İSTANBUL TECHNICAL UNIVERSITY ★ INSTITUTE OF SCIENCE AND TECHNOLOGY

ANALYSIS OF GLOBAL REGULATORY FACTORS ACTING ON THE EXPRESSION OF *ywfH* GENE IN *Bacillus subtilis* VIA ELECTROPORETIC MOBILITY SHIFT ASSAY METHOD

M.Sc. Thesis by
Seval MUTLU

Department : Advanced Technologies

Programme : Molecular Biology-Genetics and Biotechnology

OCTOBER 2011

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(521071057)

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Supervisor (Chairman) : **Assoc. Prof. Dr.Ayten Yazgan KARATAŞ (ITU)**
Members of the Examining Committee : **Assist. Prof. Dr. Fatma Neşe KÖK (ITU)**
Assoc. Prof. Dr. Melek ÖZKAN (GYTE)

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İSTANBUL TEKNİK ÜNİVERSİTESİ ★ FEN BİLİMLERİ ENSTİTÜSÜ

Bacillus subtilis'te *ywfH* GENİNİN İFADESİ ÜZERİNE ETKİ EDEN GLOBAL DÜZENLEYİCİ FAKTÖRLERİN "ELECTROPHORETIC MOBILITY SHIFT ASSAY" METODU İLE ANALİZİ

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Seval MUTLU
(521071057)

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Tez Danışmanı : Doç. Dr. Ayten Yazgan KARATAŞ (İTÜ)
Diğer Jüri Üyeleri : Yrd. Doç. Dr. Fatma Neşe KÖK (İTÜ)
Doç. Dr. Melek ÖZKAN (GYTE)

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FOREWORD

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October 2011 Seval MUTLU

TABLE OF CONTENTS

Page

ABBREVIATIONS

AHL	**:** N-achylhomoserine Lactone
bp	**:** Base pair
CSF	**:** Competence and Sporulation Factor
dH_2O	**:** Distilled water
DNA	**:** Deoxyribonucleic acid
EDTA	**:** Ethylenediaminetetraacetic acid
EMSA	**:** Electrophotetic Mobility Shift Assay
SDS-PAGE	**:** Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
EtBr	**:** Ethidium bromide
kb	**:** Kilobase
UV	**:** Ultraviolet
LB broth	**:** Luria Bertani broth
OD	**:** Optical density
PCR	**:** Polymerase Chain Reaction
QS	**:** Quorum Sensing
TAE	**:** Tris acetate EDTA
Tris	**:** Hydroxymethyl aminomethane

LIST OF TABLES

Page

LIST OF FIGURES

Page

ANALYSIS OF GLOBAL REGULATORY FACTORS ACTING ON THE EXPRESSION OF *ywfH* GENE IN *Bacillus subtilis* VIA ELECTROPORETIC MOBILITY SHIFT ASSAY METHOD

SUMMARY

Bacilysin is a dipeptide antibiotic (125 kDa) composed of L-alanin and L-anticapsin in *Bacillus subtilis*. As an antibacterial agent, it is active against wide range of bacteria even *Candida albicans.* Recently, it was found that a polycistronic operon: *ywfBCDEFG* and adjacent monocistronic gene: *ywfH* were essential for the production of bacilysin. Previously it was shown that biosynthesis of bacilysin is under the positive control of quorum sensing global regulatory circuit through the action of ComQ/ComX, PhrC(CSF), ComP/ComA in a Spo0K (opp)-dependent manner. Additionally, bacilysin production is also positively regulated by global regulatory factor Spo0A and negatively regulated by transition state regulators, CodY and AbrB. Very recently, the effects of global regulatory factors ComA, Spo0A, CodY and AbrB on the expression of *bac* operon and their interaction with the promoter region of *bac* operon were investigated. It was shown that ComA and Spo0A act as a positive regulator which participates in the transition state induction of *bac* operon both directly by interacting with the *bac* promoter while AbrB and CodY are negative regulators which directly repress the *bac* promoter.

As a further study, the present study aims to identify whether *ywfH* promoter is direct target for the global regulators; ComA, CodY, AbrB, Spo0A and DegU by using "Electrophoretic Mobility Shift Assay (EMSA)". For this purpose, the 414 bp DNA fragment harboring the promoter region of *ywfH* were incubated with the various concentrations of the purified C-terminal His_6-tagged-ComA, -Spo0A, -AbrB, -CodY, -DegU proteins. Moreover, the gel mobility shift assays were repeated in the presence of both regulatory proteins to determine whether AbrB-CodY, Spo0A-ComA, ComA-ComK and ComA-DegU could bind to *ywfH* promoter simultaneously or compete for binding.

*Bacillus subtilis'*te *ywfH* GENİNİN İFADESİ ÜZERİNE ETKİ EDEN GLOBAL DÜZENLEYİCİ FAKTÖRLERİN "ELECTROPHORETIC MOBILITY SHIFT ASSAY" YÖNTEMİ İLE ANALİZİ

ÖZET

Basilisin, *Bacillus subtilis* tarafından sentezlenen, L-alanin ve L-antikapsin'den oluşan bir dipeptit (125 kDa) antibiyotiktir. Bir antibakteriyal ajan olarak, basilisin pek çok bakteri türüne hatta *Candida albicans*'a etki göstermektedir. Yakın zamanda, *B. subtilis*'in basilisin biyosentezinde bir polisistronik operon *ywfBCDEFG* ve bitişiğindeki monosistronik gen *ywfH* in görev aldığı bulunmuştur. Önceden, basilisin biyosentezi, ComQ/ComX, PhrC (CSF), ComP/ComA' nın Spo0K (Opp)'ye bağlı bir biçimde hücre yoğunluğu sinyali döngüsünün kontrolü altında gerçekleştiği gösterilmiştir. Ek olarak, basilisin üretiminin global düzenleyici Spo0A tarafından direk olarak, pozitif yönde düzenlenirken, geçiş evresi düzenleyicileri CodY ve AbrB tarafından direk olarak, negatif yönde düzenlendiği bulunmuştur. Çok yakın zaman içinde bu global düzenleyicilerin *bac* operonunun ifadesi üzerindeki etkisi ve bu global düzenleyicilerin *bac* promoter bölgesi ile olan etkileşimleri keşfedilmiştir. AbrB ve CodY proteinleri *bac* promotörünü doğrudan baskılayan negatif düzenleyicilerken, ComA ve Spo0A, *bac* operonun geçiş evresinde direk uyarılmasını sağlayan, pozitif yönde hareket eden düzenleyiciler olduğu gösterilmiştirdir. Bir sonraki çalışma olarak, bu çalışmada, *ywfH* promotörünün ComA, CodY, AbrB, Spo0A, ve DegU global düzenleyiciler için doğrudan hedef olup olmadığını "Electrophoretic Mobility Shift Assay (EMSA)" yöntemi kullanarak tanımlanması amaçlanmıştır. Bu amaçla, 414bç DNA parçasını içeren *ywfH* promotör bölgesi, saflaştırılmış C-ucu His_6 kuyruklu ComA, Spo0A, AbrB, CodY ve DegU proteinlerinin çeşitli konsantrasyonları ile inkübe edilmiştir. Bunun dışında, AbrB-CodY, Spo0A-ComA, ComA-ComK ve ComA-DegU'nun eş zamanlı mı veya yarışmacı olarak mı P_{ywfH}'ne bağlandığını belirlemek amacıyla iki düzenleyici proteinin varlığında "jel mobility shift" yöntemi tekrarlanmıştır.

1. INTRODUCTION

1.1 *Bacillus subtilis*

Bacillus subtilis is a widely studied model species in terms of biochemistry, genetics and molecular biology. This endospore-forming rhizobacterium is an aerobic, rod-shaped gram-positive microorganism that can produce ribosomally and non-ribosomally more than twenty-four types of antibiotics that show antibacterial, antifungal and antimetabolic properties. This bacterium is a remarkably diverse bacterial species that is capable of growth within many environments such as soil on plant root surfaces, aquatic habitats, even gastrointestinal tracts of marine and terrestrial animals. It is also found in several commercially available fermented food products, including soybeans fermented with *B. Subtilis* natto, which is popular in Japan. Additionaly, it was found that *B. subtilis* has genes encoding a putative respiratory nitrate reductase. This indicated that it could grow anaerobically using nitrate instead of oxygen as an electron acceptor (Glaser *et al.,* 1995; Ramos *et al.*, 1995; Earl *et al.,* 2008).

B.subtilis is a chemoorganotroph microorganism that is able to survive very simple growth conditions like media including only salt, glucose or one of the other sugars for carbon and energy source and also nitrogen with enough oxygen level (Nicholson and Setlow, 1990). On the other hand, *B.subtilis* cells can exhibit adaptive responses under nutritionally limited growth conditions or other environmental stresses such as formation highly resistant dormant endospore (Earl *et al.*, 2008; Harwood *et al.*, 1996; Glaser *et al.*, 1995; Ramos *et al.*, 1995; Stein *et al.*, 2005) also synthesis of extracellular macromolecules-degrading enzymes, secretion of antibiotics, induction of motility and chemotaxis (Msadek, 1999; Hardman et al., 1998). This model bacterium is essential due to its metabolic diversity, non-pathogenity, ability to produce hydrolytic enzymes (e.g. alkaline proteases, amylases), peptide antibiotics (e.g. bacilysin, bacitracin), biochemicals (e.g. nucleosides for conversion to flavour enhancers) and insecticides (e.g. 8-endotoxins) particularly after the elucidation of its complete genome (Kobayashi and Ogasawara, 2002; Nicholson *et al*., 1990). In

addition, thanks to its crucial feature of genetic competence gram-positive bacterium *B. subtilis* is preferred for molecular and genetic surveys (Marten *et. al.,* 2000; Stein, 2005).

As an alternative to antibiotics, this microorganism also exhibits probiotic property and is considered as a good candidate for a novel prophylactic, therapeutic, and growth promoting agent (Hong *et al.,* 2004; Williams, 2007)

In 1997, the complete genome sequence of *B. subtilis* was published by an international consortium that contains Japanese and European laboratories. Genome of this species consist of 4.214.810 base pair and include 4106 protein-coding genes (Kunst *et al.*, 1997, Kobayashi and Ogasawara, 2002). Also 86 tRNA genes, 30 rRNA genes and three small stable RNA genes was found in its genome. Currently 63% (2379) of genes was known to have a function (Kobayashi and Ogasawara, 2002), however, 4% of these coding genes were observed to have unknown functions (Kobayashi *et. al.*, 2003).

1.2 Quorum Sensing Mechanism in B. subtilis

Bacteria are omnipresent and highly successful life forms. Their success is predominantly a consequence of their ability of adapting to a great diversity of environments. In order to overcome the strong conditions entry into stationary phase such as nutritional and physical fluctuations related to such an environment, *B. subtilis* displays a variety of survival strategies. Under batch-fermentation conditions, these processes can be readily monitored in the laboratory by growing *B. subtilis* cells. When nutrients become reducing for optimal growth at the end of the exponential growth phase, *B. subtilis* cells initiate to synthesize a complex motility and chemotaxis system that would enable them to look for nutrients in the environment. If nutritional limitation proceeds, these motile cells start to secrete degradative enzymes by going into the stationary growth phase. These degradative enzymes provide cells with nutrients from alternative resources that are normally difficult to reach. Moreover, cells start to synthesize antibiotics to struggle possible competitors. Extended nutritional stress ends up the development of competence, and eventually sporulation where in the bacterial population is able to survive harsh environmental conditions (Hamoen *et al.,* 2003; Levdikov *et al*., 2006; Msadek, 1999). *B. subtilis* properly regulates these adaptations using quorum sensing system

to modulate gene expression in response to changes in cell-population density. The term ‘quorum sensing’ (Fuqua *et al*., 1994) has been used to define the accumulation of a diffusible low molecular weight signal molecule to distinguish between low and high cell population density and to control gene expression in response to changes in cell number (Hardman *et al*., 1998).

Cell–cell signaling is a fundamental activity conducted by most cell types (Lazazzera, 2000). Bacteria often use it to monitor their population density producing and detecting small signalling hormone-like molecules, called “autoinducers” (Reading and Sperandio, 2005; Gobbetti *et al*., 2007; Chen *et al*., 2002). These signalling molecules, in turn, accumulate and stimulate cascade events when a “quorum” (e.g., a certain threshold concentration) is reached; therefore the name of “quorum sensing” describes this mechanism of cell–cell communication (Gray, 1997; Gobbetti *et al*., 2007; Hooshangi and Bentley, 2008; Miller *et al*., 2001). The discovery of cell–cell communication among bacteria has led to the realization that bacteria have the capacity to behave collectively as a group that was once believed to be restricted to multicellular organisms (Hardman *et al*., 1998).

Quorum sensing (QS) comprises the production, release and subsequent detection of chemical signaling molecules (Henke and Bassler, 2004; Lazazzera, 2000). The extracellular concentration of QS signaling molecules that are produced and secreted into the environment by the bacterial cells increases with increasing cell number. When the QS signaling molecules reach a critical threshold level, the group coordinately respond the signaling molecules by altering their gene expression (Schauder and Bassler, 2001; Reading and Sperandio, 2005). The alterations in gene expression due to the presence of signaling molecules give bacteria specific behaviors only when living in a community, not when living as induviduals (Henke and Bassler, 2004).

Quorum-sensing mechanisms can be divided into three general classes depending on the type of signalling molecules and their cognate apparatus used for its detection (Henke and Bassler, 2004). Firstly, Gram negative bacteria typically have two regulatory proteins LuxI/R quorum-sensing systems. LuxI-type enzymes catalyze the formation of a spesific acylated homoserine lactone (AHL) signals. The LuxR-type proteins bind their cognate signals and control transcription of target genes. LuxI/R systems are general mechanism that have been identified in over 70 species of Gram-

negative bacteria. As an example luciferase enzyme complex is responsible for light production in *V. fischeri*. Bioluminescence only occurs when *V. fischeri* is at high cell density, and this process is managed by quorum sensing LuxI/R systems (Bassler, 1999; Henke and Bassler, 2004; Reading and Sperandio, 2005).

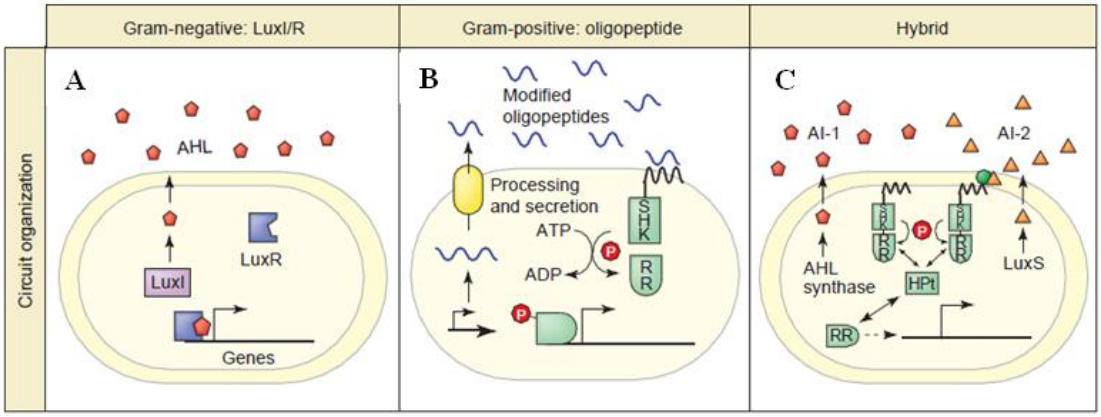


Figure 1.1 : Types of quorum-sensing systems. The three general classes of quorum- sensing systems are: A) Gram-negative LuxI/R-type, B) Gram-positive oligopeptide–two-component-type, and C) A hybrid type that has features of both Gram-negative- and Gram-positive-type systems (Bassler and Henke, 2004).

The second class of quorum-sensing system is in Gram positive bacteria modified small peptides as signals that is different from that used by Gram-negative bacteria. The signals are modified oligopeptides that are synthesized, processed and secreted into the enviroment and accumulate at high cell density. Two-component signal transduction proteins called sensor histidine kinases are used to detect fluctuations in environmental stimuli and relay the information regarding these changes into the cell and these proteins autophosphorylate to transmit sensory information via phosphorylation. Thus, the DNA binding activity is modified by phosphorylation of the response regulator, and enables it to control transcription of quorum-sensing target genes (Henke and Bassler, 2004; Reading and Sperandio, 2005).

A third class of quorum-sensing circuit is a hybrid between the canonical Gram-negative and Gram-positive systems. This hybrid system was initially identified in the bioluminescent marine bacterium *Vibrio harveyi* that uses quorum sensing to control bioluminescence like *V. fischeri,* but *V. harveyi* QS system constitutes a mix between components of Gram-positive and Gram negative systems. Particularly, *V. harveyi* uses an acyl-HSL autoinducer similar to other Gram-negative quorum sensing bacteria, but the signal detection and relay apparatus consists of two

component proteins similar to the quorum sensing systems of Gram-positive bacteria (Henke and Bassler, 2004; Reading and Sperandio, 2005).

In *B. subtilis*, like most Gram-positive bacteria quorum responses are controlled by two extracellular signaling peptides, ComX pheromone and CSF (for competence and sporulation factor), to regulate the activity of the transcription factor ComA. ComA activates the expression of several genes, including one for the development of genetic competence (Solomon *et al*., 1995 and 1996; Lazazzera *et al*., 1997; Hamoen *et al*., 2003).

The ComX pheromone, the major extracellular signaling peptide, is translated as an inactive form, which is then cleaved as a modified decapeptide with the addition of a hydrophobic modification on a tryptophan residue. It is noteworthy that *ComQ* gene is required for the production of active ComX pheromone (Magnuson *et al*., 1994; Solomon *et al*., 1995). When cells become crowded, ComX pheromone is secreted into the medium to activates the signal transduction system composed of the two-component regulatory proteins ComP and ComA. The protein kinase ComP has eight putative membrane-spanning helices and seems to be the direct receptor for ComX pheromone. The hydrophobic modification on the ComX pheromone is necessary for its function and may assist to increase the local concentration of ComX pheromone at the membrane where it can interact with and activate ComP. The activated form of histidine protein kinase ComP autophosphorylates and donates phosphate to the phosphorilation-dependent transcription factor ComA (Lazazzera *et al*., 1998; Magnuson *et al*., 1994; Solomon *et al*., 1995; Weinrauch *et al*., 1990; Griffith and Grossman, 2008; Ansaldi *et al*., 2002).

