep 96-A L D G T P N K G K L G A N A I L G V S I A V A R A A A D Y L E I P L Y S-132
Neiss 96-E L D G T E N K G N L G A N A T L A V S M A V A R A A A E D S G L P L Y R-132
Myco 95-D L D G T P D K S R L G A N A I L G V S L A V S K A A A E S A G L P L F R-131
Homo 96-E M D G T E N K S K F G A N A I L G V S L A V C K A G A V E K G V P L Y R-132
α-helix

C


Whole cells
Medium
EnoBs
EnoBsΔH

D


Whole cells
Medium
EnoFLAG
EnoBs
EnoFLAG
Eno
EMR
Eno
EMR

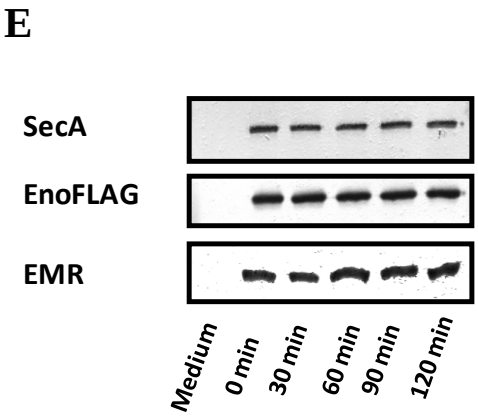


Figure 1.5. A hydrophobic domain required for enolase secretion. (A) Predicted molecular structure of enolase and its mutants. Only the N-terminal domain of enolase is shown here. The hydrophobic α-helix is highlighted in light dark ribbon. The EM domain replaced by β-galactosidase sequence of *E. coli* is highlighted by dark ribbon. The figure was drawn using the PyMOL v0.99 program. No apparent conformational change is observed by EM replacement. (B) Multiple sequence alignment of various enolases. Numbers at the ends of each line represent residues from the amino termini: *B. subtilis* (Bacil), *E. coli* (Ecoli), *Saccharomyces cerevisiae* (yeast), *Listeria monocytogenes* (Lister), *Streptococcus pneumonia* (Strep), *Neisseria meningitides* (Neiss), *Mycobacterium smegmatis* (Myco) and *Homo sapiens* (Homo). The sequences of *B. subtilis* hydrophobic α-helix and the EM domain region are indicated. (C) Loss of enolase secretion with a deletion of the α-helix domain. Strain WB600BHM harboring pDGEnoBsΔH was induced by 1 mM IPTG, and intracellular and extracellular enolase from the 10^{th} hr samples were examined by immunoblots with anti-enolase antibodies. EnoBsΔH, the mutated enolase ; EnoBs,

chromosomal-coded enolase. (D) Differential secretion of plasmid-coded enolase (EnoF-LAG) and chromosomal-coded enolase (EnoBs). The whole cell and medium samples were collected from 10^{th} hr cultures of strains WB600BHM harboring pGTN-Eno (Eno) and pGTN-EMR (EMR) and subjected to immunoblot analysis. The results with anti-enolase and anti-FLAG antibodies were shown in the upper panel and lower panel, respectively. (E) Stability of SecA, EnoFLAG and EMR in the medium. The samples were examined by immunoblots with SecA and FLAG antibodies respectively. Lane 1, control sample from medium lacking SecA, EnoFLAG and EMR, Lanes 2-6 were medium samples with whole cell extracts at 0, 30, 60,90, and 120 min.

Table 1.1. Bacterial strains and plasmids

Bacterial strains, and Plasmids	Genotype or description	Source or reference
E. coli DH5 α	F⁻ 80 Δ*lac* ΔM15 Δ(*lacZYAargF*) U169 *deo*R *rec*A1	
	*end*A1*hsad*R17 ($r_k m_k$) *supE44* λ⁻thi-1*gyr*A96*rel*A1	BRL
G. stearothermophilus	ATCC7954	ATCC
B. subtilis		
WB600 BHM	*trpC2 npr*E Δ*apr*E Δ*bpf* Δ*epr mpr*::*ble npr*B::*bsr.*	(46)
168	*trpC2*	Laboratory stock
1A304	*trpC2metB5 xin-1 SPβ* (s)	(7)
SH128	*trpC2metB5 xin-1 SPβ* (s) *lytC* :: *ble lytD* :: *spc*	(7)
L16601	*B. subtilis* 168; SPP1 indicator strain	(34)
Plasmids		
pDG148	*bla, kan*, shuttle vector	(47)
pCOS4	*bla*, *est30*, *est55*	(15)
pNW33N	*bla, cat, lacI ,* shuttle vector	(De Rossi 1994)
pHE550	*bla*, *kan*, *lacI*, *est55*	This study
pDGEnoBsΔH	*bla*, *kan*, *lacI*, *enoBsΔH*	This study
pGTN	*bla, cat, lacI ,* shuttle vector	This study
pGTN-FLAG	*bla, cat, lacI ,* shuttle vector	This study
pEnoFLAG	*bla, cat, lacI , eno-FLAG*	This study
pEMRFLAG	*bla, cat, lacI , EMR-FLAG*	This study

CHAPTER 2

A highly conserved internal hydrophobic region of *Bacillus subtilis* enolase is involved in secretion:

A putative signal for secretion in *B. subtilis* and *E. coli.*

Chun-Kai Yang, Xiao-Zho Zhang, Chung-Dar Lu, Phang C Tai*

Department of Biology, and Center of Biotechnology and Drug Design, Georgia State University, Atlanta, GA 30303

Running title: A highly conserved region of *Bacillus subtilis* enolase for secretion

* Corresponding author.
Mailing address: Department of Biology, Georgia State University, Room 492, Petite Science Center, 161 Jesse Hill Drive, Atlanta, Georgia, 30303.
Phone: 404-413-5303
Fax: 404-413-5301
Email: biopct@gsu.edu

Abbreviation used: IPTG, isopropyl b-D-1-thiogalactopyranoside; LB, Luria–Bertani; OMVs, outer membrane vesicles; SD, Shine-Dalgarno; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis. EM domain, Embedded membrane domain. HC, hydrophobic core

ABSTRACT

Many cytoplasmic proteins without a cleavable signal peptide, including enolase, are secreted during the stationary phase in *Bacillus subtilis* in the absence of cell lysis (Yang et al, 2011). The molecular mechanism of enolase secretion is not yet clear. We have identified a hydrophobic helix of enolase that may play an important role in its secretion. Within this helix a highly conserved embedded membrane (EM) domain located in the internal region is critical for the secretion of enolase. Genetic alternation by deletion and substitution showed that this domain is required for the secretion. On the other hand, mutations on the conserved basic residues flanking EM region showed no effect. *Bacillus* enolase is exported in *Escherichia coli* while the replacement of EM domain blocks secretion indicating that the secretion of enolase is not species-specific. GFP fusion experiments showed that minimal length of N-terminus 140 amino acids and its tertiary structure of EM domain are required to serve as a functional signal for the secretion of enolase in *Bacillus*. We conclude that the internal hydrophobic core of enolase is essential but it is not sufficient for its secretion, and the whole long N-terminus is required to serve as an internal signal for secretion.

INTRODUCTION

Enolases (EC 4.2.1.11) are essential cytoplasmic enzymes that catalyze the reversible conversion of 2-phosphoglycerate into phosphoenolpyruvate. Although enolases lack a classical signal sequence and typical motifs for membrane or cell wall anchoring, many reports showed that enolases can be exported to the cell surface or released to the culture medium in eukaryotic and prokaryotic organisms (Dudani 1993; Pancholi 1998; Pancholi 2001; Lamonica 2005; Lopez-Villar 2006; Antikainen 2007; Knaust 2007; Wang 2011). Extensive studies indicated that these proteins are multifunctional and play important roles in diseases (Pancholi 2001). It was demonstrated that the surface-exposed enolase could enhance the virulence of pathogenic bacteria by binding to plasminogen, which in turn facilitates bacterial persistence, colonization and the invasion of host tissues (Bergmann 2001; Jin 2005). Enolases are considered to be evolutionarily conserved due to its universal functions in glycolysis and gluconeogenesis (Hosaka 2003). It has been speculated that there might be an unknown signal for enolase export conserved over a long period in evolution (Knaust 2007). In the last decade, many bacterial cytoplasmic proteins have been found to be secreted without any cleavable signal peptides, which were termed as the non-classical secretion. Such proteins, which display two unrelated functions in different sub-cellular locations, have been termed “moonlighting” proteins (Jeffery 2003; Bendtsen 2005).

The molecular mechanism of enolase transport through the membrane is mostly unknown. It has been proposed that this release could be attributed to the cell lysis (Guiral 2005; Antelmann 2006). However, *E. coli* enolase was found to be exported into

the medium and the export may depends on covalent binding of its substrate, 2-phosphoglycerate, to Lys341 (Boel 2004). A recent study showed that the N-terminal fragment of 169 amino acids of yeast enolase can target a cytoplasmic invertase to the cell surface (Lopez-Villar 2006). Biochemical, immunological and electron microscopic evidence from different groups consistently demonstrated that enolases associate with the membranes (Pancholi 2001; Sha 2003; Sijbrandi 2005; Lopez-Villar 2006; Pal-Bhowmick 2007; Agarwal 2008). It was reported that *Bacillus anthracis* enolase was detected in the right-side out membrane vesicles (Agarwal 2008). Ferrari *et al*. found that enolase of *Neisseria meningitidis* can be found in the spontaneously released outer membrane vesicles (Ferrari 2006). Yu *et al*. showed that the enolase secretes via exosomes in the eukaryotic cells (Yu 2006). Recent study on *Clonorchis sinensis* demonstrated that enolase is an excretory/secretory protein and is also a multifunctional metabolic enzyme (Wang 2011). Extensive studies from different groups in the last ten years support the presence of an alternative secretion mechanism other than the classical pathway to drive enolase through the membrane to the cell surface or into the extracellular medium (Nombela 2006; Antikainen 2007; Knaust 2007). *E. coli* α-haemolysin is exported through the ABC transporter but apparently some via the spontaneously released outer membrane vesicles. Moreover, the export signal of the α-haemolysin resides on a 23 kDa C-terminal domain, which is sufficient for the export through the membranes (Balsalobre 2006).

B. subtilis enolase was found in the extracellular compartment by many research groups (Tjalsma 2004; Vitikainen 2004; Antelmann 2006; Zanen 2006), but how and why the enolase of this non-pathogenic strain is secreted remains unknown. We have re-

ported that enolase and other cytoplasmic proteins without a cleavable signal sequence can be secreted from *Bacillus* cells into the medium in the absence of cell lysis, and a hydrophobic helix domain may be important for the secretion (Yang 2011). Using the crystal structure of *Enterococcus hirae* enolase (Hosaka 2003) as the template, a predicted molecular structure of enolase was modeled by Swiss-Model (Arnold 2006). *Bacillus* enolase is composed of one small N-terminal domain (P_2-N_{138}) and one large C-terminal domain (S_{139}-K_{430}). According to Swiss-Prot database (Entry No. **37869**), the C-terminal barrel domain contains four phosphorylation sites, substrate and Mg^{2+} binding sites and two catalytic motifs. A long unbent α-helix (A_{108}-L_{126}) of enolase resides in the N-terminal domain, in which a hydrophobic core region (A_{110}-C_{118}) is predicted as the membrane-embedded (EM) domain by the PSSM_SVM scheme (Hu 2007).

In this work, we use the cloned enolase and a series of mutations in the hydrophobic EM domain to examine their effects on their secretion in *B. subtilis*. In addition, we found that the cloned enolase can also be exported in *E. coli*, indicating that the secretion of enolase is not species-specific. Moreover, we have identified a N-terminal domain of *B. subtilis* enolase that facilitates the secretion of a reporter protein. Taken all data together, we conclude that this highly conserved hydrophobic α-helix is unique and involved in enolase secretion.

MATERIALS AND METHODS

Bacterial strains, plasmids, culture conditions and growth. Bacterial strains and plasmids are listed in Table 2.1. *Bacillus subtilis* WB600BHM and *E. coli* strain DH5α were grown in Luria-Bertani (LB) broth or agar plates containing 0.2% glucose at 37^{o}C. The following antibiotics were used as required: ampicillin (100 μg/ml), chloramphenicol (100 μg/ml), and kanamycin (10 μg/ml).

Cloning of *B. subtilis* enolase mutants. Constructions of the wild-type *Bacillus eno* gene and other mutations on shuttle vector pDG148 using overlapping PCR were described previously (Yang 2011). To express FLAG-tagged enolase and mutants, the wild-type and mutated *eno* genes were PCR amplified and cloned into the *Sac*I/*Pst*I sites of pGTN-FLAG (Yang 2011) (Fig. 2.1).

Expression of *Bacillus* enolase and its mutants. The overnight cultures of *E. coli* DH5α or *B. subtilis* WB600BHM transformants containing *Bacillus* enolase gene were inoculated into fresh LB medium and cultured with orbital shaking at 250 rpm and 37 ℃. 1 mM IPTG was added when O.D. reached 0.2 to induce the protein expression. In *B. subtilis*: After 10 hr of cultivation, cell pellets were collected by centrifugation, resuspended in 10 mM Tris-HCl (pH 8.0) buffer containing 1 mM EDTA (TE buffer) and followed by French Press to break the cells. The whole cell lysates were centrifuged at 12,000 × *g*, for 10 min to remove cell debris and insoluble materials, and the supernatants were collected as the soluble whole cell lysates. The spent media were filtered through a

0.45 μm-pore-size filter (Fisher Science, MA) and the proteins were precipitated using DOC-TCA method (CSHProtocols 2006). The precipitated protein pellets from media were washed once with ice-cold 100% acetone, dissolved in TE buffer and used as the culture filtration fraction. In *E. coli*: After 8 hrs of incubation, cells were processed followed the same procedures described above. All prepared samples were mixed with the equal amount of 2X sample buffer. Protein profiles of *E. coli* or *B. subtilis* were analyzed by SDS-electrophoresis and followed by immunoblots with *B. subtilis* enolase antibodies under condition in which *E.coli* enolase was not detectable.

Construction of Green Fluorescent Protein fusions with enolase. Green Fluorescent Protein (GFP) was amplified from pBAD-gfpuv (Clontech, Mountain View, CA) and the restriction sites *XbaI* and *SphI* were introduced on 5' and 3' sites of GFP, respectively. The PCR product was digested by *XbaI/SphI* and subcloned onto the corresponding sites of *E. coli*/*B. subtilis* shuttle vector pDG148 in which the cloned gene was placed under the control of P_{spac} promoter and resulted in a plasmid pDGGFP. To construct the different lengths N-terminus fusion with GFP, nine PCR-amplified N-terminus fragments were inserted into pDGGFP. Nine different plasmids include pDGGFP-140Eno (whole N-terminus), pDGGFP-ΔC14 (1-126), pDGGFP-ΔC22 (1-118), pDGGFP-ΔC31(1-109), pDGGFP-sp (102-126), pDGGFP-ΔN16 (17-140), pDGGFP-ΔN40 (41-140), pDGGFP-ΔN54 (55-140), and pDGGFP-ΔN81 (82-140). The sequences of the entire inserts in the pDGGFP-derived plasmids were confirmed by DNA sequencing.

Gel electrophoresis and Western blots. Protein samples were mixed with an equal volume of 2x loading buffer and analyzed by standard Laemmli sodium dodecyl sulfate (SDS) electrophoresis in 12.5 % polyacrylamide gels (Laemmli 1970). Gels were stained with Coomassie Blue R-250. For Western blots analysis, proteins on the gel were transferred to polyvinylidene difluoride (PVDF) membrane (Problot, Applied Biosystems) (Towbin 1979). The primary antibody against *Bacillus* enolase was from the laboratory stock and anti-FLAG serum F7425 was purchased from Sigma-Aldrich (Sigma-Aldrich, St. Louis, MO). The alkaline phosphatase linked goat anti-rabbit antibodies (Bio-Rad) were used as secondary antibodies for all reactions. Membranes were developed according to manufacturer's instructions.

RESULTS

A highly conserved hydrophobic α-helix domain affects secretion of enolase. We previously demonstrated the recombinant enolase (EnoBs-FLAG) and EM replacement mutant (EMR-FLAG) were expressed well as soluble forms but failed to secret, indicating the involvement of EM region on enolase secretion (Yang 2011).

We examined whether the hydrophobicity of this EM region is important for the export process. We constructed three double or triple substitutions in the hydrophobic region (#110-116) by glycines, which provides the conformational flexibility and is less hydrophobic including ^{110}AIL→ ^{110}GGG (AIL), ^{114}VS→ ^{114}GG (VS), ^{116}MAC→ ^{116}GGG (MAC) on pGTN-FLAG. The expression levels of mutant proteins were significantly affected by glycine-substitution mutation, especially the ^{110}AIL→ ^{110}GGG (Fig. 2.2) which

had no detectable band in the intracellular sample. There was a minor band detected from the MAC mutant, while GVS was expressed normally (Fig. 2.2), indicating the differential levels of expression/stability. None of these mutant proteins could be recovered from extracellular samples, showing that the hydrophobicity or sequence specificity of this region of enolase plays a critical role on its secretion in *Bacillus*.

To further understand how this helix affects the export of enolase, additional mutants were constructed which extended from the EM domain region including ^{108}AN→ ^{108}GG (AN), ^{119}ARA→ ^{119}GGG (ARA), ^{122}AAD→ ^{122}GGG (AAD), and ^{125}FL→ ^{125}GG (FL) that cover the whole hydrophobic helix region. Mutation on AN, ARA and AAD affected secretion of recombinant enolases while chromosomal enolase secreted normally (Fig. 2.2). Similar to the AIL, the AN mutant protein was undetectable in either intra- or extracellular portions. On the other hand, the FL mutation, located opposite to the AN mutation on the other end of helix, showed a similar expression level as the chromosomal copy and the mutation on FL did not affect the export of enolase (Fig. 2.2). These data indicates that the hydrophobicity of the α-helix between A^{108} and F^{125} plays an important role for the stability and secretion of the enolase.

The conserved basic residues flanking the HC region are not crucial for the export. Positively charged amino acid residues on transmembrane domain could provide additional interactions between membrane proteins and the negatively charged phospholipids (von Heijne 1992; Heximer 2001; Thiyagarajan 2004). There are three positively charged residues flanking the HC region of enolase, and these residues are highly conserved in enolases of different species (Fig. 2.3a, labeled with "+"). Three mutations

(K103G, K105G & R120G) were constructed on pGTN-FLAG to determine the possible roles of these positively charged residues in the export process. The results showed glycine-substituted single mutations had no effect on exporting enolase (Fig. 2.3b). We further constructed three double-mutations of enolase that two of the three positively charged residues had been changed to glycine, and resulted in three-double mutants including K103G+K105G, K103G+R120G, and K105G+R120G. When the effect of double-mutation was evaluated on the secretion of enolase, we found that none of the mutants affected secretion procedure (Fig. 2.3b). Taken together, the flanking positive charges within this region do not affect enolases interaction with membrane and export in *B. subtilis*.

The hydrophobic α-helix is unique for enolase secretion. Although the importance of hydrophobic helix on secretion has been demonstrated, it is possible the impact of secretion is the consequence of mutation on the N-terminus of enolase. To test its unique role on secretion, we constructed another enolase mutant in which the last helix in N-terminus was broken by the substituting a proline (Q132P). However, the mutated enolase was expressed and secreted as shown in Fig. 2.4a. Thus, the hydrophobic helix is unique and plays a very important role on enolase secretion.

The hydrophobic core dependent secretion is a general phenomenon. We have demonstrated that deletion or replacement of this entire helix reduces the secretion of enolase in *Bacillus*. It has been reported that *E.coli* enolase can be exported out of the cells into the medium (Boel 2004). We also found that the cloned *Bacillus* enolase could

also be secreted in *E. coli* (Fig. 2.4a). We next determined whether the importance of EM domain on enolase secretion is specific for *Bacillus* or is also important in *E. coli*. As shown in Fig. 2.4b, the *Bacillus* enolase deleted or replaced of EM domain were expressed and remained soluble inside cells but the deletion also impaired the secretion in *E. coli*. Thus, this EM domain is also important in the secretion in *E. coli*, indicating a general phenomenon for the role of EM domain in the secretion.

The minimal length of N-terminus of enolase necessary for fusion protein secretion. The enolase sequence alignments of *E. coli*. and *B. subtilis* revealed that the hydrophobic α-helix as described above is highly conserved. Based on the general notion that a hydrophobic α-helix is important for interactions between membrane proteins and the membrane (Kyte 1982; Engelman 1986), we hypothesized that the highly conserved hydrophobic α-helix of enolase may play an important role for targeting the protein to the membrane or interacting with the membrane. To determine whether this putative helix can be a signal for secretion or the helix itself can be secreted, the helix was cloned onto pGT and expressed in *B. subtilis*. The result indicated that the helix itself is not enough for secretion (data not shown). Sequentially, the EM domain or extended region were used to determine if it could guide and promote the reporter protein to secret in *Bacillus*.

It has been reported that 169 amino acids on N-terminus of enolase in yeast sufficiently targeted the fused invertase to the cell surface (Lopez-Villar 2006). To test this possibility, plasmid pDG148 harboring the reporter *gfp* gene was constructed, resulting in a new plasmid pDGGFP. The N-terminus of EnoBs was cloned onto pDGGFP to test if it can be used as a signal to direct GFP across membranes and secrets into the growth me-

dium (p140EnoGFP, 140EnoGFP). Indeed, the N-terminus is capable of directing fusion protein across the *Bacillus* membrane (Fig. 2.5), as in the case of yeast enolase. We further dissected the N-terminus of enolase to determine the minimal length of carrying GFP to export. Nine hybrid proteins were constructed with different lengths of enolase N-terminus on pDGGFP. The nine constructs include pΔN16GFP (Δ1-16, N16), pΔN40GFP (Δ1-40, N40), pΔN54GFP (Δ1-54, N54), pΔN81GFP (Δ1-81, N81), pΔN99GFP (Δ1-99, N99), pΔC14GFP (Δ127-140, C14), pΔC22GFP (Δ119-140, C22), pΔC31GFP (Δ110-140, C31), and pSpGFP (102-126, Sp).

