İSTANBUL TECHNICAL UNIVERSITY ★ GRADUATE SCHOOL OF SCIENCE ENGINEERING AND TECHNOLOGY

REGULATORY EFFECT OF TRANSCRIPTIONAL FACTORS ComK AND SinR ON THE EXPRESSION OF *bacABCDEywfG* OPERON IN *Bacillus subtilis*

M.Sc. THESIS

Ali Mohammed Nasser HAIDER

Department of Molecular Biology-Genetics and Biotechnology

Molecular Biology-Genetics and Biotechnology Programme

JUNE 2016

İSTANBUL TECHNICAL UNIVERSITY ★ GRADUATE SCHOOL OF SCIENCE ENGINEERING AND TECHNOLOGY

REGULATORY EFFECT OF TRANSCRIPTIONAL FACTORS ComK AND SinR ON THE EXPRESSION OF *bacABCDEywfG* OPERON IN *Bacillus subtilis*

M.Sc. THESIS

Ali Mohammed Nasser HAIDER
(521121161)

Department of Molecular Biology-Genetics and Biotechnology

Molecular Biology-Genetics and Biotechnology Programme

Thesis Advisor: Prof. Dr. Ayten YAZGAN KARATAŞ

JUNE 2016

İSTANBUL TEKNİK ÜNİVERSİTESİ ★ FEN BİLİMLERİ ENSTİTÜSÜ

Bacillus subtilis'DE ComK VE SinR TRANSKRİPSİYONEL FAKTÖRLERİNİN *bacABCDEywfG* OPERONU ÜZERİNE ETKİSİ

YÜKSEK LİSANS TEZİ

Ali Mohammed Nasser HAIDER
(521121161)

Moleküler Biyoloji-Genetik ve Biyoteknoloji Anabilim Dalı

Moleküler Biyoloji-Genetik ve Biyoteknoloji Programı

Tez Danışmanı: Prof. Dr. Ayten YAZGAN KARATAŞ

HAZİRAN 2016

Ali Mohammed Nasser HAIDER, a **M.Sc**. student of **ITU Graduate School of Science Engineering and Technology** student ID **521121161**, successfully defended the thesis entitled "**REGULATORY EFFECT OF TRANSCRIPTIONAL FACTORS ComK AND SinR ON THE EXPRESSION OF *bacABCDE* OPERON IN *Bacillus subtilis***", which he prepared after fulfilling the requirements specified in the associated legislations, before the jury whose signatures are below.

Thesis Advisor :	**Prof. Dr. Ayten YAZGAN KARATAŞ** İstanbul Technical University	.....
Jury Members :	**Assoc.Prof. Dr. Fatma Neşe KÖK** İstanbul Technical University	.....
	Prof. Dr. Sedef TUNCA GEDİK Gebze Technical University	.....

Date of Submission : 06 May 2016
Date of Defense : 03 June 2016

Dedicated to

my mother Haylah, my wife Isimhan

and my children, Amjed, Melek and Fadhl.

FOREWORD

Few moments in our life when words stand unable to express our gratitude or to explain our feelings toward somebody. When it comes to talk about Prof.Dr. Ayten YAZGAN KARATAŞ, my great supervisor is one of these moments. To say thank you, surely is not enough and cannot be. It is a real honor for me to be a student under your guidance. I am thankful for all the opportunities you have given me. I appreciate your valuable advice, you were and still a constant source of support, encouragement and motivation. I also have to thank you for being a patient teacher, for always giving me time amidst your fully engaged schedule, for dealing with success and failure in research work with a calm, encouraging approach and for being a wonderful person to work for. You have not only helped me appreciate science, but have also set a model of what it means to be a good, responsible scientist. Your honesty, proficiency, nobility, kind-heartedness and willingness to help anyone who comes to you, your commitment and dedication to see people succeed are in all honesty ideal characters. As a scientist and as a person, I hope to model myself on some of these wonderful traits. I also hope that your guidance will continue for years to come. Your high expectations gave a true motivation to push myself further. You are the kind of professor who makes this university a great place to learn. If truth to be told, I really would not have made it without your help and support.

My thank is due to my lab-mate Hasan DEMİRCİ who has helped out in so many occasions and for always doing it with so much kindness and courtesy. I am also thankful to Dicle MALAYMAR for her endless help, for her constant support. Dear Dicle, working with you is an unforgettable experience for me. You are the soul of our lab. Special thanks to Büşra ÖZTÜK for her help and for coming when needed despite her responsibilities, wishing her a prosperous future.

I am also thankful to all the past and present members of our Lab for being a wonderful lab family (Betim KARAHODA, Bilal TETİK, Orkün PİNAR, Öznur PEHLİVAN, Koray MALCI and Berivan TEMİZ). My thanks are due to Dr. Murat Kemal AVCI, no proper words to convey my deep gratitude and respect to him, wishing him a constant success and happiness in both work and personal life. Thank you doesn't look sufficient but it is said with respect and appreciation to Dr. Öykü İrigül SÖNMEZ for her valuable help and for coming when needed in spite of her numerous responsibilities.

Last but not least, I am extremely indebted to my family for their encouragement, care, understanding and financial support.

June 2016 Ali HAIDER

TABLE OF CONTENTS

Page

ABBREVIATIONS

AMP : Ampicillin
bp : Base pair
Cm : Chloramphenicol
dH_2O : Distilled water
DNA : Deoxyribonucleic acid
EB : Elution Buffer
EDTA : Ethylenediaminetetraacetic acid
EMSA : Electrophoretic Mobility Shift Assay
Erm : Erythromycin
EtBr : Ethidium bromide
IPTG : Isopropyl-b D- thiogalactopyranoside
kb : Kilo base
LB broth : Luria Bertani broth
lacZ : Structural gene for β-galactosidase
OD : Optical density
PCR : Polymerase Chain Reaction
PA : Perry and Abraham Medium
QS : Quorum Sensing
SDS-PAGE : Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
TAE : Tris Acetate EDTA
Tris : Hydroxymethyl aminomethane
UV : Ultraviolet transilluminator

LIST OF TABLES

Page

LIST OF FIGURES

Page

Page

REGULATORY EFFECT OF TRANSCRIPTIONAL FACTORS ComK AND SinR ON THE EXPRESSION OF *bacABCDEywfG* OPERON IN *Bacillus subtilis*

SUMMARY

Bacillus subtilis is a rhizosphere-colonizing bacteria which have the ability to stimulate plant growth and also suppressing the growth of plant-pathogenic organisms. Being non-pathogenic, easy to deal with make it the organism of choice for biological and genetic laboratory research. It can grow in different environmental conditions, in soil and on the surface of plant root.

B. subtilis exhibits assortment of survival approaches such as undergoing to stationary phase due to nutritional oscillations. The moment that nutrients turn out to be reduced for optimum growth at the end of the exponential growth phase, *B. subtilis* cells start producing a complicated motility and chemotaxis system which facilitates looking for nutrients in the surroundings. If nutritional restrictions continue, the motile cells resort to entering the stationary phase and secreting degradative enzymes. These degradative enzymes make available nutrients to cells from alternative sources that are ordinarily unreachable.

Likewise, cells initiate producing antibiotics to fight probable competitors. Extended nutritional stress ends up by the development of competence, and ultimately sporulation by which the bacterial population is capable to live in such severe environmental circumstances. *Bacillus subtilis* appropriately directs these adjustments utilizing quorum sensing system to regulate gene expression in light of changes in cell-population density.

Bacillus subtilis has the ability to produce antibiotics either ribosomally and undergo posttranslational modification such as lantibiotics and lantibiotic-like peptides or non-ribosomally such as polypeptides.

Bacilysin is a peptide non-ribosomally synthesized antibiotic composed of L-alanine in the N-Terminus and L-anticapsin in the C-Terminus. Despite its simple structure, bacilysin has an impressive antimicrobial activity against a wide range of bacteria and some fungi.

Recently, in *Bacillus subtilis*, *bacABCDEywfG* operon and monocistronic gene *ywfH* were proven to be the basic component carrying the bacilysin biosynthetic core function.

Bacilysin production is under a complicated regulatory network by which several global regulators can directly or indirectly regulate its operon in negative or positive manners such as the quorum-sensing response-regulator ComA, the transition state regulator AbrB. Moreover, the bacilysin biosynthesis is regulated by quorum sensing through ComQ/ComX, PhrC (CSF) ComP/ComA pathway and stimulation of *bacABCDEF* expression is tightly regulated by ComA, Spo0A, AbrB and CodY DegU proteins.

Expression of ComK, the main transcriptional factor of competence, is under multiple controls of several proteins both negatively and positively such as DegU, SinR, CodY, AbrB, Rok and ComK itself in addition to quorum sensing regulation.

SinR, a pleiotropic DNA binding protein, is the master regulator that governs the Gram-positive model organism *Bacillus subtilis* life style whether to be a free-living, planktonic lifestyle or a biofilm-forming, is essential for the late-growth processes of competence and motility and also is a repressor of sporulation and subtilisin synthesis. The activity of the transcriptional regulator SinR is regulated by its antagonists, SinI, SlrA, and SlrR

In this study, the regulatory effect of ComK and SinR on the expression of *bacABCDEywfG* operon were determined. For this purpose, *comK* mutant, *sinR* mutant and *comK-sinR* double mutant strains were constructed. Subsequently, OGU1, and its *comK* and *sinR* and *comK-sinR* derivatives were grown in PA medium and *bac* promoter-directed β-galactosidase activities were measured. Moreover, with the purpose of make known how ComK and SinR proteins effect each other's binding to *bac* promoter, Electrophoretic Mobility Shift Assays (EMSAs) were also implemented.

To sum up, the results revealed that, in *Bacillus subtilis, comK* and *sinR* genes' products affect the expression of *bac* operon in a positive manner by binding to the *bac* operon promoter in a competitive manner.

Bacillus subtilis'DE COMK VE SINR TRANSKRİPSİYONEL FAKTÖRLERİNİN *bacABCDEywfG* OPERONU ÜZERİNE ETKİSİ

ÖZET

Bacillus subtilis, bitki büyümesini teşvik yeteneğine sahip bir rizosfer kolonizasyon bakterisidir. Ayrıca bitkideki patojen organizmaların büyümesini bastıran, patojenik olmayan gram pozitif çubuk şeklinde bir bakteridir. Farklı çevre koşullarında, toprakta ve bitki kökünün yüzeyi üzerinde büyüyebilir. *Bacillus subtilis,* genomunun tamamı dizilenmiştir. Ayrıca patojen özellikte olmaması sebebiyle kolay kontrol edilebilir bir türdür. Bölünme ve büyüme hızı, kolay transforme olabilme yeteneği, yüksek adaptasyon kabiliyeti, çeşitli antibiyotikleri, enzimleri ve biyokimyasalları üretebilmesi sayesinde bilimsel araştırmalarda sıklıkla yer alan bir model organizmadır. 4,2 Mbp uzunluğundaki genomunun tamamının dizilenmesi sonucunda, organizma genomunun yaklaşık 4,100 genin kodlanmasından sorumlu olduğu görülmüştür. Bu genlerin haritalanması ile türe özgü fonksiyonların gerçekleştirilmesinden sorumlu olan kısmın, toplam bakteri genomunun üçte birlik bir kısmı olduğu anlaşılmıştır. Haritadaki geri kalan üçte ikilik kısmın sahip olduğu fonksiyonları aydınlatma amacıyla yapılan çalışmalar hala sürmektedir.

Bacillus cinsine ait mikroorganizmalar bazı önemli özellikleri nedeniyle gıda endüstrisinde biyolojik gıda kontrol ajanı olarak kullanılmaktadırlar. Bu özelliklerinin başında GRAS (Generally Recognized as Safe) etiketine sahip olmaları, toksisitelerinin düşük olması, biyo-bozunabilirliliğinin yüksek olması ve çevreye yararlı olmaları sıralanabilir.

B. subtilis besin sıkıntısı nedeniyle hayatta kalmak için çeşitli stratejiler geliştirmiştir. Logaritmik büyüme fazının sonunda besin elementleri azaldığı zaman *B. subtilis* hücreleri ortamda besin elementleri aramayı kolaylaştırmak için karmaşık bir motilite ve kemotaksis sistemi geliştirmeye başlar. Beslenme kısıtlamaları devam ederse, hareket ederek besin bulmayı hedefleyen bakteride, bu sefer de ortamdaki diğer mikroorganizmaları bu yarıştan uzaklaştırmak için antimetabolik, antifungal ve antimikrobiyal özellikteki antibiyotiklerin üretimi tetiklenir. Besin kıtlığıyla baş edebilme çözümlerine ek olarak, aynı şekilde ortamdaki besin seviyesinde azalma söz konusu olduğunda, farklı besinleri kaynak olarak kullanabilmek için parçalayıcı enzim üretimine, kompetans geliştirerek yabancı DNA'nın hücre içine alımına ya da sporlanmaya yönelebilen *B. subtilis*, olumsuz şartlarda geliştirdiği çeşitli mekanizmalarla hayatta kalmayı başarabilen bir mikroorganizmadır.

Bunun yanı sıra sahip olduğu kemotaksis kabiliyetinin sonucu olarak katı yüzeylerde kayarak yer değiştirebilmesi, olumsuz şartlarda sporlanması ve kompetans geliştirilmesi *B. subtilis* türünü, rakipleriyle mücedelesinde öne çıkaran avantajlar arasındadır. Aynı şekilde, hücreler muhtemel rakipleri ile mücadele etmek için antibiyotik üretmeye başlar. *B. subtilis* rakipleriyle mücadele edebilme konusunda birçok avantaja sahiptir. On ikiden fazla çeşitlilikte antibiyotik üretebilmesi en çarpıcı özellikleri arasındadır. *Bacillus subtilis* antibiyotikleri, ya ribosomal olarak

sentezlenen ve translasyon sonrası modifikasyon geçiren lantibiyotikler ve lantibiyotik benzeri peptidlerdir, ya da ribosomal olmayan yolak ile üretililen çok dallı halkasal peptitlerdir.

B. subtilis hücreleri, gen ifadesini "hücre yoğunluğu algılama mekanizması" ile genom çapında kontrol ederek çevresel şartlara göre metabolizmasını hızlı ve etkin bir şekilde düzenler. Hücre yoğunluğunun algılama mekanizması, mikroorganizmaların "otoindukleyici" denilen bir takım sinyal molekülleri aracılığıyla, üreme ortamındaki hücrelerin ve ilgili sinyal moleküllerinin yoğunluğunu algılamak suretiyle hücredeki bütün genlerin koordineli bir şekilde kontrolünü sağlayan ve bu sayede ortam şartlarının gerektirdiği optimum metabolik akışı temin eden bir iletişim mekanizmasıdır. *Bacillus subtilis*, hücre yoğunluğu algılama sistemini kullanarak uygun bir şekilde bu ayarlamaları yönlendirir ve hücre popülasyonu yoğunluğundaki değişiklikler ışığında gen ekspresyonunu düzenler.

Basilisin, geniş bir bakteri yelpazesi ve bazı mantarlara karşı aktif bir antibakteriyel ajan olan ve ribozomal olmayan bir mekanizma ile sentezlenen L-antikapsin ile L-alanin'den oluşan *Bacillus subtilis* tarafından üretilen bir dipeptid antibiyotiktir.

Basilisin biyosentezinin, *bacABCDEFywfG* operonu ve *ywfH* gen ürünleri tarafından gerçekleştirildiği kısa bir süre önce keşfedilmiştir. Öncelikle, basilisin biyosentezi, *Bacillus subtilis*'te, ComQ/ComX, PhrC (CSF), ComP/ComA'nın Spo0K (Opp)'ye bağlı bir biçimde gerçekleşen, hücre yoğunluğu algılama kontrol mekanizması altında gerçekleşmektedir. Bu mekanzşmanın yanı sıra, aynı zamanda diğer global düzenleyici Spo0A ile pozitif olarak, CodY ve AbrB global düzenleyiciler ile de negatif olarak düzenlenmektedir.

SinR proteini *B. subtilis*'in büyüme eğrisinde, geçiş fazı döneminde etki gösteren önemli düzenleyicilerden biridir. SinR, geçiş fazı döneminde kompetans ve motilite gelişimini aktive ederken sporulasyon ve ekzoproteaz üretimini engelleyerek çift fonksiyon gösteren önemli bir düzenleyicidir. SinR proteininin N-terminal bölgesi helix-turn-helix motifi içerir ve buna bağlı olarak DNA bağlanma yüzeyi oluşturur. SinR'ın fonksiyonu dikkate alındığında kompetans ve motiliteyi nasıl düzenlediği halen bilinmezken ekzoporeteaz ve sporulasyon üzerindeki negatif etkisi daha iyi anlaşılmıştır.

SinR proteininin bir diğer özelliği de ComK proteininin negatif düzenlenmesinden sorumlu Rok proteininin transkripsiyonunu negatif bir şekilde etkilemesidir. Rok proteininin baskılanması ile SinR'nin ComK ekspresyonu süresindaki rolü pozitif bir şekilde düzenlenmiş olur. Aktif ComA~P, *comS* geni de dâhil olmak üzere birçok genin ekspresyonunu aktive eder. *comS* geni *srf*A operonunda, *srfB* (srfORF2) geni içinde yer alır. ComS, kompetans transkripsiyon faktörü ComK'nın ClpC (Chaperon benzeri protein) ve MecA'nın bulunduğu proteolitik kompleksten ayrılmasını sağlar ve parçalanmasını önler. Serbest kalan ComK kendi ekspresyonunu teşvik eder ve hücre içindeki konsantrasyonunu arttırır ayrıca ardından ilgili genlerin promotor bölgelerine bağlanar ve transkripsiyonlarını aktive eder. Hücre içindeki konsantrasyonu hızla artan ComK bu otoindükleyici döngü sayesinde DNA bağlanma, DNA alımı ve DNA integrasyondan sorumlu olan geç kompetans genlerini aktive eder.

DegU ve DegS proteinleri kompetans gelişimde rol alan diğer önemli düzenleyicilerdir. Birlikte kompetans gelişiminin aynı sıra degredatif enzim sentezinin düzenlenmesini de sağlarlar. Fosfatlanmış DegU~P degredatif enzim üretimini artırırken, fosfatlanmamış DegU *comK* geninin transkripsiyonunu düzenlemek için tetikleyici bir şekilde hareket eder. ComK konsantrasyonunun artması ile de

kompetans gelişimi başlar. DegU proteinine ek olarak dört farklı transkripsiyon faktörü *comK* promotorunun trankripsiyonunun kontrolünde doğruca etkili olur. Bu düzenleyici proteinler AbrB, CodY, Rok ve *comK* genin kendi ürünü olan ComK'dır. AbrB proteini *comK* promotor transkripsiyon başlama bölgesine bağlanarak *comK* promotor bölgesi ile RNA polimeraz etkileşimini engeller.Sonuç olarak, AbrB, *comK*'nın prematüre ekspresyonunu engel çıkarak kompetans gelişimini negatif yönde etkilemiş olur.

CodY logaritmik büyüme fazında negatif regülatör olarak hareket eder ve besin yoksulluğuna adaptasyon için gerekli genlerin ekspresyonunu temin eder. CodY proteininin aktivitesi GTP ve izolösin- valin gibi dallanmış amino asitlerin kontrolü altındadır. Bakteri hücreleri besin sıkıntısı ile karşılaştıklarında, GTP'nin bağlanması ile aktif hale gelen CodY' ın GTP düzeyinin düşmesine bağlı olarak aktivitesi hızlıca kısılır Karbon kaynağına ve amino asit limitasyonuna bağlı bir şekilde gelişen "stringent response" regülasyon mekanizması aktifleşir. Bu hâl GTP'nin pppGppp ve ppGpp moleküllerine dönüşmesine yol açar. Böylece, hücre içinde (p)ppGpp toplanması veya intrasellüler GTP'nin hızlıca tükenmesi ve buna bağlı olarak da CodY'in inaktif hale dönüşmesi sağlanır.

CodY proteini ComK promotörü bölgesinde, RNA prolimerazın bağlanma bölgesini istilâ ederek kompetans gelişimini baskılar. *ykuW* geni (*rok* geni adıyla tanılır) tarafından Rok proteini *comK* transkripsiyonunu baskılayarak kendi sentezini de baskılar. ComK konsantrasyonu arttığı zamanda ise *rok* ekspresyonu baskılanır.

Bu çalışmada ComK ve SinR'nin *bacABCDEywfG* operonu ekpresyonu üzerindeki etkisinin belirlenmesi amaçlanmıştır. Bunun için, bacA lokusunda *lacZ* füzyonu taşıyan, *B. subtilis* OGU1 (*bacA::lacZ::erm*) suşunda *comK, sinR tekli ve comK-sinR* ikili mutant türevleri oluşturulmuştur. Bir sonraki adımda ise, OGU1, *comK, sinR* tekli mutant *ve sinR-comK* ikili mutant türevleri PA besi yerinde büyütülmüş ve *bac* promotoruna bağlı β-galaktosidaz aktiviteleri belirlenmiştir. Bu çalışmalara ilaveten, ComK ve SinR proteinlerinin *bac* promotor bölgesine bağlanma eğilimleri ve birbirlerinin bağlanmasını nasıl etkiledikleri "Elektroforetik Hareketlilik Kaydırma Analizi" (EMSA) deneyi ile test edilmiştir. Sonuç olarak ComK ve SinR transkrisiyonel faktörlerinin, *bac* operonuna birbirleri ile yarışmalı şekilde bağlanarak bac operonunu pozitif yönde etkilediği anlaşılmıştır.

1. INTRODUCTION

1.1 *Bacillus subtilis*

Bacillus subtilis is a rhizosphere-colonizing bacteria which have the ability to stimulate plant growth and also suppressing the growth of plant-pathogenic organisms (Koumoutsi et al, 2004; Liming et al, 2014; Chena et al, 2009; Idriss et al, 2002). In addition to be non-pathogenic, gram-positive and rod-shaped bacterium, it is a notably diverse bacterial species that can grow in different environmental conditions such as in soil and on the surface of plant root (Ashlee et al, 2008).

Bacillus subtilis previously was known by the name of *Vibrio subtilis* in 1835, until it was renamed *Bacillus subtilis* in 1872 by the German biologist Ferdinand Cohn. Furthermore, it is one of the first bacteria to be studied and became a good model for cellular development and differentiation in addition to have a high capacity to respond easily to the environmental changes which in turn exert its effects on the phenotype and morphology. These causes can be attributed to the instability of growth rate, temperature, and nutrients availability (Sonenshein et al, 1993).

Despite being non-pathogenic, *Bacillus subtilis* bacteria can contaminate food, nevertheless, they seldom result in food poisoning. On the other hand, they are used on plants and on agricultural seeds as a fungicide. *Bacillus subtilis* cells colonized on root system compete with disease-causing fungal organism and secrete various antibiotics. Moreover, *Bacillus subtilis* used as a fungicide does not affect humans (Andersson et al, 1995).

Bacillus subtilis survive under severe environmental conditions such as starvation by means of its ability for the formation of stress-resistant endospores and the competence development. Depletion of carbon, nitrogen or phosphorus causes starting the sporulation. Yet, the process needs to start with the entire exhaustion of nutrients (Perez, 2000).

Although *Bacillus subtilis* had been thought to be a strict aerobe, it also can live in anaerobic environment utilizing nitrate as a terminal electron acceptor (Glaser et al,

1995; Preis, 1993). Recent studies pointed out that *Bacillus subtilis* can grow in anaerobic conditions utilizing nitrate or nitrite as a terminal electron acceptor or by fermentation (Glaser et al, 1995).