The phosphorylated form of ComA activates expression of several genes, including *comS* that is embedded in the large *srfA* operon. ComS provides dissociation of the competence transcription factor, ComK from ternary complex with ClpC (chaperon like protein) and MecA hence releasing active ComK activates transcription of its cognate gene by binding to the promoter region. Owing to this autostimulatory loop, rapidly increasing ComK activates transcription of late competence genes encoding the DNA binding, -uptake and –integration machinery (Susanna *et al*., 2006; Turgay *et al*., 1997 and 1998; Hamoen *et al*., 2003 and 1998). That is only known direct link between quorum sensing and competence devolopment (Lazazera and Grossman, 1998; van Sinderen *et al*., 1995).

The activity of ComA is regulated by at least four other density-dependent signaling pathways. PhrC, PhrF, PhrH, and PhrK are pentapeptides that are secreted into the growth medium and then are transported back into the cell through the oligopeptide permease system. They bind to and inhibit the activity of their cognate Rap proteins RapC, RapF, RapH, and RapK. RapC, RapF, RapH, and RapK inhibit ComA binding to its target sites (Griffith and Grossman, 2008).

Besides the *srfA* operon, the transcription factor ComA also directly regulates *degQ* encoding a regulator of degradative enzyme production, *rapA*, *rapC*, *rapE* and *rapF* each of which encodes a regulatory protein (Nakanno *et al*., 1991). According to the bioinformatics and overexpression experiments performed by *Natalia Comella* and *Alan D. Grossman* indicates that at least 89 genes in 35 operons are affected by ComA, independently of the effects of ComA on the competence transcription factor ComK.

According to the genetic reseach, several response regulators like DegU play a role in the development of competence. It was found that DegU and the histidine protein kinase DegS that participate in the synthesis of degredative enzymes are involved in the initiation of competence devolopment. When the ComK concentrations are low, unphosphorilated DegU acts as a priming protein to support *comK* transcription. It has been suggested that degredative enzyme production is activated by DegU-P, while unphosphorilated DegU protein is necessary for competence devolopment (Hamoen *et al*., 2000; Hameon *et al*., 2003; Susanna *et al*., 2006).

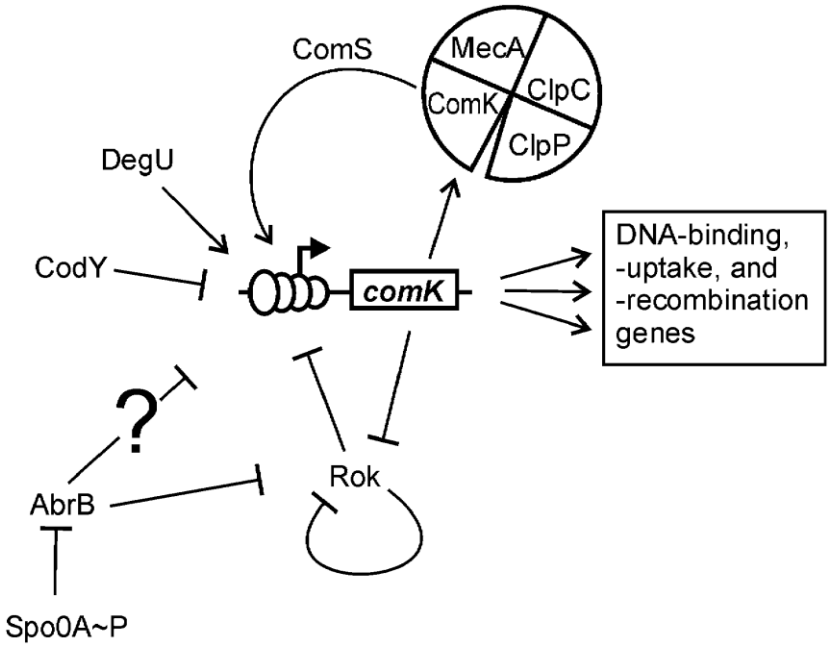


Figure 1.2 : Control of *comK* expression

DegU is not the only regulator that controls the transcription of the *comK* promoter. it is also under the direct control of four different transcription factors: AbrB, CodY, Rok and ComK itself. It was revealed that AbrB inhibits interaction of RNA polymerase to the *comK* promoter by binding transcription initiation site of it. AbrB not only inhibits premature expression of *comK*, but also has a stimulatory effect on comptence devolopment (Hameon *et al*., 2003). Amino acid mediated repression of several *B. subtilis* genes depends on a GTP-binding transcription factor, CodY regulating many stationary growth phase genes. During rapid growth CodY acts as a negative regulator, it represses genes required for adaptation to nutrient limitation (Levdikov *et al*., 2006). Two types of small-molecule effectors are now known to modulate the activity of CodY protein. One of them is GTP that interacts with CodY and activates it as a repressor, the other one is the branched-chain amino acids (BCAAs) isoleucine and valine that also bind to CodY and enhance its binding to target sites (Joseph *et al*., 2005). When the bacterial cells become limited for nutrients, CodY appears to be positive regulator and its activity diminishes. Due to the carbon source or amino acid limitation, the stringent response is activated. This leads to conversion of GTP to pppGpp and ppGpp. Thus, accumulation of (p)ppGpp or a decrease in intracellular GTP might be the signal that inactivates CodY and derepresses CodY-regulated 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). DNA footprinting analysis demostrated that CodY covers to the RNA polymerase binding region to repress comptence devolopment (Hameon *et al*., 2003; Serror and Sonenshein, 1996). *ykuW*,renamed as *rok*, encoding Rok protein also is a reppressor for *comK* promoter. In an important study (Hoa *et al*., 2002), it is stated that Rok protein both inactivates *comK* transcription by bindig to the *comK* promoter region and represses its own synthesis. When ComK concentration is high, *rok* expression is inhibited (Hameon *et al*., 2003).

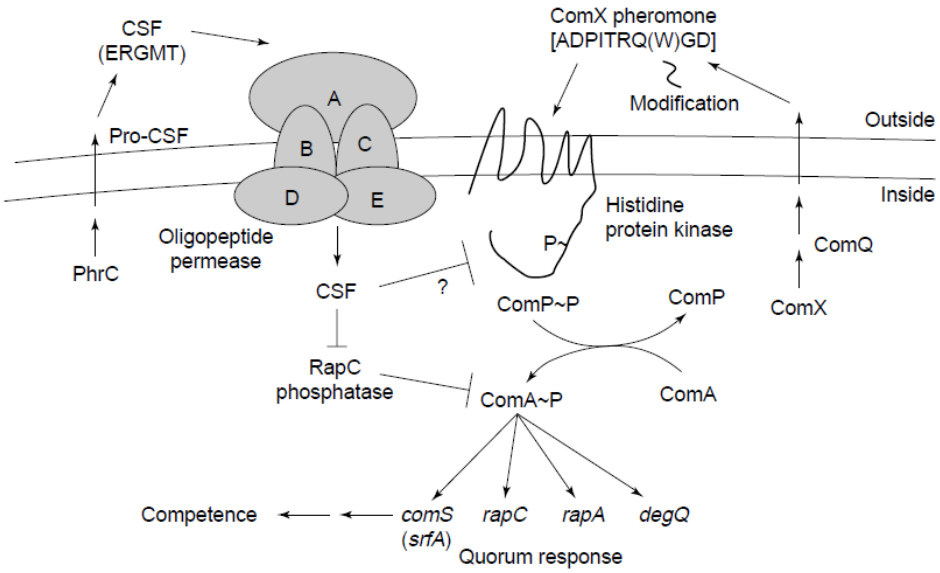


Figure 1.3 : Quorum sensing and regulation of genetic competence by competence and sporulation factor (CSF) and ComX pheromone in B. subtilis.

CSF(competence and sporulation factor) is the second secreted peptide signal having a five amino-acid. It controls the activity of transcription factor ComA in a intracellular concentration dependent manner. Unlike ComX, when CSF reaches a critical level due to the increasing cell number, it is transported back into the bacterial cell by an oligopeptide permease (Opp), which is an ATP-binding cassette transporter belonging ABC-type oligopeptide transporter family. The low internal concentration of CSF promotes competence development by inhibiting the activity of an aspartylphosphate phosphatase, RapC, whereas the high internal concentration of CSF eliminates competence and induces sporulation by interacting with histidine-protein kinase ComP, hence, inhibits the expression of ComA-controlled genes. Moreover, indirectly inhibition of ComS expression causes ComK proteolysis which stimulates expression of the proteins needed for transformation. On the other hand,

CSF, at high concentrations, also stimulates sporulation remarkably by repressing the activity of an alternate aspartyl-phosphate phosphatase, RapB (Pottahil *et al.*, 2008; Solomon *et al.*, 1995 and 1996; Lazazzera and Grossman, 1998; Lazazzera 1997; Perego, 1997).

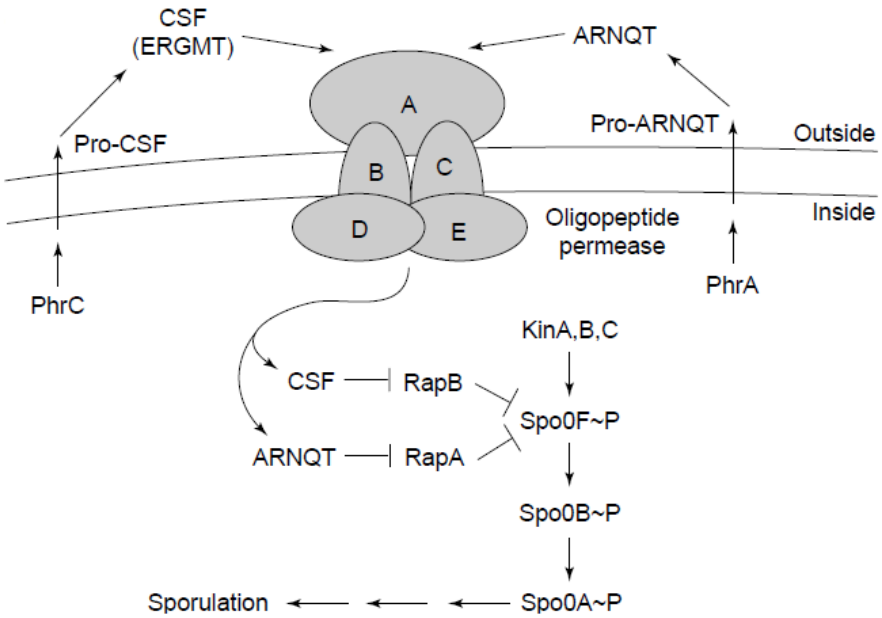


Figure 1.4 : A model for the regulation of sporulation.

Phosphorylation mediated signal transduction pathway, known as phosphorelay, regulates the initiation of sporulation in *B. subtilis*. Besides, CSF, PhrA participates in the quorum sensing mediated sporulation mechanism by positively regulating phosphorylation of Spo0A which is the master regulatory protein responsible for the initiation of sporulation. RapB and RapA downregulate this process via dephosphorylation of the response regulator Spo0F, a protein which serves as an intermediate in the phoshorelay. The phosphatase activity of RapB and RapA are inhibited by CSF and PhrA respectively, indicating that CSF and PhrA contributes sporulation by directly inhibiting these phosphatases. Additionally, Spo0F is also phosphorylated through KinA, KinB, KinC, KinD and KinE. Phosphorylated Spo0F donates its phosphate group to Spo0B which is a phosphotransferase leading to the activation of Spo0A transcription factor via phosphorylation. When transcription factor Spo0A-P accumulates, cells are directed to the sporulation pathway (Stephens, 1998, Perego *et al.*, 1994).

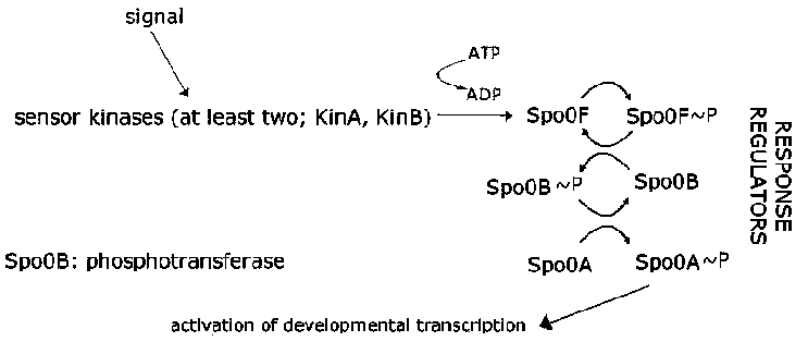


Figure 1.5 : Phosphorelay signal transduction system

1.3 Bacilysin: A Dipeptide Antibiotic

Bacilysin is one of the simpliest peptide antibiotics produced and secreted extracellularly by certain strains of *B. subtilis*. Its small (125 kDa) and basic structure contains two amino acids L-alanine at the N-terminus and L-anticapsin, an unusual amino acid, at the C terminus (Walker and Abraham, 1970). It is antimicrobially effective against a wide range of bacteria and some fungi especially *Candida albicans* (Steinborn *et al.*, 2005).

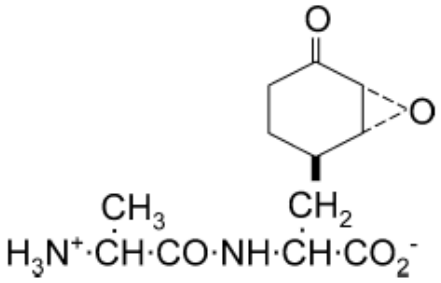


Figure 1.6 : Chemical structures of bacilysin

The anticapsin moiety of bacilysin is crucial for its antibiotic activity. When bacilysin is uptaken into susceptible cells by a distinct peptide permease system, it is hydrolyzed to L-alanine and L-anticapsin by peptidases. Then released anticapsin inhibits the activity of glucosamine synthetase that is necessary for bacterial peptidoglycan or fungal mannoprotein biosynthesis (Kenig *et al.*, 1976; Perry and Abraham, 1979; Chmara *et al.*, 1981). These effects of anticapsin leads to cell protoplasting and lysis. According to its metabolic target, anticapsin becomes specifically antagonized by glucosamine or N-acetylglucosamine (Walton and Rickes, 1962; Kenig and Abraham, 1976).

Hilton et al. (1988) was found that prephenate which belongs to the aromatic amino acid pathway is the primary precursor of anticapsin. Ligation of the peptide bound with L-alanine proceeds in an enzymatic reaction by catalysing via an amino acid ligase, bacilysin synthetase (Sakajoh *et al.,* 1987).

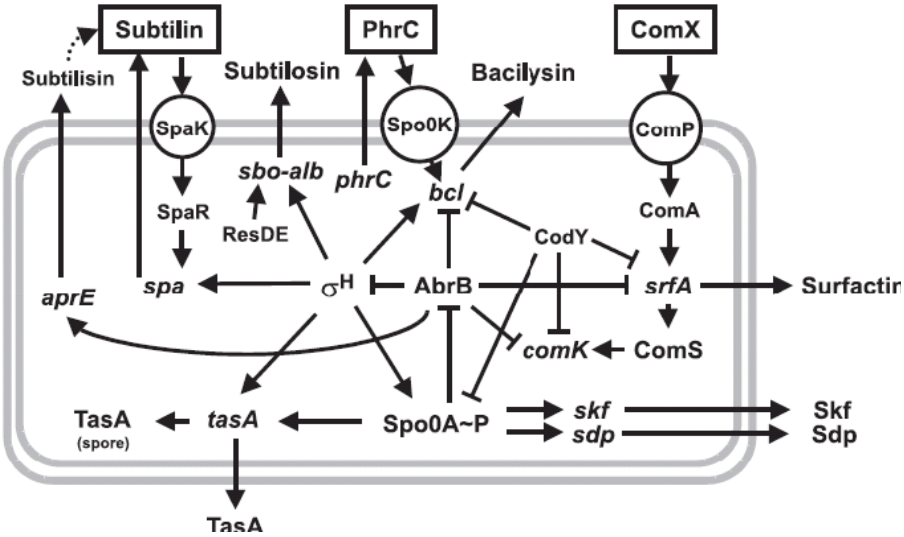


Figure 1.7 : Antibiotic biosynthesis pathway of B. subtilis involved in subtilin, subtilosin, surfactin and bacilysin.

Bacilysin production is related to the active growth in a synthetic media and is eliminated by certain growth conditions, particularly in the presence of certain nutrients, glucose or casaminoacids, and/or physiological factors, like pH, temperature (Özcengiz *et al*., 1990; Özcengiz and Alaeddinoglu, 1991; Basalp *et al*., 1992). It was demostrated that its synthesis seems to be under stringent response (Inaoka *et al*., 2003) and feedback regulation (Özcengiz and Alaeddinoğlu, 1991) as well as proved more recently is under the control of a quorum sensing regulation system through the action of ComQ/ComX, PhrC (CSF), ComP/ ComA and in a Spo0K (Opp)-dependent manner in *B. subtilis* (Yazgan *et al*., 2001; Yazgan-Karatas, *et al*., 2003). The *bacABCDE* genes of bacillus subtilis (renamed from *ywfBCDEF)* carries biosynthetic core functions on bacilysin biosynthesis. Besides, *Inaoka et al*. (2003) reveals that a monocistronic gene (*ywfH*) is also essential for its production. Each gene of *bac* operon has particular 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, respectively, which are assigned in anticapsin production from prephenate of the

aromatic amino acid pathway (Hilton *et al.*, 1988; Inaoka *et al.*, 2003; Steinborn *et al*., 2005). Moreover, the fuction of *ywfH* gene is still unknown but it can be employed to be involved in the alanine-anticapsin ligation (Inaoka *et al.*, 2003).

