**İSTANBUL TECHNICAL UNIVERSITY ★ INSTITUTE OF SCIENCE AND TECHNOLOGY**

# THE EFFECT OF GLOBAL CONTROL ELEMENTS ON THE EXPRESSION OF A NOVEL GNTR TYPE REGULATOR *yvfI* IN *B.SUBTILIS*

**M.Sc. Thesis by**
**Emine Canan ÜNLÜ ÖZKURT**

**Department : Advanced Technologies in Engineering**

**Programme : Molecular Biology-Genetics and Bioechnology**

**JANUARY 2009**

**İSTANBUL TECHNICAL UNIVERSITY ★ INSTITUTE OF SCIENCE AND TECHNOLOGY**

# THE EFFECT OF GLOBAL CONTROL ELEMENTS ON THE EXPRESSION OF A NOVEL GNTR TYPE REGULATOR *yvfI* IN *B.SUBTILIS*

**M.Sc. Thesis by**
**Emine Canan ÜNLÜ ÖZKURT**
**(521051225)**

**Date of submission : 29 December 2008**
**Date of defence examination: 22 January 2009**

**Supervisor (Chairman) : Assoc. Prof. Dr. Ayten YAZGAN KARATAŞ (ITU)**

**Members of the Examining Committee : Assoc. Prof. Dr. Gamze KÖSE (YEDITEPE UNIVERSITY)**
**Assis. Prof. Dr. Fatma Neşe KÖK (ITU)**

**JANUARY 2009**

**İSTANBUL TEKNİK ÜNİVERSİTESİ ★ FEN BİLİMLERİ ENSTİTÜSÜ**

# GLOBAL KONTROL ELEMANLARININ *B. SUBTİLİS'* TE YENİ BİR GNTR TİPİ DÜZENLEYİCİ GEN OLAN *yvfI'* NIN GEN İFADESİ ÜZERİNDEKİ ETKİLERİ

**YÜKSEK LİSANS TEZİ**

**Emine Canan ÜNLÜ ÖZKURT**
**(521051225)**

**Tezin Enstitüye Verildiği Tarih : 29 Aralık 2008**
**Tezin Savunulduğu Tarih : 22 Ocak 2009**

**Tez Danışmanı : Doç. Dr. Ayten YAZGAN KARATAŞ (İTÜ)**

**Diğer Jüri Üyeleri : Doç. Dr. Gamze KÖSE (YEDİTEPE ÜNİVERSİTESİ)**
**Yrd. Doç. Dr. Fatma Neşe KÖK (İTÜ)**

**OCAK 2009**

## FOREWORD

The presented work is a yield of hard, disciplined, and elaborate study. The biggest moiety of my outcome belongs to my unique guide. It is a great honor to present my deep gratefulness to my supervisor Assoc. Prof. Dr. Ayten YAZGAN KARATAŞ for her huge confidence, support at all conditions, unequalled advices and interest, and sharing her immense knowledge with me. It would not be possible to learn much more than now, if I did not meet her and work with her.

I am thankful to my dear friends who are more than just workmates to me; firstly, my angels Öykü İRİGÜL and Türkan Ebru KÖROĞLU. They always shared their knowledge, responded to my limitless and repeatead questions, supported me during the overall study from beginning to the end with a deep patience. I think it was a great chance to meet and share the same working area with them. The laboratory became such as a sweet home with their enjoyable personality. I would like to thank Orkun PİNAR, because of his kind friendship in and out of lab, unlimited support in troubled times, and at each point of my study. The long and sleepless working nights would not be such funny times without him. Special thanks to Günseli KURT-GÜR and Esra YÜCA for their lovely friendship. They never refused or let me down when I needed their opinion and association at any subject. In addition, I want to thank to specially Hüseyin TAYRAN, Ahmet Can BERKYÜREK, and Elif KARACA for their infinite help and priceless amity.

My enormous thankfulness is for my family. I am indebted to my father Süleyman ÜNLÜ, my mother Nilgün ÜNLÜ, my brother Cüneyt ÜNLÜ and his wife Ayşegül ÜNLÜ and lastly my pretty sister Nihan ÜNLÜ. If there were not their deep and limitless love and support, I would not be able to become this powerful and successful woman who I turned into today.

I am sincerely greateful to my husband, Cem ÖZKURT for his endless love, kindly approach during my bored and worried times, his inexpressible patience without any complain and his strong encouragment before and during my study period. I feel I am very lucky for catching a rare chance for having such a unique man.

January 2009 E. Canan ÜNLÜ ÖZKURT

Molecular Biologist

**TABLE OF CONTENTS**

**Page**

**ABBREVIATIONS** ..... **v**
**LIST OF TABLES** ..... **vi**
**FIGURES** ..... **vii**
**SUMMARY** ..... **xii**
**ÖZET** ..... **xiii**
**1. INTRODUCTION** ..... **1**
1.1. *Bacillus subtilis* ..... 1
1.2. Quorum Sensing Mechanism As A Regulatory System of Gene Expression ..... 2
1.3. Bacilysin: A Dipeptide Antibiotic ..... 8
1.4. The Aim of The Present Study ..... 13
**2. MATERIALS AND METHODS** ..... **14**
2.1. Materials ..... 14
2.1.1. Bacterial Strains and Plasmids ..... 14
2.1.2. Culture Media ..... 14
2.1.3. Buffers and Solutions ..... 14
2.1.4. Chemicals and Enzymes ..... 14
2.1.5 Maintenance of Bacterial Strains ..... 16
2.1.6. pDrive Cloning Vector ..... 16
2.1.7 pMUTIN T3 Vector ..... 17
2.1.8. pDR66 Vector ..... 17
2.2. Methods ..... 18
2.2.1. DNA Techniques and Manipulation ..... 18
2.2.1.1. Plasmid DNA Isolation ..... 18
2.2.1.2. Chromosomal DNA Isolation ..... 19
2.2.1.3. Polymerase Chain Reaction (PCR) ..... 19
2.2.1.4. Agarose Gel Electrophoresis ..... 21
2.2.1.5. Gel Extraction ..... 21
2.2.1.6. Ligation of PCR Products Into pDrive Cloning Vector ..... 22
2.2.1.7. Ligation of pMUTIN T3 Vector ..... 22
2.2.1.8. Restriction Enzyme Digestion ..... 22
2.2.2. Transformation ..... 22
2.2.2.1. Preparation of *E. coli* Electrocompetent Cells and Transformation of Electrocompetent *E. coli* Top10F' Cells ..... 22
2.2.2.2. Preparation of *B. subtilis* Competent Cells and Transformation ..... 23
2.2.2.3. Induction of MLS Gene ..... 23
2.2.2.4. Beta-Galactosidase Activity Assay ..... 24
**3. RESULTS AND DISCUSSION** ..... **26**
3.1. Construction of *yvfI* Insertional Plasmid ..... 26
3.2. Construction of *yvfI*::*lacZ* Transcriptional Fusion in *B. subtilis* ..... 28
3.3. Expression of Transcriptional *yvfI*::*lacZ* Fusion in PA Medium ..... 29

3.4. Deletion of Regulatory Genes and Their Effects on The Expression of *yvfI* Gene in *B. subtilis* ..... 30
3.4.1. Deletion of *srfA* Gene and Its Effects on The Expression of *yvfI* Gene in *B. subtilis* ..... 30
3.4.2. Deletion of *oppA* Gene and Its Effects on The Expression of *yvfI* Gene in *B. subtilis* ..... 32
3.4.3. Deletion of Pheromone Peptides Genes (*phrC, phrK, phrF*) and Their Effects on The Expression of *yvfI* Gene in *B. subtilis* ..... 34
3.4.4. Deletion of *comQ(comX),comP, comA* and *spo0A* Genes and Their Effects on The Expression of *yvfI* Gene in *B. subtilis* ..... 37
3.4.5. The Effects of *spo0A* and *abrB* Null Mutations on The Expression of *yvfI* Gene in *B. subtilis* ..... 41
3.4.6. Deletion of *codY* Gene and Its Effects on The Expression of *yvfI* Gene in *B. subtilis* ..... 45
3.4.7. Deletion of *degU* Gene and Its Effects on The Expression of *yvfI* Gene in *B. subtilis* ..... 51
3.4.8. Deletion of *sigB* Gene and Its Effects on The Expression of *yvfI* Gene in *B. subtilis* ..... 53
**4. CONCLUSION ..... 55**
**REFERENCES ..... 57**
**APPENDICES ..... 69**
**CURRICULUM VITA ..... 80**

## ABBREVATIONS

| | | |
|---|---|---|
| **AHL** | : | N-achylhomoserine Lactone |
| **Amp** | : | Ampicillin |
| **Bp** | : | Base pair |
| **Cm** | : | Chloramfenicol |
| **CSF** | : | Competence and Sporulation Factor |
| **$dH_2O$** | : | Distilled water |
| **DNA** | : | Deoxyribonucleic acid |
| **DSM** | : | Difco's Sporulation Medium |
| **EDTA** | : | Ethylenediaminetetraacetic acid |
| **Erm** | : | Erythromycin |
| **EtBr** | : | Ethidium bromide |
| **GntR** | : | DNA-binding transcriptional repressor of the gluconate operon |
| **Kan** | : | Kanamycin |
| **kb** | : | Kilobase |
| **LB broth** | : | Luria Bertani broth |
| **Ln** | : | Lincomycin |
| **µl** | : | Mikroliter |
| **Neo** | : | Neomycin |
| **OD** | : | Optical Density |
| **ONPG** | : | 2-Nitrophyl β-D-galacto pyranoside |
| **PA** | : | Perry and Abraham Medium |
| **PCR** | : | Polymerase Chain Reaction |
| **QS** | : | Quorum Sensing |
| **Rpm** | : | Revolution per minute |
| **Spc** | : | Spectinomycin |
| **TAE** | : | Tris acetate EDTA |
| **Tris** | : | Hydroxymethylaminomethane |

## LIST OF TABLES

**Page**

**Table 2.1** : Bacterial strains and their genotypes used in this study ..... 15
**Table 2.2** : Sequences of oligonucleotide primers ..... 20
**Table 2.3** : List of materials used for preparation of PCR mixture ..... 20
**Table 2.4** : Agarose gel concentration for different samples ..... 21

**LIST OF FIGURES**

**Page**


**Figure 1.1**: Regulation mechanism of quorum responses triggered by environmental signals and initiation of signal transduction cascade through ComX and CSF diffusible peptides (Lazazzera, 2000) ..... 5

**Figure 1.2**: A model for efficiency mechanism of signaling peptides and phosphatates involved in quorum response in *B. subtilis* (Pottathil and Lazazzera, 2003) ..... 7

**Figure 1.3**: Chemical structures of bacilysin and respectively anticapsin (Walker and Abraham, 1970) ..... 8

**Figure 1.4**: The bacilysin gene cluster organisation, *bacABCDE*, relative to openreading frames *ywfABCDEFG* of *Bacillus subtilis* 168 DNA sequence is among 3875148-3867678 bp in the chromosome obtained from SubtiList database R.16.1 (Kunst *et al.*, 1997). Proposed terminator (T0) elements are indicated according the SubtiList database. Sigma A promoter (P) elements_35 (TTGACA) and _10 (TAAAATT) were detected 56 bp and 33 bp upstream of the ATG codon of the *bacA* gene (Steinborn *et al.*, 2005) ..... 10

**Figure 1.5**: Antibiotic biosynthesis pathway of *B. subtilis* involved in subtilin, subtilosin, surfactin and bacilysin. Skf acts as a the killing factor and TasA is the spore-associated antimicrobial polypeptide (Stein, 2005) ..... 11

**Figure 2.1:** Genomic map of pDrive Cloning Vector including the functional genes in the structure as well as the restriction map (www1.qiagen.com/literature/pDrive/pcr_cloning21.pdf) ..... 16

**Figure 2.2:** Genomic map of pMUTIN T3 vector showing the restriction map and the functional genes (Vagner *et al.*, 1998) ..... 17

**Figure 2.3:** Shematic presentation of 9,3 kb pDR66 vector used for transformation due to $Cm^R$ ($Cat^R$) region facilitating the selection of mutant strains (Ireton *et al.,* 1993) ..... 18

**Figure 3.1:** 1-2; PCR products of 525 bp *yvfI* gene fragment amplified with spesific primers *yvfI* F and *yvfI* R and M; Marker 3: Lambda DNA/*Eco*RI+*Hind*III ..... 26

**Figure 3.2:** The confirmation of *yvfI* gene cloning into pMutinT3 vector. 2:the *Hind*III linearized recombinant pMUTIN T3 plasmid carrying *yvfI*::*lacZ* fusion, and 1: the linearized original pMUTIN T3 vector, M: Fast Ruller High Range DNA Marker .... 27

**Figure 3.3:** The confirmation of *yvfI::lacZ::erm* in *B. subtilis* chromosome. 1: PCR product amplified with specific primers to *erm*

resistance gene within pMutinT3 vector using chromosomal DNA of *yvfI::lacZ::erm* mutant as template; 2: PCR product amplified with specific primers to *yvfI* gene using chromosomal DNA of *yvfI::lacZ::erm* mutant as template; M: Marker 3: Lambda DNA/*Eco*RI+*Hind*III ..... 28

**Figure 3.4:** Growth and bacilysin activity of *B. subtilis* PY79 and TEK7 (*yvfI*::*lacZ*::*erm*) strains grown in PA medium. Specific Miller Units was calculated with formula as denoted in Section 2.3 (2.1). The symbols used for the strains are; (○) PY79 (wild type) and (●)TEK7(*yvfI*::*lacZ::erm*) ..... 29

**Figure 3.5:** Growth curves of mutant strains grown in PA medium. The symbols used for the strains are; (□) TEK7 (*yvfI*::*lacZ::erm*) and (▲)ECU13(*yvfI*::*lacZ*::*erm*Δ*srfA*::*erm*) ..... 31

**Figure 3.6:** β-Galactosidase activities of mutant strains grown in PA medium and their effects on *yvfI-lacZ* expression. Specific Miller Units was calculated with formula as denoted in Section 2.3. The symbols used for the strains are; (■) TEK7 (*yvfI*::*lacZ::erm*) and (▲)ECU13(*yvfI*::*lacZ*::*erm* Δ*srfA*::*erm*) ..... 31

**Figure 3.7:** Growth curves of TEK7 (*yvfI*::*lacZ*::*erm*) and TEK10 (*yvfI*::*lacZ*::*erm* Δ*oppA*::*spc*) strains grown in PA medium. The symbols used for the strains are; (■) TEK7(*yvfI*::*lacZ::erm*) and (▲)TEK10 (*yvfI*::*lacZ*::*erm* Δ*oppA*::*spc*)..... 33

**Figure 3.8:** β-Galactosidase activities of TEK7 and *oppA*-deleted mutant strain grown in PA medium and effect of deletion on *yvfI-lacZ* expression. The symbols used for the strains are; (■) TEK7 (*yvfI*::*lacZ::erm*) and (▲) TEK10 (*yvfI*::*lacZ*::*erm* Δ*oppA*::*spc*)... 33

**Figure 3.9:** Growth curves of TEK7 (*yvfI::lacZ::erm*), ECU5 (*yvfI::lacZ::erm* Δ*phrK::spc*), ECU6 (*yvfI::lacZ::erm* Δ*phrF163::cm*) and ECU12 (*yvfI::lacZ::erm* Δ*phrC::erm*) strains grown in PA medium. The symbols used for the strains are; (Δ) TEK7 (*yvfI*::*lacZ::erm*), (♦) ECU5 (*yvfI::lacZ::erm* Δ*phrK::spc*), (●) ECU6 (*yvfI::lacZ::erm* Δ*phrF163::cm*) and (■) ECU12 (*yvfI::lacZ::erm* Δ*phrC::erm*)..... 35

**Figure 3.10:** β-Galactosidase activities of TEK7 and *phrC*-deleted mutant strain grown in PA medium and effect of deletion on *yvfI-lacZ* expression. The symbols used for the strains are; (♦) TEK7 (*yvfI*::*lacZ::erm*) and (■) ECU12 (*yvfI::lacZ::erm* Δ*phrK::spc*)... 36

**Figure 3.11:** β-Galactosidase activities of TEK7 and *phrK*-deleted mutant strain grown in PA medium and effect of deletion on *yvfI-lacZ* expression. The symbols used for the strains are; (♦) TEK7 (*yvfI*::*lacZ::erm*) and (■) ECU5 (*yvfI::lacZ::erm* Δ*phrK::spc*)..... 36

**Figure 3.12:** β-Galactosidase activities of TEK7 and *phrF163*-deleted mutant strain grown in PA medium and effect of deletion on *yvfI-lacZ* expression. The symbols used for the strains are; (♦) TEK7 (*yvfI*::*lacZ::erm*) and (■) ECU6 (*yvfI::lacZ::erm* Δ*phrF163::cm*). β-Galactosidase activities of ECU11 and TEK7 in PA medium..... 37

**Figure 3.13:** Growth curves of TEK7 (*yvfI::lacZ::erm*), ECU3 (*yvfI*::*lacZ*::*erm* Δ*comQ*::*spc*), ECU4 (*yvfI*::*lacZ*::*erm* Δ*comP*::*spc*), ECU11 (*yvfI*::*lacZ*::*erm* Δ*comA*::*cat*) and TEK11 (*yvfI*::*lacZ*::*erm* Δ*spo0H*::*cat*) strains grown in PA medium. The symbols used for the strains are; (■) TEK7 (*yvfI::lacZ::erm*), (♦) ECU3 (*yvfI*::*lacZ*::*erm* Δ*comQ*::*spc*), (▲) ECU4 (*yvfI*::*lacZ*::*erm* Δ*comP*::*spc*), (◊) ECU11 (*yvfI*::*lacZ*::*erm* Δ*comA*::*cat*) and (○) TEK11 (*yvfI*::*lacZ*::*erm* Δ*spo0H*::*cat*).β-Galactosidase activities of ECU4 and TEK7 in PA medium ..... 39

**Figure 3.14:** β-Galactosidase activities of TEK7 and *comA*-deleted mutant strain grown in PA medium and effect of deletion on *yvfI-lacZ* expression. The symbols used for the strains are; (♦) TEK7 (*yvfI::lacZ::erm*) and (■) ECU11 (*yvfI::lacZ::erm* Δ*comA*::*cat*) ... 39

**Figure 3.15:** β-Galactosidase activities of TEK7 and *comP*-deleted mutant strain grown in PA medium and effect of deletion on *yvfI-lacZ* expression. The symbols used for the strains are; (♦) TEK7 (*yvfI*::*lacZ::erm*) and (■) ECU4 (*yvfI*::*lacZ*::*erm* Δ*comP*::*spc*) ..... 40

**Figure 3.16:** β-Galactosidase activities of TEK7 and *comQ*-deleted mutant strain grown in PA medium and effect of deletion on *yvfI-lacZ* expression. The symbols used for the strains are; (♦) TEK7 (*yvfI*::*lacZ::erm*) and (■) ECU3 (*yvfI*::*lacZ*::*erm* Δ*comQ*::*spc*) ..... 40

**Figure 3.17:** β-Galactosidase activities of TEK7 and *spo0H*-deleted mutant strain grown in PA medium and effect of deletion on *yvfI-lacZ* expression. The symbols used for the strains are; (♦) TEK7 (*yvfI*::*lacZ::erm*) and (■) TEK11 (*yvfI*::*lacZ*::*erm* Δ*spo0H*::*cat*) ... 41

**Figure 3.18:** Growth curves of TEK7 (*yvfI::lacZ::erm*), TEK9 (*yvfI*::*lacZ*::*erm* ΔabrB::*cat*), TEK12 (*yvfI*::*lacZ*::*erm* Δ*spo0A*::*spc*) and ECU1 (*yvfI::lacZ::erm* Δ*spo0A::*Δ*abrB::*cm) strains grown in PA medium. The symbols used for the strains are; (Δ) TEK7 (*yvfI::lacZ::erm*), (▲) ECU1 (*yvfI::lacZ::erm* Δ*spo0A::* Δ*abrB::cm*), (■) TEK9 (*yvfI*::*lacZ*::*erm* ΔabrB::*cat*) and (◊) TEK12 (*yvfI*::*lacZ*::*erm* Δ*spo0A*::*spc*) ..... 43

**Figure 3.19:** β-Galactosidase activities of TEK7 and *abrB*-deleted mutant strain grown in PA medium and effect of deletion on *yvfI-lacZ* expression. The symbols used for the strains are; (♦) TEK7 (*yvfI::lacZ::erm*) and (■) TEK9 (*yvfI*::*lacZ*::*erm* ΔabrB::*cat*) ..... 43

**Figure 3.20:** β-Galactosidase activities of TEK7 and *spo0A*-deleted mutant strain grown in PA medium and effect of deletion on *yvfI-lacZ* expression. The symbols used for the strains are; (♦) TEK7 (*yvfI::lacZ::erm*) and (■) TEK12 (*yvfI*::*lacZ*::*erm* Δ*spo0A*::*spc*) .. 44

**Figure 3.21:** β-Galactosidase activities of TEK7 and *spo0A-abrB*-deleted mutant strain grown in PA medium and effect of deletion on *yvfI-lacZ* expression. The symbols used for the strains are; (♦) TEK7 (*yvfI::lacZ::erm*) and (■) ECU1 (*yvfI::lacZ::erm* Δ*spo0A::*Δ*abrB::*cm) ..... 44

**Figure 3.22:** Growth curves of TEK7 (*yvfI*::*lacZ*::*erm*), ECU2 (*yvfI*::*lacZ*::*erm trpC2 unkU*::*spc* Δ*codY*::Δ*abrB*::*cm*) and ECU14 (*yvfI*::*lacZ*::*erm trpC2 unkU*::*spc* Δ*codY*) straing grown in PA medium. The symbols used for strains are; (■) TEK7 (*yvfI*::*lacZ*::*erm*), (▲) ECU2 (*yvfI*::*lacZ*::*erm trpC2 unkU*::*spc* Δ*codY*::Δ*abrB*::*cm*) and (◊) ECU14 (*yvfI*::*lacZ*::*erm trpC2 unkU*::*spc* Δ*codY*) ..... 47

**Figure 3.23:** β-Galactosidase activities of TEK7 and *codY*-deleted mutant strain grown in PA medium and effect of deletion on *yvfI-lacZ* expression. The symbols used for the strains are; (▲) TEK7 (*yvfI*::*lacZ*::*erm*) and (■) ECU14 (*yvfI*::*lacZ*::*erm trpC2 unkU*::*spc* Δ*codY*) ..... 47

**Figure 3.24:** β-Galactosidase activities of TEK7 and *codY-abrB*-deleted mutant strain grown in PA medium and effect of deletion on *yvfI-lacZ* expression. The symbols used for the strains are; (■) TEK7 (*yvfI*::*lacZ*::*erm*) and (▲) ECU2 (*yvfI*::*lacZ*::*erm trpC2 unkU*::*spc*Δ*codY*::Δ*abrB*::*cm*) ..... 48

**Figure 3.25:** Growth curve of TEK7 (*yvfI*::*lacZ*::*erm*) strain grown in PA medium within and without casein, seperately. The symbols used for the strains are; (▲) TEK7 (*yvfI*::*lacZ*::*erm*) without casein, (Δ)TEK7 (*yvfI*::*lacZ*::*erm*) within casein. β-Galactosidase activities of TEK7 in PA medium within and without casein, separately ..... 49

**Figure 3.26:** Growth curve of ECU14 (*yvfI*::*lacZ*::*erm trpC2 unkU*::*spc* Δ*codY*) strain grown in PA medium within and without casein. The symbols used for the strains are; (■) ECU14 (*yvfI*::*lacZ*::*erm trpC2 unkU*::*spc* Δ*codY*) without casein, (□) ECU14 (*yvfI*::*lacZ*::*erm trpC2 unkU*::*spc* Δ*codY*) within casein..... 49

**Figure 3.27:** β-Galactosidase activities of TEK7 (*yvfI*::*lacZ*::*erm*) strain grown in PA medium within and without casein, seperately. The symbols used for the strains are; (■) TEK7 (*yvfI*::*lacZ*::*erm*) without casein, (▲)TEK7 (*yvfI*::*lacZ*::*erm*) within casein ... 50

**Figure 3.28:** β-Galactosidase activities of ECU14 (*yvfI*::*lacZ*::*erm trpC2 unkU*::*spc* Δ*codY*) strain grown in PA medium within and without casein, seperately. The symbols used for the strains are; (■) ECU14 (*yvfI*::*lacZ*::*erm trpC2 unkU*::*spc* Δ*codY*) without casein, (▲) ECU14 (*yvfI*::*lacZ*::*erm trpC2 unkU*::*spc* Δ*codY*) within casein..... 50

**Figure 3.29:** Growth curves of TEK7 (*yvfI*::*lacZ*::*erm*) and ECU8 (*yvfI*::*lacZ*::*erm* Δ*degU*::*erm*) strains grown in PA medium. The symbols used for the strains are; (▲) TEK7 (*yvfI*::*lacZ*::*erm*) an (Δ) ECU8 (*yvfI*::*lacZ*::*erm* Δ*degU*::*erm*) ..... 52

**Figure 3.30:** β-Galactosidase activities of TEK7 and *degU*-deleted mutant strain grown in PA medium and effect of deletion on *yvfI-lacZ* expression. The symbols used for the strains are; (■) TEK7 (*yvfI*::*lacZ*::*erm*) and (▲) ECU8 (*yvfI*::*lacZ*::*erm* Δ*degU*::*erm*) .... 52

**Figure 3.31:** Growth curves of TEK7 (*yvfI*::*lacZ*::*erm*) and ECU7 (*yvfI*::*lacZ*::*erm* Δ*ML6*::*cm*) strains grown in PA medium. The symbols used for the strains are; (□) TEK7 (*yvfI*::*lacZ*::*erm*) an (■) ECU8 (*yvfI*::*lacZ*::*erm* Δ*ML6*::*cm*) ..... 53

**Figure 3.32:** β-Galactosidase activities of TEK7 and *sigB*-deleted mutant strain grown in PA medium and effect of deletion on *yvfI-lacZ* expression. The symbols used for the strains are; (■) TEK7 (*yvfI*::*lacZ*::*erm*) and (□) ECU7 (*yvfI*::*lacZ*::*erm* Δ*sigB*::*cm*)..... 54

**THE EFFECT OF GLOBAL CONTROL ELEMENTS ON THE EXPRESSION OF A NOVEL GNTR TYPE REGULATOR *yvfI* IN *B. SUBTILIS***

## SUMMARY

Bacilysin is a dipeptide antibiotic consisted of L-alanin and L-anticapsin, and produced extracellularly by certain species of *Bacillus subtilis*. Previously, we have shown that bacilysin biosynthesis was under the control of quorum sensing mechanism involving in the actions of ComQ/ComX, ComP/ComA, PhrC(CSF) and Spo0K(OppA). The disruption of lipopeptide antibiotic surfactin biosynthetic (*srfA*) operon in the bacilysin producer resulted in a bacilysin-negative phenotype, thus our study verified that the *srfA* operon functions directly in the production of bacilysin. The loss of bacilysin production in *spo0H* and or *spo0A*-blocked mutants as well as an increase in the production of bacilysin in *abrB*-disrupted mutants and the suppression of bacilysin-negative phenotype by an *abrB* mutation in *spo0A*-blocked mutants revealed that the transcription of some gene(s) involved in bacilysin formation is under the negative control of *abrB* gene product which is relieved by Spo0A protein. Recently, we identified a novel gene, namely *yvfI* required for the production of dipeptide antibiotic bacilysin. This gene encode a product resembling GntR family transcriptional regulator.

As a further work, the main purpose of the present study is to elucidate the effects of previously identified regulatory genes *oppA, comP*, *comA*, *phrC*, *phrK, phrF, comQ* (*comX*), *srfA*, *codY*, *degU*, *sigB*, *spo0A*, *spo0H* and *abrB* on the transcriptional factor encoding gene *yvfI*. Firstly, to analyze the expression of *yvfI*, a *B. subtilis* strain, namely TEK7, containing a transcriptional $P_{yvfI}$-*lacZ* fusion at the *yvfI* locus was constructed. Subsequently, each of the regulatory genes indicated above was disrupted in the transcriptional *yvfI–lacZ* fusion bearing strain TEK7. The resulting mutant strains and TEK7 as the control were cultured in PA medium and *yvfI*-directed β-galactosidase activities were monitored. Mutations in *comP, comA*, *comQ(comX), phrC, phrK ,phrF, srfA, spo0H* and *spo0A* genes completely abolished *yvfI-lacZ* expression. *abrB* null mutation gradually relieved the repression of *yvfI* during exponential phase while decreasing the induced level of expression in the stationary phase. However complete inhibition of *yvfI* expression in Δ*spo0A* strain was not restored by *abrB* mutation. During exponential phase *yvfI-lacZ* expression in *codY* mutant strain was not significantly affected, but *yvfI* expression in the stationary phase was not induced to maximal level as in the case of wild type. However, *abrB-codY* double mutations resulted in a significant elevation in *yvfI-lacZ* expression. In this study, we also found that *yvfI* expression is subject to nutritional repression mediated by Casamino acids. The effects of a transition regulator gene d*egU* and general stress control element *sigB* gene on *yvfI* expression were also investigated and we found *yvfI* expression to be positively regulated by DegU. However, SigB had no effect on *yvfI* expression.

