ISTANBUL TECHNICAL UNIVERSITY ★ INSTITUTE OF SCIENCE AND TECHNOLOGY

IDENTIFICATION OF ABRB AND SPO0A BINDING SITES ON *B. SUBTILIS YVFI* PROMOTER

M.Sc. Thesis by
Hüseyin TAYRAN

Department : Advanced Technologies

Programme: Mol. Bio. Genetics & Biotechnology

Thesis Supervisor: Assoc. Prof. Dr. Ayten YAZGAN KARATAŞ

SEPTEMBER, 2010

ISTANBUL TECHNICAL UNIVERSITY ★ INSTITUTE OF SCIENCE AND TECHNOLOGY

IDENTIFICATION OF ABRB AND SPO0A BINDING SITES ON *B. SUBTILIS YVFI* PROMOTER

M.Sc. Thesis by
Hüseyin TAYRAN
(521071039)

Date of submission : **7 May 2010**
Date of defence examination : **7 June 2010**

Supervisor (Chairman) : **Assoc. Prof. Dr. Ayten YAZGAN KARATAŞ (ITU)**
Members of the Examining Committee : **Assist. Prof. Dr. Fatma Neşe KÖK (ITU**
Assoc. Prof. Dr. Sedef TUNCA GEDİK (GYTE)

MAY, 2010

<u>İSTANBUL TEKNİK ÜNİVERSİTESİ ★ FEN BİLİMLERİ ENSTİTÜSÜ</u>

ABRB VE SPO0A TRANSKRİPSİYON FAKTÖRLERİNİN *B. SUBTILIS YVFI* PROMOTORUNDAKİ BAĞLANMA BÖLGELERİNİN BULUNMASI

YÜKSEK LİSANS TEZİ

Hüseyin TAYRAN
(521071039)

Tezin Enstitüye Verildiği Tarih : 7 Mayıs 2010
Tezin Savunulduğu Tarih : 7 Haziran 2010

Tez Danışmanı : Doç. Dr. Ayten YAZGAN KARATAŞ (İTÜ)
Diğer Jüri Üyeleri : Yrd. Doç. Dr. Fatma Neşe KÖK (İTÜ)
Doç. Dr. Sedef TUNCA GEDİK (GYTE)

MAYIS, 2010

FOREWORD

I would like to express my sincerest gratitude to my supervisor Assist. Prof. Dr. Ayten YAZGAN KARATAŞ for her thoughtful guidance, encouragement and invaluable time with me of course for her stimulating advices.

A huge bunch of thanks goes to Öykü for sharing her ideas, different point of views and sources with me not only in the lab but also outside the lab. I also want to express my deep appreciation to Türkan Ebru KÖROĞLU for offering me her experience non-hesitantly. I appreciate my special thanks to other lab assistants Esra YÜCA and Günseli KURT for spending long days for this project with me and having made it more enjoyable and efficient for me.

I would like to thank to my lab mates who have contributed to this project for their precious support and friendship during this project.

September, 2010 **Hüseyin TAYRAN**

Molecular Biolog

TABLE OF CONTENTS

Page

ABBREVIATIONS **ix**
LIST OF TABLES **xi**
LIST OF FIGURES **xiii**
SUMMARY **xv**
ÖZET **xvii**

1. INTRODUCTION 1
1.1. *Bacillus subtilis* 1
1.2. *Bacillus subtilis* Genome........ 2
1.3. *B. subtilis* Antibiotics 2
1.3.1. Ribosomal synthesis: Lantibiotics........ 3
1.3.2. Non-ribosomal Biosynthesized Peptides 4
1.4. Roles of *spo0A* and *abrB* in Antibiotic Biosynthesis........ 5
1.5. Bacilysin........ 8
1.5.1. Genetic Mechanism of Bacilysin Biosynthesis 9
1.5.2. Genetic Regulation of Bacilysin 11
1.6. Electromobility Shift Assay (EMSA) 12
1.7. DNase I Footprinting Assay........ 13
1.8. Aim of the Project........ 14

2. MATERIALS AND METHODS........ 15
2.1. Materials........ 15
2.1.1. Bacterial strains 15
2.1.2. Bacterial Culture Media 15
2.1.3. Buffers and Solutions 15
2.1.4. Chemicals and Enzymes........ 15
2.1.5. Laboratory Equipment........ 15
2.1.6. Maintenance of Bacterial Strains........ 16
2.2. Methods........ 16
2.2.1. Construction of pJET-*abrB* and pJET-*spo0A* Cloning Vectors 16
2.2.1.1. Amplification of *abrB* and *spo0A* Genes by PCR........16
2.2.1.2. Agarose Gel Electrophoresis of PCR Products........17
2.2.1.3. Ligation of the PCR Product Into pJET 1.2 / Blunt Cloning Vector 17
2.2.1.4. Preparation of Electrocompotent E. coli Top 10F' Cells........ 18
2.2.1.5. Transformation of pJET-*abrB* and pJET-*spo0A* to Electrocompotent *E.coli* Top10 Cells 18
2.2.1.6. Plasmid DNA Isolation 19
2.2.1.7. Agarose Gel Electrophoresis........20

2.2.2. Construction of pQE60-*abrB* and pQE60-*spo0A* Expression Vectors……………………………………………………………..20
2.2.2.1. Enzymatic Digestion…………………………………………20
2.2.2.2. Gel Extraction………………………………………………...21
2.2.2.3. Ligation of *abrB* and *spo0A* Genes Into pQE60 ExpressionVector…………………………………………………...22
2.2.2.4. Transformation of pQE60-*abrB* and pQE60-*spo0A* to Electrocompotent *E.coli* Top10 Cells…………………………………23
2.2.3. Expression and Purification of 6xHis-tagged Spo0A and AbrB Proteins…………………………………………………...23
2.2.3.1. Expression of 6xHis-tagged AbrB and 6xHis-tagged Spo0A Proteins…………………………………………………...23
2.2.3.2. Purification of 6xHis-tagged AbrB and 6xHis-tagged Spo0A Proteins…………………………………………………...24
2.2.3.3. SDS-Polyacrylamide Gel Electrophoresis of AbrB and Spo0A…………………………………………………………..25
2.2.4. Electromobility Shift Assay of AbrB and Spo0A Proteins…………….27
2.2.4.1. Cloning of *yvfI* Promoter Into pJET 1.2 / Blunt Cloning Vector………………………………………………………27
2.2.4.2. *yvfI* Promoter Synthesis from pJET-yvfIprom for EMSA…......29
2.2.4.3. Purification of the *yvfI* Promoter………………………………30
2.2.4.4. Binding Reactions of Protein-*yvfI* Promoter DNA……………30
2.2.4.5. Non-denaturing Polyacrylamide Gel Analysis………………..31
2.2.5. DNase I Footprinting of AbrB and Spo0A Proteins……………….…32
2.2.5.1. Synthesis and Purification of FAM-Labelled Probe……….....32
2.2.5.2. Binding Reactions of Protein-Probe DNA………………….....33
2.2.5.3. DNase Treatment……………………………………………...34
2.2.5.4. DNA Fragment Analysis………………………………………35

3. RESULTS AND DISCUSSION..37
3.1. Construction of pJET-*abrB* and pJET-*spo0A* Cloning Vectors……………...37
3.2. Construction of pQE60-*abrB* and pQE60-*spo0A* Expression Vectors………39
3.3. Expression and Purification of 6xHis-tagged Spo0A and AbrB Proteins…...41
3.4. Electromobility Shift Assays of AbrB and Spo0A Proteins…………………43
3.5. DNase I Footprinting of AbrB and Spo0A Proteins…………………………45

4. CONCLUSION 49

REFERENCES 51
APPENDIX A 59
APPENDIX B...61
APPENDIX C 63
APPENDIX D 65
APPENDIX E...67
APPENDİX F………………………………………………………………………69
APPENDİX G………………………………………………………………………71

CV 73

ABBREVIATIONS

bp	: Base pair
dH_2O	: Distilled water
DNA	: Deoxyribonucleic acid
IPTG	: Isopropyl-b D- thiogalactopyranoside
EtBr	: Ethidium bromide
kb	: Kilobase
LB- broth	: Luria Bertani broth
OD	: Optical density
TAE	: Tris acetate EDTA
NRPS	: Non-Ribosomal Peptide Synthetase
EMSA	: Electromobility Shift Assay

LIST OF TABLES

Page

Table 2.1 : Bacterial strains used through the project……………………………15
Table 2.2 : The volume of PCR components……………………………………16
Table 2.3 : Volume of components used in ligation of the PCR product into pJET 1.2 / blunt cloning vector…………………………...18
Table 2.4 : The components of the enzymatic digestion reaction…………………..20
Table 2.5 : The components of the pJET-*abrB* enzymatic digestion……………….21
Table 2.6 : The components of the pQE60 enzymatic digestion…………………...21
Table 2.7 : Components of Lower Resolving Gel…………………………………26
Table 2.8 : Components of Upper Stacking Gel..26
Table 2.9 : The volume of PCR components……………………………………27
Table 2.10 : Volume of components used in ligation of the PCR product into pJET 1.2 / blunt Cloning Vector………………………...28
Table 2.11 : The volume of PCR components…………………………………...29
Table 2.12 : Binding Reactions for Electromobility Shift Assay of Spo0A Protein…………………………………………………....31
Table 2.13 : Binding Reactions for Electromobility Shift Assay of AbrB Protein…………………………………………………..31
Table 2.14 : The volume of TBE Polyacrylamide Gels Components……………...32
Table 2.15 : The volume of PCR components…………………………………...33
Table 2.16 : Binding Reactions for DNase I Footprinting of AbrB Protein………..34
Table 2.17 : Binding Reactions for DNase I Footprinting of Spo0A Protein………34

LIST OF FIGURES

Page

Figure 1.1 : A few *Bacillus subtilis* lantibiotics, lantibiotic-like peptides and specifying gene clusters……………………………………………4
Figure 1.2 : The Phosphorelay……………………………………………………6
Figure 1.3 : Regulatory pathways of antibiotic biosynthesis in *B. subtilis*…………8
Figure 1.4 : Structural formula of bacilsin…………………………………………..9
Figure.1.5 : Organization of the bacilysin gene cluster bacABCDE………………10
Figure 1.6 : A schematic diagram of EMSA………………………………………13
Figure 1.7 : Schematic representation of footprinting assay………………………14
Figure 3.1 : Amplification of *abrB* gene by PCR……………………………….....37
Figure 3.2 : Amplification of *spo0A* gene by PCR………………………………..38
Figure 3.3 : Plasmid DNAs containing *abrB* gene isolated from *E.coli* Top10 Amp^R transformants……………………………………………38
Figure 3.4 : Plasmid DNAs containing *spo0A* gene isolated from *E.coli* Top10 Amp^R transformants……………………………………………39
Figure 3.5 : Double digestions of pJET vector including *abrB* and *spo0A* genes with *BamHI* and *Nco*I..………………………………………………39
Figure 3.6 : Double digestions of pQE60 vectors with *BamH*I and *Nco*I…………40
Figure 3.7 : The double digestion of a selected plasmid including *abrB* gene isolated from *E.coli* Amp^R transformants………………………40
Figure 3.8 : The double digestion of a selected plasmid including *spo0A* gene isolated from *E.coli* Amp^R transformants………………………41
Figure 3.9 : Purified AbrB protein…………………………………………………42
Figure 3.10 : Purified Spo0A protein………………………………………………43
Figure 3.11 : Non-denaturing polyacrylamide gel image of electromobility shift assay of purified AbrB with *yvfI* promoter of *B. subtilis*..……………44
Figure 3.12 : Non-denaturing polyacrylamide gel image of electromobility shift assay of purified Spo0A with *yvfI* promoter of *B. subtilis*…………….45
Figure 3.13 : Electropherograms shows the protection pattern of the *yvfI* promoter in the presence (blue) or absence (red) of AbrB…………46
Figure 3.14 : Electropherograms shows the protection pattern of the *yvfI* promoter in the presence (blue) or absence (red) of Spo0A………...47

IDENTIFICATION OF ABRB AND SPO0A BINDING SITES ON *B. SUBTILIS YVFI* PROMOTER

SUMMARY

The model organism for the gram-positive bacterium, *B.subtilis*, can produce more than 20 antibiotics which are predominantly peptides. Bacilysin is a dipeptide containing L-alanine at its N-terminal and the unusual amino acid L-anticapsine at its C terminal. It is produced by certain strains of *B.subtilis* against some bacteria and fungi and interferes with the synthesis of microbial cell walls. The unusual amino acid L-anticapsin moiety of bacilysin is generated through the action of a prephenate dehydratase and an aminotransferase, products of *ywfBG* genes, respectively. The biosynthesis of the dipeptide bacilysin depends on the *ywfBCDEF* gene cluster which was renamed as *bacABCDE.* Furthermore, disruption of these genes by plasmid integration was shown to cause loss of the ability to produce bacilysin, and also a lack of bacilysin synthetase activity in the crude extract. While *bacABC* genes carry the anticapsin synthesis function, the *bacD* and *bacE* genes encode for the function of amino acid ligation and self-protection to bacilysin, respectively.
Distruption of *phrC, comA* and *oppA* by Tn10 transposon mutagenesis and introduction of *comQ::cat* mutation resulted in the elimination of bacilysin biosynthesis, demonstrating that bacilysin biosynthesis is under the feedback regulation by the components of global quorum sensing control system. Also, it was shown that bacilysin production is regulated on different levels negatively by GTP via the transcriptional regulator CodY and AbrB.

During the transition-state from vegetative to stationary phase, a wide variety of two component signal transduction systems and global regulators of *B. subtilis* are activated in response to the environmental signals. These regulators have been termed transition-state regulators and AbrB, Spo0A, ScoC and Cody are some important members of this regulatory protein class. It is undoubtedly known that transition state regulator proteins, AbrB and Spo0A have crucial roles in the biosynthesis of antibiotics and toxins in *Bacillus* species. When it comes to the bacilysin, it has been proved that bacilysin is also under the regulation of *abrB* and *spo0A* genes. Insertional mutation in *abrB* gene resulted in an increase in bacilysin production, indicating that bacilysin synthesis is under the negative control of *abrB* gene and moreover it was demonstrated that *spo0A* blockage resulted in a crucial decrease in bacilysin synthesis.

Very recently, through Tn10 mutagenesis studies that were performed to reveal related genes with bacilysin biosynthesis in *Bacillus subtilis* PY79 strain, *yvfI* gene which is similar to transcriptional regulator (GntR family) was found responsible in bacilysin production. On the other hand, since *yvfI* gene is essential for the bacilysin biosynthesis, any gene involved in the regulation of *yvfI* were considered as candidates in the regulation of bacilysin production indirectly. Subject to the

foregoing provisions, in this study, *yvfI* gene promoter by DBTBS database was examined in order to find putative cis-elements in the promoter region of the *B. subtilis yvfI* gene for candidate regulatory proteins and found putative binding sites for AbrB and Spo0A regulatory proteins. AbrB and Spo0A proteins were tested for their abilities to bind the *yvfI* promoter DNA in electromobility shift experiment. Furthermore, DNase I footprinting of the *yvfI* promoter was applied in order to discover the exact regions occupied by AbrB and Spo0A trans-acting regulatory factors. According to the electropherograms obtained from the capillary-based instrument and then aligned using GeneMapper software, binding sites for each regulatory protein on *yvfI* promoter were determined. Data obtained in this study suggested that AbrB and Spo0A regulatory proteins have roles in the bacilysin biosynthesis by regulating *yvfI* gene expression on transcriptional level.

Key words: *B. subtilis*, bacilysin, *yvfI* gene, AbrB regulator protein, Spo0A regulator protein, Electromobility shift assay, DNase I footprinting.

ABRB VE SPO0A TRANSKRİPSİYON FAKTÖRLERİNİN *B. SUBTILIS* *YVFI* PROMOTORUNDAKİ BAĞLANMA BÖLGELERİNİN BULUNMASI

ÖZET

Gram-pozitif organizmalar için model organizma olarak kabul edilen *B.subtilis*, ağırlıklı olarak peptid olan 20'den fazla antibiyotik üretebilmektedir. Basilisin, N ucunda L-alanine ve C ucunda doğal olmayan bir aminoasit olan L-anticapsine bulunduran iki aminoasitten oluşan bir dipeptidtir. *B. Subtilis*'un bazı şuşları tarafından üretilen basilisin, bazı bakteri ve mayalara karşı kullanılmaktadır ve mikrobiyal hücre duvarı sentezini engellemektedir. Basilisinin doğal olmayan L-antikapsin amino asidinin, sırasıyla *ywfBG* genlerinin ürünleri olan prefenat dehidrataz ve aminotransferaz enzimleri aracılığıyla üretildiği düşünülmektedir. Basilisinin biyosentezi, daha sonra *bacABCDE* olarak adlandırılan *ywfBCDEF* gen kümesi tarafından gerçekleşmektedir. Söz konusu gen kümesinin, plazmid entegrasyonu ile bozulması sonucu basilisin üretimi durmuş, ayrıca hücre özütünde basilisin sentetaz aktivitesi kaybolmuştur. Daha sonra yapılan çalışmalarda, *bacABC* genlerinin antikapsin üretiminden sorumlu olduğu, *bacD* ve *bacE* genlerinin ise sırasıyla amino asid ligasyonu ve basilisine karşı korunma fonksiyonlarını gerçekleştirdiği kanıtlanmıştır.

phrC, *comA* and *oppA* genlerinin Tn10 mutagenez yolu ile bozulması veya *comQ::cat* mutasyonunun oluşturulması, basilisin biyosentezinin durmasıyla sonuçlanmıştır. Bu çalışmalar basilisin biyosentezinin global quorum sensing kontrol sistemi tarafından regule edildiğini göstermektedir. Ayrıca, basilisin üretiminin AbrB ve GTP ile negatif olarak düzenlediği gösterilmiştir.

B. subtilis, vegetatif formdan durgun faza geçerken, çevresel sinyallere tepki olarak iki bileşenli sinyal iletim sitemlerinin birçoğunu ve çeşitli global düzenleyicilerini aktif hale getirir. Bu düzenleyiciler, geçiş (dönüşüm)- düzenleyicileri olarak isimlendirilir. AbrB, Spo0A, ScoC ve Cody proteinleri bu düzenleyici protein sınıfının en önemli üyelerinden birkaçıdır. AbrB ve Spo0A geçiş-düzenleyici proteinlerinin Basillus türlerinde antibiyotik ve toksin biyosentezinde önemli görevleri olduğu kesin olarak bilinmektedir. Sözkonusu basilisine geldiğimizde, basilisinin de *abrB* ve *spo0A* genleri tarafından regule edildiği gösterilmiştir. *abrB* geninde oluşturulan mutasyonlar sonucu basilisin üretiminde meydana gelen artış, basilisin sentezinin *abrB* geninin negative kontrolü altında olduğunu göstermiştir. Aynı çalışmada, *spo0A* geni bloke edildiğinde basilisin üreteminde büyük bir düşüş olduğu gösterilmiştir.