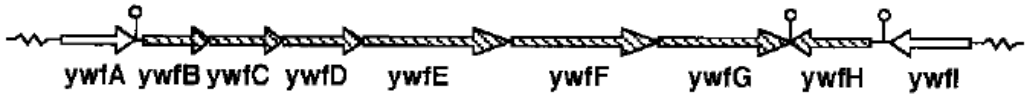


Figure 1.8 : Organization of the bacilysin gene cluster *ywfABCDEFG* and *ywfH* gene of *Bacillus subtilis* 168 (Inoaka *et al.*, 2003)

Large multienzyme complex functioning in a thiotemplate mechanism in a pathway initiated by a protein template is particular to prokaryotic organisms for producing antimicrobial peptide synthesis but still ribosomal peptide synthesis, driven by aminoacyl tRNA synthetase, is conserved for all cellular organisms. Until recently, it was thought that formation of bacilysin was conducted multiple-carrier thiotemplate mechanism but its biosynthesis mechanism is not completely matched with non-ribosomal peptide synthetase (NRPS) mechanism since adenylation and thiolation are evident only for L-alanine and not for L-anticapsin (Marahiel, 1997; vonDöhren *et al*., 1999).

The CodY regulon encodes extracellular degradative enzymes, transport proteins, catabolic enzymes, factors involved in genetic competence, antibiotic synthesis pathways, chemotaxis proteins, and sporulation proteins whose 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; Joseph *et al*., 2005). Inaoka *et al.* (2003) was demostrated that bacilysin biosynthesis is under synergistic regulation of guanosine 5’-diphosphate 3’-diphosphate (ppGpp) and guanosine triphosphate (GTP) affecting *bacABCDE* operon with CodY-mediated respression system. Additionaly, it was also shown that the expression of *ywfH* gene is dependent upon the level of intracellular GTP rather than ppGpp. More recently, two types of small-molecule effectors was identified regulating the fuction of CodY protein one of which is GTP that interacts with CodY and activates it as a repressor, the other one is the branched-chain amino acids (BCAAs) isoleucine and valine that also bind to CodY and enhance its binding to target sites (Joseph *et al*., 2005). In addition, CodY binding site in the *srfA* promoter region covers the putative RNA polymerase binding site but not with the ComA binding sites indicating that *srfA*

expression is repressed by CodY in the presence of Casamino Acids whether or not ComA is active (Serror and Sonenshein, 1996; Roggiani and Dubnau, 1993).

During transition state between active growth and stationary phase, under poor environmental conditions *B. subtilis* activates expression of many of genes which are needed for changing metabolism and energy production pathway. This process is controlled by the special proteins which are called transition state regulators such as AbrB, Hpr and Sin (Strauch and Hoch, 1993).

AbrB might behave in three different ways to affects gene expression: repression, activation and prevention. It represses expression of the *Spo0E* (Perego and Hoch, 1991) and *tycA* (Marahiel *et al.* 1987) genes, also acts as a preventer to other genes like *spoVG* (Zuber and Losick, 1987) and *aprE* (Ferrari *et al.*, 1988). As an activator, AbrB not only increases the expression of *hpr* gene, but also it activates some competence pathway regulators (Dubnau, 1991). In wilde type cells *abrB*-disrupted mutant cells caused an increase in the production of bacilysin but bacilysin negative phenotype in insertionally inactivated *spo0A* mutant cells was affected positively by the *abrB* mutation. This observation elucidated that AbrB has a repressive effect on the expression of genes which are related to bacilysin production (Yazgan-Karatas, *et al*., 2003).

Hpr is the DNA binding transition state regulator which has wide variety of function in cell mechanism. It is necessary for production of proteases, oxidative stress responses (Strauch and Hoch, 1993) and also has a crucial role in the initiation of sporulation (Perego and Hoch, 1988). Additionaly, it downregulates oligopeptide transport by repressing transcription of two operon; *opp* and *app* (Koide, Perego and Hoch, 1999). On the other hand, its transcriptional regulation is arranged by AbrB protein (Strauch and Hoch, 1993). More recently Inaoka and his colleagues concluded that *hpr* acts as a repressor in bacilysin synthesis. The expression of *ywfBCDEFG* increased during exponential phase in absence of Hpr and it was also shown that, Hpr protein directly binds to the promotor region of *bac* operon in order to create this effect. Under the light of this finding, it was identified that bacilysin biosythesis of *Bacillus subtilis* was negatively regulated by transition state regulator Hpr (ScoC) together with AbrB and CodY. Besides, it was revealed that *scoC* was not involved in regulation of *ywfH* expression (Inaoka *et al*., 2009).

In addition to mentioned global regulatory genes, recently, a novel gene, *yvfI*, is reported as required to bacilysin biosynthesis (Köroğlu *et al.*, 2008).

1.4 The Aim of The Present Study

Bacilysin is a simple dipeptide antibiotic produced and secreted by certain species of *Bacillus subtilis*, which consists of L-alanine and L-anticapsin. Its biosynhesis is under quorum sensing global regulation through the action of ComQ/ComX, PhrC (CSF), ComP/ComA and in a Spo0K (Opp)-dependent manner. Biosynthesis of bacilysin depends on the *bacABCDEywfG* operon (*bac* operon) and the adjacent *ywfH* gene. Recently, distinct effects of quorum sensing-signal transduction regulator ComA and the global transition state regulators Spo0A, AbrB and CodY on the expression of *bac* operon was revealed. Under the light of these findings, the present study aims to determine the interaction of global regulatory factors ComA, CodY, AbrB, Spo0A, DegU, and ComK on the promoter region of *ywfH*, as an essential bacilysin biosynthetic gene, by using "Electrophoretic Mobility Shift Assay (EMSA)".

2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Bacterial strains

Strains used in this project are listed in (Table 2.1). pGEM-T vector (Figure 2.1) was used for cloning of PCR products. pQE60 was used for the expression of desired proteins (Figure 2.2).

Table 2.1: Bacterial strains and their genotypes used in the project

Strain or plasmid	**Relevant Genotype, phenotype, and/or characteristics**	**Construction, source or reference**
B. subtilis PY79	wild type, BSP cured prototrophic derivative of *B.subtilis* 168	P.Youngman
E. coli Top10F'	*lac*Iq TN10 (Tetr)}, *mcrA* Δ (*mrr-hsdRMS-mrcBC*), *f80lacZΔM15* Δ*lacX74, deoR, recA1, araD139* Δ(*ara-leu*)*7697, galU, galK, rsL,(strr), endA1, nupG*	M.A. Marahiel
pHis$_6$-*comK*	*ComK in pQE60*	This study
pHis$_6$-*abrB*	*AbrB in pQE60*	H. Tayran
pHis$_6$-*spo0A*	*Spo0A in pQE60*	H. Tayran
pHis$_6$-*degU*	*DegU in pQE60*	T. E. Köroğlu
pHis$_6$-*comA*	*ComA in pQE60*	A.Berkyürek
pHis$_6$-*codY*	*CodY in pQE60*	T. E. Köroğlu

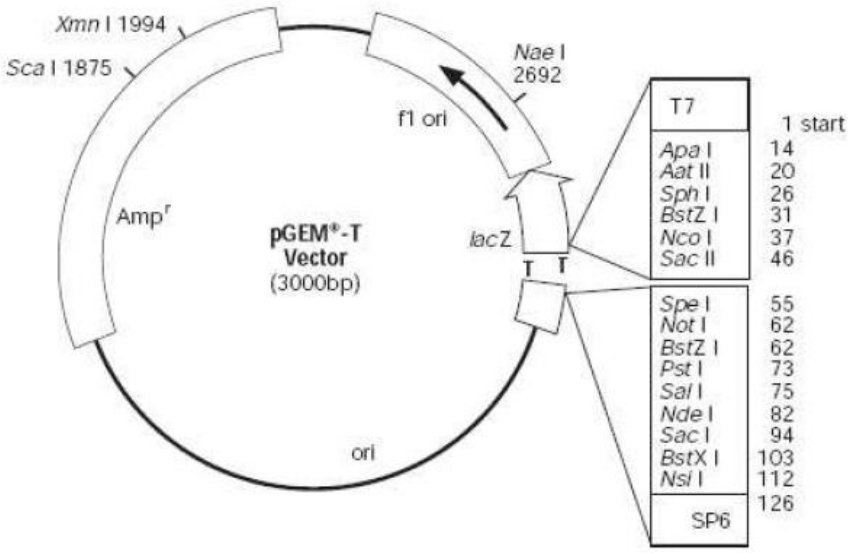


Figure 2.1 : Genomic map of the pGEM-T vector.

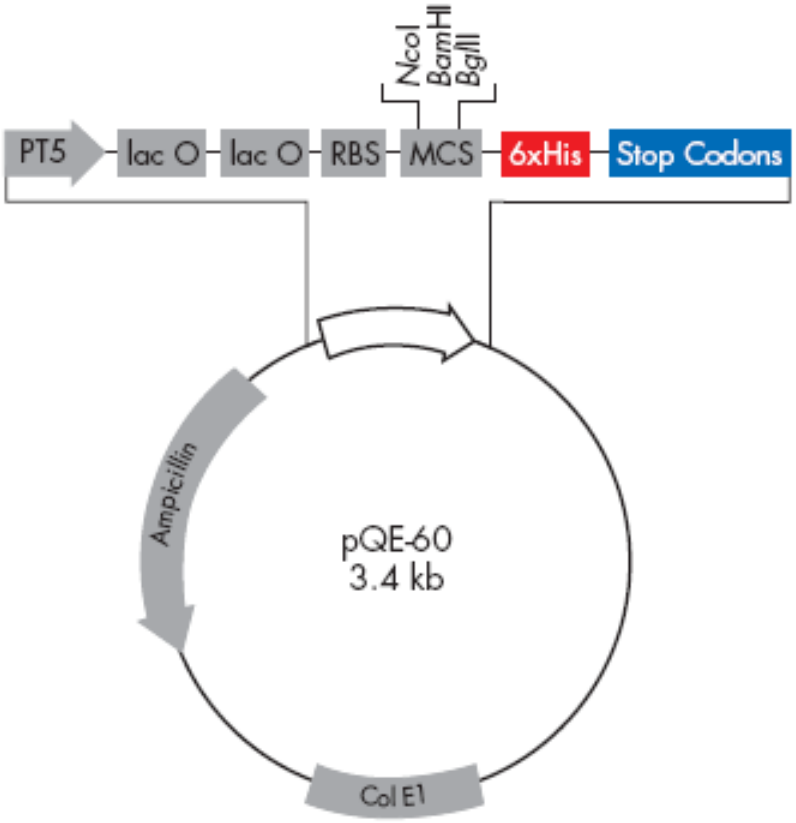


Figure 2.2 : Genomic map of the pQE60 expression vector.

2.1.1 Bacterial culture media

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

2.1.2 Buffers and solutions

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

2.1.3 Chemicals and enzymes

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

2.1.4 Laboratory equipment

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

2.1.5 Maintenance of bacterial strains

B. subtilis PY79 and *E.coli* strains were grown in Luria-Bertani (LB) liquid medium and kept on Luria-Bertani (LB) agar plates at +4°C. All strains were subcultured monthly. 10% LB glycerol stock was prepared for pHis$_6$-*comK* and other strains for long time storage at -80°C. Amp (100 μg/ml) and Tetracycline (Tet) (15 μg/ml) were used for *E.coli* Top 10 F' strain as the selective antibiotics.

2.2 Methods

2.2.1 DNA techniques and manuplations

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, pellet was resuspended in 300 μl P1 buffer (Appendix B). Following that, 300 μl P2 (Appendix B) buffer was added and solution mix was then incubated at room temperature for 5 minutes. After incubation time, 300 μl P3 (Appendix B) buffer was added and mixed through inverting the tubes until the lysate is no longer viscous. The tubes were then incubated on ice for 15 min. Then spinned 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 by applying centrifugation at 13.000 rpm for 5 minutes. 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* was isolated and purified by using a standart procedure devised for *Bacillus* species (Cutting and Horn, 1990).

1,5 ml of overnight culture of *B. subtilis* PY79 strain was centrifuged at 13000 rpm for 5 min. After discarding the supernatant obtained pellet was resuspended in 567 µl of TE (Appendix B) by repeated vortexing. Then, 10 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 mixture was incubated for 1 hour at 37°C in a water bath or in a thermomixer. Then, 100 µl of 5M NaCl solution was added and the sample was mixed without vortexing until the mucosal white substance become visible. Following, 80 µl of CTAB / NaCl (Appendix B) (prewarmed at 65°C because of viscosity) solution was added into the mixture and it was incubated for 10 min in 65°C water bath or thermomixer. The sample was then extracted with the same volume of freshly prepared phenol/chloroform/isoamyl alcohol (25:24:1) solution and centrifuged at 13000 rpm for 10 min. At a later stage, the upper phase was transferred to a new 1.5 ml microfuge tube and 0.7 volume isopropanol was added. After mixing shortly, the sample was centrifuged at 13000 rpm for 15 min. The pellet was washed with 1ml of 70% ethanol centrifuged at 13000 rpm for 5 min. Subsequently, the pellet was dried at 37°C for 1 hour and dissolved in 10 µl of TE buffer and 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 listed in Table 2.2. In order to obtain full length ComK sequences Expand High Fidelity PCR System (Roche) was used. 30 cycles lasted for 3,5 minute. The denaturation temperature was 94°C and the extention

temperature was 72^{o}C. The annealing temperature was 53^{o}C depending on the primer. The oligonucleotide primers were used at 1-20 pM (equimolar) and deoxyribonucleoside 5'triphosphates (dNTPs) were used at 0.2 mM final concentration.

Table 2.2: Oligunucleotide Primer Sequences

Primer	**Oligunucleotide Primer Sequences**
ywfH promoter Forward	5'- CCCTTGACTGGCTCCCATAAC -3'
ywfH promoter Reverse	5'- AGCCACGGTTTAATCGGCCGC -3'
RecA promoter Forward	5'- TTCACGAGTTTCACTTTGCGG-3'
RecA promoter Reverse	5'-AAGAGCCATATCTAAGGCTGC -3'
SdpA promoter Forward	5'-AGCAATATTTCACCTCAGAA -3'
SdpA promoter Reverse	5'-CGGGAATTCAATGTTTTCTTCTGTAGGGCT -3'
AtpI promoter Forward	5'-TTGGCAGTGTATGTACTGGGT -3'
AtpI promoter Reverse	5'-CGGGAATTCCATTCTTCTGACGAGCAGTAA -3'
YufO promoter Forward	5'-CAGTCGTTGAAGATGTAACGA -3'
YufO promoter Reverse	5'- CGCCTTGCGGATATTGAGCAT-3'
ComK F	5'- CGG GTATCCATGGGAGTCAGAAAACAGACGCA-3'
ComK R	5'- GCC GGATCCATACCGTTCCCCAGCTCACG -3'

A master mix composed of the materials listed below (Table 2.3) was prepared. Then, the master mix was aliquoted into for each PCR tubes and 4 µl of chromosomal DNA of *Bacillus subtilis* PY79 was added into each tube as template DNA. Finally, 0.5 µl of Expand High Fidelity enzyme was added for each tube.

Table 2.3: List of materials used for preparation of Expand High Fidelity PCR mixture

Reverse primer	2 µl
Forward primer	2 µl
10x Expand High Fidelity Buffer	10 µl
dNTP Mix (10 mM)	1 µl
Template DNA	4 µl
Expand High Fidelity Enzyme	0.5 µl
dH_2O	30.5 µl

Besides, the other PCR reactions were performed using *pfU* DNA polymerase 10x reaction buffer from Fermentas. *pfU* PCR conditions and materials used for preparation of *pfU* PCR mixture were listed below (Table 2.4)

Table 2.4: *pfU* PCR conditions

	Temperature	**Time**	**Cycles**
Initial Temperature	94 °C	2 min	
Denaturation	94 °C	30 s	
Annealing	50-55 °C	30 s	30 cycles
Extension	72 °C	2 min 30 s	
Final Extension	72 °C	10 min	

Table 2.5: List of materials used for preparation of PCR mixture

Reverse primer	0.5 µl
Forward primer	0.5 µl
10x Buffer (-$MgSO_4$)	5 µl
$MgSO_4$	5 µl
dNTP Mix (10 mM)	1 µl
Template DNA	1.5 µl
pfU DNA polymerase	0.5 µl
dH_2O	36 µl

2.2.1.4 Agarose gel electrophoresis

According to 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 gel system composed of ~1% agarose gel (different gel concentration for different samples) (Table 2.4) containing 1xTAE buffer (Appendix B) and ethidium bromide of a 0.2 µg/mL final concentration. 6X Loading dye 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*I+*HindIII* digested DNA marker and Middle range DNA Marker (Appendix D) were used to determine the molecular weights of DNA bands for desired purposes.

Table 2.6: Agarose gel concentration for different samples.

Sample	Agarose Concentration
Chromosomal DNA	0,6%
Plasmid DNA	0.8%
Digestion products of plasmid	1%
PCR products with	1.5% - 2%

2.2.1.5 Gel extraction

The desired fragments were extracted from the gel by using "MinElute Gel Extraction Kit" (Qiagen Inc., Valencia, CA). The gel slice containing the DNA band was excised from the gel and weighed. 3 volumes of buffer QG were added depending on the weight of the fragment. If the color of the solution was not yellow, 10 µl of 3M sodium acetate (pH 5.0) was added. Following, the solution was incubated for 10 min at 50°C by shortly vortexing every 2-3 min, until the gel was dissolved completely. After addition of 1 volume of isopropanol, the sample was applied to the MinElute column and centrifuged at 13000 rpm for 1 minute. Then the flow through was discarded and the MinElute column was placed back into the same collection tube. Later, 0.5 ml of buffer QG was added to the column and centrifuged at 13000 rpm for 1 minute. Subsequently, the flow through was discarded and 0.75 ml of buffer PE was added to wash. The column was standed for 2-5 min and then centrifuged at 13000 rpm for 1 minute, which was followed with an additional 1 minute at 13000 rpm. Eventually, the column was placed into a clean 1.5 ml microfuge tube and 10 µl from EB buffer was dropped to the center of the MinElute membrane within the column and it was let to stand for 1 minute and then centrifuged for 1 minute. The resulting solution within the 1.5 microfuge containing the plasmid DNA was stored at –20°C.