When *Bacillus subtilis* is exposed to stress whether physically or in nutrients, it begins developing a series of morphological and physiological responses including the trigger of macromolecular hydrolysis (proteases and carbohydrases), chemotaxis, motility, and development of competence (Colin, 2013). When these reactions fail to restore growth, sporulation is initiated in a part of the population (≤ 10 %) while the other part is imposed to cell lysis (cannibalism) to supply the nutrients needed for sporulation to continue (Veening et al, 2009). Sporulation of *Bacillus subtilis* which is regulated by Spo0A protein is induced by nutritional deficiency where cells undergoing sporulation produce sporulation killing factor (SKF) and sporulation delay protein (SDP) by expression of their operon which prevents the other cells from sporulation and cause them death to feed on their lysis (Pastor et al, 2003).

After demonstrated as a transformable organism in 1958 by Spizizen, *Bacillus subtilis 168* has been gaining an increasing importance in the field of synthetic biology, biotechnology and genetic studies. As a result of this accumulated knowledge, *B. subtilis* came to be one of the most studied microorganism (Colin et al, 2013) and became one of the most known prokaryotes in terms of molecular biology and cell biology. In addition, its diverse genetic responsiveness has offered the effective implements needed to explore a bacterium by the entire potential perspectives (Zweers et al., 2008). Moreover, research on *B. subtilis* remains at the pole position of bacterial molecular biology and cytology, and the organism represents the mode of choice for investigating cell differentiation and gene regulation bacteria. Furthermore, its ability to secrete the secondary metabolites to culture media makes *B. subtilis* one of the best fermenter prokaryote from biotechnological point of view comparing to *E. coli*. (Zweers et al., 2008).

1.1.2 *Bacillus subtilis* Genome

Bacillus subtilis has one circular chromosome with 4.2 Mb total size of genome (Frangeul et al, 1999). The sequence of its genome was completed in 1997 by an international collaboration (Kunst et al., 1997). Its genome also involves 17 sigma

factors and almost 250 DNA binding transcriptional regulators besides to 4106 protein-coding, 86 tRNA, 30 rRNA and 3 small stable RNA genes (Ando et al.,2002).

Bacillus subtilis duplicates its single circular chromosome by starting DNA replication at a single locus(Ori C), where replication proceeds bidirectionally and two replication forks proceed in the clockwise and counterclockwise directions alongside the chromosome halves and chromosome replication is ended when the forks attain the terminus region, that is positioned opposite to the origin on the chromosome map, and includes a number of short DNA sequences (Ter sites) which promote replication arrest.

1.1.3 Quorum sensing mechanism in *B. subtilis*

Bacteria are prevalent and tremendously prosperous life appearances. Their achievement is principally owing to their capability for familiarizing to enormous assortments of conditions. With the purpose of prevailing over the tough circumstances, *B. subtilis* exhibits assortment of survival approaches such as undergoing stationary phase due to nutritional oscillations. The moment that nutrients turn out to be reduced for optimum growth at the end of the exponential growth phase, *B. subtilis* cells start producing a complicated motility and chemotaxis system which facilitates looking for nutrients in the surroundings. If nutritional restrictions continue, the motile cells resort to entering the stationary phase and secreting degradative enzymes. These degradative enzymes make available nutrients to cells from alternative sources that are ordinarily unreachable. Likewise, cells initiate producing antibiotics to fight probable competitors. Extended nutritional stress ends up the developing competence, and ultimately sporulation by which the bacterial population can adapt to live in such severe environmental circumstances (Hamoen et al., 2003; Levdikov et al., 2006; Msadek,1999).

Bacillus subtilis appropriately directs these adjustments utilizing quorum sensing system to regulate gene expression in light of changes in cell-population density. Quorum sensing was initially defined in the *Vibrio fischeri* bacterium, a marine organism, due to its role as a control mechanism of light production and many various characterizations (Engebrecht and Silverman, 1984, 1987; Antunes et al, 2007). Quorum sensing regulates many important processes such as bioluminescence, sporulation, biofilm formation and virulence factors secretion (Novick & Geisinger,

2008; Ng & Bassler, 2009; Williams & Camara, 2009). Quorum sensing systems differ in regulatory components and molecular mechanism. However, they are governed by three essential determinants. First, the signaling molecules AIs. Second, the receptors located in the membrane or the cytoplasm. Third, activating related genes and AI production (Novick et al, 1995. Seed et al, 1995).

Cell–cell signaling is a basic activity performed by nearly all cell sorts (Lazazzera, 2000). Bacteria regularly manipulate it to sensor their population density generating and sensing small signaling hormone-like molecules, designated as "autoinducers" (Reading and Sperandio, 2005; Gobbetti et al., 2007; Chen et al., 2002). These signaling molecules, consecutively, accumulate and incite cascade incidents when a "quorum" (e.g., a particular onset intensity) is accessed; for that reason, the term of "quorum sensing" designates the cell–cell communication process (Gobbetti et al., 2007; Hooshangi and Bentley, 2008). Finding cell–cell communication among bacteria has led to understand that bacteria behave cooperatively as a group which previously thought to be confined to multicellular organisms (Hardman et al., 1998).

Quorum sensing encompasses the production, release and consequent recognition of chemical signaling molecules (Henke and Bassler, 2004; Lazazzera, 2000). The extracellular concentration of QS signaling molecules that are yielded and excreted into the surroundings by bacterial cells intensifies with raising cell number. When the QS signaling molecules reach a critical threshold level, the group coordinately respond to the signaling molecules by altering their gene expression (Schauder and Bassler, 2001; Reading and Sperandio, 2006). The adjustments in gene expression owing to the existence of signaling molecules offer bacteria certain performances only when living in a population, not when living as individuals (Henke and Bassler, 2004).

The signaling molecules and receptors are divided into three classes, N-acyl homoserine/lactones (AHLS) produced by Gram-negative bacteria. Oligo peptides or autoinducing peptides (AIP), used by Gram-positive bacteria (Kweon et al, 2014).

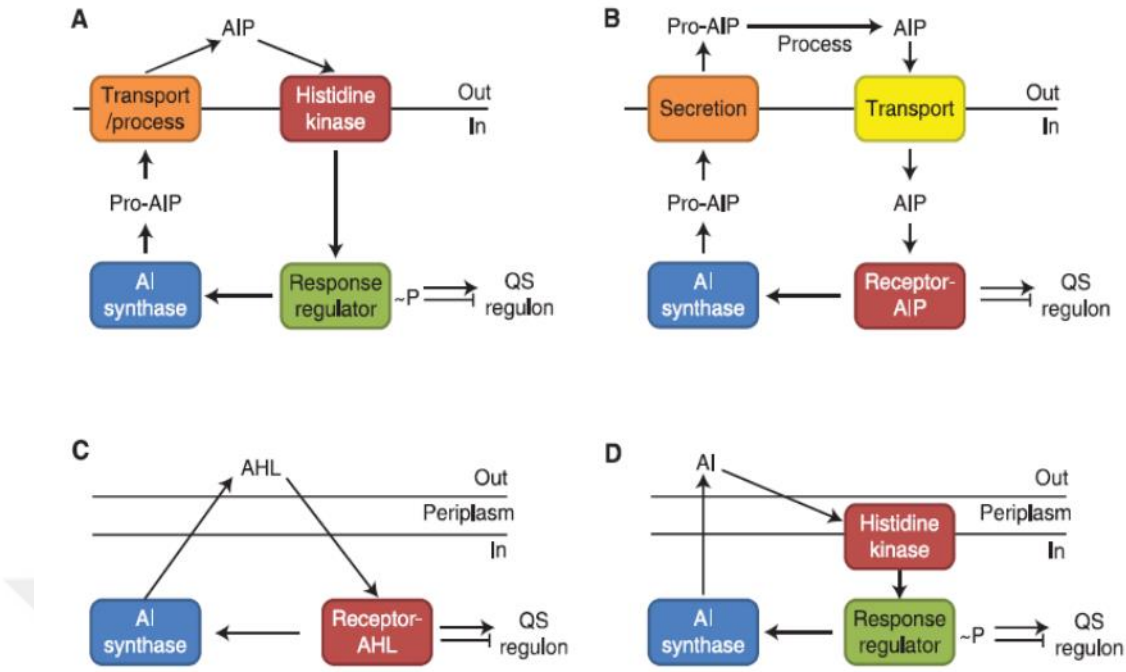


Figure 1.1: Canonical bacterial quorum-sensing (QS) circuits. Autoinducing peptide (AIP) QS in Gram-positive bacteria by (A) two-component signaling, or an AIP-binding transcription factor. Small molecule QS i. Gram-negative bacteria by (C) a LuxI/LuxR-type system, or two-component signaling. (http://www.ncbi.nlm.nih.gov/pubmed/10081580).

When it comes to the difference between quorum sensing in Gram-positive and Gam-negative bacteria. In gram negative bacteria, the signaling protein is termed acyl-homoserine lactone (AHL) functions as the key signaling molecules. Its formation is catalyzed by LuxI. LuxR binds to AHL at onset level Fig.1. These autoinducers molecules come to be able to diffuse freely through the cell membrane and spark cell response once concentration attains a certain level. As a consequence, this complex surveys Lux boxes within the promoter section to trigger specific genes.

Likewise, Gram-positive bacteria, on the other hand, employ peptides or modified peptides as the main process of signaling; and also contrary to gram-negative bacteria, there are two main quorum sensing systems obviously as follows: (i)Two-component signal transduction where the peptide signal operates by means of attaching to a sensor protein, histidine kinase, placed in the cell membrane. The histidine kinase activation directs phosphorylation of response-regulating protein, and interaction with another regulatory protein smooth the progress of transcriptional activation.

In *Bacillus subtilis*, the main physiological processes such as sporulation, biofilm formation, competence, proteolytic enzymes synthesis and peptide antibiotic production are under the control of quorum sensing (ComX/PA) pathway (Grossman, 1995). On the other way, the multicomponent regulatory circuits that control sporulation and competence feature two crucial regulators, namely Spo0A, the master transcriptional regulator of sporulation and AbrB, the transition state regulator. Spo0A, under unfavorable condition such as stress and high cell density is activated by phosphorylation initiated by histidine kinase KinB and KinC and KinA producing Spo0A-P regulon genes and control their transcription. Spo0A-P plays a dual role as a regulator both positive and a negative regulator. Spo0A-P represses the *abrB* gene thereby derepressing a number of stationary phase/sporulation genes (Stranch et al, 1990; Sonenshein, 2000).

Genetic competence can be defined as a physiological state that enable bacterial cells to bind and take up exogenous DNA (transformation). In *Bacillus subtilis*, competence is a physiological state which occur at the inception of late exponential growth within certain nutritive condition (Kürşad et, al 1998) by which the cells enter stationary phase as a response to cell density as well as growth phase and also to the accumulation of products secreted in the culture (e.g. competence factors). These particular conditions are called competence regime (Akos et al, 2009). However, only few cells can become competent (Hahn and Dubnau, 1991). A cell that has the capability to take exogenous DNA and become transformed is called competent.

Bacillus subtilis and *Streptococcus pneumoniae* theoretically have the ability to take up any linear DNA but to be replicated it should be recombined with the recipient chromosome which requires similarity in some region of the chromosome. In addition, there are specific proteins play an important role in this process including a membrane-associated DNA-binding proteins, a cell wall autolysin and different nucleases. *In Bacillus subtilis*, competence development is regulated by quorum sensing. During their growth, cells excrete a small peptide, as this peptide accumulates to high concentrations it triggers them to be competent. However, not all cells can develop competence about 20 % of the *Bacillus subtilis* cells can develop competence and remain for several hours. On the contrary, in *Streptococcus* 100 % of the cells can become competent but for short period during the growth cycle (Michael et al, 2015).

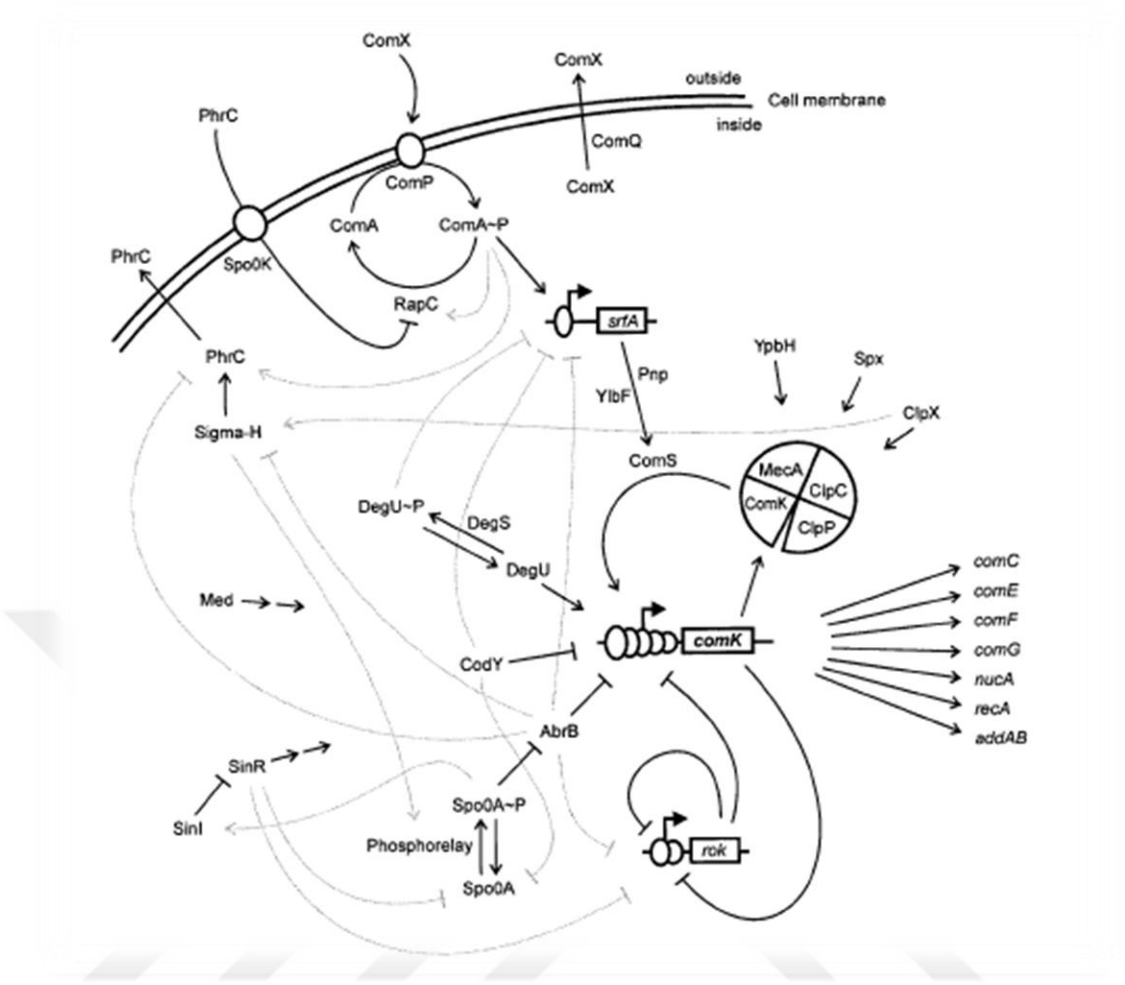


Figure 1.2: Overview of the different regulation pathways involved in competence development. The main pathways are indicated by solid black lines. The grey lines illustrate a number of regulatory connections. The *srfA*, *comK* and rok genes are schematically depicted with the promoter regions to which several transcription factors are bound (circles). The ComP and Spo0K proteins are depicted in the cell membrane. The ComK/MecA/ClpC/ClpP protein complex is encircled. Arrows and T-bars indicate positive and negative regulation, respectively.

(Hamoen et al, 2003).

Competence in *Bacillus subtilis* is regulated by quorum sensing where ComX represents the main competence pheromone Fig.1.2. ComQ encodes protein (protease enzyme) responsible of cutting and modification of competence pheromone ComX. ComP, the membrane protein, is a sensor protein. Once the active form of ComX bind to ComP which then undergo autophosphorylation leading to activation of ComA by transfer the phosphate to ComA. Consequently, ComA induce the transcription of several genes. Conversely, ComA-P can be activated by indirect pathway by competence sporulation factor (CSF) resulting from PhrC protein. CSF is attracted to

the cell by oligopeptide permease Spo0K. Once imported into the cell, CSF inhibits RapC protein which is in the case of being activated binds to the C-Terminus DNA-binding domain of ComA-P and hinders its binding to DNA leading to transcription. Fig.1.3 (Snyder and Champness, 2007).

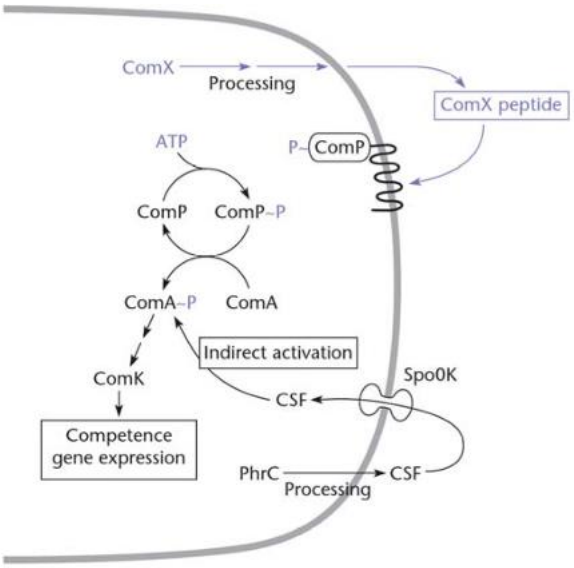


Figure 1.3: Regulation of competence development in *Bacillus subtilis* by quorum sensing. (Snyder and Champness, 2007).

There is at least one other such pathway; these pathways' purpose may be to coordinate competence with sporulation and other functions when the cell runs out of nutrients and enters stationary phase.

Expression of ComK is under multiple controls of several proteins both negatively and positively such as DegU, SinR, CodY, AbrB, Rok and ComK itself in addition to quorum sensing. ComK and DegU act positively by binding directly to *comK* promoter (P*comK*) forming a positive feedback loop (Sinderen & Venema, 1994) where DegU binds to P*comK* it rises the attraction between ComK and its promoter (Hamoen et al, 2000). However, AbrB and CodY repress ComK expression by binding directly to P*comK* (Hoa et al, 2002; Serror & Sonenshein, 1996). However, within competence development, AbrB functions in a positive manner on *comK* transcription (Hahn et al, 1996).

The stability of ComK is adjusted by ClpCP protease which acts by degrading ComK prior to stationary phase and when avoiding competence state Fig.1.4. There is no direct contact between ComK and ClpCP, however, the connection is mediated by

MecA protein which acts as an adaptor connecting ComK and ClpCP to its N-Terminus and C-Terminus domains respectively (Turgay et al, 1998; Persuh et al, 1999). ComS, a small protein formed via series of phosphorylation reactions started by quorum sensing (Lazazzera et al, 1999) releases ComK by binding to the N-Terminus domain of MecA thus protects it from degradation. YlbE plays a positive role on the expression of ComK by increasing the stability of ComS (Tortosa et al, 2000).

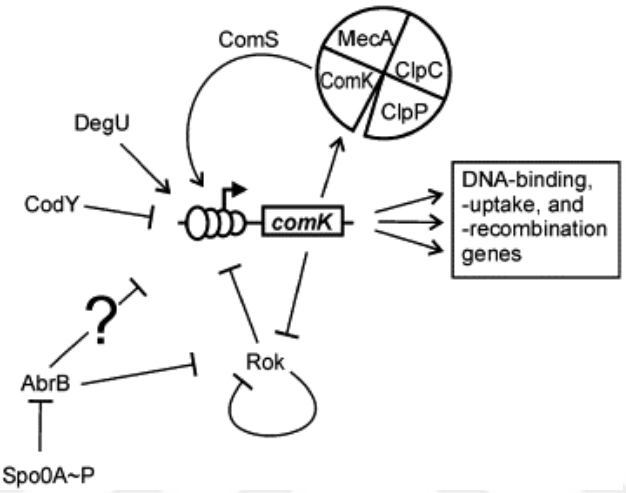


Figure 1.4: Schematic presentation of the control of *comK* expression. *comK* is schematically depicted as gene with the promoter region to which several transcription factors are bound (circles) (Hamoen et al, 2003).

The competence transcription factor encoding gene *com*K is negatively regulated by Rok (Ykuw) protein as transcription of *comK* is inhibited by Rok when it is found in extra amount without affecting its stability which is regulated by MecA where Rok exerts its effect on *comK* by binding directly to DNA portion containing *comK* promoter Fig.1.5. However, *rok* transcription is negatively regulated by SinR and AbrB proteins where they act as repressors for its transcription (Hoa et al, 2002).

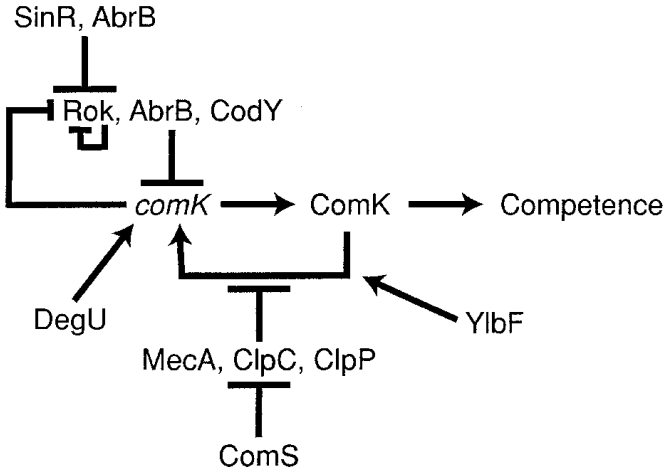


Figure 1.5: Competence regulation. In this simplified summary diagram, arrowheads and perpendiculars represent positive and negative effects respectively. These effects are variously exerted on the transcriptional or post transcriptional level. (Hoa et al, 2002).

SinR is a pleiotropic DNA binding protein that is essential for the late-growth developments of competence and motility in *Bacillus subtilis* and also is a repressor of sporulation and subtilisin synthesis besides acting as an inhibitor of sporulation (Mandic-Mulec et al, 1995). Mainly, SinR is the master regulator that governs the Gram-positive model organism *Bacillus subtilis* life style whether to be a free-living or a biofilm-forming lifestyle. The activity of SinR, is regulated by its antagonists, SinI, SlrA, and SlrR Fig.1.6. (Newman et al, 2013). When abundant SinI is existent, an SinR-SinI complex is created that inhibits the SinR-DNA interaction, triggering derepression (Lewis et al, 1996;1998).

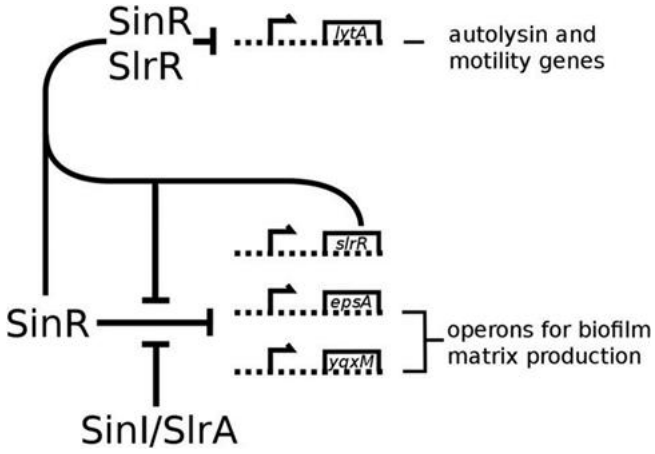


Figure 1.6: A schematic view of the control network of SinR. SinR is a tetrameric repressor of two major biofilm operons, *epsA-O* and *yqxM-sipW-tasA*. The repression is relieved by the action of SinI and SlrA, which bind to and inactivate SinR. SlrR, itself under control by SinR, repurposes SinR to become a repressor of motility genes. (Newman et al, (2013).