Our results showed that the whole 140 N-terminus indeed can serve as a signal to direct the secretion of fused GFP to the medium (Fig. 2.5). Further truncation of various N-terminus constructs showed an unstable pattern as detected by immunoblot analysis, and only N40 showed a reduced expression in the intracellular medium (Fig. 2.5). On the other hand, truncation from C-terminal of the 140 residues N-terminus showed a similar expression level as whole N-terminus fusion 140EnoGFP (Fig. 2.5). Nevertheless, none of them was active, indicating truncation on N-terminus of enolase lost the ability of fusion GFP to cross the membrane and that the whole 140 N-terminus is required to direct GFP for secretion.

DISCUSSION

Enolase is known to be a cytoplasmic protein and it converts PEG to pyruvate in glycolysis. We have previously reported that the secretion of many cytoplasmic proteins, including enolase, without classical signal peptides in the extracellular media during the stationary phase are not due to cell lysis, and a hydrophobic helix of enolase may be im-

portant for its secretion (Yang, 2011). In this study, we further analyze the role of this region in secretion. There is increasing evidence that extracellular enolase associate with the cell membrane (Pancholi 2001; Sha 2003; Sijbrandi 2005; Balsalobre 2006; Lopez-Villar 2006; Pal-Bhowmick 2007; Agarwal 2008). Moreover, several groups have recently reported that enolase was found on the cell surface to interact with host's plasminogen (Hurmalainen V 2007; Mirjam E. Meltzer 2010; Wang 2011).

By computational approaches, we have identified an EM domain buried in a hydrophobic region of the N-terminus of enolase which is predicted to integrate into membranes (Hu 2007). Genetic alternations and structural replacements of the EM domain abolished the secretion of the mutated enolases while the chromosomal enolase still secreted without any impact, indicating the presence of the EM domain is necessary for enolase secretion (Yang, Ewis et al. 2011). However, in that study, the replaced EM domain possessing a lower hydrophobicity than that of the original sequence, hence, we investigate the importance of the hydrophobicity of this region for secretion of enolase.

To address this, the hydrophobicity of mutants were calculated and monitored using method adopted from Kyte-Doolittle (Kyte 1982) followed by substitution of glycines on the HC core region. Regardless of hydrophobicity changes, no secretion was observed on all glycine substituted mutants. Though mutants ARA and AAD have similar hydrophobicity and stability as the wild-type enolase, both mutations blocked the secretion. A possible explanation on this phenomenon is the substitutions on ARA and AAD resulting in losing sequences or structures that may be required to interact with an unknown secre-

tion apparatus. On the other hand, glycine substitution on ARA and AAD may result in unexpected structure changes, hence, affected the secretion of mutated enolase. Among these substitution mutants, only FL mutant showed no effect on enolase secretion although it is a part of the important helix. The data suggest that residues FL are not important on either structure or sequence specificity.

Mutations of the positively charged residues demonstrated no significant effects on enolase secretion, indicating that they are not important factor on enolase secretion in *Bacillus*. Chen and other researchers had reported that positively charged residues in *Bacillus* may not be essential for export, and longer hydrophobic sequences may compensate the lack of positively charged residues on *Bacillus* signal peptide (Chen 1994). The core of hydrophobic helix in the EM domain of enolase contains 25 amino acids which is longer than a typical signal peptide and it may render the positively charged residues flanking the HC region negligible. On the other hand, this unique secretion is a general phenomenon as mutation on EM domain abolished its secretion in *E. coli*. Moreover, although the helix plays an important role on enolase secretion, the helix itself is not sufficient to secret.

GFP experiments showed that the N-terminus 140 amino acids of enolase could act as a signal to direct the GFP export and further deletions from both N- and C-termini impair the export. It was noted that truncation from N-terminus of this region affected protein stability. Three β-sheets were predicted at N-terminus from amino acid position 1 to 40, and modification of these β-sheets may destabilize the structure as the result of on-

ly very few truncated enolases can be recovered in the intracellular compartment. To confirm this possibility, those unstable mutants were expressed at 25^{O}C to prevent protein degradation. The unstable mutants including N16, N40, N54, and N81were successfully expressed at 25^{O}C, but N99 still failed to be recovered from the cell lysate indicated this construct is extremely unstable (data not shown). However, a cleaved GFP band was also detected by immunoblots under these constructs implied their instabilities under this condition. On the other hand, GFP alone without any fusion was found in the medium implied the presence of cell lysis at this low temperature in stationary phase. This result was further confirmed by the dropping $O.D._{600}$ reading in stationary phase.

In contrast, truncation from the C-terminal of N-terminus showed a normal expression at 37^{O}C, but none of the mutants can be recovered in the medium. Therefore, the N-terminus of enolase is structurally unique and is needed to be maintained to function as a signal.

Recent reports showed that enolase of *C. sinensis* functions not only as a key enzyme of glycolysis but also reacts with plasminogen of the host cell to facilitate host tissue invasion (Wang 2011). In addition, two trans-membrane domains have been predicted and the second domain near C-terminus most likely is related to plasminogen binding (Wang 2011), while the first domain is conserved and may be required for enolase export. Since *B. subtilis* is not a pathogenic bacterium, conceivably, the second domain is not required and is dispensable during evolution.

Taken together, we provide evidence that the hydrophobic helix is required for enolase secretion in *B. subtilis* and *E. coli,* suggesting this is a general phenomenon. In addition, to export protein across the membrane, N-terminus structure of enolase has to be maintained and the whole N-terminus sequence is needed to promote the secretion of a reporter such as GFP fusion. However, the interactions between the hydrophobic helix of enolase and membranes remain unclear. Although the extracellular function of enolase is not yet identified, numerous reports have proposed enolase as a multifunction protein in various species. Therefore, it is possible that its extracellular function may be required during the stationary phase.

ACKNOWLEDGEMENTS

This work was supported by a NIH research grant GM 34766. We thank Hae-Jin Hu and Yi Pan for providing the profile of EM domain in enolase, Hsiuchin Yang for discussions and Ping Jiang for the DNA sequencing service in the GSU Biology Core Facility which is supported by the Georgia Research Alliance, Molecular Basis of Disease Program and Center for Biotechnology and Drug Design.

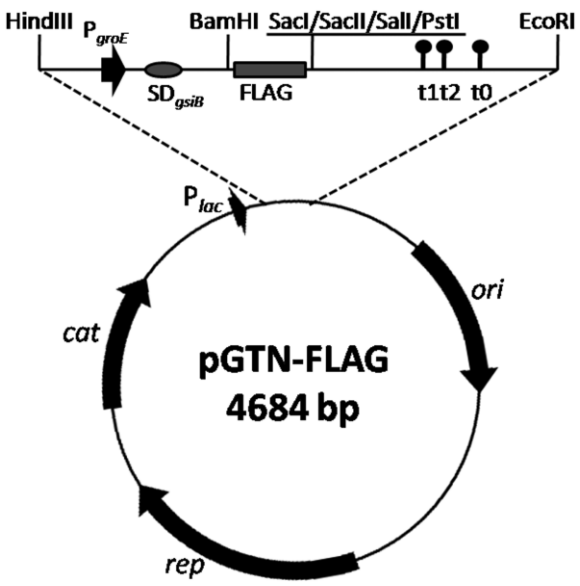


Figure 2.1. Genetic and restriction map of the expression vector pGTN-FLAG. P_{groE}, P_{lac} and SD_{gsiB} represent the *B. subtilis groE* promoter, *E. coli lac* promoter and the SD sequence of *B. subtilis gsiB* gene, respectively. t1t2 and t0 are the tandem terminators cloned from pMUTIN4. *ori*, *rep*, and *cat* represent the sequences coding for the ColE1 replication origin, replicase and chloramphenicol resistance marker, respectively. The arrows show the transcription directions for these genes.

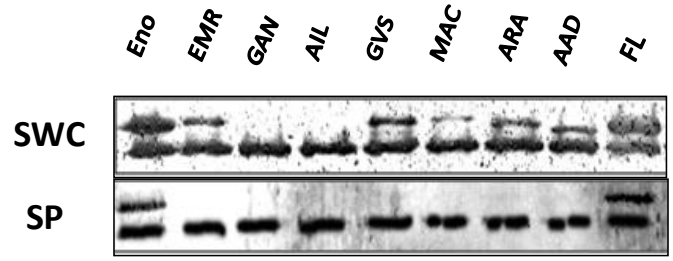


Figure 2.2. The substitution mutations in the hydrophobic core region affected the secretion of enolase. Different glycine substituted mutants were expressed in *B. subtilis* WB600BHM and intra- and extracellular samples were harvested at 10 hrs after incubation. Soluble whole cell fraction (SWC) and supernatant (SP) were studied by immunoblots. Eno: wild type enolase; EMR: helix replaced enolase; GAN: GAN→GGG mutant; AIL: AIL→GGG mutant; VS: VS→GG mutant; MAC: MAC→GGG mutant; ARA: ARA →GGG mutant; AAD: AAD→GGG mutant; FL: FL→GGG mutant.

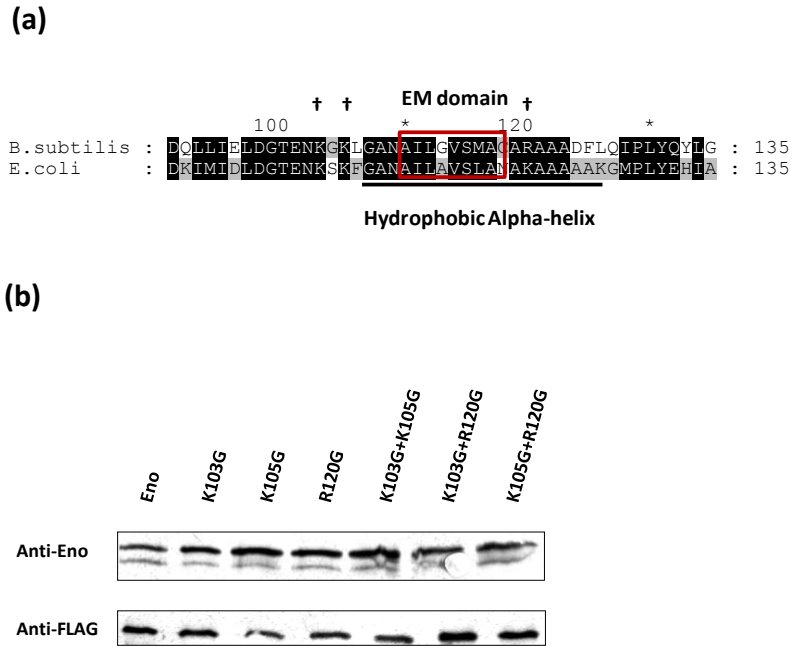


Figure 2.3. Substitution mutations of positively charged residues flanking the HC region did not affect enolase secretion. (a). Multiple sequence alignment of various enolases. Numbers at the ends of each line represent residues from the amino termini: *B. subtilis* (Bacil), and *E. coli* (Ecoli). The sequences of *B. subtilis* hydrophobic α-helix and the EM domain region are indicated. Three basic residues (K_{103}, K_{105}, R_{120}) flanking the hydrophobic core region are labeled with a "+" on the top. (b). Substitution mutants were expressed and harvested at 10 hrs after inoculation. Supernatants were used to study secretion of mutants. Enolase was detected and visualized by Western blotting using anti-EnoBs antibody (Anti-Eno) and anti-FLAG tag antibodies (Anti-FLAG). Lanes:1, Wild type enolase (eno). 2, K103G; 3, K105G; 4, R120G; 5, K103G+K105G; 6, K103G+R120G; 7, K105G+R120G.

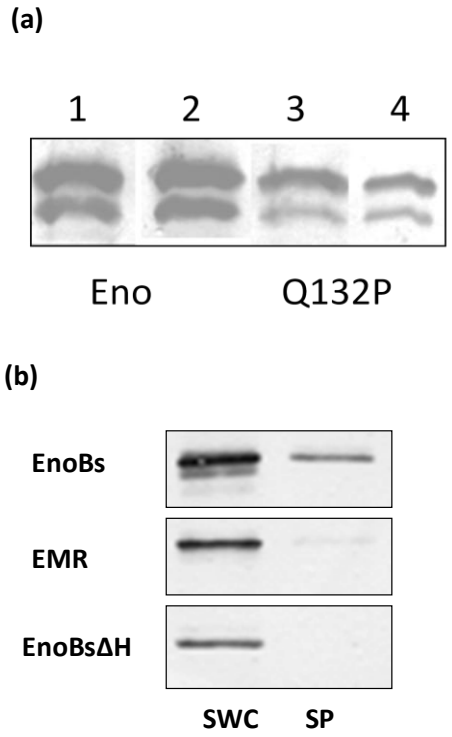


Figure 2.4. Hydrophobic core of enolase is unique for secretion. (a). Mutation of the small helix did not affect secretion. The small helix locates on the N-terminus of enolase from L^{130} to L^{134}, Intra- and extracellular samples were collected at 10hrs during growth. Soluble cell lysates (SWC) and supernatant (SP) were used to study secretion of mutants. Enolase was detected and visualized by Western blotting using anti-enolase antibody. Lane 1: Wild type enolase SWC, 2: wild type enolase SP, 3: Q132P SWC, 4: Q132P SP. (b). *Bacillus* enolase can be secreted in *E. coli* and mutation on EM domain abolished secretion. *E. coli* that harboring pDG-EnoBs/ pDGEMR/ pDGEnoBsΔH were cultured and secretion of these recombinant proteins was monitored. Soluble cell lysates (SWC) and supernatant (SP) were harvested as described in material and methods. Enolase was detected and visualized by Western blotting using anti-enolase antibody. EnoBs: *Bacillus enolase*, EMR: EM domain repelcement, EnoBsΔH: EM domain deletion mutant.

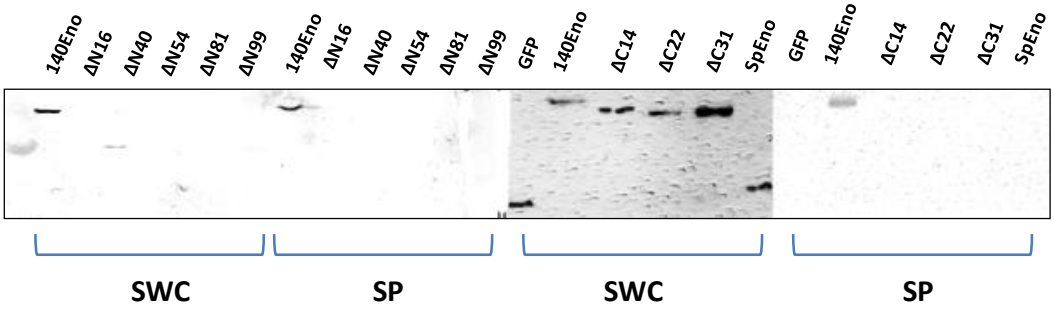


Figure 2.5. Whole N-terminus of EnoBs is capable to work as a signal for recombinant GFP. Series deletion of N-terminus from its N-terminal and C-terminal were prepared and fused with GFP. Recombinant GFPs were expressed in *B. subtilis*, and intracellular and extracellular samples were harvested at 12 hrs after inoculation. Soluble fractions of intracellular samples were prepared as aforementioned. The secretion patterns of GFPs were investigated using immunoblots with GFP antibodies.

Table 2.1. Bacterial strains and plasmids

Bacterial strains, and Plasmids	Genotype or description	Source or reference
E. coli DH5 α	F⁻ 80 Δ*lac* ΔM15 Δ(*lacZYAargF*) U169 *deo*R *rec*A1	
	*end*A1*hsad*R17 ($r_k m_k$) *supE44* λ⁻thi-1*gyr*A96*rel*A1	BRL
B. subtilis		
WB600 BHM	*trpC2 npr*E Δ*apr*E Δ*bpf* Δ*epr mpr*::*ble npr*B::*bsr*.	(Yang et al., 2011)
Plasmids		
pDG148	*bla, kan*, shuttle vector	(Yang et at., 2011)
pDGGFP	*bla, kan*, *gfp*, shuttle vector	This study
pDGGFP-140Eno	*bla, kan*, *140-eno*, shuttle vector	This study
pDGGFP-ΔC14	*bla, kan*, *eno* ΔC14, shuttle vector	This study
pDGGFP-ΔC22	*bla, kan*, *eno* ΔC22, shuttle vector	This study
pDGGFP-ΔC31	*bla, kan*, *eno* ΔC31, shuttle vector	This study
pDGGFP-sp	*bla, kan*, *sp-eno*, shuttle vector	This study
pDGGFP-ΔN16	*bla, kan*, *eno* ΔN16, shuttle vector	This study
pDGGFP-ΔN40	*bla, kan*, *eno* ΔN40, shuttle vector	This study
pDGGFP-ΔN54	*bla, kan*, *eno* ΔN54, shuttle vector	This study
pDGGFP-ΔN81	*bla, kan*, *eno* ΔN81, shuttle vector	This study
pDGEnoBsΔH	*bla*, *kan*, *lacI*, *enoBsΔH*	(Yang et at., 2011)
pGTN	*bla, cat, lacI ,* shuttle vector	This study
pGTN-FLAG	*bla, cat, lacI ,* shuttle vector	(Yang et at., 2011)

pEnoFLAG	*bla, cat, lacI , eno-FLAG*	(Yang et at., 2011)
pEMRFLAG	*bla, cat, lacI , EMR-FLAG*	(Yang et at., 2011)

CHAPTER 3

Growth phase related transcriptome analysis of *Bacillus subtilis* 168

Chun-Kai Yang, Chung-Dar Lu, and Phang C. Tai*

Department of Biology, Georgia State University, Atlanta, GA, 30303

Running Title: Growth phase related transcriptome analysis

* Corresponding author.
Mailing address: Department of Biology, Georgia State University, 161 Jesse Hill Drive, Atlanta, Georgia, 30303.
Phone: 404-413-5303
Fax: 404-413-5301
Email: biopct@gsu.edu

ABSTRACT

Transcriptome analysis revealed several interesting patterns in gene expression when the cell growth switches from exponential phase into the stationary phase. As expected, once cell growth was toward the transition phase, expressions of most SigA-dependent house-keeping genes (e.g. syntheses of ATP, amino acids, nucleotides, ribosomes), and *secY* and *yidC* genes in the Sec-dependent general protein secretion system were significantly decreased. Surprisingly, *secA, ffh* (signal recognition particle) and *sipT* (signal peptidase) were progressively induced from exponential phase while *secY* was repressed. Besides, *rpsF, rpsL, rpmF, rpsG, fusA* and *tufA* genes which function on ribosome synthesis and elongation after decreased in the transition phase were found progressively induced in the stationary phase suggesting special functions of these proteins. The *sigB* gene and the SigB regulon of 124 genes for stress response exhibited a distinct pattern of transient induction with a peak in the transition phase. A total of 68 genes were induced by at least three fold after cessation of SigB-dependent surge including *sigW*, SigW-depedent detoxification genes, and oxidative stress response genes. In addition, induction of *fruRAB* and repression of *pgi*, *pfk* and *fabA* suggested fructose utilization occurred and a sequential switch of carbon source from glucose to fructose. These results indicate a complex adaptation as *Bacillus* cells change from the fast growing exponential phase toward the stationary phase.

INTRODUCTION

Bacillus subtilis has been the model organism to study sporulation and development of Gram-positive bacterium. In addition, it is widely used by pharmaceutical and biotech companies to produce various enzymes and antibiotics. The processes of secondary metabolite synthesis, protein secretion, and sporulation occur when the cells enter into the stationary phase of growth. Although the overall cell numbers remain static, the stationary phase is considered the most dynamic period in turns of gene expression in response to ever-changing growth environments. Depending on the specific physical and chemical growth conditions, the genetic circuits of the cells are designed to make prioritized decisions for adjustments when facing multiple stress factors arising from growth. Accordingly, the nature of this design makes it a very challenging task to predict the status of the cells at the genetic level in the past. Equipped with a complete and annotated genome sequence as well as modern genomic tools, it is now feasible to conduct systematic analysis of this complicated phase of bacterial growth.

Bacillus has developed a sophisticated system when encounters stress from the environment or nutritional limitation. A set of 17 sigma factor have been identified in *Bacillus* (Haldenwang 1995) that works with RNA polymerase core enzyme to provide differential gene expression when a specific stress is detected. Among these sigma factors, 10 of them have identified functions (Haldenwang 1995), which include six vegetative-cell related sigma factors (SigA, SigB, SigC, SigD, SigH, and SigL) and four sporu-

lation-specific sigma factors (SigE, SigF, SigG, and SigK). Other than those major sigma factors, seven extra sigma factors were identified during whole genome sequencing of *B. subtilis* (Kunst 1997) including SigM, SigV, SigW, SigX, SigY, SigZ, and YlaC. They belong to the family of extracytoplasmic function sigma factors (ECF) that in response to external signals including denature of protein, oxidative stress, salt, heat and cold stress in *B. subtilis* (Asai, Yamaguchi et al. 2003). However, the functions of these ECF sigma factors are not all known (X Huang 1997; Asai, Yamaguchi et al. 2003; Cao and Helmann 2004; Yoshimura, Asai et al. 2004; Pietiäinen, Gardemeister et al. 2005; Luo, Asai et al. 2010). Only three ECF sigma factors (SigW, SigM and SigX) have designated functions which mediate responses to cell wall envelope-active antibiotics (Luo, Asai et al. 2010).

To better understand the physiological status of *B. subtilis* during growth, DNA microarrays (GeneChip® by Affymetrix) were employed to conduct transcriptome analysis. Although many publications report investigation of bacterial responses to specific stresses or genetic mutations by this approach, however, none of them focused on how *B. subtilis* adapts to growth phase changes in a temporal manner. Therefore, the major aim of this work was to study transcriptional profiling when the cells shift from the exponential phase to stationary phase by a series of snapshots in this specific time window.