2.2.1.6 Ligation of PCR products

Ligation of PCR products into pGEM®-T easy vector

Ligation of PCR products to pGEM®-T Easy Vector was performed as follows: 5 µl 2X Rapid Ligation Buffer, T4 DNA Ligase, 1 µl (50 ng/µl) pGEM®-T Easy 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 overnight at 4°C. After ligation was completed, the mixture was used for transformation into *E. coli* Top10F'.

Ligation of pQE60 expression vector

Ligation procedure for clonning into the pQE60 expression vector was carried out using 8 µl of *ywfH* PCR products as insert fragments and 2 µl of pQE60 vector. Vector and fragment were mixed in a clean eppendorf tube and incubated for 10 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. In last step, the mixture was centrifuged for a short spin and incubated at 16°C for 16 h.

2.2.1.7 Restriction enzyme digestion

Restriction enzymes (*BamH*I and *Nco*I) were held in a suitable buffer such that enzyme amount added would be appropriately 2 Units per µg of DNA. The mixture was incubated at a temperature appropriate for that restriction enzyme for 1,5-2 h. The sample was stored at -20°C until needed.

2.2.1.8 Preparation of *E.coli* electrocompetent cells and transformation of electrocompetent *E.coli* top10F' cells

The overnight culture of *E.coli* Top10F' was diluted 1:100 fold into 400 ml 2xYT medium which contained Tetracyclin (15 µg/ml). The culture was incubated at 37°C with vigorous shaking (200 rpm) in an orbital shaker until reached to 0.6 of OD_{600} (Optical Density at 600 nm). After that, incubated cells were stayed on ice for 30 minutes. Cells were harvested by centrifugation at 5000 rpm for 5 minutes. Subsequent to discard supernatant, 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. Then, it was dispensed into aliquots of 40 µl into eppendorf tubes. These 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. 10 µl of ligation products or 0.5 µg of appropriate plasmid DNA was added into the tube of competent cell and were mixed gently. Then, mix was transferred into pre-cold electroporation tube. The sample was placed onto electroporator 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. Transformant cells were incubated at 37^{o}C for 60 minutes by shaking at 200 rpm. Following, the cells were centrifuged at 5000 rpm for 10 min and supernatant was discarded. Obtained pellet was resuspended in 100 µl 0.85 % saline solution (NaCl) (Apendix B). Transformed cells were plated on selective medium (LB) containing appropriate antibiotic (100µg/ml ampicillin). For the purpose of blue-white colony selection, 40 mg/ml X-gal and 1 mM IPTG were used.

2.2.2 Protein synthesis

2.2.2.1 Purification of 6xHis-tagged proteins from *E. coli* under native conditions

Overexpression of histidine tagged proteins in *E.coli*

The overnight culture of *E.coli* Top10F' that carrying the desired protein sequences in pQE60 expression vector was diluted 1:50 fold into 1liter LB broth medium which contains Amp (100 µg/ml). Culture was incubated at 37^{o}C shaking with 200 rpm until reached to 0.6 of OD_{600}. At this moment, 1 ml of bacterial sample was transferred into a microcentrifuge tube and harvested by centrifugation at 13 000 rpm for 1 minutes. Then bacterial culture was immediately induced with 1mM of IPTG final concentration and was incubated at 37 ^{o}C shaking with 200 rpm for 4-5 hours. At the end of this time, 1 ml of induced cell sample was taken into a microcentrifuge tube and harvested by centrifugation at 13 000 rpm for 1 minutes. Remaining bacterial cell culture was harvested by centrifugation at 5000 rpm for 15 minutes. After removal of supernatant, pellet was resuspended in 4 ml Lysis buffer (Appendix B). Cell culture was stored at -80 ^{o}C.

Purification of 6xHis-tagged proteins expressed in *E.coli*

In order to disrupt the cells, following the freeze-thaw, lysozyme (1 mg/ml) treatment was performed on ice for 30 min. The crude extracts were then fractionated by sonication three times at 55 watt for 3 min. After sonication, the cell lysates were centrifuged for 15 min at 15,000 x G to remove the insoluble materials and transferred the supernatant to a fresh microcentrifuge tube. To immobilize the proteins, Ni-NTA resin (10 µl resin has a capacity for 50–100 µg 6xHis-tagged protein) was added at the ratio of 10:1 to each microcentrifuge tube containing 1ml supernatant. The mixture (the supernatant and resin) was gently rotated for 30 min at 4°C. After that, samples were centrifuged for 1 min at 1000 x G to precipitate the resin. Resin pellets were washed three times with 1 ml wash buffer (Appendix B). After washing step, his_6-proteins were eluted with 100 µl elution buffer (Appendix B) 3 times. Histidine tagged proteins were eluted against imidazole concentration under native conditions.

2.2.3 SDS-PAGE analysis

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) or denaturing gel electrophoresis was used to monitor the development of the purification process, to determine homogenity and the relative molecular mass of the proteins. SDS-PAGE was carried on a 12% separating gel and a 4% stacking gel according to the protocol of Laemmli (1970).

Molecular weight of the proteins were determined by comparing their profiles with unstained protein molecular weight marker (Fermentas) (Appendix D).

To verify the protein induction, 50 µl SDS loading dye was added to the uninduced and induced bacterial samples and lysozyme treatment was applied for 30 min at 4°C. Besides, in order to check the purity of the proteins, 5 µl SDS loading dye was used for 10 µl extracted fractions. All samples and unstained protein marker were denatured at 99°C for 5 min. After denaturation, they were loaded onto SDS-PAGE gel and run at 80-120V for 2 h. At the next step, polyacrylamide gels were stained with SDS-PAGE staining solution (Appendix B) at 37°C for 1 h, washed overnight with destain buffer (Appendix B), and then visualized.

2.2.3.1 Preperation of SDS gel

Separating gel:

	12%
dH_2O	1,6 ml
30% Acrylamide	2 ml
1,5 mM Tris pH 8,8	1,3 ml
10% SDS	0,05 ml
TEMED	4 µl
10% APS	0,05 ml

Stacking gel:

	4%
dH_2O	1,4 ml
30% acrylamide	0,33 ml
1 M Tris pH 6,8	0,25 ml
10% SDS	0,02 ml
10 % APS	0,02 ml
TEMED	2 µl

2.2.3.2 Determination of protein concentrations with Bradford assay

Protein concentration was determined using the Bradford Protein Assay with (Bradford, 1976). 0.125, 0.25, 0.5, 0.75, 1, 1.5, 2mg/ml bovine serum albumin(BSA) were used as standarts in order to obtain a correct standart curve. Proteins and standart solutions were assayed in duplicate in a 96 wells microplate. Bradford assay was performed using 5 µl of each standart solutions and proteins with 250 µl Bradford reagent.

2.2.4 EMSA (Electrophoretic mobility shift assay)

ywfH promoter region, 414 bp length, was amplified by proof-reading PCR using *B.subtilis* PY79 chromosomal DNA as template with oligonucleotide primers 5'-CCCTTGACTGGCTCCCATAAC -3' and 5'- AGCCACGGTTTAATCGGCCGC -3' for gel retardation assay. 50 mM KCl, 5 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTT, 8% glycerol in 10mM Tris-HCl, pH 8 (Appendix B); reaction buffer which was

supplemented with poly [d(I-C)] (1μg/ml), BSA (0,1 μg/ml) and 75 nanograms of promoter DNA was used for gel retardation of CodY, AbrB and ComA, only for Spo0A 10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol (DTT) and 5% (v/v) glycerol (Appendix B) was used as binding buffer. In CodY reactions leucin-valine-isoleucine (10 mM each) and dGTP (2 mM) were additionally added. 25 μl total reaction mixtures were incubated at room temperature for 30 minutes. Following that, loading dye buffer [0,25xTBE, 60%; glycerol, 40%; bromophenol blue, 0,2%(w/v)] was added to each reaction. 6% native polyacrylamide gel was pre-run (80V) for 30 min at 4°C. After pre-running, acquired mixtures were load 6% native polyacrylamide gel (Table 2.7) and electrophoresis was run in 43 mM imidazole and 35 mM HEPES at 80 V for nearly 2 hours at 4°C. Following electrophoresis, gel was incubated at room temparature for 20 minutes with SYBR Green I Nucleic Acid Gel Stain (1/10000, v/v) (Roche) and visualized with UV-transulliminator to monitor the migrated DNA fragments.

Table 2.7: Native EMSA gel

	6%
dH_2O	5.9 ml
30% Acrylamide	2 ml
5x Hepes-imidazole buffer	2 ml
10% APS	0.1ml
TEMED	8 μl

3. RESULTS AND DISCUSSION

3.1 Construction of *E. coli* pComK-His_6-strain

To purify the ComK protein easily, an *E.coli* ComK-His_6 *strain* was constructed. Firstly, *comK* gene, 576 bp in length, was amplified with *PfU* DNA polymerase using chromosomal DNA of *Bacillus subtilis* PY79 wild type strain as a template by PCR. The primers *comK* forward 5'-CGG CCATGGGT ATGAGTCAGAAAACAGACGCA-3'and *comK* reverse 5'- GCC GGATCC ATACCGTTCCCCAGCTCACG -3'including extra residues for *Nco*I and *BamH*I recognition sites were used for the amplification of *comK* gene.

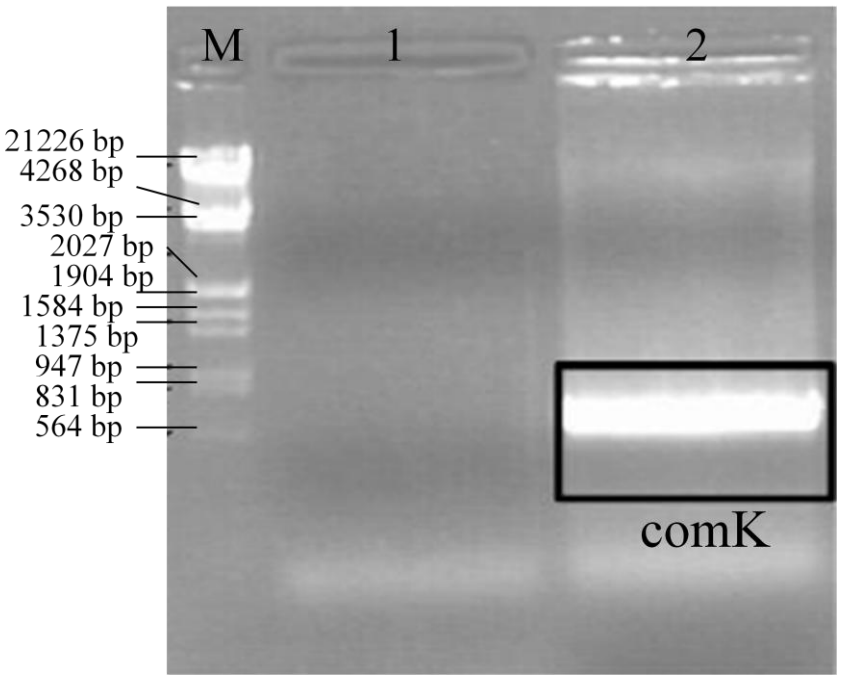


Figure 3.1 : Amplification of *comK* gene by PCR. Lane 2; 576bp PCR product *comK* gene amplified by using specific primers *comK* F and *comK* R and PY79 chromosomal DNA as template DNA, lane 1; Negatif control (no template DNA in PCR reaction) and M; Middle Range DNA Marker (Fermentas) (Appendix D).

The amplified PCR product was purified after running in agarose gel (Fig. 3.1). Extracted fragment was ligated into pGEM®-T Easy Cloning Vector and

transformed into *E. coli* Top10F' electro-competent cells. Transformants were selected on X-gal/IPTG and ampicillin-containing agar plates. Plazmids isolated from selected white colonies were first screened with *EcoRI* digestion in order to confirm the cloning of 576 bp insert into pGEM-T vector (Figure 3.2) and then sequenced to confirm that cloned 576 bp inserts belongs to *comK* gene.

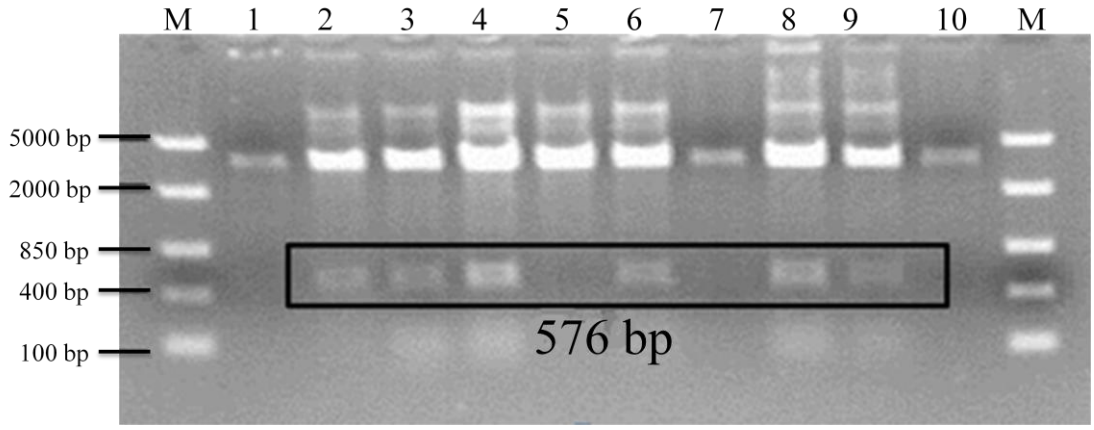


Figure 3.2 : Confirmation of cloning of 576 bp DNA fragment into pGEM®-T Easy Vector. M; Middle Range DNA Marker (Fermentas)

Isolated pGEM-T-*comK* recombinant plasmid and pQE60 expression vector were double digested with *Nco*I and *BamH*I restriction enzymes (Fig. 3.3). Following, digested *comK* fragment from pGEM®-T Easy and digested pQE60 expression vector were extracted from agarose gel and ligated with T4 ligase overnight at 4^{o}C. Then obtained ligation product was transformed into electrocompetent *E. coli* Top10F' cells and colonies were selected on 100 μg/ml ampicillin containing agar plates.

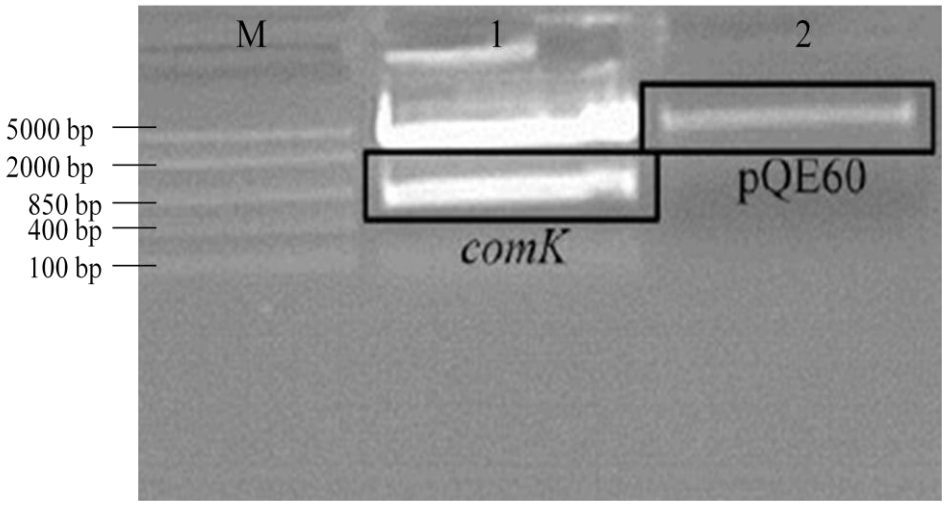


Figure 3.3 : Digestion of *comK* gene cloned into pGEM®-T Easy Vector. Lane 1; *comK* gene fragment digested with *Nco*I and *BamH*I restriction enzymes lane 2; linear pQE60 expression vector digested with *Nco*I and *BamH*I restriction enzymes M; Middle Range DNA Marker (Fermentas) (Appendix D).

Then, plasmid DNAs isolated from transformants were digested with *Nco*I and *BamH*I restriction enzymes in order to confirm the cloning of 576 bp *comK* gene into pQE60.

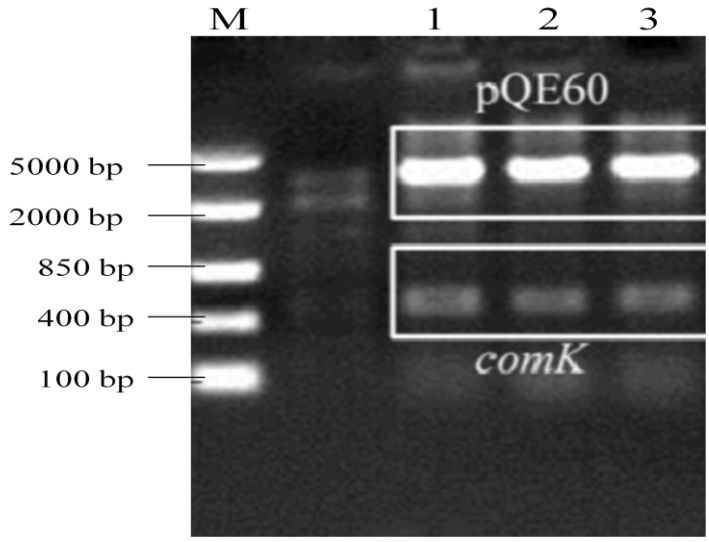


Figure 3.4 : The confirmation of the colonies including *comK* gene in pQE60 expression vector via *Nco*I and *BamH*I restriction enzyme digestion (lane 1-3). M; Middle Range DNA Marker (Fermentas) (Appendix D).