**GLOBAL KONTROL ELEMANLARININ *B. SUBTİLİS'* TE YENİ BİR GNTR TİPİ DÜZENLEYİCİ GEN OLAN *yvfI*' NIN GEN İFADESİ ÜZERİNDEKİ ETKİLERİ**

## ÖZET

Belirli bazı Bacillus subtilis suşları tarafından sentezlenen hücre dışı bir antibiyotik olan bacilysin L-alanin ve L-antikapsin'den oluşmuş bir dipeptiddir. Daha önceki çalışmalarımızda, basilisin biyosentezinin, ComQ/ComX, ComP/ComA, PhrC(CSF) ve Spo0K(OppA)' nın aktivasyonunu etkileyen quorum sensing mekanizmasının kontrolü altında olduğu gösterilmiştir. Basilisin üretebilen suşta lipopeptid antibiyotik surfactin biyosentetik (*srfA*) operonunun inaktive edilmesi sonucu basilisin üretemeyen fenotipin oluşmasıyla, bizim çalışmamızda *srfA* operonunun basilisin üretiminde doğrudan görev aldığı doğrulanmıştır. *spo0H* ve/veya *spo0A* genlerinin bloke edildiği mutantlarda basilisin üretilememesi, *abrB* geni bozulmuş mutantlarda basilisin üretiminde artış gözlenmesi, *spo0A* bloke edilmiş mutantlarda *abrB* geninin mutasyonu sonucu basilisin üretemeyen fenotipin ortadan kalkması, basilisin oluşumunda görev alan bazı gen veya genlerin transkripsiyonunun, *abrB* gen ürününün negatif kontrolü altında olduğunu ve bu negatif etkinin Spo0A tarafından düzeltilebileceğini ortaya koymuştur. Yakın bir zamanda, çalışma grubumuz dipeptid antibiyotik basilisin üretimi için gerekli olduğu belirlenen ve *yvfI* olarak adlandırılan yeni bir gen tanımlamıştır. Bu gen GntR transkripsiyonal düzenleyici ailesine benzeyen bir ürün kodlamaktadır.

Yapılan bu çalışmaların devamı olan şimdiki çalışmamızın temel amacı, daha önce etkileri tanımlanmış olan *oppA, comP*, *comA*, *phrC*, *phrK, phrF, comQ* (*comX*), *srfA*, *codY*, *degU*, *sigB*, *spo0A*, *spo0H* ve *abrB* regülator genlerinin *yvfI* tarafından kodlanan transkripsiyonel faktör üzerindeki etkileri aydınlatılmaya çalışılmıştır. Öncelikle, *yvfI* gen ifadesini analiz etmek için, *yvfI* lokusunda transkripsiyonel $P_{yvfI}$-*lacZ* füzyonu içeren ve TEK7 olarak adlandırılan bir *B. subtilis* suşu oluşturulmuştur. Daha sonra, yukarıda belirtilen düzenleyici genlerden her biri transkripsiyonel *yvfI–lacZ* füzyonu taşıyan TEK7 suşunda bloke edilmiştir. Elde edilen mutantlar ve kontrol grubu olan TEK7, PA besiyerinde büyütülmüş, *yvfI*' ya bağlı spesifik β-galaktozidaz aktivititeleri gözlenmiştir. *comP, comA*, *comQ(comX), phrC, phrK, phrF, srfA, spo0H* ve *spo0A* genlerindeki mutasyonlar *yvfI-lacZ* gen ifadesinin tamamen bozulmasıyla sonuçlanmıştır. *abrB* mutasyonu eksponensiyel büyüme fazı sırasında *yvfI* baskılanmasını kısmen hafifletmiş, durağan fazdaysa var olan gen ifadesi baskılanmıştır, buna karşılık Δ*spo0A* mutant suşunda tamemen engellenen *yvfI* gen ifadesi *abrB* mutasyonuna rağmen eski haline getirilememiştir. *codY* mutant suşunda *yvfI-lacZ* gen ifadesinin eksponensiyel büyüme fazı sırasında belirgin olarak değişmemiş olduğu, fakat durağan faz sırasında yabani suşun ulaştığı ekspresyon seviyesine ulaşamadığı görülmüştür. Bununla birikte, *abrB-codY* çift mutasyonu *yvfI-lacZ* gen ifadesinde önemli bir artış ile sonuçlanmıştır. Bu çalışmada, *yvfI* gen ifadesinin kazamino asit varlığında besinsel baskılamaya da maruz kaldığı bulunmuştur. Bir geçiş evresi düzenleyici gen olan d*egU* ve genel stres kontrol elemanı olan *sigB* genlerinin de *yvfI* gen ifadesi üzerine etkileri araştırılmış ve DegU ürünün *yvfI* gen ifadesi üzerinde pozitif düzenleyici etkiye sahip olduğu bulunmuştur.

Bunun yanısıra, SigB sigma faktörünün *yvfI* gen ifadesi üzerine hiçbir etkiye sahip olmadığı görülmüştür.

# 1. INTRODUCTION

## 1.1 *Bacillus subtilis*

*Bacillus subtilis* is an unusual endospore-forming rhizobacterium that can produce ribosomally and non-ribosomally more than twenty-four types of antibiotics along with other secondary metabolites that have antibacterial, antifungal and antimetabolic properties. *Bacillus subtilis* shows excellent genetic adaptional abilities that allow the bacterium to grow up in many diverse environments such as soil on plant roots, aquatic habitats, even gastrointestinal tracts of marine and terrestrial animals. Until recently, it was accepted as an obligate aerob organism but these new studies showed that it can be isolated from animal's feces that are fed with plants associated with *B.subtilis*. Furthermore, observation of putative respiratory nitrat reductase genes has strengthened the proposition that *B.subtilis* can live in anaerob conditions (Glaser *et al.,* 1995; Ramos *et al.*, 1995; Earl *et al.,* 2008).

Following the sequencing of its genome, *B.subtilis* became a very widely studied model organism due to its non-pathogenicity, its characteristic of being practical for harvesting and its ability for yielding plenty of industrially important products such as macrolmolecular hydrolases (proteases and carbohydrases), other specific enzymes (e.g. α-amylases, β-amylases) and many antibiotics (Kunst *et al.*, 1997). Beside of its well-known genome structure and basic nutritional requirements, *Bacillus subtilis* represents a preferred Gram-positive bacterium for molecular and genetic surveys because of its crucial feature of genetic competence (Marten *et. al.,* 2000; Stein, 2005).

Additionally, exhibiting probiotic property, *B.subtilis* is also cosidered as a good candidate for a novel prophylactic, therapeutic, and growth promoting agent as an alternative to antibiotics (Hong *et al.*, 2004; Williams 2007).

Moreover*, B. subtilis* is a chemoorganotroph which has surviving ability when exposed to limited growth conditions such as media including only salt, glucose or one of the other sugars for carbon and energy source and also nitrogen (Nicholson

and Setlow, 1990). Furthermore, *B. subtilis*, as similar to many other members of this genus, is mesophilic and may grow as normal-sized colonies within a day in a suitable temperature, 37$^{o}$C (Harwood *et al*., 1990).

In case of nutritional limitation or other unfavorable environmental conditions, cells initiate many distinct adaptative responses in order to survive. Under those conditions, cells of *B.subtilis* become not only organized for motility, chemotaxis and genetic competence allowing uptake of exogenious DNA, but also use its 'weapons' such as degradative enzymes and antibiotics in order to metabolize alternative nutrients and to eliminate other bacteria to take advantage for nutrient competition, respectively (Hamoen *et al.*, 2003). The sporulation process is the last resort of cells to keep surviving, such that sporulating cells are divided as a mother cell and smaller forespore cell, then as a result of complex regulatory circuits, resistant mature spore is generated (Banse *et al.*, 2008).

Total genome of *Bacillus subtilis* is 4.214.810 bp long consisted of 4.100 protein-coding genes (Kunst *et al.*, 1997). 271 (%6,6) of these genes are indispensable including 25 (%4) genes with unknown function, 3830 (%94,4) are non-essential (Kobayashi *et al.*, 2003). The genome of *Bacillus subtilis* encodes for 17 sigma factors and about 250 transcriptional regulators that bind to DNA, in addition, 86 tRNA, 30 rRNA and 3 small stable RNA encoding genes (Kobayashi and Ogasawara, 2002).

### 1.2 Quorum Sensing Mechanism As A Regulatory System of Gene Expression

Bacteria was thought as a non-cooperative, unable to connect to each other unicellular organism until an hormone-like extracellular product has been discovered that is produced to assist regulation of competence in *Streptococcus pneumoniae* (Tomasz, 1965; Bodman *et al.*, 2008). When intercellular communication called as "quorum sensing" was detected firstly in bioluminescent marine bacterium *Vibrio fischeri* by following surveys of Nealson and colleagues in 1970 (Nealson *et al.*, 1970; Nealson and Hasting, 1979), the vision for understanding of bacterial existence has tended to shift from that bacterial cells are non-interactive to the acception that they can act as multicellular organisms and exhibit social behaviors (Williams, 2007; Bodman *et al.,* 2008).

Quorum sensing mechanism is used by many diverse bacterial species for establishing different bacterial behaviors such as coordination of population to search for nutrients and to adapt to special environmental conditions, to defend itself aigainst other competitors, and to run away from the locations where vitae of population is at risk (Lazdunski *et al.*, 2004). Cellular processes that regulated by quorum sensing, exhibit differences in each bacterial species. For instance, the QS system is used for virulence and biofilm formation *in Pseudomonas aeruginosa*, bioluminescence in *Vibrio fischeri* and *Vibrio harveyi*, antibiotic production in *Erwinia carotovora*; virulence developing in *Staphylococcus aureus* and *Enterococcus faecalis*, genetic competence development in *Streptococcus pneumoniae* and sporulation, also genetic competence in *Bacillus subtilis* (Lazazzera, 2000, Bodman *et al.*, 2008). Several fundamental genes for signal synthesis and transduction and also diffusable small signal molecules(called as pheromones or autoinducers) are assigned in QS circuit (Camara, 2006). Because of diffusable features of the signal molecules, QS mechanism regulate not only its own cells, intraspeciesly, but also the cells of the other species, interspeciesly, and the cells of bacteria and higher organisms, inter-kingdomly (Diggle *et al.*, 2007). Quorum sensing is a density-dependent cell-signaling mechanism, therefore, when QS signaling molecules are secreted from the cell into the external environment, they are sensed by the 'neighbouring cells'. Depending on increased bacterial population density, produced QS signal molecules accumulates and their concentration reaches to a threshold level. After this point, a population-wide response is generated by signal transduction cascade which activates repression or induction of QS-dependent target genes (Schauder *et al.*, 2001; Winans and Bassler, 2002).

QS signals employed in QS circuits are chemically different elements although their functions are same. In Gram(-) bacteria very small molecules(<1000 Da), called as acylated homoserine lactone derivatives are attended in QS circuits. In contrast, small oligopetides with 5 - 20 aminoacids that usually contain chemical modification are used by Gram(+) bacteria as signaling. After reaching the adequate cell density, a target sensor kinase or response regulator is activated.

Among Gram-positive bacteria, *Bacillus subtilis* is a well known example in order to understand the signaling and regulation mechanism managed through a cell-density dependent manner (Griffith and Grossman 2008). At the end of the exponential

growth phase, if nutrients are limited for optimal growth, *B. subtilis* cells use a complex motility and chemotaxis system in order to search for nutrients in the environment. If nutritional limitation continues, after transition to the stationary growth phase, degradative enzyme production is initiated, such as proteases to liberate nutrients from alternative resources that are normally difficult to access. In addition to proteases, also antibiotics are synthesized in order to eliminate possible competitors. Prolonged nutritional stress is resulted with genetic competence development and lastly sporulation of the bacterial population (Hamoen *et al.*, 2003). These general quorum responses are regulated by extracellular signaling peptides accumulated in growth medium (Lazazzera, 2000). Until now, identified signaling peptides for *Bacillus subtilis* are classified as three classes (Auchtung *et al.*, 2006); ComX, a modified 10-amino-acid peptide interacting with its receptor, extracellularly (Magnuson *et al.,* 1994; Piezza *et al.*, 1999), lantibiotic peptides which interact extracellularly with their receptors (Stein 2005) and lastly pentapeptides, also called as Phr peptides inhibiting the activity of their target proteins, known as Rap proteins (Lazazzera 2001; Perego *et al.*, 2001).

Although ComX pheromone was firstly described as a part of competence development regulation, now it appears not only to be a general indicator of high cell density, but also the a regulatory protein involved in regulation of many genes (Dunny and Winans, 1999). ComX pheromone is a ten amino acid peptide **(**ADPITRQWGD) including a hydrophobic modification on tryptophan residue (Magnuson *et al.,* 1994). ComX is encoded by *comX* as a 55-amino acid precursors. *comQ* located upstream of *comX* gene in the chromosome, is speculated as responsible for modification or processing of ComX pheromone precursors to 10-amino acids ComX pheromone (Schneider *et al.*, 2002). After cleavage and modification by ComQ, then it is exported to the extracellular environment when population density is increased. *comQ* and *comX* are required for fully activation and maturation of ComX pheromone (Ansaldi *et al.*, 2002). ComX pheromone requires two-compenent system encoded by *comP* and *comA* (Weinrauch *et al.*, 1990). ComX binds to its cognate membrane-bound histidine kinase receptor ComP, concluded in the activation of ComP via autophosphorulation at a conserved histidine residue. Activated ComP~P then donates its phosphate to ComA, a phosphorylation-dependent response regulator transcription factor, on a conserved aspartate residue

(Pottathil *et al.*, 2008). Then, phosphorilated ComA transcription factor initiates many biological processes as a result of transcription of at least nine operons involved in the development of competence and in the production of degradative enzymes and antibiotics in response to increasing population density. Extracellular peptide signaling is used to coordinate the activity of ComA according to concentration level (Tortosa and Dubnau, 1999). At low signal peptide concentration ComA is inactive. When increasing of signaling peptide concentration then ComA is actived (Griffith and Grossman, 2008).

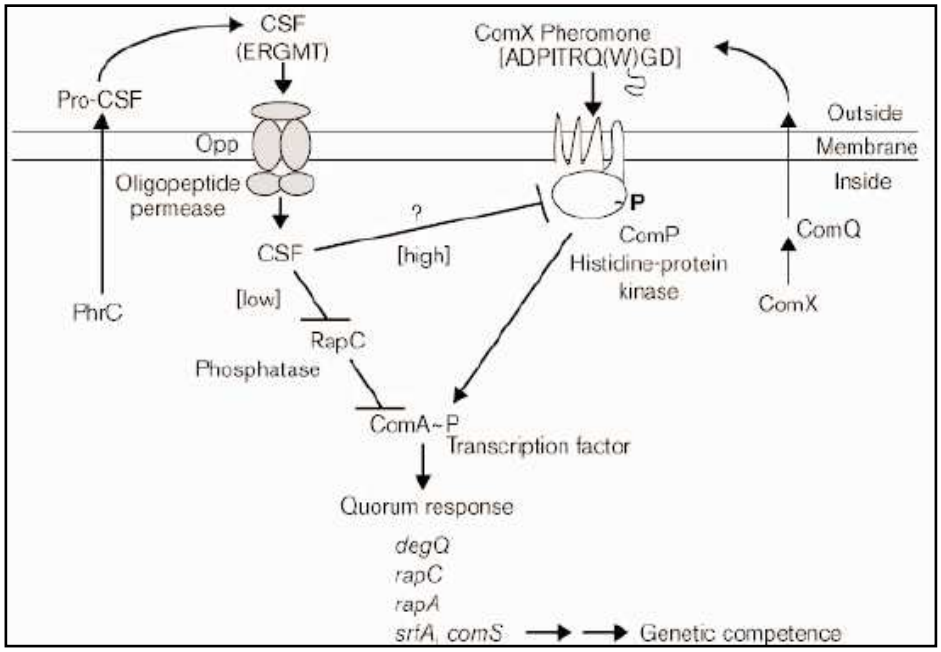



**Figure 1.1 :** Regulation mechanism of quorum responses triggered by environmental signals and initiation of signal transduction cascade through ComX and CSF diffusible peptides (Lazazzera, 2000).

There are also the other cell-density dependent signaling peptides effective on ComA activity which are secreted into growth medium and transported via oligoppeptide permease in order to act on their target intracellularly (Griffith and Grossman, 2008). ComA activation is tightly regulated by those pentapeptides and their target proteins (phosphatates) so that at low concentration of signaling peptides, ComA could not be activated for initiation of target genes expression (Pottathil and Lazazzera, 2003). A set of Phr peptides including PhrA, PhrC, PhrE, PhrF, PhrG, PhrH, PhrI and PhrK and also a set of Rap proteins consisted of 11 members from RapA to RapK are encoded by *B.subtilis* via an autoinducible operon system. Seven of this *rap* genes are located in an operon with a downstream overlapping *phr* genes so that each Phr

protein is encoded together with a cognate Rap protein at the same time (Perego *et at.*, 1996; Kunst *et al.*, 1997; Jiang *et al.*, 2000; Bongiorni *et al.*, 2005). In addition, *phr* genes include a promoter in the upstream region of *phr* providing recognition site for RNA polymerase carrying an alternative subunit, $\sigma^H$, encoded by *spo0H*. So expression of *phr* genes via $\sigma^H$ leads to accumulation of Phr peptides in the transition state from exponential phase to stationary phase (Auchtung *et al.*, 2006).

In addition to master extracellular signaling peptide ComX, the CSF peptide (PhrC) also play a key role on stimulating ComA activity. The effects of CSF on ComA regulation is more complicated than that of ComX pheromone. PhrC (ERGMT), also known as CSF, is a diffusable pentapeptide encoded from *rapC-phrC* operon, encoding CSF and its cytoplasmic receptor RapC. When CSF reaches a critical concentration, it is transported back into the cell by an oligopeptide permease (Spo0K), an ATP-binding casette transporter (Pottathil *et.al.*, 2008). Transported CSF then binds to two different intracellular receptors to modulate the activity of the ComA transcription factor according to intracellular CSF concentration level (Perego, 1997; Lazazzera and Grossman, 1998). While CSF concentration is lower (1–5 nM) at the onset of growing, CSF stimulates the activity of ComA for the expression of ComA~P dependent genes by inhibiting the activity of a putative ComA~P phosphatase, RapC (Schneider *et al.*, 2002). At higher concentrations (>20 nM), CSF interacts with its cognate receptor to prevent the expression of ComA-dependent genes (Lazazzera *et al.*, 1997). Beside these two functions of CSF, at high concentrations, sporulation is also triggered by CSF by inhibiting the activity of an alternate aspartyl-phosphate phosphatase, RapB (Lazazzera, 2000). On the other hand, it was proposed that CSF might be responsible for modulating the quorum response rather than induce it (Lazazzera and Grossman, 1998). Excistance of a binding site for sigma H ($\sigma^H$) on the promoter region of *phrC-rapC* operon signals that its production is regulated by starvation (Carter *et al.*, 1991) and sigma H is active during the transition state from exponential phase to stationary phase (Healy *et al.*, 1991), so that under starvation conditions, ComA-dependent genes which might be required for transition into stationary phase could be controlled even though in case of low cell density and therefore, CSF is an indicator not only for cell-density but also for starvation (Lazazzera *et al.*, 1999; Lazazzera 2000).

Also sporulation is regulated by Spo0A transcription factor as a response to changing signal transduction (Hoch, 1993), like a reaction against depletion of a carbon or nitrogen source, or against phosphate starvation under some conditions (Waldburger *et al.*, 1993). In order to activate this protein, phosphorylation is required and the level of Spo0A phosphorylation is regulated by the phosphorelay signal transduction system (Burbulys *et al.*, 1991).

On the quorum sensing mediated sporulation mechanism, signaling peptides have key roles and act as spore factors (Waldburger et al., 1993). After a threshold level for PhrA (ARNQT) and PhrC (CSF) (>20nM), which are encoded as precursors and activated when exported from the cell, they are imported by the oligopeptide transport system (Opp) into the cell again in order to inhibit RapA and RapB targetting Spo0F~P (Perego *et al.*, 1994) and also PhrE supports for inhibition of RapE acting on Spo0F. Among at least seven extracellular peptides encoded by *phr* genes, the function of extracellular PhrC is obvious; it is an indicator not only for cell density, but also the other physiological conditions. It is also obvious PhrA is a part of cell-cell signaling but it is suggested that PhrA involved in a timing mechanism via cell-autonomous signaling for the control of initiation of sporulation rather than critical extracellular accumulation (Perego *et al.*, 1997)

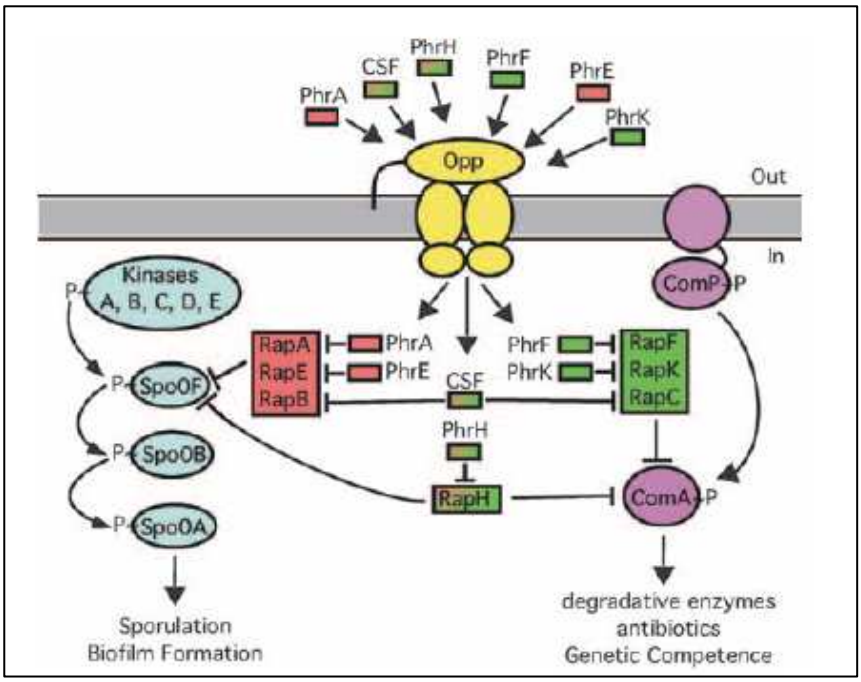



**Figure 1.2 :** A model for efficiency mechanism of signaling peptides and phosphatates involved in quorum response in *B.subtilis* (Pottathil and Lazazzera, 2003).

### 1.3. Bacilysin : A Dipeptide Antibiotic

Bacilysin is one of the simplest peptide antibiotic produced and secreted extracellularly by certain strains of *Bacillus subtilis.* Its small (125 kDa) and basic structure is consisted of L-alanine at N-terminus and L-anticapsin, an unususal aminoacid, at C terminus and (Walker and Abraham, 1970). Antibiotic activity is effective against bacteria and fungi, mainly *Candida albicans.*

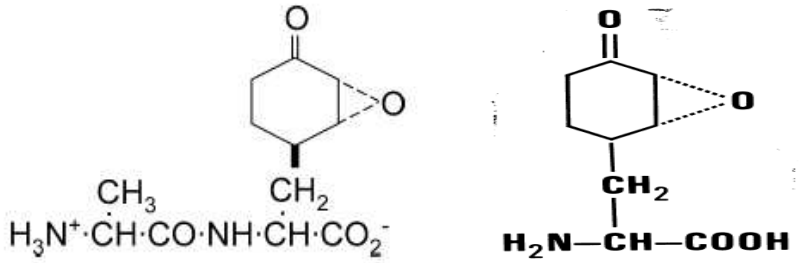



**Figure 1.3 :** Chemical structures of bacilysin and anticapsin respectively (Walker and Abraham, 1970).

Anticapsin moiety of bacilysin is the basis of its antibiotic activity (Whitney *et al.*, 1972). The antibiotic is transported into susceptible cells by a special peptide permease system and hydrolyzed to L-alanine and L-anticapsin by peptidases. Then intracellular anticapsin prevents the activity of glucosamine synthetase assigned for bacterial peptidoglycan and fungal mannoprotein biosynthesis (Perry and Abraham 1979; Chmara *et al.*, 1981). This blockage is resulted with protolasting and lysing of host cells (Whitnney and Funderburk 1970; Kenig *et al.*, 1976; Chmara *et al.*, 1982; Chmara 1985). On the basis of its metabolic target, anticapsin becomes specifically antagonized by glucosamine or N-acetylglucosamine (Walton and Rickes 1962; Kenig and Abraham 1976).

Prephenate belonging to the aromatic amino acid pathway is the primary precursor of anticapsin biosynthesis (Hilton *et al.*, 1988) and ligation of the peptide bound with L-alanine proceeds in an enzymatic reaction for catalysation by an amino acid ligase, bacilysin synthetase (Sakajoh *et al.* 1987).

Bacilysin production of *B.subtilis* is a growth-dependent phenomenon in a synthetic media and under the control of nutritional and feedback regulation. In the presence of certain nutrients, such as glucose or casaminoacids, and/or physiological factors, like

pH, temperature, biosynthesis of bacilysin is inhibited (Özcengiz *et al.*, 1990; Özcengiz and Alaeddinoglu, 1991; Basalp *et al.*, 1992). Also its biosynthesis is under global quorum-sensing control system via the OppA (Spo0K), the transporter element, and Opp-imported peptide pheromone PhrC, which is necessary for efficient sporulation and competence development (Yazgan *et al.*, 2001). Besides ComQ/ComX, PhrC (CSF), ComP/ComA and also their unique transporter Spo0K (Opp), products of *srfA, spo0A, spo0H* and *abrB* genes are defined as parts of quorum-sensing mechanism and have key roles on regulatory circuit of bacilysin biosynthesis (Karataş *et al*., 2003).

The *ywfBCDEFG* gene cluster of *B.subtilis,* renamed as *bacABCDE*, carries biosynthetic core functions on bacilysin production (Steinborn and Hofemeister 1998/2000; Inaoka *et al.*, 2003). Each gene of the operon has specific functions; *bacABC* (*ywfBCD*) encode proteins functioning in the biosynthesis of anticapsin, *bacD* (*ywfE* ) in the (amino acid) ligation of anticapsin to alanine and *bacE* (*ywfF*), in self-protection from bacilysin. *ywfB* and *ywfG* encode a prephenate dehydratase and an aminotransferase which are employed in anticapsin production because of prephenate of the aromatic amino acid pathway (Hilton *et al.*, 1988; Inaoka *et al.*, 2003; Steinborn *et al*., 2005).

Even though ribosomal peptide synthesis, driven by aminoacyl tRNA synthetase is conserved for all cellular organisms, large multienzyme complex functioning in a thiotemplate mechanism in a pathway initiated by a protein template is specific to prokaryotic organisms for producing antimicrobial peptie synthesis. Until recentely, it was thought that bacilysin formation was carried out by multiple-carrier thiotemplate mechanism. However, its biosynthesis mechanism was not fully coupled to non ribosomal peptide synthetase (NRPS) mechanism due to the fact that adenylation and thiolation were evident only for L-alanine, but not for L-anticapsin (Marahiel, 1997; von Döhren *et al.*, 1999). Besides, very recently *ywfE* was announced as a novel gene synthesizing L-amino acid ligase belonging to ATP-dependent carboxylate-amine/thiol ligase superfamily which is known to contain enzymes catalyzing the formation of various types of peptide.

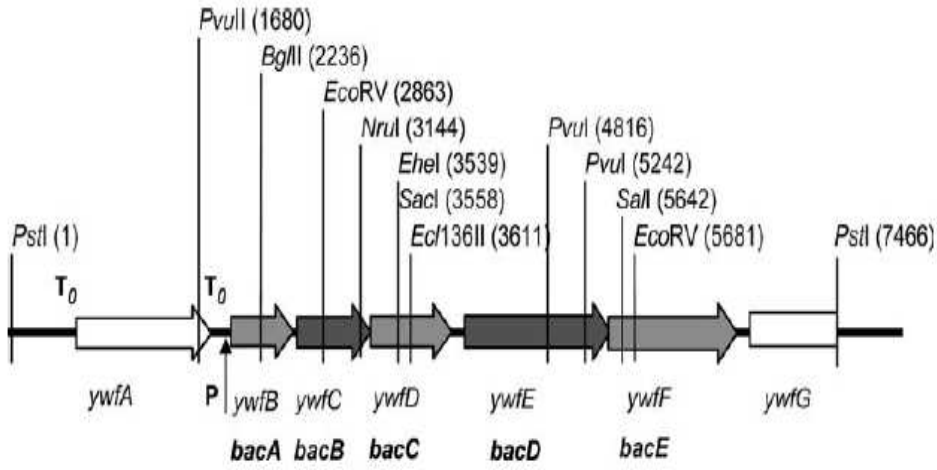



**Figure 1.4 :** The bacilysin gene cluster organisation, *bacABCDE,* relative to open reading frames *ywfABCDEFG* of *Bacillus subtilis* 168. DNA sequence is among 3875148–3867678 bp in the chromosome obtained from SubtiList database R16.1 (Kunst *et al*., 1997). Proposed terminator (T0) elements are indicated according the SubtiList database. Sigma A promoter (P) elements_35 (TTGACA) and _10 (TAAAATt) were detected 56 bp and 33 bp upstream of the ATG codon of the *bacA* gene (Steinborn *et al*., 2005).