Bacillus subtilis PY79 şuşunda basilisin üretimiyle ilgili genleri açığa çıkarmak için son zamanlarda yapılan Tn10 mutagenesis çalışmaları, transkripsiyonel düzenleyiciye benzeyen *yvfI* geninin (GntR ailesi) basilisin biyosenteziyle ilgisi olduğunu kanıtlamıştır. Bununla birlikte, *yvfI* geninin basilisin üretiminden sorumlu

olması nedeniyle, *yvfI* geninin düzenlenmesinde görev alan herhangi bir gen dolaylı olarak basilin üretiminden de sorumludur. Yukardaki çıkarımdan hareketle, DBTBS veritabanı (Sierro et al, 2008) kullanılarak *B. subtilis yvfI* geninin promoter dizisi, aday düzenleyici proteinlerin bağlanabileceği cis-elementlerinin bulunması için incelendi, ve AbrB ile Spo0A düzenleyici proteinleri için olası bağlanma bölgeleri bulundu. Elektromobility shift deneyi ile AbrB ve Spo0A proteinlerinin *yvfI* promoter DNA dizisine bağlandıkları bulunduktan sonra, söz konusu düzenleyici proteinlerin (trans-acting protenlerin) *yvfI* promoter dizisinde bağlandıkları bölgeleri açığa çıkarmak için DNase I footprinting deneyi gerçekleştirildi. Kapiler tabanlı araçtan elde edilen ve sonrasında GeneMapper programı kullanılarak düzenlenen electrophoregramlar incelendi ve her bir düzenleyici protein için *yvfI* promoter dizisi üzerinde bir bağlanma bölgesi bulundu. Elde edilen sonuçlar, AbrB ve Spo0A düzenleyici proteinlerinin, basilisin üretimini *yvfI* geninin ekspresyonunu transkripsiyon düzeyinde değiştirerek düzenlediklerini açığa çıkarmıştır.

Anahtar Sözcükler : *B. subtilis*, basilisin, *yvfI* geni, AbrB düzenleyici protein, Spo0A düzenleyici protein, Elektromobility shift, DNase I footprinting.

1. INTRODUCTION

1.1. *Bacillus subtilis*

Bacillus subtilis is an endospore-forming rhizobacterium that has always been a popular model system for genetic and biochemical investigations due to the identification and mapping of most of the genetic loci in *B.subtilis* (Stein, 2005; Harwood et al., 1990).

Ferdinand Cohn identified and named the bacterium *Bacillus subtilis* in 1872. The organism was placed in the family *Bacillaceae*. As a member of the genus Bacillus, *B. subtilis* is characterized as rod shaped, gram positive, aeobic or facultative bacteria and has the ability of forming a protective endospore, a highly refractile resting structure formed within the bacterial cell and helps the bacterium survive in extreme environmental conditions (Sonenshein et al.,1993 and Harwood et al., 1990). In 1961, by the mean of developed fluorescent antibodies against the different proteins produced in vegetative and spore forming *B. subtilis*, it was observed that this species was mostly in its vegetative form (Norris and Wolf).

Due to the increase in sequence information in DNA technology, it was revealed that *B. subtilis* 168 strain has no genes encoding the virulance factor, which is also compatible with the knowledge that bacterium is not pathogen (Kunst et al., 1997)

Contrary to the knowledge that *B. subtilis* is an obligate aerobe, recent studies have revealed putative nitrate reductase genes, which explains the source of nitrate molecules used as an electron acceptor molecule rather than oxygen when bacterium faces to anaerobic conditions (Kunst et al., 1997). In an attempt to identify the fermentation pathway in *B. subtilis* under anaerobic conditions, fermentation end products were analyzed by using in vivo nuclear magnetic resonance scans of whole cultures and demonstrated anaerobic growth of *B. subtilis* in the presence of nitrate (Nakano et al., 1997).

1.2. *Bacillus subtilis* Genome

Bacillus subtilis is the most studied gram-positive bacteria in genome level. The complete sequencing of the *Bacillus subtilis* 168 genome has been published, which was done by a European and a Japanese sequencing consortium and a Korean laboratory. Its genome was proposed to consist of 4,214,810 corresponding to 4100 protein coding genes. G+C content of the *B.subtilis* genome is about %43 and more than a quarter of the genome is predicted to encode several gene families. Moreover, a remarkable proportion of its genome was found to be responsible for the utilization of different carbon sources, including many plant-derived molecules and synthesis of secondary metabolites such as antibiotics which are especially related with *Streptomyces* species. More than ten prophages or prophage remnants in genome demonstrated the crucial role of bacteriophage infection on the evolutinary of horizontal gene transfer (Kunst et al., 1997, Ghim et al., 1998). 271 of 4200 genes are considered as essential genes which are required for sustaining a living cell and roughly half of them are directly involved in DNA, RNA and protein metabolism (Kobayashia et. al., 2003).

Revealing of *B. subtilis* 168 strain sequence showed that about % 4 of its genome, corresponding to nearly 350 kb, is devoted to produce secondary metabolites and antimicrobial compounds which serve as effective inhibitors like antibiotics against the fungi, bacteria and plants. They are proposed to help the bacterium to compete for the nutrients in extreme environmental conditions (Stein, 2005; Kunst et al., 1997).

1.3. *B. subtilis* Antibiotics

Antibiotics produced by the bacteria of the genus *Bacillus* can be divided in two groups in general; first group includes small and cyclic lipopeptides whose structures involves uncommon amino acids such as ornithine or D-amino acids and sometimes beta amino acids (Lebbadi et al. 1994; Munimbazi and Bullerman 1998). Second group contains hydrophilic antibiotics such as the dipeptide bacilysin and phosphonooligopeptide rhizocticin (Walker and Abraham 1970; Kugler et al. 1990).

Through the genus *Bacillus, B. subtilis* is considered as the major source for antibiotic production and produces more than twenty antibiotics with unusual amino

acids (Asaka and Shoda 1996). Post-translational modification, proteolytic processing in ribosomal way and non-ribosomal synthesis by the large megaenzymes called non-ribosomal peptide synthetases (NRPSs) stand for the main reasons of unusual aminoacids in *B.subtilis* antibiotics (Moszer,1998 and Stein *et al*., 2005).

Recently, *B. subtilis,* among gram-positive members of the aerobic, spore-forming genus *Bacillus* is used as a biocontrol agent thanks to its great abundance of antibiotics showing the broad spectrum activity (Stein, 2005; Moszer, 1998; Expert and Digat, 1995). Most of the *B. subtilis* antibiotics are peptides and produced by ribosomal or non-ribosomal way. Spectrum action, resistance to peptidase and protease hydrolysis are the parameters which stipulate their effectiveness (Souto *et al*., 2004; Stein, 2005).

In general, *B. subtilis* antibiotics are resistant to high temperature, a wide range of pH and many hydrolytic enzymes. Recently, it has been proved that most of the in vivo *B. subtilis* antibiotics are stable and do not show any significant difference in activity when they were treated with high temperature (121°C for 20 min), a broad range of pH (from 4 to 10) and hydrolytic enzymes such as trypsin, proteinase K, and lipase A (Souto *et al*., 2004).

Some antibiotics of *B. subtilis* display completely dinstinct roles in addition to their anti-microbial mission, especially have roles on the morphology and physiology of *B. subtilis.* For instance, *n*on-ribosomal produced antibiotics are not only involved in antimicrobial action, but also have roles on colony formation and biofilm. Furthermore, lantibiotics act as effective factors in programmed cell death and quarum sensing mechanism in *B. subtilis* (Stein, 2005).

1.3.1. Ribosomal Synthesis: Lantibiotics

Lantibiotics are a group of peptide bacteriocins including unusual 3-methyllanthionine and thioether amino acids lanthionine due to post-translational modifications; dehydration of serine and threonine residues and then addition of cysteine thiol groups. The name lantibiotics stands for lanthionine-containing antibiotic peptides (Chatterjee et al., 2005). They are synthesized as procursors molecules by ribosomal pathway and after the activation by proteolysis, are exported out of the cell (Figure 1.1). The genes responsible in biosynthesis and pathway of lantibiotics are found in gene clusters (Guder *et al,* 2000).

According to the structural properties, lantibiotics are seperated in two groups: Type A lantibiotics, consisting of 21–38 amino acid residues, exhibit a more linear secondary structure and kill gram-positive target cells by forming voltage-dependent pores into the cytoplasmic membrane. On the contrary, type B lantibiotics have a more globular structure and have comparatively small charges (Breukink *et. al,* 1999; Mannanov et al., 2001).

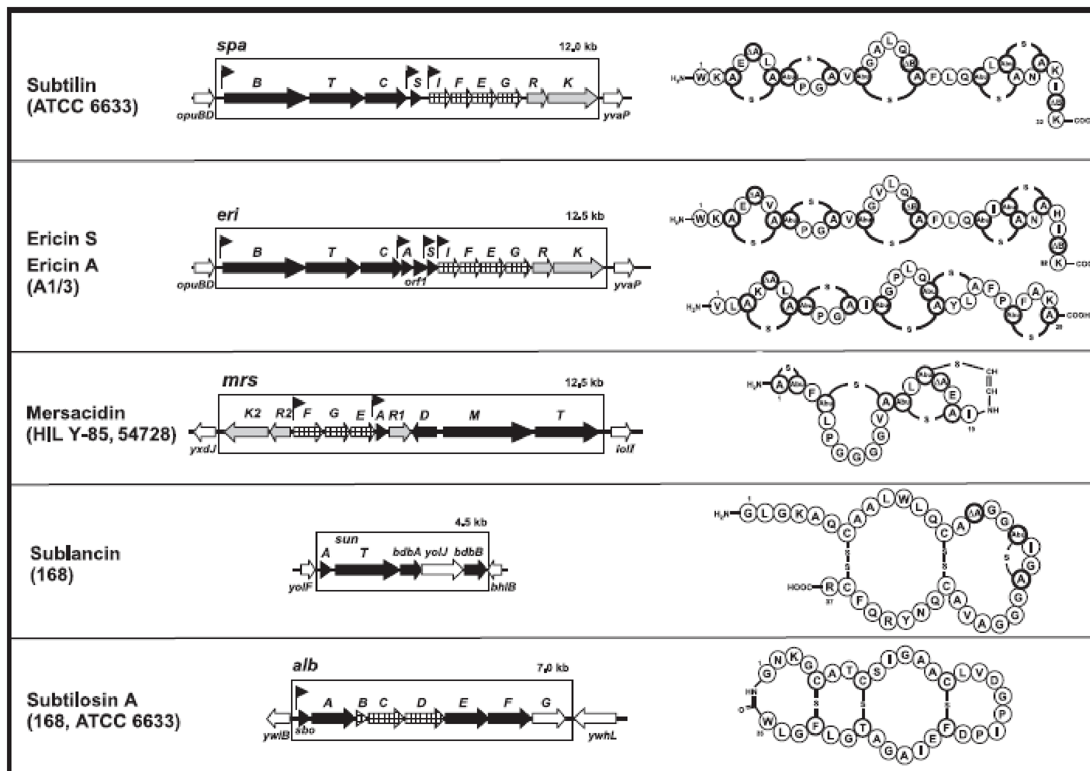


Fig. 1.1: A few *Bacillus subtilis* lantibiotics, lantibiotic-like peptides and specifying gene clusters (Guder *et al,* 2000).

1.3.2. Non-ribosomal Biosynthesized Peptides

Some of the peptide antibiotics of B. subtilis are synthesized through the large multienzymes called the non-ribosomal peptide synthetases (NRPSs). Selection and condensation af the amino acids in peptide are performed by the three catalytic domains of the NRPSs according to the multiple-carrier thiotemplate mechanism. Each elongation cycle in non-ribosomal peptide biosynthesis needs the cooperation of three core domains. Three catalytic domains of the NRPSs; adenylation domain, peptidyl carrier domain and condensation domain selects the amino acid, tranfers the adenylated amino acid substrate to its prosthetic group and finally catalyses the formation of a new peptide bond respectively. The unit of these three core domains

which catalyse all the necessary reactions in peptide biosynthesis form a structure called module. The arrangement of the modules constitutes the NRPSs (Walker et al., 1970; Lambalot et al., 1996; Stein et al., 2005).

1.4. Roles of *spo0A* and *abrB* in Antibiotic Biosynthesis

Under unfavorable conditions like nutrient deprivation and high cell density, *B. subtilis* undergoes a cellular differentiation process called sporulation, leading to the formation of a dormant spore (Phillips and Strauch, 2002). During this transition state from vegetative to sporulation phase, a wide variety of two component signal transduction systems of *B. subtilis* are activated in response to the environmental signals. Also, small peptide pheromones are excreted and imported by *B. subtilis* in order to sense the environmental situation (Fabret et al., 1999: Stephenson and Hoch 2002). According to the environmental conditions and signals, various antibiotics and antimicrobial factors are excreted out of the cell in order to outcompete other microbial species. Production of and resistance to antibiotics are regulated through *spo0-abrB* control system (Figure 1.2). Signals, indicating the hard conditions such as high cell density or nutritional stress, pump into cell and promote the Spo0 phosphorelay, eventually causing the accumulation of Spo0A phosphate (Spo0A-P), which activates sporulation gene transcription. Phosphorylated Spo0A (Spo0A-P) has a higher DNA binding affinity to the *abrB* promoter and thus represses the transcription of the transition state regulatory gene, *abrB*. The concentration of the AbrB drops below its critical threshold value, and thus AbrB-dependant repressive effects over the production of different antibiotics and other stationary phase related products are lifted (Burbulys et al, 1991; Ireton et al, 1993; Strauch et al, 1990).

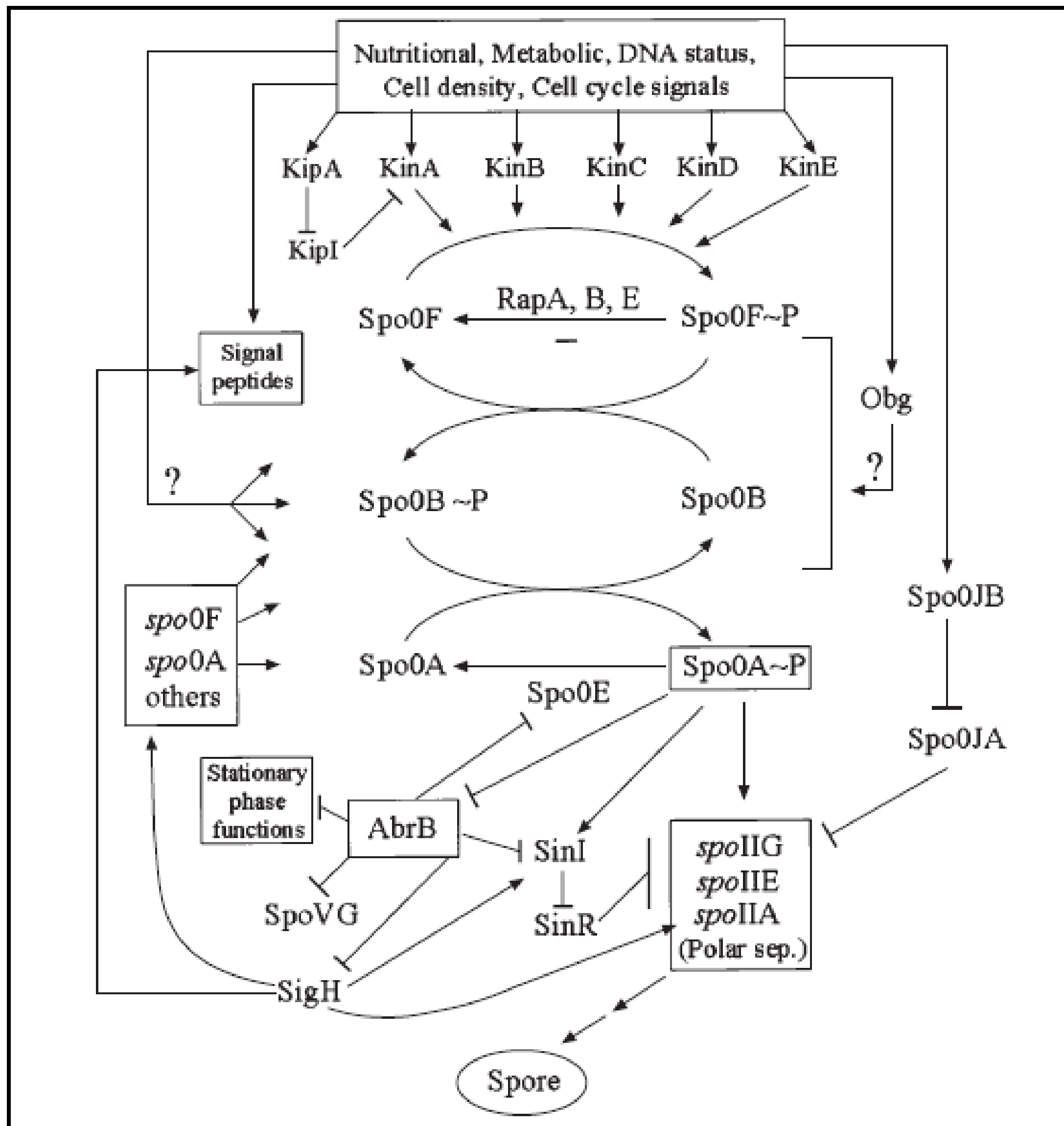


Fig. 1.2: The Phosphorelay. The main interactions that occur within and associated with the phosphorelay are illustrated. Arrows indicate activation, and barred lines indicate repression. Phosphorylated forms of proteins are indicated by ~P (Phillips and Strauch, 2002).

AbrB protein regulates more than 40 genes directly by binding their promoters or regulatory regions (Strauch 1993, Strauch 1995a). Additionally, many regulator proteins are also under the control of AbrB such as SinR and Abh regulatory proteins, since it is also a regulator molecule of other regulator proteins (Fawcett et al, 2000; Strauch et al, 2007). The genes which are controlled directly or indirectly by AbrB take part in a wide variety of metabolic processes such as antibiotic synthesis, nitrogen utilization, amino acid metabolism, development of competence and sporulation (Hahn et al, 1995; Robertson et al, 1989).

It is known that AbrB protein regulate several genes which are expressed in the transition state from exponential growth to stationary phase by binding directly on their promoter regions (Robertson et al, 1989; Strauch et al, 1989). Dimerization and multimerization of the AbrB protein are crucial steps for its binding to target DNA. As a result of dimerization of two identical monomeric subunits, a saddle-like cleft is formed into which target DNA fits and AbrB protein contact one face of the DNA helix (Xu and Strauch, 1996b; Strauch, 1995a; Vaughn et al, 2000).

AbrB changes the gene expression in three ways after binding on regulatory regions of DNA. AbrB can be a repressor (negative regulator) for some genes, and these genes are expressed constantly in *abrB* mutants since repression of AbrB over these genes is eliminated. Secondly, and most commonly, AbrB can act as inhibitor for some genes. In this manner, AbrB protein is a inhibitory factor which act on redundant regulatory network to prevent the control of other regulators over the genes that must be inactive. Thirdly, and finally, AbrB can be an activator for some genes. In fact, up to now, there has not been reported any case of RNA polymerase activation by AbrB. It is thought that AbrB protein is a negative regulator of other negative regulators; so that eliminate the negative control by binding the DNA region which is also available for other negative regulators (Strauch, 1993; Strauch and Hoch, 1993; Strauch, 1995b).

It is undoubtedly known that transition state regulator protein AbrB and sporulation initiation gene Spo0A have crucial roles in the biosynthesis of antibiotics and toxins in *Bacillus* species (Figure 1.3). So far, much more is known about the antimicrobial encoding genes and operons which are regulated by Abrb and Spo0A. For instance, it has been found that AbrB negatively regulates cyclic bacteriocin subtilosin through the antilisterial bacteriocin operon (*alb*) and *sbo* gene, which encodes the precursor of subtilosin (Zheng et al, 1999). Also, *tasA* operon whose product is an antimicrobial protein called TasA is under the control of transition state regulatory genes *spo0A* and *abrB* positively and negatively, respectively (Stover and Driks, 1999). In addition to these findings, recently, it was proven that transcription of σ^W regulon in *B. subtilis*, which activates the genes responsible in the production of antimicrobials and detoxification functions is subject to *abrB* negative control (Qian et al, 2002). Moreover, mutations in *spo0A* gene makes the *B. subtilis* unable to

produce some certain antibiotics and sensitive to some antibiotics produced by wild-type *B. subtilis* (Zuber et al, 1987).