1.2 *Bacillus subtilis* and Plant

Bacillus subtilis belong to plant growth-promoting rhizobacteria (PGPR) which are soil useful microorganisms that inhabit plant roots for nutritive reasons and consequently do good to plant by enhancing its growth or decreasing disease and most commonly found in soil environment and on plant undergrowth. They are likely to be found in O and A surface soil horizon where the concentration of oxygen is most abundant and temperature are relatively mild. These microbes form spores in times of nutrients exhaustion. However, when the nutrients required for the bacteria to grow are abundant, they exhibit metabolic activity.

Currently, biological control agents for plant diseases have obtained substantial considerations as substitutions to chemical pesticides.

From plant or agricultural point of view, *B. subtilis* is an important microorganism living in the rhizosphere -the soil area surrounding the plant root- as it enhances plant growth and acts as a biocontrol agent (Emmert and Handelsman,1999) in addition to be used as a biofertilizer (Beauregard et al, 2013). Additionally, these organisms can produce antibiotics during sporulation and many of the *Bacillus* microbes can degrade polymers such as protein, starch and pectin, therefore, they are thought to be important contributor to carbon and nitrogen cycle. However, quite a few of the *Bacillus subtilis* organisms are primarily responsible for the spoilage of food.

Proliferation of *Bacillus subtilis* in the soil needs availability of nutrient source such as breaking down organic material or plant roots (Eariam et al, 2008). Plant roots are directly surrounded by region of soil called the rhizosphere. This region is rich in plant secretions that can provide bacteria with nutrients (Danhorn et al 2007; Lugtenberg et al, 2009 and Ramy et al, 2004). Figure 1.7 shows communication network within rhizosphere microflora represented by bacteria, fungi and plant roots which play an important role in plant development and adaptation to stressful conditions where by signal exchanges is regulated by quorum sensing (Choudhary and Johri, 2009).

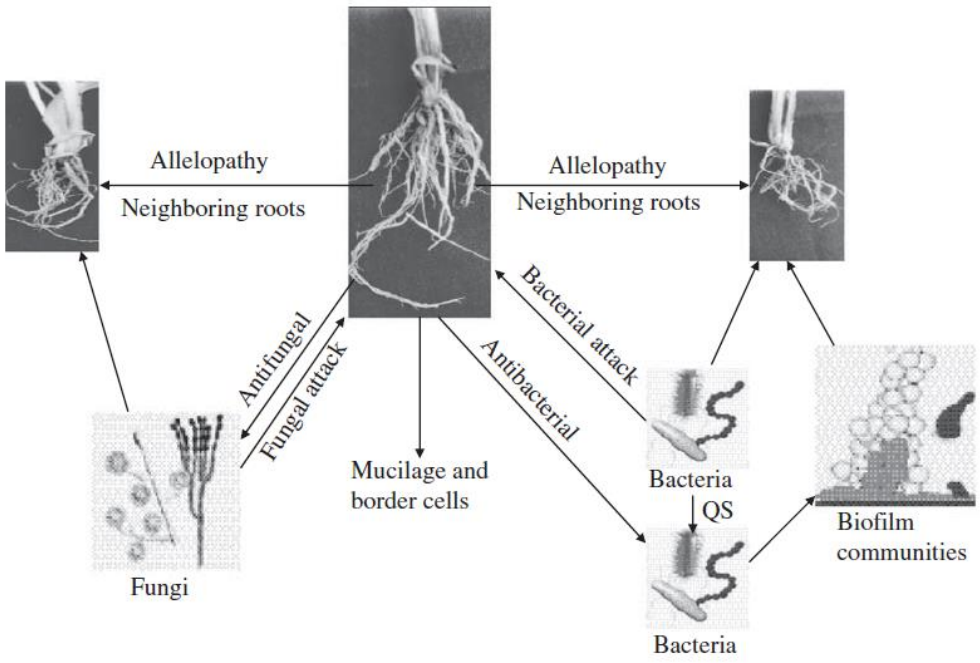


Figure 1.7: Schematic representation of complex interactions mediated by root exudates that take place in the rhizosphere between plant roots and other organisms. QS, quorum sensing. (Walker et al. (2003).

Bacillus species have the ability to enhance plant growth and can protect plants from infection by pathogenic bacteria, fungi and nematode. This protection can be achieved by the antimicrobial compounds secreted by *Bacillus subtilis* Fig.1.8. Additionally, plant systemic resistance induced responding to *Bacillus subtilis* enhance the ability of plant to be more resistant against diverse pathogens. (Emmert, & Handelsman,1999; Kloepper et al, 2004; Ryu et al, 2004; Choudhary & Johri, 2009; Srivatsan et al, 2008; Perez-Garcia et al, 2011). *Bacillus* species - including *B. subtilis* - are marketed commercially as biological control agents for agriculture (Danhorn & Fuqua 2007; Ramey et al, 2004; and Morikawa, 2006).

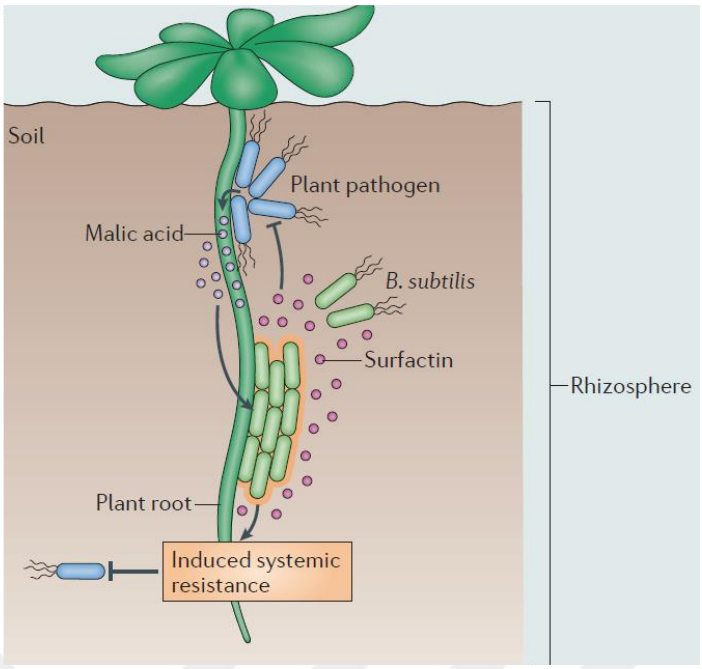


Figure 1.8: Schematic illustration of *B. subtilis* colonizing a plant root. *B. subtilis* secretes the lipopetide surfactin, which is required for *B. subtilis* biofilm formation on the root. A second trigger for *B. subtilis* matrix gene expression is malic acid, which is constitutively secreted in the rhizosphere by tomato plants but secreted by *A. thaliana* only when the plant is infected with the pathogen *P. syringae*. *B. subtilis* exerts beneficial effects on the plant by promoting its growth and helping to fight of pathogens (such as *P. syringae*), directly via the secretion of surfactin and other antimicrobials and indirectly by eliciting induced systemic resistance in the plant. (Hera Vlamakis et al, 2013. nature).

B. subtilis is easily isolated from the rhizosphere of plants and the bulk of root-associated strains are able to form vigorous biofilms in laboratory conditions (Vessey,2003 and Fall et al, 2004). Moreover, quite a lot of other *Bacillus* species develop biofilms on plant roots (Chen et al, 2012 and Rudrappa et al, 2008). Establishment of biofilm on plant roots equivalents in vitro biofilm establishment in that the matrix exopolysaccharide is necessary (Shank, et al, 2011 and Fall et al, 2004).

Likewise, the master regulator Spo0A and the antirepressor SinI are also essential for root colonization. In many wild isolates, the existence of these genes, and consequently the capability to develop a biofilm on the root, is also requisite for the strain to make use of its utmost biocontrol influence (Fall et al, 2004).

Bacillus species yield a diversity of antimicrobial agents that were suggested to be significant in plant biocontrol in view of the fact that these molecules could inhibit growth of particular pathogenic soil microorganisms Table 1.1. (Bais et al., 2004;

Stein, 2005; Nagorska et al., 2007). For example, *Bacillus thuringiensis* (Bt) is amounted for over 90 % of all marketed bioinsecticides and *Bacillus subtilis kodiak* strains highly effective for crop protection from the pathogen *Fusarium* and *Rhizobacteria* and it also stimulates plant growth.

Strains of *B. subtilis* have been commercially sold as biocontrol agents for fungal diseases of crop Table 1.2. For example, Serenade, a biofungicide produced from *B. subtilis* (QST713 strain) Payer, is used successfully to fight fungal and bacterial diseases (Morikawa, 2006). *Bacillus subtilis B25* isolated from banana rhizosphere soil was confirmed to have an antifungal protein that acts against *Fusarium oxysporum f.sp.cubense* (FOC), which is a destructive disease of banana (Tan et al, 2013). In their work on biocontrol of tomato wilt disease by *Bacillus subtilis*, Chen and others (2012) showed the importance of biofilm formation to the same degree of the production of antimicrobial agents, and their results suggested that surfactin production participates to plant biocontrol through two mechanisms by acting as antimicrobial agent and as a signaling molecule that triggers biofilm establishment on plant roots.

The antibiotic difficidin and bacilysin from *Bacillus amyloliquefaciens FZB48* have the ability to suppress bacterial blight and bacterial leaf streak, serious diseases of rice resulted from *Xanthomonas oryzae* bacterium (Liming et al, 2015). Difficidin, and bacilysin downregulated expression of genes related to *Xanthomonas virulence*, cell division, protein and cell wall synthesis. These results point out the activity of difficidin and bacilysin as antimicrobial compounds.

The bacilysin was at least five times more active against *Staphylococcus aureus* when using Dulbecco agar medium containing free amino acids comparison to the Lemco medium containing peptides which reflects the responsibility of peptides in decreasing the activity of bacilysin. Bacilysin has a wide broad activity against bacteria and *Candida albicans*, however, its activity is intensely subject to the components of the nutritive substances (Keing & Abraham, 1975). Liming Wu et al (2014) showed that, the bacilysin produced by *Bacillus amyloliquefaciens FZB42* causes lysis of algal cell and also downregulates genes involved in essential processes in the cell peptidoglycan synthesis, photosynthesis, mycosystin synthesis and cell division besides it could be developed to be used as a biological agent to alleviate the effect of damaging algal bloom produced by huge and extraordinary overgrowth of microalgae and cyanobacteria, as they represent a worrying global environmental problem.

Bacillus amyloliquefaciens FZB24, which is known by its activity against nematodes which make severe damages to agricultural crops around the world, is recorded in Germany as a plant- reinforcing agent (Krebs et al, 1998). Plantazolicin (PZN) isolated from *Bacillus amyloliquefaciens FZB24,* was discovered and named by Scholz and others (2011) due to the correlation of a number of producing organisms with plants and the integration of azole heterocycles, originated from Cys, Ser, and Thr residues of the precursor peptide. It was also described and characterized by its ability as a nematicidal agent by Zhongzhong (2013).

Table 1.1: Examples of Bacillus-based biocontrol in plants against pathogens.

Plant	Disease	Pathogen	Biocontrol Bacillus	Ref
Sugarbeet	Cercospo leaf spot	*Cercospora beticola*	*B. pumilus strains 203-6 & 203-7, B. mycoides strain Bac J*	Bargabus et al. (2002, 2004)
Radish	Bacterial leaf spot	*Xanthomonas campestris armoraciae*	*Bacillus spp.*	Krause et al. (2003)
Cucumber	Root rot	*Pythium spp.*	*Bacillus spp.*	Zhang et al.
Peanut	Late leaf spot	*Cercosporidium*	*Bacillus spp.*	Zhang et al. (2001)
Tobacco	Blue	*Peronospora tabacina*	*B. pasteurii C-9, B. pumilus SE34 and T4*	Zhang et al. (2002,
Cucumber	Bacterial wilt	*Erwinia tracheiphila*	*B. pumilus INR7*	Zehnder et al. (1997)
Tomato	Leaf spot	*Cucumber mosaic virus (CMV)*	*B. subtilis IN937b, B. pumilus SE34, B. amyloliquefaciens IN937a*	Zehnder et al. (2000)
	Late	*Phytophthora infestans*	*B. pumilus SE34*	Yan et al.
Loblolly pine	Fusiform rust	*Cronartium quercuum, Miyabe exsirai f. sp. fusiforme*	*B. sphaericus SE56 and B. pumilus INR7, SE34, SE49, SE52*	Enebak et al. (1998)
Peanut	Crown	*Aspergillus niger*	*B. subtilis AF1*	Sailaja et al.
Cucumber	Angular spot	*P. syringae*	*B. pumilus INR7*	Wei et al.
	Anthracn	*Colletotrichum arbiculare*		
Tomato	Leaf spot	*Tomato mottle virus (ToMoV)*	*B. amyloliquefaciens IN937a, B. subtilis IN937b, and B. pumilus SE34*	Murphy et al. (2000)
Tobacco	Wild fire	*P. syringae pv. tabaci*	*B. pumilus strain T4*	Park and Kloepper (2000)
Tomato	Bacterial wilt	*Ralstonia solanacearum*	*Bacillus spp.*	Jetiyanon and Kloepper (2002)
Long pepper	Anthracn	*Colletotrichum (Penz.) Penz and Sacc.*		
Green kuang Futsoi	Damping -off	*Rhizoctonia solani*		
Cucumber	Leaf spot	CMV		

Table1.2: Commercially available Bacillus-based plant disease biocontrol products.

Product name	Company	Bacillus component	Formulatin type	Primary target
Serenade	AgraQuest, Davis, CA	*B. subtilis QST 713*	WP, aqueous. Suspension	Fungi, bacteria on multiple fruits
EcoGuard	Novozymes, Salem, VA	*B. licheniformis SB3086*	Flowable	Sclerotinia homoeocarpa on turf
Kodiak	Gustafson, Plano, TX	*B. subtilis GB03*	WP, Flowable	Fungi on cotton, legumes, soybeans
Yield Shield	Gustafson	*B. pumilus GB34*	WP	Fungi on soybeans
BioYield	Gustafson	*B. amyliliquefaciens GB99+ B. subtilis GB122*	Dry flake	Fungi on bedding plants in potting mixes
Subtilex	Beker Underwood, Ames, IA	*B. subtilis MB1600*	WP	Fungi on cotton, legumes, soybeans
Hi Stick	Beker Underwood	*B. subtilis*	Flowable	Fungi on soybeans, peanut
L+Subtilex		*MB1600+Rhizobium*		

Source: Schisler et al. (2004).

aWP ¼ wettable powder.

1.3 *Bacillus subtilis* Antibiotics

Bacillus subtilis is an endospore-forming rhizobacterium which has the ability to produce more than two dozen antibiotics (Stein, 2005). According to Stein (2005), 4-5% of *Bacillus subtilis* genome is allocated to produce antibiotics. Antibiotics produced by *B. subtilis* have been characterized by being low toxic and high biodegradable, thus *B. subtilis* has been awarded GRAS (Generally Recognized As Safe) status by the US Food and Drug Administration. (Zweers et al, 2008).

As stated by Stein (2005), non-proteinaceous residues production is conducted by two distinguished pathways (i) Non- ribosomal pathway by which peptides are synthesized by non-ribosomal synthetases (NRPSs), substantial multienzymes distinguished by their ability to catalyze a series of reactions. (ii) Ribosomal pathway by which linear precursors are synthesized and undergo post-translation modification and processed by proteolytic enzymes. The non-ribosomal synthesis pathway of peptides is common within bacteria and fungi (Sieber & Marahiel, 2003.Finking & Marahiel, 2004 and Walsh, 2004).

1.3.1 Lantibiotics

Lantibiotics are bacterial peptides produced ribosomally by many gram-positive bacteria. They contain lanthionin and or methyllanthionine (Me Lan) (Bierbaum & Sahl, 2009) where the presence of lanthionin residue make them resistant against protease (Bierbaum et al, 1996; Chatterjee et al, 2005). They are encoded by gene clusters containing structural gene (*lanA*)-lantibiotic peptide-LanM biosynthetic enzyme to perform modification or LanB and LanC. For these bacteria, they produce variety of immunity protein to protect themselves such as LanI lipoproteins (Hacker et al, 2015).

Lanthionine-containing antibiotics are peptide antibiotics characterized by inter-residual thioether bonds (Schnell et al,1998). Lanthionine formation occurs within two steps. The first step includes dehydration of L-thionine residues in preplantibiotic peptide produced ribosomally forming 2,3 didehydoalanine and 2,3-didehydrobutyrin respectively. In the second step, the formation of inter-residual thioether linkage through stereospecific Michael-like addition of neighbored L-cysteine sulfhydryl group yielding meso-lanthionine and 3-methyl lanthionine respectively (Guder et al, 2000; Jack & Jung, 2000; McAuliffe et al, 2001).

Lantibiotics are classified into two types according to their structural properties Fig. 1.9 (i)Type A Lantibiotics, composed of 21-38 amino acid residues, are linear secondary structures and have the ability to kill Gram-positive cells by forming pores into the cytoplasmic membrane. Examples of them, Subtilisin, EricinA, EricinS. (ii) Type B lantibiotics are globular peptides function by attaching to lipidII, cell wall precursor consequently, inhibit the cell wall biosynthesis. Examples of them, mersacidin, sublanicin produced by *B. subtilis 168*, haloduracin, produced by *Bacillus halodurans*. However, not all lantibiotics have an antibacterial activity (Kodnai, 2004).

Subtilisin: a 32 amino-acids pentacyclic lantibiotic (Stein, 2005) yielded by *Bacillus subtilis ATCC6633*. It has a wide range activity against bacteria by increasing permeability of the cytoplasmic membrane of target bacteria. It consists of two types, subtilin and N-succinylated subtilin (Parisot et al 2008).

Ericin, (S and A): distinctive lantibiotic peptides produced by *Bacillus subtilis A1/3* (Stein et al, 2002). However, EricinA has a different ring organization and 16 amino

acids substitutions compared with Ericin S. For both Ericin A and Ericin S, a single Eri BC synthase is needed (Stein 2005).

Sublancin 168 is one of the unusual Lantibiotics, discovered by Paik et al, (1998). It is a novel lantibiotic produced by *Bacillus subtilis 168*. It contains a disulfide bridge. In addition, its microbial activity is similar to other lantibiotics by its ability to inhibit gram- positive bacteria but not gram-negative ones and it behaves like the lantibiotics nisin and subtilisin by inhibiting both bacterial spore outgrowth and vegetative growth. Furthermore, it is a distinguished stable lantibiotic and can be stored in aqueous solutions at room temperature for 2 years (Paik et al 1998). Furthermore, it is endogenous to *Bacillus subtilis 168* and characterized by possessing disulfide bridge and its extraordinary stability compared to other lantibiotics.

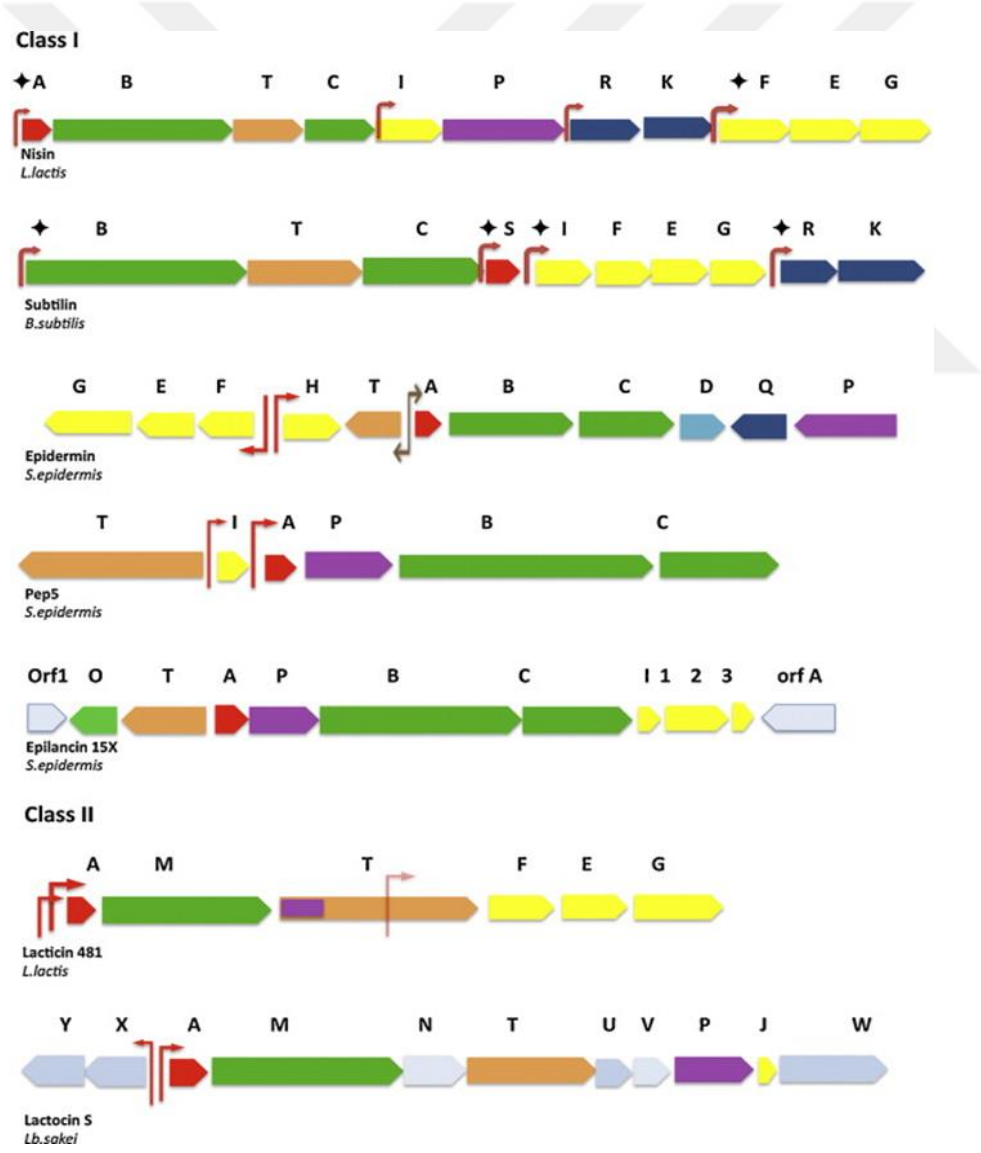


Figure 1.9: Representative gene clusters of selected class I and class II lantibiotics. (Alkhatib et al, 2012).

Subtilosin is a microcyclic peptide antibiotic characterized by interresidue bridge (Marx et al, 2001; Kawulka et al, 2003). Subtilosin A, a modified antimicrobial peptide produced by *Bacillus subtilis 186*, originally identified by Babasaki and coworkers (1985). Its encoding gene also occurs in the same *alb* gene operon. It consists of 32 common amino acids and several atypical residues developing from the posttranslational modification (Zheng et al, 1999). The production of nature subtilosin relies on expression of the *sbo-alb* gene cluster containing the subtilosin structural gene *sbo* and genes requisite in immunity (Zheng et al, 2000; Stein et al, 2004).

As for mode of action, lantibiotics are mainly produced by gram-positive bacteria, in spite of that, they exert their effects mostly on them (Ree et al, 2007). However, the outer membrane of gram-negative bacteria provides them resistance to lantibiotics as it hinders access the cytoplasmic membrane (Kordel et al, 1988; Stevens et al, 1991). In addition to the modification of peptides undertaken by LanM enzyme or LanB and LanC besides elimination of exporter LanT and the leader sequence N-terminal (Kaletta et al, 1994. Xie et al, 2004).