Additionally, bacilysin formation is under dual regulation of guanosine 5'-diphosphate 3'-diphosphate (ppGpp) and guanosine triphosphate (GTP) affecting *bacABCDE* operon as a part of stringent response via Cod-Y mediated manner (Inaoka *et al.*, 2003). The CodY regulon encodes extracellular degradative enzymes, transporter proteins, catabolic enzymes, factors involved in genetic competence, antibiotic synthesis pathways, chemotaxis proteins, and sporulation proteins and those genes and/or operons are under negative regulation of CodY in the presence of excessive glucose or casamioacids (Ratnayake-Lecamwasam *et al.*,2001; Shivers and Sonenshein 2004). On the other hand, CodY regulatory protein also acts as a positive regulator by sensing intracellular level of GTP under carbon source or amino acid limitation conditions, then stringent response is activated. In the stringent response, the product of *relA* gene called as stringent factor (ppGpp synthetase) synthesizes hyperphosphorylated guanosine nucleotides [(p)ppGpp] from GTP, namely GTP is conversed to pppGpp and ppGpp. Accumulation of (p)ppGp leads to inhibition of the activity of IMP dehydrogenase, the first enzyme involved in GMP synthesis pathway, so that GTP production level is blocked (Cashel et al. 1996). This transient increase of (p)ppGpp results with induction of spore formation in $relA^{+}$ (stringent)

cells but not in *relA*$^-$ (relaxed) mutant cells (Ochi *et al.*, 1982; Ochi and Freese, 1983). Furthermore, this decrease in intracellular GTP level creates a signal for inactivation of CodY and activation of CodY-repressed genes whose products allow adaptation to nutrient depletion (Serror and Sonenshein, 1996; Ratnayake-Lecamwasam *et al.*, 2001; Inaoka *et al.*, 2003; Molle *et al.*, 2003).

Beside CodY, the pleiotropic AbrB protein is known to play a key role in regulating numerous transition state genes expressed at the onset of entry into stationary phase, at the end of exponential phase which is also involving antimicrobials production in *B. subtilis,* such as sublancin, subtilosin A, bacilysocin, bacilysin, the SdpC sporulation delay toxin, the SkfA sporulation killing factor, and the spore-associated antimicrobial polyeptide TasA (Zheng *et al.*, 1999; Stöver and Driks, 1999; Philips and Strauch, 2002; Karataş *et al.*, 2003; Stein 2005; Strauch *et al.*, 2007). Another regulatory protein, Spo0A, is phosphorylated by a complex phosphorelay system during the transition state (Burbulys *et al.*, 1991; Hoch, 1995;). Increased level of activated Spo0A~P by phosphorulation results with higher DNA-binding affinity to the *abrB* promoter and hence more efficient repression of abrB transcription (Strauch *et al.*, 1990; Klein and Marahiel, 2002). Dropping of intracellular level of AbrB below a critical threshold rate is resulted in relieving of AbrB-dependent repression

upon the production of the various antimicrobials, antibiotic and the other stationary phase-associated products (Strauch 1993; Strauch an Hoch, 1993).

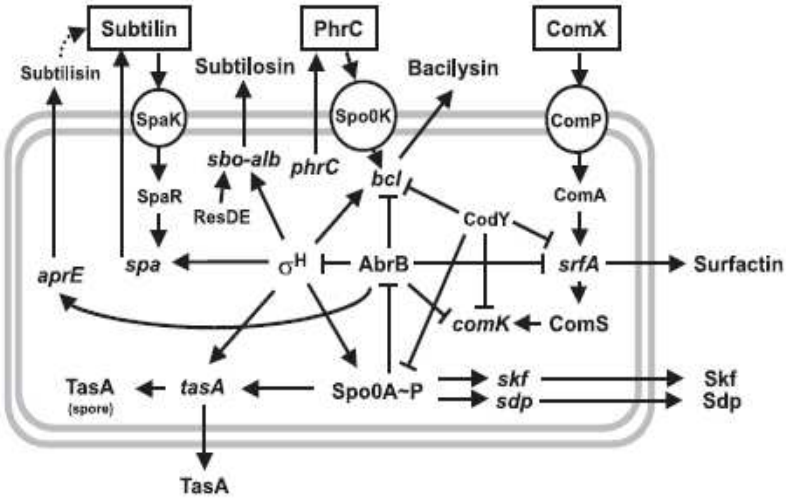



**Figure 1.5 :** Antibiotic biosynthesis pathway of *B. subtilis* involved in subtilin, subtilosin, surfactin and bacilysin. Skf acts as a the killing factor and TasA is the spore-associated antimicrobial polyeptide (Stein, 2005).

*srfA* is an operon required for the production of the lipopeptide antibiotic surfactin, competence development, and efficient sporulation in *Bacillus subtilis.* Expression of this operon is induced following the onset of stationary phase and regulated by specific regulatory genes *comP*, *comA*, and *spo0K*. Based on the location of *srf* in the regulatory pathway, it gives rise to ideas that it is an intermediate which senses an environmental condition and responds to it by transmitting a signal to the apparatus that controls the expression of genes involved in competence development and cellular differentiation (Roggiani *et al.*, 1990; Dubnau 1991; Nakano *et al.*, 1991). Repression of *srfA* operon in the presence of excess glucose and glutamine in the medium cues that its transcription is under nutritional regulation mechanism that requires CodY (Nakano and Zuber, 1989; Nakano *et al.*, 1991).

DegS and DegU are also sensor and effector proteins that form a two-component signal transduction regulatory system, transcribed from *sacU* locus (Stock *et al.*, 1989) and they are involved in the production of many types of commercially valuable degradative enzymes such as extracellular proteases, α-amylase, proteases, intracellular serine protease and levansucrase, etc. (Tanaka *et al.*, 1991).

DegS protein kinase also acts as a DegU phosphatase. DegU response regulator has two activities according to phosphorylation state; phosphorylated form which is necessary for degradative enzyme synthesis and nonphoshorylated form required for expression of genetic competence (Dahl *et al.*, 1992).

DNA-dependent RNA polymerase has central importance in bacterial gene expression of diverse regulatory mechanisms. This effect is supplied with the association of core RNA polymerase with alternative sigma (σ) factors which allow polymerase holoenzyme for recognition of different promoter and this alternativity regulates the pattern of gene expression in response to environmental, cell cycle, and morphological signals (Helmann and Chamberlin, 1988).

As an alternative subunit, Sigma-B ($\sigma^B$) responds through a complex, multibranched signal transduction pathway. Sigma-B control the transcription of operons which are responsible for diverse functions and a particular set of environmental conditions are required for its exppression such as, excessive heat, ethanol, salt, or acid (Price *et al.*,

2002). Alternative transcription factor Sigma-B of *Bacillus subtilis* controls a stationary-phase regulon induced under growth conditions that do not favor sporulation.

In addition to mentioned global regulatory genes, very recently, a novel gene, *yvfI*, is reported as required to bacilysin biosynthesis, which was identified by transposon mutagenesis method employed for disruption of potential bacilysin-related genes and screening loss of antibiotic activity against *Staphylococcus aureus* (Köroğlu *et al.*, 2008). *yvfI* is identified as encoding an unknown protein belonging to GntR familytranscriptional regulators, typically respond to metabolite effector molecules, consisted of conserved N-terminal domain that is involved in the DNA binding and C-terminal domain involved in the effector binding and/or oligomerization (Vindal *et al.*, 2007; Hoskisson *et al.*, 2006; Marchler-Bauer *et al.*, 2005). The diverge small molecules are triggered by these proteins and very distinct set of regulons are regulated (Marchler-Bauer *et al.*, 2005).

### 1.4. The Aim of The Present Study

The direct relation of *yvfI* expression with bacilysin biosynthesis raised the possibility that *yvfI* expression might be under the control of global regulatory circuits related with the competence development, sporulation and antibiotic production in *B. subtilis*. Therefore, the aim of the present study was focused on the identification of the effects of global regulatory genes *srfA*, *oppA*, *comA*, *phrC*, *phrF*, *phrK*, *comQ* (*comX*), *comP*, *spo0H*, *spo0A*, *abrB, codY*, *degU* and s*igB* on the expression of *yvfI* gene in *B. subtilis*.

# 2. MATERIALS AND METHODS

## 2.1. Materials

### 2.1.1 Bacterial Strains and Plasmids

*B. subtilis* PY79, a prototrophic derivative of standart strain *B. subtilis* 168, was used as wild type throughout this study. The strains and their genotypes that were used in the study are listed in Table 2.1. *E. coli* Top10F' [*lac*Iq TN10 (Tetr)}, *mcrA* Δ (*mrr hsdRMS-mrcBC*), *f80lacZΔM15 ΔlacX74, deoR, recA1, araD139* Δ(*ara-leu*)*7697, galU, galK, rsL,(strr), endA1, nupG*) was used as a host for cloning of *B. subtilis* chromosomal DNA.

### 2.1.2. Culture Media

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

### 2.1.3. Buffers and Solutions

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

### 2.1.4. Chemicals and Enzymes

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

**Table 2.1 :** Bacterial strains and their genotypes used in this study

| Strain | Genotype | Source |
|---|---|---|
| *Bacillus subtilis* PY79 | Wild type BPS cured protothropic derivative of *Bacillus subtilis* 168 | P.Youngman |
| TEK7 | *yvfI::LacZ::erm* | T. E. Köroğlu |
| TEK9 | ΔabrB::*cat*, *yvfI*::*lacZ*::*erm* | T. E. Köroğlu |
| TEK10 | ΔoppA::spc, *yvfI*::*lacZ*::*erm* | T. E. Köroğlu |
| TEK11 | Δ*spo*0H::*cat*, *yvfI*::*lacZ*::*erm* | T. E. Köroğlu |
| TEK12 | Δ*spo*0A::*cat*, *yvfI*::*lacZ*::*erm* | T. E. Köroğlu |
| KE10 | Δ*srfA*::*erm* | K. Appelman |
| JMS315 | *trpC2 pheA1* Δ*comQ*::*spc* | A. D. Grossman |
| BD1658 | Δ*comP*::*spc* | D. Dubnau |
| JRL192 | Δ*comA*::*cat* | A. D. Grossman |
| BAL373 | *trpC2 pheA1* Δ*abrB*::*cat* | A. D. Grossman |
| TMH307 | *trpC2 unkU*::*spc* Δ*codY* | A. D. Grossman |
| CAL7 | Δ*phrK7*::*spc* | A. D. Grossman |
| JMA163 | Δ*phrF163*::*cat* | A. D. Grossman |
| JMS751 | Δ*phrC*::*erm* | A. D. Grossman |
| CU741 | Δ*degu*::*kan* | A. D. Grossman |
| ML6 | Δ*ML6::cm (sigma B)* | A. D. Grossman |
| ECU1 | *yvfI::lacZ::erm* Δ*spo0A::*Δ*abrB::cm* | This study |
| ECU2 | *yvfI::lacZ::erm* Δ*codY::*Δ*abrB::cm* | This study |
| ECU3 | *yvfI::lacZ::erm* Δ*comQ::cm* | This study |
| ECU4 | *yvfI::lacZ::erm* Δ*comP::spc* | This study |
| ECU5 | *yvfI::lacZ::erm* Δ*phrK::spc* | This study |
| ECU6 | *yvfI::lacZ::erm* Δ*phrF163::cm* | This study |
| ECU7 | *yvfI::lacZ::erm* Δ*ML6::cm (sigmaB)* | This study |
| ECU8 | *yvfI::lacZ::erm* Δ*deg U::kan* | This study |
| ECU11 | *yvfI::lacZ::erm* Δ*comA::cm* | This study |
| ECU12 | *yvfI::lacZ::erm* Δ*phrC::erm* | This study |
| ECU13 | *yvfI::lacZ::erm* Δ*srfA::erm* | This study |
| ECU14 | *yvfI::lacZ::erm* Δ*codY::spc* | This study |
| *E. coli* Top10F' | *lac*Iq TN10 (Tetr)}, *mcrA* Δ (*mrr-hsdRMS-mrcBC*), *f80lacZ*Δ*M15* Δ*lacX74, deoR, recA1, araD139* Δ(*ara-leu*)*7697, galU, galK, rsL,(strr), endA1, nupG* | M.A. Marahiel |

### 2.1.5. Maintenance of Bacterial Strains

*B. subtilis* strains were grown in Luria-Bertani (LB) liquid medium and kept on Luria-Bertani (LB) agar plates at cool (+4 °C). *E. coli* strains were kept on Luria-Bertani (LB) agar plates (+4 °C). All cultures were subcultured monthly. 10 % LB glycerol stock was prepared for each strain and kept at -80°C.

### 2.1.6. pDrive Cloning Vector

pDrive cloning vector is supplied by a QIAGEN in a linear form with a 3' and 5' U overhangs, also carrying *amp* and *kan* resistance genes and it is constructed to be used for direct-cloning of PCR products that were generated by non-proofreading DNA polymerases just like *Taq* Polymerase. Additionally, blue-white colony screening is possible while using this vector for cloning purposes. pDrive also contains several unique restriction endonuclease recognition sites around the cloning site that allows easy restriction analysis of recombinant plasmids. T7 and SP6 promoters on either sides of the cloning site are important in order to carry out transcription of cloned PCR products, as well as sequence analysis. (http://www1.qiagen.com/HB/CRCloning).

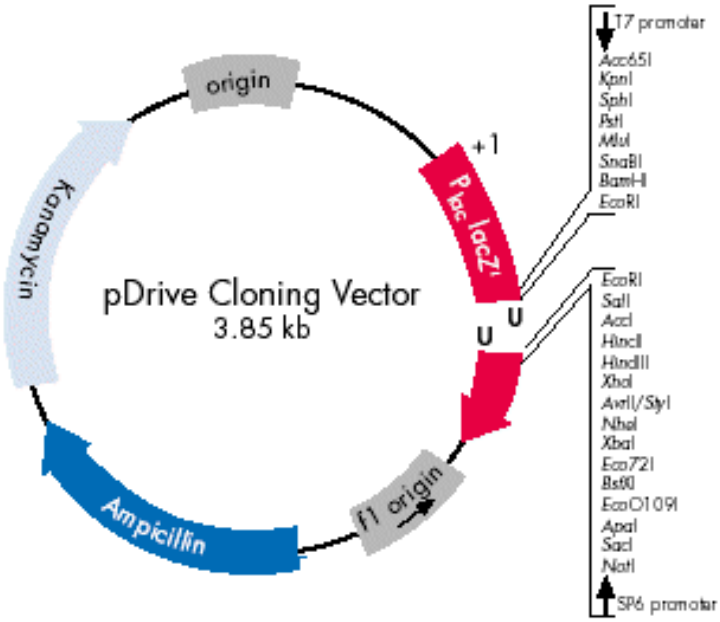



**Figure 2.1 :** Genomic map of pDrive Cloning Vector including the functional genes in the structure as well as the restriction map (www1.qiagen.com/literature/pDrive/pcr_cloning21.pdf).

### 2.1.7. pMUTIN T3 Cloning Vector

pMutin T3 is used for insertional gene inactivation in *B. subtilis* in order to characterize unknown open reading frames and to observe transcripional changes of constructed strains. This vector is a 8834 bp long plasmid and carries a reporter *lacZ* gene allowing to measure gene expression through β-galactosidase enzyme activity. Also pMUTIN T3 plasmid has an inducible promoter, Pspac, which can be induced by IPTG while normally suppressed by product of *lacI* gene. Besides, the plasmid carries *amp* and *erm* resistance genes expressed in *E.coli* and *B. subtilis*, respectively.

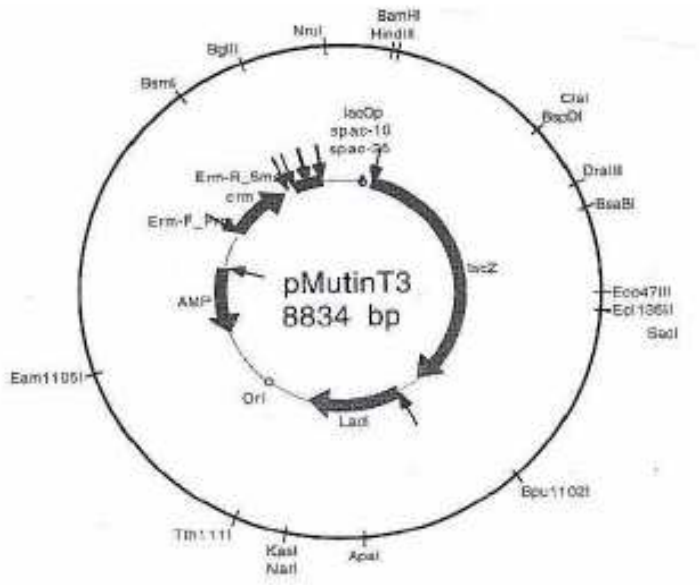



**Figure 2.2 :** Genomic map of pMUTIN T3 vector showing the restriction map and the functional genes (Vagner *et al.*, 1998).

### 2.1.8. pDR66 Cloning Vector

pDR66 including *cm* resistance gene is used for co-transformation of *B. subtilis* cell during transformation period to make easy selection of transformants which have same antibiotic resistance gene with competent cell.

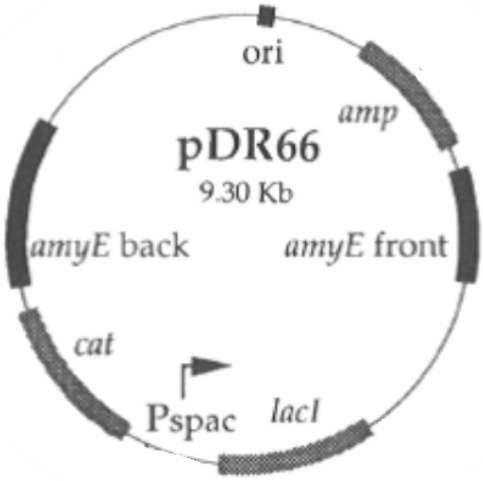



**Figure 2.3 :** Shematic presentation of 9,3 kb pDR66 vector used for transformation due to $Cm^R$ ($Cat^R$) region facilitating the selection of mutant strains (Ireton *et al.*, 1993).

## 2.2. Methods

### 2.2.1. DNA Techniques and Manipulation

#### 2.2.1.1. Plasmid DNA Isolation

Qiagen Plasmid Purification Mini and Midi Kits (Qiagen Inc., Valencia, CA) were mostly used for isolation of *E. coli* plasmid DNA as specified by the manufacturers.

Bacterial cells were harvested by centrifugation at 13.000 rpm for 5 minutes. After removing supernatant, the pellet was resuspended in 300 µl P1 buffer (Appendix B). The pellet an buffer mixed complately by vortexing. Following step, 300 µl P2 (Appendix B) buffer was added and solution mix was then incubated at room temperature for 5 minutes. After incubation, 300 µl P3 (Appendix B) buffer was added and mixed through inverting the tubes until the lysate is no longer viscous.The sample was incubated for 15 minutes on ice. Then centrifuged at 13.000 rpm for 15 minutes. Supernatant was transferred to a new 1.5 ml eppendorf tube and plasmid DNA was precipitated following the addition of 0,7 volume isopropanol and collected by centrifugation at 13.000 rpm for 30 minutes. Therefore obtained pellet was washed with 1 ml of 70% ethanol. Ethanol was dried out of 37 °C for 15 minutes after removing the supernatant. Lastly, the pellet was dissolved in 15 µl elution buffer (EB) at 37°C and 200 rpm, and stored at -20°C. The isolated DNA was run on 0,8 % agarose gel.

#### 2.2.1.2. Chromosomal DNA Isolation

Chromosomal DNA of *B. subtilis* strains was isolated and purified by using a standart procedure devised for *Bacillus* species (Cutting and Horn, 1990).

Overnight culture (1.5 ml) was harvested by centrifugation at 13000 rpm for 5 minutes. After discarding the supernatant obtained the pellet was resuspended in 567 µl of TE buffer (Appendix B) by repeated vortexing. Ten µl of proteinase K (20 mg/ml), 6 µl of RNase (10 mg/ml), 24 µl of lysozyme (100 mg/ml) and 30 µl of 10% SDS were added one by one and the solution mix was incubated for 1 hour at 37°C in a water bath or in a termomixer. In the following step, 100 µl of 5M NaCl solution was added and the sample was mixed by inverting the tubes without vortexing until the mucosal white substance can be seen. After then, 80 µL of CTAB/NaCl (Appendix B). The solution was added into the mixture and it was incubated for 10 minutes in 65°C water bath or termomixer. Freshly prepared phenol/chloroform/isoamyl alcohol (25:24:1) was then added to the mixture with the same volume of solution for extraction and it was centrifuged at 13000 rpm for 10 minutes. In the last step of isolation, the upper phase was transferred to a new 1.5 ml microfuge tube and 0.7 volume isopropanol was added. After up-down for 5-6 times, the sample was centrifuged at 13000 rpm for 15 minutes. The supernatant was removed and the pellet was washed with 1 ml 70% ethanol and centrifuged at 13000 rpm for 5 minutes. Subsequently, the pellet was dried at 37°C for 1 hour and dissolved in 10 µl of TE buffer. Obtained chromosomal DNA was stored at 4°C. Finally, the isolated DNA was made run on 0.6% agarose gel.

#### 2.2.1.3. Polymerase Chain Reaction (PCR)

The oligonucleotide primers were purchased from OPERON, Co. (Table 2.2). PCR was performed using Taq polymerase 10x reaction buffer from Roche. All cycles lasted for 1 minute. The denaturation temperature was 94°C and the extention temperature was 72°C. The annealing temperature for the first 5 cycles was 55°C and 60°C for the next 25 cycles. The concentration of chromosomal DNA was 0.01 to 0.001 ng/µl. The oligonucleotide primers were used at 1 - 10 pM (equimolar) and deoxyribonucleoside 5'triphosphates (dNTPs) were used at a final concentration of 2 mM.

Also the other oligonucleotide primers given in Table 2.2 used for confirmation of deletions.

**Table 2.2 :** Sequences of oligonucleotide primers (OPERON, Co.)

| **Primer** | **Oligunucleotide Sequence** |
|---|---|
| *yvfI* HindIII Forward | 5'-GCC AAG CTT ATG AAA CAG GGA GAA GGC-3' |
| *yvfI Bam*HI Reverse | 5'-GCG GAT CCG AAT ATC CCG AAA GCA CAT-3' |
| *srfA* Forward | 5'-TAT TTG TAC AGG GTC CGC CG-3' |
| *srfA* Reverse | 5'-AAG CAG CTT CTC TTT CTC CGC-3' |
| *phrC Pst*I Forward | 5'-GCC CTG CAG GCG GTC TCC ACA TTT GAA AGC-3' |
| *phrC Bam*HI Reverse | 5'-CGG GGA TCC TAG AAA GTA GGA AGC AGA CAG-3' |
| *codY Nco*I Forward | 5'-CGG CCA TGG GTA TGG CTT TAT TAC AA-3' |
| *codY Bam*HI Reverse | 5'-GCC GGA TTC ATG AGA TTT TAG ATT TT-3' |

**Table 2.3 :** List of materials used for preparation of PCR mixture (www.roche-applied-science.com/pack-insert/4738250a.pdf).

| **Content of PCR Mixture** | **Amount** |
|---|---|
| Forward primer | 1 µl |
| Reverse primer | 1 µl |
| 10x Buffer (- $MgCl_2$) | 5 µl |
| $MgCl_2$ | 5 µl |
| dNTP Mix ( 10 mM) | 1 µl |
| Template DNA | 1 µl |
| Taq polymerase | 0,5 µl |
| $dH_2O$ | 35,5 µl |

A master mix composed of the materials listed below (Table 2.3) was prepared. Then, the master mix was divided into separate PCR tubes and 1 µl of chromosomal DNA of *Bacillus subtilis* PY79 was added into each tube as template DNA. Finally, 0.5 µl of Taq polymerase was added separately.

#### 2.2.1.4. Agarose Gel Electrophoresis

According to basis of the purpose of electrophoresis, different concentration of agarose gels were used, which were given in Table 2.4. Electrophoresis was carried out on a horizontal submarine electrophoresis apparatus and in a neutral gel system composed of 1% agarose gel containing 1xTAE buffer (Appendix B) and ethidium bromide of a 0.2 µg/mL final concentration. Loading dye (6X) was added into the samples. Electrophoresis was performed at 90-120 Volts for 20-30 minutes. The DNA bands were visualized on a shortwave UV transilluminator (UVP) and photographed by using Gel Imaging System. *EcoR*1 digested lambda DNA marker (Appendix D) was used to determine the molecular weights of DNA bands.

**Table 2.4 :** Agarose gel concentration for different samples

| Sample | Concentration |
|---|---|
| Chromosomal DNA | 0,6 % |
| Plasmid DNA | 0,8 % |
| Digestion products of plasmid | 1 % |
| PCR products | 1.5 % - 2 % |

#### 2.2.1.5. Gel Extraction

The desired fragments were extracted from the gel by using a Qiaquick Gel Extraction kit (Qiagen Inc., Valencia, CA). The gel slice containing the DNA band was excised from the gel and DNA extraction from gel was performed according to the Qiagen's instructions. After obtaining DNA, an aliquot was run on agarose gel to monitore the DNA concentration.

#### 2.2.1.6. Ligation of PCR Products into pDrive Cloning Vector

Ligation of PCR products to pDrive Vector was performed as follows: Ligation master mix buffer (5 µl), 1 µl (50 ng/µl) pDrive vector, 2 µl insert DNA (PCR product) and 2 µl $dH_2O$ was added and total volume was completed to 10 µl. Reaction mixture was incubated at 16°C for 1.5 hours then ligase was denaturated at 70°C for 10 minutes. After ligation was completed, the mixture was used for transformation into *E.coli* Top10F'.

#### 2.2.1.7. Ligation of pMutinT3 Vector

Ligation procedure for cloning into the pMutinT3 vector was carried out using 9.5 µl of *yvfI* PCR products as insert fragments and 0.5 µl of pMutinT3 vector. Vector and fragment were mixed in an eppendorf tube and incubated for 5 min at 65°C. Then, the tube was cooled on ice. Following cooling step, 2 µl of ligation 10x buffer, 2 µl of Polyethylene glycol (50% PEG 8000), 2 µl of T4 DNA ligase, 4 µl of $dH_2O$ were added into the same eppendorf tube. Finally, the mixture was again centrifuged for a short spin and incubated at 4°C for 16 h.

#### 2.2.1.8. Restriction Enzyme Digestion

Digestion reactions were carried out as the constructerer's manufacture, in a way that the amount of 10X digestion buffer was 1/10 of the total reaction mix. The reaction mix was incubated for 1-4 hours at 37°C, then enzyme denaturation at 65°C for 10 minutes. The sample was stored at -20°C when was needed.

### 2.2.2. Transformation

#### 2.2.2.1. Preparation of *E.coli* Electrocompetent Cells and Transformation of Electrocompetent *E.coli* Top10F' Cells

The overnight inoculum of *E.coli* Top10F' was diluted 1:100 fold into 400 ml 2xYT medium which was contain Tetracyclin (20 µg/ml). Then incubated at 37°C with 250 rpm by shaking until $OD_{600}$ (Optical Density at 600 nm) reached to 0,6. Then incubated cells were stayed on ice for 30 minutes and after cold incubation period cells were harvested by centrifugation at 5000 rpm for 5 minutes. Subsequent to discard supernatant, tha pellet was resuspended in 40 ml of cold distilled water and centrifuged at 5000 rpm for 15 minutes. Supernatant was removed and pellet was resuspended in 20 ml cold distilled water and centrifuged at 5000 rpm for 15 minutes

again. Lastly, supernatant was discarded and pellet was resuspended in 1 ml of cold steril 10 % glycerol. After then, it was dispensed into aliquots of 40 µl into eppendorf tubes. Aliquots were frozen immediately by immersing within liquid nitrogen and stored at -80°C.

For transformation, competent *E. coli* cells were incubated on ice shortly. Ligation products (10 µl) or 0.5 µg of appropriate plasmid DNA was added into the tube of competent cell and were mixed by hand gently. Then, mix was transferred into pre-cold electroporation tube. The sample was placed onto electroporation device and the process was carried out at 1800V. After addition of 1ml LB broth into the mixture, it was transferred into a 2 ml eppendorf tube. Incubation was made at 37°C for 60 minutes by shaking at 200 rpm. After incubation, the cells were centrifuged at 5000 rpm for 10 min and supernatant was discarded. Obtained pellet was resuspended in 100 µl 85 % saline solution (NaCl)(Apendix B). Transformed cells were plated on selective medium containing appropriate antibiotic (100µg/ml ampicillin). For the purpose of blue-white colony selection, they were plated on LB agar media containing 40 mg/ml X-gal, 1 mM IPTG and 100µg/mL ampicillin.

#### 2.2.2.2. Preparation of *B. subtilis* Competent Cells and Transformation

Preparation of *B. subtilis* competent cells and transformation were performed as described by Klein *et al.* (1992). HS and LS (Appendix B) mediums were used for the preparation of *B. subtilis* competent cells. Appropriate amount of *B. subtilis* cells were inoculated into 3 ml of HS medium and it was cultured overnight at 37°C by shaking at 250 rpm. 0,5 ml of this overnight inoculum was transformed into 20 ml of freshly prepared LS medium and incubated at 30°C by shaking at 200 rpm until $OD_{600}$ reached to 0,55. Then, 1 ml of competent cells was transferred into 2 ml eppendorf tube inwhich contained 1 µl of chromosomal DNA or suitable plasmid was added. The cells were then incubated at 37°C for 2 hours by shaking at 200 rpm and incubated cells were harvested by centrifugation at 5000 rpm for 10 minutes. Lastly, the pellet was resuspended in 100 µl of sterile saline solution and was spread out on LB agar plates containing selective antibiotic and incubated at 37°C for 16 h.