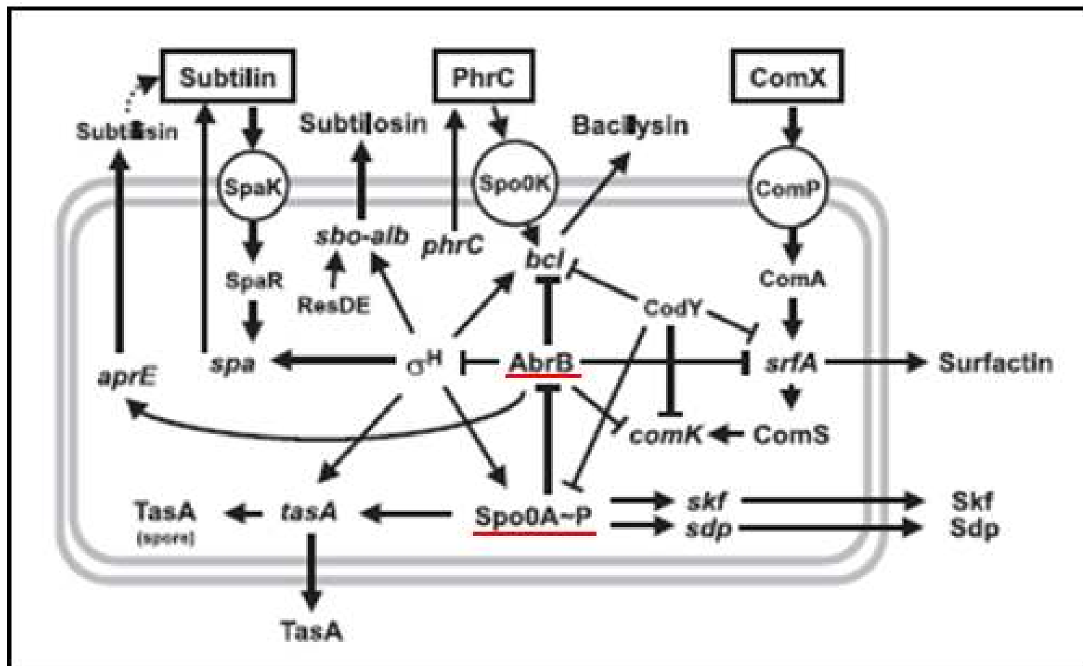


Fig. 1.3: Regulatory pathways of antibiotic biosynthesis in *B. subtilis*. Positive and negative regulation of gene expression is indicated by arrows and T-bars respectively. For clarity, the repression of AbrB on *sbo-alb* and *tasA* was omitted (Stein, 2005).

1.5. Bacilysin

Bacilysin [L-alanyl-(2.3-epoxycyclohexanone-4)-L-alanine] is one of the simplest and smallest peptide antibiotics synthesized and excreted by some bacteria of the genus *Bacillus* such as *B . amyloliquefaciens, B. licheniformis* and *B. pumilus* and *B. subtilis* 168 strain (Figure 1.4). Bacilysin is formed during the second phase of slower growth, and there is little production during the stationary phase (Loeffler *et al*., 1986; Walker *et al.* 1970; Stein *et al.* 2005). It is a dipeptide that consist of an L-alanine residue at the N terminus and a non proteinogenic amino acid, L-anticapsin, at the C terminus (Chatterjee et al. 2005).

Antibiotic activity of bacilysin depends on the L-anticapsin moiety, which is released by peptidases after uptaken of bacilysin into the cell by a peptide permease system (Kenig *et al.* 1976; Chmara *et al*. 1982; Perry and Abraham 1979). L-anticapsin, then, blocks the glucosamine synthetase, thereby, bacterial peptidoglycan or fungal mannoprotein synthesis leading cell to protoplasting and cell lysis (Kenig *et al.* 1976; Chmara *et al.* 1982; Chmara 1985; Milewski 1993).

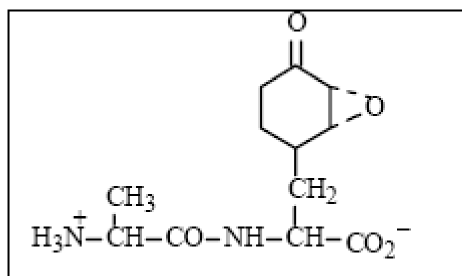


Fig. 1.4: Structural formula of bacilsin (Mannanov et al., 2001).

1.5.1. Genetic Mechanism of Bacilysin Biosynthesis

The biosynthesis of the dipeptide bacilysin depends on the *ywfBCDEF* gene cluster and renamed *bacABCDE* (Figure 1.5). It has been proved that the disruption of these genes by plasmid integration causes the loss of the ability to produce bacilysin, and also a lack of bacilysin synthetase activity in the crude extract (Inaoka *et al*., 2003). The unusual amino acid anticapsin moiety of bacilysin is probably generated through the action of a prephenate dehydratase and an aminotransferase, products of *ywfBG* genes, respectively (Roscoe and Abraham 1966; Hilton *et al*., 1988). According to the similarity features of the *bacABC* genes, mentioned proteins are most similar to following proteins; BacA to prephenate dehydratases, BacB to isomerase/guanylyl transferases and BacC to different oxidoreductases. Therefore, the products of *bacABC* genes are good candidates for catalysing the conversion of prephenate to anticapsin (Inaoka *et al*., 2003; Roscoe and Abraham 1966; Hilton et al. 1988). So as to investigate the involved genes in anticapsin production, the candidate *bacABC* genes were transformed into a *B. subtilis* Δ (*ywfA-bacABCDE*) deletion mutant and resulted in the accumulation of the anticapsin. Furthermore, individually disruption of the each chromosomal genes *bacA, bacB* or *bacC,* in three different mutants (*bacA::catR-bacBCD, bacA*-Δ*bacB::catR-bacCD and bacAB*-Δ*bacC::catR-bacD*), blocked anticapsin synthesis in each mutant, strongly suggesting the active roles of *bacABC* genes in anticapsin production (Steinborn, *et al.* 2005).

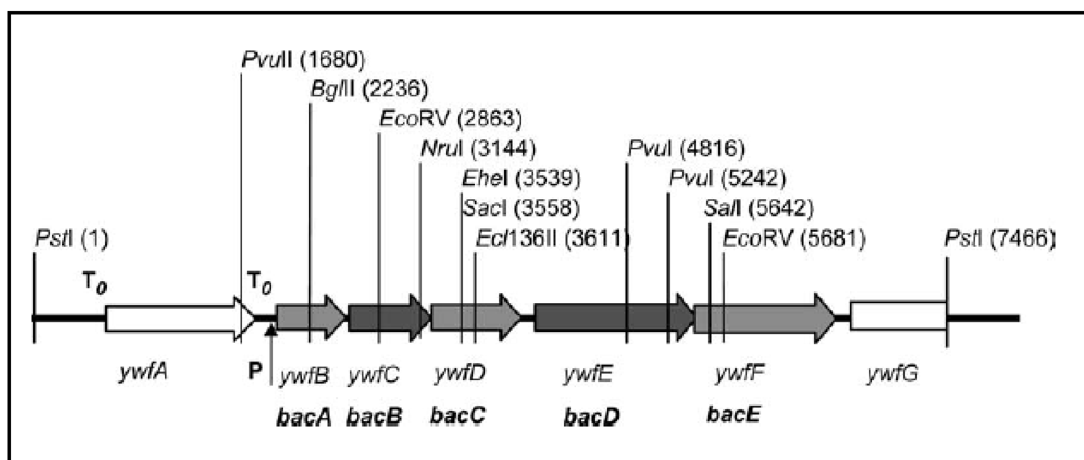


Fig. 1.5: Organization of the bacilysin gene cluster bacABCDE relative to open reading frames ywfABCDEFG of *Bacillus subtilis* 168. Restriction sites used are indicated at the base pair counted from the upstream PstI site. The DNA comprises the sequence from 3875148–3867678 bp of the SubtiList database R16.1 (Kunst et al. 1997). Proposed terminator (T_0) elements are indicated according the SubtiList database. Sigma A promoter (P) elements -35 (TTGACA) and -10 (TAAAATt) were tentatively detected 56 bp and 33 bp upstream of the ATG codon of the bacA gene (Steinborn, *et al.* 2005).

On the other hand, bacilysin synthesis is proposed not to depend on the multiple carrier thiotemplate model requiring non-ribosomal peptide synthetases (Yazgan, et al., 2001), the peptide bound between the L-alanine and L-anticapsin must be proceeded in a non-ribosomal mode, most likely by an amino acid ligase referred as bacilysin synthetase (Sakajoh *et al*. 1987). In addition to this, since BacD protein was most similar to D-alanine - D-alanine ligase, the deduced protein was a good candidate to catalyse the proposed peptide bound ligation between the L-alanine and L-anticapsin (Inaoka *et al*., 2003). After complementation of *bacD*-deficient mutant (*bacABC*-Δ*bacD::catR*) by the plasmid with *bacD* gene, bacilysin-deficient mutant was converted to bacilysin production indicating that mutation in *bacD* blocked the conversion of anticapsin to bacilysin; therefore, blocked the gene to encode the proposed ligase function (Steinborn, *et al.* 2005). In addition to this report, by in silico screening, in 2005, it was found that BacD protein catalyzes the formation of dipeptide forming L-alanyl-L-glutamine from L-alanine and L-glutamine in an ATP dependent manner. ADP and phosphate were formed in the ligation reaction catalyzed by BacD protein (Tabata et al., 2005), whereas aminoacyl-tRNA synthetase or the adenylation domain of NRPS forms acyl-AMP as a reaction products (Cane *et al.* 1999). Besides, BacD protein does not include any motif of

aminoacyl-tRNA synthetase or the adenylation domain of NRPS (Tabata et al., 2005). So that, in accordance with the previous research (Sakajoh *et al*. 1987), it has been proved once more again that bacilysin synthesizing reaction is carried out on a amino acid ligase, which is now known as BacD protein encoded by *bacD* gene, rather than NRPS-like manner (Tabata *et al.,* 2005; Steinborn, *et al.* 2005; Yazgan *et al.,* 2001).

By the way, over-production of recombinant anticapsin in Δ(*ywfA-bacABCDE*)::*catR* mutant strain caused cell protoplasting and later cell lysis. This demonstrated that chromosomal *bacE* gene is responsible in self-protection to bacilysin (Steinborn, *et al.* 2005).

To sum up, *bacABC* genes carry the anticapsin production functions, while the *bacD* and *bacE* genes encode the functions of amino acid ligation and self-protection to bacilysin, respectively (Inaoka *et al*., 2003; Steinborn *et al.* 2005; Tabata *et al.,* 2005).

1.5.2. Genetic Regulation of Bacilysin

When it comes to the bacilysin, it has been proved that even the bacilysin is under the regulation of *abrB* and *spo0A* genes. Insertional mutation in *abrB* gene resulted in an increase in bacilysin production, indicating that bacilysin synthesis is under the negative control of *abrB* gene. In the same research, *spo0A* blocked mutant cells could not produce bacilysin (Yazgan *et al.,* 2003).

Bacilysin production is regulated on different levels negatively by GTP via the transcriptional regulator CodY and AbrB (Mascher *et. al.,* 2004 and Yazgan *et al.,* 2003). Distruption of *phrC, comA* and *oppA* by Tn10 transposon mutagenesis and resulting bacilysin-negative mutants demonstrated the reationship between the Spo0K dependent quorum sensing regulation system and bacilysin biosynthesis in *B. subtilis* (Yazgan *et al.,* 2001). In other words, bacilysin biosynthesis is directly related with the ComA and PhrC in a Spo0K-dependent manner and under the regulation of quorum sensing mechanism in *B. subtilis* (Yazgan *et al.,* 2001). Moreover, the second pathway of quorum sensing system called as two-component signal transduction system was also found to be essential for bacilysin biosythesis since the introduction of *comQ::cat* mutation resulted in the elimination of bacilysin biosynthesis (Yazgan *et al*., 2003).

Recent researches have showed that disruption of *srfA* operon in the bacilysin producer caused the bacilysin-negative phenotype. Therefore, it is thought that bacilysin and surfactin biosynthesis is under control of same regulation factors (Yazgan *et al.,* 2003).

Positive regulation occurs by guanonsine 5'-diphosphate 3'-diphosphate (ppGpp) and a quorum-sensing mechanism through the peptide pheromone PhrC (Inaoka *et al*., 2003; Yazgan *et al.,* 2001).

1.6. Electromobility Shift Assay (EMSA)

Electromobility shift assay, which is also called as mobility shift electrophoresis or gel retardation assay is a common electrophorosis technique which is used in molecular biology for the protein-DNA or protein-RNA interactions**.** This technique determine whether a protein or protein mixture binds to its target DNA (or RNA) or not. While protein-RNA (or protein-DNA) mixture is run on a agarose or polyacrylamide gel for a period, control lane is run only with DNA probe (or RNA probe). EMSA protocol can be divided three major steps; labelling and isolation of the nucleic acids (DNA or RNA), incubation of protein-DNA binding reaction, and finally running the sample on a native poliacrylamide or agarose gel (Figure 1.6). If the protein has the capability of binding to its target, the lane with the protein-DNA will move more slowly compared the control lane which includes only the free DNA and therefore, cause a shift detection (Hellman and Fried,2007; Holden and Tacon, 2010). Molecule size, molecular weight and charge are the considerable parameters that make samples run on nondenaturing gel matrix in different mobilities (Gaudreault, M. et al, 2009; Hellman and Fried,2007).

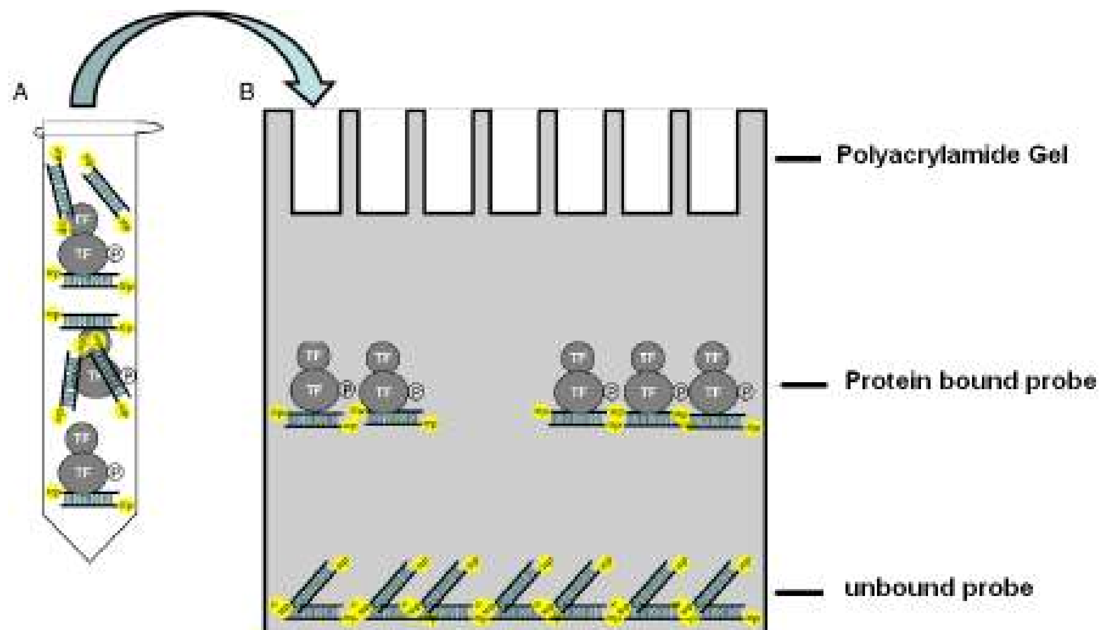


Fig. 1.6: A schematic diagram of EMSA (Holden and Tacon, 2010).

1.7. DNase I Footprinting Assay

Transcription of DNA to RNA requires many factors including enhancer, silencer elements and transcription factors that bind to regulatory regions on DNA. So as to identify the DNA regions on which trans-acting molecules bind, recently being common method called DNase footprinting assay has been employed mostly. In this assay, one end of the double-stranded DNA is labelled wtih radiactive molecules or a fluorescein dyes. Labelled DNA is incubated with the DNA-binding protein and then the labelled DNA in sample is cut by chemical (hydroxyl radicals) or enzymatic cleavage (DNase 1) agent. Resulted DNA fragments are separated on sequencing gel. There will be gaps on the sequencing gel due to the binding proteins which bind to DNA and protect the binding region of DNA being cleaved by DNase 1 or hydroxyl radicals. The protected region of DNA will be missing (Figure 1.7) and will be an evident as a gap which also called as footprint on the separation gel (Hampshire, et al. 2007; Wilson et al., 2001). Recently, instead of sequencing gel, capillary electrophoresis is used for separation of the DNA fragments (Wilson et al., 2001; Zianni, 2006).

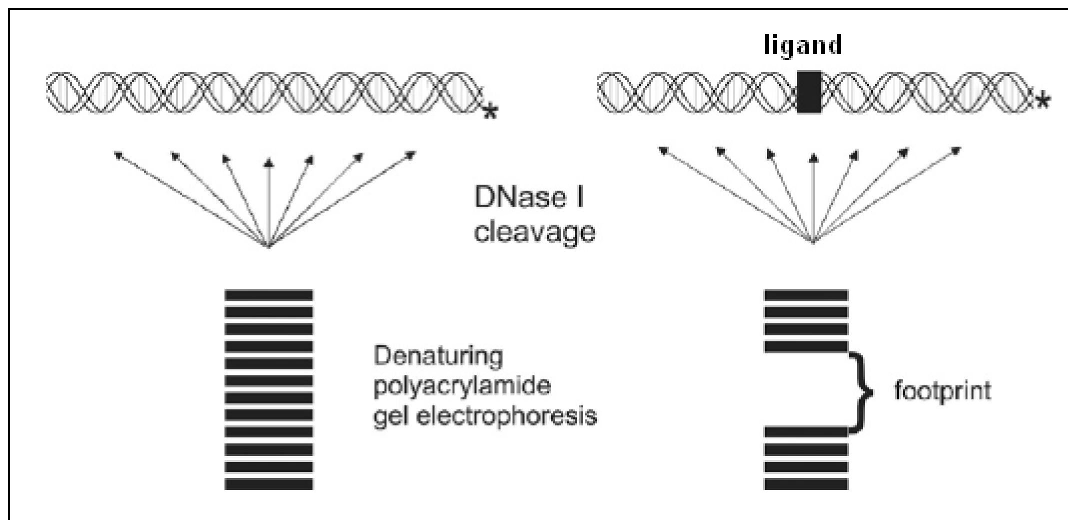


Fig. 1.7: Schematic representation of footprinting assay (Hampshire et al., 2007).

1.8. Aim of the Project

There are strong evidences showing the essential roles of AbrB and Spo0A on the biosynthesis of antibiotics and toxins in *Bacillus* species. It has been demonstrated that even the bacilysin is under the regulatin of *abrB* and *spo0A* genes. After the recent findings concerning the responsibility of *yvfI* gene whose product is similar to transcriptional regulator (GntR family) in bacilysin production in *Bacillus subtilis* PY79 strain, there needs to find the other genes regulating bacilysin production through *yvfI* gene, since any gene involved in the regulation of *yvfI* is also responsible in the regulation of bacilysin production indirectly. Subject to the foregoing provisions, our studies are focused on determining the cis-elements in the promoter region of the *B.subtilis yvfI* gene to which the candidate regulatory proteins are bound.