MATERIALS AND METHODS

Bacterial strains, culture conditions, and growth. *Bacillus subtilis* strains 168 was grown in Luria-Bertani (LB) broth or agar plates containing 0.2% glucose at 37 ^{o}C. The overnight seed cultures of *B. subtilis* 168 was inoculated into fresh LB medium at a

dilution ratio of 1% (v/v) and cultured with orbital shaking 300 rpm at 37 ℃. At various times, 4 mL of *B. subtilis* cells were collect by centrifugation at 16,000 x *g*. The collected cells were then subjected to total RNA extraction.

RNA isolation, generation of cDNA, hybridization of GeneChip, and data analysis. Cells that harvested at time points aforementioned were used to extract total RNA as described previously (Kwon and Lu 2006) and sequentially purified by QIA-GEN spin columns (Chatsworth, Calif.) after DNase I treatment. Preparation of cDNA, hybridization of GeneChip (Affymetrix, Santa Clara, CA 95051), and data analysis were performed as previously described (Lu, Yang et al. 2004). To systematically study gene expressions upon different time points, the antisense *Bacillus subtilis* genome arrays were utilized to monitor the gene expression. In addition, to have better sensitivity and reveal different patterns from transcriptome data, some restrictions have been set in GeneSpring including: First, gene expression level below 100 at all times was adjusted to 100, Second, expression level must be above 300 at 3 different time points. Third, one of the time points must be above 500. Gene must fit all criteria to be remained to further analysis. Then the remaining data was collected and threefold change was used as a cut-off value and applied to genechip results to be considered significant.

RESULTS AND DISCUSSION

Clusters of time course-dependent transcriptional profiling. The changes of gene expression when the cells shift from the exponential phase to stationary phase of growth were studied. *Bacillus subtilis* was grown in Luria-Bertani (LB) medium with 0.2% of glucose as supplement to prevent sporulation. Total RNA samples were taken at 200 min (-T60), 220 min (-T40), 240 min (-T20), 260 min (T0), 300 min (T40), 360 min (T100), and 420 min (T160) after inoculation (Fig. 3.1a). With the criteria for data mining described in Materials and Methods, the total number of genes that were considered active during any time point of this study was minimized from 4108 to 2231. Within these genes, 1638 of them exhibited less than three-fold change during growth in this time window, and therefore were excluded for further analysis. For the remaining 726 genes, in general they can be differentiated into three clusters based on time course-dependent expression patterns as described below.

Genes that are turned off in the stationary phase. The first pattern was shown in Fig. 3.1b (p-1), where gene expression was high at time points –T60 and –T40, followed by a drastic downturn at T0, and remained low for the rest of time points. A list of 226 genes belongs to this cluster, including operons for ribosome components, cell wall synthesis, de novo nucleotide biosynthesis, ATP synthase, and ABC transporters. Most of genes within this pattern are transcribed by the SigA-dependent RNA polymerase. SigA is the primary sigma factor that is responsible for transcription of most genes in vegetative stage. It is also named “housekeeping” sigma factor to emphasize the importance on

maintaining *Bacillus* physiology (Jarmer, Larsen et al. 2001). More than 400 genes have been reported that are regulated by SigA (Sierro, Makita et al. 2008). The expression profile of *sigA* during growth was shown in Fig. 3.2a. The *sigA* gene along with the upstream *yqxD* and *dnaG* genes form an operon, and expression of which was turned on in the exponential phase (-T60) but was repressed to its basal level at time point T0 and points after.

As expected, genes in this cluster were turned on during the exponential phase to ensure supply of basic building blocks and assembly of macromolecules to sustain bacterial growth, and were turned off when the cell growth stopped in the stationary phase. Strikingly, *rpsF, rpmF, rpsL,* and *rpsG* encoding ribosomal components showed significant induction from transition phase which is quite different from other ribosomal genes (Fig. 3.2a). In addition, *fusA* and *tufA* (*fusA* encodes EF-G and *tufA* encodes EF-Tu) forming an operon with upstream *rpsG* also showed a similar pattern, suggesting induction of this specific set of housekeeping genes was not due to experimental artifacts. However, why these ribosomal genes got induced in transition phase is not clear.

Genes that are transiently induced at the transition stage. The second pattern displayed a surge of gene expression at T0 as shown in Fig. 3.1b (p-2). There are 43 genes categorized into this group, including *sigB* (Fig. 3.1a) and its regulatory genes *rsbVWX*. All genes in this cluster have been reported as members of the SigB regulon (Akbar, Gaidenko et al. 2001; Brigulla, Hoffmann et al. 2003; Zhang and Haldenwang 2003; Kuo, Zhang et al. 2004; Pané-Farré 2005).

In response to a variety of stress and starvation stimuli, *B. subtilis* has the capacity to post a drastic induction of more than 150 SigB-dependent general stress genes (Akbar, Gaidenko et al. 2001; Brigulla, Hoffmann et al. 2003; Zhang and Haldenwang 2003; Kuo, Zhang et al. 2004; Pané-Farré 2005). The activity of SigB itself is subjected to a very complicated regulatory cascade triggered by either physical stress (e.g. salt, ethanol, temperature) or nutritional stress (e.g. phosphate, carbon source, nitrogen source). The SigB protein can be inactivated when bound by the regulatory protein RsbW, and which itself can be sequestered by RsbV. The antagonist activity of RsbV was lost when phosphorylated by RsbW. When the cells encounter stress, RsbU or RsbP (YvfP) can dephosphrylate RsbV to facilitate its association with RsbW, and as the consequence, release SigB from the RsbW-SigB complex.

The *sigB* gene is in an eight-gene operon in the order of *rsbRSTUVW-sigB-rsbX* (Kunst 1997). As shown in Fig. 3.2a & 3.2b, expression of *rsbVW-sigB-rsbX* exhibited the distinct surge pattern of this cluster while expression of *rsbRSTU* remained constitutive. This result is consistent to the reports that transcription of the *rsb* operon is initiated by a SigA-dependent promoter upstream of *rbsR* and a SigB-dependent promoter upstream of *rsbV* (Wise 1995; Kunst 1997).

Since the SigB regulon responds to stress, its unique pattern of a surge expression suggests that the cells encountered stress of unknown nature when reaching T0, and this stress was released soon after a surged response by the SigB regulon. A possible candidate of this stress stimulus is low ATP level. It has been reported that during nutrient li-

mitation the intracellular ATP level was reduced (Stülke, Hanschke et al. 1993; U Voelker 1995), and the low level of ATP, but not GTP or the redox state, can specifically trigger SigB expression (Zhang and Haldenwang 2005). Consistent to this hypothesis was the observation that expression of ATP synthase genes was drastically reduced when the cells enter into time point T0 (Fig. 3.2a). If low ATP level indeed is the trigger, returning of *sigB* expression to the basal level at T20 and points after would suggest that the cells regain a relatively high ATP level once past T0, perhaps through reorganization of metabolic pathways to cut ATP consumption.

From this study it was noted that 12 genes in the reported SigB regulon display expression patterns that are different from that of *sigB*. While many of these genes were constitutively expressed, the following genes caught our attention with unique patterns - *yceCDEFGH, sodA, ydbP, yjbD,* and *yraA*. Based on their expression pattern, these genes were grouped into the third cluster as described below.

Genes that are induced in the stationary phase. In general, genes in this cluster exhibited a pattern of strong induction in the stationary phase as shown in Fig. 3.1b (p-3), and they were further divided into three subgroups based on physiological functions.

(i) toxin-antitoxin and membrane stress response. In this subgroup, 20 genes in 11 operons were reported as members of the SigW regulon (Table 3.1). SigW has been reported that regulates detoxification response and the production of antimicrobial compounds (Turner and Helmann 2000; Cao, Wang et al. 2002), and its regulon contains sev-

eral membrane-localized proteins which can be activated under membrane stress (Kingston, Subramanian et al. 2011). The activity of SigW is controlled by anti-sigma factor RsiW (YbbM) which traps SigW and prevents it from binding to RNA polymerase. The release of RsiW from SigW is mediated by regulated in-transmembrane proteolysis (RIP) (Ellermeier and Losick 2006; Koichi YANO 2011), which takes two steps to degrade RsiW and as consequence activated SigW and its regulon.

The following 14 genes were found to function related to antibiotic activities - *yqeZ-yqfAB, yknW, yfhL, yokD, yceCDEF*, and *yvlABCD*. The *yqeZ-yqfAB* genes participate in detoxification of sublancin, a prophage-encoded bacteriocin (Butcher and Helmann 2006). YfhL has been proposed to encode SdpI which can immune the toxic peptide SdpC, a toxic peptide expressed in Spo0A-activated cells that kills bacillus sibling cells that have not activated Spo0A, thereby delaying the onset of sporulation (Butcher and Helmann 2006; Stragier 2006). Similar function has also been elucidated on *yknW*. *yokD,* which encodes an enzyme aminoglycoside acxetyltransferase, has been found to be an effective inhibitor for anthrax lethal factor (Klimecka, Chruszcz et al. 2011). The *yce* operon may play a role in survival of ethanol stress while the *yvl* operon has putative functions on cell envelope stress response. Other than those genes directly defend antibiotic compounds, the *lytR* gene was recently renamed as *tagU* and has a function on anionic cell wall synthesis (Kawai, Marles-Wright et al. 2011) while *yoeB* is an inhibitor that could prevent cell lysis caused by antibiotic (Salzberg and Helmann 2007) implied a release of cell wall stress.

Four other monocistronic genes were also found in the regulon including *ybbM, ysdB, yozO,* and *ythP*. The *ybbM* and *ysdB* genes encode peptides in regulation of the SigW activity while *ythP* has been predicted as an ABC transporter but *yozO* currently has not been elucidate on its function.

(ii) Oxidative stress and repair. Other than genes belong to SigW regulon, 16 genes are found to have functions on oxidative stress response including *resE, yjbD, mrgA, ygaG, yurUVWX, trxB, yjbI, srfAA-srfAB-comS-srfAC-srfAD,* and *yutM* and are listed in Table 3.2. Among these genes, *ygaG* has been renamed as *perR* and functions as a major regulator on peroxide stress (Fuangthong, Herbig et al. 2002) while *yjbD* has been renamed *spx* which is a general stress regulator for oxidative stress (Zuber 2009). Since most of oxidative response genes were governed by *perR* and *spx,* induction of this set of genes implied a strong oxidative stress in the stationary phase. PerR responds to the presence of peroxide especially H_2O_2 while Spx is sensitive to thiol-depleting agents. As transcriptional regulators, PerR controls its regulon by binding to the Per-box while Spx is a RNA polymerase-binding protein (Fuangthong, Herbig et al. 2002; Zuber 2004). The strong induction of PerR and Spx regulons suggested that cells in stationary phase encountered a much harsh condition where more reactive oxygen species or toxic electrophiles are accumulated. It could lead to macromolecular damage, increase mutagenesis, and even induce cell death (Zuber 2009). Indeed, we also found several genes that have potential functions on DNA and protein repair and degradation in this cluster. This indicated that cells not only removed the oxidant and thiol depleting agents but also repaired the damage from those stress to survive during stationary phase.

sodA, *ydbP*, and *yraA* where previously found in SigB regulon but the expression profile is far different from *sigB*. SodA is a superoxide dismutase in detoxification of oxygen radicals. YdbP was predicted as a thioredoxin enzyme while YraA is a cystein protease in degradation of damaged thio-containing proteins. Zuber reported that these genes should be regulated by either PerR or Spx during growth (Zuber 2009). In addition, *perR* and *spx* displayed a very similar pattern with that of *sodA, ydbP*, and *yraA*, also suggesting that these genes could be under regulation by PerR or Spx.

(iii) Nutrient supply and proteins of unknown function. There are 22 genes listed in this subgroup as shown in Table 3.3. The *pstSCABAB* operon has been reported to encode components for a phosphate uptake system, and its expression is induced by phosphate starvation (Antelmann, Scharf et al. 2000). Although the *pst* operon is considered as a member of the PhoP-PhoR regulon (Allenby, O'Connor et al. 2004), its induction in the stationary phase in this study were independent of PhoP as no other genes in the regulon were co-induced.

The *fruRBA* operon encoding a transcriptional regulator, fructose-1-phosphate kinase, and fructose-specific phosphotransferase system were also found induced in the stationary phase. Induction of the *fruRBA* operon and repression of *pgi*, *pfk*, and *fbaA* (data not shown) in glucose utilization strongly indicated that the cells in the early stationary phase have switched from glucose to fructose as carbon source. A constitutive high level of *gap, pgk, tpi, pgm*, and *eno* expression would ensure fructose metabolism through glycolysis.

Other than these polycistronic genes, a monocistronic gene *yvgP* induced progressively and significantly increased for 10 fold from –T60 to T160. This gene has been renamed *nhaK* due to its function on transporting cation and H^+ and maintains cation concentration and osmosis (Fujisawa, Kusumoto et al. 2005), and it could be induced in stationary phase due to high concentration of Na^+/H^+. Similar expression profile was found in our data suggested high concentration of cation presents in stationary phase and induction of this anti-porter gene could be used to maintain cell physiology.

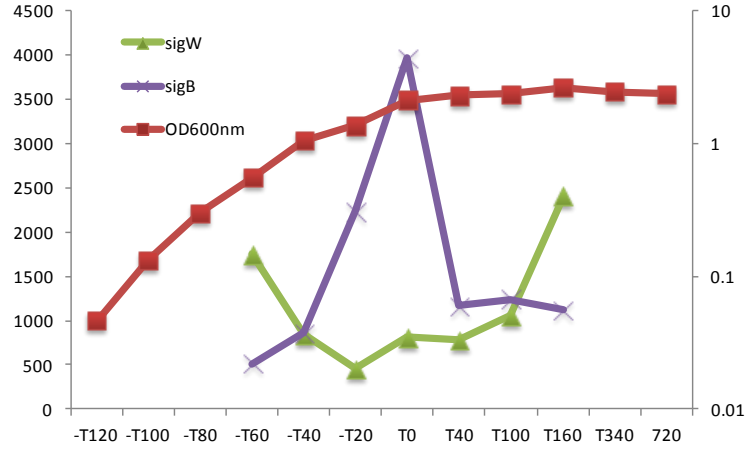
The expression profile of general secretion pathways. Secretion is an important feature for bacteria to maintain or survival during growth condition. Several secretion mechanisms have been identified in *B. subtilis* (Tjalsma 2000; Antelmann 2001; Tjalsma 2004; Yang 2011), and the majority of the extracellular proteins are secreted by Sec translocase. We observed a downturn on *secY* expression from –T40 and remained at basal level in stationary phase (Fig. 3.3a). In addition, *sppA* is predicted as protease which could degrade lipoprotein signal peptide shared a similar expression pattern as *secY* also implied the dispensability of sec secretion system in stationary phase. Besides, *spoIIIJ* which was predicted as *yidC* homolog behaved similarly as *secY*. On the contrary, *secA* was induced from exponential phase and induction was continued into stationary phase. Similarly, *sipT and sipS* encoding signal peptide peptidase I were gradually induced from –T20 to T160 (Fig. 3.3b). The late induction of *secA* and signal peptide peptidase I in stationary phase suggested the lack of *secY* could trigger the expression of *secA* and signal peptidase I. In addition, SRP gene *ffh* was induced in stationary phase also implied a stress occurred by the absence of secYEG. Since the SecYEG apparatus was

dispensable in an *in vitro secA*-liposomes translocation system (Ying-Hsin Hsieh and Hao Zhang, unpublished data), perhaps an induced level of SecA in the stationary phase suggests a possible SecA-alone translocase activity for protein secretion.

ACKNOWLEDGEMENTS

This work was supported by a NIH research grant GM 34766. We thank Dr. Hsi-uchin Yang for discussions and Sonja Yang for the Microarray service in the GSU Biology Core Facility which is supported by the Georgia Research Alliance, Molecular Basis of Disease Program and Center for Biotechnology and Drug Design.CKY is the fellow of the Program in Molecular Basis of Diseases at Georgia State University.

(a)



(b)

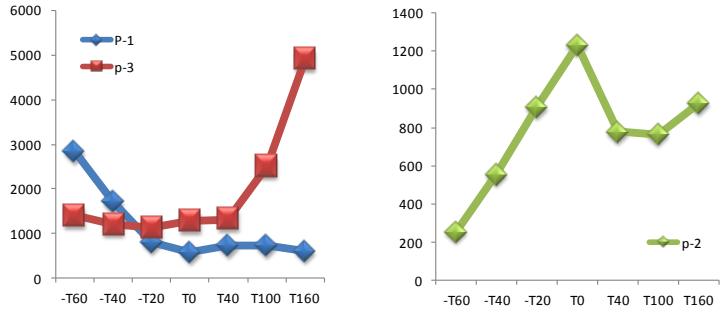
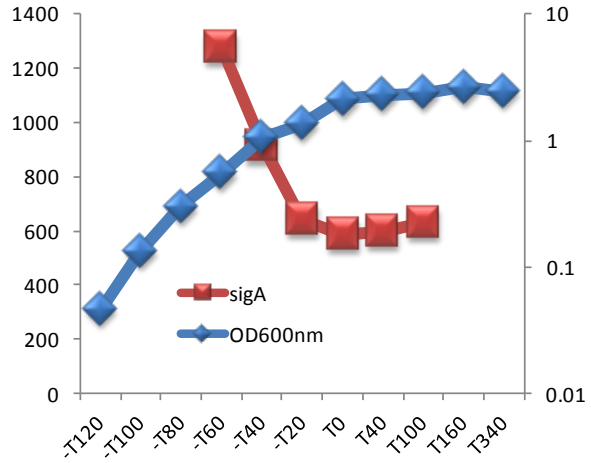
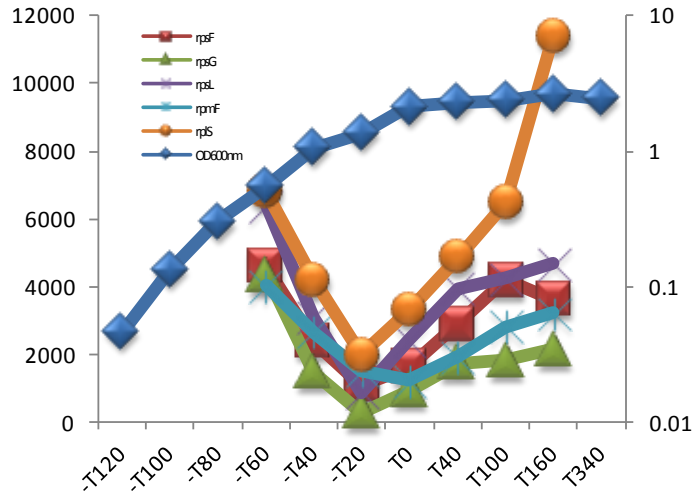


Figure 3.1. The growth profile and gene expression of *B. subtilis 168*. (a). The growth curve of *B. subtilis 168* in LB with supplement of 0.2 % glucose was monitored. Total RNA samples were collected from –T120 to T720. The gene expression profiles of sigma factors sigB (purple) and sigW (green) were also shown in the figure. (b). Three major expression patterns during growth. The pattern one (p-1) expressed in exponential phase and stayed at basal level from transition phase (blue). The pattern two (p-2) showed a significant induction at transition phase (T0) (green, left figure). The pattern three (p-3) displayed a significant induction from T40 (red).

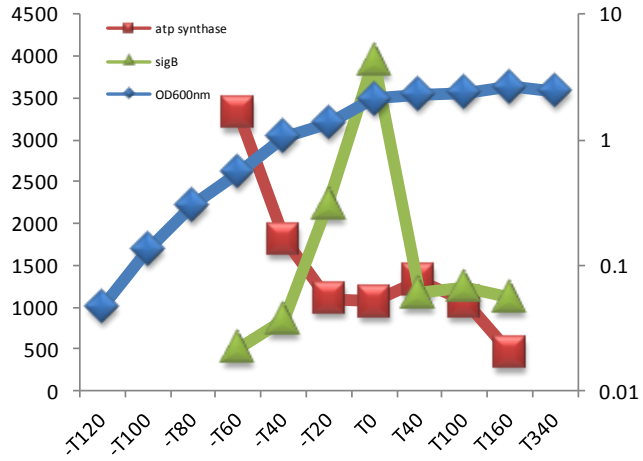
(a)


1400
1200
1000
800
600
400
200
0
10
1
0.1
0.01
sigA
OD600nm
-T120
-T100
-T80
-T60
-T40
-T20
T0
T40
T100
T160
T340

(b)


12000
10000
8000
6000
4000
2000
0
10
1
0.1
0.01
rpsF
rpsG
rpsL
rpmF
rpiS
OD600nm
-T120
-T100
-T80
-T60
-T40
-T20
T0
T40
T100
T160
T340

(c)



(d)

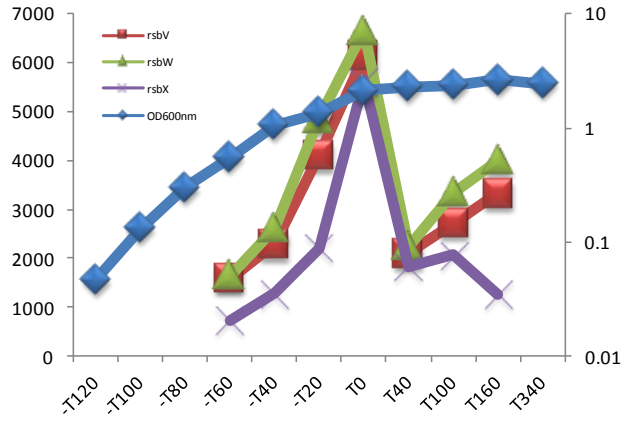
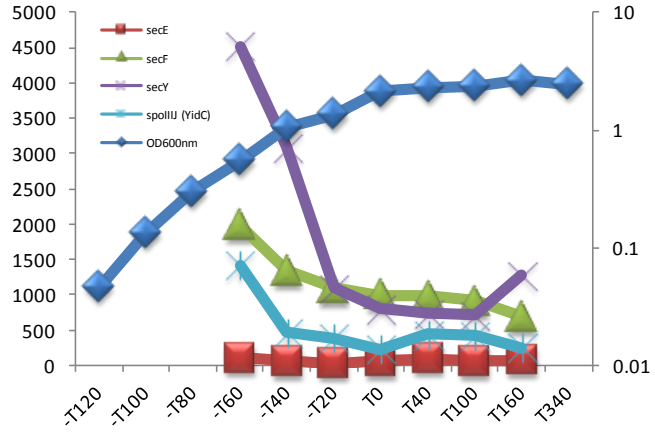


Figure 3.2. The expression profiles of different sigma factors. (a). *sigA* expression during growth. (b). *rpsF*, *rpsG*, *rpmF*, *rplS*, and *rpsL* expression pattern during growth. (c). *sigB* and ATP synthase expression profile. (d). Three regulatory genes rsbV, rsbW, and rsbX of *sigB* and their expression profile.