#### 2.2.2.3. Induction of MLS Gene

Competent *B. subtilis* cells were incubated in HS medium until $OD_{600}$ reached to 0,55 by the same conditions given the Section 2.2.2.2 and then incubated for 2 hours.

In the overlay step, firstly, 2 µl of 2 mg/ml erythromycin antibiotic solution was added into a small test tube containing 2,5 ml % 0.7 LB agar which was melted and kept in 50°C. Then, immediately, all amount of transformed cells were added into same tube, mixed quickly and were poured onto an antibiotic-free LB agar plate containing 25 ml LB agar. Following incubation at 37°C for 2 hours, a second overlay was performed. This second overlay has contained 2.5 ml of melted % 0.7 LB agar, 20 µl (2 mg/ml) erythromycin antibiotic solution and 20 µl (50 mg/ml) lincomycin antibiotic solution. The agar plate was incubated at 37°C for 1-2 days.

**2.3. Beta-Galactosidase Activity Assay**

Initially, all strains were grown overnight at 37°C in PA medium (Appendix A). Then, they were used to inoculate 100 ml of PA medium to an initial optical density of about 0,1 at 595 nm ($OD_{595}$). The cultures were incubated at 37°C shaking at 250 rpm for 30 h. 1 ml of culture was collected as double eppendorf tubes for each hour for checking up of assay. If it was needed, suitable dilution was made for measurement.

After each sampling, the culture was centrifuged at 13000 rpm for 5 minutes and the supernatant was discarded. Then, the pellet was washed with 500 µl of ice-cold 25 mM Tris-Cl (pH 7.4) by centrifugation. Following, the removal of the supernatant, the pellet was resuspended in 650 µl Z-buffer by vortex and 170 µl of lysozyme was added. Later, the solution was vortexed for a second and incubated at 37°C for 5 min. Subsequently, the samples were taken on ice and 9 µl of 10% Triton-X 100 was added. After vortexing for a while, the extracts were incubated at 30°C for 5 min. Subsequent to this, 200 µl of ONPG solution was added, incubated at 30°C and the solution was watched for the progression of the yellow color. Following, the reaction was stopped by the addition of 1 M $Na_2CO_3$ and reaction time was recorded. Reaction time refers to a period that was started by adding ONPG and continued until formation of the yellow color, and so this period was recorded. At last, the samples were centrifuged at 13000 rpm for 5 min. and the supernatant was taken to measure $A_{420}$ and $A_{550}$ sepearately. Calculations for β-galactosidase activity were carried out according to the formulation (Miller, 1972) below and a graph for both the β galactosidase activity and log $OD_{595}$ was drawn seperately.

$$\text{Miller units} = \frac{A_{420} - (1.75 \times A_{550})}{\text{Reaction time (min)} \times OD_{595}} \times 1000 \qquad \textbf{(2.1)}$$

## 3. RESULTS AND DISCUSSION

### 3.1. Construction of *yvfI* Insertional Plasmid

To achieve integration of pMUTIN T3 vector, firstly, a *yvfI* gene fragment, 508 bp in lenght, was amplified using chromosomal DNA of *Bacillus subtilis* PY79 wild type strain as a template by PCR. The primers *yvfI* Forward 5' GCCAAGCTTATGAAACAGGGAGAAGGC 3' and *yvfI* Reverse 5' GCGGATCCGAATATCCCGAAAGCACAT 3' including extra residues for *BamH*I and *Hind*III recognition sites were used for the amplification of *yvfI* gene fragment. Hence, a total of 525 bp PCR product was obtained by PCR (Fig. 3.1).

The amplified PCR product was purified after running in agarose gel. Extracted fragment was ligated into pDrive Cloning Vector (Qiagen) and transformed into *E. coli* Top10F' electro-competent cells. Suitable plasmid carrying 525 bp insert within pDrive vector was selected and the sequence analysis was performed. The deduced nucleotide sequence data was compared to *B. subtilis* genome sequence, using Blast search, at National Center for Biotechnology Information (NCBI) database.

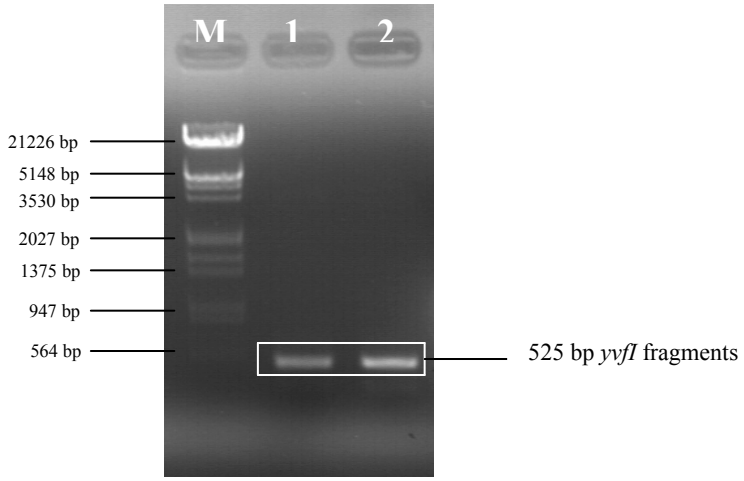



**Figure 3.1 :** 1-2; PCR products of 525 bp *yvfI* gene fragment amplified with spesific primers *yvfI* F and *yvfI* R and M; Marker 3: Lambda DNA / *Eco*RI+*Hind*III (Appendix D).

This recombinant pDrive and pMUTIN T3 vectors were digested with the same restriction enzymes, *BamH*I and *Hind*III, and were run in agarose gel. Following that *yvfI* fragment from pDrive and digested pMUTIN T3 were extracted from gel and these were ligated overnight with T4 ligase. Transformation of ligation product into *E. coli* Top10F' competent cells was carried out. Transformants were selected on 100 µg/ml ampicillin containing agar plates. Plasmid DNAs were isolated from the selected colonies, which were expected to carry the recombinant pMUTIN T3 plasmid, and run on agarose gel in order to verify the expected size (9359 bp). Then, one of the suitable sized plasmids was selected and digested with *BamH*I and *Hind*III restriction enzymes for confirmation of existing of *yvfI* fragment in the pMUTIN T3 vector (Figure 3.2A).

In order to verify the existence of *yvfI* fragment and also *erm* casette, the selected recombinant plasmid was used as template in the PCR with specific primers and expected bands were obtained on agarose gel as can be seen in the Figure 3.2B.

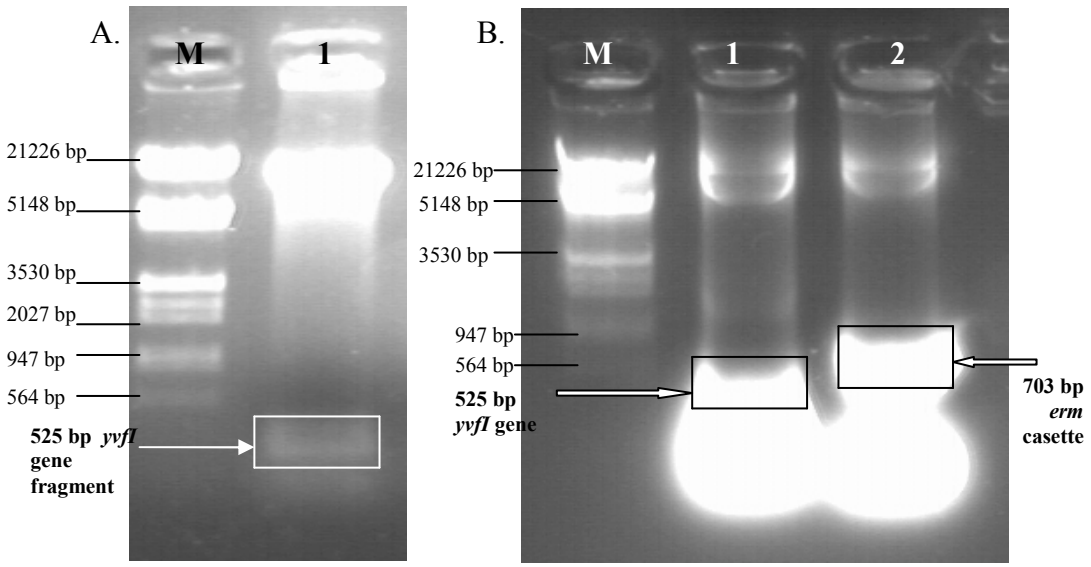



**Figure 3.2 :** The confirmation of *yvfI* gene cloning into pMutinT3 vector. A: *Hind*III ve *Bam*HI double digestion of recombinant plasmid (1), B: PCR products for *yvfI* gene (1) and *erm casette* (2) amplified using DNA of recombinant plasmid as template, M: Marker 3: Lambda DNA / *Eco*RI+*Hind*III.

### 3.2. Construction of *yvfI*::*lacZ* Transcriptional Fusion in *B. subtilis*

The selected recombinant pMUTIN T3 plasmid carrying 525 bp *yvfI* fragment was transformed into *B. subtilis* PY79 wild type strain in order to construct *yvfI*::*lacZ*::*erm* transcriptional fusion in its chromosome. The integration was as a result of a single cross-over event at *yvfI* locus, in the other name Campbell-like insertion.

The selection according to erythromycin resistance on LB plates containing erythromycin (1µg/mL) could not be fully performed because of the generation of the spontaneous erythromycin resistant *B. subtilis* PY79 cells. In order to eliminate this problem, an alternative selection method, Macrolide-Lincosamide-Streptogramin B-resistance ($MLS^R$) conferred by *erm* gene, was used. Chromosomal DNA of the resultant $MLS^R$ transformant was isolated and screened with PCR analysis by using primers specific to *erm* resistance gene on pMUTIN T3 in order to confirm the insertion of the plasmid into the *B. subtilis* chromosome. 703 bp *erm* gene was amplified by using the chromosomal DNA of $MLS^R$ *B. subtilis* transformant as template (Fig 3.3). The corresponding $MLS^R$ *B. subtilis* PY79 transformant was designated as TEK7.

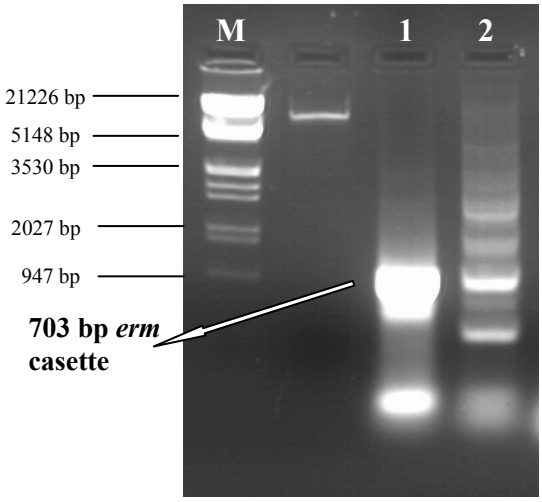



**Figure 3.3 :** The confirmation of *yvfI::lacZ::erm* in *B. subtilis* chromosome. 1: PCR product amplified with specific primers to *erm* resistance gene within pMutinT3 vector using chromosomal DNA of *yvfI::lacZ::erm* mutant as template; 2: PCR product amplified with specific primers to *yvfI* gene using chromosomal DNA of *yvfI::lacZ::erm* mutant as template; M: Marker 3: Lambda DNA / *Eco*RI+*Hind*III.

### 3.3. Expression of Transcriptional *yvfI*::*lacZ* Fusion in PA Medium

In order to compare the expression level of *yvfI* both in *yvfI*::*lacZ* mutant strain TEK7 and wild type *B. subtilis* PY79, as control, cells were grown in PA medium, which is a defined medium stimulating bacilysin production, at 37° C with 200 rpm and samples were collected at every hour till the end of thirty hours, and also growth of cells was monitored. Collected cells was assayed for β-galactosidase assay, using ONPG as substrate, enzyme activity was calculated. The results of expression of *yvfI*::*lacZ* fusion and *B. subtilis* PY79 wild type strain were compared as can be seen in the Figure 3.4. As growth of both strains were similar, expression of *yvfI* in TEK7 mutant strain was increased in the exponential phase slowly, then was increased in the transition state between exponential and stationary phase steadily and reached to its maximal level (detected as 62 Miller units) upon entry into stationary phase (Fig. 3.4).

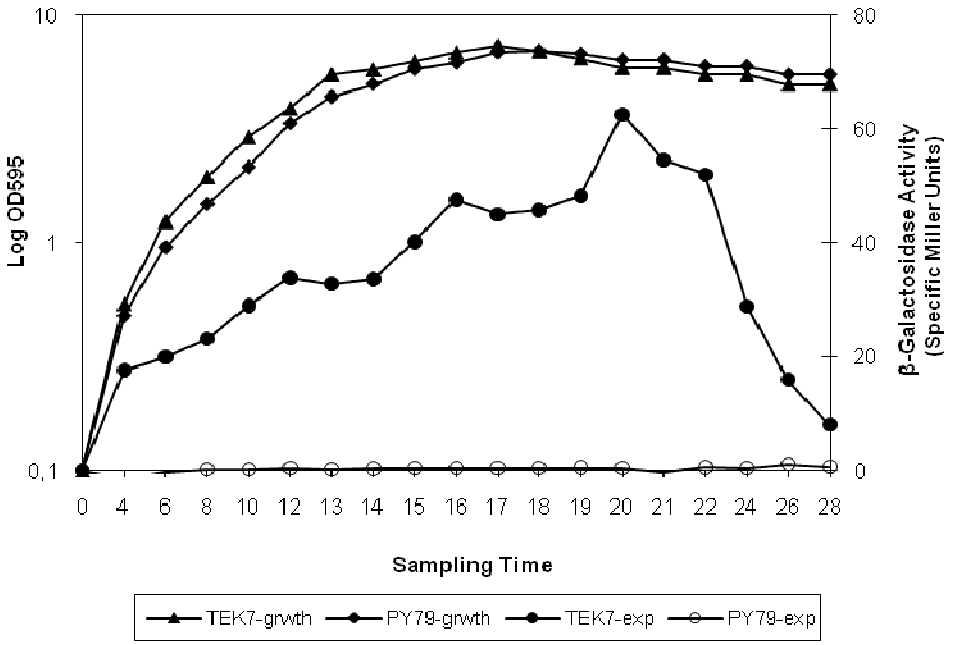



**Figure 3.4 :** Growth and bacilysin activity of *B. subtilis* PY79 and TEK7 (*yvfI*::*lacZ*::*erm*) strains grown in PA medium. Specific Miller Units was calculated with formula as denoted in Section 2.3 (2.1). The symbols used for the strains are; (○) PY79 (wild type) and (●) TEK7 (*yvfI*::*lacZ*::*erm*).

### 3.4. Deletion of regulatory Genes and Their Effects on the Expression of *yvfI* gene in *B. subtilis*

#### 3.4.1. Deletion of *srfA* Gene and Its Effect on the Expression of *yvfI* Gene in *Bacillus subtilis*

*srfA* operon is consisted of four modular open reading frames (ORFs); ORF1 (*srfA*-A),ORF2 (*srfA*-B), ORF3 (*srfA*-C) and ORF4 (*srfA*-D). It is an important operon needed for the efficient development of genetic competence which is regulated by ComA response regulatory protein. The operon is normally expressed during active growth at a low level, just before the transition to stationary phase, its transcription is increased sharply dependent on *comA*, *spo0K* and *comQ* activity (Van Sinderen *et al.*, 1990, Weinrauch *et al.*, 1991; Cosby *et al.*, 1998). Null mutations of those three genes resulted with decreased expression level of *srf.* Therefore, it seems as *srf* operon might be an intermediate component of the regulatory pathway (Hahn and Dubnau, 1991).

To monitore the effect of *srfA* knockout profile, *srfA* gene was disrupted in the transcriptional *yvfI*::*lacZ* fusion strain TEK7. For this purpose, Δ*srfA*::*erm comS*$^{+}$ mutant derivative of *B. subtilis* was used as competent cell, then was co-transformed with chromosomal DNA of TEK7 and pDR66 plasmid (used to facilitate selection on agar plates via *cm* antibiotic casette region. Transformants were selected with resistance to Erm(1µg/ml), Ln (25µg/ml) and Cm (20 µg/ml) antibiotics and then were screened with PCR using specific primers (Table 2.2). Lastly selected mutant was named as ECU13 (*yvfI*::*lacZ*::*erm* Δ*srfA*::*erm*).

The resulting strain ECU13 and TEK7, as control strain, were cultured in PA medium and were sampled in 1 h intervals for the β-galactosidase assay. Growth curves and β-galactosidase activities were shown in Figure 3.5 and in the Figure 3.6, respectively.

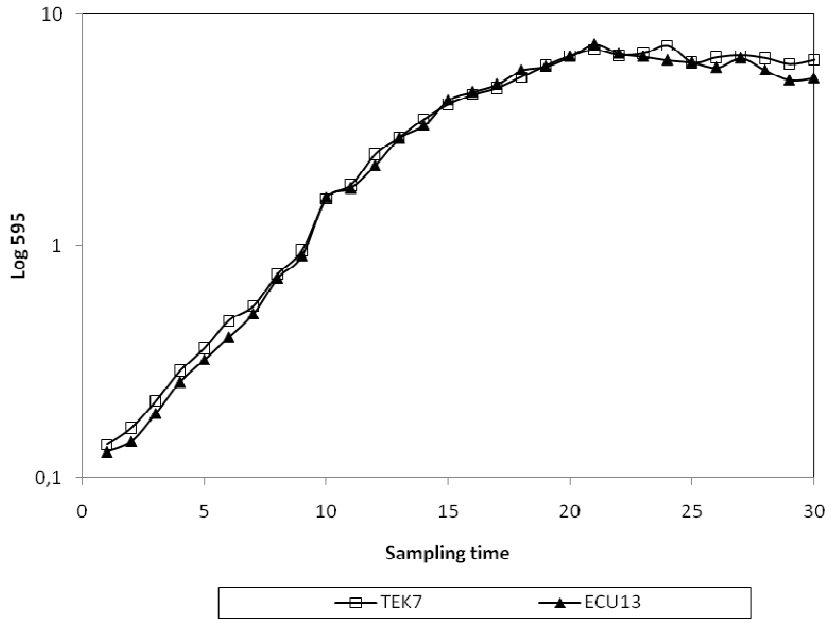



**Figure 3.5 :** Growth curves of mutant strains grown in PA medium. The symbols used for the strains are; (□) TEK7 (*yvfI*::*lacZ*::*erm*) and (▲) ECU13 (*yvfI*::*lacZ*::*erm* Δ*srfA*::*erm*).

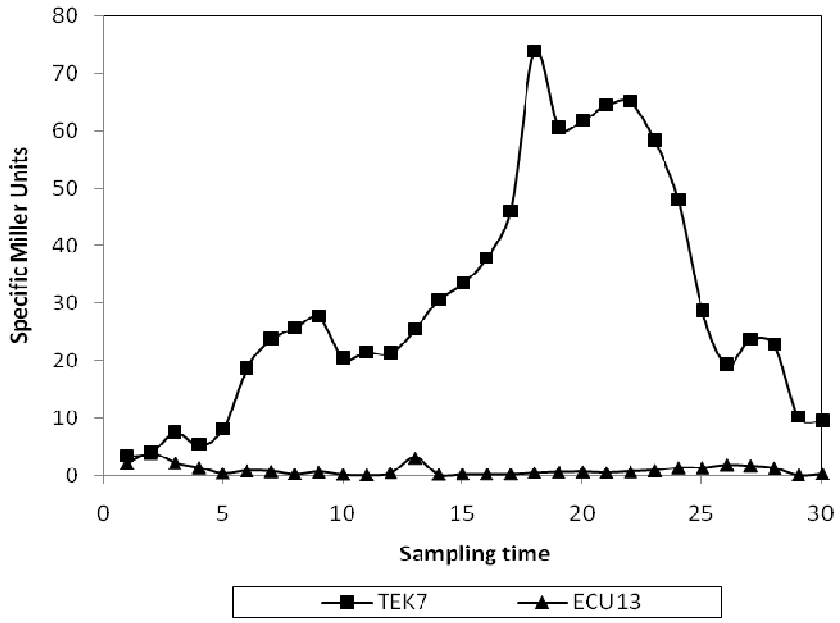



**Figure 3.6 :** β-Galactosidase activities of mutant strains grown in PA medium and their effects on *yvfI-lacZ* expression. Specific Miller Units was calculated with formula as denoted in Section 2.3. The symbols used for the strains are; (■) TEK7 (*yvfI*::*lacZ*::*erm*) and (▲) ECU13 (*yvfI*::*lacZ*::*erm* Δ*srfA*::*erm*).

As shown in Fig. 3.6, in the absence of *srfA* gene product, *yvfI* gene expression was completely disrupted. Nakano *et al.* (1991) revealed that 5' half of the *srfA* operon was essential for surfaction formation, sporulation and genetic competence, but 3' half of *srfA* operon was not required for genetic competence. So it was predicted that *srfA* gene products act in *B. subtilis* cell specialization and differentiation. In 2003, it was shown that *srfA* disrupted mutants were not able to produce bacilysin, hence *srfA* might be effective on bacilysin biosynthesis (Yazgan-Karataş *et al.,* 2003). In the present study, it was found that, *srfA* was also directly effective on yvfI expression as bacilysin biosynthesis.

### 3.4.2. Deletion of *oppA* Gene and Its Effect on the Expression of *yvfI* gene *B. subtilis*

The *spo0K* (opp) is an operon encoding oligopeptide permease, ATP-binding casette transporter. Five proteins OppA, OppB, OppC, OppD, and OppF which are products of this operon, are required for uptake of oligopeptides, development of genetic competence, and initiation of sporulation in *Bacillus subtilis* (LeDeaux *et al.*, 2006). Exported inhibitory peptides are accumulated in the culture supernatant and then they are imported by the oligopeptide permease (Lazazzera *et al.*, 1997). Firstly OppA binds to oligopeptide substrate and delivers it to the rest part of the operon (Perego *et al.*, 1991). Null mutations on *spo0KABCD* have proven that they are required for mentioned cell responses, but disruption of *spo0KE* caused a less severe phenotype than disruption of other genes of the operon (LeDeaux *et al.*, 1997).

In order to analyze the deletion effect of *oppA* gene on *yvfI* expression, it was deleted in TEK7 (*yvfI*::*lacZ*::*erm*) by transforming the competent cells of TEK7 with the chromosomal DNA of the Δ*oppA*::*Tn10(spc)* mutant strain AK3. Then, selection of transformants on agar plate containing Spc (100µg/ml) was performed. Selected mutant was named as TEK10 (*yvfI*::*lacZ*::*erm* Δ*oppA*::*spc*),

The resulting strain TEK10 and TEK7, as the control, were cultured in PA medium and were sampled in 1 h intervals for the β-galactosidase assay. Growth profiles were shown in Figure 3.7 and β-galactosidase activities were shown in the Figure 3.8.

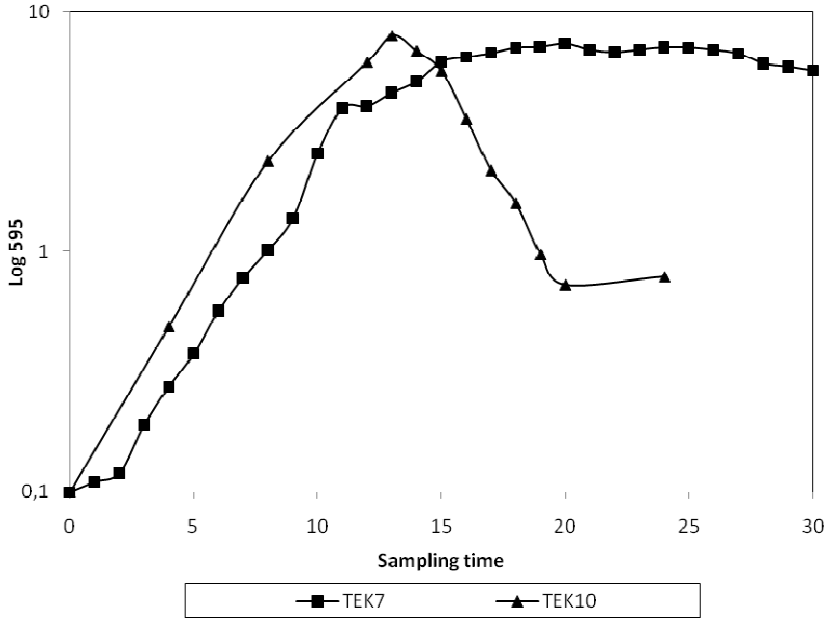



**Figure 3.7 :** Growth curves of TEK7 (*yvfI*::*lacZ*::*erm*) and TEK10 (*yvfI*::*lacZ*::*erm* Δ*oppA*::*spc*) strains grown in PA medium. The symbols used for the strains are; (■)TEK7 (*yvfI*::*lacZ*::*erm*) and (▲) TEK10 (*yvfI*::*lacZ*::*erm* Δ*oppA*::*spc*)

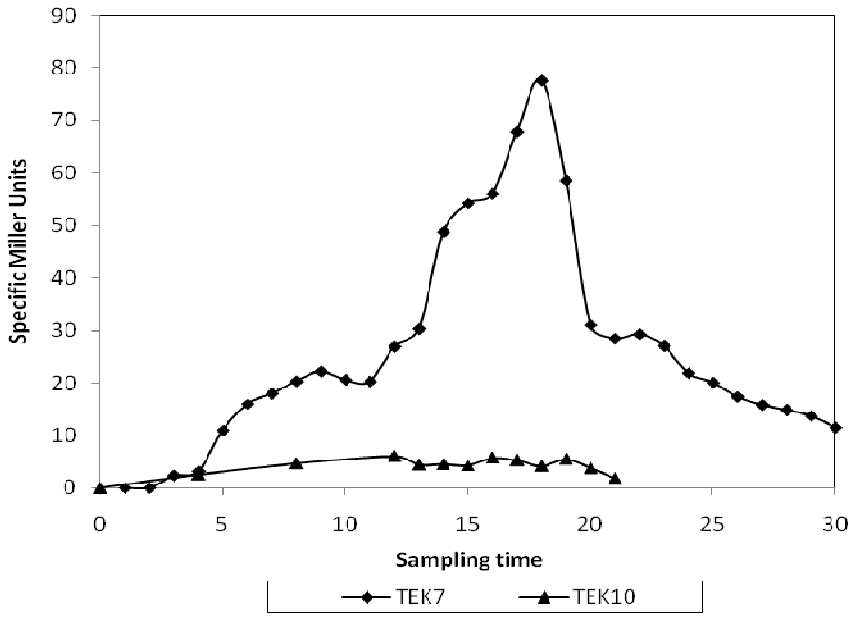



**Figure 3.8 :** β-Galactosidase activities of TEK7 and *oppA*-deleted mutant strain grown in PA medium and effect of deletion on *yvfI-lacZ* expression. The symbols used for the strains are; (♦) TEK7 (*yvfI*::*lacZ*::*erm*) and (▲) TEK10 (*yvfI*::*lacZ*::*erm* Δ*oppA*::*spc*).

Fig. 3.8 shows that *yvfI-lacZ* expression was severely disrupted in absence of *oppA* gene product both during exponential and stationary phases. It should be noted that distruption of *oppA* was resulted in the same effect on the biosynthesis of bacilysin. As being a key component of QS, *oppA* mutation creates a loss of bacilysin biosynthesis, it was suggested that bacilysin biosynthesis is under the global quorum sensing mechanism or a component of this mechanism. (Yazgan *et al.*, 2001). So, we can propose that *yvfI* expression can be regulated crucially with *oppA*, as a part of quorum sensing mechanism.

A family of phosphatases (Rap) and cognate regulators (Phr) modulate the two-component signal transduction systems in *Bacillus subtilis.* Phosphatases are encoded together with their negative regulators, pentapeptides in the other name pheromone peptides. This operon system also includes a binding site for alternative subunit of RNA polymerase (Perego *et al.*, 1994; Perego and Hoch, 1996).

The effects of deletion mutations on *phrC*, *phrF*, and *phrK* was studied by Achtung *et al.* (2006), and it was indicated that those peptides stimulate ComA-dependent gene expression within different level and in the absence of all three peptides the ComA-dependent genes could not be fully expressed and the involvement of ComA and PhrC in bacilysin biosynthesis was shown (Yazgan et al., 2001). Therefore Opp-imported other pheromones PhrF and PhrK as well as PhrC are considered to have function in *yvfI* expression and thus the expression of *yvfI-lacZ* expression was examined in the PhrC,PhrF and PhrK mutant backgrounds, as shown in the following sections.