For this purpose, *B. subtilis yvfI* promoter was examined using DBTBS database and putative binding sites on *yvfI* promoter were found for AbrB and Spo0A regulatory proteins. After the production of purified Spo0A and AbrB recombinant proteins in *E. coli,* DNA Mobility Shift Assay (EMSA) and Capillary –Based DNase I Footprinting Assay have been employed in order to find the exact binding sites of AbrB and Spo0A regulatory proteins on *yvfI* promoter and to provide further insight into the regulation of bacilysin by the means of transcriptional regulation of *yvfI* gene.

2. MATERIALS and METHODS

2.1. Materials

2.1.1. Bacterial Strains

Table 2.1: Bacterial strains used through the project

Strain	Genotype	Source
Bacillus subtilis PY79	Wild type, BSP cured prototrophic derivative of *B.subtilis* 168	P.Youngman
E.coli Top10F’	[*lac*Iq Tn10(Tet^r)], *mcrA* Δ(*mrr-hsdRMS-mcrBC*), *f80lacZΔM15 ΔlacX74*, *deoR, recA1, araD139 Δ(ara-leu)7697, galU, galK,rpsL (Strr), endA1, nupG*	M.A.Marahiel

2.1.2. Bacterial Culture Media

The compositions and preparation of bacterial culture media are given in Appendix A.

2.1.3. Buffers and Solutions

The compositions and preparation of buffers and solutions are given in Appendix B.

2.1.4. Chemicals and Enzymes

The chemicals and enzymes used and their suppliers are given in Appendix C.

2.1.5. Laboratory Equipment

The laboratory equipment used during the project is listed in Appendix G.

2.1.6. Maintenance of Bacterial Strains

PY79 strain of *B.subtilis* was grown in Luira-Bertani (LB) liquid medium and kept on Luira-Bertani (LB) agar plates. *E.coli* strains were kept on Luira-Bertani (LB) agar plates. All cultures were stored at 4 C°.

2.2. Methods

2.2.1. Construction of pJET-*abrB* and pJET-*spo0A* Cloning Vectors

2.2.1.1. Amplification of *abrB* and *spo0A* Genes by Polymerase Chain Reaction

The oligonucleotide primers *abrB* F_1, *abrB* R_1, *spo0A* F_1 and *spo0A* R_1 primers given below are the forward and the reverse primers for the amplification of *abrB* and *spo0A* genes to be cloned into pJET 1.2 / Blunt Cloning Vector, respectively. Underlined sequences represent recognition sites for *BamHI* and *NcoI* restriction enzymes on forward and reverse primers respectively.

abrB F_1 : 5' – CGGCCATGGGTATGTTTATGAAATCTACTGGT – 3'
abrB R_1 : 5' – GCCGGATCCTTTAAGGTTTTGAAGCTG – 3'
spo0A F_1: 5' – CGGCCATGGGTGAGAAAATTAAAGTTTGT– 3'
spo0A R_1: 5'– GCCGGATCCAGAAGCCTTATGCTCTAACCT – 3'

PCR components given in Table 2.2 were combined in a 0.2 ml tubes on ice.

Table 2.2 : The volume of PCR components

Component	**Exp.**	**(-) Control**
dH_2O	40,5 µl	42,5 µl
10X *pfu* Buffer	5 µl	5 µl
dNTP	1 µl	1 µl
F Primer	0,5 µl	0,5 µl
R Primer	0,5 µl	0,5 µl
Template DNA (PY79)	2 µl	-
pfu DNA polymerase	0,5 µl	0,5 µl
Total	**50 µl**	**50 µl**

The final concentrations of the primers and dNTP mix were 0,5 μM and 0,2 μM respectively. Chromosomal DNA of *B.subtilis* PY79 strain was used as template. PCR reaction was performed according to the following cycling program, enabling the heated lid:

PCR conditions

95 °C, 3 min ⟹ 1 cycle (Initial denaturation)

95 °C, 1 min
55 °C, 1 min } 30 cycles + 72 °C, 10 min } 1 cycle (Final extension)
72 °C, 2 min

2.2.1.2. Agarose Gel Electrophoresis of PCR Products

After the amplification of the desired genes, PCR products were analyzed on agarose gel electrophoresis. Firstly, for %1 gel, 0.5 gr agarose was added in 50 ml 0.5X TAE buffer (Appendix B) and then gel solution was boiled in a microwave until the agarose was completely dissolved. After cooling the solution to about 60°C, ethidium bromide was added to the gel solution to a final concentration of 0.5 ug/ml. Then, gel solution was poured into gel casting tray and a proper comb was placed in gel tray. After the gel had been solidified, the comb was removed and gel was placed in electrophoresis chamber containing the appropriate amount of 0.5X TAE buffer (Appendix B). An appropriate molecular weight marker (Marker 9, Fermentas) and PCR product samples that were mixed with the 6X loading dye depending on volume were pipeted into the gel wells. Gel was run at 80 V for 20 min. EtBr-stained DNA bands were visualized by UV trans-illuminator.

2.2.1.3. Ligation of the PCR Product Into pJET 1.2 / Blunt Cloning Vector

For the ligation of the PCR products into pJET 1.2/Blunt Cloning Vector, CloneJET PCR Cloning Kit (Fermentas) was used and the following reaction was set up for each PCR product. All components were added into a 1.5 ml eppendorf tube according to volumes that are given in Table 2.3. The eppendorf tube including all components was incubated at 25 ^{o}C for 30 min.

Table 2.3 : Volume of components used in ligation of the PCR product into pJET 1.2 / blunt cloning vector

Component	Volume/reaction
pJET1.2/blunt Cloning Vector (50 ng/ul)	1 µl
PCR product	3 µl
2X Reaction Buffer	10 µl
T4 DNA Ligase	1 ul
Water, nuclease-free	5 µl
Total volume	**20 µl**

Thereby, blunt-ended PCR products generated with the *pfu* DNA polymerase were ligated directly into the cloning vector. The newly contructed vectors were renamed as pJET-*abrB* and pJET-*spo0A*.

2.2.1.4. Preparation of Electrocompotent *E.coli* Top 10F' Cells

To make the electrocompotent cells, one night before *E.coli* Top10F' strain was inoculated into 500 ml 2xYT broth (Tet 20 µg/ml) with a 1/100 dilution rate and incubated at 37°C until OD_{600} reached 0.6. After incubation, the flask containing *E.coli* Top10F' strain was stayed on ice for 30 min. Then, cells were centrifuged in a 50 ml ependorf tube at 4000 rpm for 15 minutes. Supernatant was removed and pellet was resuspended in 40 ml of cold dH_2O and centrifuged at 4000 rpm for 15 min. After having removed the supernatant, the pellet was again resuspended in 20 ml of cold dH_2O in a 50 ml tube at 4000 rpm for 15 minutes. Then supernatant was discarded and cells were resuspended in 1 ml of 10% glycerol. Aliquots were prepared as 40 µl volume in the 1.5 ml eppendorf tubes. The samples were stored at -80°C.

2.2.1.5. Transformation of pJET-*abrB* and pJET-*spo0A* to Electrocompotent *E.coli* Top10 Cells

Initially, electrocompotent *E.coli* top 10 cells were thawed on ice. 10 µl of the pJET-abrB ligation sample which had been obtained in section 2.2.1.3, was added to the tube containing 40 µl *E.coli* top 10F’ cells and all together were transferred into the electroporater tube which had been cooled before on ice for 10 min. Then, the

electroporator tube was placed into electroporation machine and transformation processed at 1800V for 2-3 second. After addition of 1 ml of LB broth to the electroporator tube, the mixture was transferred to a 2 ml eppendorf tube and incubated at 37°C for 1 h. For negative control, 1 ml LB broth was added into the another 2 ml eppendorf tube containing only the compotent cells. After 1 hour incubation of both tubes at 37 °C, the tubes were centrifuged for 10 min. at 5000 rpm so as to harvest cells. The supernatants were removed and the pellets were resuspended in 100 µl of 0.85% $NaCl_2$. 100 µl of each suspention was spread out on LB plates containing ampicilin (100 µg/ml). Agar plates were incubated at 37°C for 16 hours. Colonies are selected and inoculated into LB broth with amp (100µg/ml) and also sketched to a LB agar plate with amp (100µg/ml). Both agar plate and broth cultures were incubated at 37°C for 16 hours.

All the protocol has been done exactly in the same way for the transformation of the pJET-*spo0A* to *E.coli* Top10 cells.

2.2.1.6. Plasmid DNA Isolation

Plasmid DNA isolation of the cultures incubated for 16 hours in LB broth was applied through using the buffers and solutions of the QIAquick Plasmid DNA Isolation Kit (Qiagen).

After centrifugation in 2 ml ependorf tubes at 13000 rpm for 5 minute, bacterial cells were harvested. The supernatant was removed and the pellet was resuspended in 300 µl P1 buffer completely by vortexing and pipetting up and down. 300 µl P2 buffer was added and the suspention was mixed by inverting tubes 4-6 times before the incubation at room temperature for 5 minutes. Then, 300 µl P3 buffer was added into the tubes and mixed by inverting until lysate is no longer viscous. After that, ependorf tubes were stayed on ice for 15 minutes before centrifugation at 13000 rpm for 15 minutes. With the help of pipette, the supernatant part of the suspention was put to new 1.5 ml eppendorf tubes. According to the volume of the supernatant, 0.7 volume of isopropanol was added into the tubes and centrifuged at 13000 rpm for 30 minutes. After the removal of supernatant, 1ml of 70% ethanol was added and centrifugation at 13000 rpm for 5 minutes was applied to wash the pellets. Supernatant was removed and pellets were dried out at 37°C for 15 minutes for the

complete removal of ethanol by evaporation. Finally, 15 µl of EB buffer was added and the tubes were incubated at 37°C for 15 minutes at 350 rpm.

2.2.1.7. Agarose Gel Electrophoresis

% 0.8 agarose gel was prepared to check the result of the plasmid isolation. Firstly, 0.4 gr agarose was added in 50 ml of 0.5X TAE buffer (Appendix B), then gel was boiled in a microwave oven until completely melted. After cooling the solution, to facilitate visualization of DNA after electrophoresis, a final concentration of 0.5 ug/ml of ethidium bromide was added to the gel solution. Then it was poured into a casting tray containing a sample comb and allowed to solidify at room temperature. After the solidification, the comb was removed and plasmid DNA samples and moleculer weight Marker 3 (Fermentas, Appendix D) were loaded into the gel wells after all samples were mixed with 6X gel loading dye depending on volume. Gel was run at 80V for 25 min. and plasmids DNA bands were visualized by UV trans-illuminator.

2.2.2. Construction of pQE60-*abrB* and pQE60-*spo0A* Expression Vectors

2.2.2.1. Enzymatic Digestion

Concentrated plasmids having the different conformations on agarose gels were chosen for enzymatic digestion. Following reaction including the components as shown in Table 2.4 was set up for each vector construct, pJET-*abrB* and pJET-*spo0A* and digestion proceeded with *BamHI* and *NcoI* restriction enzymes at 37°C for 3 hours in order to check whether *abrB* and *spo0A* genes had been cloned into the pJET1.2/blunt Cloning Vector. Then enzymes were denaturated at 65°C for 20 min.

Table 2.4 : The components of the enzymatic digestion reaction

Component	Volume/reaction
pJET-*abrB* (or pJET-*spo0A*)	2 µl
Buffer 10 X Tango	2 µl
BamHI	0.25 µl
NcoI	0.25 ul
dH_2O	15.5 µl
Total volume	**20 µl**

For constructing expression vectors containing the *abrB* (or *spo0A*) gene, pQE60 Expression Vector, pJET-*abrB* and pJET-*spo0A* were digested with same restriction enzyme, *BamHI* and *NcoI* in the same conditions provided for pJET1.2/blunt Cloning Vector. As fragments including *abrB* and *spo0A* genes and pQE60 Expression Vector both were digested with same restriction enzymes, direct ligation of *abrB* and *spo0A* genes to pQE60 Expression Vector would be applied. The components of the reactions are given in Table 2.5 and Table 2.6.

Table 2.5 : The components of the pJET-*abrB* enzymatic digestion

Component	**Volume/reaction**
pJET-*abrB* (or pJET-*spo0A*)	9 µl
Buffer 10 X Tango	4 µl
BamHI	0.5 µl
NcoI	0.5 ul
dH_2O	26 µl
Total volume	**40 µl**

Table 2.6 : The components of the pQE60 enzymatic digestion

Component	**Volume/reaction**
pQE60 Expression Vector	9 µl
Buffer 10 X Tango	4 µl
BamHI	0.5 µl
NcoI	0.5 ul
dH_2O	26 µl
Total volume	**40 µl**

2.2.2.2. Gel Extraction

After 3 hours incubation of both reactions at 37°C in thermomixer, the samples that had been digested by restriction enzymes were loaded on agarose gel in order to isolate only the linearized pQE60 and DNA fragments including *abrB* and *spo0A*

genes from the rest of the DNA fragments for direct ligation. In that purpose, QIAquick Gel Extraction Kit (Qiagen) was used for the gel extraction of the enzymatic digestion. The fragments pQE60 in lineer form and DNA fragments including *abrB* and *spo0A* genes were excised from the gel and put into the different ependorf tubes. After that, depending on the weight of the fragments, 3 volumes of buffer QG were added. In order to dissolve the gels, tubes were incubated for 10 minutes at 50°C by shortly vortexing every 2-3 minutes, until the gels were dissolved completely. Following, 1 volume of isopropanol was added into tubes. The samples were mixed by inverting several times and then applied to the QIAquick column for centrifugation at 13000 rpm for 1 minute. Then the flow through was discarded and the QIAquick column was placed back into the same collection tube. After addition of 500 µl buffer QG, the samples were centrifuged at 13000 rpm for 1 minute. Later, the flow through was removed and 750 µl buffer PE was added so as to wash the samples. The columns were standed for 2-5 minutes and then centrifuged at 13000 rpm for 1 minute. For complete removal of ethanol, the columns were centrifuged at 13000 rpm for an additional 1 minute. Finally, the columns were placed into clean 2 ml microfuge tubes and 40 µl of EB buffer was added to the center of the QIAquick membranes. The samples were standed for 1 minute and then centrifuged for 1 minute at 13000 rpm. The final solutions contaning the linearized pQE60 expression vector and DNA fragments including *abrB* and *spo0A* genes in discrete tubes were stored at –20 °C until the next ligation step.

2.2.2.3. Ligation of *abrB* and *spo0A* Genes Into pQE60 Expression Vector

To begin up, 9 µl sample of DNA fragment containing *abrB* gene as insert and 2 µl pQE60 vector were added into an eppendorf tube and incubated at 65°C for 5 minutes. After that, the tube was cooled on ice. Before having started the ligation, eppendorf tube was spanned down so as to collect the whole mixture at bottom of the tube. Following, 2µl of ligation 10 X buffer, 2µl of T4 DNA ligase (Roche), 6µl of dH_2O were added into the same eppendorf tube. The mixture was again spanned down for a quick spin and incubated at 16°C for 16 hours. After incubation, the tube was incubated at 65°C for 10 minutes for denaturation of the enzyme. Same protocol was done exactly in the same way for the ligation of *spo0A* gene into linearized pQE60 expression vector. The newly contructed vectors were renamed as pQE60-*abrB* and pQE60-*spo0A*.

2.2.2.4. Transformation of pQE60-*abrB* and pQE60-*spo0A* to Electrocompotent *E.coli* Top10 Cells

40 µl aliquot of electrocompotent *E.coli* top 10 cells was thawed on ice. 10 µl of the pQE60-*abrB* ligation sample which had been obtained in section 2.2.2.5, was added to the tube containing 40 µl *E.coli* top 10F' cells and all together were transferred into the electroporation cuvette which had been cooled on ice before. Then, the electroporator cuvette was placed into electroporation machine and transformation processed at 1800V for 2-3 second. After addition of 1 ml of LB broth to the electroporator cuvette, the mixture was mixed gently by pipetting up-down and transferred to a 2 ml eppendorf tube for the incubation at 37°C for 1 h. For negative control, 1 ml LB broth was added into the another 2 ml eppendorf tube containing only the compotent cells. After 1 hour incubation of both tubes at 37 °C, the tubes were centrifuged for 10 min. at 5000 rpm so as to harvest cells. The supernatants were removed and the pellets were resuspended in 100 µl of 0.85% $NaCl_2$. 100 µl of each suspention was spread out on LB plates containing 100 µg/ml final concentration of ampicilin. Agar plates were incubated at 37°C for 16 hours. Colonies are selected and inoculated into LB broth with amp (100µg/ml) and also sketched to a LB agar plate with amp (100µg/ml). Both agar plate and broth cultures were incubated at 37°C for 16 hours.

All the protocol has been done exactly in the same way for the transformation of the pQE60-*spo0A* vector to *E.coli* Top10 cells.

2.2.3. Expression and Purification of 6xHis-tagged Spo0A and AbrB Proteins

2.2.3.1. Expression of 6xHis-tagged AbrB and 6xHis-tagged Spo0A Proteins

Selected recombinant clones from each transformation were inoculated into the fresh 10 ml LB medium containing 100 ug/ ml amp and incubated overnight at 37°C at 200 rpm shaking. After that 3 ml of the each culture was transferred to fresh 100 ml LB medium with 100 ug/ ml amp and cells were grown until the the OD_{600} reaches 0,55. Immediately before the induction, 2 ml sample from each culture was taken into new tubes and cells collected by centrifugation at 4000 g for 20 min at 4°C and were stored at -80°C until the purification step. This samples were the noninduced controls. In order to induce T7 RNA polymerase, and hopefully our target proteins, AbrB and Spo0A, IPTG was added to a final concentration of 1 uM and cells were

grown for an additional 5 hours at 30°C with gentle shaking at 200 rpm. Then, cells were harvested by centrifugation at 4000 g for 20 min at 4°C and were stored at -80°C until the purification step.

2.2.3.2. Purification of 6xHis-tagged AbrB and 6xHis-tagged Spo0A Proteins

Since our overexpressed proteins were soluble proteins, they were purified under the native conditions. Also, to prevent the degradation of the proteins, cells and proteins solutions were kept at 0 - 4°C at all times and an air-cooled centrifuge with swinging-bucket rotor was used.

1. Cell pellets obtained in previous step by centrifugation before (noninduced samples) and after induction (induced samples) were thawed on ice for 15 min. Then, pellets of noninduced and induced samples were resuspended in 100ul and 10 ml lysis buffer, respectively (Appendix B). For further cell distruption, lysozyme to a final concentration of 0.2 mg/ml was added to each solution and incubated on ice for 30 min. So as to lyse the cells as much as possible, sonicator equipped with a microtip was used with a 10 s cooling period between six 10 s bursts at 70 W.

   20 ul of each cell lysate (induced AbrB, induced Spo0A, noninduced AbrB and noninduced Spo0A) was kept in 1.5 ml tubes for subsequent SDS-PAGE analysis. These cell lysates contain all the proteins expressed by *E.coli,* so that marked as total homogenate.

2. Insoluble materials of induced AbrB and induced Spo0A cell lysates were removed from the soluble fraction by centrifugation at 4°C for 30 min at 4000g and supernatant fraction of each culture was collected into fresh tubes.