(a)



(b)

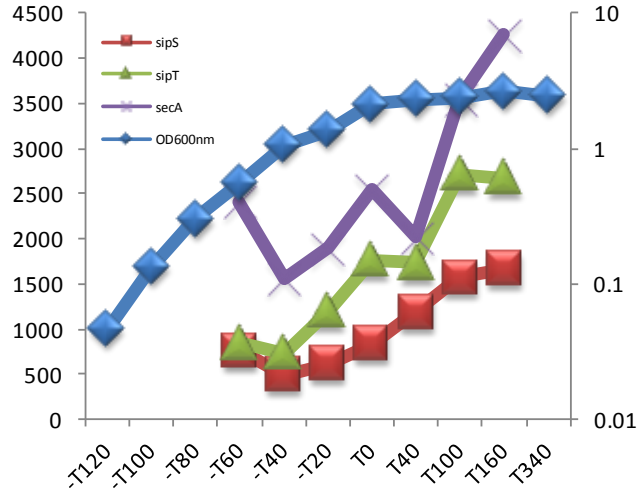


Figure 3.3. Expression of sec dependent apparatus at different stages during growth. (a). Gene expression profile of *secYEG* and *yidC* homolog *spoIIIJ* were monitored during growth. (b). *secA* and signal peptidase I genes *sipS* and *sipT* progressively induced in stationary phase.

Table 3.1. List of antitoxin resistant and membrane stress response genes from sigW regulon

Gene	-T60	-T40	-T20	T0	T40	T100	T160	Function
ybbM	555	356	305	306	315	372	944	anti-sigma(W) factor
yqeZ	839	789	757	1365	1149	2640	8538	putative membrane bound hydrolase
yqfA	802	717	650	1142	941	1870	6430	conserved hypothetical protein
yqfB	742	701	579	980	1072	1671	5888	conserved hypothetical protein
yceC	4728	3556	2673	3114	2358	4084	9588	putative stress adaptation protein
yceD	4140	2898	2588	2640	2148	3707	8650	putative stress adaptation protein
yceE	3659	2759	2545	2606	2071	3615	8871	putative stress adaptation protein
yceF	4261	3246	2968	2764	2535	4465	8641	putative stress adaptation protein
yknW	773	626	459	535	747	1309	4343	putative permease
yozO	332	344	229	438	587	521	1840	conserved hypothetical protein
ythP	306	433	472	648	622	913	2102	putative ABC transporter (ATP-binding protein)
yvlA	777	717	681	826	569	871	2984	conserved hypothetical protein
yvlB	1268	1045	930	1201	769	1428	4988	conserved hypothetical protein
yvlC	819	711	630	743	640	712	2964	putative regulator (stress mediated)
yvlD	442	488	485	437	440	526	1387	putative integral inner membrane protein
ysdB	365	510	811	1382	882	841	1621	conserved hypothetical protein
yfhL	217	603	949	1299	598	587	1284	resistence protein (against toxic peptide SdpC)
yokD	250	409	447	411	631	989	1241	aminoglycoside N3'-acetyltransferase
lytR	2823	1860	1363	1346	1541	2514	4849	membrane-bound transcriptional regulator
yoeB	1255	1382	3119	6003	4498	8472	13969	inhibitor of cell-separation enzymes

Table 3.2. Oxidative stress and repair genes expressed in stationary phase

Gene	-T60	-T40	-T20	T0	T40	T100	T160	Function
srfAA	1,154	696	573	700	1014	1922	1978	surfactin synthetase
srfAB	354	428	509	500	427	1169	2070	surfactin synthetase
srfAC	569	842	774	1067	716	1922	3712	surfactin synthetase
srfAD	446	611	714	854	649	1164	2668	surfactin synthetase
comS	825	919	1014	881	851	1806	3152	regulator of genetic competence
resE	1294	907	1044	1193	1145	2179	3695	ResE two-component sensory histidine kinase
ygaG	3720	3421	2968	4517	4129	6761	14112	transcriptional regulator (Fur family)
yurU	3084	2171	1718	1735	1686	3429	5522	FeS cluster formation protein
yurV	2646	1729	1348	1364	1396	2535	4097	iron-sulfur cluster assembly scaffold protein
yurW	3395	2189	2027	1605	1540	3220	5657	cysteine desulfurase
yurX	3610	2413	2147	1825	2033	3631	6012	FeS assembly protein SufD
trxB	968	1199	1456	3034	2253	3038	4158	thioredoxin reductase
yjbI	699	986	1595	2545	2223	2619	2743	putative thiol management oxidoreductase component
yutM	1747	1452	2394	4168	3827	6536	6964	putative chaperone involved in Fe-S cluster assembly
yjbD	3673	3324	3572	4059	3595	6606	12292	redox-sensitive regulator enzyme (spx)
mrgA	3524	3190	2472	1621	2305	5188	12421	metalloregulation DNA-binding stress protein
ymdA	3547	3192	2926	3986	4530	8775	12246	ribonuclease Y
yqfS	490	305	328	486	665	1242	2332	type IV apurinic/apyrimidinic endonuclease
yqfR	947	521	617	977	1029	2088	3264	ATP-dependent RNA helicase; cold shock
ykrL	392	429	933	1800	1419	2264	3157	membrane protease

Table 3.3. Nutrient supply and unknown function genes that expression significantly in stationary phase

Gene	-T60	-T40	-T20	T0	T40	T100	T160	Function
yqgG	100	206	563	720	1343	2987	4619	phosphate starvation protein
yqgH	100	273	595	847	1009	1960	3746	phosphate starvation protein
yqgI	111	286	637	893	1093	2239	5091	phosphate starvation protein
yqgJ	100	196	552	916	1146	2796	6201	phosphate starvation protein
yqgK	100	244	479	863	972	2393	5561	phosphate starvation protein
yxiE	1322	1891	3149	3767	4410	7152	11575	phosphate starvation protein
fruA	269	310	520	1151	758	1494	3924	fructose-specific enzyme IIABC component
fruB	192	250	376	782	648	919	2492	fructose-1-phosphate kinase
fruR	458	626	920	2105	1475	2366	5705	transcriptional regulator
yvgP	730	1200	2583	4199	2717	4450	7224	Na+/H+ antiporter
yneJ	717	652	479	678	1078	1441	2733	putative integral inner membrane protein
yqaP	532	611	463	520	1031	1549	2035	conserved hypothetical protein
yqgW	433	893	821	790	1670	3291	2944	conserved hypothetical protein
ynfC	509	408	658	728	870	2011	3112	conserved hypothetical protein
yncM	751	1385	2050	3247	3143	6032	5232	conserved hypothetical protein
ydeB	367	530	867	1499	1442	1467	2592	putative transcriptional regulator
yebE	167	251	503	652	363	180	344	conserved hypothetical protein
yfhJ	648	995	1912	2835	2070	2805	5369	conserved hypothetical protein
ylxM	1709	535	632	1589	1537	1555	1265	conserved hypothetical protein
ymzA	379	567	807	1481	1694	2265	2113	conserved hypothetical protein
yqfU	225	451	671	1225	842	882	1331	putative integral inner membrane protein
yrzF	320	535	1074	1707	1614	2670	3219	putative serine/threonine-protein kinase

grey background color indicated that protein with unknown function

GENERAL CONCLUSION

The secretion of cytoplamic proteins in the stationary phase in *B. subtilis* is controversial. In this study, we show that cell lysis cannot account for the secretion of proteins in late stationary phase by several lines of evidences: constant cell viability count, no change in cell density in the presence of chloramphenicol, the negligible amounts of SecA and EF-Tu in the medium, minimal effect on secretion using an autolysin mutant.

A recent report introduced a new prediction algorithm for the identification of non-classical secretory proteins, based on the biological and chemical properties; such as threonine contents, transmembrane helices, and protein disorder in the structure (Bendtsen 2005). It is noted that enolase, pyruvate dehydrogenase (S-complex) (Caulfield, Horiuchi et al. 1984; Caulfield, Furlong et al. 1985; Hemila, Palva et al. 1990), and GroEL are predicted to be non-secreted proteins using this prediction algorithm, while they are identified as abundant, secreted proteins in our study.

Recent publications indicated that several cytoplasmic proteins, present in extracellular environment, are associated with membrane vesicles in Gram-positive bacteria. However, the extent to which the membrane-vesicles mediate release of these proteins has not been reported, and such release is not significant for Est55, SodA and enolase.

Autolysins have been proposed to play several roles in motility, cell separation, competency, antibiotic-induced lysis, pathogenesis, cell wall synthesis and turnover, and differentiation (Blackman, Smith et al. 1998). A similar secretion pattern has been found

in the autolysin-deficient mutant and its parent strain indicating that autolysins are not a major factor.

Regardless of the mechanism of secretion, the presence of several cytoplasmic proteins such as GroEL, SodA, and enolase in the medium with defined physiological cytoplasmic functions raises an intriguing question: What is the significance of their presence in the medium? It is noted that many of these key proteins are involved in energy consumption. Perhaps "dumping" these enzymes provides a way to quickly reduce ATP consumption and re-orientate metabolism in preparation for sporulation or for cell survival. Alternatively, it would be interesting to explore whether the secreted proteins have functions that differ from their cytoplasmic counterparts. It has been reported that many proteins have "moonlighting" functions (Jeffery 1999; Jeffery 2003), and that *Streptococcus* enolase, for example, is an important factor for plasminogen binding and adhesion during host invasion (Kolberg, Aase et al. 2006).

To further understand the mechanism behinds the signal-less protein secretion, enolase was taken as a model system for investigation. Enolase is known to be a cytoplasmic protein and it converts PEG to pyruvate in glycolysis. By computational approaches, we have identified an EM domain buried in a hydrophobic region of the N-terminus of enolase which is predicted to integrate into membranes (Hu 2007) and may play an important role on its export. Genetic alternations and structural replacements of the EM domain abolished the secretion of the mutated enolases while the chromosomal enolase still secreted without any impact, indicating the presence of the EM domain is necessary for enolase secretion. Moreover, we have found that the replaced EM domain

possessing a lower hydrophobicity than that of the original sequence, hence, the hydrophobicity of this region may need to be well maintained for secretion of enolase.

However, some mutants indicated even though the hydrophobicity was maintained, the secretion still impacted. The mutants ARA and AAD have similar hydrophobicity and stability as the wild type enolase, but both mutations blocked the secretion. Among these substitution mutants, only FL mutant showed no effect on enolase secretion although it is a part of the important helix. The data suggest that residues FL are not important on either structure or sequence specificity.

In addition, the effect of positively charged residues had also been studied in *Bacillus* and it had been shown that the positively charged residues play the least important role on secretion when hydrophobicity, positively charged residue, and structure were taken into consideration (Chen 1994). Three positively charged residues were found to flank the important α-helix, however, mutations of these residues demonstrated no significant effects on enolase secretion, indicating that they are the least importance on enolase secretion in *Bacillus*. Chen and other researchers had reported that positively charged residues in *Bacillus* may not be essential for export, and longer hydrophobic sequences may compensate the lack of positively charged residues on *Bacillus* signal peptide (Chen 1994). The core of hydrophobic helix in the EM domain of enolase contains 25 amino acids which is longer than a typical signal peptide and it may render the positively charged residues flanking the HC region negligible.

GFP experiments proved that the N-terminus 140 amino acids of enolase could act as a signal to direct the GFP export and further deletions from both N and C termini impaired the export. There are three β-sheets were predicted at N-terminus, modification

of these β-sheets may destabilize the structure as the result of only very few truncated enolases can be recovered in the intracellular compartment. In contrast, truncation from the C terminal of N-terminus showed a normal expression, but none of the mutants can be secreted in the medium. Furthermore, loss of the hydrophobic helix was also failed to function as a signal peptide for fusion GFP secretion. Therefore, the N-terminus of EnoBs is structural unique and is needs to be maintained to function as a signal peptide.

We also monitored gene expression of *Bacillus subtilis* from the exponential phase to the stationary phase. Three major patterns have been found among the genes that expressed significantly during growth. The first significant pattern expressed a downturn pattern from the first time point –T60, and 227 genes were found to express similarly from our data. Several operons have been discovered from this set of data including ribosomal genes, cell wall synthesis genes, purine nucleotides de novo biosynthesis, pyrimidine synthesis, ATP synthase, and ABC transporters, It was noted that housekeeping sigma factor *sigA* expressed similarly and most of genes within this pattern are under control of SigA.

There are 43 genes showed unusual pattern which were highly induced at T0 (260min) but their expressions were repressed in stationary phase, and we categorized them as pattern 2. *sigB* and its regulatory genes displayed a similar pattern indicated SigB may play an important role in the T0 which helps cells adapt changes from transition phase to stationary phase. Within the regulon, several genes including *sodA, ydbP* and *yraA* seemed more likely under control of PerR which respond to oxidative stress.

Except the genes that aforementioned, surprisingly, 62 of *Bacillus* genes showed progressively induction in stationary phase, and those genes were grouped as pattern 3.

We have noted that several operons that in this pattern are under control of SigW, and indeed, *sigW* showed an induction in stationary phase implied the important influence of SigW in stationary phase. The genes that under control by SigW are mostly antitoxin resistance genes and membrane stress response genes. Other than SigW regulon, we also observed a set of oxidative stress response genes was highly induced in this stage suggests high concentration of oxidant and thio-depleting agents in stationary phase. Besides, phosphate starvation related genes and fructose metabolism genes were also found to be induced in stationary phase indicated a high demand on phosphate and a switch from glucose to fructose as the major carbon source.

Strikingly, *secY* and *yidC* homolog *spoIIIJ* draw a downturn on their expression while secA and signal peptide peptidase I progressively induced simultaneously in stationary phase implicated the stress response on the absence of *secYEG* and SecA may compensate it on Sec depend secretion in stationary phase. Besides, five ribosomal genes, *fusA*, and *tufA* were induced in the stationary phase suggests other functions behind ribosomal proteins or elongation in stationary phase.

Taken together, we first provide solid evidence to prove the secretion of signalless protein is not due to cell lysis, second, the requirement of the hydrophobic helix for enolase secretion in *B. subtilis* and *E. coli* demonstrated another tier of evidence that there is a mechanism involved which is needed to be further investigated. In addition, GFP fusion experiments confirmed the N-terminal of enolase is capable to work as an internal signal. On the other hand, transcriptome data provided another point of view to understand the physiology of *B. subtilis* at different growth stages.

REFERENCES

Abraham L. Sonenshein, J. A. H., and Richard M. Losick (2001). "Bacillus subtilis and Its Closest Relatives: from Genes to Cells." ASM press, Washington, D.C.

Agarwal, S., Kulshreshtha, P., Bambah Mukku, D., Bhatnagar, R. (2008). "alpha-Enolase binds to human plasminogen on the surface of *Bacillus anthracis*." Biochim Biophys Acta **1784**(7-8): 986-994.

Akbar, S., T. A. Gaidenko, et al. (2001). "New Family of Regulators in the Environmental Signaling Pathway Which Activates the General Stress Transcription Factor {sigma}B of Bacillus subtilis." J. Bacteriol. **183**(4): 1329-1338.

Allenby, N. E. E., N. O'Connor, et al. (2004). "Post-transcriptional regulation of the Bacillus subtilis pst operon encoding a phosphate-specific ABC transporter." Microbiology **150**(8): 2619-2628.

Anagnostopoulos, C., and J. Spizizen. (1961). "Requirements for transformation in *Bacillus subtilis*." J Bacteriol **81**: 741-746.

Antelmann, H., H. Tjalsma, V. Birgit, O. Steffen, S. Bron, J. M. van Dijl, and M. Hecker. (2001). "A proteomic view on genome-based signal peptide predictions." Genome Research **11**: 1484-1501.

Antelmann, H., H. Tjalsma, V. Birgit, O. Steffen, S. Bron, J. M. van Dijl, and M. Hecker. (2001). "A proteomic view on genome-based signal peptide predictions. ." Genome Research **11**: 1484-1501.

Antelmann, H., C. Scharf, et al. (2000). "Phosphate Starvation-Inducible Proteins ofBacillus subtilis: Proteomics and Transcriptional Analysis." Journal of bacteriology **182**(16): 4478-4490.

Antelmann, H., Van Dijl, J. M., Bron, S., and Hecker, M. (2006). "Proteomic survey through secretome of *Bacillus subtilis*." Methods Biochem Anal **49**: 179-208.

Antikainen, J., Kuparinen, V., Lahteenmaki, K., and Korhonen, T. K. (2007). "pH-dependent association of enolase and glyceraldehyde-3-phosphate dehydrogenase of *Lactobacillus crispatus* with the cell wall and lipoteichoic acids." J Bacteriol **189**(12): 4539-4543.

Arnold, K., Bordoli, L., Kopp, J., Schwede, T. (2006). "The SWISS-MODEL workspace: a web-based environment for protein structure homology modelling." Bioinformatics **22**(2): 195-201.

Asai, K., H. Yamaguchi, et al. (2003). "DNA microarray analysis of Bacillus subtilis sigma factors of extracytoplasmic function family." FEMS Microbiology Letters **220**(1): 155-160.

Balsalobre, C., Silvan, J. M., Berglund, S., Mizunoe, Y., Uhlin, B. E., and Wai, S. N. (2006). "Release of the type I secreted alpha-haemolysin via outer membrane vesicles from *Escherichia coli*." Mol Microbiol **59**(1): 99-112.

Bendtsen, J. D., Kiemer, L., Fausboll, A., and Brunak, S. (2005). "Non-classical protein secretion in bacteria." BMC Microbiol **5**: 58.

Bergmann, S., Rohde, M., Chhatwal, G. S., Hammerschmidt, S. (2001). "alpha-Enolase of *Streptococcus pneumoniae* is a plasmin(ogen)-binding protein displayed on the bacterial cell surface." Mol Microbiol **40**(6): 1273-1287.

Blackman, S. A., T. J. Smith, et al. (1998). "The role of autolysins during vegetative growth of *Bacillus subtilis* 168." Microbiology **144 (Pt 1)**: 73-82.

Boel, G., Pichereau, V., Mijakovic, I., Maze, A., Poncet, S., Gillet, S., Giard, J. C., Hartke, A., Auffray, Y., Deutscher, J. (2004). "Is 2-phosphoglycerate-dependent automodification of bacterial enolases implicated in their export?" J Mol Biol **337**(2): 485-496.

Bradford, M. M. (1976). "A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. ." Anal. Biochem. **72**: 248-254.

Brigulla, M., T. Hoffmann, et al. (2003). "Chill Induction of the SigB-Dependent General Stress Response in Bacillus subtilis and Its Contribution to Low-Temperature Adaptation." J. Bacteriol. **185**(15): 4305-4314.

Burts, M. L., W. A. Williams, et al. (2005). "EsxA and EsxB are secreted by an ESAT-6-like system that is required for the pathogenesis of Staphylococcus aureus infections." Proc Natl Acad Sci U S A **102**(4): 1169-1174.

Butcher, B. G. and J. D. Helmann (2006). "Identification of Bacillus subtilis σW-dependent genes that provide intrinsic resistance to antimicrobial compounds produced by Bacilli." Molecular Microbiology **60**(3): 765-782.

Cao, M. and J. D. Helmann (2004). "The Bacillus subtilis Extracytoplasmic-Function {sigma}X Factor Regulates Modification of the Cell Envelope and Resistance to Cationic Antimicrobial Peptides." J. Bacteriol. **186**(4): 1136-1146.

Cao, M., T. Wang, et al. (2002). "Antibiotics that inhibit cell wall biosynthesis induce expression of the Bacillus subtilisσW and σM regulons." Molecular Microbiology **45**(5): 1267-1276.

Caulfield, M. P., D. Furlong, et al. (1985). "Secretory S complex of Bacillus subtilis forms a large, organized structure when released from ribosomes." Proc Natl Acad Sci U S A **82**(12): 4031-4035.

Caulfield, M. P., S. Horiuchi, et al. (1984). "The 64-kilodalton membrane protein of Bacillus subtilis is also present as a multiprotein complex on membrane-free ribosomes." Proc Natl Acad Sci U S A **81**(24): 7772-7776.

Chang, S. and S. N. Cohen (1979). "High frequency transformation of *Bacillus subtilis* protoplasts by plasmid DNA." Mol Gen Genet **168**(1): 111-115.

Chen, M., V Nagarajan (1994). "Effect of alteration of charged residues at the N termini of signal peptides on protein export in Bacillus subtilis." J Bacteriol. **176**(18): 5796-5801.

Christine Eymann, G. H., Christian Scharf, and Michael Hecker. (2002). " Bacillus subtilis functional genomics: global characterization of the stringent response by proteome and transcriptome analysis. ." J Bacteriol. **184**: 2500-2520.

Cooper, H. M., and Y. Paterson. (1995). Production of polyclonal antisera. *In* Current Protocols in Immunology (J.E. Coligan, A.M. Kruisbeek, D.H. Margulies, E. M. Shevach, and W. Strober, eds.). New York, pp.2.4.1-2.4.9 John Wiley & Sons, New York.

CSHProtocols (2006). "Using Deoxycholate and Trichloroacetic Acid to Concentrate Proteins and Remove Interfering Substances." doi:10.1101/pdb.prot4258.