3.2 Expression of *comK*-his$_6$ in *E. coli* Protein

In order to purify ComK protein, firstly we checked whether *comK-his$_6$* is expressed in *E. coli* as in soluble or insoluble form. For this purpose, constructed *E.coli* p*comK-his$_6$* strain was grown in 10 mL of LB medium containing ampicillin (100 μg / ml) at overnight at 37 ^{o}C with shaking at 200 rpm. 1 mL of overnight culture was then transferred into 50 ml of LB medium. Culture was then incubated at 37 ^{o}C with shaking at 200 rpm until OD_{600} of culture reached to 0.6. At this moment, 1 ml of culture was transferred to a centrifuge tube and cells were harvested by centrifugation and stored. The remaining culture was then induced with 1mM of IPTG addition and incubated at 37 ^{o}C with shaking at 200 rpm for 5 hours. 1 ml of induced culture was then centrifuged and pellet was dissolved in 50μl SDS sample buffer. Finally, uninduced and induced cell samples were run on SDS-PAGE.

Our SDS-PAGE results indicated that ComK protein was successfully overexpressed in *E. coli* cells as in soluble form (Fig. 3.5).

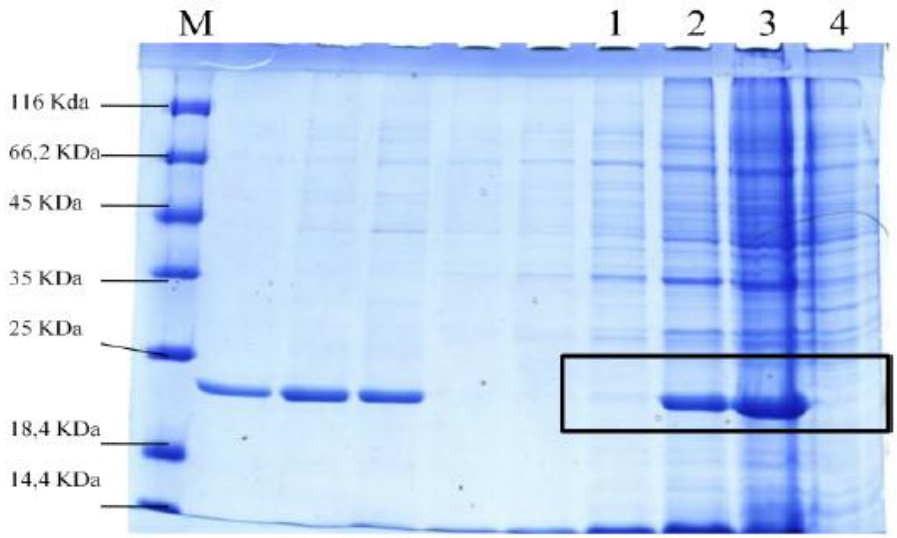


Figure 3.5 : Determination of the ComK protein localization and overexpression. M:Unstained protein molecular mass marker (Fermentas), uninduced sample (lane 4), induced sample (lane 3), flow-through (lane 2), pellet (lane 1).

3.3 Overexpression of ComK-His_6, ComA-His_6, ComK-His_6, DegU-His_6, CodY-His_6, AbrB-His_6 and Spo0A-His_6 proteins in *E.coli*

Each of the recombinant *E. coli* strains carrying the expression vector for the global regulatory proteins tested in this study were grown in 100 mL of LB medium and then diluted 50 fold into 1liter of LB medium containing ampicillin (100 μg/ml). At the beginning of the exponential phase, growing cells were induced with 1mM IPTG to overexpress proteins. After 5 hours incubation period, cells were harvested by centrifugation. Pellets were resuspended in 4 ml lysis buffer and stored at -80 ^{o}C.

3.4 Purification of ComK-His_6, ComA-His_6, ComK-His_6, DegU-His_6, CodY-His_6, AbrB-His_6 and Spo0A-His_6 proteins

In order to purify ComA, ComK, DegU, CodY, AbrB and C-terminal DNA-binding domain of Spo0A proteins, 50% slurry Ni-NTA resin (Qiagen) was used. As first step, cells were lysed by sonication. The culture was cleared by centrifugation at 15,000 G for 15min at 4^{o}C and the cell extracts were treated with Ni-NTA resin. After treatment, histidine-tagged proteins were eluted against increased imidazole concentration under native conditions.

3.5 SDS-PAGE Analysis

Histidine tagged proteins were purified by using Ni-NTA resin system. The extracted proteins were analysed by SDS-PAGE on the basis of the molecular mass of the proteins. As seen in Fig.3.6-11 ComK-His6, AbrB-His6, Spo0A-His6, CodY-His6,ComA-His6 and DegU-His6 were purified succesfully.

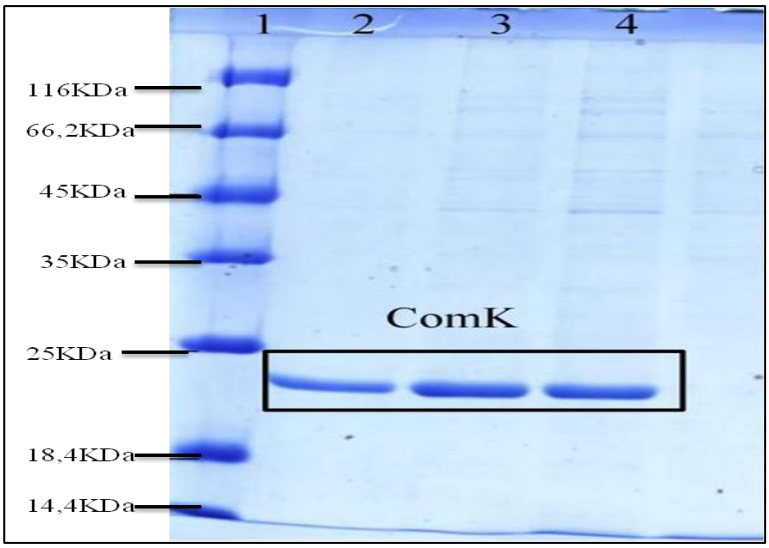


Figure 3.6 : Purified ComK (22.3KDa) protein. 1; Protein molecular mass marker (Fermentas), elutions of the purified ComK proteins (lane 2-4).

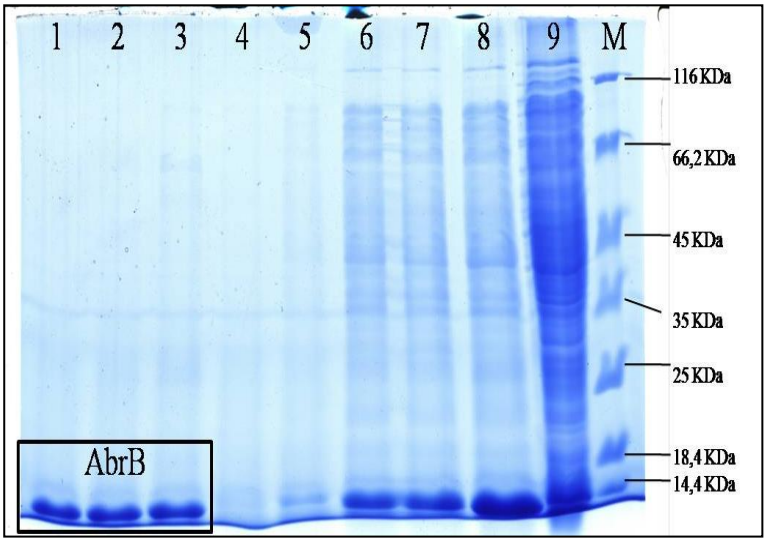


Figure 3.7 : Purified AbrB (10.6KDa) protein. M; Protein molecular mass marker (Fermentas), uninduced sample (lane 9), induced sample (lane 8), flow-through (lane 7), wash 1-2-3 samples (lane 4-6), elutions of the purified AbrB proteins (lane 1-3).

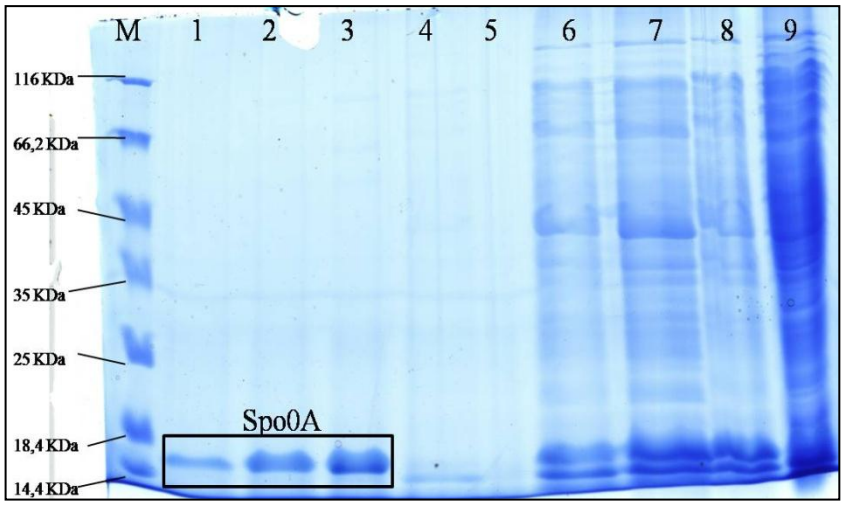


Figure 3.8 : Purified Spo0A (13.9KDa) protein. M; Protein molecular mass marker (Fermentas), uninduced sample (lane 9), induced sample (lane 8), flow-through (lane 7), wash 1-2-3 samples (lane 4-6), elutions of the purified Spo0A proteins (lane 1-3).

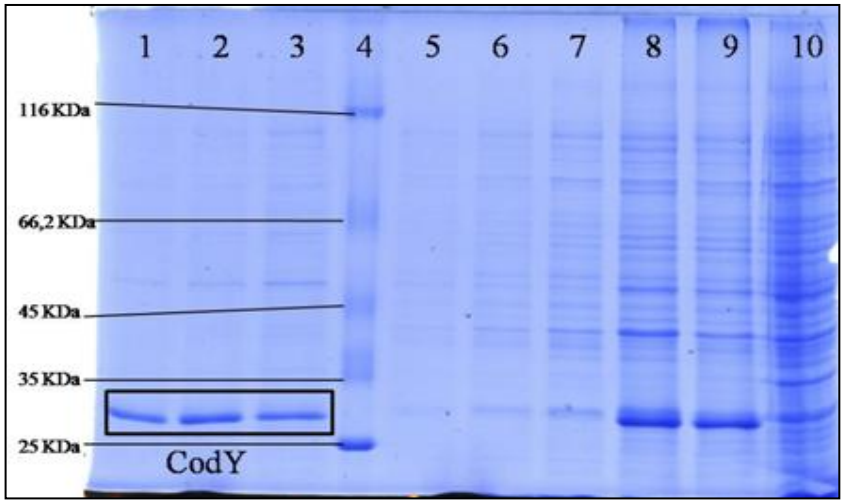


Figure 3.9 : Purified CodY (28.9 KDa) protein. Lane 4; Protein molecular mass marker (Fermentas), uninduced sample (lane 9), induced sample (lane 8), wash 1-2-3 samples (lane 5-7), elutions of the purified CodY proteins (lane 1-3).

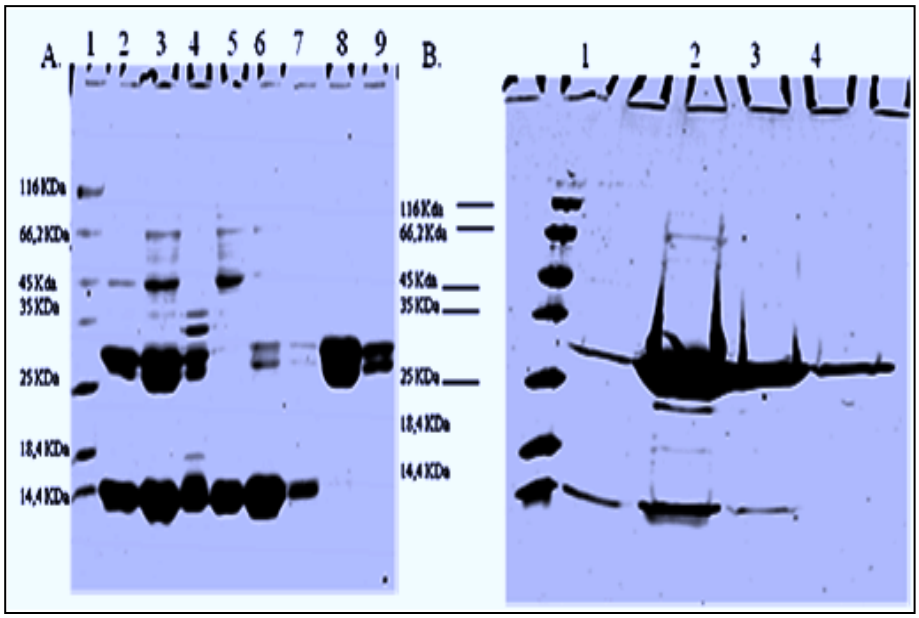


Figure 3.10 : Purified ComA (23.98KDa) protein. A. Protein molecular mass marker (Fermentas) (lane 1), wash 1-2-3 samples (lane 5-6), elutions of the purified ComA proteins (lane 2-4). B. Protein molecular mass marker (Fermentas) (lane 1), elutions of the purified ComA proteins (lane 2-4).

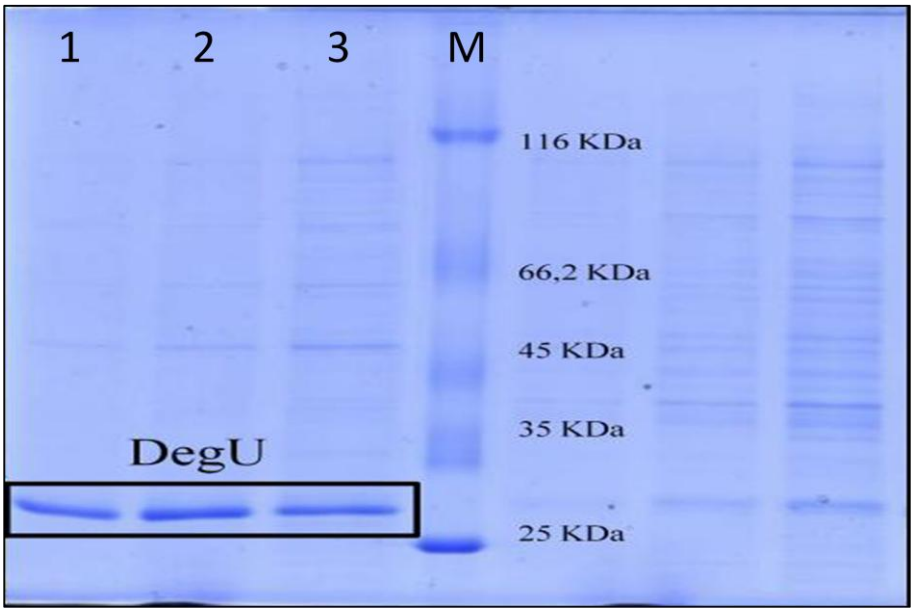


Figure 3.11 : Purified DegU (25.71KDa) protein. M; Protein molecular mass marker (Fermentas), elutions of the purified DegU proteins (lane 1-3).

3.6 EMSA (Electrophoretic Mobility Shift Assay) Experiments

3.6.1 Amplification of the promotor regions using *PfU* DNA polymerase

ywfH promotor region was amplified with *pfu* DNA polymerase using *B.subtilis* PY79 chromosomal DNA as template with oligonucleotide primers 5'-

CCCTTGACTGGCTCCCATAAC-3’ and 5’-AGCCACGGTTTAATCGGCCGC-3’ for gel retardation assay. After amplification, PCR product was loaded into the %1 agarose gel to verify size of the DNA. In addition to *ywfH* promoter region, *recA*, *atpI*, *yufO* and *sdpA* promoter regions were also amplified with PCR.

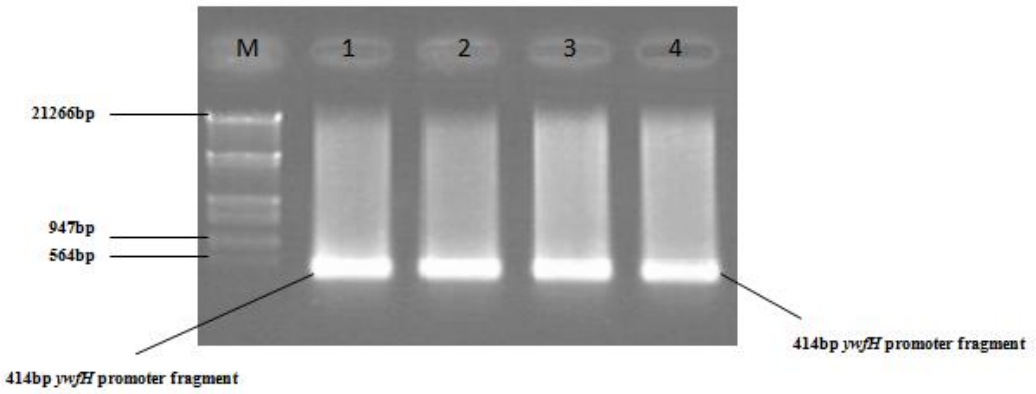


Figure 3.12 : *ywfH*, 414bp in length, pcr products (lane 1-4).

3.6.2 Gel retardation assay

To determine whether the transcriptional regulators AbrB, Spo0A, CodY, ComA and DegU exerts their effects on the *ywfH* promoter through a direct interaction or not, electrophoretic mobility shift assays were employed. For this purpose, the amplified *ywfH* promoter region, 414bp in length, were incubated with the various concentration of the purified regulatory proteins. Each binding experiment were performed in the presence of excess nonspecific competitor DNA poly [d(I-C)]. The promoter regions of arbitrary chosen genes: *recA, yufO, sdpA* and *atpI,* not known nor believed in the same regulon were also included as negative controls and bovine serum albumin (BSA, 10 mg/ ml) was used in negative control reactions as candidate protein to verify and monitor the specifity of the protein-DNA interaction.

3.6.2.1 Binding of the ComA, DegU, CodY, AbrB and Spo0A to the *ywfH* promoter

In *Bacillus subtilis*, the transcription factor ComA activates various biological processes including development of genetic competence, sporulation, production of degradative enzymes, biofilm formation and antibiotic synthesis by using the ComX-ComP-ComA signaling circuit which constituting the quorum response (Auchtung *et*

al., 2006; Griffith and Grossman, 2008; Natalia and Grossman, 2005). Two extracellular signaling peptides, ComX pheromone and CSF (for competence and sporulation factor) are used to coordinate the activity of ComA. When the concentration of signaling peptides is low, ComA is largely inactive but higher concentration of signaling peptides enables ComA to become activated and stimulate the transcription of at least nine operons (Griffith and Grossman, 2008; Lazazzera, 2000).