   20 ul of each supernatant solution (induced AbrB, induced Spo0A) was kept in 1.5 ml tube for subsequent SDS-PAGE analysis. These samples contain all the soluble proteins expressed by *E.coli*, so that marked as total supernatant.

3. 1 ml of the 50% slurry of Ni-NTA resin was added to each tube and mixed gently by shaking at 50 rpm on a rotary shaker for 30 min at 4°C. Then the tubes were centrifuged at 1000 g for 1 min to pellet the resin.

20 ul of each supernatant solution was transferred into 1.5 ml tube for subsequent SDS-PAGE analysis. These supernatant solutions contain all the proteins which have not bound to the resin, so that marked as unbound samples. Remaining supernatants were discarded.

4. Ni-NTA resins were washed twice with 1 ml of wash buffer (Appendix B). Tubes were centrifuged for 1 min at 1000 g between each wash step and the supernatants were carefully removed.

   20 ul of each supernatant solution was kept in 1.5 ml tube for subsequent SDS-PAGE analysis. These supernatant solutions were marked as wash 1 and wash 2.

5. Our interested proteins, AbrB and Spo0A, were eluted 3 times with 500 ul elution buffer (Appendix B). Tubes were centrifuged for 1 min at 1000 g between each elution step and the supernatants were carefully transferred into new fresh tubes.

   20 ul of each supernatant solution was kept in 1.5 ml tube for subsequent SDS-PAGE analysis. These supernatant solutions contain our target proteins and so that marked as elution 1, elution 2 and elution 3.

Thereby, by the way of metal chelation affinity between the Ni^{2+} metal ions on NTA and imidazole ring on histidine residues (6xHis tag), overexpressed recombinant Spo0A and AbrB proteins were purified for the electromobility shift and Dnase I footprinting assays.

2.2.3.3. SDS-Polyacrylamide Gel Electrophoresis of AbrB and Spo0A

In order to analyze the samples obtained during purification steps (section 2.2.3.2) and purification quality of the AbrB and Spo0A proteins, two SDS polyacrylamide gels were prepared one for AbrB, and other one for Spo0A protein analysis by mixing the ingredients needed for % 12 gel as shown in Table 2.7 and Table 2.8.

Table 2.7 : Components of Lower Resolving Gel (12 % Acrylamide/Bis-acrylamide)

Components	***Volume***
H_2O	3.3 ml
1.5 M Tris-HCI (pH:8.8)	2.5 ml
SDS (10%, w/v)	0.1 ml
Acrylamide:bis-acrylamide (29:1) (30%, w/v)	4.0 ml
Ammonium Persulfate (APS) (10%, w/v)	0.1 ml
TEMED	10 ul
Total	10 ml

Prepared solution was poured into the gel casting forms provided that leaving 2 cm below the bottom of the comb for the upper stacking gels. Top of the gels were layered with 200 ul isopropanol to remove the bubbles. After the polymerization of the lower resolving gels, isopropanol was washed out completely.

Table 2.8 : Components of Upper Stacking Gel (5 % Acrylamide/Bis-acrylamide)

Components	**Volume**
H_2O	1.4 ml
1 M Tris-HCI (pH:6.8)	0.25 ml
SDS (10%, w/v)	0.02 ml
Acrylamide:bis-acrylamide (29:1) (30%, w/v)	0.33 ml
Ammonium Persulfate (APS) (10%, w/v)	0.02 ml
TEMED	6 ul
Total	2 ml

Prepared upper stacking gel solution was poured on the top of the lower resolving gels and combs were inserted. After this, gels were allowed for complete polymerization. 12 ul of each sample obtained in purification steps were mixed with 6 ul of 3X SDS loading dye (Appendix B) and boiled for 5 min at 95 °C for protein denaturation. After loading all the samples and protein marker (Fermentas, Appendix D) to the gel lanes, gels were run at 80 volt for upper stacking gels and 140 volt for lower resolving gels until the loading dyes reached the bottom of the gels.

Visualization of the protein bands were accomplished by staining the gels with 100 ml of gel stain solution (Appendix B) with shaking at 40 rpm for 1 hour at 37 °C. Finally, gels were destained by 100 ml of gel destain solution (Appendix B) with gentle shaking at 30 rpm for 2 hours at 37 °C.

2.2.4. Electromobility Shift Assay of AbrB and Spo0A Proteins

2.2.4.1. Cloning of *yvfI* Promoter Into pJET 1.2 / Blunt Cloning Vector

The oligonucleotide primers *yvfI P/* F_1 and *yvfI P/* R_1 primers given below are the forward and the reverse primers for the amplification of the DNA fragments including the *yvfI* promoter to be cloned, respectively.

yvfI P/ F_1 : 5' – CGGCCATGGGTATGTTTATGAAATCTACTGGT – 3'
yvfI P/ R_1 : 5' – GCCGGATCCTTTAAGGTTTTGAAGCTG – 3'

Underlined sequences represent recognition sites for *EcoRI* and *BamHI* restriction enzymes on forward and reverse primers respectively.

Table 2.9 : The volume of PCR components

Component	Exp.	(-) Control
dH_2O	40,5 µl	42,5 µl
10X *pfu* Buffer	5 µl	5 µl
dNTP	1 µl	1 µl
F Primer	0,5 µl	0,5 µl
R Primer	0,5 µl	0,5 µl
Template	2 µl	-
pfu DNA polymerase	0,5 µl	0,5 µl
Total	**50 µl**	**50 µl**

PCR components given in Table 2.9 were combined in a 0.2 ml tubes on ice. PCR reaction was performed according to the following cycling program, enabling the heated lid:

PCR conditions

95 °C, 3 min ⟹ 1 cycle (Initial denaturation)

95 °C, 1 min
55 °C, 1 min } 30 cycles + 72 °C, 10 min } 1 cycle (Final extension)
72 °C, 2 min

The final concentrations of the primers and dNTP mix were 0,5 µM and 0,2 µM respectively. Chromosomal DNA of *B.subtilis* PY79 strain was used as template.

Having performed the PCR reaction, components needed for cloning of the *yvfI* promoter into pJET 1.2 / Blunt Cloning Vector were added into a 1.5 ml eppendorf tube according to volumes that was given in Table 2.10. The eppendorf tube including all components was incubated at 25 ^{o}C for 30 min. for ligation and then ligase was denatured at 70 °C for 10 minutes.

Table 2.10 : Volume of components used in ligation of the PCR product into pJET 1.2 / blunt Cloning Vector

Component	Volume/reaction
pJET1.2/blunt Cloning Vector (50 ng/ul)	1 µl
PCR product	3 µl
2X Reaction Buffer	10 µl
T4 DNA Ligase	1 ul
Water, nuclease-free	5 µl
Total volume	**20 µl**

After the ligation reaction, the newly constructed plasmid renamed as pJET-yvfIprom. Bacterial transformation and plasmid isolation of the pJET-yvfIprom was performed exactly in the same way as mentioned before in section 2.2.1.5 and 2.2.1.6, respectively. pJET-yvfIprom plasmid was used as a template for the synthesis of unlabelled oligonucleotide and FAM-labelled probe, needed for subsequent electromobility shift and DNase I footprinting assays, respectively.

2.2.4.2. *yvfI* Promoter Synthesis from pJET-yvfIprom for EMSA

Double-stranded oligonucleotides that encompasses bases –396 to + 18 relative to the translational start point of *yvfI* (Appendix F) were synthesized by PCR from the pJET-yvfIprom using following primers:

yvfI E/ F : 5' – CTGGCGCCATATGTAAGCGGT – 3'
yvfI E/ R : 5' – GCCTTCTCCCAGTTTCAT – 3'

The final concentrations of the primers and dNTP mix were 0,5 µM and 0,2 µM respectively. pJET-yvfIprom was used as template.

Table 2.11 : The volume of PCR components

Component	Exp.	(-) Control
dH_2O	40,5 µl	42,5 µl
10X *pfu* Buffer	5 µl	5 µl
dNTP	1 µl	1 µl
F Primer	0,5 µl	0,5 µl
R Primer	0,5 µl	0,5 µl
Template (pJET-yvfIprom)	2 µl	-
pfu DNA polymerase	0,5 µl	0,5 µl
Total	**50 µl**	**50 µl**

PCR components given in Table 2.11 were combined in a 0.2 ml tubes on ice. PCR reaction was performed for 30 cycles under the following conditions, enabling the heated lid:

PCR conditions

95 °C, 2 min ⟹ 1 cycle (Initial denaturation)

95 °C, 1 min
55 °C, 1 min } 30 cycles + 72 °C, 10 min } 1 cycle (Final extension)
72 °C, 1 min

As a result of the PCR reaction, amplification of the 414 bp DNA fragment that encompasses bases –396 to +18 of *yvfI* (according to the translational start point of *yvfI*) was accomplished.

2.2.4.3. Purification of the *yvfI* Promoter

So as to purify the PCR product, QIAquick PCR Purification Kit (Qiagen) was used.

1. 250 ml of the Buffer PBI was added to 50 ml of PCR product and mixed.
2. Sample was applied to the QIAquick column and centrifuged 1 min at 13.000 rpm.
3. Flow-through was discarded from the tube and QIAquick column was placed into the same collection tube.
4. 0.75 ml of the Buffer PE was added to the QIAquick column and centrifuged 1 min at 13.000 rpm for washing the sample.
5. Flow-through was discarded from the collection tube and QIAquick column was placed back in the same collection tube. Sample was centrifuged at 13.000 rpm for for an additional 1 min to remove the ethanol from the sample completely.
6. After replacing the collection tube with a clean 1.5 ml microcentrifuge tube, 40 ul of ddH_2O (pH:7.0-8.5) was added to the center of the QIAquick membrane and the sample was centrifuged at 13.000 rpm for 1 min.

2.2.4.4. Binding Reactions of Protein-*yvfI* Promoter DNA for EMSA

Binding reactions for Spo0A-*yvfI* promoter were employed by incubating varying amounts of Spo0A proteins ranging from 0 to 60 ug with 1 ug of DNA fragments (414 bp DNA fragments consisting of bases –396 to + 18 relative to the translational start point of the *yvfI* gene, Appendix F) and 2 µg of Poly(dI-dC) as a non-specific competitor DNA at room temperature for 25 min in binding buffer A [20 mM Hepes, 1 mM EDTA, 10 mM $(NH)_4SO_4$, 1 mM DTT, %0,2 Tween 20 and 30 mM KCI, pH:8.0]. The reaction mixtures in 0.2 ml tubes were completed to a final volume of 25 µL with ddH_2O after combining all the components as shown in Table 2.12. Control tube contained BSA protein instead of AbrB protein.

Table 2.12 : Binding Reactions for Electromobility Shift Assay of Spo0A Protein

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**
DNA fragments	1 ug	1 ug	1 ug	1 ug	1 ug	1 ug	1 ug	1 ug
Poly(dI-dC)	2 ug	2 ug	2 ug	2 ug	2 ug	2 ug	2 ug	2 ug
Spo0A protein	-	-	10ug	20ug	30ug	40ug	50ug	60ug
BSA	-	20ug	-	-	-	-	-	-
5X binding buffer A	5 ul	5 ul	5 ul	5 ul	5 ul	5 ul	5 ul	5 ul

Binding reactions of AbrB-*yvfI* promoter were performed by incubating varying amounts of AbrB proteins ranging from 0 to 42 ug with 1 ug of DNA fragments (414 bp DNA fragments consisting of bases –396 to + 18 in relation to the translational start point of the *yvfI* gene, Appendix F) and 2 ug of Poly(dI-dC) as a non-specific competitor DNA at room temperature for 20 min in binding buffer B (50 mM KCI, 5 mM MgCI2,, 0.1 mM EDTA, 1 mM DTT and % 8 glycerol, pH:8.0). The reaction mixtures in 0.2 ml tubes were completed to a final volume of 25 µL with ddH_2O after combining all the components in reaction tubes as shown in Table 2.13. Control tube contained BSA protein instead of AbrB protein.

Table 2.13 : Binding Reactions for Electromobility Shift Assay of AbrB Protein

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
DNA fragments	1 ug	1 ug	1 ug	1 ug	1 ug	1 ug	1 ug	1 ug	1 ug
Poly (dI-dC)	2 ug	2 ug	2 ug	2 ug	2 ug	2 ug	2 ug	2 ug	2 ug
AbrB protein	-	-	6ug	12ug	18ug	24ug	30ug	36ug	42ug
BSA	-	16ug	-	-	-	-	-	-	-
5X binding buffer B	5 ul	5 ul	5 ul	5 ul	5 ul	5 ul	5 ul	5 ul	5 ul

2.2.4.5. Non-denaturing Polyacrylamide Gel Analysis

In order to resolve the protein-DNA complexes from the free DNA, %6 non-denaturing TBE polyacrylamide gels were prepared according to the table below

(Table 2.14), which gives the amount of each ingredient required to make 2 gel of 0.75 mm thickness.

Table 2.14 : The volume of TBE Polyacrylamide Gels Components

Component	**Volume**
Acrylamide:bisacrylamide (29:1) (30%, w/v)	2.4 ml
H_2O	7.2 ml
5x TBE buffer	2.4 ml
Ammonium Persulfate (APS) (10%, w/v)	200 ul
TEMED	20 ul
Total volume	**12 ml**

Non-denaturing gel solution was poured into the gel casting forms and alllowed for 2 hours at room temperature for complete polymerization. Then, gels were pre-run at 60 volt for 30 min so as to remove the all the traces of ammonium persulfate and to provide an ion equilibration between the gels and running buffer. After loading all the AbrB and Spo0A binding reaction samples into the wells, gels were run in electrophoresis buffer (50 mM KCI, 5 mM $MgCI_2$, 0.1 mM EDTA) at 80 volts for 3 hours. After that, polyacrylamide gels were stained in TBE buffer (Appendix B) containing 1:10.000 diluted SYBR Green dye for 30 min with a gentle shaking in the dark. Gels stained with SYBR Green stain were visualized using the UV transilluminator source.

2.2.5. DNase I Footprinting of AbrB and Spo0A Proteins

2.2.5.1. Synthesis and Purification of FAM-Labelled Probe

pJET-yvfIprom plasmid, which contain the *yvfI* promoter, was used in PCR reaction as a template in order to prepare 414 bp single end 5'-FAM-labelled probe. The oligonucleotide primers *yvfI P/* F_2 and *yvfI P/* R_2, given below, are the forward and the reverse primers for the amplification of the FAM-labelled DNA probe comprising the putative binding regions for AbrB and Spo0A proteins.

yvfI P/ F_2 : 5' – 5'-FAM- CTGGCGCCATATGTAAGCGGT– 3'
yvfI P/ R_2 : 5' – GCCTTCTCCCAGTTTCAT– 3'

Table 2.15 : The volume of PCR components

Component	Exp.	(-) Control
ddH_2O	40,5 µl	42,5 µl
10X *pfu* Buffer	5 µl	5 µl
dNTP	1 µl	1 µl
F Primer	0,5 µl	0,5 µl
R Primer	0,5 µl	0,5 µl
Template	2 µl	-
pfu DNA polymerase	0,5 µl	0,5 µl
Total	**50 µl**	**50 µl**

PCR components given in Table 2.15 were combined in a 0.2 ml tubes on ice. The final concentrations of the primers and dNTP mix were 0,5 µM and 0,2 µM respectively. PCR reaction was cycled 30 times according to the cycling program below, enabling the heated lid:

PCR conditions

95 °C, 2 min ⟹ 1 cycle (Initial denaturation)

95 °C, 1 min
55 °C, 1 min } 30 cycles + 72 °C, 10 min } 1 cycle (Final extension)
72 °C, 1 min

After the amplification of the 414 bp FAM-labelled DNA probe that encompasses bases –396 to +18 in relation to the translational start point of the *yvfI* gene (Appendix F), probe was purified using the QIAquick PCR Purification Kit (Qiagen), as done in section 2.2.4.3 exactly in the same way.

2.2.5.2. Binding Reactions of Protein-Probe DNA

After the amplification and purification of the single end FAM-labelled probe, the following binding reactions were set up according to the Table 2.16 for the DNase I footprinting of AbrB and brought to 35 ul with the dH_2O. AbrB reaction tubes (1-4) included varying amounts of AbrB protein ranging from 0 to 45 ug were incubated at

room temperature for 25 min in binding buffer B (50 mM KCI, 5 mM $MgCI_2$,, 0.1 mM EDTA, 1 mM DTT and % 8 glycerol, pH:8.0). Reaction tube 1 including the BSA protein instead of the AbrB protein was the control tube of the footprinting assay.

Table 2.16 : Binding Reactions for DNase I Footprinting of AbrB Protein

	1	**2**	**3**	**4**
DNA fragments	1 ug	1 ug	1 ug	1 ug
Poly (dI-dC)	2 ug	2 ug	2 ug	2 ug
AbrB protein	-	15 ug	30 ug	45 ug
BSA	20 ug	-	-	-
5X binding buffer B	7 ul	7 ul	7 ul	7 ul

For the Spo0A footprinting assay, all the ingredients shown in Table 2.17 were mixed in 0.2 ml reaction tubes and brought to 35 ul with the dH_2O. Reaction tubes (1-4) containing different amounts of Spo0 protein ranging from 0 to 60 ug were incubated at room temperature for 25 min in binding buffer A (20 mM Hepes, 1 mM EDTA, 10 mM $(NH)_4SO_4$, 1 mM DTT, %0,2 Tween 20 and 30 mM KCI, pH:8.0). BSA protein was used instead of AbrB protein in reaction tube 1 (Control tube).

Table 2.17 : Binding Reactions for DNase I Footprinting of Spo0A Protein

	1	**2**	**3**	**4**
DNA fragments	1 ug	1 ug	1 ug	1 ug
Poly (dI-dC)	2 ug	2 ug	2 ug	2 ug
Spo0A protein	-	20 ug	40 ug	60 ug
BSA	16 ug	-	-	-
5X binding buffer A	7 ul	7 ul	7 ul	7 ul

2.2.5.3. DNase Treatment

After binding reaction of protein-probe DNA, 0.00816 Knutz Unit of DNase I (Roche) is added to reaction tubes and incubation proceeded for 3 min at room temperature. DNase I was inhibited by incubating the reaction tubes at 75°C for 10

min. Then, resulted DNA fragments were purified with the QIAquick PCR Purification kit and eluted with 30 µl dd H_2O, previously as done in section 2.2.4.3. 10 µl of digested DNA was added to 9.9 µl Hi-Di formamide and 0.1 µl 500 LIZ size standart.

2.2.5.4. DNA Fragment Analysis

All samples were analyzed with the 3130 DNA Sequence Analyzer (Applied Biosystems) running a default Genemapper 50-POP7-1 module with the 3 kV injection voltage and 30 seconds injection time. After running the samples, electropherograms obtained from the DNA sequence analyzer (Applied Biosystems) were aligned with Peak Scanner Software v1.0 (Applied Biosystems).

3. RESULTS AND DISCUSSION

3.1. Construction of pJET-*abrB* and pJET-*spo0A* Cloning Vectors

The insert preparation that would be cloned into cloning vectors had been achieved by the means of polymerase chain reaction using the chromosomal DNA of *B. subtilis* PY79 wild type strain as template DNA. The primers 5'– CGGCCATGGGT ATGTTTATGAAATCTACTGGT – 3' and 5' – GCCGGATCCTTTAAGGTTTTGA AGCTG – 3' were the forward and reverse primers that had been used for the amplification of 288 bp *abrB* gene. For the amplification of 375 bp *spo0A* gene, 5'– CGGCCATGGGTGAGAAAATTAAAGTTTGT– 3' and 5'– GCCGGATCCAG AAGCCTTATGCTCTAACCT – 3' forward and reverse primers were used in PCR reaction. Underlined sequences are the recognition sites for *BamHI* and *NcoI* restriction enzymes on forward and reverse primers respectively. After PCR, the amplified fragments including the *abrB* and *spo0A* genes were seperated on %1 agarose gel electrophoresis and visualized by UV trans-illuminator.