Danese, P. N. and T. J. Silhavy (1998). "TARGETING AND ASSEMBLY OF PERIPLASMIC AND OUTER-MEMBRANE PROTEINS IN ESCHERICHIA COLI." Annual Review of Genetics **32**(1): 59-94.

De Rossi, E., P. Brigidi, N. E. Welker, G. Riccardi, and D. Matteuzzi (1994). "New Shuttle Vector For Cloning in Bacillus stearothermophilus." Res. Microbiol. **145**(8): 579-583.

Denis Dacheux, O. E., Arjan de Groot, Benoit Guery, Rozen Leberre, Ina Attree, Benoit Polack, and Bertrand Toussaint. (2002). "Activation of the Pseudomonas aeruginosa Type III Secretion System Requires an Intact Pyruvate Dehydrogenase aceAB Operon." Infect Immun. **70**(3973-3977).

Dudani, A. K., Cummings, C., Hashemi, S., and Ganz, P. R. (1993). "Isolation of a novel 45 kDa plasminogen receptor from human endothelial cells." Thromb Res **69**(2): 185-196.

Ellermeier, C. D. and R. Losick (2006). "Evidence for a novel protease governing regulated intramembrane proteolysis and resistance to antimicrobial peptides in Bacillus subtilis." Genes & Development **20**(14): 1911-1922.

Engelman, D. M., Steitz, T. A., Goldman, A. (1986). "Identifying nonpolar transbilayer helices in amino acid sequences of membrane proteins." Annu Rev Biophys Biophys Chem **15**: 321-353.

Ewis, H. E., A. T. Abdelal, and C. D. Lu. (2004). "Molecular cloning and characterization of two thermostable carboxyl esterases from *Geobacillus stearothermophilus*." Gene **329**: 187-195.

Feng, Y., Pan, XZ., Sun, W., Wang, CJ., Zhang, H., Li, Z., Ma, Y., Shao, Z., Ge, J., Zheng, F., Gao, G. F., and Tang, J. (2009). "*Streptococcus suis* Enolase Functions as a Protective Antigen Displayed on the Bacterial Cell Surface." The Journal of Infectious Diseases **200**: 1583-1592.

Ferrari, G., Garaguso, I., Adu-Bobie, J., Doro, F., Taddei, AR., Biolchi, A., Brunelli, B., Giuliani, MM., Pizza, M., Norais, N., and Grandi, G. (2006). "Outer membrane vesicles from group B *Neisseria meningitidis* gna33 mutant: Proteomic and immunological comparison with detergent-derived outer membrane vesicles." Proteomics **6**(6): 1856 - 1866.

Fuangthong, M., A. F. Herbig, et al. (2002). "Regulation of the Bacillus subtilis fur and perR Genes by PerR: Not All Members of the PerR Regulon Are Peroxide Inducible." Journal of bacteriology **184**(12): 3276-3286.

Fujisawa, M., A. Kusumoto, et al. (2005). "NhaK, a novel monovalent cation/H⁺ antiporter of <i>Bacillus subtilis</i&gt." Archives of Microbiology **183**(6): 411-420.

Gabriela E. Bergonzelli, D. G., Raymond D. Pridmore, Laure F. Marvin-Guy, Dominique Donnicola, and Irène E. Corthésy-Theulaz. (2006). "GroEL of Lactobacillus johnsonii La1 (NCC 533) Is Cell Surface Associated: Potential Role in Interactions with the Host and the Gastric Pathogen." Helicobacter pylori Infect Immun. **74**: 425-434.

Guiral, S., Mitchell, T. J., Martin, B., and Claverys, J. P. (2005). "Competence-programmed predation of noncompetent cells in the human pathogen *Streptococcus pneumoniae*: genetic requirements." Proc Natl Acad Sci U S A **102**(24): 8710-8715.

Haichun Gao, X. J., Kit Pogliano, and Arthur I. Aronson. (2002). "The E1β and E2 Subunits of the Bacillus subtilis Pyruvate Dehydrogenase Complex Are Involved in Regulation of Sporulation." J Bacteriol. **184**(10): 2780-2788.

Haldenwang, W. G. (1995). "The sigma factors of Bacillus subtilis." Microbiol Rev. **59**(1): 1-30.

Hemila, H., A. Palva, et al. (1990). "Secretory S complex of Bacillus subtilis: sequence analysis and identity to pyruvate dehydrogenase." J Bacteriol **172**(9): 5052-5063.

Heximer, S. P., Lim, H., Bernard, J. L., Blumer, K. J. (2001). "Mechanisms governing subcellular localization and function of human RGS2." J Biol Chem **276**(17): 14195-14203.

Hirose, I., K. Sano, I. Shioda, M. Kumano, K. Nakamura, and K. Yamane. (2000). "Proteome analysis of *Bacillus subtilis* extracellular proteins: a two-dimensional protein electrophoretic study." Microbiology **146** (**Pt 1**): 65-75.

Hirose, I., K. Sano, et al. (2000). "Proteome analysis of *Bacillus subtilis* extracellular proteins: a two-dimensional protein electrophoretic study." Microbiology **146 (Pt 1)**: 65-75.

Ho, S. N., Hunt, H. D., Horton, R. M., Pullen, J. K., and Pease, L. R. (1989). "Site-directed mutagenesis by overlap extension using the polymerase chain reaction." Gene **77**(1): 51-59.

Hosaka, T., Meguro, T., Yamato, I., Shirakihara, Y. (2003). "Crystal structure of Enterococcus hirae enolase at 2.8 A resolution." J Biochem **133**(6): 817-823.

Hu, H.-J., Holley, J., He, J., Harrison, R. W., Yang, H., Tai, P.C., Pan, Y. (2007). "To Be or Not to Be: Predicting Soluble SecAs as Membrane Proteins." IEEE TRANSACTIONS ON NANOBIOSCIENCE **6**(2): 168-179.

Hu, H. J., Y. Pan, et al. (2004). "Improved protein secondary structure prediction using support vector machine with a new encoding scheme and an advanced tertiary classifier." IEEE Trans Nanobioscience **3**(4): 265-271.

Hurmalainen V, E. S., Antikainen J, Baumann M, Lähteenmäki K, Korhonen TK. (2007). "Extracellular proteins of Lactobacillus crispatus enhance activation of human plasminogen." Microbiology **153**(Pt 4): 1112-1122.

Jarmer, H., T. S. Larsen, et al. (2001). "Sigma A recognition sites in the Bacillus subtilis genome." Microbiology **147**(9): 2417-2424.

Jeffery, C. J. (1999). "Moonlighting proteins." Trends Biochem Sci **24**(1): 8-11.

Jeffery, C. J. (2003). "Moonlighting proteins: old proteins learning new tricks." Trends Genet **19**(8): 415-417.

Jin, H., Song, Y. P., Boel, G., Kochar, J., and Pancholi, V. (2005). "Group A *streptococcal* surface GAPDH, SDH, recognizes uPAR/CD87 as its receptor on the human pharyngeal cell and mediates bacterial adherence to host cells." J Mol Biol **350**(1): 27-41.

Jurgen, B., T. Schweder, and M. Hecker (1998). "The stability of mRNA from the gsiB gene of *Bacillus subtilis* is dependent on the presence of a strong ribosome binding site." Mol Gen Genet **258**(5): 538-545.

Kawai, Y., J. Marles-Wright, et al. (2011). "A widespread family of bacterial cell wall assembly proteins." EMBO J **advance online publication**.

Kingston, A. W., C. Subramanian, et al. (2011). "A σW-dependent stress response in Bacillus subtilis that reduces membrane fluidity." Molecular Microbiology **81**(1): 69-79.

Klimecka, M. M., M. Chruszcz, et al. (2011). "Structural Analysis of a Putative Aminoglycoside N-Acetyltransferase from Bacillus anthracis." Journal of Molecular Biology **410**(3): 411-423.

Knaust, A., Weber, M. V., Hammerschmidt, S., Bergmann, S., Frosch, M., Kurzai, O. (2007). "Cytosolic proteins contribute to surface plasminogen recruitment of Neisseria meningitidis." J Bacteriol **189**(8): 3246-3255.

Kodamaa, T., K. Endo, K. Ara, K. Ozaki, H. Kakeshita, K. Yamane, and J. Sekiguch (2007). "Effect of *Bacillus subtilis spo0A* mutation on cell wall lytic enzymes and extracellular proteases, and prevention of cell lysis." Journal of Bioscience and Bioengineering **103**(1): 13-21.

Koichi YANO, H. I., Hirokazu MORI, Lii Mien YEE, Satoshi MATSUOKA, Yoshito SADAIE and Kei ASAI (2011). "Heterologous Expression of the Oceanobacillus iheyensis SigW and Its Anti-Protein RsiW in Bacillus subtilis." Biosci. Biotechnol. Biochem. **75**: 966-975.

Kolberg, J., A. Aase, et al. (2006). "Streptococcus pneumoniae enolase is important for plasminogen binding despite low abundance of enolase protein on the bacterial cell surface." Microbiology **152**(Pt 5): 1307-1317.

Kunst, F., Ogasawara, N., Moszer, I., Albertini, A. M., Alloni, G., Azevedo, V., Bertero, M. G., Bessieres, P., Bolotin, A., Borchert, S., Borriss, R.,Boursier, L.,Brans, A.,Braun, M.,Brignell, S. C.,Bron, S.,Brouillet, S.,Bruschi, C. V.,Caldwell, B.,Capuano, V.,Carter, N. M.,Choi, S.,K.,Codani, J. J.,Connerton, I. F.,Cummings, N. J.,Daniel, R. A.,Denizot, F.,Devine, K. M.,Dusterhoft, A.,Ehrlich, S. D.,Emmerson, P. T., Entian, K. D., Errington, J., Fabret, C.,Ferrari, E.,Foulger, D.,Fritz, C.,Fujita, M.,Fujita, Y.,Fuma, S.,Galizzi, A.,Galleron, N.,Ghim, S. Y.,Glaser, P.,Goffeau, A.,Golightly, E. J.,Grandi, G.,Guiseppi, G.,Guy, B. J.,Haga, K.,Haiech, J.,Harwood, C. R.,Henaut, A.,Hilbert, H.,Holsappel, S.,Hosono, S.,Hullo, M. F.,Itaya, M.,Jones, L.,Joris, B.,Karamata, D.,Kasahara, Y.,Klaerr-Blanchard, M.,Klein, C.,Kobayashi, Y.,Koetter, P.,Koningstein, G.,Krogh, S.,Kumano, M.,Kurita, K.,Lapidus, A.,Lardinois, S.,Lauber, J.,Lazarevic, V.,Lee, S. M.,Levine, A.,Liu, H.,Masuda, S.,Mauel, C.,Medigue, C.,Medina, N.,Mellado, R. P.,Mizuno, M.,Moestl, D.,Nakai, S.,Noback, M.,Noone, D.,O'Reilly, M.,Ogawa,

K.,Ogiwara, A.,Oudega, B.,Park, S. H.,Parro,V.,Pohl, T. M.,Portetelle, D.,Porwollik, S.,Prescott, A. M.,Presecan, E.,Pujic, P.,Purnelle, B.,Rapoport, G.,Rey, M.,Reynolds, S.,Rieger, M.,Rivolta, C.,Rocha, E.,Roche, B.,Rose, M.,Sadaie, Y.,Sato, T.,Scanlan, E.,Schleich, S.,Schroeter, R.,Scoffone, F.,Sekiguchi, J.,Sekowska, A.,Seror, S. J.,Serror, P.,Shin, B. S.,Soldo, B.,Sorokin, A.,Tacconi, E.,Takagi, T.,Takahashi, H.,Takemaru, K.,Takeuchi, M.,Tamakoshi, A.,Tanaka, T.,Terpstra, P.,Tognoni, A.,Tosato, V.,Uchiyama, S.,Vandenbol, M.,Vannier, F.,Vassarotti, A.,Viari, A.,Wambutt, R.,Wedler, E.,Wedler, H.,Weitzenegger, T.,Winters, P.,Wipat, A.,Yamamoto, H.,Yamane, K.,Yasumoto, K.,Yata, K.,Yoshida, K.,Zumstein, E.,Yoshikawa, H.,Danchin, A. (1997). "The complete genome sequence of the Gram-positive bacterium Bacillus subtilis." Nature **390**(6657): 249-256.

Kuo, S., S. Zhang, et al. (2004). "Associations between Bacillus subtilis σB regulators in cell extracts." Microbiology **150**(12): 4125-4136.

Kwon, D. H. and C.-D. Lu (2006). "Polyamines Induce Resistance to Cationic Peptide, Aminoglycoside, and Quinolone Antibiotics in Pseudomonas aeruginosa PAO1." Antimicrob. Agents Chemother. **50**(5): 1615-1622.

Kyte, J., Doolittle, R. F. (1982). "A simple method for displaying the hydropathic character of a protein." J Mol Biol **157**(1): 105-132.

Laemmli, U. K. (1970). "Cleavage of structural proteins during the assembly of the head of bacteriophage T4." **Nature** **227**(**5259**): **680-685**.

Lamonica, J. M., Wagner, M., Eschenbrenner, M., Williams, L. E., Miller, T. L., Patra, G., DelVecchio, V. G. (2005). "Comparative secretome analyses of three *Bacillus anthracis* strains with variant plasmid contents." Infect Immun **73**(6): 3646-3658.

Lee, E.-Y., D.-Y. Choi, D.-K. Kim, J.-W. Kim, J. O. Park, S. Kim, S.-H. Kim, D. M. Desiderio, Y.-K. Kim, K.-P. Kim, and Y. S. Gho (2009). "Gram-positive bacteria produce membrane vesicles: Proteomics-based characterization of *Staphylococcus aureus*-derived membrane vesicles." PROTEOMICS **9**: 5425-5436.

Liu, P., H.E. Ewis, YJ. Huang, CD. Lu, PC. Tai, IT. Weber (2007). "Structure of *Bacillus subtilis* superoxide dismutase." Acta Crystallographica Section F **F63**: 1003-1007.

Lopez-Villar, E., Monteoliva, L., Larsen, M. R., Sachon, E., Shabaz, M., Pardo, M., Pla, J., Gil, C., Roepstorff, P., and Nombela, C. (2006). "Genetic and proteomic evidences support the localization of yeast enolase in the cell surface." Proteomics **6 Suppl 1**: S107-118.

Lu, C.-D., Z. Yang, et al. (2004). "Transcriptome Analysis of the ArgR Regulon in Pseudomonas aeruginosa." J. Bacteriol. **186**(12): 3855-3861.

Luo, Y., K. Asai, et al. (2010). "Transcriptomic and Phenotypic Characterization of a Bacillus subtilis Strain without Extracytoplasmic Function {sigma} Factors." J. Bacteriol. **192**(21): 5736-5745.

Manting, E. H. and A. J. M. Driessen (2000). "Escherichia coli translocase: the unravelling of a molecular machine." Molecular Microbiology **37**(2): 226-238.

Mashburn-Warren, L. M., M. Whiteley (2006). "Special delivery: vesicle trafficking in prokaryotes." Molecular Microbiology **61** 839-846.

Mirjam E. Meltzer, C. J. M. D., Philip G. de Groot, Frits R. Rosendaal and Ton Lisman (2010). "Plasma levels of fibrinolytic proteins and the risk of myocardial infarction in men " BLOOD **116**(4): 529-536.

Nombela, C., Gil, C., and Chaffin, W. L. (2006). "Non-conventional protein secretion in yeast." Trends Microbiol **14**(1): 15-21.

Oliver, D. B. and J. Beckwith (1981). "E. coli mutant pleiotropically defective in the export of secreted proteins." Cell **25**(3): 765-772.

Pal-Bhowmick, I., Vora, H. K., and Jarori, G. K. (2007). "Sub-cellular localization and post-translational modifications of the *Plasmodium yoelii* enolase suggest moonlighting functions." Malar J **6**: 45.

Pallen, M. J. (2002). "The ESAT-6/WXG100 superfamily -- and a new Gram-positive secretion system?" Trends Microbiol **10**(5): 209-212.

Pancholi, V. (2001). "Multifunctional alpha-enolase: its role in diseases." Cell Mol Life Sci **58**(7): 902-920.

Pancholi, V., Fischetti, V. A. (1998). "alpha-enolase, a novel strong plasmin(ogen) binding protein on the surface of pathogenic *streptococci*." J Biol Chem **273**(23): 14503-14515.

Pané-Farré, J., Lewis, RJ., Stülke, J. (2005). "The RsbRST Stress Module in Bacteria: A Signalling System That May Interact with Different Output Modules." J Mol Microbiol Biotechnol **9**: 65-76.

Petersohn, A., M. Brigulla, et al. (2001). "Global Analysis of the General Stress Response of Bacillus subtilis." J. Bacteriol. **183**(19): 5617-5631.

Phan, T. T., H. D. Nguyen, et al. (2006). "Novel plasmid-based expression vectors for intra- and extracellular production of recombinant proteins in Bacillus subtilis." Protein Expr Purif **46**(2): 189-195.

Pietiäinen, M., M. Gardemeister, et al. (2005). "Cationic antimicrobial peptides elicit a complex stress response in Bacillus subtilis that involves ECF-type sigma factors and two-component signal transduction systems." Microbiology **151**(5): 1577-1592.

Pugsley, A. P. (1993). "The complete general secretory pathway in gram-negative bacteria." Microbiol Rev. **57**(1): 50-108.

Rivas, L. A., V. Parro, et al. (2000). "The *Bacillus subtilis* 168 csn gene encodes a chitosanase with similar properties to a streptomyces enzyme." Microbiology **146 (Pt 11)**: 2929-2936.

Salzberg, L. I. and J. D. Helmann (2007). "An Antibiotic-Inducible Cell Wall-Associated Protein That Protects Bacillus subtilis from Autolysis." J. Bacteriol. **189**(13): 4671-4680.

Sao-Jose, C., C. Baptista, et al. (2004). "*Bacillus subtilis* operon encoding a membrane receptor for bacteriophage SPP1." J Bacteriol **186**(24): 8337-8346.

Sha, J., C. L., Pancholi, V., Popov, V. L., Zhao, Y., Houston, C. W., Chopra, A. K. (2003). "Differential expression of the enolase gene under in vivo versus in vitro growth conditions of *Aeromonas hydrophila*." Microb Pathog **34**(4): 195-204.

Sibbald, M. J., A. K. Ziebandt, et al. (2006). "Mapping the pathways to staphylococcal pathogenesis by comparative secretomics." Microbiol Mol Biol Rev **70**(3): 755-788.

Sierro, N., Y. Makita, et al. (2008). "DBTBS: a database of transcriptional regulation in Bacillus subtilis containing upstream intergenic conservation information." Nucleic Acids Research **36**(suppl 1): D93-D96.

Sijbrandi, R., Den Blaauwen, T., Tame, J. R., Oudega, B., Luirink, J., Otto, B. R. (2005). "Characterization of an iron-regulated alpha-enolase of Bacteroides fragilis." Microbes Infect **7**(1): 9-18.

Simonen, M., and I. Palva. (1993). "Protein secretion in *Bacillus* species." Microbiol Rev **57**: 109-137.

Simonen, M. and I. Palva (1993). "Protein secretion in *Bacillus* species." Microbiol Rev **57**(1): 109-137.

Spizizen, J. (1958). "Transformation of Biochemically Deficient Strains of *Bacillus Subtilis* by Deoxyribonucleate." Proc Natl Acad Sci U S A **44**(10): 1072-1078.

Stephenson, K., and C. R. Harwood. (1999). "Cellular lysis in *Bacillus subtilis*; the affect of multiple extracellular protease deficiencies. ." Letters in Applied Microbiology **29**: 141-145.

Stephenson, K. and C. R. Harwood (1999). "Cellular lysis in *Bacillus subtilis*; the affect of multiple extracellular protease deficiencies." Letters in Applied Microbiology **29**(0): 141-145.

Stragier, P. (2006). "To Kill but Not Be Killed: A Delicate Balance." Cell **124**(3): 461-463.

Stülke, J., R. Hanschke, et al. (1993). "Temporal activation of β-glucanase synthesis in Bacillus subtilis is mediated by the GTP pool." Journal of General Microbiology **139**(9): 2041-2045.

Tai, P. C., G. Tian, et al. (1991). Chapter 7 In Vitro Protein Translocation into Escherichia coli Inverted Membrane Vesicles. Methods in Cell Biology. M. T. Alan, Academic Press. **Volume 34:** 167-187.

Thiyagarajan, M. M., Stracquatanio, R. P., Pronin, A. N., Evanko, D. S., Benovic, J. L., Wedegaertner, P. B. (2004). "A predicted amphipathic helix mediates plasma membrane localization of GRK5." J Biol Chem **279**(17): 17989-17995.

Tjalsma, H., A. Bolhuis, J. D. Jongbloed, S. Bron, and J. M. van Dijl. (2000). "Signal peptide-dependent protein transport in *Bacillus subtilis*: a genome-based survey of the secretome." Microbiol Mol Biol Rev **64**: 515-547.

Tjalsma, H., H. Antelmann, J. D. Jongbloed, P. G. Braun, E. Darmon, R. Dorenbos, J. Y. Dubois, H. Westers, G. Zanen, W. J. Quax, O. P. Kuipers, S. Bron, M. Hecker, and J. M.van Dijl, (2004). "Proteomics of protein secretion by *Bacillus subtilis*: separating the "secrets" of the secretome." Microbiol Mol Biol Rev **68**(2): 207-233.

Tjalsma., H. (2006). "Feature-based reappraisal of the Bacillus subtilis exoproteome." PROTEOMICS **7**(1): 73-81.

Towbin, H., T. Staehelin, et al. (1979). "Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications." Proc Natl Acad Sci U S A **76**(9): 4350-4354.

Towbin, H., T. Staehelin, and J. Gordon. (1979). "Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. ." Proc Natl Acad Sci U S A **76**: 4350-4354.