Regarding protection from lantibiotics, Lantibiotics exert their effects on the producer's relative strains. On the other hand, the producers have genes encoding products act as self-protective by different mode of actions (Draper et al, 2008). Self-protection against Lantibiotics is acquired by ATP-binding cassette (ABC) transporter homologous proteins (Lan FEG) that conveys the Lantibiotic from the cytoplasmic membrane toward the extracellular gap (Stein et al, 2005). In addition, some Lantibiotic producers produce LanI, membrane-bound lipo protein which prevents the gathering of the Lantibiotic to high concentrations in the vicinity of the cytoplasmic membrane and hindering Lantibiotic lipid II pore formation (Koponen et al, 2004; Stein et al, 2005).

As for food industry, the major manipulation of Lantibiotics is exemplified by Nisin, which is used as a biopreservative mediator due to its ability to prevent bacterial spoilage and inhibit the clostridial spores (Delves et al, 1996). Moreover, some Lantibiotics showed their potentiality to be used in biomedical industry for example, nisin for peptic ulcers treatment, nisin and lacticin 3147 were proposed as contraceptive ingredients (Silkin et al, 2007; and Aranha et al, 2004).

1.3.2 Non-ribosomal biosynthesized peptides (NRBP)

Nonribosomal peptides stand for a large family of bioactive secondary metabolites produced by bacteria and fungi. Many of these peptides became notable antibiotics such as iturin, gramicidin, and bacitracin (Chalasani et al, 2015) Fig. 1.10. Moreover, the non-ribosomal synthesis of peptide antibiotics is widespread among bacteria and fungi (Stein, 2005).

Non-ribosomal peptide synthesis is an enzymatic mechanism undertaken by a large multienzymes complex to assemble and catalyze gradual peptide condensation (Fischbach & Walsh, 2006). The nonproteinogenic amino acids such as D-isomerase, carboxy acids and N-methylated residue represent the building blocks of substrates of these enzyme complexes. On the other hand, NRBP mechanism is associated with other common postsynthetic modifications such as Glycosylation and oxidative cross-linking (Sieber & Marahiel, 2003).

NRBP (noribosomal peptide) synthetase and polyketides synthetase widely distributed across the three domain of life (Wang et al, 2013) and they are synthesized on large nonribosomal peptide synthetase (NRPS) enzyme complex. Wang et al (2014) in their work on nonribosomal peptides and polyketides biosynthetic pathways revealed related siderophore NRPS genes clusters that encoded modular and nonmodular NRPs enzymes organized in a gradient with higher frequency gene clusters of NRPs and PKs from bacteria than archaea and Eukarya. However, the majority of these NRPs and PKs gene clusters and their products are still unknown.

NRPS are large multienzymes composed of modularly arranged domains where there are three core domains responsible for peptide biosynthesis. (i) The adenylation domain composed of 550 amino acid residues, which is similar to the amino-acylation of tRNA synthetases through ribosomal peptide biosynthesis. (ii) The thiolation or peptidyl carrier domain composed of 88 amino acids supported by 4'-phosphopantetheine (PPan) prosthetic group to which the adenylated amino acid substrate is transferred under release of AMP (Stein, 2005). (iii) The condensation domain composed of 450 amino acids flanked by every pair of adenylation and peptidyl carrier domains.

Lipopeptides can be classified to three types, Iturin, Surfactin and Fungycin (Meana & Kanwar, 2015). Surfactin, is one of biosurfactants produced by different strains of

Bacillus subtilis. It is composed of seven amino acid residues and resembles detergents in terms of effects by forming pore in cell membrane (Carrillo, 2003). The Surfactin biosynthesis regulation is related to competence development (Maracheil et al, 1993).

Iturin, is produced by some strains of *Bacillus subtilis*. Iturins are classified to Iturin A, Iturin D, Iturin E, Bacillomycins, Bacillomycins F, Bacillomycins L, Bacillomycins Lc and Mycosubtilin (Peypoux et al 1993,1977,1978, Besson et al, 1984, Besson et al 1976, Winkelmannet et al 1983, Besson & Michel 1987). Iturin A is the main inhibitor against postharvest fungal pathogens (Arrebola et al, 2010). Iturin isolated from *Bacillus amyloliquefaciens* is a promising biocontrol agent in plant defense immunity where it causes *Verticillium dahlia* cell death by affecting fungal signaling pathway (Han et al, 2015).

Mycosubtilin, pore-forming lipopeptides produced by various strains of *Bacillus subtilis* are characterized by a strong antifungal activity. However, they are active against some bacteria such as *Micrococcus luteus* (Maget-D & Peypoux 1994).

Fungycin, an antifungal cyclic lipopetide produced by *Bacillus subtilis F-29-3* strain. It has the ability to inhibit filamentous fungi by forming pores in lipid membrane (Vanittanakom et al, 1986. Fracchia et al, 2015). It can be used as an environmentally friendly agent to control the pathogenic *F.oxysporum* (Nam et al, 2015).

Mycosubtilin, an antimicrobial lipopetide produced by *Bacillus subtilis ATCC663* strain (Duitman et al, 1999) is distinguished by its effective anti-fungal performance (Nasir & Besson, 2012) and it also acts against plasma membrane of sensitive cell (Nasir et al, 2011).

Amicoumacin, produced by several strains of *Bacillus subtilis* (Pinchuk et al 2002), it has a wide range of activity such as antibacterial, anti-inflammatory in addition to their ability to act against *Helicobacter pylori* (*H. pylori*), which make it a potent agent for human treatment mainly for chronic gastritis and peptic ulcer (Pinchuk et al, 2001).

Bacilysocin, a unique phospholipid antibiotic produced by *Bacillus subtilis 168* was discovered by Tamehiro et al in 2002. Figure 1.10 shows summery of Antibiotics produced by *Bacillus subtilis*.

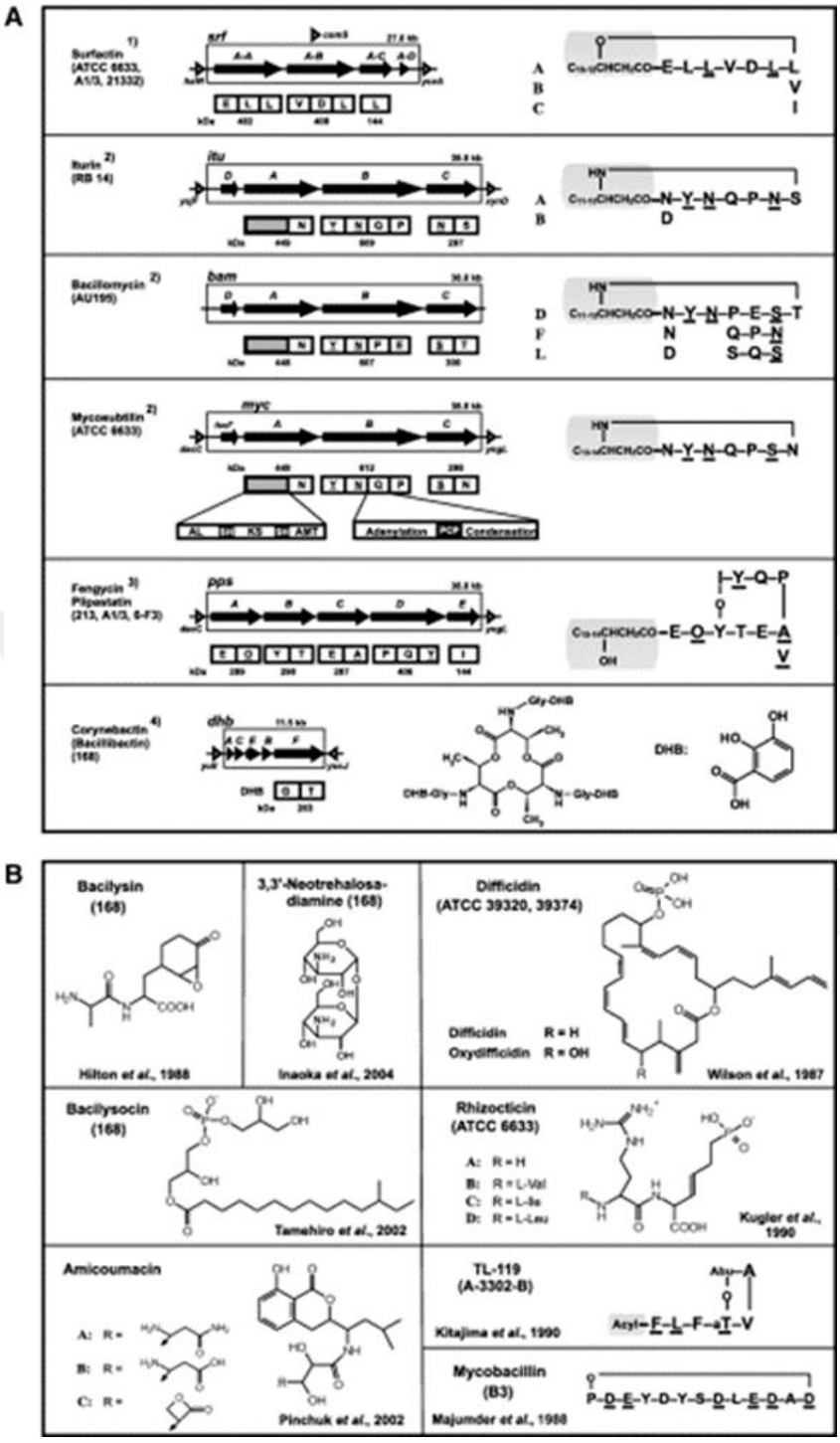


Figure 1.10: Summary of *B. subtilis* antibiotics.

1.3.2.1 Bacilysin

1.3.2.1.1 Bacilysin biosynthesis

Bacilysin is a non-ribosomal peptide antibiotic composed of L-alanine in the N-Terminus and L-anticapsin in the C-Terminus as shown in Fig.1.11. (Walker &Abraham, 1970; Kenig & Abraham, 1976).

L-anticapsin is the effective component of bacilysin which acts as an inhibitor of glucosamine synthesis. Inhibiting the glucosamine synthetase by anticapsin leads to blocking biosynthesis of bacterial peptidoglycan and fungal mannoprotein therefore causing cell protoplasting and lysis (Whitney & Funderburk, 1970; Keing et al, 1976; Chmara et al, 1982; Chmara,1985 and Milewski,1993). The releasing of L-anticapsin is achieved by the proteolysis of di-peptides by peptidase (Finking & Marahiel, 2004).

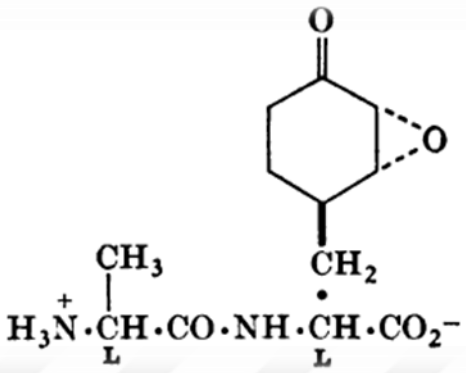


Figure 1.11: Chemical structure of bacilysin (Walker & Abraham, 1970).

Bacilysin is produced by *Bacillus subtilis* and also can be found in some strains of Bacillus species related to *Bacillus subtilis* such as *B. amyloliquefaciens, B. licheniformis* and *B. pumilus* (Loeffler et al, 1986).

Despite its simple structure, bacilysin has a strong activity against a wide range of bacteria and several fungi (Walker & Abraham, 1970; Kenig et al, 1976). Furthermore, bacilysin also has a comparatively wide-ranging, unspecific activity against gram-positive and gram-negative bacteria along with budding and filamentous fungi where it is uptaken efficiently by sensitive cells through dipeptide and oligopeptide permease systems (Diddens et al., 1979; Perry & Abraham, 1979; Chmara et al., 1981).

Inaoka et al (2003) showed that, the *ywfBCDEywfG* operon and a *ywfH* gene encode bacilysin biosynthesis machinery Fig.1.12. After demonstrated to have the essential functions in synthesizing bacilysin, the *ywfBCDEF* were renamed *bacABCDE* (Steinborn et al, 2005), and also *ywfG* and *ywfH* were proposed to renamed *bacF* and *bacG* respectively by Parker & Walsh, 2010 and 2012. Now, it is known that *bacABCDFG* are necessary for the successive reactions starting from prephenate and ending by bacilysin (Parker & Walsh, 2013).

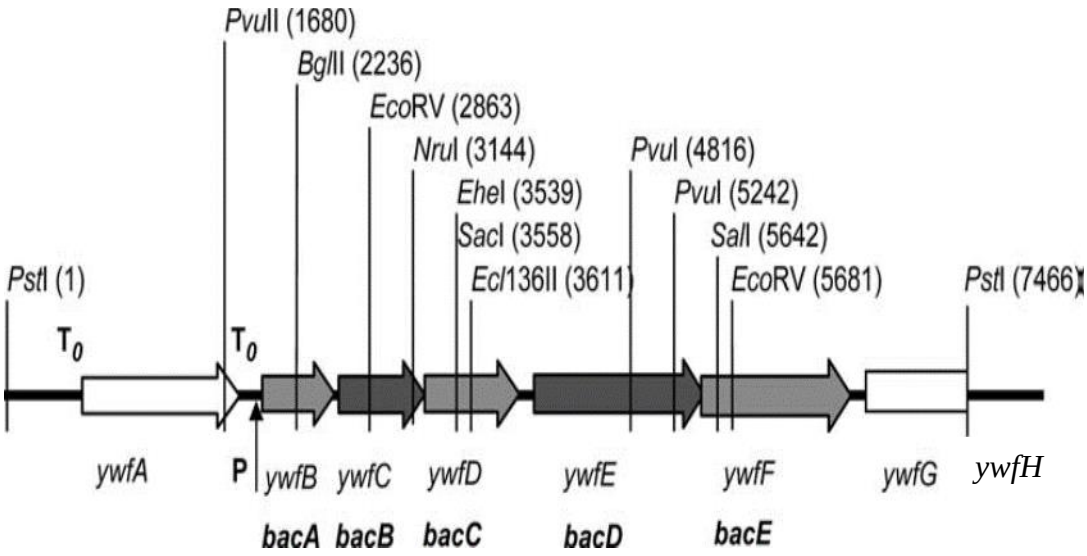


Figure 1.12: Bacilysin biosynthetic operon of *Bacillus subtilis 168* (Steinborn et al, 2005).

bacA, the first part of *bac operon,* is responsible for biosynthesis of dipeptide antibiotic bacilysin. *bacB*, carries out an isomerization process (Mahlstedt & Walsh 2010 and Rajavel et al, 2009). BacG, (*ywfH*), Phenylalanine aminotransferase forming tetrahydrotyrosine in bacilysin synthesis Fıg.13. *bacF*, bacilysin biosynthesis aminotransferase. BacC acts as oxidoreductase in bacilysin biosynthesis. BacD, an L.amino acid ligase (Lal), is responsible for dipeptide ligation acting by adding L-Ala to the amino group of anticapsin as a last step (Steinborn et al, 2005; Tabata et al, 2005; Shomura et al, 2012). Additionally, *bacD* and *bacE* genes were indicated to be responsible for amino acid ligation and self-protection to bacilysin in turn (Stein et al, 2005).

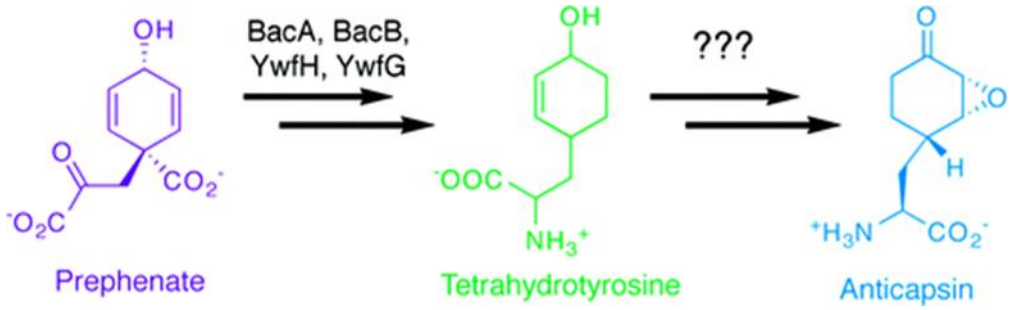


Figure 1.13: 4 enzyme (BacA, BacB, YwfH, and YwfG) pathway leading to the H (4) Tyr production and ultimately to L-anticapsin formation.

1.3.2.1.2 Regulation of bacilysin biosynthesis

Bacilysin biosynthesis is tightly related with growth media where the production of bacilysin can be repressed or inhibited by main nutritional components mostly by glucose and casamino acids (Özcengiz et al, 1990; Özcengiz and Alaeddinoğlu, 1991; Basalp et al, 1992).

On the other hand, bacilysin biosynthesis is positively regulated by quorum sensing regulatory pathway through ComQ/ComX, PhrC (CSF), ComP/ComA dependent way.

CodY, a global transcription factor, is a GTP-binding protein that has the ability to sense the intracellular GTP concentration and then regulate the transcription of early stationary phase and sporulation genes, thus regulating the expression of many genes in *B. subtilis* during transition from exponential phase to stationary phase and sporulation (Manoj et al, 2001). Transcription of *bac* operon was also regulated by the guanine nucleotides ppGpp and GTP (Inaoka et al, 2003). Additionally, ScoC, the transition state regulator jointly with CodY and AbrB regulates bacilysin production in *Bacillus subtilis* in a negative manner (Inaoka et al, 2009). The recent study of Köroğlu et al (2011) pointed out that the activation and induction of *bac* operon expression is under the firm regulation and balance of four proteins: ComA, Spo0A, AbrB and CodY. Both AbrB and CodY proteins negatively regulate the expression of *bac* operon by directly repressing its transcription while ComA and Spo0A act as positive transcriptional regulators: ComA activity is required for its basal level expression but both ComA and Spo0A activity is required for transition-state induction of *bac* operon.

On the other hand, DegU/DegS two component system has the capacity to control multi processes inside the cell such as motility exportease production, biofilm formation and genetic competence. In another study carried out by Mariappan et al, (2012) showed that DegU positively regulates the transcription of bacilysin biosynthetic operon in *Bacillus amyloliquefaciens FZB42* Fig. 1.14.

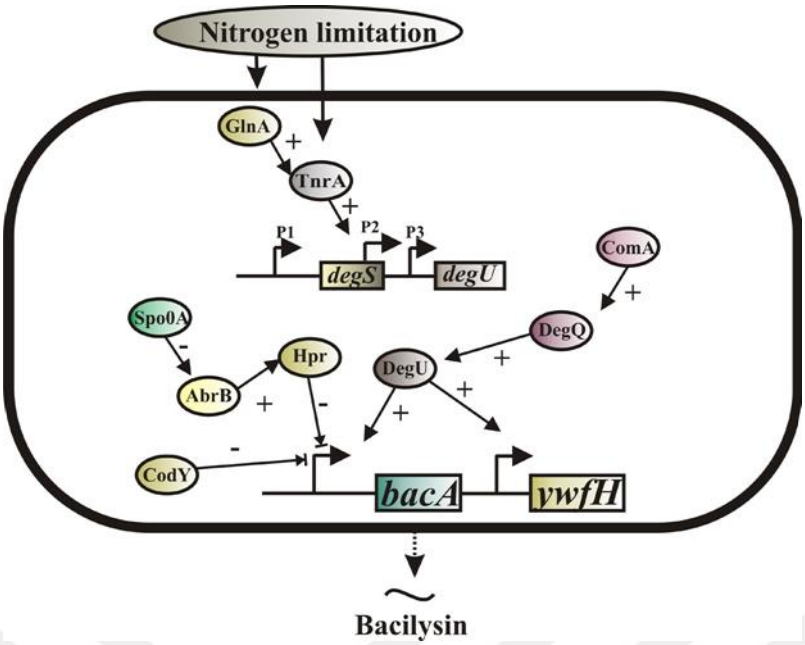


Figure1.14: Regulatory network of bacilysin production. Several global regulators are directly or indirectly regulating bacilysin. However, DegU and Hpr are directly acting on the *bacA* promoter. (Marriapan 2012).

1.4 The Purpose of Thesis

The present study aimed to investigate the regulatory effect of *comK* and *sinR* gene products on the bacilysin biosynthetic operon *bacABCDEywfG* and increasing further understanding into how they exert their effects via investigating interactions between ComK and SinR proteins within *bac* promoter region.

2. MATERIAL AND METHODS

2.1 Materials

2.1.1 Bacterial strains

Bacterial strains used within this study and their genotypes are listed in Table 2.1. *Bacillus subtilis PY79* was used as a wild type strain.

Figure 2.1 shows pGEM-T vector that was used for cloning of PCR products and Figure 2.2 shows pQE60 vector that was used for the expression of desired proteins.

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

Strains	Genotype	Source
B. subtilis PY79	wild type, BSP cured prototrophic derivative of *B. subtilis* 168	P.Youngman
OGU1	*bacA::lacz::erm*	İ.Öğülür M.Sc. Thesis
B. subtilis ComK	Δ *comK::kan*	ÁkosT.Kovács
OGU1CK	Δ *comK::kan bacA::lacz::erm*	This study
OGU1SRCK	Δ *comK::kan* Δ *sinR::spc bacA::lacz::erm*	This study
OGU1SR	Δ *sinR::cm bacA::lacz::erm*	Our laboratory
E. coli DH5α	F' Φd*lac*Z (*lac*ZY AargF), U169,*sup*E44λ-,*thi*-1,*gyr*A,*rec*A1, *rel*A1 *end*A1, *hsd*R17]	American Type Culture Collection; Hanahan (1983)
pHis$_6$-*comK*	ComK in *pQE60*	T. E. Köroğlu
pHis6-sinR	SinR in pQE60	Öykü İrigül

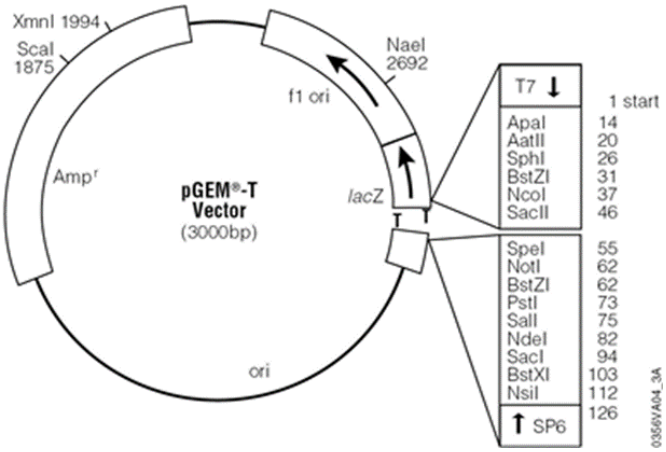


Figure 2.1: Genomic map of the pGEM-T vector.