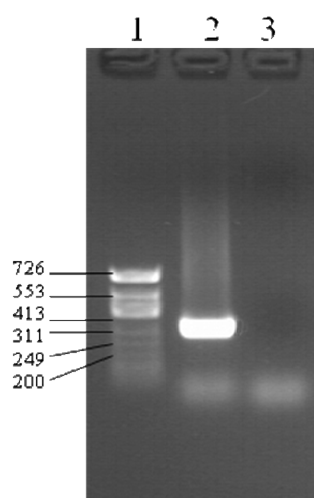


Fig. 3.1: Amplification of *abrB* gene by PCR. Marker 10 : PhiX174 DNA / *Bsu*RI (lane 1), amplified *abrB* gene fragment (lane 2), control pcr (lane3)

Agarose gel images of *abrB* and *spo0A* PCRs have demonstrated the amplifications of the 375 bp *spo0A* and 288 bp *abrB* genes (Fig. 3.1 and Fig 3.2).

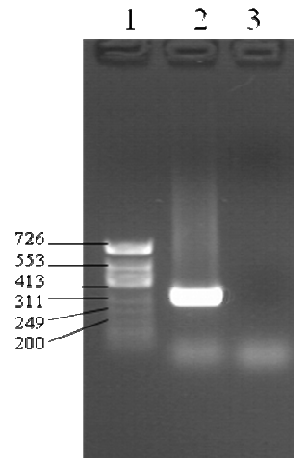


Fig. 3.2: Amplification of *spo0A* gene by PCR. Marker10 : PhiX174 DNA / *Bsu*RI (lane 1), amplified *spo0A* gene fragment (lane 2), control pcr (lane3)

Amplified DNA fragments including *abrB* and *spo0A* genes were ligated into the pJET cloning vectors (Fermentas) and the newly contructed vectors, pJET-*abrB* and pJET-*spo0A*, were transformed to electrocompotent *E.coli* Top 10F' cells via Eppendorf Electroporator 2510.

Having done the transformation of the new generated plasmids, pJET-*abrB* and pJET-*spo0A*, to the *E.coli* cells, plasmids were amplified in *E.coli* cells and they were harvested through plasmid isolation protocol, and visualized on agarose gel as shown in Fig. 3.3 and Fig. 3.4.

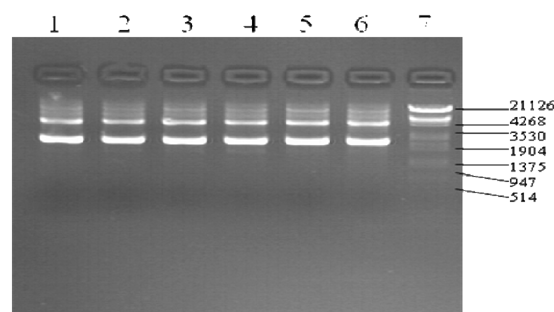


Fig. 3.3: Plasmid DNAs containing *abrB* gene isolated from *E.coli* Top10 Amp^R transformants (lane 1-6). Marker 3: Lambda DNA / *Eco*RI + *Hind*III (lane 7).

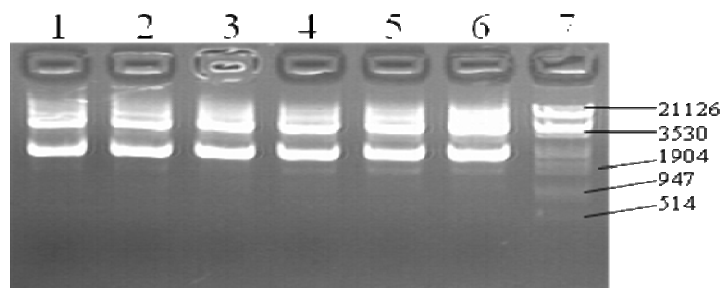


Fig. 3.4: Plasmid DNAs containing *spo0A* gene isolated from *E.coli* Top10 Amp^R transformants (lane 1-6). Marker 3: Lambda DNA / *Eco*RI + *Hind* III (lane 7).

Plasmids having different conformations and concentrations were selected and digested with *BamHI* and *NcoI* restriction enzymes so as to confirm whether *abrB* and *spo0A* genes had been cloned into cloning vector or not. Convenient plasmids including the *abrB* and *spo0A* genes (pJET-*abrB* and pJET-*spo0A*) were selected as subcloning sources for ligation into pQE60 vector.

3.2. Construction of pQE60-*abrB* and pQE60-*spo0A* Expression Vectors

Plasmids including *abrB* and *sp0A* genes (pJET-*abrB* and pJET-*spo0A*) were selected for ligation of the *abrB* and *spo0A* genes into the pQE60 vector. In this purpose, 3431 bp pQE60 expression vector and and selected plasmids were digested with the same enzymes, *BamHI* and *NcoI*, in order to create sticky ends that could be hybridized with each other. Agarose gel images of the double digestions were shown in Fig. 3.5 and Fig.3.6.

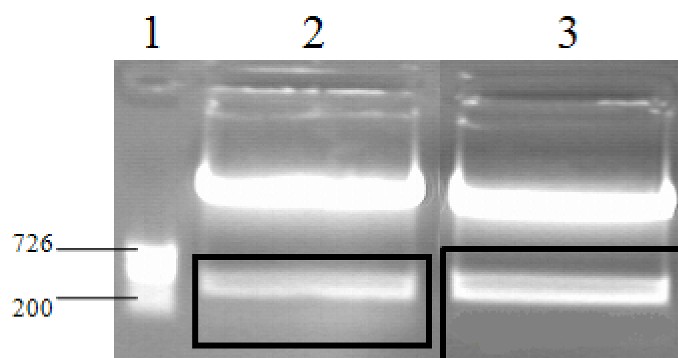


Fig. 3.5: Double digestions of pJET vector including *abrB* (lane 2) and *spo0A* genes with *BamHI* and *NcoI* (lane3). Marker 10 : PhiX174 DNA /*Bsu*RI (lane 1).

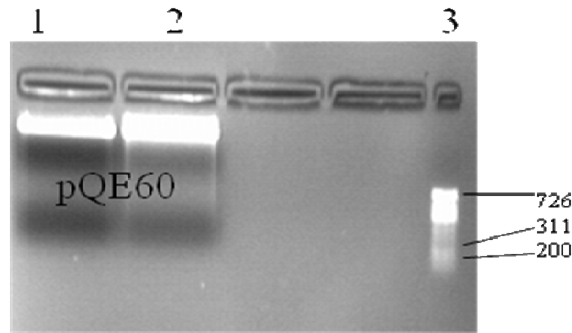


Fig. 3.6: Double digestions of pQE60 vectors with *BamHI* and *NcoI* (lane1-2). Marker 10 : PhiX174 DNA /*Bsu*RI (lane 3).

After that, linearized form of pQE60 vector and digested *abrB* and *spo0A* fragments from pJET-*abrB* and pJET-*spo0A* were exracted from agarose gel. DNA fragments including *abrB* and *spo0A* genes were ligated into linearized pQE60 with T4 DNA ligase by incubation at 16°C for overnight to create the pQE60-*abrB* and pQE60-*spo0A* plasmids. Following that, transformations of pQE60-*abrB* and pQE60-*spo0A* plasmids into the *E.coli* competent cells via Eppendorf Electroporator 2510 were performed. After 16 hours inoculaton of the cells on LB agar plate containing amp (100µg/ml), the resulting 50 transformants were picked up and used for plasmid DNA isolation to verify the cloning of *abrB* and *spo0A* genes fragments. Insertions of the *abrB* and *spo0A* genes into the pQE60 expression vectors were confirmed by double digestion of the newly constructed plasmids, pQE60-*abrB* and pQE60-*spo0A,* with *BamHI* and *NcoI* restriction enzymes. Figure 3.7 and Figure 3.8 indicated the insertions of the *abrB* and *spo0A* genes into the pQE60 expression vectors.

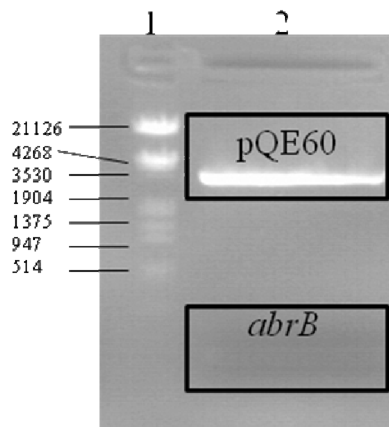


Fig. 3.7: The double digestion of a selected plasmid including *abrB* gene isolated from *E.coli* Amp^R transformants (lane2). Marker 3: Lambda DNA / *Eco*RI + *Hind*III (lane 1).

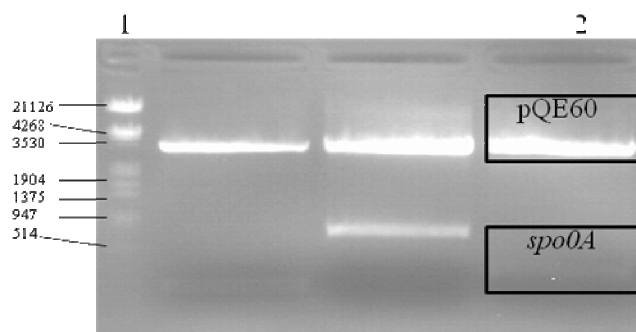


Fig. 3.8: The double digestion of a selected plasmid including *spo0A* gene isolated from *E.coli* Amp^R transformants (lane2). Marker 3: Lambda DNA / *Eco*RI + *Hind*III (lane 1).

Selected pQE60-*abrB* and pQE60-*spo0A* plasmids were sequenced by using PRISM Ready Reaction Dye-Deoxy terminator cycle sequencing kit (Applied Biosystems) and sequence results were analyzed on the ABI PRISM 3100 DNA sequencer. Obtained sequence data sets for *abrB* and *spo0A* genes were compared to the *B. subtilis* 168 genome sequence at National Center for Biotechnology Information (NCBI) database using the BLAST search.

3.3. Expression and Purification of 6xHis-tagged Spo0A and AbrB Proteins

After the transformation of pQE60-*abrB* and pQE60–*spo0A* plasmids to the *E.coli* cells, colonies harboring the convenient pQE60-*abrB* and pQE60-*spo0A* plasmids were selected according to the results of BLAST searches. After that, desired colonies were grown in LB medium and were induced with IPTG for overexpression of interested AbrB and Spo0A proteins. Overexpressied proteins were purified using Ni-NTA resin molecules. To evaluate the expression level of the desired proteins and purification quality of each step, all fractions collected during protein purification were analyzed by SDS-PAGE staining with Coomassie brilliant blue (Fig. 3.9 and Fig. 3.10).

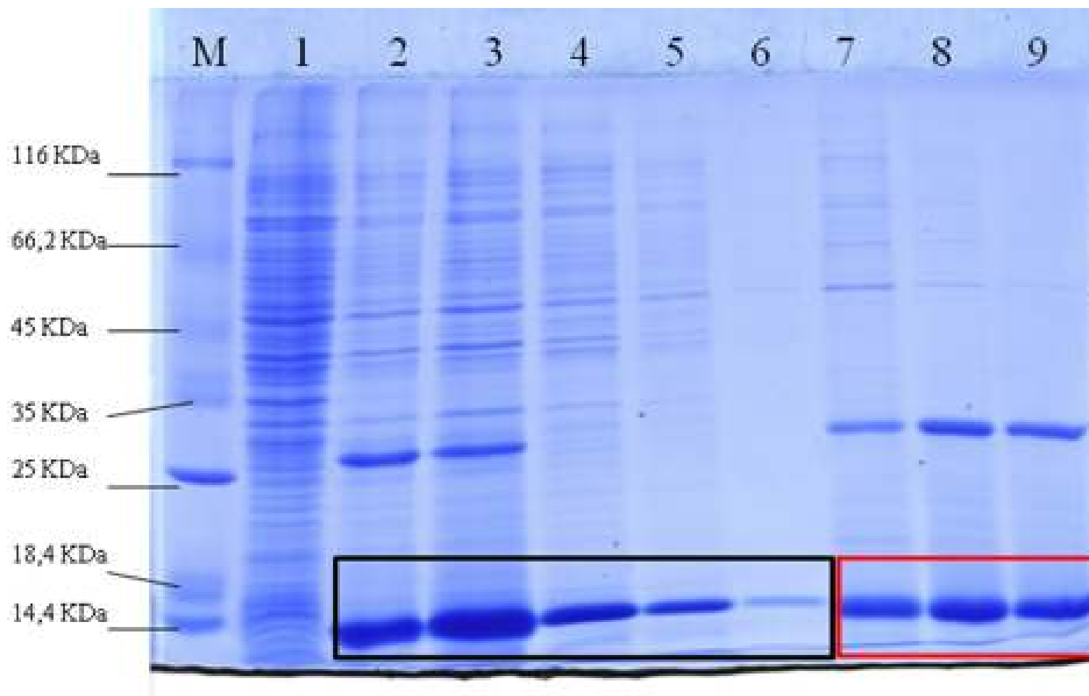


Fig. 3.9: Purified AbrB protein. Protein Molecular Weight Marker (M), total homogenate from uninduced cell (lane 1), total homogenate from induced cell (lane 2), total supernatant fraction from induced cell (lane 3), subsequent washes of supernatant fraction (lane 4-6), subsequent elutes from supernatant fraction (lane 7-9).

While lane 1 in Figure 3.9 belongs to the total homogenate of uninduced colony, lane 2 and lane 3 represent the total homogenate and total supernatant fractions that were obtained from the induced abrB colony, respectively. When compared to the induced and uninduced total homogenate fractions (lane 1 and lane 2), a notable thick band, near the bottom of the SDS-PAGE, corresponding to a protein of approximately 11 kDa implies the successful AbrB induction. The wash fractions (lane 4-6) and elution fractions (lane 7-9) indicate that unwanted proteins had been removed efficiently and most of the target AbrB protein retained and purifed. In elution fractions (lane 7-9), there is also a thick band corrosponding to the protein of about 25 kDa suggesting the existence of dimeric form AbrB protein (Fig. 3.9).

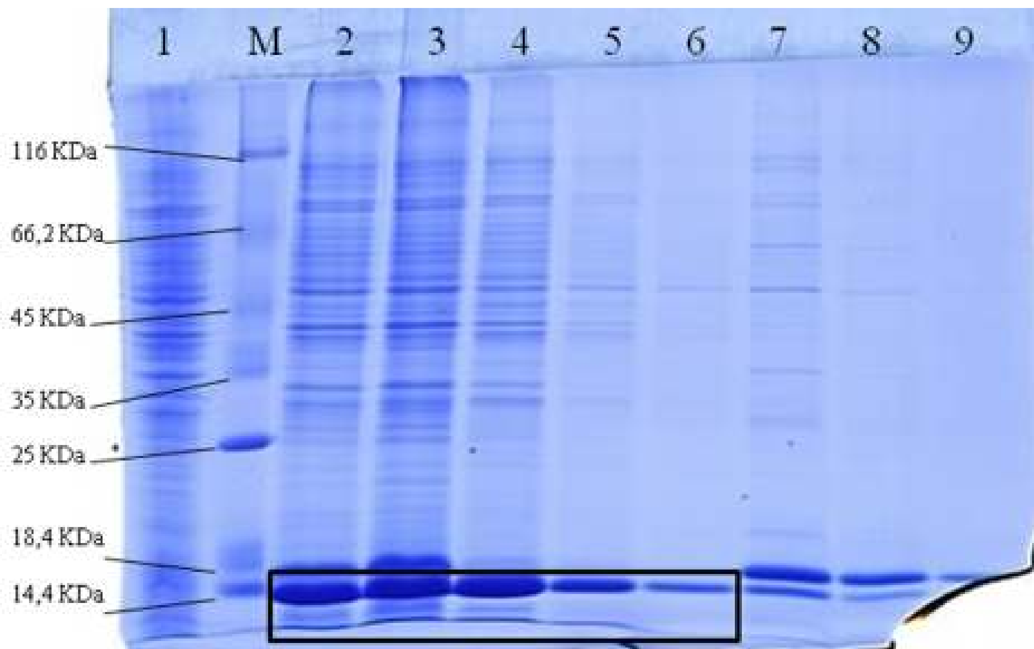


Fig. 3.10: Purified Spo0A protein. Total homogenate from uninduced cell (lane 1), protein molecular weight marker (M), total homogenate from induced cell (lane 2), total supernatant fraction from induced cell (lane 3), subsequent washes of supernatant fraction (lane 4-6), subsequent elutes from supernatant fraction (lane 7-9)

When it comes to Spo0A protein, lane 1 and lane 2 in figure 3.10 represent total homogenates from uninduced and induced colonies, respectively. A notable thick band whose size is approximately 13 kDa at the bottom of the SDS-PAGE indicates the induction of the Spo0A protein. Subsequent washes of the supernatant fraction (lane 4-6) removed most of the unwanted proteins and a remarkable amount of the desired Spo0A protein (lane 7-9) was purified efficiently (Fig. 3.10).

3.4. Electromobility Shift Assays of AbrB and Spo0A Proteins

The possible binding sites for AbrB and Spo0A regulator proteins on *yvfI* promoter were studied by electromobility shift assay. EMSA was performed using non-specific double stranded oligonucleotides (Poly [dI-dC]). The binding of purified AbrB and Spo0A proteins to the double stranded oligonucleotides is presented in Fig. 3.11 and Fig. 3.12. Clear shifts were produced for both of the AbrB and Spo0A proteins.

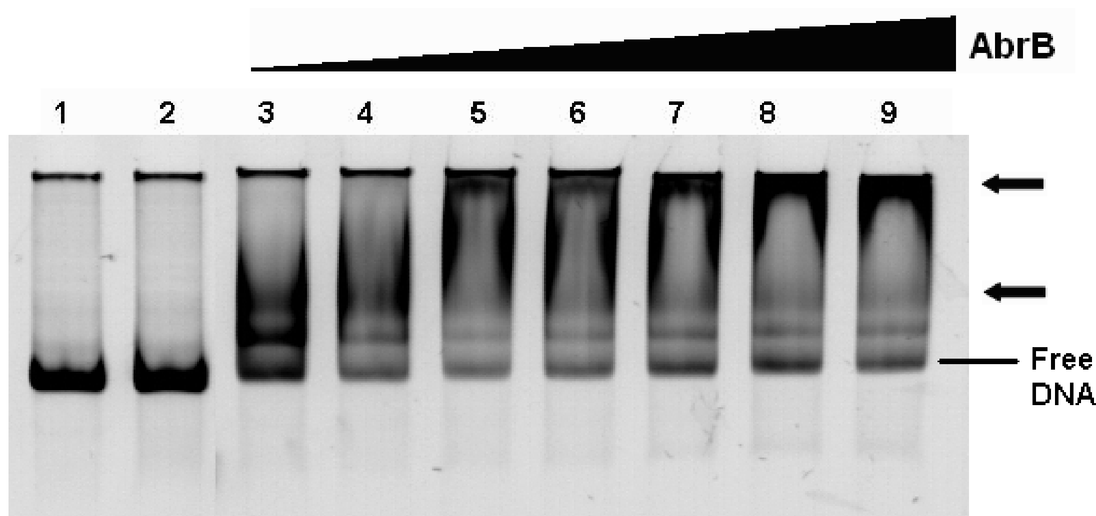


Fig. 3.11 : Non-denaturing polyacrylamide gel image of electromobility shift assay (EMSA) of purified AbrB with *yvfI* promoter of *B. subtilis.* Free DNA (*yvfI* promoter DNA, lane 1), *yvfI* promoter DNA with BSA protein (lane 2), increasing amounts of AbrB protein from 6 ug to 42 ug with *yvfI* promoter (lane 3-9). Specific DNA/protein interactions are indicated at the level of two arrows.