Turner, M. S. and J. D. Helmann (2000). "Mutations in Multidrug Efflux Homologs, Sugar Isomerases, and Antimicrobial Biosynthesis Genes Differentially Elevate Activity of the sigma X and sigma W Factors in Bacillus subtilis." J. Bacteriol. **182**(18): 5202-5210.

U Voelker, A. V., B Maul, M Hecker, A Dufour, W G Haldenwang (1995). "Separate mechanisms activate sigma B of Bacillus subtilis in response to environmental and metabolic stresses." J Bacteriol. **177**(13): 3771-3780.

Vagner, V., Dervyn, E., and Ehrlich, S. D. (1998). "A vector for systematic gene inactivation in *Bacillus subtilis*." Microbiology **144 (Pt 11)**: 3097-3104.

van Wely, K. H., J. Swaving, et al. (2001). "Translocation of proteins across the cell envelope of Gram-positive bacteria." FEMS Microbiol Rev **25**(4): 437-454.

Vitikainen, M., Lappalainen, I., Seppala, R., Antelmann, H., Boer, H., Taira, S., Savilahti, H., Hecker, M., Vihinen, M., Sarvas, M., and Kontinen, V. P. (2004). "Structure-function analysis of PrsA reveals roles for the parvulin-like and flanking N- and C-terminal domains in protein folding and secretion in *Bacillus subtilis*." J Biol Chem **279**(18): 19302-19314.

von Heijne, G. (1992). "Membrane protein structure prediction. Hydrophobicity analysis and the positive-inside rule." J Mol Biol **225**(2): 487-494.

Wang, X., W. Chen, F. Hu, C. Deng, C. Zhou, X. Lv, Y. Fan, J. Men, Y. Huang, J. Sun, D. Hu, J. Chen, Y. Yang, C. Liang, H. Zheng, X. Hu, J. Xu, Z. Wu and X. Yu (2011). "Clonorchis sinensis enolase: Identification and biochemical characterization of a glycolytic enzyme from excretory/secretory products." Molecular and Biochemical Parasitology **177**(2): 135-142.

Wise, A., Price, CW (1995). "Four additional genes in the sigB operon of Bacillus subtilis that control activity of the general stress factor sigma B in response to environmental signals." J. Bacteriol. **177**(1): 123-133.

Wood, A. N., R. Fernandez-Lafuente, et al. (1995). "Purification and partial characterization of a novel thermophilic carboxylesterase with high mesophilic specific activity." Enzyme Microb Technol **17**(9): 816-825.

Wu, X. C., W. Lee, et al. (1991). "Engineering a *Bacillus subtilis* expression-secretion system with a strain deficient in six extracellular proteases." J Bacteriol **173**(16): 4952-4958.

X Huang, A. D., A Sorokin, J D Helmann (1997). "The Bacillus subtilis sigma(X) protein is an extracytoplasmic function sigma factor contributing to survival at high temperature." J Bacteriol. **179**(9): 2915-2921.

Yang, C., Ewis, H.E., Zhang, XZ., Lu, CD., Hu, HJ., Pan, Y., Abdelal, A.T., and Tai, PC. (2011). "Non-classical protein secretion of Bacillus subtilis in the stationary phase is not due to cell lysis " J Bacteriol **193**: 5607-5615.

Yansura, D. G. and D. J. Henner (1984). "Use of the *Escherichia coli lac* repressor and operator to control gene expression in *Bacillus subtilis*." Proc Natl Acad Sci U S A **81**(2): 439-443.

Ye, B.-C., Y. Zhang, et al. (2009). "Time-Resolved Transcriptome Analysis of <italic>Bacillus subtilis</italic> Responding to Valine, Glutamate, and Glutamine." PLoS ONE **4**(9): e7073.

Yoshimura, M., K. Asai, et al. (2004). "Interaction of Bacillus subtilis extracytoplasmic function (ECF) sigma factors with the N-terminal regions of their potential anti-sigma factors." Microbiology **150**(3): 591-599.

Yu, X., Harris, S. L., and Levine, A. J. (2006). "The regulation of exosome secretion: a novel function of the p53 protein." Cancer Res **66**(9): 4795-4801.

Zanen, G., Antelmann, H., Meima, R., Jongbloed, J. D., Kolkman, M., Hecker, M., van Dijl, J. M., and Quax, W. J. (2006). "Proteomic dissection of potential signal recognition particle dependence in protein secretion by *Bacillus subtilis*." Proteomics **6**(12): 3636-3648.

Zhang, S. and W. G. Haldenwang (2003). "RelA Is a Component of the Nutritional Stress Activation Pathway of the Bacillus subtilis Transcription Factor {sigma}B." J. Bacteriol. **185**(19): 5714-5721.

Zhang, S. and W. G. Haldenwang (2005). "Contributions of ATP, GTP, and Redox State to Nutritional Stress Activation of the Bacillus subtilis {sigma}B Transcription Factor." J. Bacteriol. **187**(22): 7554-7560.

Zuber, P. (2004). "Spx-RNA Polymerase Interaction and Global Transcriptional Control during Oxidative Stress." Journal of bacteriology **186**(7): 1911-1918.

Zuber, P. (2009). "Management of Oxidative Stress in Bacillus." Annual Review of Microbiology **63**(1): 575-597.

Appendix I.

Genechip raw data of *Bacillus subtilis* at different growth stages

Table S1. SigA regulon and genes expressed in exponential phase

Transcriptional regulator	BG number	Gene name	-T40	-T20	T0	T40	T100	T160	Function
	BG10002	purA	3945	1669	381	308	221	186	adenylosuccinate synthetase (PurA)
spo0A	BG10017	yybN	1053	507	354	644	254	216	conserved hypothetical protein (YybN)
	BG10061	jag	560	523	212	582	523	394	SpoIIIJ-associated RNA/ssDNA-binding protein (Jag)
spo0A	BG10075	yaaD	1777	536	747	655	638	866	glutamine amidotransferase for pyridoxal phosphate synthesis (YaaD)
spo0A	BG10076	yaaE	1443	579	613	516	573	791	glutamine amidotransferase for pyridoxal phosphate synthesis (YaaE)
	BG10077	serS	870	505	472	386	326	345	seryl-tRNA synthetase (SerS)
	BG10110	purR	1457	559	473	395	398	368	transcriptional regulator of the purine biosynthesis operon (PurR)
	BG10111	yabJ	1641	542	686	781	834	755	putative enzyme resulting in alteration of gene expression (YabJ)
	BG10135	yacD	525	198	449	471	293	307	putative protein secretion PrsA homolog (YacD)
	BG10142	folK	579	215	521	450	376	506	7,8-dihydro-6-hydroxymethylpterin pyrophosphokinase (FolK)
	BG10153	yacN	727	457	445	420	339	290	2-C-methyl-D-erythritol-2,4-cyclodiphosphate synthase (YacN)
	BG10155	cysE	793	424	630	491	321	449	serine acetyltransferase (CysE)
	BG10156	cysS	960	385	646	604	367	465	cysteinyl-tRNA synthetase (CysS)
	BG10198	ptsG	826	405	317	399	351	295	(PTS) glucose-specific enzyme IICBA component
sigW	BG10207	pdhA	1443	1075	1347	947	801	445	pyruvate dehydrogenase (E1 alpha subunit) (PdhA)
sigD	BG10250	fliL	651	476	292	323	286	256	flagellar basal-body associated protein (FliL)
sigD	BG10252	fliY	1189	1250	648	515	420	275	flagellar motor switching and energizing phosphatase (FliY)
sigD	BG10258	cheY	1379	1654	922	657	745	546	regulator of chemotaxis and motility (CheY)
	BG10284	aroF	689	1015	375	338	349	291	chorismate synthase (AroF)
	BG10285	aroB	563	709	258	267	286	171	3-dehydroquinate synthase (AroB)
	BG10286	aroH	1505	1774	805	705	726	505	chorismate mutase (AroH)
	BG10293	tyrA	1008	992	573	579	584	360	prephenate dehydrogenase (TyrA)
	BG10321	valS	1142	675	542	538	542	404	valyl-tRNA synthetase (ValS)
	BG10338	lonA	1011	1463	1198	1060	1157	588	class III heat-shock ATP-dependent LonA protease
	BG10362	thrS	1184	1074	585	658	791	466	threonyl-tRNA synthetase (ThrS)
	BG10371	tyrS	1785	743	546	696	645	265	tyrosyl-tRNA synthetase (TyrS)
	BG10375	aroA	1341	1201	623	632	649	460	chorismate mutase-isozyme 3 (AroA)
spo0A	BG10377	yuxH	811	696	528	498	390	311	putative phosphodiesterase (YuxH)
sigH	BG10384	citG	599	487	370	366	424	190	fumarate hydratase (CitG)
sigD	BG10398	yvyF	1338	1106	626	710	608	470	putative regulator of flagella formation (YvyF)
sigD	BG10399	flgM	2870	2498	1409	1257	1300	1049	anti-sigma factor repressor of sigma(D)-dependent transcription (FlgM)
sigD	BG10400	yvyG	1236	1048	654	571	553	469	putative flagellar protein (YvyG)
sigD	BG10401	flgK	1760	1422	739	661	723	614	flagellar hook-filament junction (FlgK)
sigB	BG10402	gtaB	2849	3785	3285	2020	1854	865	UTP-glucose-1-phosphate uridylyltransferase (GtaB)
sigD	BG10405	lytA	1210	704	286	512	212	167	membrane bound lipoprotein (LytA)
sigD	BG10406	lytB	1597	1322	618	624	443	291	modifier protein of major autolysin LytC
sigD	BG10407	lytC	1089	794	368	508	379	247	N-acetylmuramoyl-L-alanine amidase
	BG10410	ctrA	1223	655	392	672	687	450	CTP synthetase
	BG10412	fbaA	7383	5913	5114	4896	6608	2346	fructose-1,6-bisphosphate aldolase (FbaA)
	BG10413	ywjH	3474	2483	2075	2386	2807	922	putative transaldolase (YwjH)

Table S1. ----*Continued*

Transcriptional regulator	BG number	Gene name	-T40	-T20	T0	T40	T100	T160	Function
	BG10415	ywjI	1835	1291	1276	1113	1447	532	fructose 1,6-bisphosphatase class II (YwjI)
	BG10422	groES	5019	3544	3500	2756	1951	543	chaperonin small subunit
	BG10423	groEL	3968	3720	2816	2007	1812	797	chaperonin large subunit
	BG10427	yhgC	1091	1580	1536	1410	960	483	heme-degrading monooxygenase (YhgC)
sigW	BG10442	rpsE	3090	768	673	536	454	970	ribosomal protein S5 (RpsE)
	BG10443	rpmD	1937	472	443	425	413	645	ribosomal protein L30 (BL27) (RpmD)
sigW	BG10444	rplO	2236	492	522	557	516	840	ribosomal protein L15 (RplO)
sigW	BG10445	secY	3094	1092	807	733	711	1272	preprotein translocase subunit
sigW	BG10446	adk	3192	1310	716	752	882	1240	adenylate kinase
	BG10447	map	3325	1670	899	975	926	1407	methionine aminopeptidase (Map)
sigD	BG10455	lytD	586	472	275	316	192	181	exported N-acetylglucosaminidase (LytD)
sigV	BG10461	thrC	649	637	361	313	261	262	threonine synthase (ThrC)
sigH	BG10463	yuxL	523	570	337	322	274	214	putative acylaminoacyl-peptidase (YuxL)
	BG10468	ald	2181	1590	725	628	598	492	L-alanine dehydrogenase
	BG10471	alsS	2901	2511	1666	1180	1202	1197	alpha-acetolactate synthase (AlsS)
	BG10472	alsD	3128	2408	1698	1244	1660	1762	alpha-acetolactate decarboxylase (AlsD)
	BG10509	serA	1372	909	387	458	357	328	3-phosphoglycerate dehydrogenase (SerA)
	BG10512	ppiB	1912	894	916	1773	1936	764	peptidyl-prolyl isomerase (PpiB)
	BG10531	resA	785	510	477	709	421	289	extracytoplasmic thioredoxin (lipoprotein) (ResA)
sigY	BG10546	ywaA	970	855	365	417	393	384	branched-chain amino acid aminotransferase (YwaA)
spo0A, sigW, sigV, sigX	BG10548	dltD	1000	549	299	505	473	544	putative D-alanine esterase (DltD)
spo0A, sigW, sigV	BG10549	dltC	1080	484	284	472	369	473	D-alanyl carrier protein (DltC)
spo0A, sigW, sigV	BG10550	dltB	1295	665	420	572	632	644	putative D-alanine esterase (DltB)
	BG10583	qoxA	2997	2196	1376	1258	1129	631	cytochrome aa3-600 quinol oxidase (subunit II) (QoxA)
	BG10585	qoxC	3174	2070	1226	1360	1338	1304	cytochrome aa3-600 quinol oxidase (subunit III) (QoxC)
	BG10634	pta	2777	1116	834	1176	1015	426	phosphotransacetylase
	BG10663	grpE	1982	1544	1287	1283	1460	532	nucleotide exchange factor for DnaK activity (GrpE)
codY, sigV	BG10670	ilvB	1350	852	270	306	213	233	acetolactate synthase (large subunit) (IlvB)
codY	BG10671	ilvN	1684	1132	301	351	288	236	acetolactate synthase (small subunit) (IlvN)
codY	BG10672	ilvC	1541	1318	528	375	283	279	acetohydroxy-acid isomeroreductase (IlvC)
codY, sigW, sigV	BG10675	leuB	596	618	325	359	263	248	3-isopropylmalate dehydrogenase (LeuB)
	BG10676	leuS	1155	645	488	516	574	272	leucyl-tRNA synthetase (LeuS)
spo0A	BG10700	purE	5313	793	590	472	377	443	phosphoribosylaminoimidazole carboxylase I (PurE)
spo0A	BG10701	purK	5410	693	400	331	230	281	phosphoribosylaminoimidazole carboxylase (PurK)
	BG10702	purB	5641	768	390	371	280	374	adenylosuccinate lyase (PurB)
sigD	BG10703	purC	6130	977	399	362	302	312	phosphoribosylaminoimidazole succinocarboxamide synthetase (PurC)
	BG10704	yexA	6124	1056	402	351	311	375	factor required for phosphoribosylformylglycinamidine synthetase (YexA)
sigD	BG10705	purL	6030	1140	467	366	354	341	phosphoribosylformylglycinamidine synthetase II (PurL)
	BG10706	purQ	5857	2062	513	394	365	380	phosphoribosylformylglycinamidine synthetase I (PurQ)
	BG10707	purF	5694	3077	700	377	344	365	glutamine phosphoribosylpyrophosphate amidotransferase (PurF)

Table S1. ----*Continued*

Transcriptional regulator	BG number	Gene name	-T40	-T20	T0	T40	T100	T160	Function
sigD	BG10709	purN	4760	2903	732	415	278	388	phosphoribosylglycinamide formyltransferase (PurN)
sigV	BG10710	purH	6472	5356	1340	547	411	513	inosine-monophosphate cyclohydrolase (PurH)
	BG10711	purD	4139	3352	1035	453	335	403	phosphoribosylglycinamide synthetase (PurD)
sigW	BG10715	pyrAA	971	443	294	704	499	324	pyrimidine-specific carbamoyl-phosphate synthetase
sigW	BG10716	pyrAB	1727	836	559	988	884	600	pyrimidine-specific carbamoyl-phosphate synthetase
sigW	BG10717	pyrDII	1335	561	347	540	681	360	dihydroorotate dehydrogenase
sigW, sigV	BG10718	pyrD	2651	1298	714	972	1272	664	dihydroorotate dehydrogenase
sigW, sigV	BG10719	pyrF	2163	976	525	690	1050	469	orotidine 5'-phosphate decarboxylase
sigW	BG10720	pyrE	1270	552	292	363	491	241	orotate phosphoribosyltransferase
	BG10726	rplL	1935	630	712	1041	1028	796	ribosomal protein L12 (BL9) (RplL)
	BG10730	rpsM	3417	1384	1328	1149	1255	1558	ribosomal protein S13 (RpsM)
	BG10731	rpsK	3153	1343	1187	1011	1393	1625	ribosomal protein S11 (BS11) (RpsK)
	BG10732	rpoA	4035	2193	1630	1557	2431	2223	RNA polymerase (alpha subunit) (RpoA)
	BG10746	kapB	776	841	788	829	548	291	signal transduction and activation of the phosphorelay to sporulation (KapB)
sigW, sigV	BG10755	rplP	2884	1202	668	637	778	1137	ribosomal protein L16 (RplP)
sigW, sigV	BG10756	rpmC	1619	551	279	299	323	471	ribosomal protein L29 (RpmC)
sigW, sigV	BG10757	rpsQ	2869	1117	551	567	730	1002	ribosomal protein S17 (BS16) (RpsQ)
sigW	BG10758	rplN	3534	1629	734	688	1035	1259	ribosomal protein L14
sigW	BG10759	rplX	2549	1108	493	451	622	776	ribosomal protein L24 (BL23) (RplX)
sigW, sigV	BG10760	rplE	2868	946	534	435	630	693	ribosomal protein L5 (BL6) (RplE)
sigW	BG10761	rpsN	2916	928	604	518	660	968	alternative ribosomal protein S14 (RpsNB)
sigW	BG10762	rpsH	1536	497	242	272	224	475	ribosomal protein S8 (BS8) (RpsH)
	BG10771	oppA	4319	2746	557	469	353	387	oligopeptide ABC transporter (binding lipoprotein) (OppA)
	BG10772	oppB	749	529	253	285	222	229	oligopeptide ABC transporter (permease) (OppB)
	BG10773	oppC	1104	802	457	349	331	330	oligopeptide ABC transporter (permease) (OppC)
	BG10774	oppD	1388	1255	568	411	382	334	oligopeptide ABC transporter (ATP-binding protein) (OppD)
	BG10775	oppF	1384	1322	505	354	396	332	oligopeptide ABC transporter (ATP-binding protein) (OppF)
	BG10799	trpS	960	670	459	427	387	425	tryptophanyl-tRNA synthetase (TrpS)
	BG10812	ytxK	966	857	609	958	649	344	putative nucleic acid methyltransferase (YtxK)
sigV	BG10813	ackA	1556	782	690	818	885	559	acetate kinase (AckA)
	BG10815	atpB	2082	1078	1232	1710	994	331	ATP synthase (AtpB)
	BG10816	atpE	2290	1242	1302	1611	1141	516	ATP synthase (AtpE)
	BG10817	atpF	1721	867	1001	1220	834	319	ATP synthase (AtpF)
	BG10818	atpH	2474	1522	1557	1721	1534	610	ATP synthase (AtpH)
	BG10819	atpA	1430	963	825	1032	889	380	ATP synthase (AtpA)
	BG10820	atpG	2640	1616	1376	1597	1530	618	ATP synthase (AtpG)
	BG10821	atpD	1868	1389	894	1214	1096	585	ATP synthase (AtpD)
	BG10835	feuA	874	417	487	467	336	316	iron hydroxamate-binding lipoprotein (FeuA)
	BG10840	pel	701	444	444	477	422	232	pectate lyase (Pel)
	BG10849	katA	4819	3233	972	897	1584	2155	vegetative catalase 1 (KatA)

Table S1. ----*Continued*

Transcriptional regulator	BG number	Gene name	-T40	-T20	T0	T40	T100	T160	Function
sigD	BG10917	flhO	1817	1873	1060	937	896	610	putative flagellar basal-body rod protein (FlhO)
sigD	BG10920	yvyC	750	475	187	329	197	100	putative flagellar protein (YvyC)
sigD	BG10921	fliD	2461	2133	854	735	735	433	flagellar hook-associated capping protein 2 (HAP2) (FliD)
sigD	BG10922	fliS	2274	1533	645	655	628	299	flagellar assembly protein FliS
spo0A, sigD	BG10923	fliT	3165	2039	1128	1040	956	565	flagellar assembly protein FliT involved in control of flagella expression
spo0A	BG10924	purT	1304	941	493	511	374	464	phosphoribosylglycinamide formyltransferase 2 (PurT)
	BG10944	glyA	5155	3517	2034	1171	1166	1317	serine hydroxymethyltransferase (GlyA)
	BG10945	upp	3277	1678	1003	1073	1237	498	uracil phosphoribosyltransferase
	BG10948	glmS	1947	697	915	672	786	594	L-glutamine-D-fructose-6-phosphate amidotransferase (GlmS)
	BG10986	ampS	1330	1301	762	780	617	415	aminopeptidase (AmpS)
	BG11023	yvcE	672	284	249	390	236	169	secreted cell wall DL-endopeptidase (YvcE)
	BG11042	rpmJ	4794	2211	1760	1786	2410	2372	ribosomal protein L36 (ribosomal protein B) (RpmJ)
	BG11043	infA	3578	1574	1568	1448	1589	1972	initiation factor IF-I (InfA)
	BG11048	yoxD	1006	366	172	279	269	263	putative oxido-reductase (YoxD)
sigW, sigV	BG11078	rplV	3371	1026	903	1074	1556	2064	ribosomal protein L22 (BL17) (RplV)
	BG11079	xpt	1998	482	271	275	277	235	xanthine phosphoribosyltransferase (Xpt)
	BG11080	pbuX	1511	437	238	290	251	225	xanthine permease (PbuX)
	BG11081	narG	856	939	549	373	289	328	nitrate reductase (alpha subunit) (NarG)
	BG11082	narH	1140	1636	719	357	281	362	nitrate reductase (beta subunit)
	BG11083	narJ	1015	1440	776	422	349	308	nitrate reductase (protein J)
	BG11084	narI	785	1230	806	505	409	459	nitrate reductase (gamma subunit)
	BG11150	yxjA	956	850	730	432	372	399	purine nucleoside transporter (YxjA)
	BG11156	yxjG	689	608	307	292	239	287	putative methyltetrahydrofolate methyltransferase (YxjG)
	BG11182	yckF	945	604	339	278	170	224	6-phospho-3-hexuloisomerase (PHI) (YckF)
	BG11183	yckG	1428	758	520	362	284	363	3-hexulose-6-phosphate synthase (HPS) (YckG)
	BG11207	dapB	995	692	652	936	682	392	dihydrodipicolinate reductase (DapB)
sigW, sigV	BG11217	rplB	2475	513	840	916	1199	1693	ribosomal protein L2 (BL2) (RplB)
sigW, sigV	BG11218	rplC	976	352	347	517	453	866	ribosomal protein L3 (BL3) (RplC)
sigW, sigV	BG11219	rplD	2011	490	572	820	1238	1614	ribosomal protein L4 (RplD)
sigW, sigV, ylac	BG11220	rplJ	1911	368	497	915	1074	539	ribosomal protein L10 (BL5) (RplJ)
sigW, sigV	BG11221	rplW	2470	437	619	874	1254	1777	ribosomal protein L23 (RplW)
	BG11234	pbpC	949	429	416	565	632	371	penicillin-binding lipoprotein 3 (PbpC)
spo0A	BG11247	tkt	1059	915	670	622	387	151	transketolase
sigB	BG11308	ywjC	512	866	768	387	247	300	conserved hypothetical protein (YwjC)
	BG11342	narK	885	795	327	377	302	286	nitrite extrusion permease (NarK)
	BG11343	fnr	948	963	425	397	272	314	transcriptional regulator (FNR/CAP family)
	BG11344	ywiC	767	540	275	359	235	311	putative integral inner membrane protein (YwiC)
	BG11345	ywiD	794	690	248	321	246	187	transcriptional regulator (YwiD)
	BG11370	opuAA	865	256	464	553	522	430	glycine betaine ABC transporter (ATP-binding protein) (OpuAA)
sigV	BG11371	opuAB	786	256	486	518	464	501	glycine betaine ABC transporter (permease) (OpuAB)