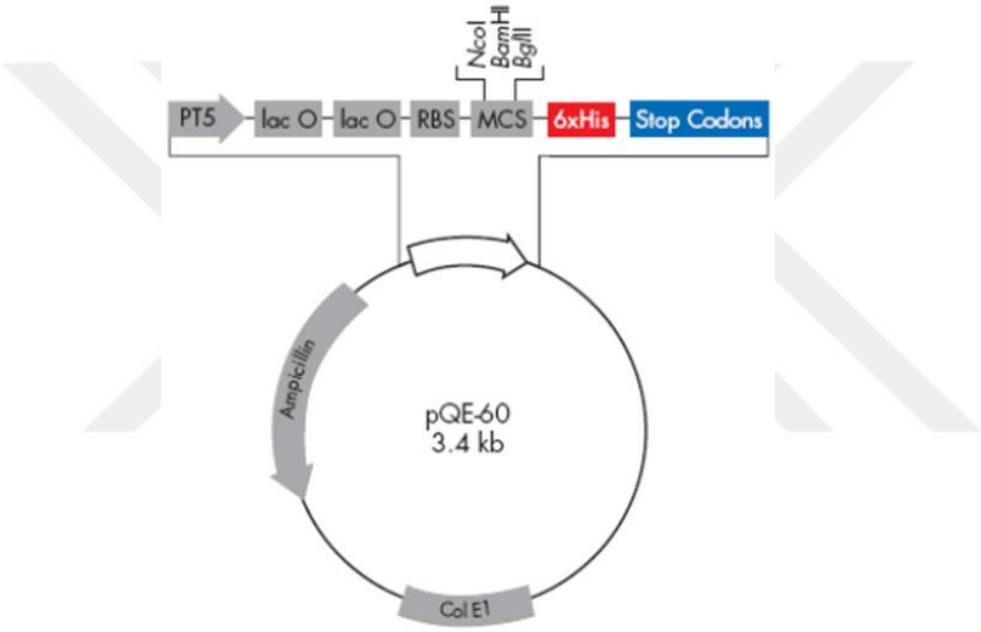


Figure 2.2: Genomic map of the pQE60 expression vector.

2.1.2 Bacterial culture media

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

2.1.3 Buffers and solutions

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

2.1.4 Chemicals and enzymes

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

2.1.5 Laboratory equipment

The laboratory equipment used during the study is listed in Appendix D.

2.1.6 Maintenance of bacterial strains

B. subtilis and *E. coli* were grown in Luria-Bertani (LB) liquid medium and were kept on Luria-Bertani (LB) agar plates at +4 °C. All strains were sub-cultured frequently. 10% LB glycerol stock was prepared for long time storage of strains at -80°C. Amp (100 µg/ml) was used for *E. coli DH5α* strain for selection with antibiotic.

2.2 Methods

2.2.1 DNA techniques and manipulation

2.2.1.1 Chromosomal DNA isolation

Chromosomal DNA of *B. subtilis* PY79 strain was isolated by using a standard procedure developed for Bacillus species (Cutting and Horn, 1990). 1,5 ml of overnight culture was pelleted by centrifugation at 13000 rpm for 5 min. The pellet was resuspended in 567 µl of TE (Appendix B) with frequent vortex. Followed by adding 10 µl of proteinase K (20mg/ml), 6 µl of RNase (10 mg/ml), 24 µl of lysozyme (100mg/ml) and 30 µl of 10% SDS. Next, the mixture was incubated for 1 hour at 37°C. After addition of 100 µl of 5M NaCl solution, the sample was mixed without vortex until the mucosal white substance turn out to be noticeable. Following, 80 µl of CTAB / NaCl (Appendix B) (prewarmed at 65°C) solution was added and the mixture was incubated for 10 min in 65°C water bath. 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. Afterward, the upper layer 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 and centrifuged at 13000 rpm for 5 min. Subsequently, the pellet was placed for dryness at 37°C for 1 hour and was dissolved in 10 µl of TE buffer and stored at 4°C. Finally, the isolated DNA was run on 1.0% agarose gel.

2.2.1.2 Polymerase chain reaction (PCR)

The sequences of primers used in PCR reactions are given in Table (2.3).

PCR components and amounts used are given in Table (2.2).

PCR was performed using *i*-Taq polymerase supplied by INTRON Biotechnology,

Inc. Reaction steps were as follows:

Initial denaturation	94 °C for 3	min	
Denaturation	94 °C for 1	min	for 5 cycles
Annealing	55 °C for 1	min	
Extension	72 °C for 1	min	
Denaturation,	94 °C for 1 min		for 25 cycles
Annealing,	60 °C for 1 min		
Extension,	72 °C for 1 min		
Final extension,	72 °C for 15		

Table 2.2: i-Taq PCR components and amounts.

Component	Amount (50 µL in total)
10X Buffer	5 µL
dNTP	5 µL
Template	1 µL
Reverse primer	1 µL
Forward primer	1 µL
i-Taq polymerase	0,5 µL
dH_2O	36,5 µL

Table 2.3: Oligonucleotide primer sequences.

Primer	Oligonucleotide sequences
bacA reverse	GCC GAG CGT GTT GAC TGT AAT
bacA forward	GGC CGT GAC ATT AGG TTC TGC
yufO reverse	CGC CTT GCG GAT ATT GAG CAT
yufO forward	CAG TCG TTG AAG ATG TAA CGA
ywbH reverse	CAG AGC GAT TTG AAT AAC GGT
ywbH forward	GCC GTT TGG TAA AAC AGTT C

2.2.1.3 Agarose gel electrophoresis

Electrophoresis was carried out on a horizontal submarine electrophoresis apparatus in a gel system comprised of ~1% agarose gel encompassing 1xTAE buffer (Appendix B). Ethidium bromide of a 0.2 μg/mL final concentration. 1X Loading dye was added into the samples. Electrophoresis was performed at 90-120 Volts for 30-35 minutes. The DNA bands were visualized on a UV transilluminator (UVP) and photographed by using Gel Imaging System. Gene Ruler DNA Ladder Mix marker (Appendix D) were used to determine the molecular weights of DNA bands for desired purposes.

2.2.2 Preparation of *B. subtilis* competent cells and transformation

Preparation of *B. subtilis* competent cells and their transformation process were implemented as illustrated by Klein et al (1992). The *B. subtilis* competent cells were prepared using a two-step procedure involving two types of minimal media, HS and LS (Appendix A) media were used. Suitable amount of *B. subtilis* cells were inoculated into 3 mL of HS medium and incubated overnight at 37°C with shaking at 250 rpm. 0,5 mL of this overnight culture was transformed into 20 mL of freshly prepared LS medium and incubated at 30°C with shaking at 200 rpm until the optical density (O.D) of the culture at 600 nm reached 0,55. Then, 1 mL of competent cells was transferred into 2 mL Eppendorf tube and 1 μL of DNA was added. Following, the cells were incubated at 37°C for 2 hours with shaking at 200 rpm and incubated cells were pellted by centrifugation at 5000 rpm for 10 minutes. Finally, the pellet was resuspended in 100 μL of sterile saline solution (%0,85 NaCl) and was spread out on LB agar plates containing selective antibiotics and incubated at 37°C for 16 h.

2.2.3 β-galactosidase assays

Bacillus subtilis cultures were grown overnight in PA medium at 37°C (Appendix A). Then, 150 mL of PA medium were inoculated with this overnight culture to an initial optical density (O.D) of about 0,1 at 595 nm (OD_{595}). After that, cultures were incubated at 37°C with shaking of 250 rpm for 30 h. 1mL of culture was taken as duplicates each hour for the later implementation of β-galactosidase assay.

For the measurement of growth at $O.D_{595}$, the necessary amount of cultures was taken as dilutions. 1 mL of culture taken as a sample each hour was centrifuged at 13000 rpm for 5 minutes and the supernatant was discarded. Subsequently, the pellet was washed with 500 μL of ice-cold 25 mM Tris-Cl (pH 7,4) and centrifuged. Following

the removal of washing buffer, the pellet was resuspended in 640 µL Z-buffer and 160 µL of lysozyme was added so as to lysis cell. Subsequent to the incubation at 37°C for 5 min, the samples were carried on ice and 8 µL of 10% Triton-X100 was added. Previous to incubation on ice, mixtures were vortexed for 2 seconds.

After the incubation, β-galactosidase assay was carried out by using o-nitrophenyl-β-d-galactopyranoside as a substrate and then initiated by prewarming the extracts to 30°C. Consequently, 200 µL of ONPG solution was added and the solution was observed for the development of yellow color. At the precise time that yellow color stabilized, the reaction was stopped by the addition of 100µL 1M Na_2CO3 and reaction time was recorded.

Reaction time refers to a period that was started by adding ONPG and continued until the steady of yellow color. Finally, the samples were centrifuged at 13000 rpm for 5 min and then transfer solution from the top of the tubes to cuvette for measuring the cell density of culture (O.D) at A_{420} and A_{550}. Calculation for β-galactosidase specific activity was carried out according to the formulation developed by Miller in (1972) as follow:

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

2.2.4 Protein synthesis

2.2.4.1 Overexpression of histidine-tagged proteins in *E. coli*

Pre-culture of *E. coli DH5α* carrying the desired protein sequences in pQE60 expression vector was grown in 50 mL medium overnight at 37 ^oC with gentle shaking and then was moved into 1 L LB broth medium containing Amp (100 µg/ml) as a selective antibiotic. Culture was incubated at 37 ^oC with shaking of 200 rpm until reached 0.6 of OD_{600}. Subsequently, 1 ml of bacterial sample was transferred to a microcentrifuge tube and was pelleted by centrifugation at 13000 rpm for 1 minute and was marked as an uninduced sample. IPTG was added into the growing cultures with a final concentration of 1 mM and incubated at 37 ^oC with shaking of 200 rpm for 5 hours. By the end of this time, 1 ml of induced cell sample was taken into a microcentrifuge tube and was pelleted by centrifugation at 13000 rpm for 1 minute

and was marked as an induced sample. Residual bacterial cell culture was harvested by centrifugation at 5000 rpm for 15 minutes. After removal of supernatant, pellet was resuspended with 2 ml Lysis buffer (Appendix B). Finally, pellet was stored at -80 ^{o}C.

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

Protein purification procedures were undertaken in + 4 ^{o}C. After the freeze-thaw of pellets, they were treated with lysozyme (1 mg/mL, 30 minutes, on ice) and then were disrupted by sonication at 50 watts for 7-10 minutes. Following, cell lysis was centrifuged at 10,000g for 15 minutes to remove insoluble materials and the supernatant was transferred to a fresh 1,5 mL Eppendorf tube. To immobilize the protein, 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. Top layer which contain alcohol was replaced by lysis buffer (Appendix B). The mixture (the supernatant and resin) was gently mixed for 45 minutes at + 4°C in a rotator. Subsequently, samples were centrifuged for 1 min at 1000 rcf to precipitate the resin. Resin pellets were washed three times with 1 ml 20 mM wash buffer and one time with 1 ml 50 mM wash buffer (Appendix B) for eliminating non-specific proteins. After washing steps, His6-proteins were eluted with 100 µl elution buffer (Appendix B) 1-2 times compared to imidazole concentration under native conditions. Purified proteins bands were examined by SDS-PAGE.

2.2.5 SDS-PAGE analysis

Histidine-tagged ComK and SinR proteins were purified by using Ni-NTA resin system. The extracted proteins were analyzed by means of SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis) technique which is commonly used for the separation of proteins in light of their molecular weight. SDS-PAGE separates proteins according to their differential rates of migration across a sieving matrix (a gel) under the influence of an applied electrical field which is determined by their molecular weight. 50 µl SDS-loading dye was added to samples and lysozyme treatment was applied for 20-30 min at + 4 ^{o}C. All samples and unstained protein marker were denatured at 99°C for 5 min. SDS-PAGE was carried on a 15% separating gel and a 4% stacking gel (Table 2.4) according to the protocol of Laemmli (1970).

Molecular weight of the proteins was determined by contrasting their profiles with unstained protein molecular weight marker (Fermentas) (Appendix D). Following denaturation, samples were loaded onto SDS-PAGE gel and run at 80V for 20-30 min until the samples pass the separating gel and soon after voltage was increased 120V for 2-3 hours. After that, polyacrylamide gels were stained with SDS-PAGE staining solution (Appendix B) at 37°C for 1 hour with a gentle shacking. Finally, gel was washed overnight with destain buffer (Appendix B), and then visualized.

Table 2.4: Preparation of SDS-gel.

15% Separating Gel	**for 5 mL**
dH_2O	1,15 mL
30% acrylamide	2,5 mL
1,5 mM Tris pH 8,8	1,25 mL
10% SDS	50 µl
TEMED	4 µl
10% APS	50 µl

4% Stacking Gel	**for 5 mL**
dH_2O	1,4 mL
30% acrylamide	330 µl
1 M Tris pH 6,8	250 µl
10% SDS	20 µl
TEMED	2 µl
10%APS	20 µl

2.2.6 Bradford assay

The Coomassie brilliant blue protein assay, universally known as the Bradford assay (Bradford, 1976), is the most widely used method that allow quick and straightforward protein quantification The Bradford Reagent was used to determine the concentration of proteins. The procedure is based on the formation of a complex between the Brilliant Blue G dye and proteins in solution. By using BSA (bovine serum albumin) as the standard protein at different concentrations 0.125, 0.25, 0.5, 0.75, 1, 1.5, 2 mg/ml respectively so as to obtain a correct standard curve. Proteins and standard solutions

were assayed in a microplate containing 96 wells. Bradford assay was performed using 5 µl of each standard solutions and proteins with 250 µl Bradford reagent. Color of this mixture was converted blue according to concentration of protein. Absorption spectrum was measured at 595 nm.

2.2.7 EMSA (Electrophoretic mobility shift assay)

For gel retardation assay, 382 bp length promoter region extends from -273 to 108 relative to the transcriptional start of *bacABCDE* operon (Inaoka et al., 2009), promoter region of *yufO* and promoter region of *ywbH* were amplified by PCR using *B. subtilis PY79* chromosomal DNA as a template with specific primers given in Appendix B. 50 mM KCl, 5 mM MgCl2, 0.1 mM EDTA, 1 mM DTT, 8% glycerol in 10mM Tris-HCl, pH 8 (Köroğlu *et al.*, 2008) (Appendix B); reaction buffer supplemented with competitor DNA poly [d(I-C)] (1µg/ml), BSA (0,1 µg/ml) and 45 nanograms of promoter DNA was used for gel retardation of ComK and SinR, 10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1 mM EDTA, 1 mM (DTT) dithiothreitol with 5% (v/v) glycerol (Appendix B) was used as a binding buffer. 25 µl total reaction mixtures were incubated at 37 °C for 10 minutes after that at 4 °C for 15 minutes. Following, 5µl of loading dye buffer [0,25xTGE, 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, samples were loaded into a non-denaturing 6 % polyacrylamide gel (Table 2.5) and an electrophoresis was run in 43mM imidazole and 35mM Hepes pH 7.4 buffer at 80 V for 75 mins at 4°C within an ice box. Following electrophoresis, gel was incubated at 37°C on the orbital shaker with low rpm for 20 minutes with SYBR® Green I Nucleic Acid Gel Stain (10,000X concentrate in DMSO) (Lonza) and were visualized with the Gel Doc XR^+ System (BioRad).

Table 2.5: 6% Native EMSA gel.

dH_2O	6.4 ml
40 % Acrylamide	1.5 ml
5x Hepes-imidazole buffer	2 ml
10 % APS	0.1ml
TEMED	8µl

3. RESULTS

3.1 Expression of *bac* operon

3.1.1 Strains construction

With the aim of observing the *bac operon* expression, *B. subtilis OGU1* strain containing a transcriptional P*bac lacZ* fusion at the *bacA* locus was used as a control. This strain was constructed previously in our lab by a transcriptional fusion between the *bac* promoter (P*bac*) and the *lacZ* gene in pMutinT3 vector.

In this study, to examine the possible effect of the key transcriptional regulator of the competence development in *Bacillus subtilis* ComK and the master regulator SinR. To investigate potential effects of ComK on the *bac* operon, the *comK* gene was disrupted in OGU1 strain. For this, chromosomal DNA of *B. subtilis comK::kan* mutant strain was used to transform competent cells of *B. subtilis* OGU1 (*bacA::lacZ::erm*). This transformation resulted in a strain which contain *comK*-disrupted mutation (Δ*comK::kan bacA::lacZ::erm)* and this mutant was designated as OGU1CK. Additionally, previously constructed *sinR*-disrupted strain OGU1SR (Δ*sinR::spc bacA::lacz::erm)* was used.

For investigating the role that *comK* and *sinR* play together in regulation of the *bac* operon expression, a *ΔcomK-ΔsinR* double mutant strain (Δ *comK::kan* Δ *sinR::spc bacA::lacz::erm*) was also constructed. to produce *comK-sinR* double mutant, competent cells of OGU1SR (Δ*sinR::spc bacA::lacz::erm)* strain was transformed with the chromosomal DNA of c*omK*-disrupted mutant and designated as OGU1SRCK

3.1.2 β-galactosidase assay

All Strains, OGU1 and its derivatives *comK*-mutant (OGU1CK), *sinR*-mutant (OGU1SK) and *comK-sinR* double-mutant (OGU1SRCK) strains were cultured in PA medium at 37°C for 30 hours and the β-galactosidase activities of the cells were measured at intervals in different stages of growth (Fig.3.1).

OGU1 strain was used as a control to compare the newly constructed mutants. As a result, β-galactosidase activities related to *bac* promoter activity of the *Bacillus subtilis* strain containing a transcriptional fusion P*bac-lacZ* termed OGU1 confirmed that the expression of *bac* operon was persistent during the exponential growth phase, however, it amplified in the course of transition from exponential to stationary phase and get to its top level at the onset of the stationary phase (Fig .3.1).

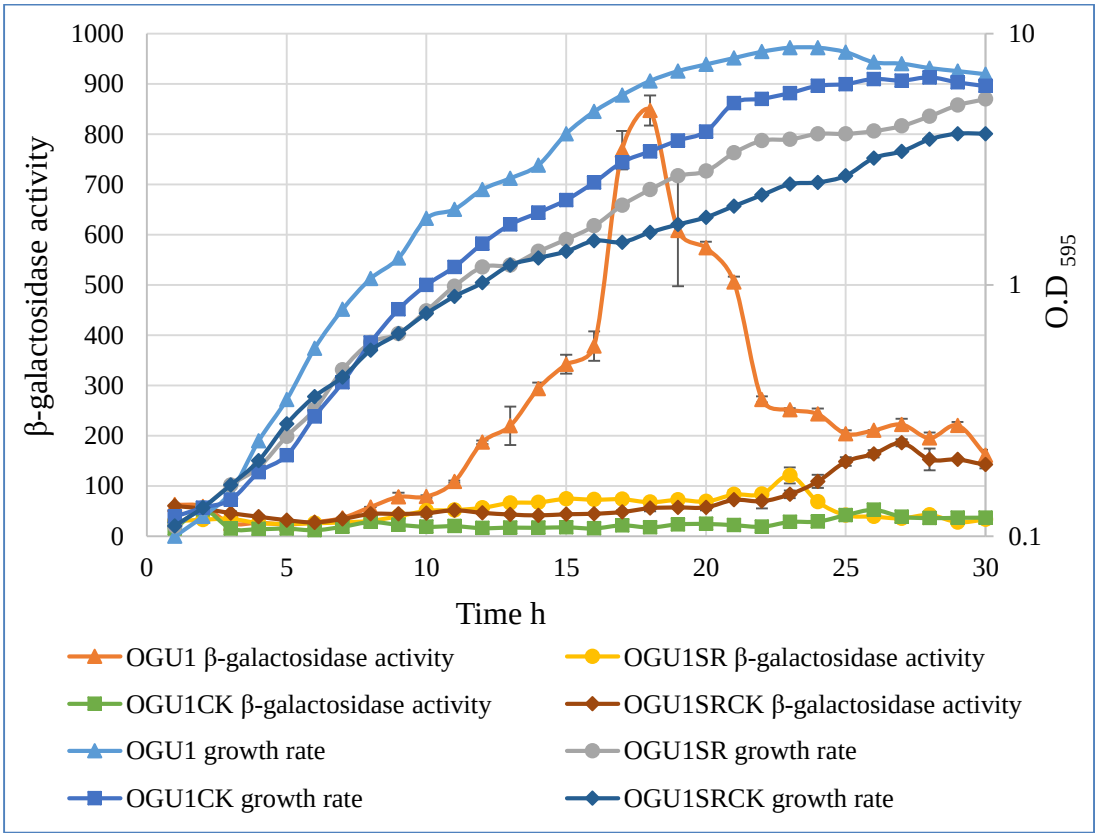


Figure 3.1: Growth and β-galactosidase activity of *bacA::lacz* fusion OGU1 and its derivatives OGU1CK mutant, OGU1SR mutant and OGU1SRCK double mutant. Error bars indicate the standard deviation.

3.1.3 Effect of *comK* null mutation on the expression of *bac* operon in *Bacillus subtilis*

As shown in Fig. 3.2, contrarily to OGU1, the deletion of *comK* gene severely affected the expression of *bac* operon despite that the growth of this mutant was approximately constant and somehow closer to that of OGU1 during the growth stages and a basal level of *bac* operon expression was observed during growth phases but no induction of *bac* expression was observed at the onset of stationary phase in this mutant.

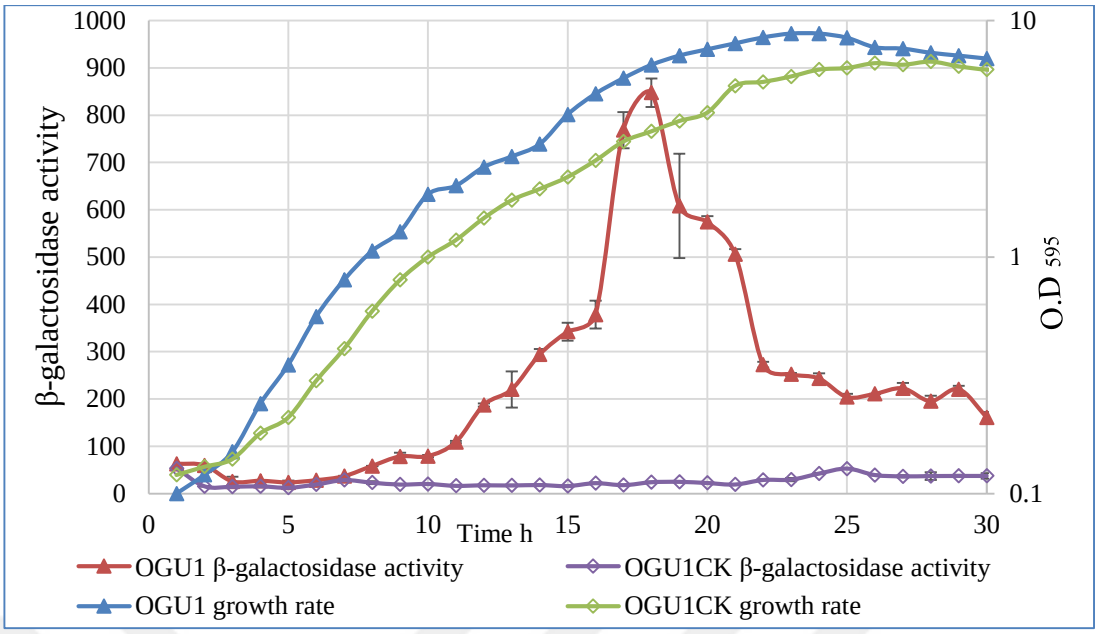


Figure 3.2: Cell growth and β-galactosidase activity of OGU1CK strain compared to OGU1 strain. Error bars indicate the standard deviation.

3.1.4 Effect of *sinR* null mutation on the expression of *bac operon* in *Bacillus subtilis*

As shown in Fig.3.3, the *sinR* mutation dramatically influenced the *bac* operon expression. *bac* expression of the *sinR*-disrupted OGU1SR strain was remain at a basal level as in the case of *comK*-disrupted OGU1CK strain. Additionally, growth profile of OGU1SR was dramatically affected. Its transition phase was extended compare to OGU1 strain.