Fig. 3.11 shows the effect of increasing amounts of AbrB protein on the electromobility of *B. Subtilis yvfI* promoter DNA. In lane 1 containing *B. Subtilis yvfI* promoter DNA alone, a single band was observed. The same band was observed for the samples containing *B. Subtilis yvfI* promoter DNA and AbrB protein (lane 3- 9) ; but the intensity of the band was lower when the amount of the AbrB protein increased. On the other hand, existence of the BSA protein instead of AbrB protein (lane 2) did not produce any shift, thereby indicating the specifity of DNA-protein complex.

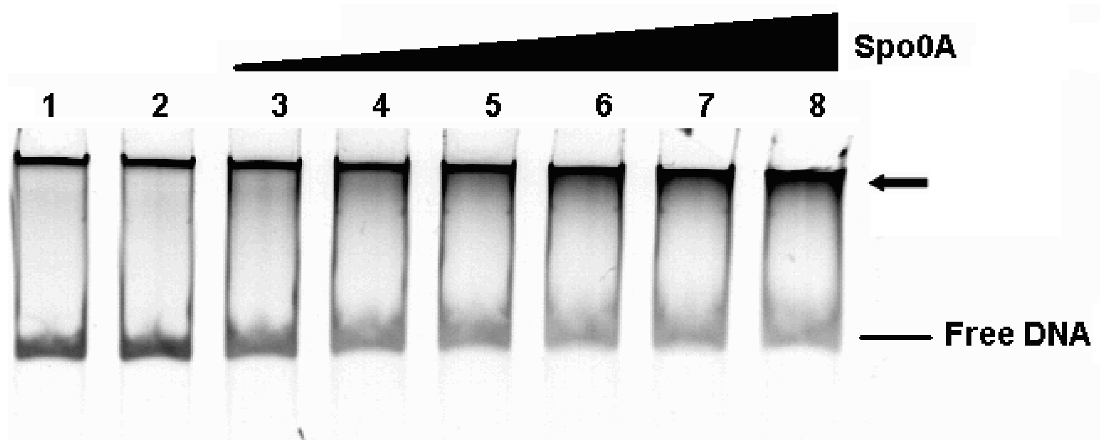


Fig. 3.12 : Non-denaturing polyacrylamide gel image of electromobility shift assay (EMSA) of purified Spo0A with *yvfI* promoter of *B. subtilis.* Free DNA (*yvfI* promoter DNA, lane 1), *yvfI* promoter DNA with BSA protein (lane 2), increasing amounts of AbrB protein from 10 ug to 60 ug with *yvfI* promoter (lane 3-8). Specific DNA/protein interactions are indicated at the level of two arrow.

In electromobility shift of Spo0A protein, mobility of the yvfI promoter DNA was not retarded and migrated to the bottom of the gel faster in the absence of the Spo0A protein (Fig. 3.12, lane 1), even in the presence of the non-specific competitor protein, BSA (Fig. 3.12, lane 2). However, when the *yvfI* promoter DNA was incubated with the Spo0A protein, mobility of the DNA was retarded because of the DNA-protein complex formation (Fig. 3.12, lane 3-8). Besides, intensities of the bands were reduced due to the increasing amount of the Spo0A protein in the samples (lane 3-8).

These results suggest that transcriptional regulators, AbrB and Spo0A proteins, bind to *yvfI* promoter and are involved in the transcriptional regulation of the *yvfI* gene expression.

3.5. DNase I Footprinting of AbrB and Spo0A Proteins

After revealing that both of the transcriptional regulator proteins, AbrB and Spo0A, bind to the *yvfI* promoter, DNase I footprinting assay was employed to verify the exact binding sites of the regulator proteins. For this aim, 414 bp single end 5'-FAM-labelled probe (-396 to +18 relative to the translational start point of the *yvfI* gene, Appendix F) was synthesized and incubated with the increasing amount of the interested protein. After the DNase I digestion, samples were run on 3130 DNA Sequence Analyzer and aligned with Peak Scanner Software v1.0 for protection

pattern. As negative control reaction, BSA protein was used instead of the AbrB and Spo0A proteins. As expected, the digested DNA patterns were very similar when comparing desired proteins to BSA-involved control fragments. In other words, BSA involvement did not produce any protected region.

In Figure 3.13, blue electropherograms represent the protected sample, while the red ones represent the unprotected sample in AbrB footprinting. After superimposing the red (in absence of the AbrB protein) and blue (in presence of AbrB protein) electropherograms for the visual analysis of the protected regions, the protected region for AbrB was determined to be 12 bases in length, from -316 to -304 relative to the translational start point of the *yvfI* gene (Appendix F) as seen in Fig. 3.13. The protected region is indicated by the horizontal bracket.

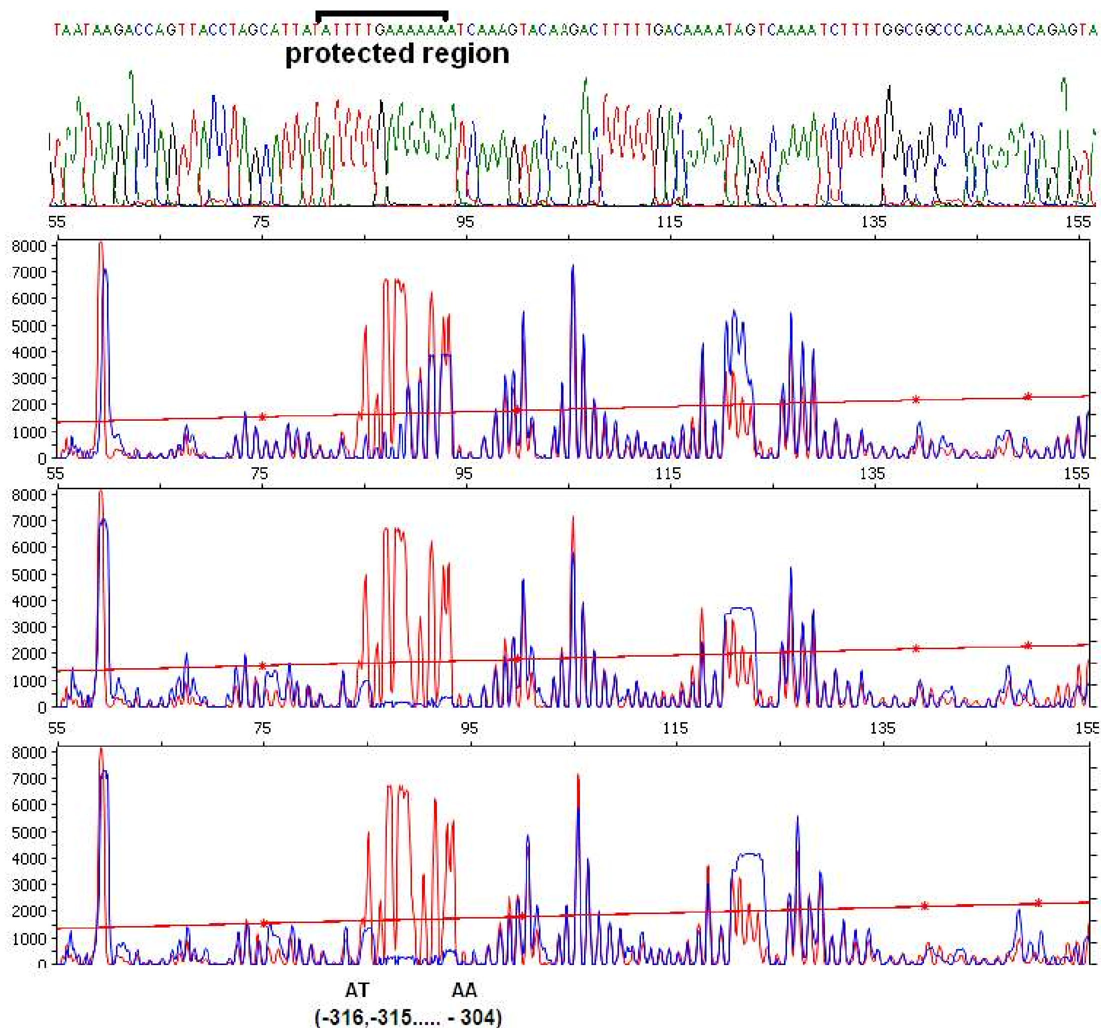


Fig. 3.13: Electropherograms shows the protection pattern of the *yvfI* promoter in the presence (blue) or absence (red) of AbrB. From top to bottom, increasing amount of the AbrB protein protects the DNA being cleaved by DNase I.

Moreover, increasing amount of the AbrB protein (from top to bottom) protects the binding location more efficiently as seen in Fig. 3.13.

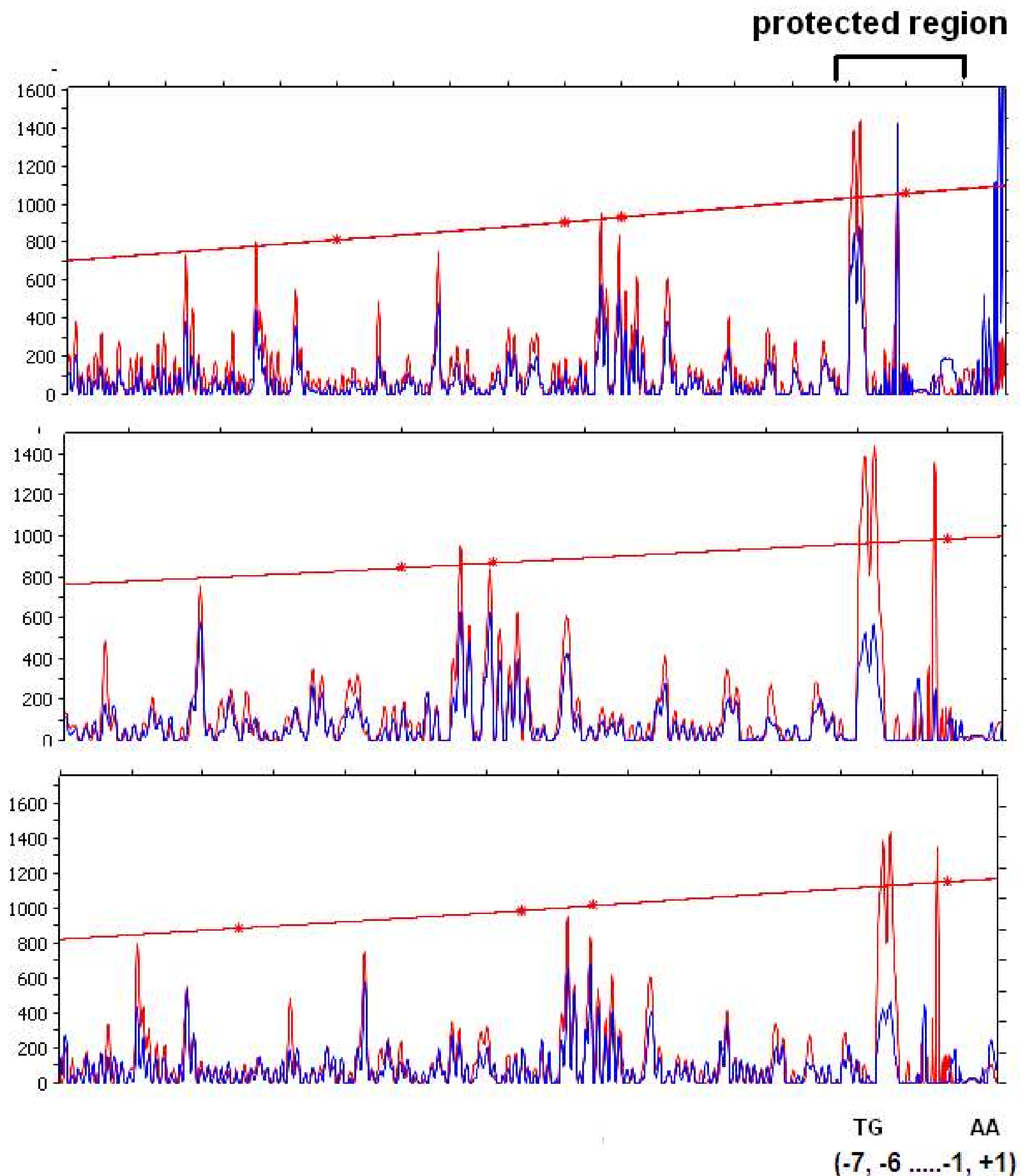


Fig. 3.14: Electropherograms shows the protection pattern of the *yvfI* promoter in the presence (blue) or absence (red) of Spo0A protein. From top to bottom, increasing amount of the Spo0A protein protects the DNA being cleaved by DNase I.

In Figure 3.14, the blue electropherograms represent the Spo0A involved samples, whereas the red electropherograms are from the BSA control. After superimposing the protected (blue electropherograms) and unprotected (red electropherograms) samples, the binding region of the Spo0A, thereby the protected region, was

corresponded to the bases -7 to +1 in relation to the translational start point of the *yvfI* gene (Appendix F). The protected region is indicated by the horizontal bracket. Also, the increasing amount of the Spo0A protein (from top to bottom) make the protected region being cleaved by the DNase I, more efficiently (Fig. 3.14).

The proteced regions for AbrB and Spo0A proteins on *yvfI* promoter are compatible with the putative binding sites exactly the same size and same location that were determined previously (Sierro et al, 2008).

4. CONCLUSION

It is known that AbrB and Spo0A regulator proteins to be responsible in antibiotic synthesis. There are so many genes and operons that are regulated by Spo0A and AbrB. *TasA* operon (Stover and Driks, 1999) and subtilosin operon (Zheng et al, 1999) are a few examples whose regulations are subject to AbrB and Spo0A proteins. Dipeptide antibiotic bacilysin is also under the regulatiom of AbrB and Spo0A proteins. *B. subtilis* whose *spo0A* gene is mutated can not produce bacilysin efficiently compared to the wild type ones. On the other hand, mutation in the *abrB* makes the *B. subtilis* produce more bacilysin indicating its negative control on bacilysin (Zuber et al, 1987; Yazgan et al., 2003). Recently, the necessity of the *yvfI* gene in bacilysin biosynthesis has been proved (Köroğlu et al, 2008).
Besides, *yvfI* gene promoter contains potential binding sites for AbrB and Spo0A transcriptional regulators. In order to determine whether AbrB and Spo0A proteins bind to the mentioned region, firstly FAM-labelled oligonucleotide encompassing the candidate binding sites, -396 to +18 in relation to the translational start point of the *yvfI* gene (Appendix F), was synthesized. Candidate proteins were subjected to the EMSA for their abilities to bind the target probe. Results obtained from the electromobility of AbrB and Spo0A revealed the shift in *yvfI* promoter, thereby binding abilities of the mentioned proteins to the *yvfI* promoter. Subsequent capillary electrophoresis based DNase I footprinting experiments demonstrated that AbrB binds to a region covering the sequence from -316 to -304 and Spo0A to -7 to +1 relative to the translational start point of the *yvfI* gene. The found binding regions of AbrB and Spo0A on *yvfI* promoter are well matched to putative DNA sequences previously shown (Sierro et al., 2008).

Based on these results, it can be said that transcription of the *yvfI* gene underlies a regulation exerted by the global regulator proteins, AbrB and Spo0A. When taken the recent findings into consideration (Yazgan et al., 2003), it is figured out that global transient-phase regulator protein, AbrB, is a repressor of the bacilysin by

hindering the *yvfI* expression, while the Spo0A is a transcritional factor acting as activator on yvfI expression so that on bacilysin production in *B. subtilis.*

REFERENCES

Burbulys, D., Trach, K.A. and Hoch, J.A.(1991). Initiation of sporulation in *B. subtilis* is controlled by a multicomponent phosphorelay. Cell. 64,545–552.

Cane, D. E., and C. T. Walsh. (1999). The parallel and convergent universes of polyketide syntheses and nonribosomal peptide synthetases. Chem. Biol.6:319–325.

Chatterjee, C., Paul, M., Xie, L. and van der Donk, W. A. (2005). Biosynthesis and mode of action of lantibiotics. *Chem. Rev.*, **105**, 633-683.

Chmara, H. et al. (1982). Epoxypeptides—a novel group of metabolic inhibitors in procaryotic and eucaryotic organisms. Drugs Exp. Clin. Res. 8, 11–12.

Chmara, H. (1985). Inhibition of glucosamine synthase by bacilysin and anticapsin. J. Gen. Microbiol., 131, 265–271.

Earl, A. M. et al. (2008) Ecology and genomics of *Bacillus Subtilis*. Trends in Microbiology. 16 (6), 269-275

Fabret, C. et al. (1999). Two-Component Signal Transduction in *Bacillus subtilis*: How One Organism Sees Its World. Journal of Bacteriology, (181):7 , 1975–1983.

Fawcett, P., Eichenberger, P., Losick, R. and Youngman P. (2000) The transcriptional profile of early to middle sporulation in *Bacillus subtilis*. Proc. Natl. Acad. Sci. USA. 97, 8063–8068

Fürbass, R., Gocht, M., Zuber, P. and Marahiel, M.A. (1991) Interaction of AbrB, a transcriptional regulator from Bacillus subtilis with the promoters of the transition state-activated genes tycA and spoVG. Mol Gen Genet. 225:(3), 347-54.

Gaudreault, M. et al . *Methods in Molecular Biology, DNA-Protein Interactions.* Tom Moss and Benoît Leblanc eds., 2009. Vol. 543; 15-35.

Ghim, S. Y. et al. (1998). Sequence analysis of the *Bacillus subtilis* 168 chromosome region between the *sspc* and *odha* loci (184◦-180◦). DNA Research, 5, 195–201

Guder, A., Wiedemann, I., and Sahl, H.G. (2000) Posttranslationally modified bacteriocins – the lantibiotics. *Biopolymers* **55:** 62–73.

Hahn, J., Roggiani, M. and Dubnau D. (1995) The major role of Spo0A in genetic competence is to downregulate *abrB*, an essential competence gene. Journal of Bacteriology. 177, 3601–360.

Hilton, M.D., Alaeddinoglu, N.G., and Demain, A.L. (1988) Synthesis of bacilysin by *Bacillus subtilis* branches from the prephenate of the aromatic amino acid pathway. Journal of Bacteriology. 170, 482–484.

Hofemeister, J., et al. (2004) Genetic analysis of the biosynthesis of non-ribosomal peptide- and polyketide-like antibiotics, iron uptake and biofilm formation by *Bacillus subtilis* A1/3. *Molecular Genetics and Genomics,* **272,** 363-378.

Inaoka, T., Takahashi, K., Ohnishi-Kameyama, M., Yoshida, M., and Ochi, K. (2003) Guanine nucleotides guanosine 5- diphosphate 3-diphosphate and GTP co-operatively regulate the production of an antibiotic bacilysin in *Bacillus subtilis*. *J Biol Chem* 278, 2169–2176.