Table S1. ----*Continued*

Transcriptional regulator	BG number	Gene name	-T40	-T20	T0	T40	T100	T160	Function
	BG11383	accB	862	622	404	603	502	316	acetyl-CoA carboxylase subunit (biotin carboxyl carrier subunit) (AccB)
	BG11384	accC	1687	1014	813	846	732	376	acetyl-CoA carboxylase subunit (biotin carboxylase subunit) (AccC)
sigW	BG11408	rplF	2353	680	306	366	332	729	ribosomal protein L6 (BL8) (RplF)
	BG11409	rplR	3301	983	645	578	591	1124	ribosomal protein L18 (RplR)
	BG11458	ypwA	670	683	456	670	496	278	metal-dependent carboxypeptidase (YpwA)
	BG11501	ypmA	987	589	353	704	695	355	conserved hypothetical protein (YpmA)
	BG11502	ypmB	2386	1192	521	1281	1356	380	conserved hypothetical protein (YpmB)
	BG11511	yqhK	1043	819	415	524	609	572	glycine decarboxylase (subunit 2) (glycine cleavage system protein P) (YqhK)
	BG11513	aspB	1950	1150	444	784	816	162	putative aspartate aminotransferase (AspB)
	BG11531	cspD	6909	2975	2350	4167	6059	5546	cold-shock protein, molecular chaperone, RNA-helicase co-factor (CspD)
	BG11536	acpA	2351	1526	1163	1588	1507	343	acyl carrier protein (AcpA)
	BG11588	yhcJ	545	667	576	496	267	415	putative ABC transporter (binding lipoprotein) (YhcJ)
	BG11601	yhcW	1566	1633	1263	826	655	813	putative phosphoglycolate phosphatase (YhcW)
	BG11657	glyQ	939	571	376	401	457	329	glycyl-tRNA synthetase (alpha subunit) (GlyQ)
	BG11658	glyS	857	491	251	307	332	229	glycyl-tRNA synthetase (beta subunit) (GlyS)
	BG11673	yqgA	3768	1443	981	2189	1528	318	hypothetical protein (YqgA)
	BG11691	yqgX	1366	1011	794	679	476	401	putative metal-binding hydrolase (YqgX)
	BG11709	yqhY	1337	1346	1072	1002	806	514	conserved hypothetical protein (YqgY)
	BG11710	yqhZ	3415	1098	617	713	576	326	transcriptional regulator of stress (YqgZ)
	BG11711	folD	1672	517	355	345	383	247	methylenetetrahydrofolate dehydrogenase
	BG11738	yqjI	4361	4093	3052	1923	2249	1368	NADP+-dependent 6-P-gluconate dehydrogenase (YqjI)
	BG11761	yqkF	664	1047	853	775	790	379	NADPH-dependent aldo-keto reductase (YqkF)
	BG11792	ileS	1778	1295	690	746	759	543	isoleucyl-tRNA synthetase (IleS)
	BG11931	czcD	3113	1548	628	678	851	809	potassium/proton-divalent cation antiporter (CzcD)
sigD	BG11936	flgL	1147	997	523	604	531	451	flagellar hook-filament junction (FlgL)
codY, sigV	BG11948	leuA	774	586	316	342	277	257	2-isopropylmalate synthase (LeuA)
	BG11968	rapF	1073	665	499	502	440	345	response regulator aspartate phosphatase (RapF)
	BG11980	trkA	1461	701	331	363	364	253	putative oxidoreductase (TrkA)
	BG12199	ydiB	650	499	515	581	245	294	ATPase, regulated by oligomerization (YdiB)
	BG12310	ysaA	786	463	420	569	616	314	putative phosphatase (YsaA)
	BG12311	ysbA	4470	1934	1029	456	376	287	antiholin factor (YsbA)
	BG12312	ysbB	3308	1466	883	516	389	374	antiholin factor (YsbB)
	BG12361	yugG	732	468	375	419	396	298	putative transcriptional regulator (Lrp/AsnC family) (YugG)
	BG12388	yulF	600	639	459	453	284	225	enzyme involved in biofilm formation (YulF)
codY	BG12392	yumD	4835	677	371	403	382	253	GMP reductase (YumD)
sigD	BG12535	ywtD	690	554	442	514	442	225	gamma-DL-glutamyl hydrolase (PGA depolymerase) (YwtD)
	BG12536	ywtE	1086	855	570	739	502	308	putative hydrolase (YwtE)
sigD	BG12541	yxkC	1067	641	306	461	325	226	conserved hypothetical protein (YxkC)
	BG12563	alaS	1015	679	538	553	651	374	alanyl-tRNA synthetase (AlaS)
	BG12572	aspS	1651	2149	1425	1105	1629	783	aspartyl-tRNA synthetase (AspS)

Table S1. ----*Continued*

Transcriptional regulator	BG number	Gene name	-T40	-T20	T0	T40	T100	T160	Function
spo0A	BG12574	cccB	2001	1236	1179	1501	1723	787	cytochrome c551 (CccB)
	BG12581	deoD	810	331	184	555	365	244	purine nucleoside phosphorylase (DeoD)
	BG12587	frr	1014	510	440	971	804	319	ribosome recycling factor (Frr)
sigY	BG12598	hisB	374	552	353	339	268	222	imidazoleglycerol-phosphate dehydratase (HisB)
sigY	BG12599	hisD	344	587	301	277	214	182	histidinol dehydrogenase (HisD)
	BG12600	hisF	462	639	372	345	256	267	imidazole glycerol phosphate synthase subunit (HisF)
	BG12602	hisH	523	856	410	417	363	333	amidotransferase (glutaminase) (HisH)
	BG12603	hisI	462	639	439	350	235	328	phosphoribosyl-ATP pyrophosphohydrolase (HisI)
	BG12605	hisS	1725	1999	882	916	920	421	histidyl-tRNA synthetase (HisS)
	BG12607	hit	1988	1486	1034	1004	702	941	Hit-family hydrolase
	BG12644	pfk	2803	1758	1473	1832	1843	486	6-phosphofructokinase (Pfk)
	BG12673	serC	1898	1327	611	595	388	331	phosphoserine aminotransferase (SerC)
	BG12675	smbA	1798	692	773	1614	1322	391	uridylate kinase (SmbA)
	BG12680	sucC	2890	2603	1480	1652	1899	803	succinyl-CoA synthetase (beta subunit)
	BG12683	sunT	640	229	147	197	172	146	sublancin 168 lantibiotic transporter (SunT)
	BG12704	ybbT	605	262	447	489	376	207	phosphoglucosamine mutase (YbbT)
	BG12811	yebB	835	186	252	226	203	258	hypoxanthine/guanine permease (YebB)
	BG12838	yerL	1462	665	461	595	355	250	glutamyl-tRNA(Gln) amidotransferase (subunit C) (YerL)
	BG12839	yerM	1378	709	460	562	389	307	glutamyl-tRNA(Gln) amidotransferase (subunit A) (YerM)
	BG12840	yerN	1116	631	380	485	386	317	glutamyl-tRNA(Gln) amidotransferase (subunit B) (YerN)
sigD	BG12863	yetG	880	756	555	417	358	439	heme-degrading monooxygenase (YetG)
	BG12877	yfhB	1265	1098	660	655	505	487	putative isomerase (YfhB)
sigD	BG12970	yfmS	1448	1679	1189	561	645	392	putative chemotaxis sensory transducer (YfmS)
sigD	BG12971	yfmT	2128	2487	1722	780	927	496	putative aldehyde dehydrogenase (YfmT)
	BG13010	yhdD	645	679	455	384	342	276	gamma-D-glutamate-meso-diaminopimelate muropeptidase (YhdD)
	BG13054	yhfI	1616	1316	1199	1158	723	474	putative metal-dependent hydrolase (YhfI)
	BG13055	yhfJ	947	800	684	814	589	319	lipoate-protein ligase (YhfJ)
	BG13141	yjbL	899	588	500	627	290	229	putative phosphatase (YjbL)
	BG13142	yjbM	911	568	538	658	463	328	(p)ppGpp synthetase (YjbM)
spo0A	BG13227	ykbA	1168	552	488	453	396	550	Ser/Thr exchanger transporter (YkbA)
	BG13236	ykgB	1972	2161	1339	1166	1055	695	putative 6-phosphogluconolactonase (YkgB)
	BG13256	ykoK	1676	1984	1820	580	531	506	magnesium transporter (YkoK)
	BG13301	ykuQ	1902	1207	557	591	438	259	tetrahydrodipicolinate N-acetyltransferase (YkuQ)
	BG13395	yloU	932	752	534	578	359	325	conserved hypothetical protein (YloU)
spo0A	BG13580	yolA	2807	1765	766	1361	1026	424	exported protein of unknown function; phage SPbeta (YolA)
spo0A	BG13581	yolB	1148	793	353	634	611	339	conserved hypothetical protein; phage SPbeta (YolB)
	BG13785	yrbF	2849	1409	719	1256	1063	595	conserved hypothetical protein (YrbF)
	BG13801	yrvB	1665	1202	1154	1271	1100	534	
	BG13814	yrzD	941	648	465	737	406	243	post-transcriptional regulator (YrzD)
sigW	BG13839	yteI	1464	863	648	686	490	422	signal peptide peptidase (YteI)

Table S1. ----*Continued*

Transcriptional regulator	BG number	Gene name	-T40	-T20	T0	T40	T100	T160	Function
	BG13851	ytgA	3465	1986	1338	1695	1750	789	manganese ABC transporter (manganese binding lipoprotein) (YtgA)
	BG13852	ytgB	2799	1627	855	1084	1050	563	manganese ABC transporter (ATP-binding protein) (YtgB)
	BG13853	ytgC	3068	1829	885	1181	1342	810	manganese ABC transporter (permease) (YtgC)
	BG13854	ytgD	1882	1187	574	666	741	530	manganese ABC transporter (permease) (YtgD)
	BG13855	ytgI	3994	4048	2731	2629	2460	1602	putative peroxiredoxin (YtgI)
	BG13934	ytwP	1026	934	693	976	982	379	negative regulator of FtsZ ring formation (YtwP)
	BG13935	ytzA	1931	1379	1058	1144	1284	552	putative integral inner membrane protein (YtzA)
	BG13965	yueJ	1835	1427	1034	948	858	527	nicotinamidase (YueJ)
spo0A	BG13966	yueK	1443	1088	636	665	623	383	nicotinate phosphoribosyltransferase (YueK)
	BG14013	yusA	1004	625	474	467	408	477	methionine ABC transporter, substrate binding lipoprotein (YusA)
	BG14019	yusG	2478	2035	1588	1540	1725	979	conserved hypothetical protein (YusG)
	BG14020	yusH	2895	2026	1541	1765	1530	807	putative glycine cleavage system protein H (YusH)
	BG14021	yusI	1767	1137	1042	1002	681	472	putative oxidoreductase with thioredoxin domain (YusI)
	BG14037	yusY	665	838	441	410	382	313	Pseudogene: yusY
	BG14042	yutF	1108	1048	734	824	622	450	putative p-nitrophenyl phosphatase (YutF)
	BG14125	yvoB	1024	561	661	747	719	323	serine/threonine protein kinase/phosphorylase (YvoB)
	BG14160	yvzD	642	559	311	482	227	219	Pseudogene
	BG14161	ywiB	1581	1312	1251	1368	1295	561	conserved hypothetical protein (YwiB)
sigW	BG19003	lctE	1445	435	514	490	420	544	L-lactate dehydrogenase (LctE)
sigW, sigV	BG19005	rpsC	1466	524	390	402	467	677	ribosomal protein S3 (BS3) (RpsC)
sigW, sigV	BG19011	rpsS	2170	357	634	753	874	1196	ribosomal protein S19 (BS19) (RpsS)
	BG19023	tig	1523	811	649	820	600	325	prolyl isomerase (trigger factor) (Tig)

Table S2. SigB regulon and genes expressed in transition phase

Transcriptional regulator	BG number	Gene name	-T40	-T20	T0	T40	T100	T160	Function
spo0A	BG10100	abrB	530	369	816	436	279	434	response regulator
sigB	BG10115	ctc	998	1728	4713	1915	1869	3546	ribosomal protein Ctc, binding 5S RNA
sigB	BG10116	spoVC	553	301	817	509	389	372	peptidyl-tRNA hydrolase (SpoVC)
sigB, sigF	BG10148	clpC	1245	2661	3239	1432	1787	3399	class III stress response-related ATPase, AAA+ superfamily
	BG10269	ylxP	508	791	1326	733	864	1124	conserved hypothetical protein
sigB	BG10302	bmrU	354	848	1305	552	557	849	putative diacylglycerol kinase
sigB	BG10303	bmr	348	645	1291	529	679	729	multidrug-efflux transporter
sigB	BG10373	ytxJ	1200	2345	3536	1193	1119	1049	conserved hypothetical protein
	BG10427	yhgC	1091	1580	1536	1410	960	483	heme-degrading monooxygenase (YhgC)
sigH	BG10469	yuxI	1148	1410	1480	951	914	571	conserved hypothetical protein (YuxI)
sigB	BG10558	gspA	772	2532	3483	1121	1001	945	putative glycosyl transferase (general stress protein)
	BG10643	kbaA	504	265	734	446	216	375	activation of the KinB signaling pathway to sporulation
sigB	BG10733	rsbV	2285	4106	6131	2100	2706	3320	sigB regulator
sigB	BG10734	rsbW	2616	4866	6657	2236	3348	4001	sigB regulator
sigB	BG10735	sigB	858	2237	3959	1167	1236	1117	Sigma factor
sigB	BG10736	rsbX	1289	2215	5616	1836	2067	1260	sigB regulator
sigD	BG10751	sigD	682	617	812	496	329	490	RNA polymerase sigma-28 factor (sigma-D) (SigD)
sigB, sigZ	BG10826	gsiB	2553	6229	8771	5425	5788	5277	general stress protein
sigW	BG10829	ylxM	535	632	1589	1537	1554	1265	conserved hypothetical protein (YlxM)
	BG10830	ffh	847	1248	2690	2103	2578	2376	signal recognition particle-like (SRP) GTPase (Ffh)
sigB	BG10850	yitT	408	807	1310	622	582	656	putative integral inner membrane protein
sigB, sigH	BG10974	ytxG	2059	3923	5160	1453	976	853	conserved hypothetical protein
sigB	BG10975	ytxH	1809	4298	5921	1594	1550	1506	conserved hypothetical protein
	BG10976	yuxJ	503	845	1247	713	738	525	putative exporter (YuxJ)
sigB	BG11102	katB	674	1585	2585	839	752	755	catalase 2
sigB	BG11148	yxiS	826	1330	2445	1071	930	930	hypothetical protein
sigB, sigF, sigG	BG11591	yhcM	338	601	846	455	374	414	hypothetical protein
sigB	BG11693	yqgZ	1368	3615	4700	1266	1585	2517	transcriptional regulator of stress
	BG11742	yqjM	398	722	986	601	507	457	NADPH-dependent flavin oxidoreductase (YqjM)
sigB, sigF, sigG	BG11922	csbX	294	585	842	390	353	267	putative permease
sigB	BG12052	ydaD	391	994	1444	496	313	349	putative dehydrogenase
sigB	BG12053	ydaE	305	602	847	401	271	334	predicted D-lyxose isomerase
sigB	BG12063	ydaP	599	999	1336	556	633	486	putative enzyme with pyruvate as substrate (YdaP)
sigB	BG12066	ydaS	378	1248	2053	735	398	440	conserved hypothetical protein
sigB	BG12067	ydaT	429	811	1328	648	601	719	conserved hypothetical protein
sigB	BG12071	ydbD	663	1424	3729	1776	1824	1502	putative manganese-containing catalase
sigB	BG12188	ydhK	425	629	766	452	289	301	hypothetical protein (YdhK)
	BG12367	yugM	552	712	1118	934	682	442	putative transporter (YugM)
sigB	BG12478	ywmG	462	977	1111	463	419	364	stress response protein (YwmG)
sigB	BG12530	ywsB	908	1832	2835	1337	1057	964	conserved hypothetical protein

Table S2. ----*Continued*

Transcriptional regulator	BG number	Gene name	-T40	-T20	T0	T40	T100	T160	Function
	BG12582	dhaS	361	483	954	695	873	629	putative aldehyde dehydrogenase
sigB	BG12584	dps	1121	2392	4426	1518	1014	753	DNA-protecting protein, ferritin
sigB	BG12641	opuE	324	606	927	307	268	245	proline transporter
sigB	BG12754	ybyB	1636	4494	6816	3228	2336	3321	conserved hypothetical protein
sigD	BG12814	yebE	251	503	652	363	180	344	conserved hypothetical protein (YebE)
sigB	BG12886	yfhK	337	778	1162	407	247	320	conserved hypothetical protein (YfhK)
sigB	BG12924	yfkH	754	1225	1517	690	599	456	putative hydrolase (YfhM)
sigB	BG12925	yfkI	589	1112	1392	554	495	401	conserved hypothetical protein (YfkI)
sigB	BG12926	yfkJ	837	1532	1920	694	701	544	conserved hypothetical protein
sigB	BG12929	yfkM	469	1301	2423	1017	737	836	putative hydrolase
sigB	BG13020	yhdN	315	666	820	350	402	386	aldo/keto reductase specific for NADPH
sigB	BG13043	yheK	680	2636	6120	2205	1842	3670	stress response protein, UspA family
sigB	BG13337	ykzI	396	887	1317	514	344	523	conserved hypothetical protein
sigB	BG13524	yocK	255	645	1024	451	535	773	putative general stress protein
sigB	BG14057	yvaA	1399	2139	2293	1230	1028	468	scyllo-inositol dehydrogenase (YvaA)
sigB	BG14066	yvaK	778	900	990	647	543	384	carboxylesterase (YvaK)
sigB	BG14162	ywzA	533	1464	2338	627	560	666	conserved hypothetical protein
sigB	BG19017	ydaG	1447	3884	6040	2208	1582	1233	putative general stress protein
sigB	BG19020	yflT	1070	4074	7470	3148	1980	2089	heat stress induced protein
sigB	BG19021	ykzA	611	2241	4198	2165	2331	2782	organic hydroperoxide resistance reductase B

Table S3. SigW regulon and genes expressed in stationary phase

Transcriptional regulator	BG number	Gene name	-T40	-T20	T0	T40	T100	T160	Function
sigV	BG10047	rpsR	1183	1025	1062	1659	2551	2622	ribosomal protein S18 (RpsR)
sigW, sigV	BG10048	ssb	2624	1865	2064	2903	4515	4675	single-strand DNA-binding protein
	BG10068	recF	445	560	694	565	971	1127	DNA repair and genetic recombination factor (RecF)
	BG10069	yaaB	300	419	618	634	862	905	regulator of the extracellular matrix
	BG10070	gyrB	624	759	926	729	1375	1848	DNA gyrase (subunit B) (GyrB)
sigW	BG10089	xpaC	369	259	570	467	436	817	putative phosphatase (XpaC)
sigW	BG10091	yaaO	377	381	678	628	685	1005	putative lysine decarboxylase (YaaO)
sigW	BG10092	tmk	311	170	495	495	407	836	thymidylate kinase (Tmk)
	BG10093	yaaQ	1804	725	2226	1672	1789	2910	conserved hypothetical protein (YaaQ)
spo0A	BG10094	yaaR	1135	537	1967	1055	1568	1979	conserved hypothetical protein (YaaR)
spo0A	BG10095	holB	643	584	1081	635	936	1669	DNA polymerase III delta' subunit (HolB)
spo0A	BG10096	yaaT	1318	1346	1889	1334	2240	3784	conserved hypothetical protein (YaaT)
spo0A	BG10107	veg	1099	1457	2286	2088	3604	5529	conserved hypothetical protein
	BG10136	cysK	1440	723	1340	865	938	2270	cysteine synthase (CysK)
sigB, sigF	BG10148	clpC	1245	2661	3239	1432	1787	3399	class III stress response-related ATPase, AAA+ superfamily (ClpC)
codY	BG10168	srfAA	696	573	700	1014	1922	1978	surfactin synthetase
	BG10169	srfAB	428	509	500	427	1168	2070	surfactin synthetase
	BG10170	srfAC	842	774	1067	716	1922	3712	surfactin synthetase
	BG10171	srfAD	611	714	854	649	1164	2668	surfactin synthetase
	BG10205	patA	808	1073	1024	875	1509	2004	aminotransferase (PatA)
ylac	BG10210	pdhD	2524	2209	2723	2212	5515	6725	dihydrolipoamide dehydrogenase (PdhD)
sigD	BG10239	fliE	1705	1849	1298	1015	1384	3206	flagellar basal body protein (FliE)
	BG10340	hemA	3391	2944	3364	3006	5933	9308	glutamyl-tRNA reductase (HemA)
	BG10341	hemX	1142	1038	1211	1066	1849	2962	negative effector of the concentration of glutamyl-tRNA reductase HemA
	BG10342	hemC	923	985	910	760	1394	2400	porphobilinogen deaminase
	BG10343	hemD	1462	1427	1660	1169	2326	3484	uroporphyrinogen III cosynthase
	BG10344	hemB	1966	2084	1990	1429	3112	5251	delta-aminolevulinic acid dehydratase
	BG10345	hemL	1750	1728	1776	1204	2378	4466	glutamate-1-semialdehyde 2,1-aminotransferase
	BG10349	uvrC	572	716	1025	925	1298	1435	excinuclease ABC (subunit C)
	BG10353	sdhB	2200	3074	3771	2943	5763	8661	succinate dehydrogenase (iron-sulfur protein) (SdhB)
sigX	BG10404	lytR	1860	1363	1346	1541	2514	4849	membrane-bound transcriptional regulator
	BG10424	glnR	260	302	511	441	675	1554	transcriptional regulator (nitrogen metabolism) (GlnR)
	BG10450	tagA	595	489	566	717	789	1283	glycosyltransferase
	BG10465	addB	508	697	899	748	1183	1332	ATP-dependent deoxyribonuclease (subunit B) (AddB)
	BG10466	addA	679	930	1251	962	1590	2132	ATP-dependent deoxyribonuclease (subunit A) (AddA)
	BG10477	cdd	814	888	1125	1233	1931	2078	cytidine/deoxycytidine deaminase (Cdd)
	BG10515	sipS	489	614	838	1185	1572	1665	type I signal peptidase (SipS)
	BG10522	ribT	1123	1380	2285	2668	4864	5526	putative acetyltransferase (RibT)
	BG10534	resD	879	997	1122	975	1702	2941	two-component response regulator
	BG10535	resE	907	1044	1192	1144	2179	3695	two-component sensory histidine kinase