In the previous studies, Yazgan *et al.* (2001) demostrated that bacilysin biosynthesis is under the control of quorum sensing system and signal transduction phosphorelay. Consistently, our recent study revealed that cell density-dependent induction of *bac* operon is directly depends on ComA.

Under the light of these findings in order to elucidate whether ComA directly binds to *ywfH* promoter (P_{ywfH}) or not, electrophoretic mobility shift assay was performed in the current study. As shown in figure 3.13 based on the increased amount of ComA-His_6 concentration, *ywfH* promoter was shifted obviously, and the degree of the retardation of the *ywfH* promoter directly depends on the ComA concentration. This results imply that more than one molecules of ComA can bind to *ywfH* promoter and the stimulatory effect of ComA on the transcripiton of *ywfH* gene is exhibited by direct binding to P_{ywfH}. This finding is consistent with our previous study in that the disruption of ComA causes basal expression level decreased to 2% of that in the parental strain, indicating that ComA activity is crucial for the activation of the *bac* operon transcription and it exerts its stimulatory effect directly by binding to *bac* promoter (Köroğlu *et al*., 2011).

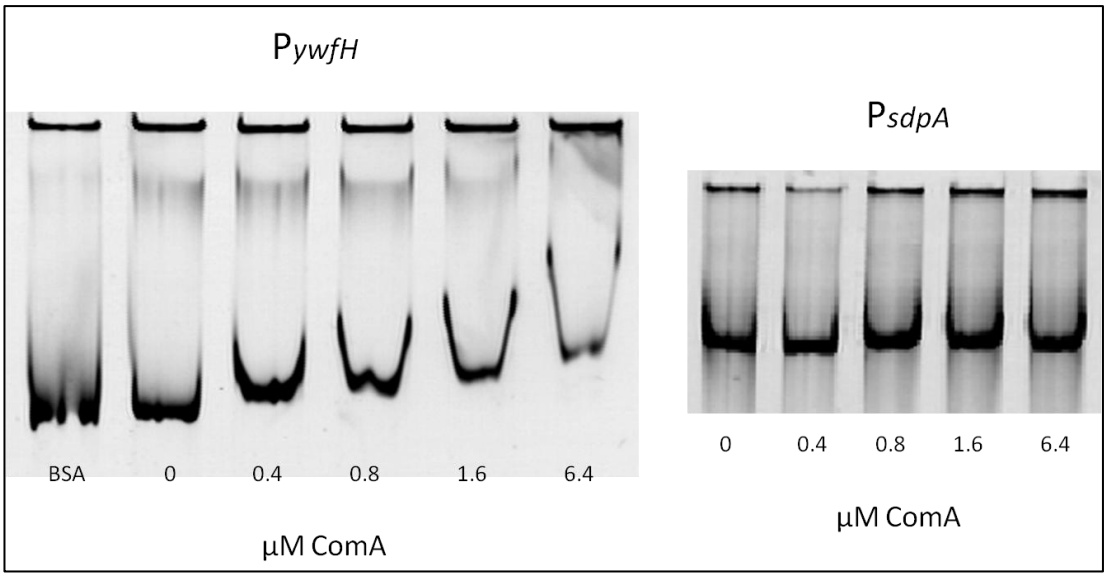


Figure 3.13 : Gel retardation assay performed by *ywfH* promoter fragments incubated with purified ComA-His_6 at the indicated concentrations. Gel shift for controls: promoter region of *sdpA* for ComA binding represent the negative control. 'BSA' lanes also represent the negative controls of the reactions where only 10 mg/ml BSA were used as the candidate protein. All of the binding reactions contain 75 ng/µl of *ywfH* promotor DNA and 1mg/ml poly[d(I-C)].

DegS and DegU are sensor and effector proteins that are transcribed from *sacU* locus and they form a two-component signal transduction regulatory system (Stock *et al.*, 1989). They take part 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).

The response regulator DegU that is responsible for degredative enzyme synthesis also participates in competence devolopment as a phosphorilation dependent manner in *Bacillus subtilis*. Transcription factor ComA-P also regulates *srfA* operon. *comS* gene that is embedded in large *srfA* operon releases ComK from ternary complex. ComK activates transcription of late competence genes (Susanna *et al*., 2006; Turgay *et al*., 1998; Hamoen *et al*., 2003, 1998). When the ComK concentrations are low, unphosphorilated DegU efficiently binds to the *comK* promoter to stimulate binding of ComK at the onset of competence development (Hamoen *et al*., 2000; Hameon *et al*., 2003; Susanna *et al*., 2006).

With this respect, in order to investigate possible effects of the response regulator DegU on the expression of *ywfH* which is a result of its direct binding to the P_{ywfH} or

via through some other regulatory proteins, gel retardation assay was performed. As shown in Figure 3.14. *ywfH* promoter fragment shifted depending on the increased amount of DegU-His_6 concentration. The degree of the retardation seems to be concentration dependent manner. This finding elucidated that response regulator DegU contributing to competence development takes a role in bacilysin formation by binding directly to P_{ywfH}. This result is not suprising because there are some clues on the effect of response regulator DegU protein on the bacilycin biosynthesis. Bacilysin and surfactin biosynthesis are under the control of same regulatory factors. In addition, in our previous study disruption of *srfA* operon is resulted in the bacilysin negative phenotype indicating that *srfA* directly plays a role in biosynthesis of bacilysin (Yazgan-Karatas, *et al*., 2003). Consistently, disruption of *degU* decreases the expression of *srfA* (Dubnau, 1991; Dahl *et al*., 1992).

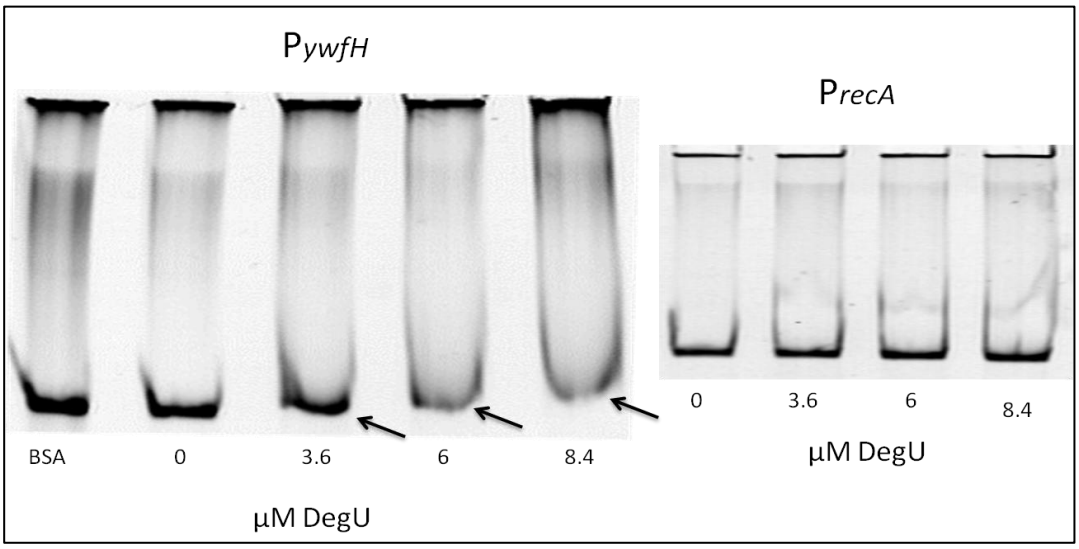


Figure 3.14 : Gel retardation assay performed by *ywfH* promoter fragments incubated with purified DegU-His_6 at the indicated concentrations. Gel shift for controls: promoter region of *recA* for DegU binding represent the negative control. 'BSA' lanes also represent the negative controls of the reactions where only 10 mg/ml BSA were used as the candidate protein. All of the binding reactions contain 75 ng/µl of *ywfH* promotor DNA and 1mg/ml poly[d(I-C)].

The other protein that regulates the transcription of early stationary phase genes is a GTP binding protein CodY. It was first identified as a repressor of the *B. subtilis* dipeptide transport (*dpp*) operon (*dppABCDE*) (Ratnayake-Lecamwasam *et al* 2001; Molle *et al*., 2003).

During rapid growth of cells in rich medium, CodY represses the early stationary phase genes whose products enable cells to adapt nutrient limitation. When the cells become limited for carbon or nitrogen or phosphorus source, CodY activity diminishes the genes repressed by CodY are transcribed. The activity of CodY depends on two effector molecules, GTP and branched-chain amino acids (Molle *et al*., 2003; Villapakkam *et al*., 2009).

In 2003 inaoka *et al*. revealed that *codY* disruption causes an increase at the level of expression of genes involved in bacilysin biosynthesis, therefore GTP-detecting transcriptional regulator CodY is effective on bacilysin production in *B. subtilis*. Furthermore, synthesis of bacilysin is controlled by dual regulation system consist of the guanine nucleotides ppGpp and GTP (Inaoka *et al*., 2003; Ratnayake-Lecamwasam *et al*., 2001).

Based on the dramatic effects CodY global regulator on bacilysin production, we checked if the *ywfH* promoter is the direct target of the CodY protein. For this purpose, electrophoretic mobility shift assay was performed using different concentrations of purified CodY-His_6. As seen in Figure 3.15, *ywfH* promoter fragment shifted apperantly with increased concentration of CodY. Thus, it can be suggested that, the effect of the CodY protein on the bacilysin biosynthesis is a result of direct binding of CodY to *ywfH* promoter region as in the case of *bac* operon. The transition state regulator CodY shows its effect on both P_{bac} and P_{ywfH} via direct interaction.

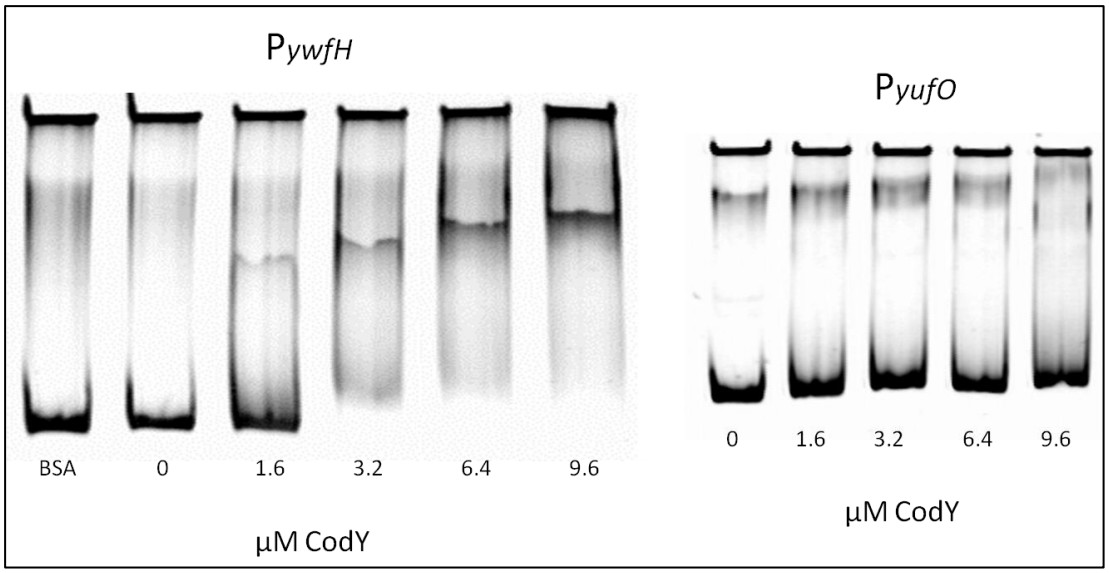


Figure 3.15 : Gel retardation assay performed by *ywfH* promoter fragments incubated with purified CodY-His_6 at the indicated concentrations. Gel shift for controls: promoter region of *yufO* for CodY binding represent the negative control. 'BSA' lanes also represent the negative controls of the reactions where only 10 mg/ml BSA were used as the candidate protein. All of the binding reactions contain 75 ng/µl of *ywfH* promotor DNA and 1mg/ml poly[d(I-C)].

AbrB is a key transcriptional regulator that represses many stationary phase processes in *Bacillus subtilis*. Genes involved in sporulation (e.g. *spo0E*, *spoVG*), degradative enzyme synthesis (e.g. *aprE*), amino acid utilization (e.g. *dpp*), and antibiotic production (e.g. *tycA*) have been shown to be under control of AbrB (Hamoen *et al*., 2003). During exponential growth, AbrB prevents premature expression of these genes. Towards the end of exponential growth, expression of *abrB* is repressed by the activated response regulator Spo0A-P (Hamoen *et al*., 2003).

Spo0A is the master regulatory protein that has been found to affect the expression of over 500 genes the early stages of stationary phase, directly or indirectly in *Bacillus subtilis* genome (Molle *et al.*, 2003). Formerly, it was shown that the SpoOA 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).

In previous study performed by Yazgan-Karataş *et al.* (2003), it was revealed that Spo0A and AbrB together regulate the synthesis of bacilysin. The loss of bacilysin

production in insertionally inactivated *spo0A* mutants was proved that biosynthesis of this antibiotic is under the control of Spo0A protein. While *spo0A*-disrupted mutants were defficient for bacilysin production, *abrB*-disrupted mutants created an increase in the production of bacilysin. Regain of antibiotic activity in *abrB* mutation in *spo0A*-blocked mutants elucidated 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.

As shown in Fig. 3.16 and 3.17 the mobility of the *ywfH* promoter was shifted remarkably upon incubation with the various amount of AbrB-His_6 and Spo0A-His_6. The degree of the retardation of the *ywfH* promoter fragment depends on the concentration of these proteins. These results showed that AbrB and Spo0A proteins specificly bind to the *ywfH* promoter region. These findings correlates with our recent study related with bacilysin biosynthetic operon. According to this study, both of these regulators have a dramatic effect on the regulation of *bac* operon through interacting directly to its promotor, P_{bac}.

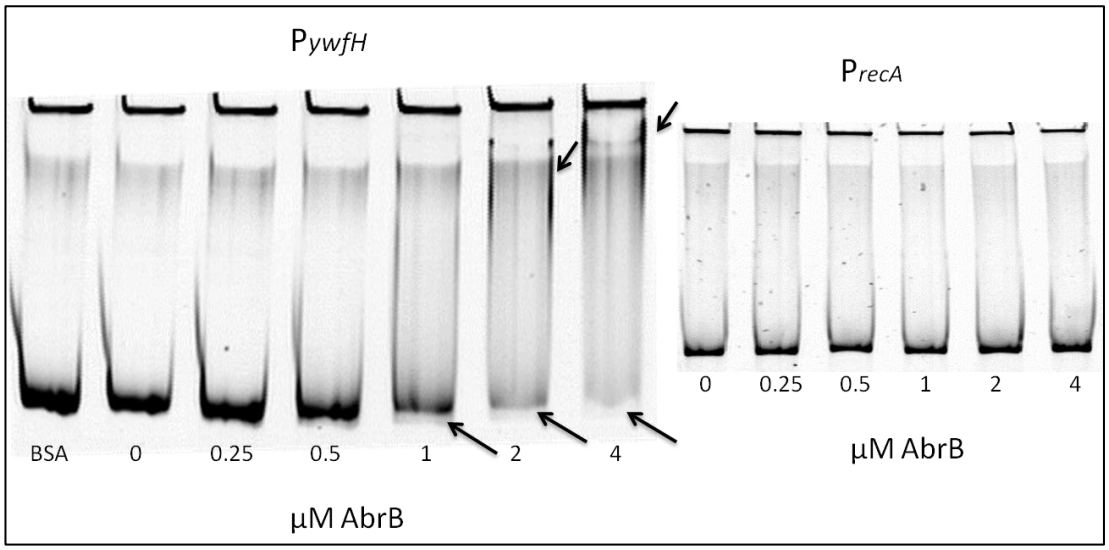


Figure 3.16 : Gel retardation assay performed by *ywfH* promoter fragments incubated with purified AbrB-His_6 at the indicated concentrations. Gel shift for controls: promoter region of *recA* for AbrB binding represent the negative control. 'BSA' lanes also represent the negative controls of the reactions where only 10 mg/ml BSA were used as the candidate protein. All of the binding reactions contain 75 ng/µl of *ywfH* promotor DNA and 1mg/ml poly[d(I-C)].

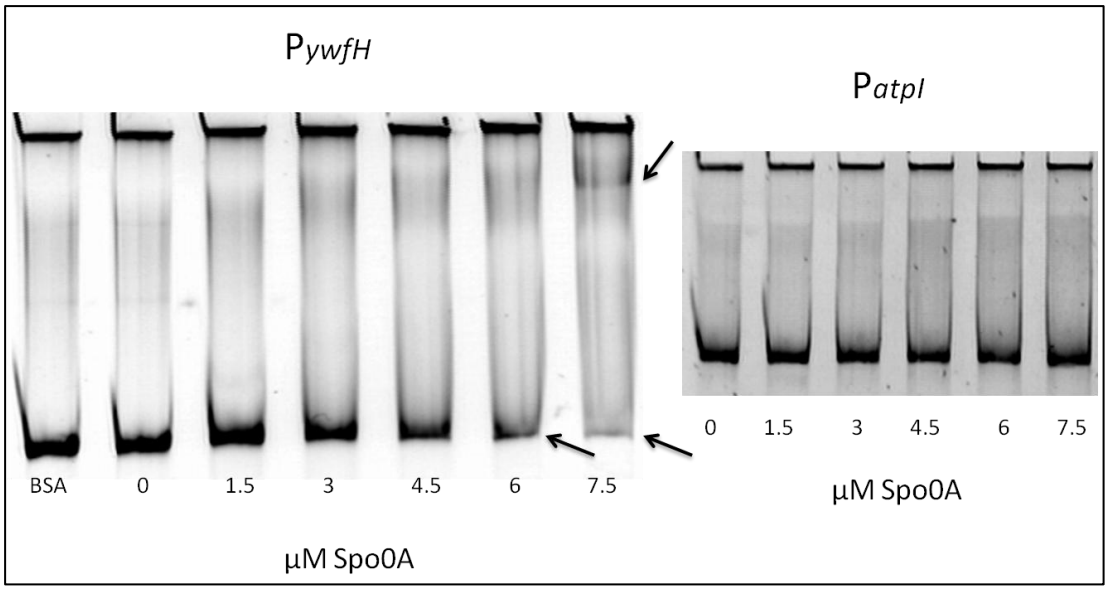


Figure 3.17 : Gel retardation assay performed by *ywfH* promoter fragments incubated with purified Spo0A-His_6 at the indicated concentrations. Gel shift for controls: promoter region of *atpI* for Spo0A binding represent the negative control. 'BSA' lanes also represent the negative controls of the reactions where only 10 mg/ml BSA were used as the candidate protein. All of the binding reactions contain 75 ng/µl of *ywfH* promotor DNA and 1mg/ml poly[d(I-C)].