Ireton, K., D. Z. Rudner, K. J. Siranosian, and A. D. Grossman. (1993) Integration of multiple developmental signals in *Bacillus subtilis* through the Spo0A transcription factor. Genes Dev. 7, 283–294.

Jaenicke, R. (2005) Abundance of cellular material and proteins in the atmosphere. Science 308, 73.

Hampshire, A. J. et al. (2007) Footprinting: A method for determining the sequence selectivity, affinity and kinetics of DNA-binding ligands. Methods 42, 128–140.

Hellman L .M. and **Fried M.G**. (2007). Electrophoretic Mobility Shift Assay (EMSA) for Detecting Protein-Nucleic Acid Interactions. *Nat. Protoc. 2(8)*: 1849–1861.

Holden, M.S. and **Tacon, C.E**. (2010). Principles and problems of the electrophoretic mobility shift assay, Journal of Pharmacological and Toxicological Methods. Doi:10.1016/j.vascn. (article in press). 2010.03.002

Kenig M., Vandamme, E., Abraham, E.P. (1976) The mode of action of bacilysin and anticapsin and biochemical properties of bacilysin-resistant mutants. J Gen Microbiol. 94, 47–45.

Kobayashia, K. et. al., (2003). Essential *Bacillus subtilis* genes**.** *PNAS.* 100, 4678-4683.

Köroğlu, T.E., Kurt-Gür, G., Ünlü E.C., Yazgan-Karataş, A. (2008). The novel gene yvfI in *Bacillus subtilis* is essential for bacilysin biosynthesis. Antonie van Leeuwenhoek. 94, 471–479

Kunst, F. et al. (1997) The complete genome sequence of the grampositive bacterium *Bacillus subtilis*. Nature 390, 249–256

Lambalot, R.H. et al., (1996) A new enzyme superfamily – the phosphopantetheinyl transferases. *Chem. Biol.* 3, 923–936.

Lazazzera, B.A. (2000) Quorum sensing and starvation: signals for entry into the stationary phase. Curr. Opin. Microbiol. 3, 177–182

Mannanov, R. N. and Sattarova, R. K. (2001). Antibiotics produced by *Bacillus* bacteria. *Chemistry of Natural Compounds,* **37**, 117-123.

Milewski, S. (1993) Chemical modification studies of the active site of glucosamine-6-phosphate synthase from baker's yeast. Biochim Biophys Acta 1161, 279–284.

Moszer, I. (1998). The complete genome of *Bacillus subtilis*: from sequence annotation to data management and analysis. *FEBS Lett* **430**, 28–36.

Nagorska, K. et al. (2007) Multicellular behaviour and production of a wide variety of toxic substances support usage of *Bacillus subtilis* as a powerful biocontrol agent. Acta Biochim. Pol. 54, 495–508.

Nakano, M.M. et al. (1997) Characterization of anaerobic fermentative growth of *Bacillus subtilis*: identification of fermentation end products and genes required for growth. Journal of Bacteriology. 179, 6749–6755.

Norris, J.R. and Wolf, J. (1961) A study of antigens of the aerobic sporeforming bacteria. J. Appl. Bacteriol. 24, 42–56

Perkel , J. M. (2003) Nonribosomal Peptide Synthesis. The Scientist, March 24; 17(6).

Perry, D. and Abraham, E.P. (1979) Transport and metabolism of bacilysin and other peptides by suspensions of Staphylococcus aureus. J. Gen. Microbiol. 115, 213–221.

Phillips, Z.E.V. and Strauch, M.A. (2002) *Bacillus subtilis* sporulation and stationary phase gene expression. Cell. Mol. Life Sci. 59, 392–402.

Qian, Q., Lee, C. Y., Helmann, J. D., Strauch, M. A. (2002) AbrB is a regulator of the 6^{W} regulon in *Bacillus subtilis*. FEMS Microbiology Letters. 211, 219-223.

Robertson, J. B., Gocht, M., Marahiel, M.A. and Zuber P. (1989) AbrB, a regulator of gene expression in *Bacillus*, interacts with the transcription initiation regions of a sporulation gene and an antibiotic biosynthesis gene. Proc. Natl. Acad. Sci. USA. 86, 8457–8461.

Roscoe, J. and Abraham, E. P. (1966) Experiments relating to the biosynthesis of bacilysin. Biochem. J.*,* 99 (3):793-800.

Sakajoh, M., Solomon, N.A., Demain, A.L. (1987) Cell-free synthesis of the dipeptide antibiotic bacilysin. J Ind Microbiol. 2, 201–208.

Sierro, N., Makita, Y., de Hoon, M.J.L. and Nakai, K. (2008) DBTBS: a database of transcriptional regulation in *Bacillus subtilis* containing upstream intergenic conservation information. *Nucleic Acids Res.*, 36, 93-96.

Sonenshein, A.L. et al. (2002) *Bacillus subtilis* and Its Closest Relatives: From Genes to Cells, ASM Press

Souto G.I. et al. (2004) Genetic and functional characterization of a *Bacillus* sp. strain excreting surfactin and antifungal metabolites partially identified as iturin-like compounds. *J Appl Microbiol* **97**: 1247–1256.

Stein, T. (2005) *Bacillus subtilis* antibiotics: structures, syntheses and specific functions. Mol. Microbiol. 56, 845–857.

Steinborn, G., Hajirezaei, M.R., Hofemeister, J. (2005) *bac* genes for recombinant bacilysin and anticapsin production in *Bacillus* host strains. Arch Microbiol., 183: 71–79.

Stephenson, K. and Hoch, J.A. (2002) Two-component and phosphorelay signal-transduction systems as the therapeutic targets. Current Opinion in Pharmacology, 2, 507-512.

Stover, A.G. and Driks A. (1999) Regulation of synthesis of the *Bacillus subtilis* transition-phase, spore-associated antibacterial protein TasA. Journal of Bacteriology. 181: 5476–5481.

Strauch, M.A., Spiegelman, G. B., Perego, M., Johnson, W.C., Burbulys, D. and Hoch, J. A. (1989) The transition state transcription regulator AbrB of *Bacillus subtilis* is a DNA binding protein. EMBO J. 8, 1615–1621.

Strauch, M.A. et al. (1990) The Spo0A protein of *Bacillus subtilis* is a repressor of the abrB gene, Proc. Natl. Acad. Sci. USA. 87:1801– 1805.

Strauch, M.A. and Hoch, J.A. (1993a) Transition-state regulators: sentinels of Bacillus subtilis post-exponential gene expression. Mol. Microbiol. 7, 337-342.

Strauch, M.A. (1993b) Regulation of *Bacillus subtilis* gene expression during the transition from exponential growth to stationary phase. Prog. Nucleic Acids Res. Mol. Biol. 46, 121–153.

Strauch, M.A. (1995a) In vitro binding affinity of the *Bacillus subtilis* AbrB protein to six different target regions. Journal of Bacteriology. 177, 4532–4536.

Strauch, M.A. (1995b) AbrB modulates expression and catabolite repression of a *Bacillus subtilis* ribose transport operon. Journal of Bacteriology. 177, 6726–6731.

Strauch, M.A. ***et al.*** (2007) Abh and AbrB Control of *Bacillus subtilis* Antimicrobial Gene Expression. Journal of Bacteriology. 189(21): 7720–7732.

Tabata, K. et al. (2005) *ywfE* in *Bacillus subtilis* Codes for a Novel Enzyme, L-Amino Acid Ligase. Journal of Bacteriology, 187 (15): 5195–5202.

Vaughn, J. L., Feher, V., Naylor, S., Strauch, M.A. and Cavanagh, J. (2000) Novel DNA binding domain and genetic regulation model of *Bacillus subtilis* transition state regulator AbrB. Nature Struct. Biol. 7, 1139–1146.

Walker, J. E. and Abraham, E. P. (1970) The structure of bacilysin and other products of *Bacillus subtilis*. *Biochem. J.,* 118, 563-570.

Walker, J. E. and Abraham, E. P. (1970) Isolation of bacilysin and a new amino acid from culture filtrates of *Bacillus subtilis*. *Biochem. J.*, 118, 557-561.

Wilson, D. O. ***et al.*** (2001) Nonradiochemical DNase I footprinting by capillary electrophoresis. *Electrophoresis*, *22,* 1979–1986.

Xu, K., Clark, D. and Strauch, M.A. (1996a) Analysis of *abrB* mutations, mutant proteins, and why *abrB* does not utilize a perfect consensus in the 35 region of its sigma A promoter. J. Biol. Chem. 271:2621–2626.

Xu, K., and Strauch, M.A. (1996b) In vitro selection of optimal AbrB-binding sites: comparison to known in vivo sites indicates flexibility in AbrB binding and recognition of three-dimensional DNA structures. Mol. Microbiol. 19,145–158.

Yazgan, A., Cetin, S. and Ozcengiz, G. (2003) The effects of insertional mutations in comQ, comP, srfA, spo0H, spo0A and abrB genes on bacilysin biosynthesis in *Bacillus subtilis*. *Biochimica et Biophysica Acta,**1626***, 51– 56

Yazgan, A., Ozcengiz, G., Ozcengiz, E., Kılınc, K., Marahield, M.A. and **Alaeddinoglu, N. G.** (2001a). Bacilysin biosynthesis by a partially-purified enzyme fraction from *Bacillus subtilis*. *Enzyme and Microbial Technology,* **29**, 400–406.

Yazgan, A., Ozcengiz, G. and **Marahiel, M. A.** (2001b) Tn10 insertional mutations of *Bacillus subtilis* that block the biosynthesis of bacilysin. *Biochimica et Biophysica Acta*. 1518, 87-94.

Zuber, P., and R. Losick. 1987. Role of AbrB in the Spo0A- and Spo0Bdependent utilization of a sporulation promoter in *Bacillus subtilis*. Journal of Bacteriology. 169, 2223–2230.

Zheng, G., Yan, L. Z., Vederas, J. C. and Zuber, P. (1999) Genes of the *sbo*-*alb* locus of *Bacillus subtilis* are required for production of the antilisterial bacteriocin subtilosin. Journal of Bacteriology. 181, 7346–7355

Zianni, M. et al. (2006) Identification of the DNA Bases of a DNase I Footprint by the Use of Dye Primer Sequencing on an Automated Capillary DNA Analysis Instrument. Journal of Biomolecular Techniques. 17, 103–113.

APPENDICES

APPENDIX A : Compositions and Preparation of Culture Media
APPENDIX B : Compositions of Buffers and Solutions
APPENDIX C : Enzymes and Chemicals
APPENDIX D : Markers
APPENDIX E : *yvfI* DNA Sequence
APPENDIX F : Mapping of the *yvfI* Promoter
APPENDIX G : Laboratory Equipment

APPENDIX A

Compositions and Preparation of Culture Media

Luria Bertani (LB) Medium (1000ml)

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

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

Luria Bertani (LB) Agar Medium (1000 ml)

Tryptone	10 g/L
Yeast Extract	5 g/L
$NaCl_2$	5 g/L
Agar	15 g/L

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

APPENDIX B

Compositions of Buffers and Solutions

Gel Stain

Methanol	50 ml
Glacial acetic acid	10 ml
H_2O	50 ml
Coomassie Brilliant Blue R	0.275 g

Destaining Solution

Methanol	75 ml
Glacial acetic acid	50 ml
H_2O	875 ml

50X TAE Buffer (Tris-Acetate-EDTA)

Tris base	242 gm
Acetic Acid	57.1 mL
0.5 M EDTA	100 mL (shake vigorously before use)

Add ddH_2O to 1 Liter and adjust pH to 8.5 using KOH.

TBE Buffer (Tris-Boric acid-EDTA)

Tris base	108g
Boric acid	55g
0.5M EDTA	40ml, (pH 8.0)

Add ddH_2O to 1 Liter.

Lysis Buffer

50 mM NaH_2PO_4
300 mM NaCl
10 mM imidazole

Adjust pH to 8.0 using NaOH.

Wash Buffer

50 mM NaH_2PO_4
300 mM NaCl
20 mM imidazole

Adjust pH to 8.0 using NaOH.

Elution Buffer

50 mM NaH_2PO_4
300 mM NaCl
250 mM imidazole
Adjust pH to 8.0 using NaOH.

3X SDS Loading Dye

200 mM Tris-HCl (pH 6.8)
100 mM NaCl
100 mM DTT
3 mM EDTA
30% Glycerol
6% SDS
0.02% Bromophenol blue

APPENDIX C

Enzymes and Chemicals

Enzyme	Supplier
Lysozyme	Sigma

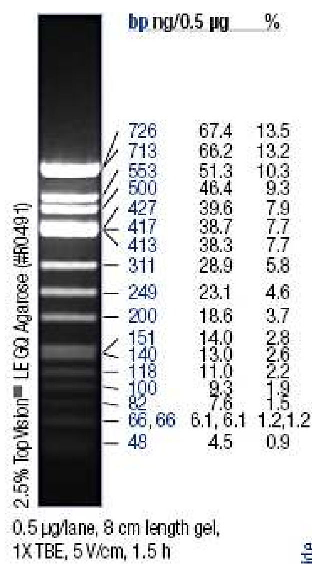
Chemical	Supplier
Acrylamide	Merck
Agar	Sigma
Alanine	AppliCHEM
Ammonium persulfate	Merck
Bis-acrylamide	Merck
Coomassie Brilliant Blue R	Sigma
Ethanol	Riedel-de Haën
Fructose	Merck
Glycial Acetic Acid	Riedel-de Haën
Glycerol	Merck
Glycine	Merck
HCl	Merck
KH_2PO_4	Merck
K_2HPO_4	Merck
KOH	Sigma
Methanol	Riedel-de Haën
$Na_2SO_4.10H_2O$	Merck
Natrum hydroxid (NaOH)	Riedel-de Haën
Nutrient broth	Merck
Potassium chloride (KCl)	Riedel-de Haën

2-propanol	Riedel-de Haën
Riboflavin	Merck
SDS	Merck
Sodium chloride (NaCl)	Riedel-de Haën
Sodium hydrogen phosphate($Na_2HPO_4.7H_2O$)	Merck
TEMED	Carlo Ederba
Tris (hydrocymethyl) aminomethane	Merck
Tryptone	Sigma
Yeast Extract	Sigma

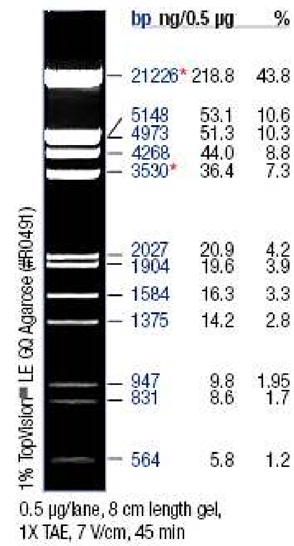
APPENDIX D

Markers……………………………………………………………Fermentas

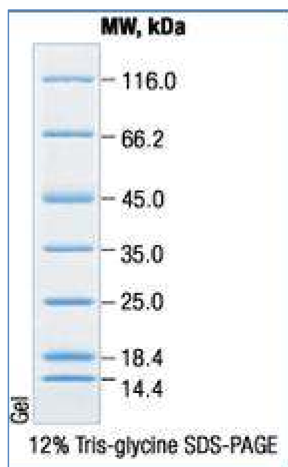
phiX174 DNA/HinfI Marker, 10



Lambda DNA/EcoRI+HindIII Marker, 3



Unstained Protein Molecular Weight Marker



APPENDIX E

yvfI DNA Sequence

```
  1 atgaaacagg gagaaggcac gtatctgaag gaatttgagc tcaatcaaat ttctcagccg
 61 ctctcagccg cccttctgat gaaaaaagag gacgtcaagc agctgctcga ggtcagaaaa
121 ctgcttgaaa tcggcgtggc ttcactagcg gctgaaaaaa ggacagaagc agatctcgaa
181 agaattcagg atgcactaaa ggaaatgggc agcattgaag cggacgggga gctgggagag
241 aaagcagact ttgcatttca tcttgcgctt gcggacgctt ctcaaaatga acttcttaaa
301 cacttgatga atcacgtgtc atcattgctg ctggaaacaa tgagggaaac gaggaaaatc
361 tggctgtttt ccaagaagac ctccgttcag cggctgtatg aggagcacga acggatttac
421 aatgctgtgg ctgccgggaa cggtgcacag gcggaagccg ccatgctggc gcatttgacg
481 aatgtggaag atgtgctttc gggatatttc gaggaaaatg tgcaataa
```

APPENDIX F

Mapping of the *yvfI* Promoter

aataatcggtacaagtgcggcaagcgctgataatagcaaatttccccctataggcgtatatgcctgtgtccattgcatccca

aacccctccttgaaacataaactggcgccatatgtaagcggtttctatagatgatatgatcatctgatgactgggtaataaga

ccagttacctagcattatattttgaaaaaaatcaaagtacaagactttttgacaaaatagtcaaaatcttttggcggccc

AbrB AbrB AbrB

acaaaacagagtataatagatcgt**a**aacagccagacctacaagttacggggtgaattgcattgaaatataaacagatt

aaaacaaaaaaatatatgaagaagtagcggatgccctattagatatgatcaaaaatggcgaattgaagccgggggata

Spo0A

aactggactctgttcaggcgcttgctgagagctttcaagtcagccgttcagcggttcgcgaagcactttctgcgctaaa

agcgatggggcttgtcgaa **A**tg aaa cag gga gaa ggc acg tat ctg

Spo0A M K Q G E G T Y L

The translational start point of the *yvfI* is indicated by bold uppercase letter. Potential cis-acting elements to which AbrB and Spo0A might bind were found by the help of the DBTBS database (Sierro et. al., 2008). These potential binding sites are indicated with names below the sequences and shown in blue and yellow background colors respectively.

APPENDIX G

Laboratory Equipment

Autoclave: Tuttnauer Systec Autoclave (2540 ml)

Balances: Precisa 620C SCS
Precisa 125 A SCS

Centrifuge: Beckman Coulter, Microfuge 18

Centrifuge rotor: F241.5P

Deep freezes and refrigerators: -80°C Heto Ultrafreeze 4410
-20°C Arçelik 209lt
+4°C Arçelik

Electrophoresis equipments: E – C mini cell primo EC320

Gel documentation system: UVI PHotoMW Version 99.05 for Windows

Incubators: Nüve EN400
Nüve EN500

Orbital shaker incubators: Sertomat S – 2
Thermo 430

Pipettes: Gilson pipetteman 10 µl, 20 µl, 200 µl, 1000 µl
Volumate Mettler Toledo 10 µl, 20 µl, 200 µl, 1000 µl
Eppendorf research 10 µl, 20 µl, 200 µl, 1000 µl

pH meter: Mettler Toledo MP220

Spectrophotometer: PerkinElmer Lambda25 UV/VIS Spectrometer

Thermomixer: Eppendorf thermomixer comfort (1.5 ml)

Transillumunator: Biorad UV transilluminator 2000

Vortexing machine: Heidolph Raax top

Waterbaths: Memmert wb-22

Ultrafiltration tube: VIVASPIN

Lyophilizator : ALPHA 1-2 LD plus

Power supply: Bio-Rad

Dialysis Tubing Cellulose Membrane: Sigma-Aldrich

SDS-PAGE Apparatus: Bio-Rad

Polyacrylamide Gel Electrophoresis Apparatus: Bio-Rad

GS-800 CALIBRATED DENSITOMETER: Bio-Rad

CURRICULUM VITAE

Candidate's full name : Hüseyin TAYRAN

Place and date of birth : Tunceli, 1982

Permanent Address : 3948 Sok. Çınar Apt., No: 9, D: 5, İzmir

University : Istanbul Technical University