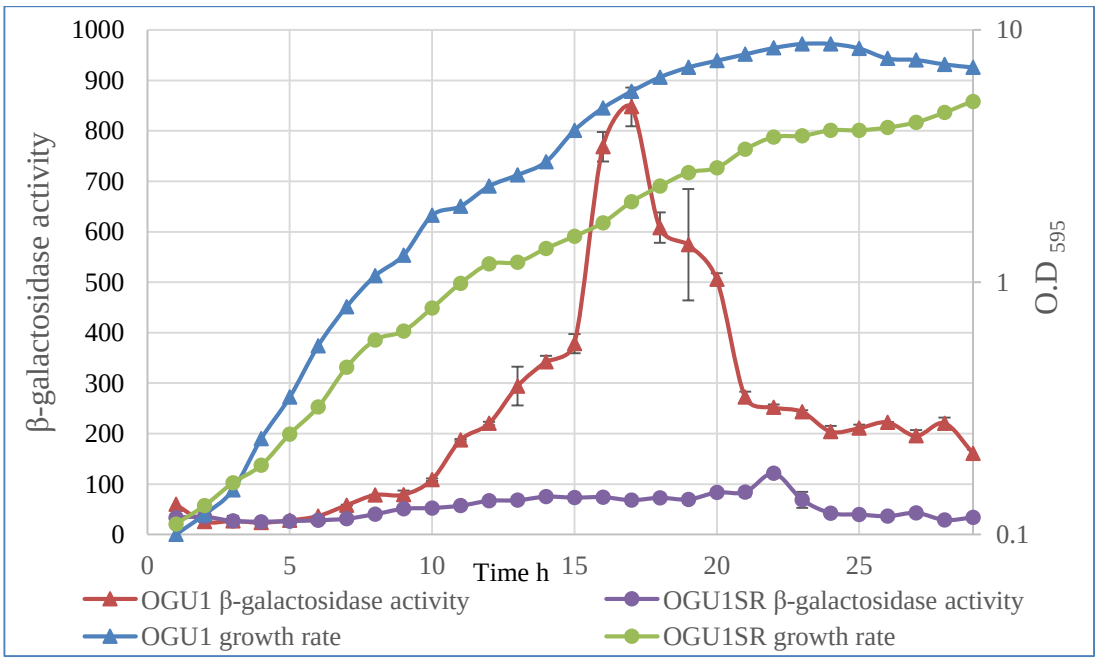


Figure 3.3: Cell growth and β-galactosidase activity of OGU1SR strain compared to OGU1 strain. Error bars indicate the standard deviation.

3.1.5 Effect of *comK- sinR* double mutation on the expression of *bac* operon in *Bacillus subtilis*

As shown in Fig. 3.4 and Fig. 3.5, *bacA-lacZ* expression was severely impaired in the *comK- sinR* double mutant strain compared to OGU1. *bac* operon expression in OGUSRCK strain was kept at same basal level with the single SinR mutant OGU1SR strain until reached to stationary phase but *bac* operon expression slightly increased and reached to basal level of parental strain OGU1 within stationary phase.

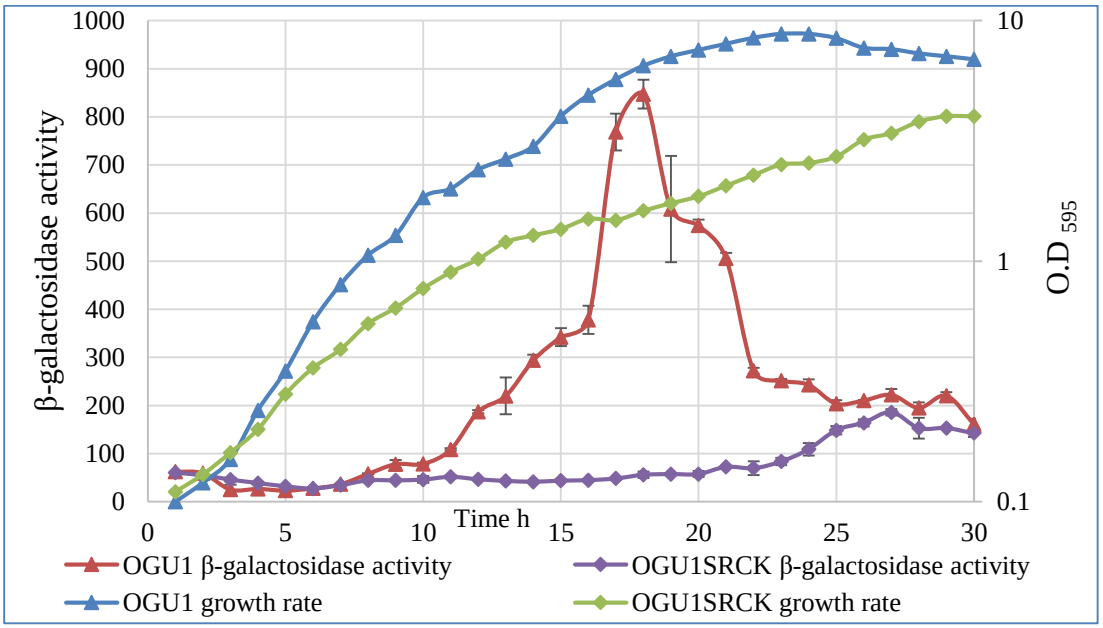


Figure 3.4: Cell growth and β-galactosidase activity of OGU1SRCK strain compared to OGU1 strain. Error bars indicate the standard deviation.

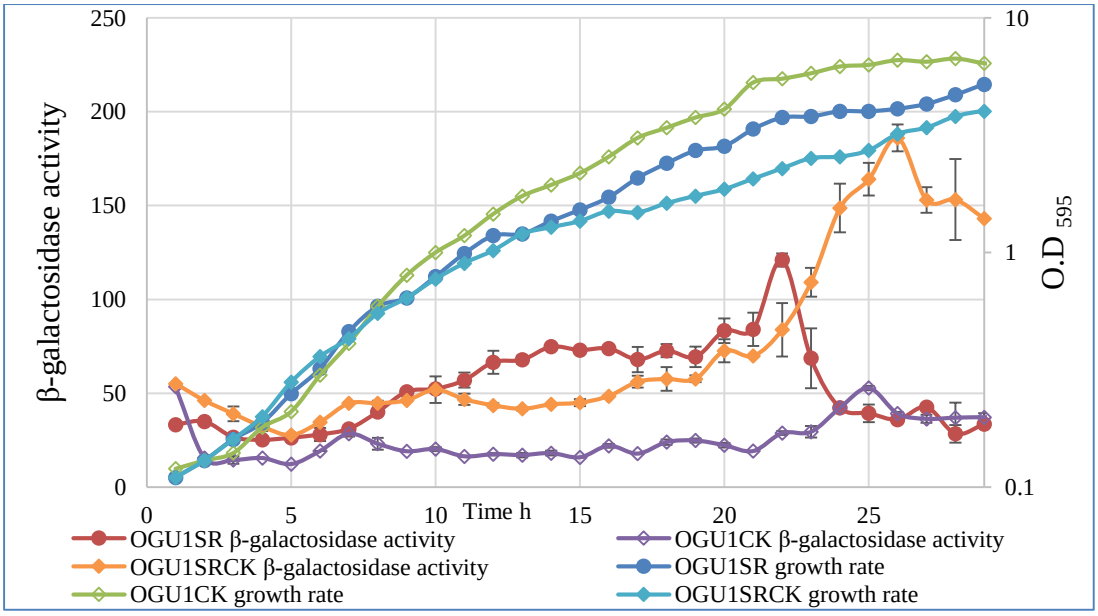


Figure 3.5 : Cell growth and β-galactosidase activity of OGU1CK mutant, OGU1SR mutant and OGU1SRCK double mutated strain. Error bars indicate the standard deviation.

3.2 EMSA (Electrophoretic Mobility Shift Assay) Experiments

3.2.1 Overexpression of 6xHis-tagged ComK and SinR Proteins in *E. coli*

Overnight culture of recombinant *E. coli* strain carrying the pQE-SinR expression vector grown in 50 mL of LB medium were moved into 1 L of LB medium containing 1 mL ampicillin (100 µg/ml). At the beginning of the exponential phase (OD_{600} 0,6) these cultures were induced with 1mM IPTG to overexpress proteins. After 5 hours' incubation period, cultures were divided into 3 Beckmann centrifuge tubes and were pelleted by centrifugation at 4000 x g for 15minutes. Pellets were resuspended in 1 ml lysis buffer and stored at -80 ºC.

3.2.2 Purification of ComK-His_6 and SinR-His_6 proteins

Purification of overexpressed proteins were performed at 4°C by using Ni-NTA Resin via the batch method based on centrifugation including collect flow-through, wash and elution fractions. Cells were lysed by lysozyme followed by sonication. The cell extracts were added to the centrifuge tubes and Ni-NTA Resin was added to each tube. Tubes were Centrifuged for 15 minute at maximum rpm and mixed slowly for 45 minutes by using rotary mixer followed by centrifugation, wash and elution. The histidine-tagged proteins were eluted in contrast to increased imidazole concentration under native conditions. The eluted proteins were analyzed by SDS-PAGE (Fig 3.6 and Fig.3.7).

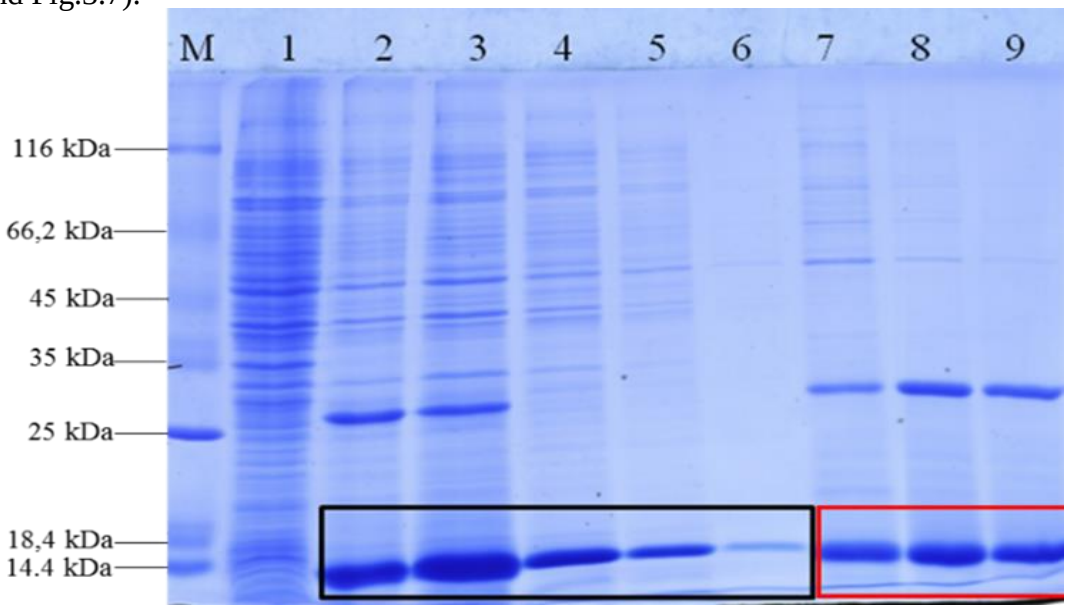


Figure 3.6: Purified SinR (12.989 KDa) protein. Washing steps (line 2,3,4,5,6), elution of the purified SinR proteins (lane 7-8-9), M; Protein molecular mass marker (Fermentas) (Appendix).

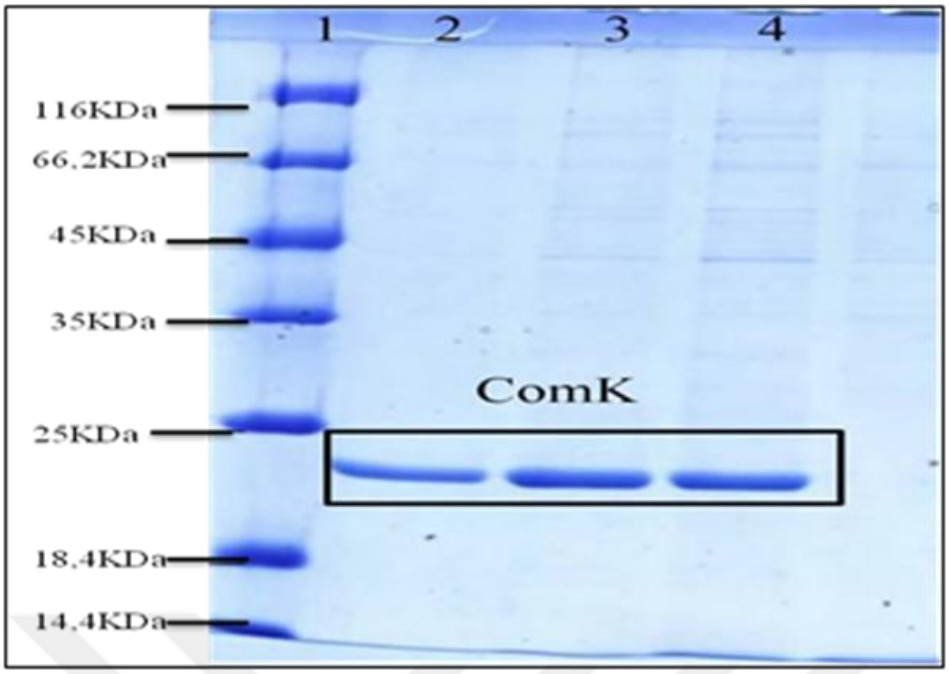


Figure 3.7: Purified ComK (22.3KDa) protein. 1; Protein molecular mass marker (Fermentas), elution of the purified ComK proteins (lane 2-4).

3.2.3 Binding of ComK and SinR to the *bac ABCDE* promoter

Based on the effect of *comK* and *sinR* mutation on *bacA* expression, it was required to check if the *bac* promoter is the direct target of these transcriptional regulators. For this, gel retardation assays EMSAs were carried out where the promoter regions of *bacA* was amplified by PCR using chromosomal DNA of *B. subtilis* PY79 as a template with specific primers (Fig.3.8 and Fig.3.9). The amplified *bacA* promoter region, 382 bp in length harboring the regions between -273 and +108 relative to the transcriptional start site (Inaoka et al, 2009) was incubated with different concentrations of the purified proteins, His6-tagged-ComK and His6-tagged-SinR.

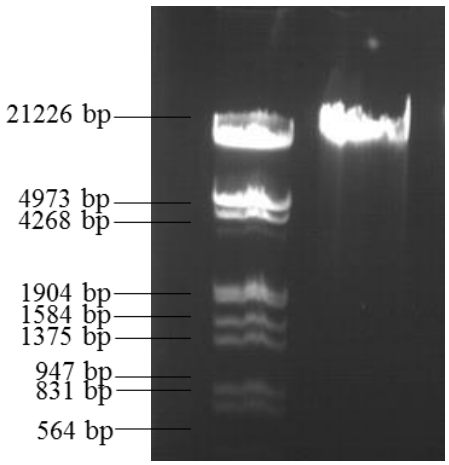


Figure 3.8 : Chromosomal DNA isolated from Bacillus subtilis PY79 (line 1) and M; Lambda DNA/EcoRI+HindIII Marker (Fermentas).

The promoter regions of *yufO* and *ywbH* were used as negative controls for ComK and SinR respectively. Fig.3.10 and Fig.3.11. As shown in Figure 3.12, the mobility of the *bac* promoter fragment was shifted apparently upon incubation with the increased concentrations of ComK-His6.

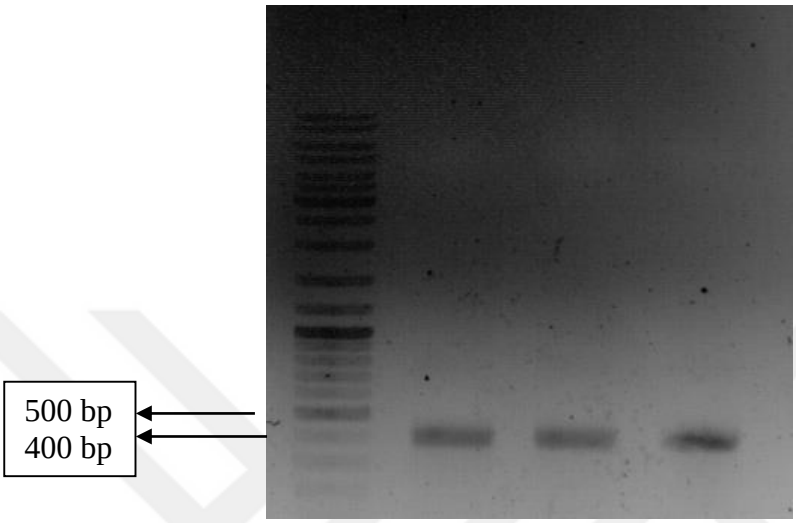


Figure 3.9 : *bacA* DNA amplified by PCR using Bacillus subtilis PY79 Chromosomal DNA as template M:marker, lanes 1,2,3, *bacA* DNA.

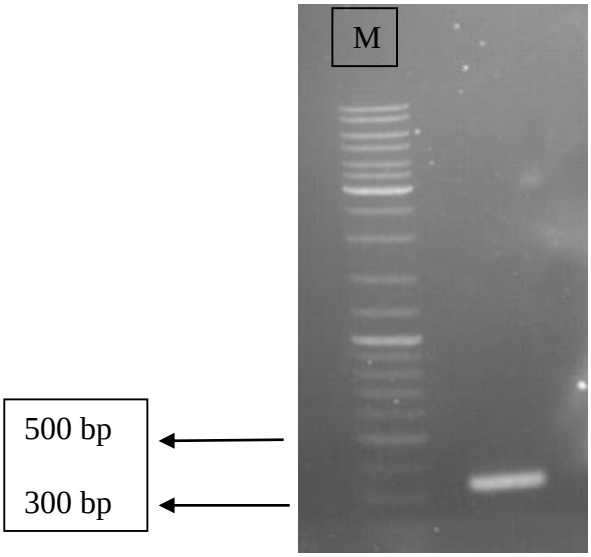


Figure 3.10 : *yufO* DNA amplified by PCR using *yufO* F and *yufO* R primers. M: marker, lane1: *yufO* DNA.

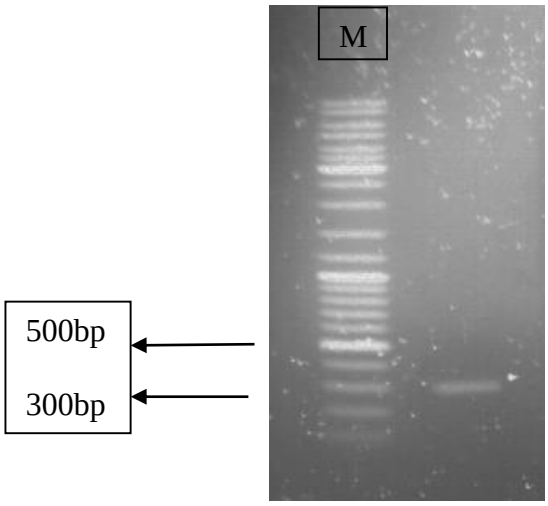
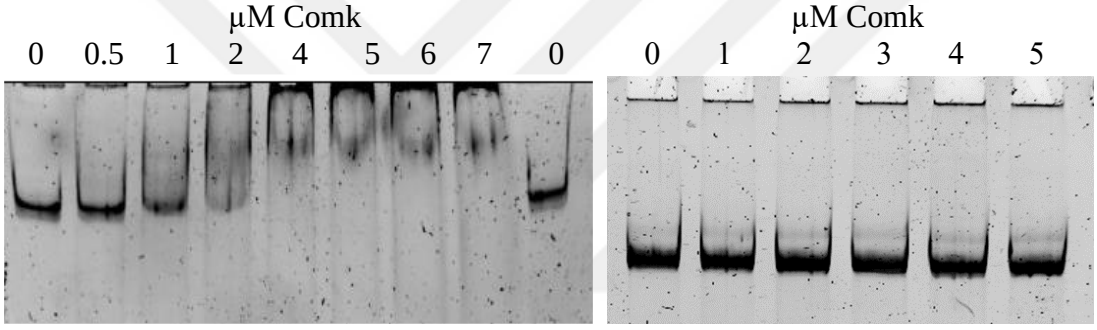


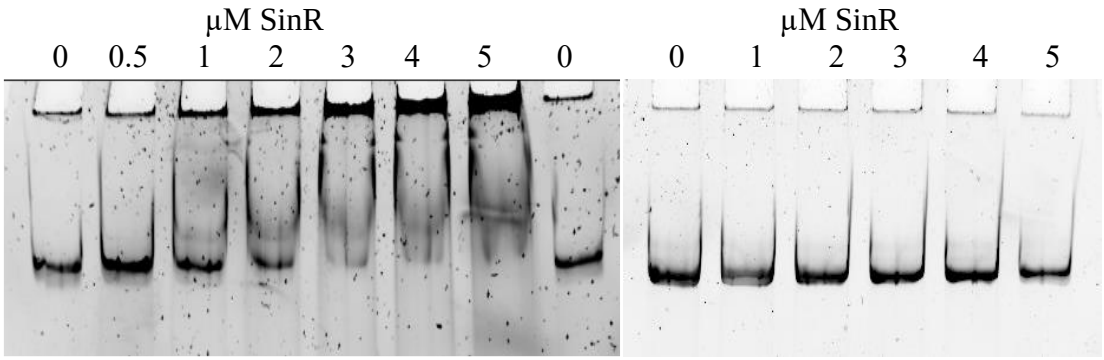
Figure 3.11 : *ywbH* DNA amplified by PCR using *ywbH* F and *ywbH* R primers. M: marker, lane1*ywbH* DNA.



(A) P*bac* **(B)** P*yufO* negative control

Figure 3.12 : (A) Gel mobility shift assay performed using *bacA* promoter fragments incubated with purified ComK-His_6 at the indicated concentrations. **(B)** Gel mobility shift assay performed using purified ComK-His_6 incubated with *yufO* DNA represent the negative control at the indicated concentrations.

Similarly, the shifted bands were observed when incubating the *bac* promoter with increasing concentrations of SinR-His_6 protein (Fig. 3.13). The level of retardation of the *bac* promoter fragment was proportional to the concentration of these proteins. These results indicate that ComK and SinR exert their effect directly by binding to P*bac.* The observed shifts were specific to ComK and SinR since these proteins caused no retardation in electrophoretic mobility with their negative controls DNA fragments (Fig. 3.12. B and Fig 3.13). B respectively in addition to the presence of nonspecific competitor DNA poly [d(I-C)].



(A) P*bac* (**B)** P*ywbH* negative control.

Figure 3.13 : (A) Gel mobility shift assay performed using *bacA* promoter fragments incubated with purified SinR-His_6 at the indicated concentrations. **(B)** Gel shift for control: purified SinR-His_6 incubated at the indicated concentrations with *ywbH* DNA represent the negative control.

3.2.4 Investigation of regulatory interaction between SinR and ComK by EMSA

To analyze whether ComK and SinR bind to P*bac* simultaneously or compete for binding, the gel retardation assays were repeated in the presence of both proteins in which the fixed amount of one of them was mixed with different concentrations of the other. As shown in Figure.3.14, purified ComK and SinR were mixed jointly with 382-bp DNA fragment, when the addition of increasing amounts of ComK with a fixed amount of SinR, no additional retardation in electrophoretic mobility of ComK-bound P*bac* fragments was observed with the increased ComK concentrations.