3.6.2.2 Gel Mobility Shift Assay Performed in the Presence of AbrB-CodY, Spo0A-ComA, ComA-ComK and ComA-DegU

To determine whether AbrB-CodY, Spo0A-ComA, ComA-ComK and ComA-DegU can bind to P_{ywfH} simultaneously or compete for binding, the gel retardation assays were repeated in the presence of both regulator proteins in which the fixed amount of one of them was mixed with various concentrations of others.

The transition state regulators AbrB and CodY negatively regulates bacilysin biosynthesis directly by binding to P_{bac} (Köroğlu et al., 2011). In this study, it was shown that those regulators also affect the expression of *ywfH* directly interacting with *ywfH* promoter region. In order to understand if AbrB-CodY can bind to P_{ywfH} simultaneously or compete for binding, EMSA were repeated in the presence of both regulator proteins.

As shown in Figure 3.18, while CodY protein was kept on a constant level, the concentration of AbrB was gradually increased and thus additional retardation in electrophoretic mobility of CodY-bound P_{ywfH} fragments was observed. Similarly, the addition of increasing amounts of CodY in the presence of a fixed amount of AbrB caused the generation of supershifted bands in the EMSA (Figure 3.18), indicating that both CodY and AbrB can bind to the P_{ywfH} simultaneously.

Recently, Köroğlu *et al*. (2011) demostrated the similar results with study of bacilysin biosythetic operon. CodY and AbrB negatively regulate the transcription of *bac* operon and exhibit their effects by binding directly and simultaneously to the promoter region of *bac* operon.

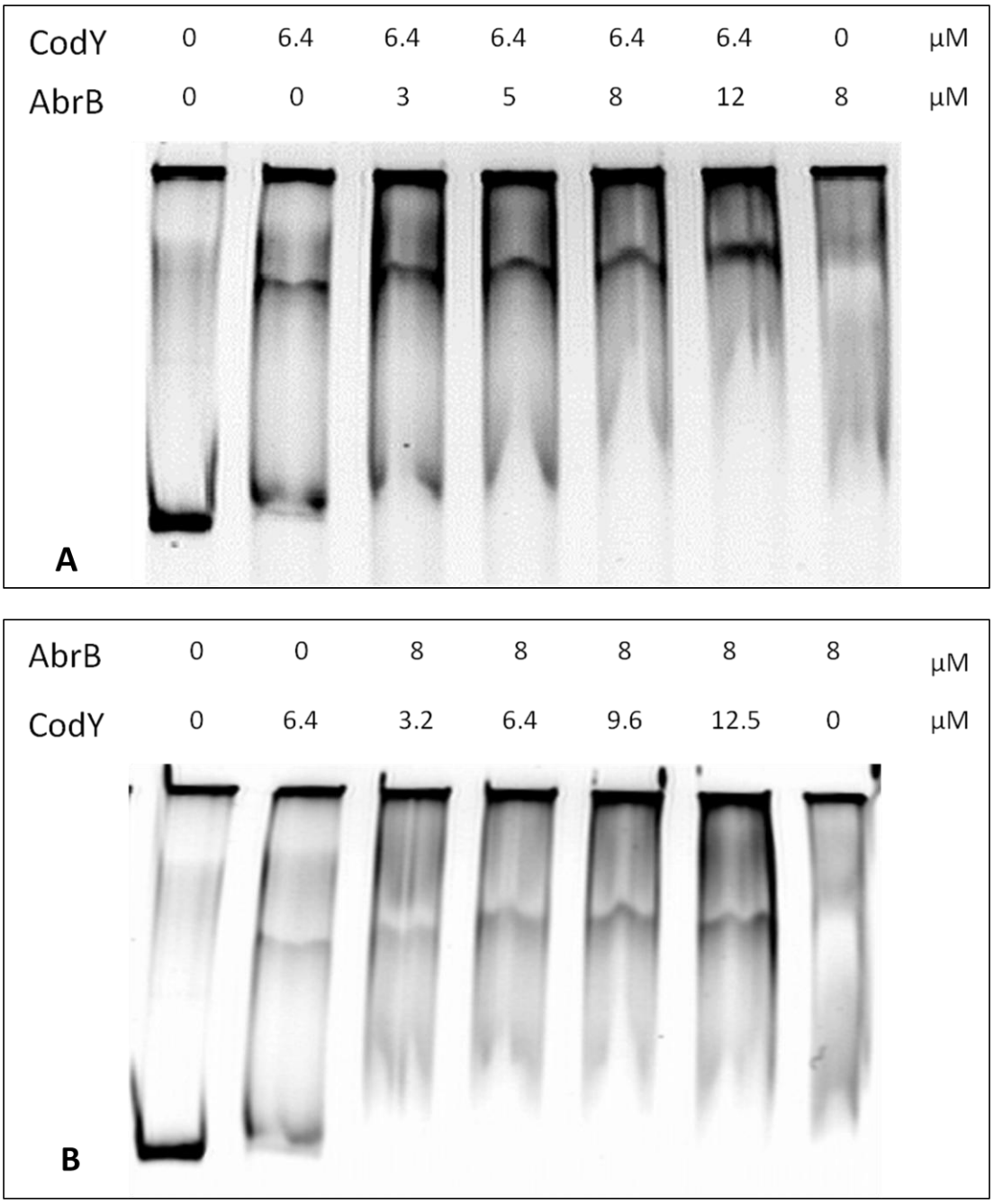


Figure 3.18 : Gel mobility shift assays performed using P*ywfH* promotor DNA incubated with increasing concentration of AbrB in the presence of 6.4 µM of CodY (A), increasing concentration of CodY in the presence of 8 µM of AbrB (B).

As shown in Figure 3.19, increasing amount of Spo0A in the presence of a constant concentration of ComA caused gradually disappearance of ComA band and resulted in shifting of the P_{ywfH} fragment towards to the well as in the case of Spo0A alone-P_{ywfH} fragment. On the other hand, when we applied EMSA with the increasing concentration of ComA up to 5 µM in the presence of constant level of Spo0A (8 µM) we couldn't observe the same result (Figure 3.19). Most probably 5 µM ComA

amount used in this reaction remained low to be able to compete with 8mM Spo0A. It was previously demonstrated that Spo0A and ComA compete with each other for binding *bac* operon promoter under regulator aspects when ComA concentration was increased up to 11,75 µM (Köroğlu *et al*. 2011). Observed data about the pattern of ComA-Spo0A binding to the *ywfH* promotor, exactly fits the binding pattern of those to the P_{bac} (Köroğlu *et al*.,2001). Therefore it is reasonable to suggest that ComA and Spo0A compete for binding to *ywfH* promotor as in the case of *bac* promoter.

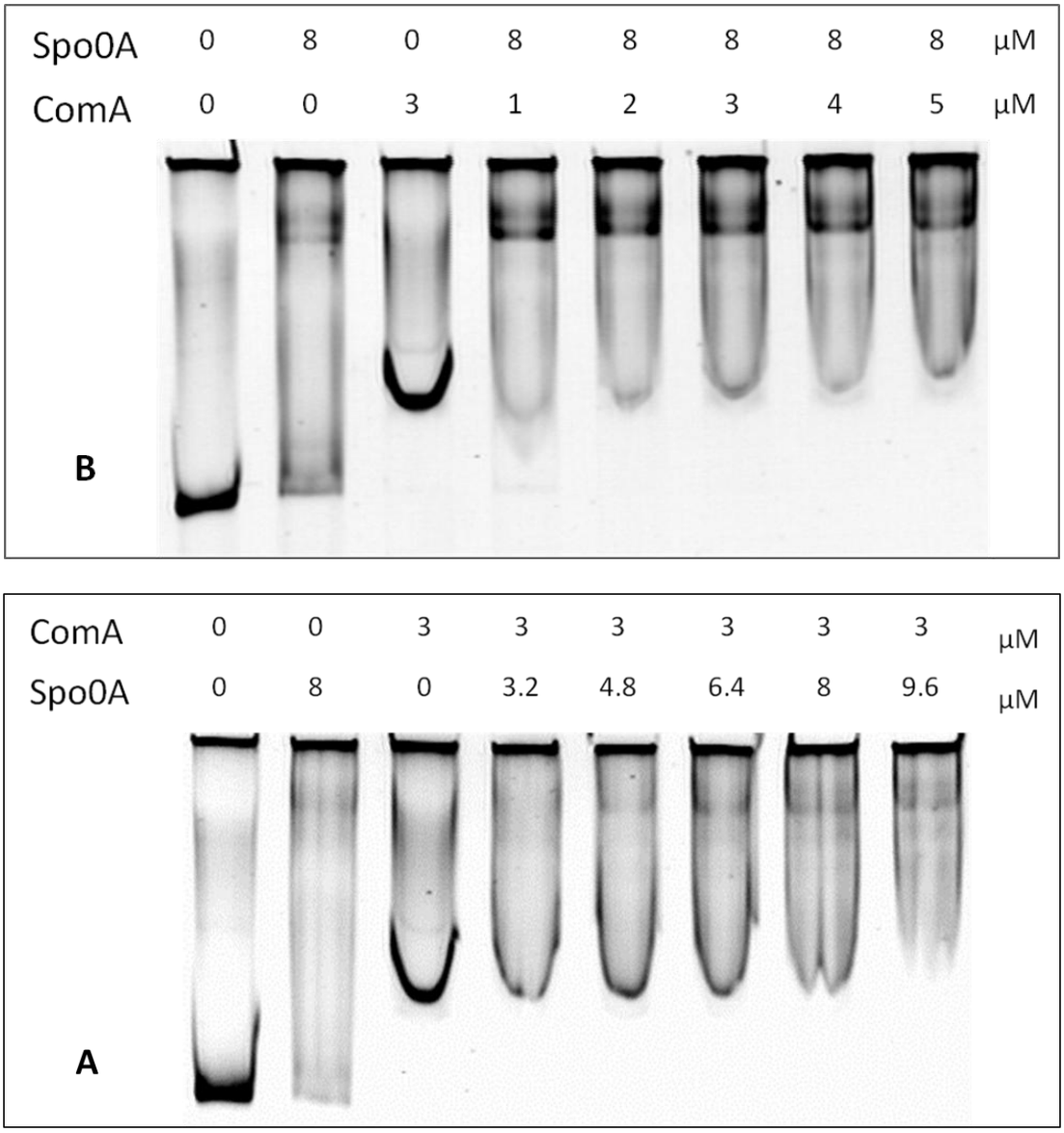
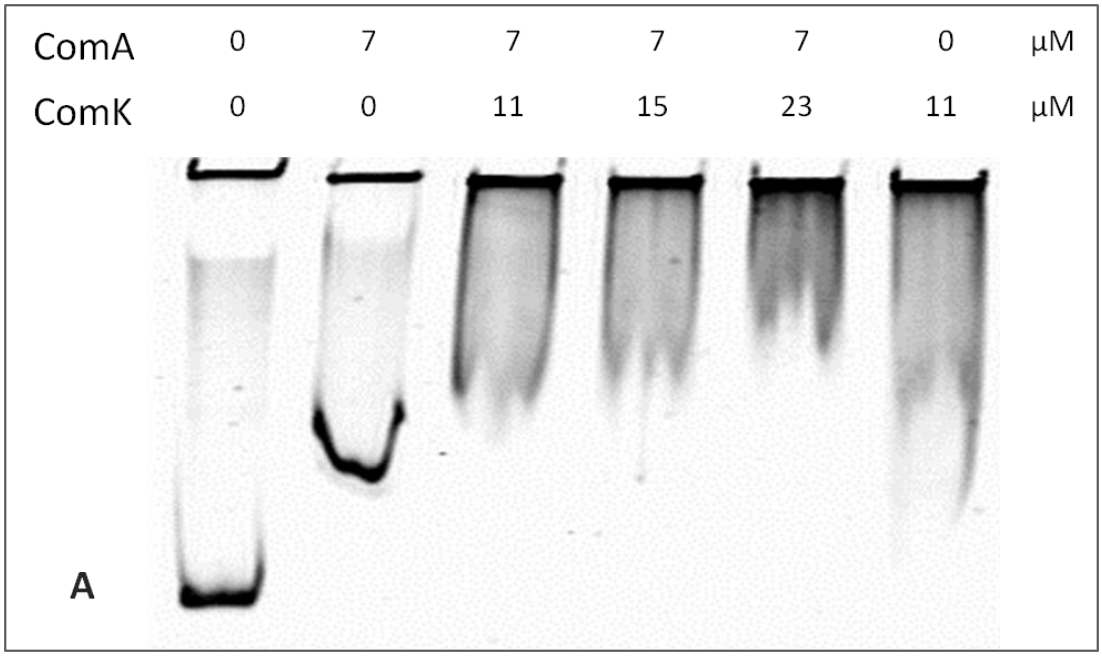


Figure 3.19 : Gel mobility shift assays performed using P*ywfH* promotor DNA incubated with increasing concentration of Spo0A in the presence of 3 µM of ComA (A), increasing concentration of ComA in the presence of 8 µM of Spo0A (B).

The phosphorylated form of ComA activates expression of several genes, including *comS* that is embedded in the large *srfA* operon. ComS releases the competence

transcription factor, ComK from ternary complex so, releasing active ComK activates transcription of late competence genes encoding the DNA binding, -uptake and –integration machinery (Susanna *et al*., 2006; Turgay *et al*., 1997 and 1998; Hamoen *et al*., 2003 and 1998). Additionaly, it was demostrated that the disruption of *comK* gene decreases the expression of *ywfH* indicating that *comK* gene has a stimulatory effect on the expression of *ywfH* gene and it shows its regulatory effect on bacilysin biosynthesis through direct binding to the *ywfH* promoter (unpublished data). In order to identify if ComA-ComK can bind to P_{ywfH} simultaneously or compete for binding, EMSA was performed in the presence of both proteins.

As shown in Fig. 3.20, when ComA was kept on constant value (7μM) and the concentration of ComK was increased up to 23μM, addition of ComK resulted in significant retardation of fragment of P_{ywfH}. Besides, addition of increasing amounts of ComA to the reactions containing fixed amount of ComK caused a difference in mobility of the *ywfH* promoter fragment (super-shift) (Figure 3.20). This indicates that ComK and ComA are able to bind simultaneously to the fragment of P_{ywfH}.



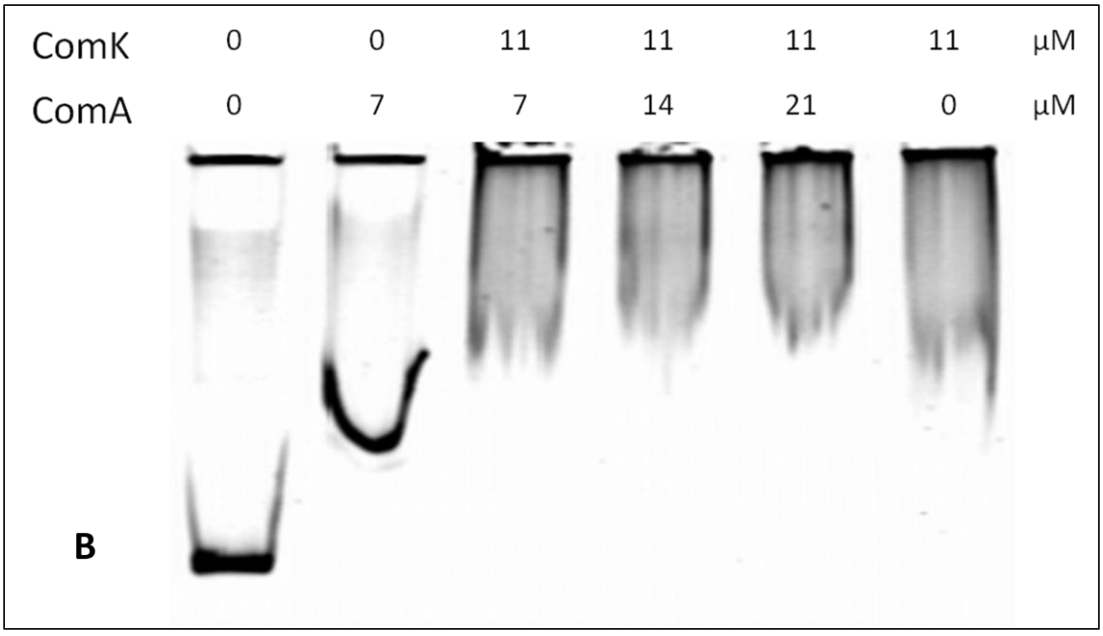


Figure 3.20 : Gel mobility shift assays performed using P*ywfH* promotor DNA incubated with increasing concentration of ComK in the presence of 7 µM of ComA (A), increasing concentration of ComA in the presence of 11 µM of ComK (B).

In the present study, we demonstrated that ComA and DegU binds directly to *ywfH* promoter suggesting that ComA and DegU control expression of *ywfH* gene. As a further study we also checked whether ComA-DegU can interact with P_{ywfH} simultaneously or compete for binding. As shown in Figure 3.21, when purified transcription factors ComA and DegU were incubated with *ywfH* promoter fragment together neither incerasing concentrations of ComA nor DegU abolishes each-others

binding shifts, but instead, additional retardation of the electrophoretic mobility of the *ywfH* promoter fragment was observed. This indicates that both proteins bind to the *ywfH* promoter simultaneously.