### 3.4.3. Deletion of Phr Peptides Genes (*phrC, phrK, phrF*) and Their Effects on the Expression of *yvfI* gene in *B. subtilis*

In order to examine the effects of blockage of *phrC*, *phrK*, and *phrF* genes on *yvfI*::*lacZ* fusion mutant strain TEK7, ECU5 (*yvfI::lacZ::erm* Δ*phrK::spc*), ECU6 (*yvfI::lacZ::erm* Δ*phrF163::cm*) mutant strains were constructed seperately via transformation of TEK7 mutant strain with JMS751 (Δ*phrC*::*erm)*, CAL7 (Δ*phrK7*::*spc)* and JMA163 (Δ*phrF163*::*cat)* chromosomal DNAs, respectively. For deletion of *phrC* gene in TEK7 (*yvfI*::*lacZ*::*erm*), the competent cells of Δ*phrC*::*erm* PY79, supplied by Grossman, was co-transformed with chromosomal DNA of TEK7 and pDR66 plasmid and selection was made depending on the resistance to

Erm(1µg/ml), Ln (25µg/ml) and Cm (20 µg/ml) antibiotics. One of transformants was selected and named as ECU12 (*yvfI::lacZ::erm* Δ*phrC::erm*). Also deletion mutations were confirmed by PCR analysis using specific primers (Table 2.2). Resulting strains were named as ECU5 (*yvfI::lacZ::erm* Δ*phrK::spc*) and ECU6 (*yvfI::lacZ::erm* Δ*phrF163::cm*) mutant strains were constructed in the sanme prosedure.

The resulting strains ECU12, ECU5, ECU6 and TEK7 were cultured in PA medium and the specific activities of the samples were measured. Also growth of mutants was followed (Fig. 3.9, 3.10, 3.11, 3.12).

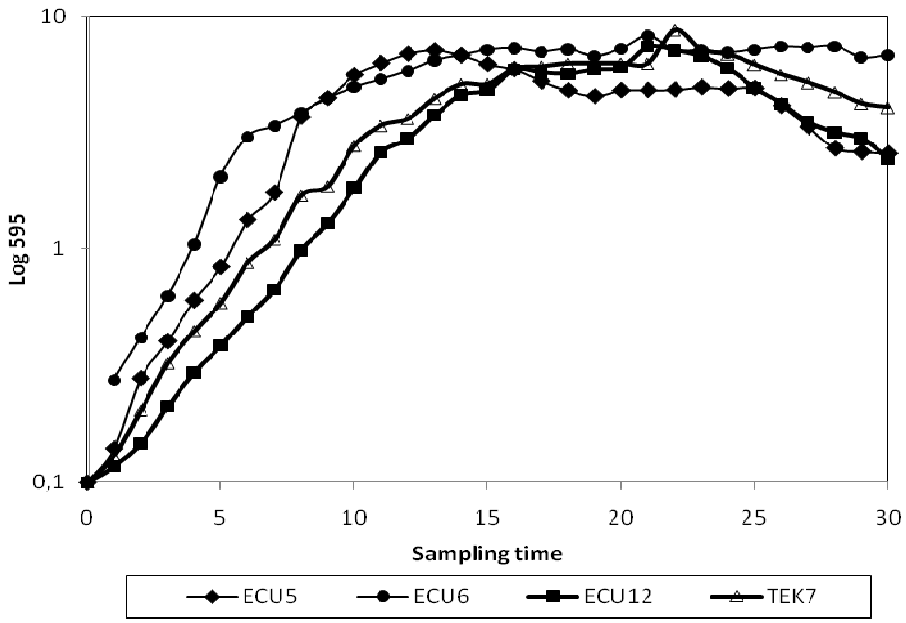



**Figure 3.9 :** Growth curves of TEK7 (*yvfI::lacZ::erm*), ECU5 (*yvfI::lacZ::erm* Δ*phrK::spc*), ECU6 (*yvfI::lacZ::erm* Δ*phrF163::cm*) and ECU12 (*yvfI::lacZ::erm* Δ*phrC::erm*) strains grown in PA medium. The symbols used for the strains are; (Δ) TEK7 (*yvfI::lacZ::erm*), (♦) ECU5 (*yvfI::lacZ::erm* Δ*phrK::spc*), (●) ECU6 (*yvfI::lacZ::erm* Δ*phrF163::cm*) and (■) ECU12 (*yvfI::lacZ::erm* Δ*phrC::erm*).

Deletion results of *phrC, phrK* and *phrF163* genes are shown in the following figures (Fig. 3.10, Fig. 3.11 and Fig 3.12).

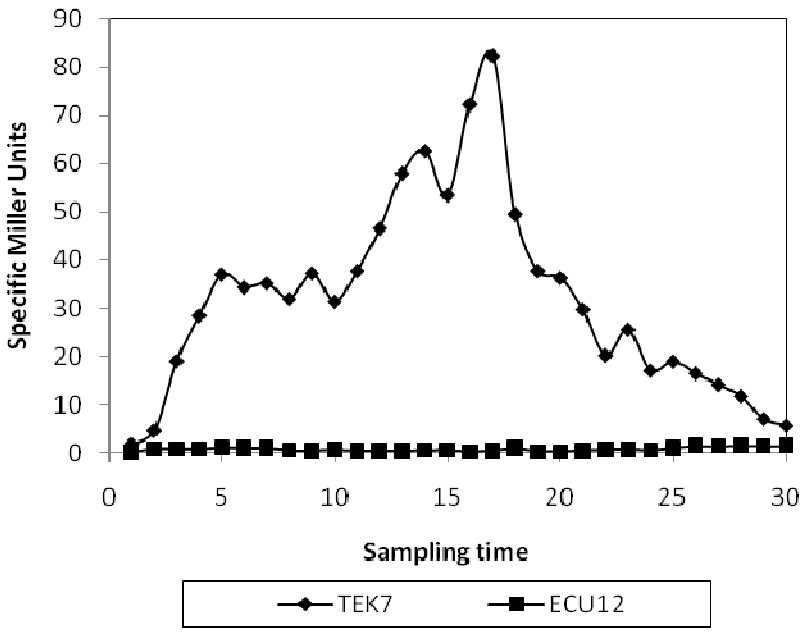



**Figure 3.10 :** β-Galactosidase activities of TEK7 and *phrC*-deleted mutant strain grown in PA medium and effect of deletion on *yvfI-lacZ* expression. The symbols used for the strains are; (♦) TEK7 (*yvfI*::*lacZ::erm*) and (■) ECU12 (*yvfI::lacZ::erm* Δ*phrC::erm*).

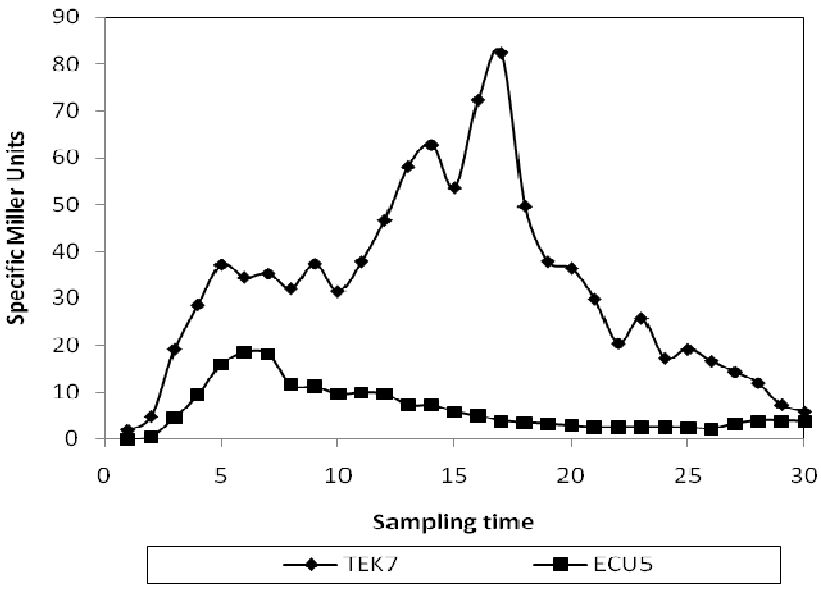



**Figure 3.11 :** β-Galactosidase activities of TEK7 and *phrK*-deleted mutant strain grown in PA medium and effect of deletion on *yvfI-lacZ* expression. The symbols used for the strains are; (♦) TEK7 (*yvfI*::*lacZ::erm*) and (■) ECU5 (*yvfI::lacZ::erm* Δ*phrK::spc*).

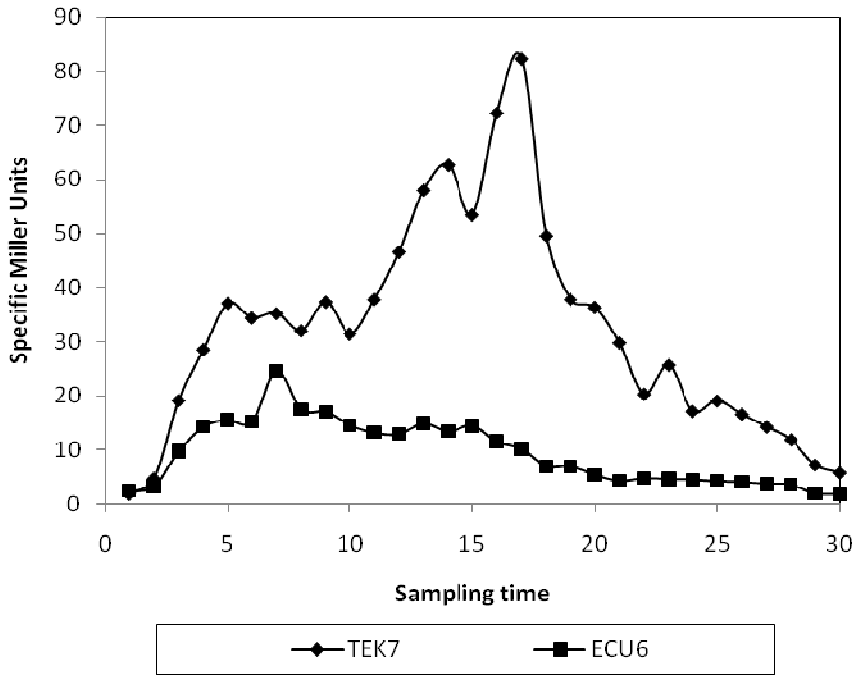



**Figure 3.12 :** β-Galactosidase activities of TEK7 and *phrF163*-deleted mutant strain grown in PA medium and effect of deletion on *yvfI-lacZ* expression. The symbols used for the strains are; (♦) TEK7 (*yvfI*::*lacZ::erm*) and (■) ECU6 (*yvfI::lacZ::erm* Δ*phrF163::cm*).

As shown in Fig 3.10, Fig. 3.11 and Fig. 3.12, the deletion mutation of *phrC*, *phrF* and *phrK* genes affected *yvfI*::*lacZ* expression seriously. Therefore, our results indicate that the Phr C, F and K peptides are required for the regulation of *yvfI* expression supposingly by enhancing the full activation of transcriptional factor ComA~P as in the case of bacilysin biosynthesis.

### 3.4.4. Deletion of *comQ(comX), comP, comA* and *spo0H* and Their Effects on the Expression of *yvfI* gene in *B. subtilis*

The *comQ*, *comX*, *comP* and *comA* genes are clustered, in this order, on the chromosome of *B. subtilis*. The upstream gene *comQ* is needed for producing a modified peptide pheromone in the system (Kleerebezem *et al.*, 1997).

*Bacillus subtilis* can use excreted peptide signals to mediate quorum sensing and to activate the transcription factor ComA (Lazazzera *et al.*, 1999; Bongiorni *et al.*, 2005). ComA is part of a two-component regulatory system and is activated by phosphorylation via membrane-bound sensor histidine-protein kinase, ComP (Weinrauch *et al.*, 1990; Core and Perego 2003).

ComX pheromone is an extracellular signaling peptide that activating the autophosphorylation of ComP and hence, activates the ComA transcriptional regulator in order to control expression of varied quorum-responsive genes, such as *srfA*, *degQ*, *rapA* and *rapC* (Magnuson *et al.*, 1994; Solomon *et al.*, 1995; Lazazzera and Grossman, 1998).

Spo0H($\sigma^H$) encoded by *spo0H* is an alternative subunit for RNA polymerase and involves in many cellular responses. It is known that the expression of the *phr* genes is controlled via sigma-H(*spo0H* gene product)-dependent promoter (P2) located at upstream of *phrC* and internal to *rapC* and most likely contribution of the effects of cell density on sporulation, also genetic competence, because of such events are more effective at high cell density as a result of accumulated peptides (Lazazzera *et al.*, 1999). Defects in production of some of signaling peptides in *spo0H* mutants are indicated that several cellular responses are modulated by population density and by controlling effect of Sigma-H on the expression of the *phr* genes by sensing extracellular peptides (Rudner *et al.*, 1991; Ireton et al., 1993; Lazazzera et al., 1997; McQuade *et al.*, 2001).

*comQ*, *comP, comA* and *spo0H* genes were examined whether they effect on *yvfI* expression or not. For this purpose, *comQ*, *comP, comA* and *spo0H* genes were insertionally deleted in TEK7. TEK7 (*yvfI*::*lacZ*::*erm*) was used as competent cell and transformed with chromosomal DNAs of corresponding null mutants. Resulting transformants were named as ECU11 (*yvfI*::*lacZ*::*erm* Δ*comA*::*cat*), ECU3 (*yvfI*::*lacZ*::*erm* Δ*comQ*::*spc*), ECU4 (*yvfI*::*lacZ*::*erm* Δ*comP*::*spc*), and TEK11 (*yvfI*::*lacZ*::*erm* Δ*spo0H*::*cat*).

The resulting strains ECU3, ECU4, ECU11, TEK11 and TEK7 were cultured in PA medium and the specific β-galactosidase activities of the samples were measured while growth profiles of mutants was followed (Fig. 3.13, 3.14, 3.15, 3.16, 3.17).

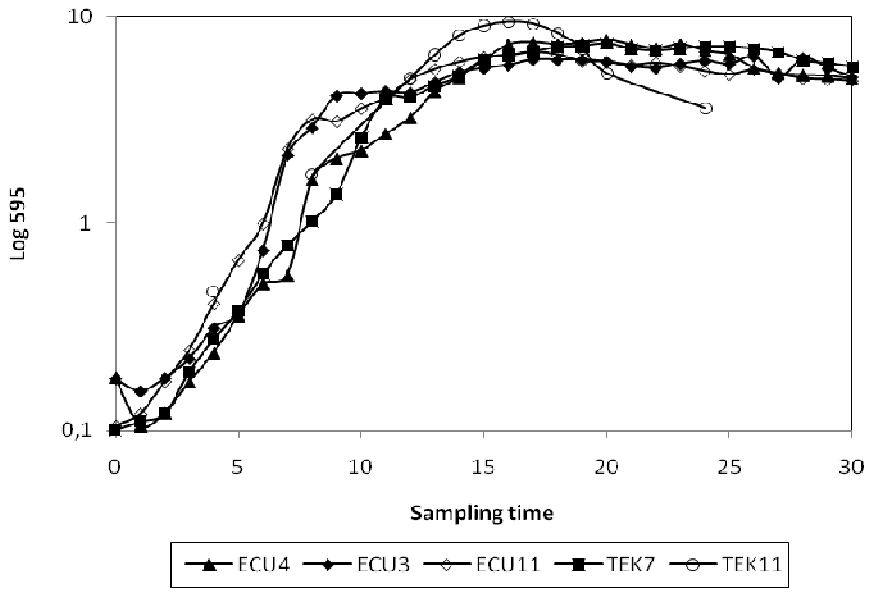



**Figure 3.13 :** Growth curves of TEK7 (*yvfI::lacZ::erm*), ECU3 (*yvfI*::*lacZ*::*erm* Δ*comQ*::*spc*), ECU4 (*yvfI*::*lacZ*::*erm* Δ*comP*::*spc*), ECU11 (*yvfI*::*lacZ*::*erm* Δ*comA*::*cat*) and TEK11 (*yvfI*::*lacZ*::*erm* Δ*spo0H*::*cat*) strains grown in PA medium. The symbols used for the strains are; (■) TEK7 (*yvfI::lacZ::erm*), (♦) ECU3 (*yvfI*::*lacZ*::*erm* Δ*comQ*::*spc*), (▲) ECU4 (*yvfI*::*lacZ*::*erm* Δ*comP*::*spc*), (◊) ECU11 (*yvfI*::*lacZ*::*erm* Δ*comA*::*cat*) and (○) TEK11 (*yvfI*::*lacZ*::*erm* Δ*spo0H*::*cat*).

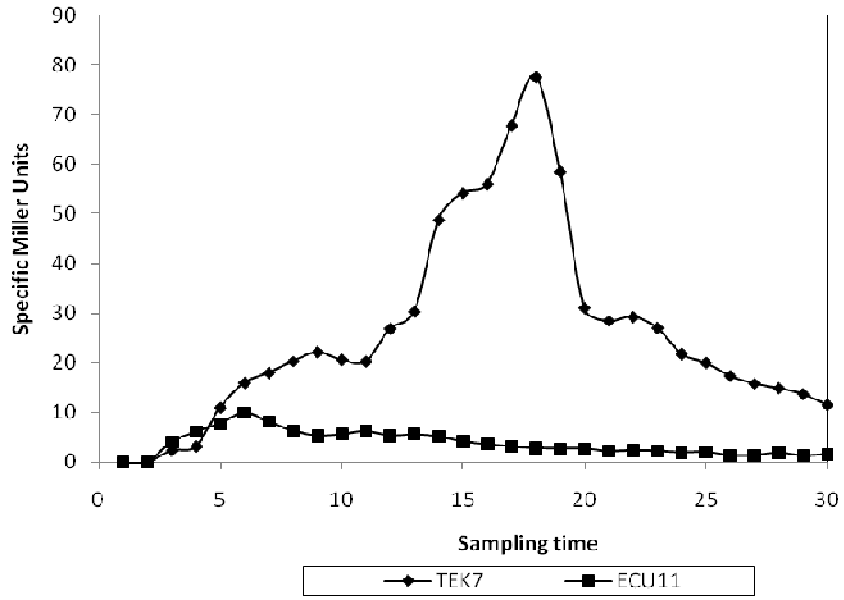



**Figure 3.14 :** β-Galactosidase activities of TEK7 and *comA*-deleted mutant strain grown in PA medium and effect of deletion on *yvfI-lacZ* expression. The symbols used for the strains are; (♦) TEK7 (*yvfI*::*lacZ::erm*) and (■) ECU11 (*yvfI::lacZ::erm* Δ*comA*::*cat*).

Results of deletion of *comP, comQ* and *spo0H* genes are given in the following figures (Fig. 3.15, Fig. 3.16 and Fig. 3.17).

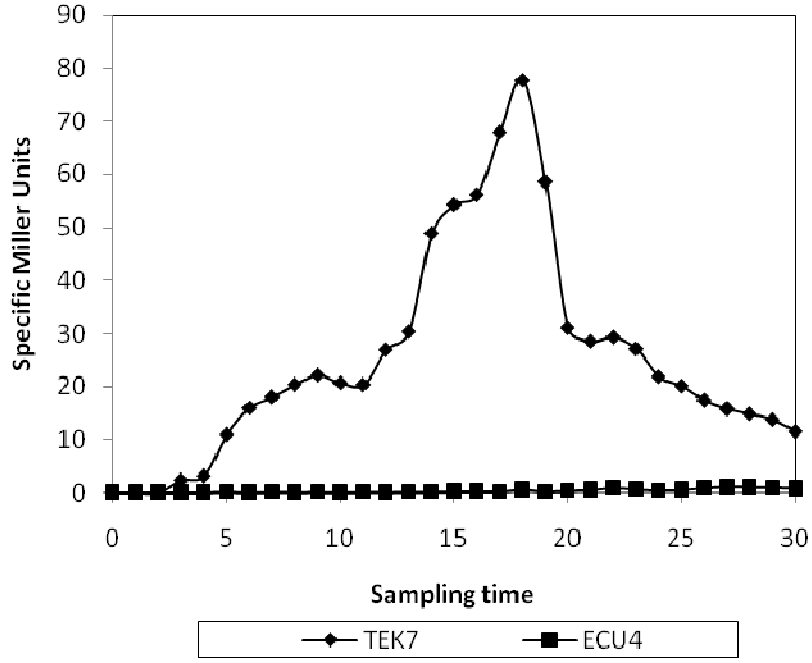



**Figure 3.15 :** β-Galactosidase activities of TEK7 and *comP*-deleted mutant strain grown in PA medium and effect of deletion on *yvfI-lacZ* expression. The symbols used for the strains are; (♦) TEK7 (*yvfI*::*lacZ*::*erm*) and (■) ECU4 (*yvfI*::*lacZ*::*erm* Δ*comP*::*spc*).

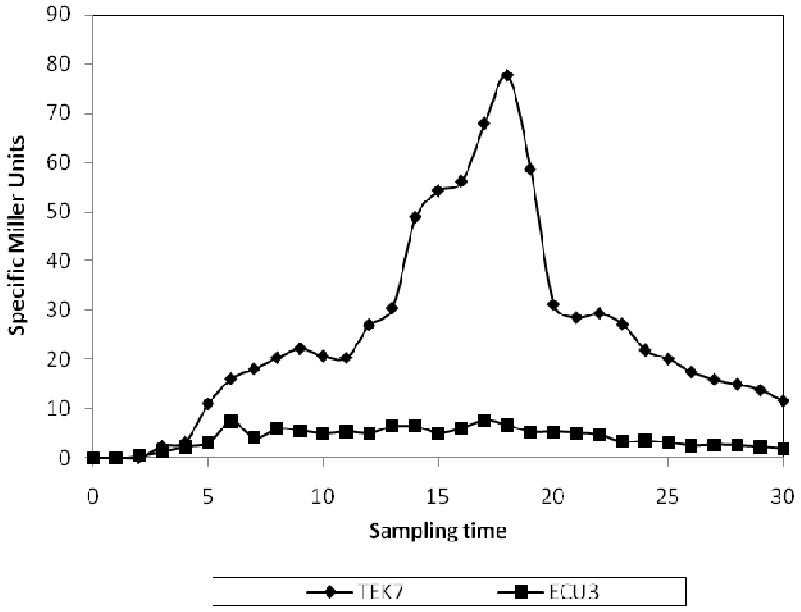



**Figure 3.16 :** β-Galactosidase activities of TEK7 and *comQ*-deleted mutant strain grown in PA medium and effect of deletion on *yvfI-lacZ* expression. The symbols used for the strains are; (♦) TEK7 (*yvfI*::*lacZ*::*erm*) and (■) ECU3 (*yvfI*::*lacZ*::*erm* Δ*comQ*::*spc*)

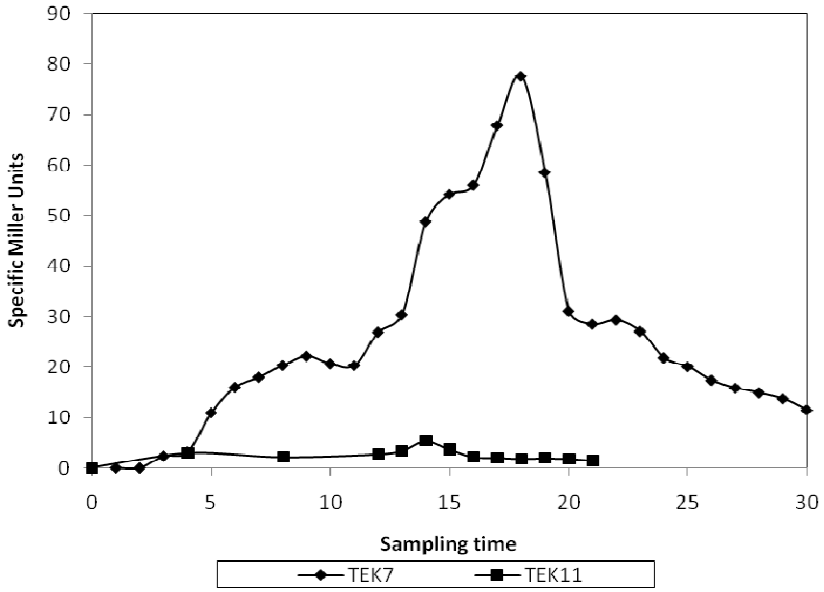



**Figure 3.17 :** β-Galactosidase activities of TEK7 and *spo0H*-deleted mutant strain grown in PA medium and effect of deletion on *yvfI-lacZ* expression. The symbols used for the strains are; (♦) TEK7 (*yvfI*::*lacZ*::*erm*) and (■) TEK11 (*yvfI*::*lacZ*::*erm* Δ*spo0H*::*cat*).

As shown in Fig. 3.14, Fig 3.15, Fig. 3.16 and Fig. 3.17, *yvfI* activity was impaired in the deletion of *comA*, *comP*, *comQ* and *spo0H* genes and *yvfI*::*lacZ* expression was repressed completely. ComQ was put forth as required for correct processing of ComX pheromone, so that ComQ effects expression of *srfA* and transformability via ComX (Magnuson *et al.*, 1994). ComP and ComX were required for signal transduction system that activate ComA and also $\sigma^H$ required for *phr* gene expression during transition state initiating signal transduction cascade for ComA~P-dependent gene expression as quorum response. In this point, it would not be wrong to suggest that expression of *yvfI* gene was regulated in a ComA-dependent manner and probably, the products of these genes might regulate bacilysin biosynthesis through affecting the expression of *yvfI* gene.

### 3.4.5. The Effects *spo0A* and *abrB* Null Mutations on the Expression of *yvfI gene* in *B. subtilis*

Spo0A is the master regulatory protein which is effective on the expression of over 500 genes the early stages of stationary phase, directly or indirectly in *Bacillus subtilis* genome (Molle *et al.*, 2003). Particularly disruption on *spo0A* in *B. subtilis* is resulted with blockage of sporulation and exhibition pleiotropic effects on the

expression of a wide range genes associated with the transition state (Strauch *et al.*, 1990).

A wide variety of antibiotics and antifungals are synthesized and secreted by *Bacillus* species upon depletion of nutrients and entry into stationary phase (Zuber *et al.*, 1993). In *spo0A* disrupted mutants of *Bacillus subtilis,* the synthesis of antimicrobials and also resistanca against other antibiotics were found to be deficient. These effects of *spo0A* mutations were associated with AbrB global regulator protein which is repressed by Spo0A (Trowsdale *et al.*, 1978; Staruch *et al.*, 1990; Strauch, 1993).It was indicated that the Spo0A protein binds to a specific region of the *abrB* gene located downstream from the transcriptional start sites and functions as a repressor of transcription in vitro (Staruch *et al.,* 1990).

To analyze the effects of *spo0A* and *abrB* null mutations, *spo0A* and *abrB* genes were inactivated in TEK7, the transcriptional *yvfI*::*lacZ* fusion strain, separately. For this knockout mutation, TEK7 competent cells were used for the transformation of chromosomal DNAs of Δ*spo0A*::*cat* mutant and Δ*abrB*::*cat* mutant derivatives. The selected mutants with regard to specific antibiotic resistance were named as TEK9 (*yvfI*::*lacZ*::*erm* ΔabrB::*cat*), TEK12( *yvfI*::*lacZ*::*erm* Δ*spo0A*::*spc*) and ECU1( *yvfI*::*lacZ*::*erm* Δ*spo0A*::Δ*abrB*::*cm*). Additionally the expression of *yvfI*::*lacZ* fusion was analyzed in *spo0A-abrB* double mutant genetic background for detection of the effect of absence of both repressor proteins that repress different genes in sequential stages. *spo0A-abrB* double mutant strain ECU1 (*yvfI*::*lacZ*::*erm* Δ*spo0A*::*spc* Δ*abrB*::*cat*) was constructed by transforming the competent cells of TEK9 with chromosomal DNA of the Δ*abrB*::*cat* mutant strain of *B. subtilis.* Transformants were selected on agar plates containing antibiotics Cm (5 µg/ml) and Spc (100µg/ml). In addition, this double mutant strain was performed on DSM (Appendix A) agar plate in order to detect sporulation ability.

The resulting strains ECU1, TEK9, TEK12 and TEK7 as the control were cultured in PA medium and were sampled in 1 h intervals. Collected samples were further assayed for β-galactosidase activity (Fig. 3.19, 3.20, 3.21). At the same time growth profiles of mutants were analyzed (Fig. 3.18).

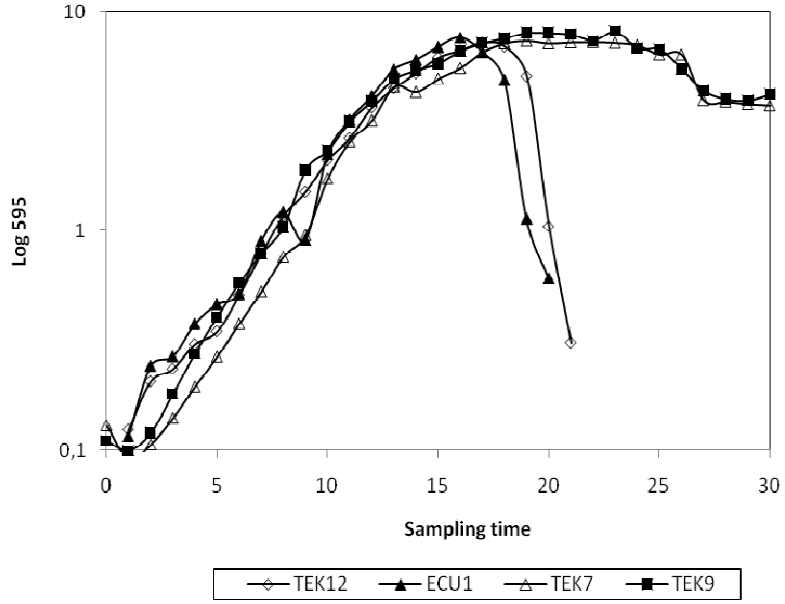



**Figure 3.18 :** Growth curves of TEK7 (*yvfI::lacZ::erm*), TEK9 (*yvfI*::*lacZ*::*erm* ΔabrB::*cat*), TEK12 (*yvfI*::*lacZ*::*erm* Δ*spo0A*::*spc*) and ECU1 (*yvfI::lacZ::erm* Δ*spo0A*::Δ*abrB*::cm) strains grown in PA medium. The symbols used for the strains are; (Δ) TEK7 (*yvfI::lacZ::erm*), (▲) ECU1 (*yvfI::lacZ::erm* Δ*spo0A*::Δ*abrB::cm*), (■) TEK9 (*yvfI*::*lacZ*::*erm* ΔabrB::*cat*) and (◊) TEK12 (*yvfI*::*lacZ*::*erm* Δ*spo0A*::*spc*).