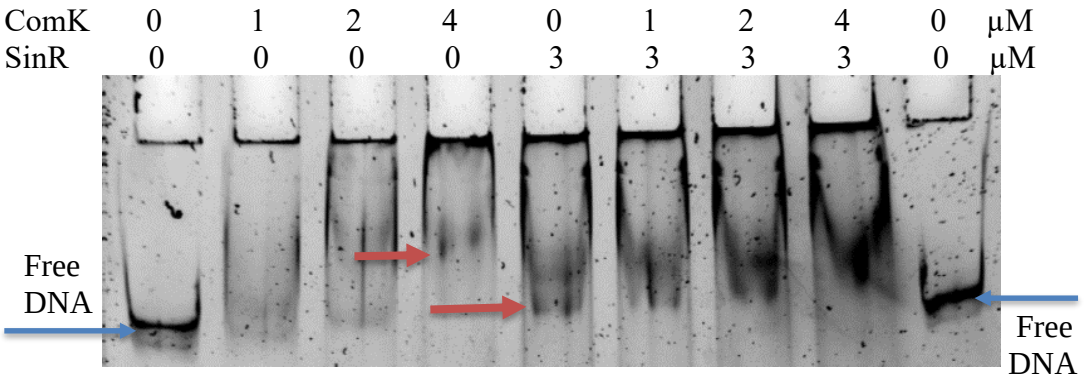


Figure 3.14: Gel mobility shift assay performed using *bacA* promoter fragments incubated with increasing concentration of ComK in the presence of 3 µM of SinR. The reaction contains 1ng/µl of *Pbac* promoter DNA,1µg/µl poly[d(I-C)]. The leftmost and rightmost lanes represent the position of unshifted DNA in the gel.

Consistently, the addition of increasing amounts of SinR in the presence of a fixed amount of ComK caused the displacement of ComK bands in the EMSA gel (Fig. 3.15). Conversely, by increasing amounts of these proteins leads to hinder retardation and the bands were displaced by the increasing concentration of proteins indicating that both ComK and SinR compete for binding to the P*bac* and they can dislocate each other from *bac* promoter in a concentration dependent way.

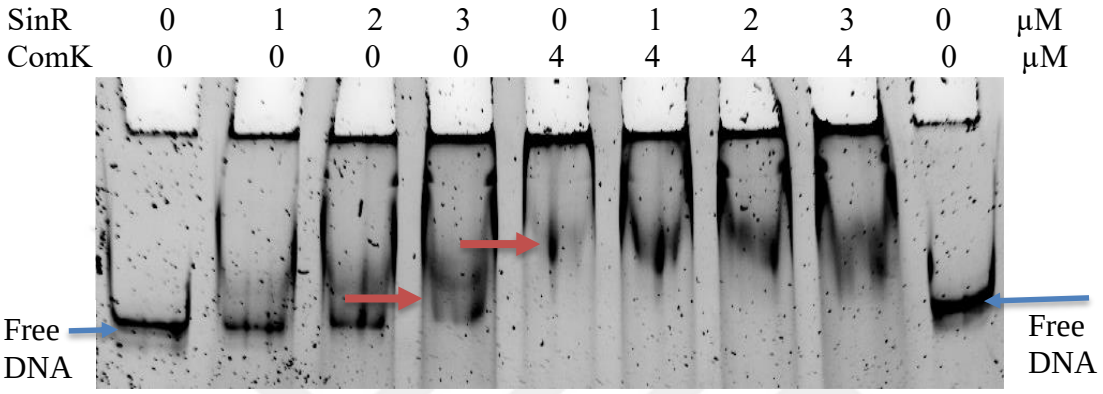


Figure 3.15: Gel mobility shift assay performed using *bacA* promoter fragments incubated with increasing concentration of SinR in the presence of 4µM of ComK. The reaction contains1ng/µl of P*bac* promoter DNA,1µg/µl poly[d(I-C)]. The leftmost and rightmost lanes represent the position of unshifted DNA in the gel.

DISCUSSION

In *Bacillus subtilis* several physiological processes such as genetic competence development, sporulation, degradative enzyme production, biofilm formation and antibiotic synthesis are organized by quorum sensing through the ComX-ComP-ComA signaling circuit as a main quorum sensing pathway (Auchtung et al,2005; Griffith & Grossman, 2008; Natalia & Grossman, 2005). Bacilysin biosynthesis is encompassed by *bacABCDEywfG* operon and the *ywfH* gene (Steinborn et al, 2005; Inaoka et al, 2003). The bacilysin operon is ruled by the quorum sensing regulator ComA and negatively controlled by AbrB, the transition state regulator (Yazgan et al, 2001). ppGpp (Guanosine 5′-diphosphate 3′-diphosphate) also has a crucial role in the transcription of bacilysin biosynthetic operon in a GTP-dependent manner (Inaoka et al, 2003).

Yazgan et al, (2003) demonstrated that, the bacilysin biosynthesis is regulated by quorum sensing through ComQ/ComX, PhrC (CSF) ComP/ComA pathway, the activation and induction of *bacABCDEtwfG* expression is under the firm regulation and balance of four proteins: ComA, Spo0A, AbrB and CodY. Both AbrB and CodY proteins negatively regulate *bacABCDEywfG* in the transcriptional level and have the ability to bind concurrently to the *bac* operon promoter (P*bac*), whereas ComA and Spo0A act as positive transcriptional regulators (Köroğlu et al 2011).

Expression of ComK is under multiple controls of several proteins both negatively and positively such as DegU, SinR, CodY, AbrB, Rok and ComK itself in addition to quorum sensing. ComK and DegU act positively by binding directly to P*comK* (comK promoter) forming a positive feedback loop (Sinderen & Venema, 1994) where DegU binding to P*comK* rises the attraction between ComK and its promoter (Hamoen et al, 2000). However, AbrB and CodY repress ComK expression by binding directly to P*comK* (Hoa et al, 2002; Serror & Sonenshein, 1996).

Whithin competence development, AbrB functions in a positive manner on *comK* transcription (Hahn et al, 1996). The stability of ComK is adjusted by ClpCP protease which acts by degrading ComK prior to stationary phase and when avoiding

competence state. There is no direct contact between ComK and ClpCP, however, the connection is mediated by MecA protein which acts as an adaptor connecting ComK and ClpCP to its N-terminal and C-terminal domains respectively (Turgay et al, 1998; Persuh et al, 1999). ComS, a small protein formed via the series of phosphorylation reactions started by quorum sensing (Lazazzera et al, 1999) releases ComK by binding to the N-terminal domain of MecA thus protects it from degradation. YlbE plays a positive role in the expression of ComK by increasing the stability of ComS (Tortosa et al, 2000).

The gene encoding competence transcription factor, *comK*, is negatively regulated by Rok (Ykuw) protein as transcription of *comK* is inhibited by Rok when it is found in extra amount without affecting its stability which is regulated by MecA. Rok exerts its effect on *comK* by binding directly to DNA portion containing *comK* promoter (P*comK*). However, *roK* transcription is negatively regulated by SinR and AbrB proteins where they act as repressor for its transcription (Hoa et al, 2002).

SinR is a pleiotropic DNA binding protein that is essential for the late-growth processes of competence and motility in *Bacillus subtilis* and is also acting as repressor in subtilisin synthesis and inhibitor in sporulation (Mandic-Mulec et al, 1995). Moreover, SinR is the master regulator that governs the Gram-positive model organism *Bacillus subtilis* life style whether to be a free-living, planktonic lifestyle or a biofilm-forming. The activity of SinR is regulated by its antagonists, SinI, SlrA, and SlrR (Newman et al, 2013). When abundant SinI is existent, an SinR-SinI complex is created that inhibits the SinR-DNA interaction, triggering derepression (Lewis et al, 1996.;1998)

On the light of these findings it was rational to investigate the potential effect of ComK and SinR on the expression of bacilysin biosynthetic operon. To do so, by using insertional inactivation, *comK*, *sinR* genes and *comK* gene together with *sinR* were disrupted in *bacA::lacZ* fusion strain OGU1 generating new mutants designated as OGU1CK, OGU1SRCK in addition to *sinR*-disrupted strain OGU1SR constructed previously in our laboratory.

Based on the results of this study, the expression of *bac* operon was persistent within the exponential growth phase in the control strain OGU1, however, it amplified in the

course of transition from exponential to stationary phases and get to its top level at the onset of the stationary phase

Contrarily, the expression of *bac* operon in *comK*-disrupted strain was severely effected. A basal level of expression was observed but there was no induction of *bac* expression during transition phase in this mutant. In the same way, the expression of *bac* operon in the *sinR* mutation severely decreased with remaining a basal level of expression. However, a very slight induction of *bac* operon expression was detected at the beginning of stationary phase. Likewise, the *bac* expression of *comK-sinR* double-mutated strain remained at a basal level during the exponential and transition phases, however, it reached its peak in the stationary phase in a level higher than both *comK* and *sinR* mutants and was shifted to the end of stationary phase. Indicating that ComK and SinR activity is crucial for *bac* operon expression.

EMSAs with the 382-bp DNA fragment harboring the regions between -273 and +108 relative to the transcriptional start site (Inaoka et al, 2009) together with various concentrations of the purified His_6-tagged ComK and were performed. The promoter regions of *yufO* and *ywbH* were used as a negative control for ComK and SinR respectively.

The mobility of the *bac* promoter fragment was shifted apparently upon incubation with the increased concentrations of ComK-His_6. Similarly, the shifted bands were observed when incubating the *bac* promoter with increasing concentrations of SinR-His_6 protein. The degree of retardation of the *bac* promoter fragment was proportional to the concentration of these proteins. These results indicated that ComK and SinR exert their effect by binding directly to P*bac*.

To determine whether ComK and SinR bind to P*bac* simultaneously or compete for binding, the gel retardation assays were repeated in the presence of both proteins in which the fixed amount of one of them was mixed with various concentrations of the other one. As shown in Fig.3.14., purified ComK and SinR were mixed jointly with 382-bp DNA fragment, when supplement of increasing amounts of purified protein ComK with a fixed amount of SinR no additional retardation in electrophoretic mobility of ComK-bound P*bac* fragments was observed. Consistently, the addition of increasing amounts of SinR with a fixed amount of ComK caused the generation of one shifted band in the EMSA gel (Fig.3.15). As a result, the increasing amounts of

SinR proteins leads to hinder retardation and generate a single shifted band. Since this band was displaced by the increasing concentration of proteins indicating that both ComK and SinR compete for binding to the P*bac*.

CONCLUSION

In this study, with the aim of elucidating the effects of *comK* and *sinR* null mutations on the expression of *bac* operon, *comK* and *sinR* genes were disrupted in the *bacA::lacZ* fusion mutant OGU1 and *bac* operon expression within the constructed mutants [OGU1CK (*comK::kan bacA::lacZ::erm*), OGU1SR (*sinR::spc bacA::lacZ::erm*), OGU1SRCK (*comK::kan, sinR::spc bacA::lacZ::erm*)] were analyzed through β- galactosidase assays.

The results of β- galactosidase assays showed that the expression of *bac* operon of OGU1strain increased during transition phase and reached to maximal level upon entry into stationary phase. The deletion of *comK* and *sinR* genes seriously affected the expression of *bac* operon and a basal level of *bac* operon expression was observed during growth phases but no induction of expression was observed at the transition phase in *comK*-disrupted strain. However, in *sinR*-disrupted strain a very slight induction of *bac* operon expression was detected during the stationary phase but it showed a lvery ow level peak within this phase. Likewise, the *bac* operon expression of *comK-sinR* double-mutated strain remained at a basal level until stationary phase however, it slightly increased and reached to basal level of parental strain OGU1 observed within stationary phase.

These results revealed that *comK* and *sinR* genes are essential for *bac* operon expression.

EMSA analysis performed in this study revealed that transcriptional key factor ComK and the master transcriptional regulator SinR exert their effect by binding directly to the promoter region of *bacA* in a competitive manner.

To sum up, the results revealed that, in *Bacillus subtilis, comK* and *sinR* gene products affect the expression of *bac* operon in a positive manner by binding to the *bac* operon promoter in a competitive manner.

REFERENCES

Abraham, E. P., Callow, D., & Gilliver, K. (1946). Adaptation of Staphylococcus aureus to growth in the presence of certain antibiotics. *Nature*, *158*(4023), 818-21.

Akamatsu, T., & Taguchi, H. (2001). Incorporation of the whole chromosomal DNA in protoplast lysates into competent cells of *Bacillus subtilis*. *Bioscience, biotechnology, and biochemistry*, *65*(4), 823-829.

Alkhatib, Z., Abts, A., Mavaro, A., Schmitt, L., & Smits, S. H. (2012). Lantibiotics: how do producers become self-protected?. *Journal of biotechnology*, *159*(3), 145-154.

Andersson, A., Rönner, U., & Granum, P. E. (1995). What problems does the food industry have with the spore-forming pathogens Bacillus cereus and Clostridium perfringens?. *International journal of food microbiology*, *28*(2), 145-155.

Ando, Y., Asari, S., Suzuma, S., Yamane, K., & Nakamura, K. (2002). Expression of a small RNA, BS203 RNA, from the yocI-yocJ intergenic region of Bacillus subtilis genome. *FEMS Microbiol Lett*. 22;207(1):29-33.

Antunes, L. C., Schaefer, A. L., Ferreira, R. B., Qin, N., Stevens, A. M., Ruby, E. G. & Greenberg, E. P. (2007). Transcriptome analysis of the *Vibrio fischeri* LuxR–LuxI regulon. *Journal of Bacteriology* 189, 8387–8391.

Aranha C, Gupta S, & Reddy KVR (2004). Contraceptive efficacy of antimicrobial peptide Nisin: in vitro and in vivo studies. *Contraception* 69:333–338.

Arrebola, E., Jacobs, R., & Korsten, L. (2010). Iturin A is the principal inhibitor in the biocontrol activity of Bacillus amyloliquefaciens PPCB004 against postharvest fungal pathogens. *Journal of Applied Microbiology*, *108*(2), 386-395.

Babasaki, K., Takao, T., Shimonishi, Y., & Kurahashi, K. (1985). Subtilosin A, a new antibiotic peptide produced by Bacillus subtilis 168: isolation, structural analysis, and biogenesis. *Journal of biochemistry*, *98*(3), 585-603.

Bais, H. P., Fall, R., & Vivanco, J. M. (2004). Biocontrol of Bacillus subtilis against infection of Arabidopsis roots by Pseudomonas syringae is facilitated by biofilm formation and surfactin production. *Plant physiology*, *134*(1), 307-319.

Barbieri, G., Voigt, B., Albrecht, D., Hecker, M., Albertini, A. M., Sonenshein, A. L., & Belitsky, B. R. (2015). CodY regulates expression of the Bacillus subtilis extracellular proteases Vpr and Mpr. *Journal of bacteriology*, *197*(8), 1423-1432.

Başalp A., Özcengiz G., & Alaeddinoğlu N.G. (1992). Changes in patterns of alkaline serine protease and bacilysin formation caused by common effectors of sporulation in *Bacillus subtilis 168*.*Current Microbiology*, 24 129–135.

Beauregard, P. B., Chai, Y., Vlamakis, H., Losick, R., & Kolter, R. (2013). *Bacillus subtilis* biofilm induction by plant polysaccharides. *Proceedings of the National Academy of Sciences*, *110*(17), E1621-E1630.

Besson, F., Peypoux, F., Quentin, M. J., & Michel, G. (1984). Action of antifungal peptidolipids from *Bacillus subtilis* on the cell membrane of *Saccharomyces cerevisiae*. *The Journal of antibiotics*, *37*(2), 172-177.

Bierbaum, G., & Sahl, H. G. (2009). Lantibiotics: mode of action, biosynthesis and bioengineering. *Current pharmaceutical biotechnology*, *10*(1), 2-18.

Bierbaum, G.; Szekat, C.; Josten, M.; Heidrich, C.; Kempter, C.; Jung, G. & Sahl, H.-G. (1996*).* Engineering of a novel thioether bridge and role of modified residues in the lantibiotic Pep5. *Applied Environmental Microbiology*. 62(2), 385-392.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical biochemistry*, *72*(1-2), 248-254.

Breen, S., Solomon, P. S., Bedon, F., & Vincent, D. (2015). Surveying the potential of secreted antimicrobial peptides to enhance plant disease resistance. *Frontiers in plant science*, *6*.

Carrillo, C., Teruel, J. A., Aranda, F. J., & Ortiz, A. (2003). Molecular mechanism of membrane permeabilization by the peptide antibiotic surfactin. *Biochimica et Biophysica Acta (BBA)-Biomembranes*, *1611*(1), 91-97.

Chalasani, A. G., Dhanarajan, G., Nema, S., Sen, R., & Roy, U. (2015). An Antimicrobial Metabolite from *Bacillus sp.*: Significant Activity Against Pathogenic Bacteria Including Multidrug-Resistant Clinical Strains. *Frontiers in microbiology*, *6*.

Chatterjee, C., Paul, M., Xie, L., & van der Donk, W. A. (2005). Biosynthesis and mode of action of lantibiotics. *Chemical reviews*, *105*(2), 633-684.

Chatterjee, S., Chatterjee, S., Lad, S. J., Phansalkar, M. S., Rupp, R. H., Ganguli, B. N., Fehlhaber H. W., & KOGLER, H. (1992). Mersacidin, a new antibiotic from Bacillus Fermentation, isolation, purification and chemical characterization. *The Journal of antibiotics*, *45*(6), 832-838.

Chen X.H., Schauder, S., Potier, N., Van Dorsselaer, A., Pelczer, I., Bassler, B. L., & Hughson, F.M., (2002**)**. Structural identification of a bacterial quorum-sensing signal containing boron. *Nature*, 415(6871), 545-9.

Chen, X. H., Koumoutsi, A., Scholz, R., & Borriss, R. (2008). More than anticipated–production of antibiotics and other secondary metabolites by Bacillus amyloliquefaciens FZB42. *Journal of molecular microbiology and biotechnology*, *16*(1-2), 14-24.

Chen, X. H., Koumoutsi, A., Scholz, R., Schneider, K., Vater, J., Süssmuth, R., Pielc J., & Borriss, R. (2009). Genome analysis of *Bacillus*

amyloliquefaciens FZB42 reveals its potential for biocontrol of plant pathogens. *Journal of biotechnology*, *140*(1), 27-37.

Chen, X. H., Scholz, R., Borriss, M., Junge, H., Mögel, G., Kunz, S., & Borriss, R. (2009b). Difficidin and bacilysin produced by plant-associated Bacillus amyloliquefaciens are efficient in controlling fire blight disease. *Journal of biotechnology*, *140*(1), 38-44.

Chmara, H. (1985). Inhibition of glucosamine synthase by bacilysin and anticapsin. *Microbiology*, *131*(2), 265-271.

Chmara, H., Milewski, S., Dzieduszycka, M., Smulkowski, M., Sawlewicz, P., & Borowski, E. (1982). Epoxypeptides a novel group of metabolic inhibitors in procaryotic and eucaryotic organisms. *Drugs Exp Clin Res* 8:11–12.

Chmara, H., Woynarowska, B., & Borowski, E. (1981). Epoxypeptide antibiotic tetaine mimics peptide in transport to bacteria. *Journal of Antibiotics*. 34, 1608-1612.

Choudhary, D. K., & Johri, B. N. (2009). Interactions of *Bacillus spp*. and plants–with special reference to induced systemic resistance (ISR). *Microbiological research*, *164*(5), 493-513.

Cladera-Olivera, F., Caron, G. R., & Brandelli, A. (2004). Bacteriocin-like substance production by Bacillus licheniformis strain P40. *Letters in Applied Microbiology*, *38*(4), 251-256.

Claverys, J. P., Prudhomme, M., & Martin, B. (2006). Induction of competence regulons as a general response to stress in gram-positive bacteria. *Microbiology*, *60*(1), 451.

Contente, S., & Dubnau, D. (1979). Characterization of plasmid transformation in Bacillus subtilis: kinetic properties and the effect of DNA conformation. *Molecular and General Genetics MGG*, *167*(3), 251-258.

Coote, J. G. (1972). Sporulation in *Bacillus subtilis*. Characterization of oligosporogenous mutants and comparison of their phenotypes with those of asporogenous mutants. *Microbiology*, *71*(1), 1-15.

Danhorn, T., & Fuqua, C. (2007). Biofilm formation by plant-associated bacteria. *Annu. Rev. Microbiol.*, *61*, 401-422.

Draper, L. A., Ross, R. P., Hill, C., & Cotter, P. D. (2008). Lantibiotic immunity. *Current protein & peptide science*, *9*(1), 39-49.

Dubnau, D. (1976). Genetic transformation of *Bacillus subtilis*: a review with emphasis on the recombination mechanism. *Microbiology*, *1976*, 14-27.

Dubnau, D. (1991). Genetic competence in *Bacillus subtitles. Microbiological reviews*. 55 (3), 395-424.

Dubnau, D. (1997). Binding and transport of transforming DNA by Bacillus subtilis: the role of type-IV pilin-like proteins–a review. *Gene*, *192*(1), 191-198.

Duitman, E.H., Hamoen, L.W., Rembold, M., Venema, G., Seitz, H., & Saenger W. (1999). The mycosubtilin synthetase of Bacillus subtilis ATCC

6633: a multifunctional hybrid between a peptide synthetase, an amino transferase, and a fatty acid synthase. *Proc Natl Acad Sci* USA. 13294–13299.

Earl, A. M., Losick, R., & Kolter, R. (2008). Ecology and genomics of *Bacillus subtilis*. *Trends in Microbiology*. 16, 269–275**.**

Eberhard, A., Burlingame, A. L., Eberhard, C., Kenyon, G. L., Nealson, K. H., & Oppenheimer, N. J. (1981). Structural identification of autoinducer of *Photobacterium fischeri luciferase*. *Biochemistry*, *20*(9), 2444-2449.

Engebrecht, J., & Silverman, M. (1984). Identification of genes and gene products necessary for bacterial bioluminescence. *Proceedings of the National Academy of Sciences*, *81*(13), 4154-4158.

Engebrecht, J., & Silverman, M. (1984). Identification of genes and gene products necessary for bacterial bioluminescence. *Proceedings of the National Academy of Sciences*, *81*(13), 4154-4158.

Engebrecht, J., Nealson, K., & Silverman, M. (1983). Bacterial bioluminescence: isolation and genetic analysis of functions from *Vibrio fischeri*. *Cell*, *32*(3), 773-781.

Finking, R., & Marahiel, M. A. (2004). Biosynthesis of nonribosomal peptides 1. *Annu. Rev. Microbiol.*, *58*, 453-488.

Fischbach, M. A., & Walsh, C. T. (2006). Assembly-line enzymology for polyketide and nonribosomal peptide antibiotics: logic, machinery, and mechanisms. *Chemical reviews*, *106*(8), 3468-3496.

Foster, J. W., & Woodruff, H. B. (1946). Bacillin, a new antibiotic substance from a soil isolate of Bacillus subtilis. *Journal of bacteriology*, *51*(3), 363.

Fracchia, L., Banat, J. J., Cavallo, M., Ceresa, C., & Banat, I. (2015). Potential therapeutic applications of microbial surface-activecompounds. *AIMS Bioengineering*, *2*(3), 144-162.

Frangeul, L., Nelson, K. E., Buchrieser, C., Danchin, A., Glaser, P., & Kunst, F. (1999). Cloning and assembly strategies in microbial genome projects. *Microbiology*, *145*(10), 2625-2634.

Glaser, P., Danchin, A., Kunst, F., Zuber, P., & Nakano, M. M. (1995). Identification and isolation of a gene required for nitrate assimilation and anaerobic growth of *Bacillus subtilis*. *Journal of bacteriology*, *177*(4), 1112-1115.

Gobbetti, M., De Angelis, M., Di Cagno, R., Minervini, F., & Limitone, A. (2007). Cell–cell communication in food related bacteria. *International journal of food microbiology*, *120*(1), 34-45.