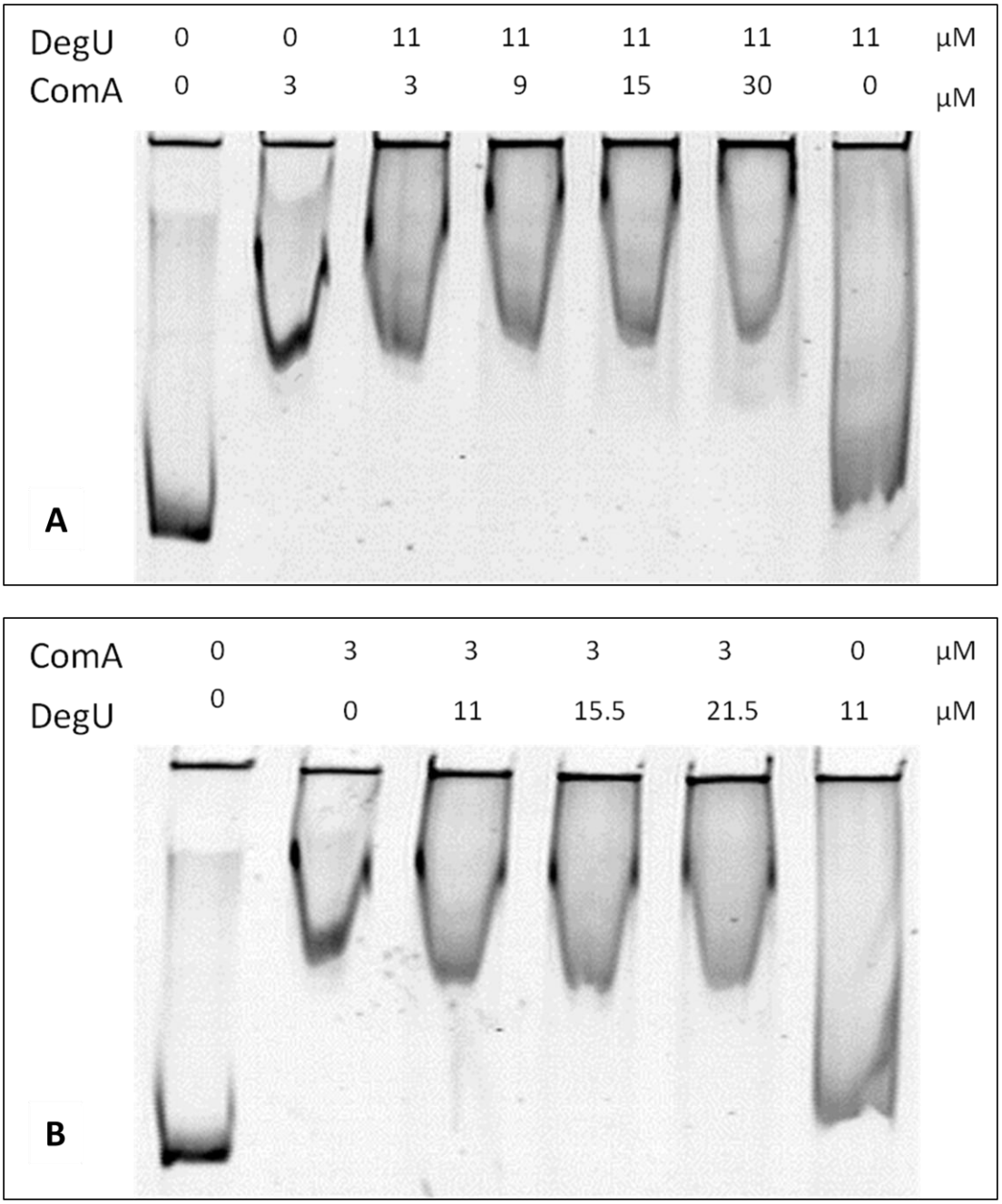


Figure 3.21 : Gel mobility shift assays performed using P*ywfH* promotor DNA incubated with increasing concentration of ComA in the presence of 11 µM of DegU (A), increasing concentration of DegU in the presence of 3 µM of ComA (B).

4. CONCLUSION

In this study, based on the dramatic effects of global regulators ComA, DegU, CodY, AbrB and Spo0A on bacilysin production, in order to determine whether the promotor region of *ywfH*, which is an essential biosynthetic gene adjacent to *bac* operon in *B.subtilis*, is a direct target of these proteins, electrophoretic mobility shift assay (EMSA) was performed in the presence of increasing concentrations of the purified proteins and *ywfH* promoter fragment. The observed DNA shifts on the gel retardation assays indicated that ComA, DegU, CodY, AbrB and Spo0A exert their effect directly by binding to P_{ywfH}. To analyze whether AbrB-CodY, Spo0A-ComA, ComA-ComK and ComA-DegU can bind to P_{ywfH} simultaneously or compete for binding, gel retardation assays were repeated in the presence of both regulators in which one of them was kept at a fixed concentration while the concentration of the other was gradually increased. Our results indicate that regulatory proteins CodY-AbrB, ComA-ComK and ComA-DegU are able to bind P_{ywfH} simultaneously. However, when ComA and Spo0A examined together for binding to P_{ywfH}, it was demostrated that these proteins compete for binding to P_{ywfH} and Spo0A is capable to displace the ComA from P_{ywfH} in a concentration dependent manner

Finally, this present study clearly showed that expression of *ywfH* is tightly regulated and delicately balanced by six proteins: ComA, ComK, DegU, CodY, AbrB and Spo0A.

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APPENDIXES

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 : *ywfH* DNA sequence
APPENDIX F : Laboratory equipment

APPENDIX A

Luria Bertani (LB) Medium (1000 ml)

Tryptone	10 g
Yeast Extract	5 g
NaCl2	5 g
Agar	15 g (Added before autoclaving for solid LB medium)

Distilled H_2O was added up to 1L 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)

Distilled H_2O was added up to 1L and then autoclaved for 4 minutes.

APPENDIX B

P1 Buffer (pH 8)

Tris-base	6.06 gr
EDTA.2H_2O	3.72 gr

Dissolve Tris-base and EDTA with 800 ml dH_2O. Adjust pH to 8 with HCl. Adjust volume to 1 lt dH_2O. Add 100 mg RNase A per liter of P1.

P2 Buffer

NaOH	8 gr
SDS solution (20%)	50 ml

Dissolve NaOH in 950 ml dH_2O. Add 50 ml SDS solution.

P3 Buffer (pH 5.5)

Potassium acetate	294.5 gr

Dissolve in 500 ml dH_2O. Adjust pH to 5.5.

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	100 ml (0.5 M, pH 8.0)

Dissolve in 800 ml dH_2O and adjust pH to 8.0 using HCl. Adjust volume to 1 lt dH_2O.

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 dH2O. 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

Dissolved in 1L distilled H_2O and autoclave.

Buffers for Purification Under Native Conditions

Lysis buffer (1 liter)

NaH_2PO_4	6.90 g
NaCl	17.54 g
Imidazole (10 mM)	0.68 g

Dissolve in 800 ml dH_2O and adjust pH to 8.0 using NaOH. Adjust volume to 1 lt dH_2O.

Wash buffer (1 liter):

NaH_2PO_4	6.90 g
NaCl	17.54 g
Imidazole (20 mM)	1.36 g

Dissolve in 800 ml dH_2O and adjust pH to 8.0 using NaOH. Adjust volume to 1 lt dH_2O.

Elution buffer (1 liter):

NaH_2PO_4	6.90 g
NaCl	17.54 g
Imidazole (250 mM)	17.00 g

Dissolve in 800 ml dH_2O and adjust pH to 8.0 using NaOH. Adjust volume to 1 lt dH_2O.

SDS-PAGE and Native Gel solutions and buffers

Monomer solution for SDS-PAGE (12%)

Acrylamide	29.5 g
Bisacrylamide	0.5 g

Dissolve in 100 ml of dH_2O and stored at 4^0C in the dark.

Separating gel buffer for SDS-PAGE

Tris base (1.5 M)	18.2 g

Dissolve in 80 ml dH_2O and adjust pH to 8.8. Add distilled H_2O up to 100 ml.

Stacking gel buffer for SDS-PAGE

Tris-HCl (1 M)	12.1 g

Dissolve in 80 ml dH_2O and adjust pH to 6.8. Add distilled H_2O up to 100 ml.

3X Loading (Sample) buffer for SDS-PAGE

Tris base	5 ml (0.135 M, pH 6.8)
Glycerol	3 ml
SDS	0.3 g
DTT	231 mg
Bromophenol blue	0.03 % (w/v)

Dissolve and add distilled water up to 10 ml.

10x Tank Buffer (Running) for SDS-PAGE

Tris base	30.3 g
Glycine	144 g
SDS	5 g

Add distilled H_2O up to 1L.

Gel Staining Solution for SDS-PAGE

Methanol	40% (v/v)
Glacial acetic acid	20% (v/v)
Coomassie Brillant Blue	0,08% (m/v)

Dissolve in 1L of distilled water.

Gel Destaining Solution for SDS-PAGE

Methanol	10%
Glacial acetic acid	10%

Dissolve in 1L of distilled water.

Ammonium persulphate (APS) concentration 10% (w/v)

Sodium dodecyl sulphate (SDS) concentration 10% (w/v)

5x Native Gel Buffer for EMSA

Hepes	0.83 g
Imidazole	0.29 g

Dissolve in 80 ml dH_2O and adjust pH to 7.4. Add distilled H_2O up to 100ml.

1x Tank Buffer (Running) for EMSA

Hepes (35mM)	8.34 g
Imidazole (43mM)	2.93 g

Dissolve in 800 ml dH_2O and adjust pH to 7.4. Add distilled H_2O up to 1L.

10x Binding Buffer Stock Solution A for EMSA

NaCl	292 mg
EDTA	37 mg
Tris base	8 ml (100 mM, pH 7.5)

Final volume was made up to 10 mL with dH_2O.

5x Binding Buffer A Preperation

10x Binding Buffer Stock solution A	0.5ml
Glycerol	0.25 ml
DTT (0.1M)	0.05 ml

Add sterile distilled H_2O up to 1ml.

10x Binding Buffer Stock Solution B for EMSA

KCl	372 mg
$MgCl_2$	102 mg
EDTA	3.7 mg
Tris base	8 ml (100 mM, pH 8.0)

Final volume was made up to 10 mL with dH_2O.

5x Binding Buffer B Preperation

10x Binding Buffer Stock solution B	0.5ml
Glycerol	0.4 ml
DTT (0.1M)	0.05 ml

Add sterile distilled H_2O up to 1ml.

Loading (Sample) buffer for EMSA

0.25x Native gel buffer	60%
Glycerol	40%
Bromophenol blue	0.2% (w/v)

APPENDIX C

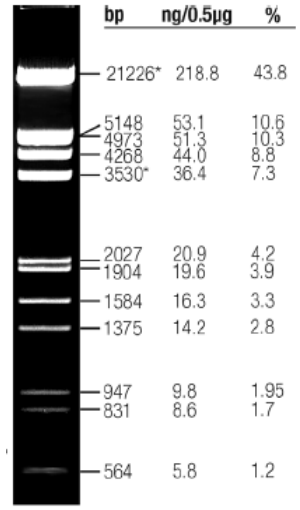
Chemical	**Supplier**
Acrylamide	Merck
Agar	Sigma
Agarose	Prona
Ammonium persulfate	Merck
Bis-acrylamide	Merck
Bromophenol blue	Sigma
Coomassie Brilliant Blue R	Sigma
CTAB	Sigma
EDTA	Sigma
Ethanol	Riedel-de Haën
Ethidium bromide	Sigma
DTT	Sigma
Gilicial Acetic Acid	Riedel-de Haën
Glycerol	Merck
Glycine	Merck
HCl	Merck
Hepes	AppliChem
Imidazole	Merck
IPTG	AppliChem
KCl	Carlo Erba
Methanol	Riedel-de Haën
$MgCl_2.6H_2O$	Carlo Erba
NaCl	Merck
NaOH	Carlo Erba
NaH_2PO_4	Merk
Phenol-chloroform-isoamylalcohol	Fluka
SDS	Merck
TEMED	Carlo Erba
Tris-base	Merck
X-Gal	Sigma

Yeast Extract	Acumedia

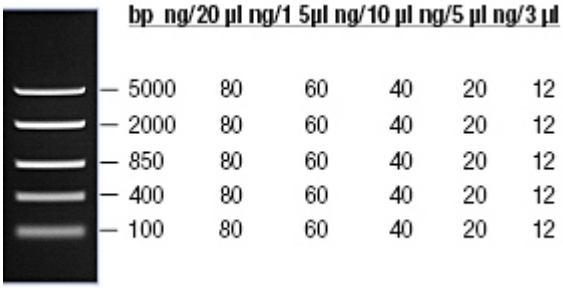
Enzymes	**Supplier**
*Bam*HI	Fermentas
NcoI	Fermentas
Lysozyme	AppliChem
Proteinase K	Sigma
RNAse A	Sigma
Expand High Fidelity Enzyme	Roche
Pfu DNA Polymerase	Fermentas
T4 DNA Ligase	Roche

APPENDIX D

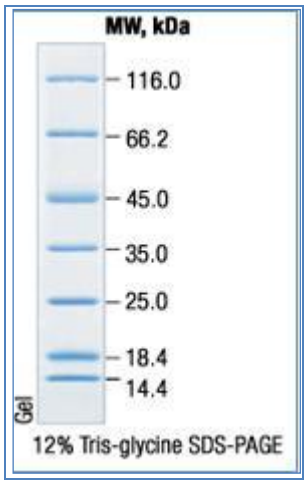
Marker	**Supplier**
Lambda DNA/*Eco*RI+ *Hind*III Marker (Marker 3)	MBI Fermentas



Middle Range DNA Ladder	Fermentas



Unstained Protein Weight Marker	Fermentas



APPENDIX E

ywfH Promoter Region Sequence

AGCCACGGTTTAATCGGCCGCAGCTATGCGGGCAAAGTCAAACAGATCA
TCACCGGCTCTGTCGGCTTTGACGATTACGAGTGGGGCGTTACATTATTT
AGCGATGATGTCCTTCAATTCAAGAAGCTAGTGTACGAAATGCGTTTTGA
CGAAGTCAGCGCTCGCTATGGCGAATTTGGTTCATTCTTTGTCGGCAATC
ACCTTTCGCTTGATACACTTCCTCAATTCTTATATGTATAATATCCCCGCC
CGCCCTATCCGGCGGGAGTTTTTCAATTCTCCTTTATCAGAATTTAAATAA
AATTTGCTATTTTCTTCGTTTTTTCTTACGATGTAGATAATAATCCATTTTT
TTCAAAGGGGTGCAGTTATTTGTCAAAACGAACCGCATTTGTTATGGGAG
CCAGTCAAGGG

APPENDİX F

LABORATORY EQUIPMENT

Autoclave	Tuttnauer Systec Autoclave (2540 ml) Nüve OT 4060 Steam Sterilizer
Centrifuge	Microfuge 18, Beckman Coulter AllegraTM25R Centrifuge, Beckman Coulter
Deep freezes and refrigerators	-80°C Heto Ultrafreeze 4410 -20°C Arçelik 209lt +4°C Arçelik
Electrophoresis equipments	E – C mini cell primo EC320
Gel documentation system	UVI PHotoMW Version 99.05 for Windows
Ice machine	AF 10, Scotsman
Incubators	Nüve EN400 Nüve EN500
Laminar flow cabinet	Biolab Faster BH-EN2003
Magnetic stirrers	AGE 10.0164, Velp Scientifica srl. AGE 10.0162, Velp Scientifica srl.
Microplate reader	Model 3559 UV Microplate, Bio-Rad
Micropipettes	Eppendorf AG, 1000µl, 200µl, 100µl, 20µl, 1µl, Gilson pipetteman 10 µl, 20µl,200µl, 1000µl, Volumate Mettler Toledo 10µl, 20µl, 200µl, 1000µl
Orbital shakers	Shell lab 1575R-2E Thermo Electron Corporation B. Braun Biotech Internationnal GmbH
Power supply	Bio-Rad
pH meter	Mettler Toledo MP220
Pure water systems	USF Elga UHQ-PS-MK3, Elga Labwater

SDS-PAGE Apparatus	Bio-Rad
Spectrophotometer	PerkinElmer Lambda25 UV/VIS Shimadzu UV-Pharmaspec 1700
Thermomixer	Eppendorf thermomixer comfort (1.5 ml)
Vortexing machine	Heidolph Raax top
Waterbaths	Memmert wb-22
Polyacrylamide Gel Electrophoresis Apparatus	Bio-Rad

CURRICULUM VITAE

Candidate's full name: Seval MUTLU

Place and date of birth: Kayseri, 01.01.1985

Permanent Address: Altkaynarca Mah. İstasyon Cad Hatboyu Sok Mehmetoğlu Apt. 4/4 Pendik/İSTANBUL

Universities and Colleges attended: Istanbul Technical University, Advanced Technologies, Molecular Biology-Genetic & Biotechnology Program, Istanbul/TURKEY, M.Sc., 2009-2011.
Ege University, Faculty of Science, Department of Biology (Basic and Industrial Microbiology), Izmir/TURKEY,
BSc., 2002-2007.

Publications:

- Koroğlu, TE.; Ogulur, I.; **Mutlu, S.**; Yazgan-Karatas, A.; Ozcengiz, G. 2011. Global Regulatory Systems Operating in Bacilysin Biosynthesis in Bacillus subtilis, **Journal of Molecular Microbiology and Biotechnology**
- **Mutlu, S.**; Koroglu, TE.; Tayran, H,; Karatas, A. 2010. Regulatory profile of CodY protein on bacilysin biosynthetic operon, **35th FEBS Congress Molecules of Life, Gothenburg-Sweden**
- Koroglu, TE.; Tayran, H.; **Mutlu, S.**; Karatas, A. 2010. Determination of regulatory mechanism of AbrB protein on bac operon, **35th FEBS Congress Molecules of Life, Gothenburg-Sweden**