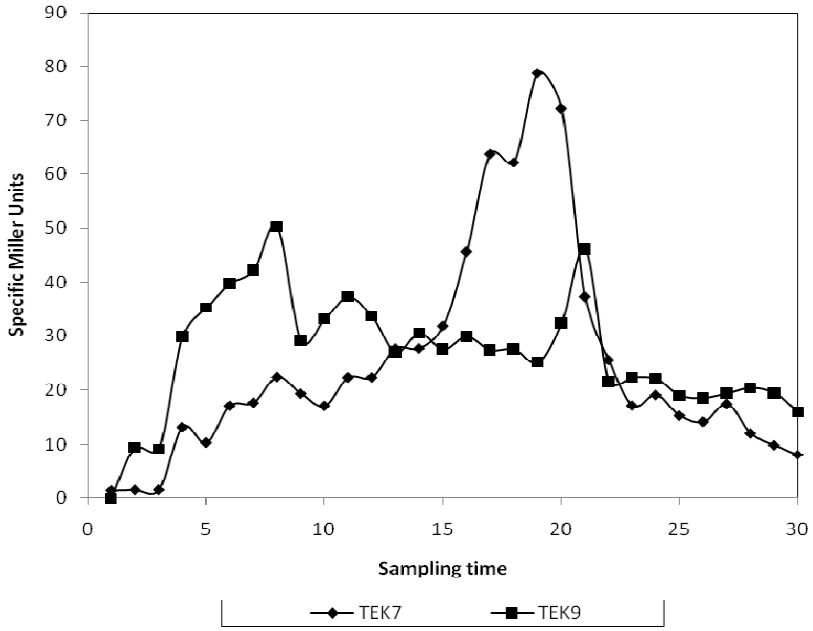



**Figure 3.19 :** β-Galactosidase activities of TEK7 and *abrB*-deleted mutant strain grown in PA medium and effect of deletion on *yvfI-lacZ* expression. The symbols used for the strains are; (♦) TEK7 (*yvfI::lacZ::erm*) and (■) TEK9 (*yvfI*::*lacZ*::*erm* ΔabrB::*cat*).

Deletion results of *spo0A* and *spo0A-abrB* genes are shown in the following figures (Fig. 3.20 and Fig 3.21).

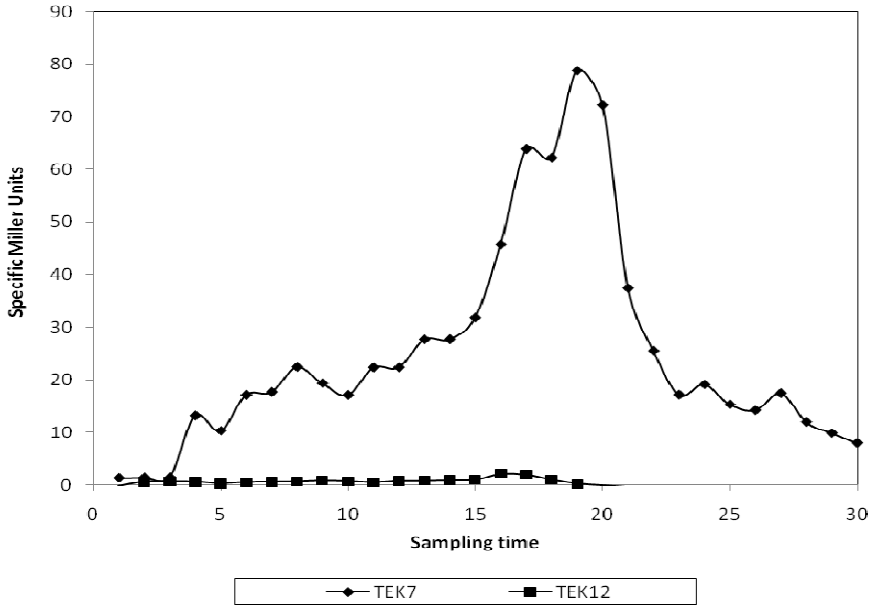



**Figure 3.20 :** β-Galactosidase activities of TEK7 and *spo0A*-deleted mutant strain grown in PA medium and effect of deletion on *yvfI-lacZ* expression. The symbols used for the strains are; (♦) TEK7 (*yvfI::lacZ::erm*) and (■) TEK12 (*yvfI*::*lacZ*::*erm* Δ*spo0A*::*spc*).

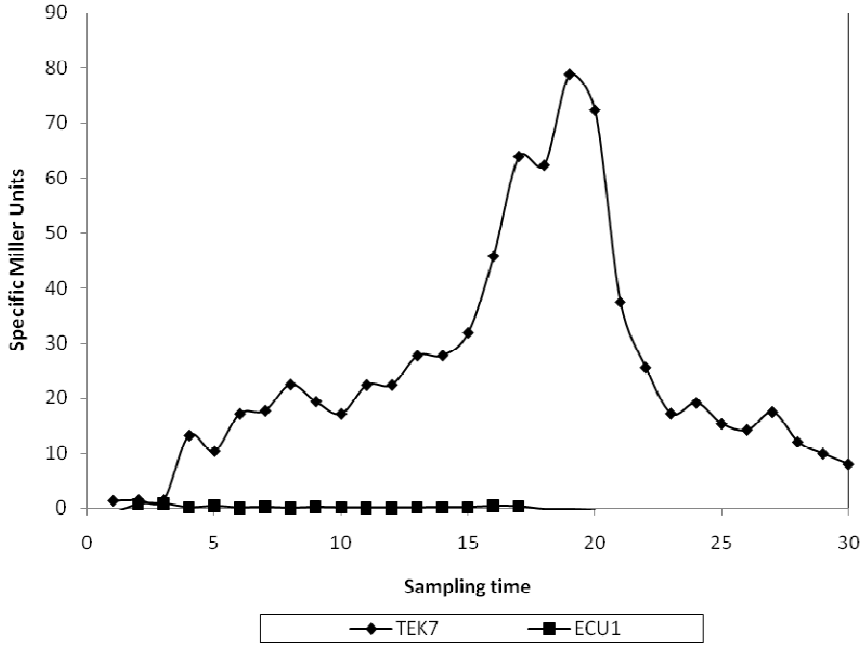



**Figure 3.21 :** β-Galactosidase activities of TEK7 and *spo0A-abrB*-deleted mutant strain grown in PA medium and effect of deletion on *yvfI-lacZ* expression. The symbols used for the strains are; (♦) TEK7 (*yvfI::lacZ::erm*) and (■) ECU1 (*yvfI::lacZ::erm* Δ*spo0A::*Δ*abrB::*cm).

As seen in Fig. 3.20 the expression of *yvfI*::*lacZ* was fully eliminated by deletion of *spo0A* gene. In *abrB* null mutation, a relief was monitored in the expression profile during exponential growth, on the other hand, in stationary phase severe repression of *yvfI*::*lacZ* expression was observed (Fig. 3.19). Besides, it was encountered an unexpected result with *yvfI*::*lacZ* expression in *spo0A-abrB* double mutant strain (Fig. 3.21). It was suggested that abrogated *yvfI* expression could be relieved by null mutation in *abrB* in *spo0A* mutation background in TEK7. But in contrarily, not only *yvfI*::*lacZ* expression was abolished completely, but also life span was shortened due to double mutation in *spo0A-abrB* double mutant strains ECU1. In light of these data, it was proposed that *spo0A* was needed for *yvfI* gene expression and AbrB was required for the induction for maximum *yvfI* expression at the onset of stationary phase as well as Spo0A. It was also suggested that beside to *abrB* there might be other regulator(s) interferring in *yvfI*::*lacZ* expression profile in the absence of *abrB*.

### 3.4.6. Deletion of *codY* Gene and Its Effect on the Expression of *yvfI* in *Bacillus subtilis*

The *B. subtilis* CodY protein has a global regulator effect on gene expression. CodY as a GTP-binding repressor of several genes is susceptive to the concentration of GTP in growing medium (Ratnayake-Lecamwasam *et al.*, 2001). When concentration of GTP is high, then CodY becomes active. On the other hand, when growth rate of *B. subtilis* slows down due to limitation of the carbon or nitrogen or phosphorus source, then GTP level reduces (Lopez *et al.*, 1979 and 1981). In this case CodY loses repressing activity, and hence the genes which are under CodY repression become actively transcribed.

CodY represses early-stationary phase genes under excess nutrition condition and ability of *codY* mutant strains for sporulation and genetic competence development even though there is a nutrition excess, points out that CodY has a key role in regulation (Inaoka *et al.,* 2002; Molle *et al.*, 2003). CodY binding site in the *srfA* promoter region overlaps with the presumed RNA polymerase binding site but not with the ComA binding sites (Roggiani and Dubnau, 1993). *codY-null* mutation in which *srfA* and *comK* were intact, CodY seems to be the inhibitor of *srfA* expression by blocking RNA polymerase activity. This suggestion fits to the supposition that

*srfA* expression is repressed by CodY in the presence of Casamino acids whether or not ComA is active (Serror and Sonenshein, 1996).

In order to determine the effect of *codY-null* mutation on *yvfI* gene expression, *codY* was insertionally inactivated in *Bacillus subtilis* PY79 wild type strain via transformation. PY79 wild type strain was used as competent cell, and was transformed by chromosomal DNA of strain of TMH307 (Δ*codY*::*spc)*. The transformants were then screened both on agar plates containing Spc (100μg/ml) antibiotic and also with PCR with specific primers for *codY* (primers were listed in Table 2.2). Then selected Δ*codY* transformant was used as competent cell and was transformed by chromosomal DNA of TEK7 (*yvfI*::*lacZ*::*erm*). Then the transformants were screened on X-gal (40 μg/ml) IPTG containing agar plates for checking of *lacZ* activity, and also for $Spc^R$ (100 μg/ml). The selected mutant was named as ECU14 (*yvfI*::*lacZ*::*erm trpC2 unkU::spc* Δ*codY*).

Then *codY-abrB* double mutant strain was constructed by using ECU14 as competent cell. ECU14 was transformed with chromosomal DNA of Δ*abrB*::*cat* mutant strain derivative of *B. subtilis.* The transformants were screened for double antibiotic resistance to Spc (100 μg/ml) and Cm (5 μg/ml). The selected mutant was named as ECU2 (*yvfI*::*lacZ*::*erm trpC2 unkU::spc* Δ*codY*::Δ*abrB*::*cm*).

The resulting strain ECU14, ECU2 and TEK7, as the control, were cultured in PA medium and were sampled in 1 h intervals for the β-galactosidase activity assay. Growth curves were shown in Figure 3.22 and data of β-galactosidase activities was shown in the Figure 3.23, and Fig. 3.24.

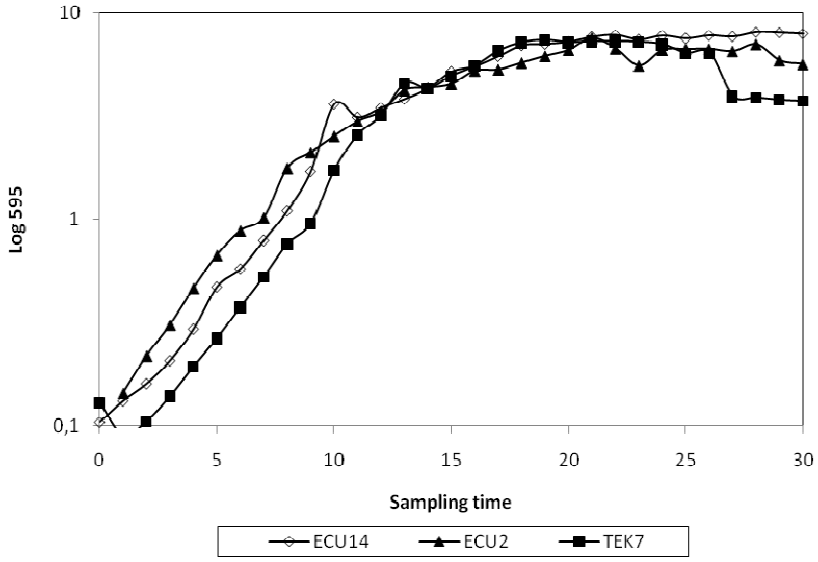



**Figure 3.22 :** Growth curves of TEK7 (*yvfI*::*lacZ*::*erm*), ECU2 (*yvfI*::*lacZ*::*erm trpC2 unkU*::*spc* Δ*codY*::Δ*abrB*::*cm*) and ECU14 (*yvfI*::*lacZ*::*erm trpC2 unkU*::*spc* Δ*codY*) straing grown in PA medium. The symbols used for strains are; (■) TEK7 (*yvfI*::*lacZ*::*erm*), (▲) ECU2 (*yvfI*::*lacZ*::*erm trpC2 unkU*::*spc* Δ*codY*::Δ*abrB*::*cm*) and (◊) ECU14 (*yvfI*::*lacZ*::*erm trpC2 unkU*::*spc* Δ*codY*).

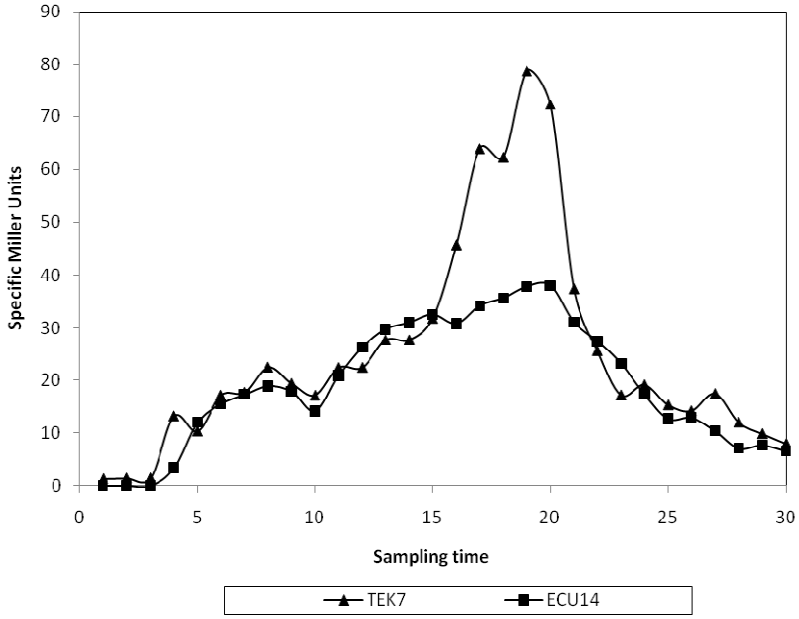



**Figure 3.23 :** β-Galactosidase activities of TEK7 and *codY*-deleted mutant strain grown in PA medium and effect of deletion on *yvfI-lacZ* expression. The symbols used for the strains are; (▲) TEK7 (*yvfI::lacZ::erm*) and (■) ECU14 (*yvfI*::*lacZ*::*erm trpC2 unkU*::*spc* Δ*codY*).

Deletion result of *codY-abrB* genes is shown in the following figure (Fig. 3.24).

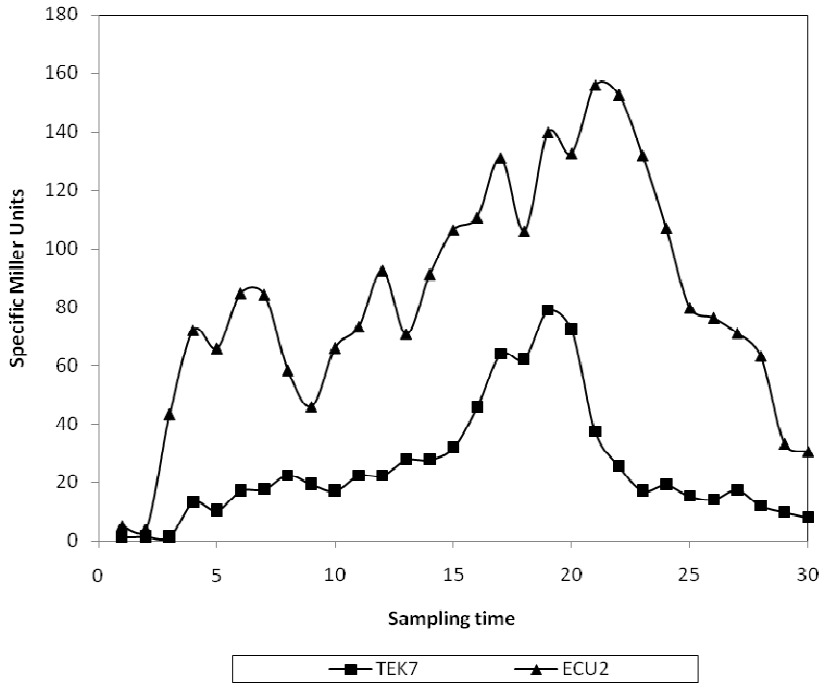



**Figure 3.24 :** β-Galactosidase activities of TEK7 and *codY-abrB*-deleted mutant strain grown in PA medium and effect of deletion on *yvfI-lacZ* expression. The symbols used for the strains are; (■) TEK7 (*yvfI::lacZ::erm*) and (▲) ECU2 (*yvfI::lacZ::erm trpC2 unkU::spc* Δ*codY*::Δ*abrB*::*cm*)

As seen in Fig. 3.23 in *codY*-disrupted strain, *yvfI*::*lacZ* expression has decreased exponentially, but the most strong supression was monitored during the stationary phase. Similarly *abrB* deletion resulted in nearly same repression profile in yvfI expression (Fig. 3.19). However, in the case of deletion of both *codY* and *abrB* genes, overproducing of *yvfI* was observed (Fig. 3.24). This data has given rise to the opinion that *yvfI* gene might be under mutual control of those both genes.

On the other hand, in order to examine the nutritional regulation system on both conditions, in presence and absence of *codY* gene, casaminoacid (casein) addition was applied. So casein was added into growth medium of ECU14, and also TEK7 was used as control group.

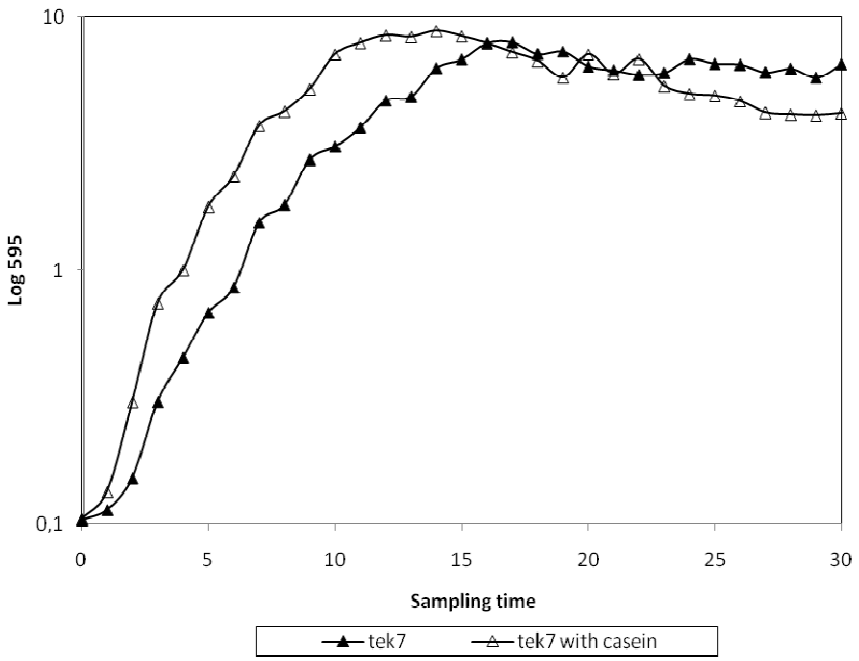



**Figure 3.25 :** Growth curve of TEK7 (*yvfI::lacZ::erm*) strain grown in PA medium within and without casein, seperately. The symbols used for the strains are; (▲) TEK7 (*yvfI::lacZ::erm*) without casein, (Δ)TEK7 (*yvfI::lacZ::erm*) within casein.

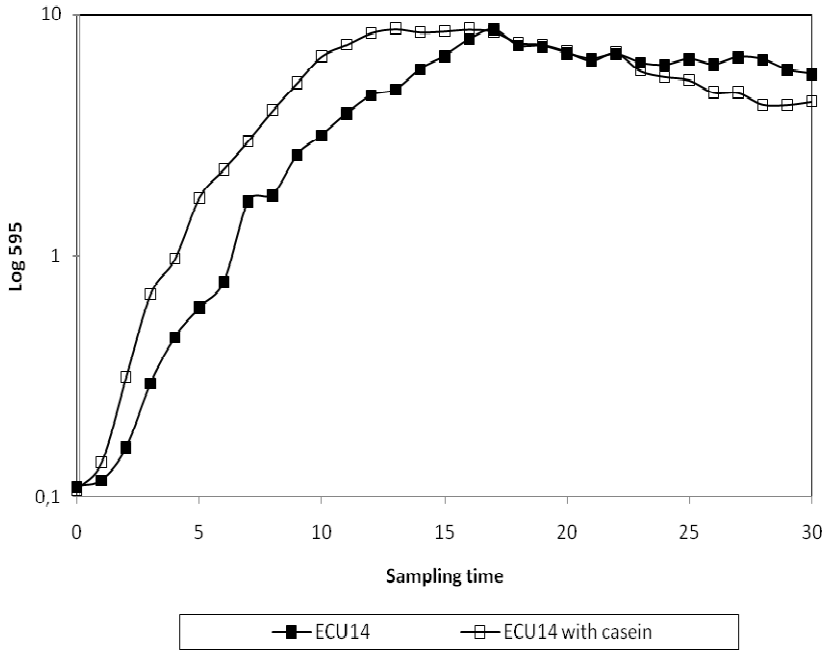



**Figure 3.26 :** Growth curve of ECU14 (*yvfI::lacZ::erm trpC2 unkU::spc* Δ*codY*) strain grown in PA medium within and without casein. The symbols used for the strains are; (■) ECU14 (*yvfI::lacZ::erm trpC2 unkU::spc* Δ*codY*) without casein, (□) ECU14 (*yvfI::lacZ::erm trpC2 unkU::spc* Δ*codY*) within casein.

The effects of casein addition are shown in the following figures ( Fig. 3.27 and Fig. 3.28).

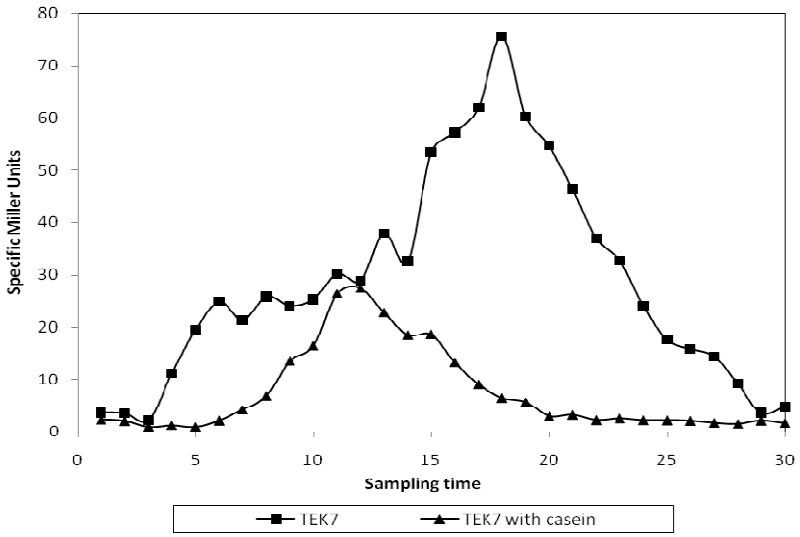



**Figure 3.27 :** β-Galactosidase activities of TEK7 (*yvfI::lacZ::erm*) strain grown in PA medium within and without casein, seperately. The symbols used for the strains are; (■) TEK7 (*yvfI::lacZ::erm*) without casein, (▲)TEK7 (*yvfI::lacZ::erm*) within casein.

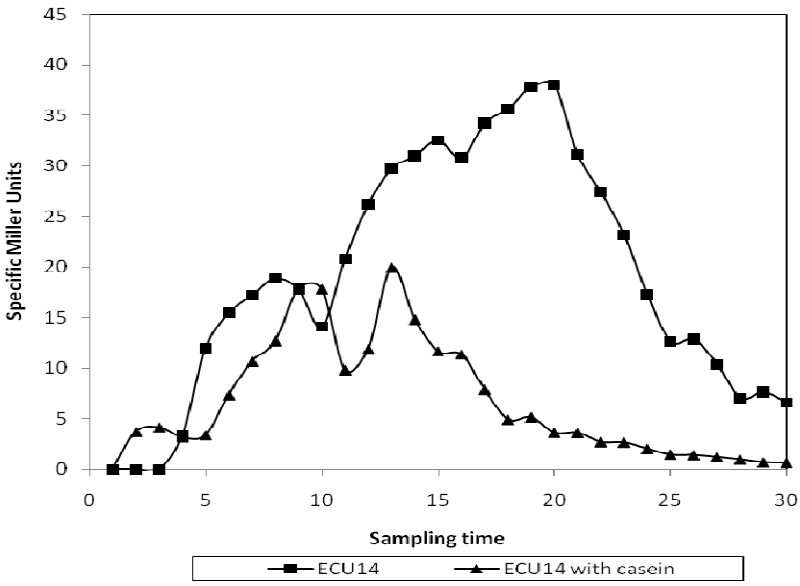



**Figure 3.28 :** β-Galactosidase activities of ECU14 (*yvfI::lacZ::erm trpC2 unkU::spc* Δ*codY*) strain grown in PA medium within and without casein, seperately. The symbols used for the strains are; (■) ECU14 (*yvfI::lacZ::erm trpC2 unkU::spc* Δ*codY*) without casein, (▲) ECU14 (*yvfI::lacZ::erm trpC2 unkU::spc* Δ*codY*) within casein.

As can be seen in Fig. 3.25 and Fig. 3.26, casein supplementation has accelerated growth during the exponential phase in both mutant strain, TEK7 and ECU14. Nevertheless, both in TEK7 and ECU14 mutants, *yvfI* expression was observed to be supressed with the addtion of extra casamino acid (Fig. 3.27, Fig. 3.28). Knowing that CodY is a very strong element of nutritional regulation circuit, repression of *yvfI* expression was expected in TEK7 when the growth medium is supplemented with casein considering the presence of intact CodY protein. On the other hand, a relief in supresion was expected in Δ*codY* strain, ECU14. In contrast with this expectation, specific expression of *yvfI* in ECU14 was severely abolished in stationary phase even though *codY* was deleted. Therefore, it might be an evidence for the presence of another strong supressor, which is effective on *yvfI* gene regulation. When overexpression of *yvfI* in ECU2 mutant strain (Δ*codY*- Δ*abrB* mutant) is considered, then AbrB might be thought to be the second regulator assisting to CodY for repression of *yvfI* gene.

### 3.4.7. Deletion of *degU* Gene and Its Effect on the Expression of *yvfI* Gene in *Bacillus subtilis*

The *B.subtilis* DegS–DegU two-component system positively regulates many cellular processes including exoprotease production, competence development and motility, dependent on DegU response regulator. Some target genes expression is stimulated by phosphorylated DegU, while some is triggered by unphosphorylated DegU (Msadek *et al.*, 1995).

Furthermore, *degU* and *degS* mutations stimulate degradative enzyme synthesis and inhibit competence (Msadek *et al.*, 1990; Dahl *et al.*, 1991). While one class of mutations leads to deficiency of degradative enzyme synthesis (degS or degU mutations), the second one leads to overproduction of degradative enzymes causing cells to sporulate even though there is presence of glucose, loss of flagella, and decreased genetic competence (Msaek *et al.*, 1990). Also *degU* mutation decreases the expression of *srfA,* an essential competence regulatory gene (Dubnau, 1991; Dahl *et al.*, 1992).

In order to examine the effect of *degU* null mutation, *degU* gene was deleted in the transcriptional *yvfI*::*lacZ* fusion strain TEK7. For this purpose, TEK7 was used ascompetent cell, then was transformed with the chromosomal DNA of Δ*degU*::*kan*

mutant derivative of *B. subtilis.* The transformants were selected with regard to specific antibiotic resistance for Kan (10 µg/ml). As a consequence, selected mutant was named as ECU8 (*yvfI*::*lacZ*::*erm* Δ*degU*::*erm*).