Table S3. ----*Continued*

Transcriptional regulator	BG number	Gene name	-T40	-T20	T0	T40	T100	T160	Function
	BG10589	ywcG	1849	2706	4280	3081	6083	10260	FMN-containing NADPH-linked nitro/flavin reductase (YwcG)
spo0A	BG10590	ywcH	617	854	1320	1090	1788	2179	putative monooxygenase (YwcH)
spo0A, codY	BG10652	rapA	657	1074	2060	1382	1345	2597	response regulator aspartate phosphatase (RapA)
spo0A	BG10653	phrA	815	1213	2524	1585	1556	2984	secreted inhibitor of the activity of phosphatase RapA (PhrA)
	BG10680	mecA	930	1636	2186	2080	2684	4694	adaptor protein controlling oligomerization of the AAA+ protein ClpC (MecA)
	BG10721	recA	907	751	1240	1633	2338	2693	multifunctional SOS repair factor (RecA)
sigV	BG10729	rpoC	1462	1166	1151	948	2101	2839	RNA polymerase (beta' subunit) (RpoC)
sigF	BG10741	secA	1562	1899	2543	2016	3549	4256	translocase binding subunit (ATPase) (SecA)
	BG10742	prfB	1616	1688	2085	1762	3256	4004	peptide chain release factor 2 (PrfB)
	BG10827	gap	8031	7976	10197	7350	13024	20207	glyceraldehyde-3-phosphate dehydrogenase (Gap)
	BG10855	citZ	1064	1670	2213	1569	2493	2673	citrate synthase II (CitZ)
	BG10864	mrgA	3190	2472	1621	2305	5188	12421	metalloregulation DNA-binding stress protein (MrgA)
	BG10899	eno	6363	6322	7560	5563	10132	14054	enolase
	BG10942	ywlF	1908	2160	2048	1705	2918	4278	ribose 5-phosphate epimerase (YwlF)
	BG10943	ywlG	1130	1357	1268	904	1551	2572	conserved hypothetical protein (YwlG)
spo0A, sigX	BG10988	abh	678	408	300	409	614	909	transcriptional regulator (Abh)
	BG11013	psd	828	893	994	823	1587	2471	phosphatidylserine decarboxylase (Psd)
	BG11018	yvmB	352	400	624	699	1588	1663	putative transcriptional regulator (YvmB)
	BG11045	comS	919	1014	881	851	1806	3152	regulator of genetic competence (ComS)
	BG11056	tufA	8034	5577	6960	5476	8468	13516	elongation factor Tu
	BG11090	rsbR	1066	883	1292	949	1537	2340	positive regulation of sigma(B) activity in response to salt and heat stress (RsbR)
	BG11091	rsbS	824	593	885	682	870	1754	antagonist of RsbT (RsbS)
	BG11092	rsbT	1054	842	1149	863	1275	2341	controls the activity of the piezosome (stressosome) (RsbT)
	BG11134	yxiE	1891	3149	3767	4410	7152	11575	phosphate starvation protein (universal stress protein A family) (YxiE)
	BG11188	bex	851	932	1249	1319	2284	2386	GTP-binding protein (Bex)
	BG11204	ahpF	7617	7451	5580	3328	5918	9961	alkyl hydroperoxide reductase (large subunit) (AhpF)
	BG11251	yneJ	652	479	678	1078	1441	2733	putative integral inner membrane protein (YneJ)
	BG11267	yqaP	611	463	520	1031	1549	2035	conserved hypothetical protein; skin element (YqaP)
	BG11330	pnp	904	1641	2096	1952	3506	4401	purine nucleoside phosphorylase (Pnp)
	BG11331	drm	1556	2449	2704	2489	4130	4392	phosphopentomutase (Drm)
	BG11362	yxcE	477	682	932	825	1265	1358	conserved hypothetical protein (YxcE)
nonW	BG11375	yqgG	206	563	720	1343	2987	4619	phosphate starvation protein
nonW	BG11376	yqgH	273	595	847	1009	1960	3746	phosphate starvation protein
nonW	BG11377	yqgI	286	637	893	1092	2239	5091	phosphate starvation protein
nonW	BG11378	yqgJ	196	552	916	1146	2796	6201	phosphate starvation protein
nonW	BG11379	yqgK	244	479	863	972	2393	5561	phosphate starvation protein
	BG11385	ahpC	7316	6175	5874	4530	7560	11337	alkyl hydroperoxide reductase (small subunit) (AhpC)
	BG11496	ypiA	417	620	792	904	1449	2362	conserved hypothetical protein (YpiA)
sigH	BG11497	ypiB	1305	2528	2591	1544	3140	5121	conserved hypothetical protein (YpiB)
sigH	BG11521	phrE	872	677	808	957	1686	1659	regulator of the activity of phosphatase RapE (PhrE)

Table S3. ----*Continued*

Transcriptional regulator	BG number	Gene name	-T40	-T20	T0	T40	T100	T160	Function
sigW	BG11573	sigW	841	456	813	778	1051	2403	ECF type sigma factor W (SigW)
sigW	BG11574	ybbM	356	305	306	315	372	944	anti-sigma(W) factor (YbbM)
	BG11619	yplP	421	512	1024	897	995	1458	transcriptional enhancer (YplP)
	BG11620	yplQ	284	344	434	497	624	806	putative membrane hydrolase (YplQ)
	BG11626	ypoP	432	576	659	722	1075	1497	putative transcriptional regulator (MarR family) (YpoP)
	BG11627	yppP	1025	2167	2518	1812	2931	5202	peptide methionine S-sulfoxide reductase (YppP)
	BG11628	yppQ	520	1176	1251	1147	1884	3292	peptide methionine R-sulfoxide reductase (YppQ)
sigW	BG11650	yqeZ	789	757	1365	1149	2640	8538	putative membrane bound hydrolase (YqeZ)
sigW	BG11651	yqfA	717	650	1142	941	1870	6430	conserved hypothetical protein (YqfA)
sigW	BG11652	yqfB	701	579	980	1072	1670	5888	conserved hypothetical protein (YqfB)
	BG11664	yqfR	521	617	977	1029	2088	3264	ATP-dependent RNA helicase; cold shock (YqfR)
	BG11665	yqfS	305	328	486	665	1242	2332	type IV apurinic/apyrimidinic endonuclease (YqfS)
sigG	BG11667	yqfU	451	671	1225	842	882	1331	putative integral inner membrane protein (YqfU)
	BG11668	yqfV	223	253	356	471	566	688	transcriptional regulator (Fur family) (YqfV)
sigB	BG11676	sodA	4679	4158	7009	4689	7560	10658	superoxide dismutase (SodA)
sigF	BG11704	yqhP	850	1127	1414	1062	1825	2323	conserved hypothetical protein (YqhP)
sigB, sigF	BG11705	yqhQ	730	1000	1232	1027	1675	1886	conserved hypothetical protein (YqhQ)
	BG11716	yqiG	854	1252	1954	2202	3706	3584	putative NADH-dependent flavin oxidoreductase (YqiG)
	BG11736	yqjG	575	660	861	755	1223	1841	Sec-independent factor for membrane protein insertion (YqjG)
	BG11759	yqkD	209	342	402	385	450	558	putative hydrolase (YqkD)
	BG11778	yrkL	754	1089	1238	1175	1942	2661	putative NAD(P)H oxidoreductase (YrkL)
	BG11825	yneR	549	984	891	887	1388	1601	conserved hypothetical protein (YneR)
	BG11826	yneS	295	362	464	618	708	881	acylphosphate:glycerol-3-phosphate acyltransferase (YneS)
	BG11827	yneT	500	702	821	907	1247	1839	putative CoA-binding protein (YneT)
	BG11828	ynfC	408	658	728	870	2011	3112	conserved hypothetical protein (YnfC)
	BG11938	fruA	310	520	1151	758	1494	3924	fructose-specific enzyme IIABC component (FruA)
sigW, sigV	BG11939	fusA	2142	456	1146	1704	2812	2974	elongation factor G (FusA)
sigW	BG11944	infC	4249	2675	3685	4033	6524	7014	initiation factor IF-3 (InfC)
	BG11972	rpmI	3944	2370	3055	3704	5940	6817	ribosomal protein L35 (RpmI)
	BG11977	sipT	736	1200	1767	1735	2713	2676	type I signal peptidase (SipT)
	BG12065	ydaR	3245	3341	4093	2653	4717	7091	manganese transporter (YdaR)
	BG12076	ydbI	694	614	993	906	1191	1845	putative integral inner membrane protein (YdbI)
sigB	BG12083	ydbP	1417	1961	3647	3413	5712	8453	putative thioredoxin or thiol-disulfide isomerase (YdbP)
	BG12129	ydeB	530	867	1499	1442	1466	2592	putative transcriptional regulator (YdeB)
	BG12206	ydiI	1220	1473	2768	2297	4042	6060	twin-arginine pre-protein translocation pathway (YdiI)
	BG12207	ydiJ	1089	1580	2518	2076	3403	5455	twin-arginine pre-protein translocation pathway (YdiJ)
	BG12223	ygaC	1158	1669	2027	1657	2402	3379	putative factor (YgaC)
	BG12227	ygaG	3421	2968	4517	4129	6761	14112	transcriptional regulator (Fur family) (YgaG)
sigH, sigW	BG12229	ygaI	1256	997	1299	986	1476	3484	sporulation-control gene (YgaI)
	BG12247	yitW	1164	1684	2430	1906	3011	3994	conserved hypothetical protein (YitW)

Table S3. ----*Continued*

Transcriptional regulator	BG number	Gene name	-T40	-T20	T0	T40	T100	T160	Function
	BG12255	ynaC	270	341	455	721	986	1093	conserved hypothetical protein (YnaC)
	BG12306	yrpC	435	578	734	1038	1639	1884	glutamate racemase (YrpC)
sigW	BG12316	ysdB	510	811	1382	882	841	1621	conserved hypothetical protein (YsdB)
	BG12320	ysfC	339	369	433	520	1002	974	glycolate oxidase subunit (YsfC)
	BG12321	ysfD	278	335	360	397	763	727	glycolate oxidase iron-sulfur subunit (YsfD)
	BG12370	yugP	633	642	871	767	1059	1986	putative metal-dependent protease/peptidase (YugP)
	BG12371	yugS	514	695	823	627	571	1470	membrane protein involved in divalent ion export (YugS)
	BG12398	trxB	1199	1456	3034	2253	3038	4158	thioredoxin reductase (TrxB)
	BG12402	yvcL	1362	1562	1620	1158	1849	2922	putative morphogen
	BG12484	ywnF	955	1588	2193	1474	1848	2837	conserved hypothetical protein (YwnF)
	BG12505	ywqA	310	342	338	283	409	707	putative ATP-binding SNF2 helicase or protein kinase (YwqA)
	BG12588	fruB	250	376	782	648	919	2492	putative ATP-binding SNF2 helicase or protein kinase (YwqA)
spo0A	BG12589	fruR	626	920	2105	1475	2366	5705	transcriptional regulator (DeoR family) (FruR)
	BG12608	htrA	499	687	704	744	1173	1332	membrane bound serine protease
	BG12667	rplS	4188	1987	3303	4857	6493	11375	ribosomal protein L19 (RplS)
	BG12668	rpmF	2662	1511	1227	1935	2819	3220	ribosomal protein L32 (RpmF)
	BG12674	sipV	586	748	1129	1114	1398	1770	type I signal peptidase (SipV)
sigK	BG12677	spoIISA	485	699	1010	1199	1358	1778	two-component apoptosis factor (SpoIISA)
	BG12678	spoIISB	673	969	1193	1198	1550	1877	two-component apoptotic control system component B
	BG12682	sunA	5115	4773	6028	4293	7904	11837	lantibiotic antimicrobial precursor peptide (SunA)
	BG12697	uvrA	512	481	837	809	1220	1827	excinuclease ABC (subunit A) (UvrA)
	BG12745	ybfQ	1043	1031	1592	1593	2636	3054	putative enzyme with rhodanese domain (YbfQ)
sigB, sigW, sigM	BG12767	yceC	3556	2673	3114	2358	4084	9588	putative stress adaptation protein (YceC)
sigB, sigW, sigM	BG12768	yceD	2898	2588	2640	2148	3707	8650	putative stress adaptation protein
sigB, sigW	BG12769	yceE	2759	2545	2606	2071	3615	8871	putative stress adaptation protein
sigB, sigW	BG12770	yceF	3246	2968	2764	2535	4465	8641	putative stress adaptation protein
	BG12803	ydjL	5435	4683	7586	5626	9954	18555	acetoin reductase/2,3-butanediol dehydrogenase (YdjL)
	BG12824	yeeI	2223	1692	2381	2309	3631	4788	conserved hypothetical protein (YeeI)
sigW	BG12835	yerG	475	485	524	544	812	1268	DNA ligase (YerG)
sigM	BG12836	yerH	376	222	287	349	477	716	putative lipoprotein (YerH)
	BG12885	yfhJ	995	1912	2835	2070	2805	5369	conserved hypothetical protein (YfhJ)
	BG12995	yhaS	475	811	1305	1081	1104	1525	K+/H+ antiporter for K+ efflux (YhaS)
	BG13022	yhdP	1001	1166	1463	1245	1923	3824	K+/H+ antiporter for K+ efflux
	BG13023	yhdQ	1640	1517	1958	1733	2673	4424	copper efflux transcriptional regulator (YhdQ)
	BG13042	yheJ	230	260	324	456	510	633	hypothetical protein; orphan (YheJ)
	BG13056	yhfK	801	1015	1284	1190	1576	2120	putative epimerase (YhfK)
	BG13072	yhjE	614	843	1220	1046	1550	2093	putative integral inner membrane protein (YhjE)
sigB, sigW, sigM	BG13133	yjbD	3324	3572	4059	3595	6606	12292	redox-sensitive regulator enzyme (YjbD)
	BG13137	yjbH	578	922	1233	1164	1308	1555	regulator of Spx proteolysis (YjbH)
	BG13138	yjbI	986	1595	2545	2223	2619	2742	putative thiol management oxidoreductase component (YjbI)

Table S3. ----*Continued*

Transcriptional regulator	BG number	Gene name	-T40	-T20	T0	T40	T100	T160	Function
spo0A, sigW	BG13216	yjoB	421	508	603	518	489	1066	ATPase possibly involved in protein degradation (YjoB)
sigW	BG13243	yknW	626	459	535	747	1309	4343	putative permease (YknW)
	BG13270	ykpA	827	769	981	1036	1484	2024	ABC efflux transporter (ATP-binding protein) (YkpA)
	BG13274	ykrL	429	933	1800	1419	2264	3157	membrane protease (YkrL)
	BG13308	ykvA	528	764	1139	1285	1402	2928	conserved hypothetical protein (YkvA)
	BG13309	ykvD	461	601	577	638	757	1206	linking spore formation to extracellular matrix (YkvD)
	BG13325	ykvW	654	808	1045	728	1200	1852	Zn transporter (YkvW)
	BG13343	ylaF	745	873	1141	1315	2310	2737	conserved hypothetical protein (YlaF)
	BG13368	ylbP	436	617	621	661	934	1456	putative acetyltransferase (YlbP)
sigW	BG13383	yloA	350	473	693	676	966	1246	putative persistent RNA/DNA binding protein (YloA)
	BG13417	ymcA	914	1147	1533	1746	3429	3454	master regulator for biofilm formation (YmcA)
	BG13420	ymdA	3192	2926	3986	4530	8775	12246	ribonuclease Y (YmdA)
	BG13440	yndB	594	540	749	1151	1855	1829	conserved hypothetical protein (YndB)
sigX, sigM	BG13454	yngC	420	515	643	904	1136	1117	putative integral inner membrane protein (YngC)
	BG13511	yobT	185	320	318	410	495	590	putative metal-dependent hydrolase (YobT)
	BG13549	yoeB	1382	3118	6002	4498	8472	13969	inhibitor of cell-separation enzymes (YoeB)
	BG13571	yokD	409	447	411	631	989	1241	aminoglycoside N3'-acetyltransferase (YokD)
	BG13584	yolF	1105	1111	1744	1758	2578	2991	protein of immunity to sublancin (YolF)
	BG13631	yonU	221	232	321	546	649	892	hypothetical protein; phage SPbeta (YonU)
sigW	BG13762	yozO	344	229	438	587	521	1840	conserved hypothetical protein (YozO)
	BG13767	ypzE	279	464	683	657	823	1013	hypothetical protein (YpzE)
	BG13772	yqzF	614	760	1178	1395	2504	2422	hypothetical protein (YpzF)
sigB, sigM	BG13776	yraA	1662	2793	3830	3895	7454	10441	general stress protein (YraA)
	BG13816	yrzF	535	1074	1707	1614	2670	3219	putative serine/threonine-protein kinase (YrzF)
	BG13817	yrzG	848	1320	1969	1841	2646	3651	putative serine/threonine-protein kinase (YrzG)
	BG13837	ytdI	697	1381	1652	1123	1607	2040	inorganic polyphosphate/ATP-NAD kinase (YtdI)
sigW	BG13860	ythP	433	472	648	622	913	2102	putative ABC transporter (ATP-binding protein) (YthP)
	BG13918	ytrP	399	474	641	609	816	1247	putative diguanylate cyclase-related enzyme (YtrP)
sigW	BG13946	yuaF	431	425	455	426	455	1296	membreane integrity integral inner membrane protein
sigW	BG13947	yuaG	279	297	335	272	373	1403	putative flotillin-like protein (YuaG)
sigW	BG13948	yuaI	325	316	351	356	381	851	putative acetyl-transferase (YuaI)
	BG14007	yurU	2171	1718	1735	1686	3429	5522	FeS cluster formation protein (YurU)
	BG14008	yurV	1729	1348	1364	1396	2535	4097	iron-sulfur cluster assembly scaffold protein (YurV)
	BG14009	yurW	2189	2027	1605	1540	3220	5656	cysteine desulfurase (YurW)
	BG14010	yurX	2413	2147	1825	2033	3631	6012	FeS assembly protein SufD (YurX)
	BG14011	yurY	4243	3198	3212	2990	5577	7836	sulfur mobilizing ABC protein, ATPase (YurY)
	BG14049	yutM	1452	2394	4168	3827	6536	6964	chaperone involved in Fe-S cluster assembly (YutM)
sigW	BG14085	yvbQ	4137	4897	6604	4814	8690	12438	transcriptional regulator of gapA (YvbQ)
	BG14098	yvgP	1200	2583	4199	2717	4450	7224	Na+/H+ antiporter (YvgP)
sigD, sigW	BG14116	yvlA	717	681	826	569	871	2984	conserved hypothetical protein (YvlA)

Table S3. ----*Continued*

Transcriptional regulator	BG number	Gene name	-T40	-T20	T0	T40	T100	T160	Function
sigW	BG14117	yvlB	1045	930	1200	769	1428	4988	conserved hypothetical protein (YvlB)
sigW	BG14118	yvlC	711	630	743	640	712	2964	putative regulator (stress mediated) (YvlC)
sigW	BG14119	yvlD	488	485	437	440	526	1387	putative integral inner membrane protein (YvlD)
	BG14120	yvmA	532	628	787	712	1602	2232	putative efflux transporter (YvmA)
	BG14121	yvmC	461	430	521	624	1156	1185	cyclodipeptide synthase (YvmC)
sigW, sigV	BG19006	rpsG	1461	291	934	1735	1813	2149	ribosomal protein S7 (BS7) (RpsG)
sigW, sigV	BG19008	rpsJ	1011	359	388	760	742	1067	ribosomal protein S10 (BS13) (RpsJ)
sigW, sigV	BG19009	rpsL	3092	868	2426	3937	4254	4680	ribosomal protein S12 (BS12) (RpsL)
	BG19010	rpsO	2155	1366	2103	2975	3203	5988	ribosomal protein S15 (BS18) (RpsO)