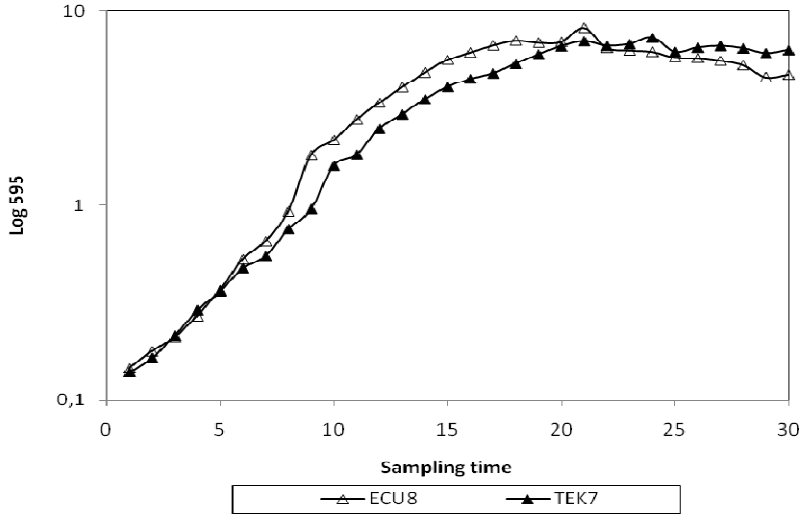



**Figure 3.29 :** Growth curves of TEK7 (*yvfI*::*lacZ*::*erm*) and ECU8 (*yvfI*::*lacZ*::*erm* Δ*degU*::*erm*) strains grown in PA medium. The symbols used for the strains are; (▲) TEK7 (*yvfI*::*lacZ*::*erm*) an (Δ) ECU8 (*yvfI*::*lacZ*::*erm* Δ*degU*::*erm*).

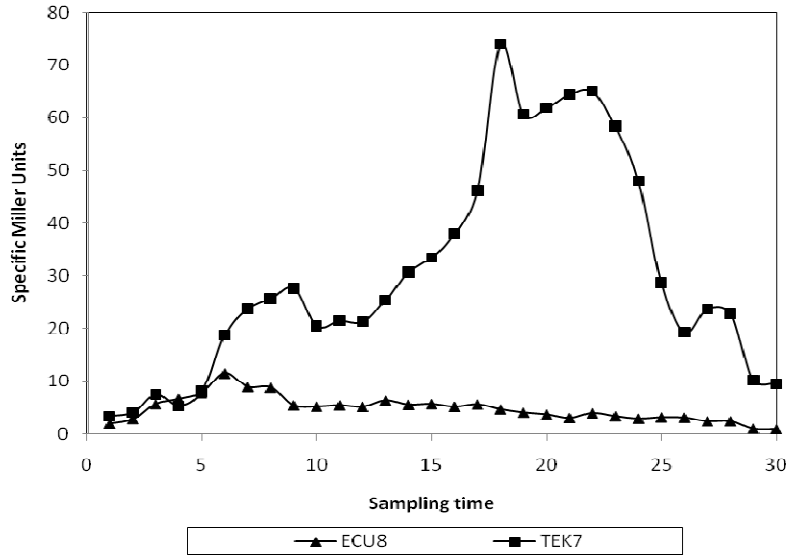



**Figure 3.30 :** β-Galactosidase activities of TEK7 and *degU*-deleted mutant strain grown in PA medium and effect of deletion on *yvfI*-*lacZ* expression. The symbols used for the strains are; (■) TEK7 (*yvfI*::*lacZ*::*erm*) and (▲) ECU8 (*yvfI*::*lacZ*::*erm* Δ*degU*::*erm*).

As seen in Fig 3.30, *yvfI* expression was almost fully blocked when degU gene was targeted for deletion. DegU is an effective positive regulator of *srf* operon, therefore, it is proposed that expression of *yvfI* has required *degU* gene as well as *srfA* gene.

### 3.4.8. Deletion of *sigmaB* Gene and Its Effect on the Expression of *yvfI* Gene in *Bacillus subtilis*

The *sigB* gene product, $\sigma^B$ is activated by metabolic stress conditions or environmental signals at the end of logarithmic growth. In order to monitor the effect of *sigB* null mutation, *sigB* gene was deleted in the transcriptional *yvfI*::*lacZ* fusion strain TEK7. For this purpose, TEK7 was used as competent cell, then was transformed with thw chromosomal DNA of Δ*ML6*::*cm* (*sigB*::*cm*) mutant derivative of *B. subtilis.* The transformants were selected with regard to specific antibiotic resistance for Cm (5µg/ml). Later on, the selected mutant was named as ECU7 (*yvfI*::*lacZ*::*erm* Δ*ML6*::*cm*).

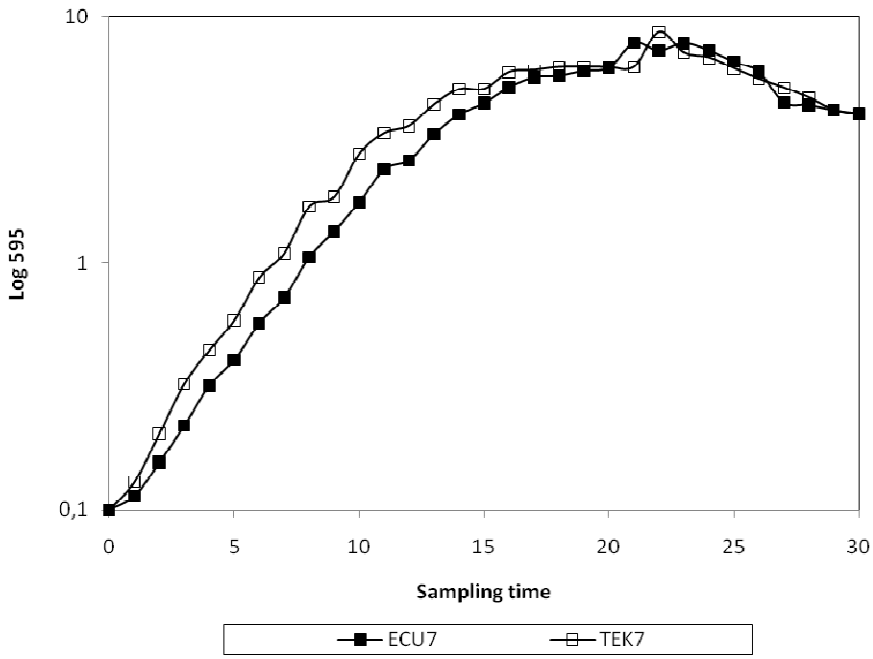



**Figure 3.31 :** Growth curves of TEK7 (*yvfI*::*lacZ*::*erm*) and ECU7 (*yvfI*::*lacZ*::*erm* Δ*ML6*::*cm*) strains grown in PA medium. The symbols used for the strains are; (□) TEK7 (*yvfI*::*lacZ*::*erm*) an (■) ECU8 (*yvfI*::*lacZ*::*erm* Δ*ML6*::*cm*).

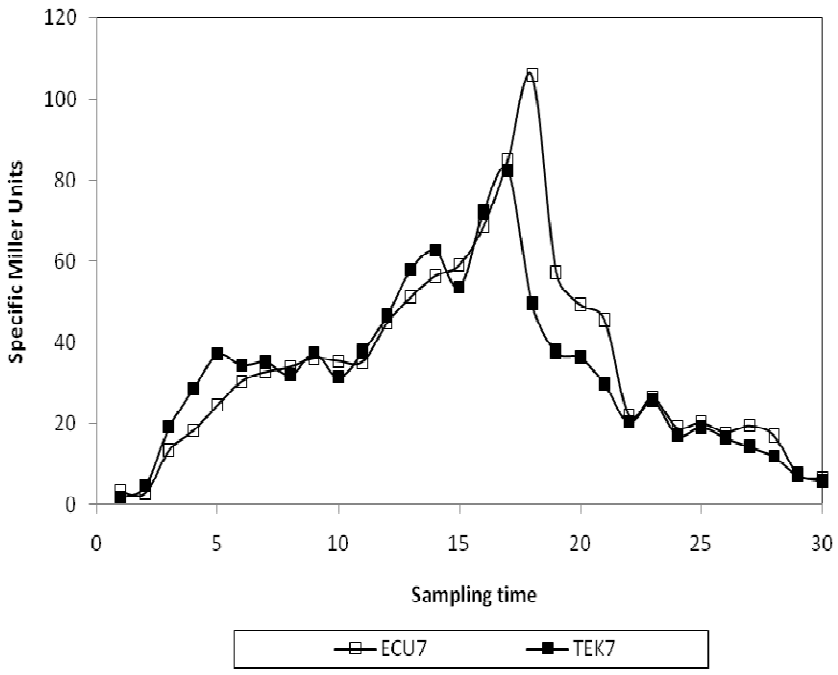



**Figure 3.32 :** β-Galactosidase activities of TEK7 and *sigB*-deleted mutant strain grown in PA medium and effect of deletion on *yvfI-lacZ* expression. The symbols used for the strains are; (■) TEK7 (*yvfI*::*lacZ*::*erm*) and (□) ECU7 (*yvfI*::*lacZ*::*erm* Δ*sigB*::*cm*).

As observed in Fig. 3.32, *sigB* deletion mutation did not seriously affect *yvfI* gene expression. Product of this gene, SigmaB, was required in response to excessive conditions such as salt, acid, heat. Therefore, this observation indicated that SigmaB was not an alternative subunit needed for *yvfI* expression.

## 4. CONCLUSION

In this present study, in order to monitor expression of *yvfI,* the *yvfI::lacZ* fusion mutant was constructed (TEK7) and the *yvfI::lacZ* expression was measured using beta-galactosidase activity assay in PA medium. In the result of assay, *yvfI* expression was slowly increased during the exponential growth and reached to maximum level during the transition upon entry into stationary phase.

In order to identify the effects of global regulatory genes on the expression of yvfI gene, these genes were deleted via transformation of competent cells of TEK7 with chromosomal DNAs of the corresponding blocked mutant strains and *yvfI*-directed β-galactosidase activities were subsequently analyzed.

The deletion of *srfA* gene resulted in the completely elimination of *yvfI-lacZ* expression, suggesting that fully expression of *yvfI* gene requires intact surfactin operon.

In disruption of *oppA* gene, *yvfI-lacZ* expression severely abolished, indicating that product *of oppA* gene regulates *yvfI* gene expression as a part of quorum sensing mechnanism

The blockage of *phrC, phrF* and *phrK* genes affected *yvfI-lacZ* expression seriously. Depending on this result, it was suggested that the pheromone peptides Phr C, F and K are essential for *yvfI* gene expression, most likely by enhancing the full activation of transcriptional factor ComA~P.

In the result of *comA*, *comP*, *comQ* and *spo0H* genes deletion, *yvfI-lacZ* expression entirely impaired, indicating that products of those genes has regulating effects on the expression of *yvfI*. Considering that ComP and ComX (ComQ) involved in ComA phosphorylation and activation, in addition, $\sigma^H$ required for increased level of *phr* gene during transition state in order to induce activation of ComA, it is proposed that *yvfI* might be transcribed in a ComA~P-dependent manner.

*spo0A* and *abrB* genes were examined through the disruption of those genes in TEK7 strain. In the expression of *yvfI-lacZ* fully eliminated in *spo0A* mutant strain, a relief was monitored during exponential growth, but contrarily, in stationary phase severe repression of *yvfI-lacZ* expression was observed in the expression profile of *abrB* mutant strain, and *yvfI-lacZ* expression was abolished completely in *spo0A-abrB* double mutant strain. Depending on these findings, we indicated that both AbrB and Spo0A activities are required for the induction and maximum *yvfI* expression at the onset of stationary phase.

In *codY* gene deletion, decreased *yvfI-lacZ* expression during exponential phase, and strongly decreased *yvfI-lacZ* expression during the stationary phase was monitored in the *codY*-disrupted strain. Moreover, deletion of both *codY* and *abrB* genes, resulted with overproduction of *yvfI* gene. In order to identify the nutritional regulation in the presence and absence of *codY* gene, casein was added as supplementery nutrient source in PA medium, resulted in the similar *yvfI* expression profile in *yvfI-lacZ* fusion strain and *codY*-disrupted strain. Thereupon, we proposed that those results might be an evidence for presence of another strong supressor effective on *yvfI* gene regulation, having a good candidate as AbrB.

As a result of *degU* gene deletion in TEK7 strain, *yvfI* expression was almost fully blocked. Therefore, we suggested that DegU positively regulates the expression of *yvfI.*

Finally, *sigB* deletion mutation did not seriously affect *yvfI* gene expression, indicating that SigmaB was not an alternative subunit needed for *yvfI* expression.

## REFERENCES


**Abrahart, R. J., and See, L.,** 1998: Neural Network vs. ARMA Modelling: Constructing Benchmark Case Studies of River Flow Prediction. In GeoComputation '98. Proceedings of the Third International Conference on GeoComputation, University of Bristol, United Kingdom, 17–19 September (CD-ROM).

**Ansaldi, M., Marolt, D., Stebe, T., Mandic-Mulec, I., and Dubnau, D.,** 2002. Specific activation of the *Bacillus* quorum-sensing systems by isoprenylated pheromone variants. *Molecular Microbiology*, 44, 6, 1561-1573.

**Auchtung, J. M., Lee, C. A., and Grossman, A. D.,** 2006. Modulation of the ComA-Dependent Quorum Response in *Bacillus subtilis* by Multiple Rap Proteins and Phr Peptides. *Journal of Bacteriology*, Vol. 188, No. 14, 5273-5285.

**Banse, A. V., Chastanet, A., Rahn-Lee, L., Hobbs, E. C., and Losick, R.,** 2008. Parallel pathways of repression and antirepression governing the transition to stationary phase in *Bacillus subtilis*. *Proc Natl Acad Sci*., 7, 105, 40, 15547-52.

**Basalp, A., Özcengiz, G., and Alaeddinoglu, N. G.,** 1992. Changes in patterns of alkaline serine protease and bacilysin formation caused by common effectors of sporulation in *Bacillus subtilis* 168. *Curr Microbiol*., 24, 129-135.

**Bodman, S. B., Willey, J. M., and Diggle, S. P.,** 2008. Cell-Cell Communication in Bacteria: United We Stand. *Journal of Bacteriology*, Vol. 190, no. 13, 4377–4391.

**Bongiorni, C., Ishikawa, S., Stephenson, S., Ogasawara, N., and Perego, M.,** 2005. Synergistic regulation of competence development in *Bacillus subtilis* by two Rap-Phr systems. *J. Bacteriol*., 187, 4353-4361

**Boylan, S. A., Rutherford, A., Thomas, S. M., and Price, C. W.,** 1992. Activation of *Bacillus subtilis* Transcription Factor 0FB by a Regulatory Pathway Responsive to Stationary-Phase Signals. *Journal of Bacteriology*, Vol. 174, No. 11, 3695-3706

**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.

**Camara, M.,** 2006. Quorum Sensing: A Cell-Cell Signalling Mechanism Used to Coordinate Behavioral Changes in Bacterial Populations. *Workshop on Membrane Computing 2006,* 42-48.

**Carter, H. L., Tatti, K. M., and Moran, C. P.,** 1990. Cloning of a promoter used by sigma-H RNA polymerase in *Bacillus subtilis*. *Gene,* 96, 101-105.

**Cashel, M., Gentry, D., Hernandez, V., and Vinella, D.,** 1996. The stringent response. In *Escherichia coli and Salmonella*, (pp. 1458–1496), *ASM Press*.

**Chmara, H.,** 1985. Inhibition of glucosamine synthase by bacilysin and anticapsin. *J Gen Microbiol*., 131, 265-271.

**Chmara, H., Woynarowska, and B., Borowski, E.,** 1981. Epoxypeptide antibiotic tetaine mimics peptides in transport to bacteria. *J Antibiot*., 34, 1608-1612.

**Core, L., and Perego, M.,** 2003. TPR-mediated interaction of RapC with ComA inhibits response regulator -DNA binding for competence development in *Bacillus subtilis*. *Mol. Microbiol*., 49, 1509-1522.

**Cosby, W. M., Vollenbroich, D., Lee, O. H., and Zuber, P.,** 1998. Altered *srf* Expression in *Bacillus subtilis* Resulting from Changes in Culture pH Is Dependent on the Spo0K Oligopeptide Permease and the ComQX System of Extracellular Control.*Journal of Bacteriology*, Vol. 180, No. 6, 1438-1445.

**Dahl, M. K., Msadek, T., Kunst, F., and Rapoport, G.,** 1991. Mutational analysis of the *Bacillus subtilis* DegU regulator and its phosphorylation by the DegS protein kinase. *J. Bacteriol*., 173, 2539-2547.

**Dahl, S., Msadek, T., Kunst, F., and Rapoport, G.,** 1992. The Phosphorylation State of the DegU Response Regulator Act as a Molecular Switch Allowing Either Degradative Enzyme Synthesis or Expression of Genetic Competence in *Bacillus subtilis. J. Biol. Chem.,* Vol. 267, No. 20, 14509-14514.

**Diggle, S. P., Gardner, A., West, S. A., and Griffin, A. S.,** 2007. Evolutionary theory of bacterial quorum sensing: when is a signal not a signal?. *Phil. Trans. R. Soc. B*, 1-9

**Dubnau, D.,** 1991. Genetic competence in *Bacillus subtilis*. *Microbiol.Mol. Biol. Rev.,* 55, 3, 395-424.

**Dubnau, D.,** 1991. The regulation of genetic competence in *Bacillus subtilis. Mol. Microbiol.,* 5, 11-18.

**Dunny, G. M., and Winans, S. C.,** 1999. Cell-cell Signaling in Bacteria(pp. 31-33). *ASM Press.*

**Earl, A. M., Losick, R., and Kolter, R.,** 2008. Ecology and genomics of *Bacillus subtilis*. *Trends Microbiol*., 16/6, 269-275

**Ferrari, E., Jarnagin, A. S., and Schmidt, B. F.,** 1993. Commercial production of extracellular enzymes. In: *Bacillus subtilis and other Gram-positive bacteria. Biochemistry, physiology, and molecular genetics*, (pp. 917–937), *American Society for Microbiology.*

**Fujita, M., Gonzalez-Pastor, J. E., and Losick, R.,** 2005. High- and Low-Threshold Genes in the Spo0A Regulon of *Bacillus subtilis*. Journal of Bacteriology, Vol. 187, No. 4, 1357-1368.

**Fujiya, M., Musch, M. W., Nakagawa, Y., Hu, S., Alverdy, J., Kohgo, Y., Schneewind, O., Jabri, B., and Chang, E. B.,** 2007. The Bacillus subtilis Quorum-Sensing Molecule CSF Contributes to Intestinal Homeostasis via OCTN2, a Host Cell Membrane Transporter, *Cell Host & Microbe* 1, 299–308.

**Fuma, S., Fujishima, Y., Corbell, N., D'Souza, C., Nakano, M. M., Zuber, P., and Yamane, K.,** 1993. Nucleotide sequence of 59 portion of *srfA* that contains the region required for competence establishment in *Bacillus subtilis*. *Nucleic Acids Res*., 21, 93-97.

**Galli, G., Rodriguez, F., Cosmina, P., Pratesi, C., Nogarotto, R., de Ferra, F., and Grandi, G.,** 1994. Characterization of the surfactin synthetase multienzyme complex. *Biochim. Biophys. Acta*., 120, 19-28.

**Glaser, P., Danchin, A., Kunst, F., Zuber, P., and Nakano, M. M.,** 1995. Identification and Isolation of a Gene Required for Nitrate Assimilation and Anaerobic Growth of *Bacillus subtilis. Journal Of Bacteriology*, 177, 4, 1112-1115.

**Griffith, L. K., and Grossman, A. D.,** 2008. A Degenerate Tripartite DNA-Binding Site Required for Activation of ComA-Dependent Quorum Response Gene Expression in *Bacillus subtilis*. *J. Mol. Biol*., 381, 261–275.

**Guespin-Michel, J. F.,** 1971. Phenotypic Reversion in Some Early Blocked Sporulation Mutants of *Bacillus subtilis*: Isolation and Phenotype Identification of Partial Revertants. *Journal of Bacteriology*, Vol. 108, No. 1, 241-247.

**Hahn, J., and Dubnau, D.,** 1991. Growth Stage Signal Transduction and the Requirements for *srfA* Induction in Development of Competence. *Journal of Bacteriology.,* Vol. 173, No. 22, 7275-7282.

**Havarstein, S. L.,** 2003. Intercellular Communication in Gram-Positive Bacteria Depends on Peptide Pheromones and Their Histidine Kinase Receptors. *Chapter of Histidine Kinases in Signal Transduction*, chapter no. 16, 341-363.

**Helmann, J. D., and Chamberlin, M. J.,** 1988. Structure and function of bacterial sigma factors. *Annu. Rev. Biochem*., 57, 839-872.

**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. *J Bacteriol.,* 170, 482-484.

**Hoch, J. A.,** 1993. Regulation of the phosphorelay and the initiation of sporulation in Bacillus subtilis. *Annu. Rev. Microbiol,*. 47**,** 441–465.

**Hoch, J.A.,** 1995. Control of cellular development in sporulating bacteria by the phosphorelay two-component signal transduction system. In: Two-component Signal Transduction (pp. 129-144). *American Society for Microbiology*.

**Hong, H.A., Duc, L.H., and Cutting, S.M.,** 2005. The use of bacterial spore formers as probiotics. *FEMS Microbiol. Rev*., 29, 813–835.

**Hoskisson, P. A., Rigali, S., Fowler, K., Findlay, K. C., and Buttner, M. J.,** 2006. DevA, a GntR-Like Transcriptional Regulator Required for Development in *Streptomyces coelicolor. Journal of Bacteriology*, Vol. 188, No. 14, 5014-5023.

**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.

**Inaoka, T., and Ochi.,** 2002. RelA Protein Is Involved in Induction of Genetic Competence in Certain *Bacillus subtilis* Strains by Moderating the Level of Intracellular GTP. *Journal of Bacteriology*, Vol. 184, No. 14, 3923-3930.

**Inouye, M., and Dutta, R.,** 2002.Histidine Kinases in Signal Transduction, (pp 313-340), *Academic Press.*

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

**Isenberg, H. D., Hoch, J. A., Sonenshein, A. L., Losick, R.,** 1993. *Bacillus subtilis* and other gram-positive bacteria: biochemistry, physiology and molecular genetics. (pp 747-755). *American Society for Microbiology*

**Jaacks, K. J., Healy, J., Losick, R., and Grossman, A. D.,** 1989. Identification and characterization of genes controlled by the sporulation-regulatory gene *spo0H* in *Bacillus subtilis*. *J. Bacteriol*., 171, 4121-4129.

**Jiang, M., Grau, R., and Perego, M.,** 2000. Differential processing of propeptide inhibitors of Rap phosphatases in *Bacillus subtilis*. *J. Bacteriol*., 182**,** 303-310

**Kalman, S., Duncan, M. L., Thomas, S. M., and Price, C. W.,** 1990. Similar organization of the *sigB* and *spoIIA* operons encoding alternate sigma factors of *Bacillus subtilis* RNA polymerase. *J. Bacteriol*., 172, 5575-5585.

**Kenig, M., and Abraham, E. P.,** (1976) Antimicrobial activities and antagonists of bacilysin and anticapsin. *J Gen Microbiol*., 94, 37-45.

**Kenig, M., Vandamme, E., and Abraham, E. P.,** 1976. The mode of action of bacilysin and anticapsin and biochemical properties of bacilysin-resistant mutants. *J Gen Microbiol*., 94, 46-54.

**Kleerebezem, M., Quadri, L. E., Kuipers, O. P., de Vos, W. M.,** 1997. Quorum sensing by peptide pheromones and two-component signal transduction systems in Gram-positive bacteria. *Mol Microbiol*., 24, 895-904.

**Klein, W., and Marahiel, M. A.,** 2002. Structure-Function Relationship and Regulation of Two *Bacillus subtilis* DNA-Binding Proteins, HBsu and AbrB *J. Mol. Microbiol. Biotechnol.,* 4, 3, 323-329.

**Kobayashi, K., and Ogasawara, N.,** 2002. Genome biology of a model bacterium, *Bacillus subtilis*, *International Congress Series*, 1246, 15–25

**Kobayashi, K., Ehrlich, S. D., Albertini, A., Amati, G., Andersen, K. K., Arnaud, M., Asai, K., Ashikaga, S., Aymerich, S., Bessieres, P., Boland, S. C., Brignell, Bron, S., Bunai, K., Chapuis, J., Christiansen, L C., Danchin, A., De´ barbouille, M., Dervyn, E., Deuerling, E., Devine, K., Devine, S. K., Dreesen, O., Errington, J., Fillinger, Foster, S. J., Fujita, Y., Galizzi, A., Gardan, R., Eschevins, C., Fukushima, T., Haga, K., Harwood, C. R., Hecker, M., Hosoya, D., Hullo, M. F., Kakeshita, H., Karamata, D., Kasahara, Y., Kawamura, F., Koga, K., Koski, P., Kuwana, R., Imamura, D., Ishimaru, M., Ishikawa, S., Ishio, I., Le Coq, D., Massona, A., Mauel, C., Meima, R., Mellado, R. P., Moirk, A., Moriya, S., Nagakawa, E., Nanamiya, H., Nakai, S., Nygaard, P., Ogura, M., Ohanan, T., O'Reilly, M., O'Rourke, M., Pragai, Pooley, Z., Rapoport, H. M., Rawlins, G., J. P., Rivas, L. A., Rivolta, C., Sadaie, A., Sadaie, Y., Sarvas, M., Sato, T., Saxild, H. H., Scanlan, E., Schumann, W., Seegers, J. F. M. L., Sekiguchi, J., Sekowska, A., Seror, S. J., Simon, M., Stragier, P., Studer, R., Takamatsu, H., Tanaka, T., Takeuchi, M., Thomaides, H. B., Vagner, V., van Dijl, J. M., Watabe, K., Wipat, A., Yamamoto, H., Yamamoto, M., Yamamoto, Y., Yamane, K., Yata, K., Yoshida, K., Yoshikawa, H., Zuber, U., and Ogasawara, N.,** 2003. Essential *Bacillus subtilis* genes. *PNAS*, Vol.100, no. 8, 4678-4683

**Köroğlu, T. E., Kurt-Gür, G., Ünlü, E. C., and Yazgan-Karataş, A.,** 2008. The novel gene yvfI in Bacillus subtilis is essential for bacilysin biosynthesis. *Antonie van Leeuwenhoek,,* 94, 3, 471-479.

**Kunst, F., Ogasawara, N, Moszer, I., Albertini, A.M., Alloni, G., Azevedo, V., Bertero, M.G., Bessiéres, P., Bolotin, A., Borchert, S., Borriss, R., Boursier, L., Brans, A., Braun, M., Brignell, S.C., Bron, S, Brouillet, S., Bruschi, C.V., Caldwell, B., Capuano, V., Carter, N.M., Choi, S.-K., Codani, J.-J., Connerton, I.F., Cummings, N.J., Daniel, R.A., Denizot, F., Devine, K.M., Düsterhöft, A., Ehrlich, S.D., Emmerson, P.T., Entian, K.D., Errington, J., Fabret, C., Ferrari, E., Foulger, D., Fritz, C., Fujita, M., Fujita, Y., Fuma, S., Galizzi, A., Galleron, A., Ghim, S.-Y., Glaser, P., Goffeau, A., Golightly,E.J., Grandi, G., Guiseppi, G., Guy, B.J., Haga, K., Haiech, J., Harwood, C.R., Hénaut, A., Hilbert, H., Holsappe, S., Hosono, S., Hullo, M.-F., Itaya, M., Jones, L., Joris, B., Karamata, D., Kasahara, Y., Klaerr-Blanchard, M., Klein, C., Kobayashi, Y., Koetter, B., Koningstein, G., Krogh, S., Kumano, M., Kurita, K., Lapidus, A., Lardinois, S., Lauber, J., Lazarevic, V., Lee, S.-M., Levine, A., Liu, H., Masuda, S., Mauel, C., Médigue, C., Medina, N., Mellado, R.P., Mizuno, M., Moest, D., Nakai, S., Noback, M., Noone, D., O'Reilly, M., Ogawa, K., Ogiwara, A., Oudega, B., Park, S.-H., Parro, V., Pohl, T.M., Portetelle, D., Porwollik, S., Prescott, A.M., Presecan, E., Pujic, P., Purnelle, B., Rapoport, G., Rey, M., Reynolds, S., Rieger, M., Rivolta, C., Rocha, E., Roche, B., Rose, M., Sadaie, Y., Sato, T., Scanlan, E., Schleich, S., Schroeter, R., Scoffone, F., Sekiguchi, J., Sekowska, A., Seror, S.J., Serror, P., Shin, B.-S., Soldo, B., Sorokin, A., Tacconi, E., Takagi, T., Takahashi, H., Takemaru, K., Takeuchi, M., Tamakoshi, A.,Tanaka, T., Terpstra, P., Tognoni, A., Tosato, V., Uchiyama, S., Vandenbol, M., Vannier, F., Vassarotti, A., Viari, A., Wambutt, R., Wedler, E., Wedler, H., Weitzenegger, T., Winters, P., Wipat, A., Yamamoto, H., Yamane, K., Yasumoto, K., Yata, K., Yoshida, K., Yoshikawa, H.-F., Zumstein, E., Yoshikawa, H., and Danchin, A.,** 1997. The complete genome sequence of the Gram-positive bacterium *Bacillus subtilis*. *Nature,* 390, 249-256.

**Lazazzera, B. A.,** 2001. The intracellular function of extracellular signaling peptides. *Peptides*, 22**,** 1519-1527

**Lazazzera, B. A.,** 2000. Quorum sensing and starvation: signals for entry into stationary phase. *Current Opinion in Microbiology*, 3, 177-182

**Lazazzera B. A., Grossman, A. D.,** 1998. The ins and outs of peptide signaling. *Trends Microbiol,* 6, 288-294.

**Lazazzera, B. A., Kurtser, I. G., McQuade, R. S., Grossman, A. D.,** 1999. An autoregulatory circuit affecting peptide signaling in *Bacillus subtilis*. *J Bacteriol.,* 181, 5193-5200.

**Lazazzera, B. A., Solomon J. M., Grossman A.D.,** 1997. An exported peptide functions intracellularly to contribute to cell density signaling in *B. subtilis*. *Cell* 89, 917-925.

**Lazdunski, M A., Ventre' I., and Sturgis' J. N., 2004.** Regulatory Circuits and Communication in Gram-Negative Bacteria. *Nature Reviews Microbiology* **2**, 581-592.

**LeDeaux, J. R., Solomon, J. M., and Grossman, A. D.,** 1997. Analysis of non-polar deletion mutations in the genes of the *spo0K* (opp) operon of *Bacillus subtilis FEMS Microbiology Letters*, Vol. 153, No. 1**,** 63-69.

**LeDeaux, J. R., Yu, N., and Grossman, A. D.,** 1995. Different Roles for KinA, KinB, and KinC in the Initiation of Sporulation in *Bacillus subtilis. Journal of Bacteriology*, Vol. 177, No. 3, 861-863.

**Lopez, J. M., Dromerick, A., and Freese, E.,** 1981. Response of guanosine 5'-triphosphate concentration to nutritional changes and its significance for *Bacillus subtilis* sporulation*. J. Bacteriol*., 146, 605-613.

**Lopez, J. M., Marks, C. L., and Freese, E.,** 1979. The decrease of guanine nucleotides initiates sporulation of *Bacillus subtilis*. *Biochim. Biophys. Acta.,* 587, 238–252.

**Magnuson, R., Solomon, J., and Grossman, A. D.,** 1994. Biochemical and genetic characterization of a competence pheromone from *B. subtilis Cell,* Vol. 77, 2, 207-216

**Marahiel, M. A.,** 1997. Protein templates for the biosynthesis of peptide antibiotics. *Chem. Biol*., 4, 561-567.

**Marchler-Bauer, A., Anderson, J. B., Cherukuri, P. F., DeWeese-Scott, C., Geer, L. Y., Gwadz, M., He, S., Hurwit,z D. I., Jackson, J. D., Ke, Z., Lanczycki, C. J., Liebert, C. A., Liu, C., Lu, F., Marchler, G. H., Mullokandov, M., Shoemaker, B. A., Simonyan, V., Song, J. S., Thiessen, P. A., Yamashita, R. A., Yin, J. J., Zhang, D., an d Bryant, S. H.,** 2005. CDD: a Conserved Domain Database for protein classification. *Nucleic Acids Res.,*33, 192-196.

**Marten, P., Smalla, K., and Berg, G.,** 2000. Genotypic and phenotypic differentiation of an antifungal biocontrol strain belonging to *Bacillus subtilis. Journal of Applied Microbiology*, 89, 463-471.

**Msadek T., Kunst, F., and Rapoport, G.,** 1995. A signal transduction network in *Bacillus subtilis* includes the DegS/DegU and ComP/ComA two-component systems. In: *Two-Component Signal Transduction*, (pp. 447-471), *American Society for Microbiology.*

**Msadek, T., Kunst, F., Henner, D., Klier, A., Rapoport, G., and Dedonder, R.,** 1990. Signal transduction pathway controlling synthesis of a class of degradative enzymes in *Bacillus subtilis*: expression of the regulatory genes and analysis of mutations in *degS* and *degU*. *J. Bacteriol*., 172, 824-834.

**Molle, V., Fujita, M., Jensen, S. T., Eichenberger, P., González-Pastor, J. E., Liu, J. S., and Losick, R.,** 2003. The Spo0A regulon of *Bacillus subtilis. Molecular Microbiology*, 50, 5, 1683-1701.

**Molle, V., Nakaura, Y., Shivers, R. P., Yamaguchi, H., Losick, R., Fujita, Y., and Sonenshein, A. L.,** 2003. Additional Targets of the *Bacillus subtilis* Global Regulator CodY Identified by Chromatin Immunoprecipitation and Genome-Wide Transcript Analysis. *Journal of Bacteriology*, Vol. 185, No. 6, 1911-1922.

**Nakano, M. M., and Zuber, P.,** 1989. Cloning and characterization of *srfB,* a regulatory gene involved in surfactin production and competence in *Bacillus subtilis*. *J. Bacteriol*., 171, 5347-5353.

**Nakano, M. M., Magnuson, R., Myers, A., Curry, J., Grossman, A. D., and Zuber, P.,** 1991. *srfA* is an operon required for surfactin production, competence development, and efficient sporulation in *Bacillus subtilis*. *J. Bacteriol*., Vol. 173, No. 5, 1770-1778.

**Nakano, M. M., Xia, L., and Zuber, P.,** 1991. Microbiology Transcription Initiation Region of the *srfA* Operon, Which Is Controlled by the *comP-comA* Signal Transduction System in *Bacillus subtilis*. *Journal of Bacteriology*,. Vol. 173, No. 17, 5487-5493.

**Nealson, K. H., and Hastings, J. W.,** 1979. Bacterial Bioluminescence: Its Control and Ecological Significance *Microbiological Reviews*, Vol. 43, no. 4, 496-518

**Nealson, K. H., Platt, T., and Hasting, W.,** 1970. Cellular Control of the Synthesis and Activity of the Bacterial Luminescent System. *Journal of Bacteriology*, Vol.104, no. 1, 313-322.

**Ochi, K., and Freese, E.,** 1983. Effect of antibiotics on sporulation caused by the stringent response in *Bacillus subtilis*. *J. Gen. Microbiol*., 129, 3709-3720.

**Ochi K., Kandala, J. C., and Freese, E.,** 1982. Evidence that *Bacillus subtilis* sporulation induced by the stringent response is caused by the decrease in GTP or GDP. *J. Bacteriol.*, 151, 1062-1065.

**Ogura, M., Yamaguchi, H., Yoshida, K. I., Fujita, Y., and Tanaka, T.,** 2001. DNA microarray analysis of *Bacillus subtilis* DegU, ComA and PhoP regulons: an approach to comprehensive analysis of *B.subtilis* two-component regulatory systems. *Nucleic Acids Res.,*29, 18, 3804-3813.

**Özcengiz, G., Alaeddinoglu, N. G., and Demain, A. L.,** 1990. Regulation of biosynthesis of bacilysin by *Bacillus subtilis*. *J Ind Microbiol.,* 6, 91-100.

**Özcengiz, G., and Alaeddinoglu, N. G.,** 1991. Bacilysin production by *Bacillus subtilis*: effects of bacilysin, pH and temperature. *Folia Microbiol*., 36, 522-526.

**Özcengiz, G., and Alaeddinoglu, N. G.,** 1991. Bacilysin production and sporulation in *Bacillus subtilis*. *Curr Microbiol*., 23, 61-64.

**Parsek, M. R., Greenberg, E. P.,** 2000. Acyl-homoserine lactone quorum sensing in Gram-negative bacteria: A signaling mechanism involved in associations with higher organisms. *PNAS*, Vol. 97, no. 16, 8789-8793.

**Perego, M.,** 1997. A peptide export-import control circuit modulating bacterial development regulates protein phosphatases of the phosphorelay. *Proc Natl Acad Sci*, 94, 8612-8617.

**Perego, M., and Brannigan, J. A.,** 2001. Pentapeptide regulation of aspartylphosphate phosphatases. *Peptides,* 22**,** 1541-1547

**Perego, M., and Hoch, J. A.,** 1996. Cell-cell communication regulates the effects of protein aspartate phosphatases on the phosphorelay controlling development in *Bacillus subtilis*. *Proc. Natl. Acad. Sci. USA,* 93, 1549-1553.

**Perego, M., and Hoch, J. A.,** 1996. Protein aspartate phosphatases control the output of two-component signal transduction systems. *Trends Genet*., 12, 97-101.

**Perego, M., Hanstein, C. G., Welsh, K. M., Djavakhishvili, T., Glaser, P., and Hoch, J. A.,** 1994. Multiple protein-aspartate phosphatases provide a mechanism for the integration of diverse signals in the control of development in *B. subtilis Cell* 79, 6, 1047–1055.

**Perego, M., Glaser, P., and Hoch, J. A.,** 1996. Aspartyl-phosphate phosphatases deactivate the response regulator components of the sporulation signal transduction system in *Bacillus subtilis*. *Mol. Microbiol*., 19, 1151-1157.

**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., and Strauch, M. A.,** 2002. *Bacillus subtilis* sporulation and stationary phase gene expression. *Cell. Mol. Life Sci*., 59, 392-402.

**Piazza, F., Tortosa, P., and Dubnau, D.,** 1999. Mutational analysis and membrane topology of ComP, a quorum-sensing histidine kinase of *Bacillus subtilis* controlling competence development. *J. Bacteriol*., 181, 4540-4548.

**Pottathil, M., Jung, A., and Lazazzera, B. A.,** 2008. CSF, A Species-Specific Extracellular Signaling Peptide for Communication among Strains of *Bacillus subtilis* and *Bacillus mojavensis. Journal of Bacteriology*, Vol. 190, no. 11, 4095–4099

**Price, C. W.,** 2002. The general stress response. *In Bacillus subtilis* and its closest relatives: from genes to cells, (pp. 369–384), *ASM Press*.

**Ramos, H. C., Boursier, L., Moszer., Kunst, F., Danchin, A., and Glaser, P.,** 1995. Anaerobic transcription activation in *Bacillus subtilis*: identification of distinct FNR-dependent and -independent regulatory mechanisms. *The EMBO Journal*, 14, 23, 5984-5994.

**Ratnayake-Lecamwasam, M., Serror, P., Wong, K. W., and Sonenshein, A. L.,** 2001. *Bacillus subtilis* CodY represses early-stationary-phase genes by sensing GTP levels *Genes & Dev.,* 15, 1093-1103.

**Roggiani, M., and Dubnau, D.,** 1993. ComA, a phosphorylated response regulator protein of *Bacillus subtilis*, binds to the promoter region of *srfA*. *J. Bacteriol.*, 175, 3182-3187.

**Roggiani, M., Hahn, J., and Dubnau, D.,** 1990. Suppression of early competence mutations in *Bacillus subtilis* by mec mutations. *J. Bacteriol*., 172, 4056-4063.

**Rudner, D. Z., LeDeaux, J. R., Ireton, K., and Grossman, A. D.,** 1991. The *spo0K* locus of *Bacillus subtilis* is homologous to the oligopeptide permease locus and is required for sporulation and competence. *J. Bacteriol*., 173, 1388-1398.

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

**Schauder, S., Shokat, K., Surette, M. G., and Bassler, B. L.,** 2001. The LuxS family of bacterial autoinducers: biosynthesis of a novel quorum-sensing signal molecule *Molecular Microbiology*, 41(2), 463-476

**Schneider, K. B., Palmer, T. M., and Grossman, A. D.,** 2002. Characterization of *comQ* and *comX*, Two Genes Required for Production of ComX Pheromone in *Bacillus subtilis. Journal of Bacteriology,* Vol. 184, no. 2, 410-419.

**Serror, P., and Sonenshein, A. L.,** 1996. CodY Is Required for Nutritional Repression of *Bacillus subtilis* Genetic Competence. *Journal of Bacteriology*, Vol. 178, No. 20, 5910-5915.

**Shafikhani, S. H., and Leighton, T.,** 2004. AbrB and Spo0E Control the Proper Timing of Sporulation in *Bacillus subtilis. Current Microbiology,* Vol. 48, No. 4, 262-269.

**Shapiro, J. A.,** 1998. Thinking about bacterial populations as multicellular organisms. *Annu. Rev.* Microbiol, 52*,* 81–104.

**Shivers, R. P., and Sonenshein, A. L.,** 2004. Activation of the Bacillus subtilis global regulator CodY by direct interaction with branched-chain amino acids. *Mol. Microbiol.* 53, 2, 599-611.

**Solomon, J. M., Lazazzera, B. A., and Grossman, A. D.,** 1996. Purification and characterization of an extracellular peptide factor that affects two different developmental pathways in *Bacillus subtilis*. *Genes Dev.,* 10, 2014-2024.

**Solomon, J. M., Magnuson, R., Srivastava, A., Grossman, A. D.,** 1995. Convergent sensing pathways mediate response to two extracellular competence factors in *Bacillus subtilis*. *Genes Dev.,* 9, 547-558.

**Stein, T.,** 2005. *Bacillus subtilis* antibiotics: structures, syntheses and specific functions. *Molecular Microbiology*, 56(4), 845-857.

**Steinborn, G., and Hofemeister, J.,** 1998/2000. Genes for the biosynthesis of anticapsin and bacilysin, their isolation and use. PCT/DE99/ 02179, WO 00/03009.

**Stephenson, S., Mueller, C., Jiang, M., and Perego M.,** 2003. Molecular Analysis of Phr Peptide Processing in *Bacillus subtilis. J. Bacteriol*., 185, 16, 4861-4871.

**Stock, J. B., Ninfa, A. J., and Stock, A. M.,** 1989. Protein phosphorylation and regulation of adaptive responses in bacteria. *Microbiol. Rev*., 53, 450-490.

**Stöver, A. G., and Driks, A.,** 1999. Regulation of synthesis of the *Bacillus subtilis* transition-phase, spore-associated antibacterial protein TasA. *J. Bacteriol*., 181, 5476-5481.

**Strauch, M. A.,** 1993. AbrB, a transition state regulator. In: *Bacillus subtilis and other gram-positive bacteria: biochemistry, physiology and molecular genetics*, (p. 757–764), *American Society for Microbiology*.

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

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

**Strauch, M. A., Bobay, B. G., Cavanagh, J., Yao, F., Wilson, A., and Le Breton, Y..** 2007. Abh and AbrB Control of *Bacillus subtilis* Antimicrobial Gene Expression. *Journal of Bacteriology*, Vol. 189, No. 21, 7720-7732.

**Strauch, M.A., Webb, V., Spiegelman, G., and Hoch, J. A.,** 1990. The Spo0A protein of *Bacillus subtilis* is a repressor of the *abrB* gene. *Proc. Natl. Acad. Sci*., 87, 1801-1805.

**Tanaka, T., Kawata, M., and Mukai, K.,** 1991. Altered Phosphorylation of *Bacillus subtilis* DegU Caused by Single Amino Acid Changes in DegS. *Journal of Bacteriology*, Vol. 173, No. 17, 5507-5515.

**Tomasz, A.,** 1965. Control of the Competent State in *Pneumococcus* by a Hormone-Like Cell Product: An Example for a New Type of Regulatory Mechanism in Bacteria. *Nature*, 208, 155-159.

**Tortosa, P., and Dubnau, D.,** 1999.Competence for transformation: a matter of taste. *Current opinion in microbiology*, 2(6), 588-592.

**Trowsdale, J., Chen, S. M. H., and Hoch, J. A.,** 1978. Genetic analysis of phenotype revertants of *spo0A* mutants in *Bacillus subtilis*: a new cluster of ribosomal genes. In: Spores VII (pp. 131-135). *American Society for Microbiology*.

**vanSinderen, D., Withoff, S., Boels, H., and Venema, G.,** 1990. Isolation and characterization of *comL*, a transcription unit involved in competence development in *Bacillus subtilis*. *Mol. Gen. Genet*., 224, 396-404.

**vonDöhren, H., Dieckmann, R., and Pavela-Vrancic, M.,** 1999. Chem. Biol. 6, 273-279**Weinrauch, Y., Penchev, R., Dubnau, E., Smith, I., and Dubnau, D.,** 1990. A *Bacillus subtilis* regulatory gene product for genetic competence and sporulation resembles sensor protein members of the bacterial two-component signal-transduction systems. *Genes Dev.,* 4, 860-872.

**Waldburger, C., Gonzalez, D., and Chambliss, G. H.,** 1993. Characterization of a New Sporulation Factor in *Bacillus subtilis*. *Journal of Bacteriology*, Vol. 175, No. 19, 6321-6327.

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

**Walton, R. B., and Rickes, E. L.,** 1962. Reversal of the antibiotic bacillin by N-acetylglucosamine. *J Bacteriol*., 84, 1148-1151.

**Weir, J., Predich, M., Dubnau, E., Nair, G., and Smith, I.,** 1991. Regulation of *spo0H*, a gene coding for the *Bacillus subtilis* σH factor. *J. Bacteriol*., 173, 521-529.

**Whitney, J. G., and Funderburk, S. S.,** 1970. Anticapsin, a new biologically active metabolite. IV. Mechanism of action. In: Abstracts for the X international congress for microbiology, Mexico City, p. 101.

**Whitney, J. G., Funderburk, S. S., Westhead, J. E., Lively, D. H., Solenberg, J. M., and Denney, J. W.,** 1972. Anticapsin, a New Biologically Active Metabolite: Screening and Assay Procedures. *Applied Microbiology*, Vol. 24, No. 6, 907-910.

**Williams, P.,** 2007. Quorum sensing, communication and cross-kingdom signalling in the bacterial world. *Microbiology*, 153, 3923-3938.

**Williams, P.,** 2007. Bacillus subtilis: A Shocking Message from a Probiotic *Cell Host & Microbe*, Vol. 1, no. 4, 248-24.

**Winans, S. C., and Bassler, B. L.,** 2002. *Journal of Bacteriology*, Vol. 184, no. 4, 873-883.

**Yazgan, A., Özcengiz, G., and Marahiel, M. H.,** 2001. Tn 10 insertional mutations of *Bacillus subtilis* that block the biosynthesis of bacilysin. *Biochim Biophys Acta*., 1518, 87-94.

**Yazgan, A., Özcengiz, G., Özcengiz, E., Kılınc, K., Marahiel, M. A., and Alaeddinoğlu, N. G.,** 2001. Bacilysin biosynthesis by a partially-purified enzyme fraction from *Bacillus subtilis*. *Enzyme Microb Technol*., 9, 400-406.

**Yazgan-Karatas, A. Y., Cetin, S., and Özcengiz, G.,** 2003. The effects of insertional mutations in *comQ, comp, srfA, spo0H, spo0A* and *abrB* genes on bacilysin biosynthesis in *Bacillus subtilis*. *Biochim Biophys Acta.,* 1626, 51-56.

**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. *J. Bacteriol*., 181, 7346-7355.

**Zuber, P., Nakano, M. M., and Marahiel, M. A.,** 1993. Peptide antibiotics. In: *Bacillus subtilis and Other Gram-positive Bacteria: Biochemistry, Physiology, and Molecular Genetics* (pp. 897-916), *American Society for Microbiology*.

**APPENDICES**

**APPENDIX A :** Composition and Preperation of Culture Media
**APPENDIX B :** Composition and Preparation of Buffers and Solutions
**APPENDIX C :** Chemicals and Enzymes
**APPENDIX D :** Markers
**APPENDIX E :** *yvfI* DNA Sequence

## APPENDIX A

**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 1000ml and then autoclaved for 4 minutes.

**2xYT Medium (1000ml)**

| | |
|---|---|
| Tryptone | 16 g |
| Yeast Extract | 10 g |
| NaCl | 5 g |
| Agar | 15 g (Added before autoclaving for solid 2xYT medium) |

**HS medium (30 ml)**

| | |
|---|---|
| 10X-S-base | 3 ml |
| Glucose (50%) | 300 µl |
| Yeast Extract (10%) | 300 µl |
| Casaminoacid (2%) | 300 µl |
| Arg (8%) + His (0.4%) | 3 ml |
| Tryptophan (0.5%) | 300 µl |
| Phenylalanine (0.3%) | 450 µl |

Completed up to 30 ml with sterile distilled $H_2O$ and store at cold (+4°C) for a week.

**LS Medium (20 ml)**

| | |
|---|---|
| 10X-S-Base | 2 ml |
| Glucose | 200 µl |
| Tryptophan | 200 µl |
| Phenylalanine | 30 µl |
| Casaminoacid | 100 µl |
| Yeast Extract | 200 µl |
| Spermine (50mM) | 200 µl |
| $MgCl_2$ (1M) (filter steriled) | 50 µl |

Freshly prepared and completed up to 20 ml with sterile distilled $H_2O$.

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

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

*Autoclave separately

**Filter sterilization

**DSM Medium (Difco's Sporulation Medium)**

| | |
|---|---|
| Nutrient Broth | 8 g |
| KCl (10 % w/v) | 10 ml |
| $MgSO_4.7H_2O$ (1.2 %) | 10 ml |
| NaOH (1 M) | 0.5 ml |

Autoclaved for 30 min. and cooled own to 50 ˚C.

| | |
|---|---|
| $Ca(NO_3)_4$ (1 M) | 1 ml |
| $MnCl_2$ (0.01 M) | 1 ml |
| $FeSO_4$ (1 mM) | 1 ml (resuspended before use) |

1.5 % Agar was added if needed.

## APPENDIX B

**P1 Buffer (pH 8)**

| | |
|---|---|
| Tris-base | 6.06 gr |
| EDTA.2$H_2O$ | 3.72 gr |

Dissolve Tris-base and EDTA with 800 ml d$H_2O$. Adjust pH to 8 with HCl. Adjuste volume to 1 lt d$H_2O$. Add 100 mg RNase A er liter of P1.

**P2 Buffer**

| | |
|---|---|
| NaOH | 8 gr |
| SDS solution (20%) | 50 ml |

Dissolve NaOH in 950 ml d$H_2O$. Add 50 ml SDS solution.

**P3 Buffer (pH 5.5)**

| | |
|---|---|
| Potassium acetate | 294.5 gr |

Dissolve in 500 ml d$H_2O$. Adjust pH

**TE Buffer (pH 7)**

| | |
|---|---|
| Tris base | 10 mM |
| EDTA | 1 mM |

Adjusted pH 7 with HCl

**TAE Buffer (50X)**

| | |
|---|---|
| Tris base (2 moles) | 242 g |
| Glacial acetic acid (57.1 ml) | 57.1 ml |
| EDTA (100mL 0.5M) | 100 ml (0.5 M, pH 8.0) |

Add d$H_2O$ up to 1L and adjust pH to 8 by HCl

**Low Melting Agarose Gel (1%)**

Agarose 0.5 g

TAE buffer (1X) 50 ml

Add 1.5µl EtBr (final concentration: 0.5 µg/ml) before pouring the gel into tray.

**CTAB/NaCl Solution (10 % CTAB/ 0.7 M NaCl)**

4.1 g of NaCl was dissolved in 80 ml of $dH_2O$. Then, 10 g of CTAB (hexadecyl trimethyl ammonium bromide) was added and dissolved with vigorously shaking and gentle heating up to 65 ° C. Final volume was made up to 100 ml with $dH_2O$.

**Physiological Sodium Chloride Solution (0.85%) (1000 ml)**

$NaCl_2$ 8.5 g

Dissolve in 1000 ml distilled water and autoclave.

**Tris-Cl Solution (25mM, 1000 ml, pH 7.4)**

Tris (hydromethyl)aminomethane 3.03 g

Dissolve in 1000 ml distilled water and adjust pH to 7.4 with HCl (1 M).

**Z Buffer (500 ml, pH 7.0)**

$Na_2HPO_4.7H_2O$ (60mM) 5.33 g

$NaH_2PO_4$ (40 mM) 3.12 g

$KCl_2$ (10 mM) 0.373 g

$MgSO_4.7H_2O$ (1 Mm) 0.123 g

All mixed and dissolved within 500 ml distilled water and the pH was adjusted to a value of 7.0.

β-mercaptoethanol : 6,75µl was added into 50 ml of Z buffer, immediately before using.

Triton X-100 : 100 µl was added into 900 µl Z buffer including β-mercaptoethanol.

Lysozyme final concentration: 2.5 mg/ml

ONPG final concentration: 4.0 mg/ml

**APPPENDIX C**

| **Chemicals** | **Suppliers** |
|---|---|
| Agar Bacteriological | AppliChem |
| Agarose | Prona |
| Ammonium persulfate | Carlo Erba |
| Arginine | Merck |
| $CaCl_2.2H_2O$ | Merck |
| Casamino acid | AppliChem |
| Cloramphenicol | Sigma |
| $CoCl_2.6H_2O$ | Carlo Erba |
| CTAB | Sigma |
| $CuSO_4.5H_2O$ | Carlo Erba |
| EDTA | Sigma |
| Ethanol | Botafarma |
| Ethidium bromide | Sigma |
| Glucose | Riedel deHaen |
| Glycerol | Merck |
| HCl | Carlo Erba |
| Histidine | Merck |
| IPTG | Sigma |
| $K_2HPO_4$ | J.T.Baker |
| KCl | Carlo Erba |
| $KH_2PO_4$ | J.T.Baker |
| Mercaptoethanol | Merck |
| $MgCl_2.6H_2O$ | Carlo Erba |
| $MgSO_4.7H_2O$ | Carlo Erba |
| $MnCl_2.4H_2O$ | Carlo Erba |
| $Na_2CO_3$ | Riedel deHaen |
| $Na_3$citrate.$2H_2O$ | Riedel deHaen |
| NaCl | Carlo Erba |
| NaOH | Carla Erba |

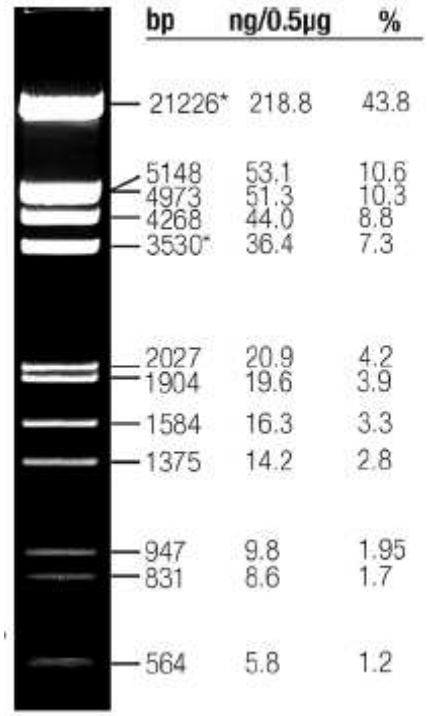
| | |
|---|---|
| 2-Nitrophyl β-D-galacto pyranoside (ONPG) | Sigma |
| Nutrient Broth | Merck |
| PEG 8000 | Merck |
| Phenol-chloroform-isoamylalcohol | Fluka |
| Phenylalanin | Merck |
| SDS | Merck |
| Spermine | Fluka |
| Sucrose | Merck |
| Tris-base | Merck |
| Triton-X100 | Sigma |
| Trypton | Acumedia |
| Tryptophan | Merck |
| X-Gal | Sigma |
| Yeast Extract | Acumedia |

**Enzymes**

| | |
|---|---|
| *Bam*HI | Fermentas |
| *Hin*dIII | Fermentas |
| Lysozyme | AppliChem |
| Proteinase K | Sigma |
| RNAse A | Sigma |
| *Taq* DNA Polymerase | Roche |
| T4 DNA Ligase | Roche |

**APPENDIX D**

| **Marker** | **Supplier** |
|---|---|
| Lambda DNA/*EcoR*I+ *Hind*III Marker (Marker 3) | MBI Fermentas |



## APPENDIX E

### *yvfI* DNA Sequence

atgaaacagggagaaggcacgtatctgaaggaatttgagctcaatcaaatttctcagccgctctca
gccgcccttctgatgaaaaaagaggacgtcaagcagctgctcgaggtcagaaaactgcttgaaat
cggcgtggcttcactagcggctgaaaaaaggacagaagcagatctcgaaagaattcaggatgca
ctaaaggaaatgggcagcattgaagcggacggggagctgggagagaaagcagactttgcatttc
atcttgcgcttgcggacgcttctcaaaatgaacttcttaaacacttgatgaatcacgtgtcatcattgc
tgctggaaacaatgagggaaacgaggaaaatctggctgttttccaagaagacctccgttcagcgg
ctgtatgaggagcacgaacggatttacaatgctgtggctgccgggaacggtgcacaggcggaag
ccgccatgctggcgcatttgacgaatgtggaagatgtgctttcgggatatttcgaggaaaatgtgca
a

**CURRICULUM VITA**

**Candidate's full name** : E. Canan ÜNLÜ ÖZKURT

**Place and date of birth** : İstanbul, 24.09.1982

**Permanent address** : İlk mektep Sk. No: 19 D. 5

Kocamustafapaşa, İstanbul, TÜRKİYE

**Universities and**

**Colleges attended** :

**Publications** :

- Köroğlu, T., E., Kurt-Gür , G., Unlu, E., C., Yazgan-Karatas, A., 2008. The novel gene *yvfI* in *Bacillus subtilis* is essential for bacilysin biosynthesis. *Antonie van Leeuwenhoek*, Vol. 94/3, 471-479.