González-Pastor, J. E., Hobbs, E. C., & Losick, R. (2003). Cannibalism by sporulating bacteria. *Science*, *301*(5632), 510-513.

Gray K.M. (1997). Intercellular communication and group behavior in bacteria. *Trends in microbiology*. 5(5), 184-8.

Griffiths, A.J., Miller, J.H., & Suzuki, D.T., (2000). An Introduction to Genetic Analysis. *7th edition. New York: W. H. Freeman.* Available from: http://www.ncbi.nlm.nih.gov/books/NBK21766/.

Guder, A., Schmitter, T., Wiedemann, I., Sahl, H.G., & Bierbaum, G. (2002) Role of the single regulator MrsR1 and the two-component system MrsR2/K2 in the regulation of mersacidin production and immunity. *Applied Environmental Microbiology.* 68: 106–113**.**

Guder, A., Wiedemann, I., & Sahl, H. G. (2000). Posttranslationally modified bacteriocins—the lantibiotics. *Peptide Science*, *55*(1), 62-73.

Hacker, C., Christ, N. A., Duchardt-Ferner, E., Korn, S., Göbl, C., Berninger, L., & Entian, K. D. (2015). The solution structure of the lantibiotic immunity protein NisI and its interactions with nisin. *Journal of Biological Chemistry*, *290*(48), 28869-28886.

Hahn, J., & Dubnau, D. (1991). Growth stage signal transduction and the requirements for srfA induction in development of competence. *Journal of bacteriology*, *173*(22), 7275-7282.

Hahn, J., Luttinger, A., & Dubnau, D. (1996) Regulatory inputs for the synthesis of ComK, the competence tran- scription factor of *Bacillus subtilis*. *Molecular Microbiology*. 21: 763–775.

Hamoen L.W., Van Werkhoven, A.F., Bijlsma.J.E., Dubnau, D., & Venema, G. (1998). The competence transcription factor of *Bacillus subtilis* recognizes short AT-rich sequences arranged in a unique, flexible pattern along the DNA helix. *Genes Dev*. 12: 1539–1550.

Hamoen, L. W., Eshuis, H., Jongbloed, J., Venema, G., & Sinderen, D. (1995). A small gene, designated comS, located within the coding region of the fourth amino acid-activation domain of srfA, is required for competence development in *Bacillus subtilis*. *Molecular microbiology*, *15*(1), 55-63.

Hamoen, L. W., Kausche, D., Marahiel, M. A., van Sinderen, D., Venema, G., & Serror, P. (2003). The Bacillus subtilis transition state regulator AbrB binds to the—35 promoter region of comK. *FEMS microbiology letters*, *218*(2), 299-304.

Hamoen, L. W., Van Werkhoven, A. F., Venema, G., & Dubnau, D. (2000). The pleiotropic response regulator DegU functions as a priming protein in competence development in *Bacillus subtilis*. *Proceedings of the National Academy of Sciences*, *97*(16), 9246-9251.

Hardman, M., Stewart, G. S., & Williams, P., (1998). Quorum sensing and the cell-cell communication dependent regulation of gene expression in pathogenic and non-pathogenic bacteria. *Antonie van Leeuwenhoek*. 74(4):199-210.

Harwood, C. R. (1992). Bacillus subtilis and its relatives: molecular biological and industrial workhorses. *Trends in biotechnology*, *10*, 247-256.

Harwood, C. R., Pohl, S., Smith, W., & Wipat, A. (2013). Bacillus subtilis: model Gram-positive synthetic biology chassis. *Methods in microbiology. Academic Press: Amsterdam, The Netherlands*, 87-117.

Henke, J. M., & Bassler, B. L. (2004). Bacterial social engagements. *Trends in cell biology*, *14*(11), 648-656.

Hilton M.D., Alaeddinoglu N.G., & Domain A.L. (1988). Synthesis of bacilysin by Bacillus subtilis branches from prephenate of the aromatic amino acid pathway, *Journal Bacteriology*. 170(1): 482–484.

Hoa Tran Thu, Tortosa Pablo, Albano Mark, & Dubnau David (2002). Rok (YkuW) regulates genetic competence in Bacillus subtilis by directly repressing comK. *Molecular Microbiology*. 43(1), 15–26.

Idriss, .E.S., Makarewicz, O., Farouk, A., Rosner, K., Greiner, R., Bochow H., Richter, T., & Borriss, R. (2002). Extracellular phytase activity of *Bacillus amyloliquefaciens FZB45* contributes to its plant-growth promoting effect. *Microbiology*. 148: 2097–2109.

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

Jack, R. W., & Jung, G. (2000). Lantibiotics and microcins: polypeptides with unusual chemical diversity. *Current opinion in chemical biology*, *4*(3), 310-317.

Jansen, E. F., & Hirschmann, D. J. (1944). Subtilin, an antibacterial product of Bacillus subtilis: culturing conditions and properties. *Arch. Biochem*, *4*(297), 37-58.

Johnson, B.A., Anker, H., & Meleney, F.L. (1945). Bacitracin: A new antibiotic produced by a member of the *B. subtilis* group. *Science*. 12;102(2650):376–377.

Joseph, P., Manoja, R.L., Abraham, L., & Sonenshein (2005). A Region of Bacillus subtilis CodY Protein Required for Interaction with DNA. *J Bacteriol*. 187(12): 4127–4139.

Karataş, A.Y., Cetin, S., & Özcengiz, G. (2003). The effects of insertional mutations in comQ, comp, srfA, spo0H, spo0A and abrB genes on bacilysin biosynthesis in Bacillus. *Biochim Biophys Acta*. 1626:51–56.

Kawulka, K., Sprules, T., McKay, R.T., Mercier, P., Diaper, C.M., Zuber, P., & Vederas, J.C. (2003). Structure of subtilosin A, an antimicrobial peptide from Bacillus subtilis with unusual posttranslational modifications linking cysteine sulfurs to alpha-carbons of phenylalanine and threonine. *J. Am. Chem. Soc*. 125:4726-4767.

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

Kenig, M., & Abraham, E. P. (1976). Antimicrobial activities and antagonists of bacilysin and anticapsin. *Microbiology*, *94*(1), 37-45.

Kloepper, J.W., Ryu, C.M., & Zhang, S. (2004). Induced systemic resistance and promotion of plant growth by *Bacillus Spp*. *Phytopathology 94*, 1259–1266**.**

Kobayashi, K., Ehrlich, S. D., Albertini, A., Amati, G., Andersen, K. K., Arnaud, M., & Boland, F. (2003). *Essential Bacillus subtilis* genes. *Proceedings of the National Academy of Sciences*, *100*(8), 4678-4683.

Kodani, S., Hudson, M.E., Durrant, M.C., Buttner, M.J., Nodwell J.R., & Willey, J.M. (2004). The SapB morphogen is a lantibiotic-like peptide derived from the product of the developmental gene ramS in Streptomyces coelicolor. *Proceedings of the National Academy of Sciences of the United States of America*, 101(31).

Koponen, O., Takala, T.M., Saarela, U., Qiao, M., & Saris, P.E. (2004) Distribution of the NisI immunity protein and enhancement of nisin activity by the lipid-free NisI. *FEMS Microbiol Lett* 231: 85–90.

Köroğlu, T.E., Öğülür, İ., Mutlu, S., Yazgan-Karataş, A., & Özcengiz, G. (2011). Global regulatory systems operating in Bacilysin biosynthesis in *Bacillus subtilis*. *J Mol Microbiol Biotechnol*. 20:144–155**.**

Koumoutsi, A., Xiao-Hua, C., Anke, H., Heiko L., Gabriele H., Peter F., Joachim V., & Rainer B. (2004). Structural and Functional Characterization of Gene Clusters Directing Nonribosomal Synthesis of Bioactive Cyclic Lipopeptides in *Bacillus amyloliquefaciens* Strain *FZB42. J. Bacteriol*.186: 4 1084-1096.

Kovács, Á. T., Smits, W. K., Mirończuk, A. M., & Kuipers, O. P. (2009). Ubiquitous late competence genes in Bacillus species indicate the presence of functional DNA uptake machineries. *Environmental microbiology*, *11*(8), 1911-1922.

Krebs, B., Höding, B., Kübart, S.M., Workie, A., Junge, H., Schmiedeknecht G., Grosch, R., Bochow, H., & Hevesi, M. (1998). Use of Bacillus subtilis as biocontrol agent. Activities and characterization of *Bacillus subtilis* strains. *Journal of Plant Disease Protection*. 105, 181-197.

Kunst, F., Ogasawara, N., Moszer, I., Albertini, A. M., Alloni, G., Azevedo, V., Bertero, M. G., Bessieres, P., Bolotin, A., Borchert, S., Borriss, R., Boursier, L., Brans, A., Braun, M., Brignell, S. C., Bron, S., Brouillet, S., Bruschi, C. V., Caldwell, B., Capuano, V., Carter, N. M., Choi, S. K., Codani, J. J., Connerton, I. F., Cummings, N. J., Daniel, R. A., Denizot, F., Devine, K. M., Dusterhoft, A., Ehrlich, S. D., Emmerson, P. T., Entian, K. D., Errington, J., Fabret, C., Ferrari, E., Foulger, D., Fritz, C., Fujita, M., Fujita, Y., Fuma, S., Galizzi, A., Galleron, N., Ghim, S. Y., Glaser, P., Goffeau, A., Golightly, E. J., Grandi, G., Guiseppi, G., Guy, B. J., Haga, K., Haiech, J., Harwood, C. R., Henaut, A., Hilbert, H., Holsappel, S., Hosono, S., Hullo, M. F., Itaya, M., Jones, L., Joris, B., Karamata, D., Kasahara, Y., KlaerrBlanchard, M., Klein, C., Kobayashi, Y., Koetter, P., Koningstein, G., Krogh, S., Kumano, M., Kurita, K., Lapidus, A., Lardinois, S., Lauber, J., Lazarevic, V., Lee, S. M., Levine, A., Liu, H., Masuda, S., Mauel, C., Medigue, C., Medina, N., Mellado, R. P., Mizuno, M., Moestl, D., Nakai, S., Noback, M., Noone, D., Oreilly, M., Ogawa, K., Ogiwara, A., Oudega, B., Park, S. H., Parro, V., Pohl, T. M., Portetelle, D., Porwollik, S., Prescott, A. M., Presecan, E., Pujic, P., Purnelle, B., Rapoport, G., Rey, M., Reynolds, S., Rieger, M., Rivolta, C., Rocha, E., Roche, B., Rose, M., Sadaie, Y., Sato, T., Scanlan, E., Schleich, S., Schroeter, R., Scoffone, F., Sekiguchi, J., Sekowska, A., Seror, S. J., Serror, P., Shin, B. S., Soldo, B., Sorokin, A., Tacconi, E., Takagi, T.,

Takahashi, H., Takemaru, K., Takeuchi, M., Tamakoshi, A., Tanaka, T., Terpstra, P., Tognoni, A., Tosato, V., Uchiyama, S., Vandenbol, M., Vannier, F., Vassarotti, A., Viari, A., Wambutt, R., Wedler, E., Wedler, H., Weitzenegger, T., Winters, P., Wipat, A., Yamamoto, H., Yamane, K., Yasumoto, K., Yata, K., Yoshida, K., Yoshikawa, H. F., Zumstein, E., Yoshikawa, H., & Danchin, A. (1997). The complete genome sequence of the Gram-positive bacterium *Bacillus subtilis*. *Nature 390,* 249-256.

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

Lazazzera, B.A., Palmer, T., Quisel, J., & Grossman, A.D. (1999). Cell density control of gene expression and development in Bacillus subtilis. In Cell–Cell Signaling in Bacteria. Dunny G.M. and Winans S.C. Washington, DC: *American Society of Microbiology Press*, pp. 27–46.

Levdikov, V.M., Blagova, E., Joseph, P., Sonenshein, A.L., & Wilkinson, A.J. (2006). The structure of CodY, a GTP- and isoleucine-responsiveregulator of stationary phase and virulence in gram-positive bacteria. *The Journal of biological chemistry*, 281(16), 11366-73.

Liming, W., Huijun, W., Lina, C., Shanshan, X., Haoyu, Z., Rainer, B., & Xuewen, G. (2014). Bacilysin from *Bacillus amyloliquefaciens FZB42* Has Specific Bactericidal Activity against Harmful Algal Bloom Species. *Appl Environ Microbiol*. 80(24): 7512–7520.

Liming, W., Huijun, W., Lina C., Xinfang, Y., Rainer B., & Xuewen G. (2015). Difficidin and bacilysin from *Bacillus amyloliquefaciens FZB42* have antibacterial activity against *Xanthomonas oryzae* rice pathogens, *Scientific Reports* 5, Article number: 12975.

Loeffler, W., Tschen, J.S., Vanittanakom, N., Kugler, M., Knorpp, E., Hsieh, T.F., & Wu, T.G. (1986) Antifungal effects of bacilysin and fengycin from *Bacillus subtilis F-29-3*. A comparison with activities of other Bacillus antibiotics. *J Phytopathol* .115:204–213.

Lugtenberg, B., Kamilova, F. (2009), Plant-growth-promoting rhizobacteria, *Annual Review of Microbiology*, 63:541-556.

Maget-Dana, R., & Peypoux, F. (1994). Iturins, a special class of pore-forming lipopeptides: biological and physicochemical properties. *Toxicology*, *87*(1-3), 151-174.

Mahlstedt, S., Fielding, E.N., Moore, B.S., & Walsh, C.T. (2010). Prephenate decarboxylases: a new prephenate-utilizing enzyme family that performs nonaromatizing decarboxylation en route to diverse secondary metabolites. *Biochemistry*. 49. 9021–9023.

Mahlstedt, S. A., & Walsh, C. T. (2010). Investigation of anticapsin biosynthesis reveals a four-enzyme pathway to tetrahydrotyrosine in Bacillus subtilis. *Biochemistry*, *49*(5), 912-923.

Malligarjunan, R., Ashima, M., & Balasubramanian, G. (2009). Role of Bacillus subtilis BacB in the Synthesis of Bacilysin. *The Journal of Biological Chemistry*. 284, 31882-31892.

Marahiel, M.A., Nakano, M.M., & Zuber, P. (1993). Regulation of peptide antibiotic production in Bacillus. *Mol Microbiol.* 7: 631–636.

Mariappan, A., Makarewicz, O., Chen, X.H., & Borriss, R. (2012). Two-component response regulator DegU controls the expression of bacilysin in plant-growth-promoting bacterium *Bacillus amyloliquefaciens FZB42*. *Journal of Molecular Microbiology and Biotechnology*.22(2):114-25.

Marx, R., Stein, T., Entian, K.D., & Glaser, S. J. (2001). Structure of the Bacillus subtilis peptide antibiotic subtilosin A determined by 1H-NMR and matrix assisted laser desorption/ionization time-of-flight mass spectrometry. *J. Protein Chem.* 6:501-506.

Mattick, A.T.R., & Hirsch, A. (1947). Further observations on an inhibitory substance (nisin) from lactic streptococci. *Lancet* Volume 250, No. 6462, p5–8.

McAuliffe, O., Ross, R.P., & Hill, C. (2001) Lantibiotics: structure, biosynthesis and mode of action. *FEMS Microbiol Rev.* 25: 285–308.

Meena, K. R., & Kanwar, S. S. (2015). Lipopeptides as the antifungal and antibacterial agents: applications in food safety and therapeutics. *BioMed research international*, *2015*.

Michael, T., Martinko, J.M.; Bender, K.S.; Buckley, D.H.; & Stahl, D. A. (2015). Brock Biology of Microorganisms. Boston: Pearson,2015.

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

Miller, M. B., & Bassler, B. L. (2001). Quorum sensing in bacteria. *Annual Reviews in Microbiology*, *55*(1), 165-199.

Morikawa, M. (2006). Beneficial biofilm formation by industrial bacteria *Bacillus subtilis* and related species. *Journal of Bioscience and Bioengineering.* 101, 1–8.

Msadek, T. (1999). When the going gets tough: survival strategies and environmental signaling networks in *Bacillus subtilis*. *Trends in microbiology*, 7(5),201-7.

Nagorska, K., Bikowski, M., & Obuchowskji, M. (2007). Multicellular behaviour and production of a wide variety of toxic substances support usage of *Bacillus subtilis* as a powerful biocontrol agent. *Acta Biochimica Polonica*.;54:495–508.

Nakano, M.M., Xia, L.A, & Zuber, P. (1991). Transcription initiation region of the srfA operon, which is controlled by the comP-comA signal transduction system in *Bacillus subtilis*. *J Bacteriol*.173:5487–5493.

Nakano, M. M., & Zuber, P. (1998). Anaerobic growth of a "strict aerobe"(Bacillus subtilis). *Annual Reviews in Microbiology*, *52*(1), 165-190.

Nasir, M. N., & Besson, F. (2012). Interactions of the antifungal mycosubtilin with ergosterol-containing interfacial monolayers. *Biochimica et Biophysica Acta (BBA)-Biomembranes*, *1818*(5), 1302-1308.

Newman, J. A., Rodrigues, C., & Lewis, R. J. (2013). Molecular Basis of the Activity of SinR Protein, the Master Regulator of Biofilm Formation in *Bacillus subtilis. J Biol Chem*. 12; 288(15): 10766–10778.

Newton, G. F. (1949). Antibiotics from a Strain of *B. subtilis*: Bacilipin A and B and Bacilysin. *Br J Exp Pathol*. 30(4): 306–319.

Ng, W. L., & Bassler, B. L. (2009). Bacterial quorum-sensing network architectures. *Annual review of genetics*, *43*, 197.

Novick, R.P., Projan, S.J, Kornblum J., Ross, H.F., Kreiswirth B., Vandenesch F., & Moghazeh, S. (1995.). The agr P2 operon: An autocatalytic sensory transduction system in *Staphylococcus aureus*. *Mol Gen Genet*. 248: 446 – 458.

Novick, R. P., & Geisinger, E. (2008). Quorum sensing in staphylococci. *Annual review of genetics*, *42*, 541-564.

Özcengiz, G., Alaeddinoglu, N.G. & Domain, A.L. (1990). Regulation of biosynthesis of bacilysin by *Bacillus subtilis. J Ind Microbiol*, 6. 91–100.

Özcengiz, G., & Alaeddinoglu, N. G. (1991). Bacilysin production byBacillus subtilis: Effects of bacilysin, pH and temperature. *Folia microbiologica*, *36*(6), 522-526.

Paik, S.H., Chakicherla, A., & Hansen, J.N. (1998) Identification and characterization of the structural and transporter genes for, and the chemical and biological properties of, sublancin 168, a novel lantibiotic produced by *Bacillus subtilis 168. J Biol Chem.* 273: 23134–32142.

Parisot J., Sarah, C., Eefjan, B., Weng, C., Chan, A. N. & Boyan, B. (2008). Molecular Mechanism of Target Recognition by Subtilin, a Class I Lanthionine. *Antibiotic. vol*. 52 no. 2 612-618.

Parker, J. B., & Walsh, C. T. (2012). Olefin isomerization regiochemistries during tandem action of BacA and BacB on prephenate in bacilysin biosynthesis. *Biochemistry*, *51*(15), 3241-3251.

Parker, J. B., & Walsh, C. T. (2013). Action and timing of BacC and BacD in the late stages of biosynthesis of the dipeptide antibiotic bacilysin. *Biochemistry*, *52*(5), 889-901.

Perez, A.R, Abanes-De M.A, & Pogliano, K. (2000). SpoIIB Localizes to Active Sites of Septal Biogenesis and Spatially Regulates Septal Thinning during Engulfment in *Bacillus subtilis. J Bacteriol.*182(4): 1096–1108.

Perry, D., & Abraham, E. P. (1979). Transport and metabolism of bacilysin and other peptides by suspensions of Staphylococcus aureus. *Microbiology*, *115*(1), 213-221.

Persuh, M., Turgay, K., Mandic-Mulec, I., & Dubnau, D. (1999). The N- and C-terminal domains of MecA recognize different partners in the competence molecular switch. *Mol Microbiol.* 33: 886–894.

Peypoux, F., Besson, F, Michel, G., & Delcambe, L. (1979). Preparation and antibacterial activity upon Micrococcus luteus of derivatives of iturin

A, mycosubtilin and bacillomycin L, antibiotics from *Bacillus subtilis. J Antibiot* (Tokyo). 32(2):136-40.

Pinchuk, I.V., Bressollier, P., Verneuil, B., Fenet, B., Soroku-lova, I.B., Megraud, F., & Urdaci, M.C. (2001). In vitro anti-Helicobacter pylori activity of the probiotic strain Bacil- lus subtilis 3 is due to secretion of antibiotics. *Antimicrob Agents Chemother*. 45: 3156–3161.

Pinchuk, I.V., Bressollier, P., Sorokulova, I.B., Verneuil B., & Urdaci, M.C. (2002) Amicoumacin antibiotic production and genetic diversity of Bacillus subtilis strains isolated from different habitats. *Res Microbiol*. 153: 269–276.

Priest, F.G. (1993). Systematics and Ecology of Bacillus, p 3-16. In Sonenshein A, Hoch J, Losick R, *Bacillus subtilis* and Other Gram-Positive Bacteria. *ASM Press*, Washington, DC. doi: 10.1128/9781555818388.ch1

Rainer, B. (2011). Use of Plant-Associated *Bacillus Strains* as Biofertilizers and Biocontrol Agents in Agriculture. In *Bacteria in Agrobiology*: *Plant Growth Responses*. Chapter 3. 41-76.

Rajavel, M., Mitra, A., & Gopal, B. (2009). Role of *Bacillus subtilis* BacB in the Synthesis of Bacilysin. *The Journal of Biological Chemistry*, 284(46), 31882–31892. http://doi.org/10.1074/jbc.M109.014522.

Ramey, B. E., Koutsoudis, M., von Bodman, S. B., & Fuqua, C. (2004). Biofilm formation in plant-microbe associations. *Curr. Opin. Microbiol*. 7, 602–609.

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

Reading, N. C., & Sperandio, V. (2006). Quorum sensing: the many languages of bacteria. *FEMS microbiology letters*, *254*(1), 1-11.

Rogers, H.J., Newton, G.G, & Abraham, E.P (1965). Production and purification of bacilysin. *Biochem J*. 97:573–578.

Rogers, L. A., & Whittier, E. O. (1928). Limiting factors in the lactic fermentation. *Journal of Bacteriology*, *16*(4), 211.

Roscoe, J., & Abraham, E. P. (1966). Experiments relating to the biosynthesis of bacilysin. *Biochemical Journal*, *99*(3), 793.

Sahl, H. G., & Bierbaum, G. (1998). Lantibiotics: biosynthesis and biological activities of uniquely modified peptides from gram-positive bacteria. *Annual Reviews in Microbiology*, *52*(1), 41-79.

Saito, Y.; Taguchi, H.; & Akamatsu, T. (2006). Fate of transforming bacterial genome following incorporation into competent cells of *Bacillus subtilis*. A continuous length of incorporated DNA. *Journal of Bioscience and Bioengineering*. 101 (3):257-62.

Schauder, S., & Bassler, B. L. (2001). The languages of bacteria. *Genes & Development*, *15*(12), 1468-1480.

Schnell, N., Entian, K.D., Schneider, U., Götz, F., Zahner, H., Kellner, R., & Jung, G. (1988) Prepeptide sequence of epidermin, a ribosomally synthesized antibiotic with four sulphide-rings. *Nature*. 333: 276–278.

Scholz, R., Molohon, K. J., Nachtigall, J., Vater, J., Andrew, L., Markley, R.D., Süssmuth, D.A., & Mitchelland, R. B. (2011). Plantazolicin, a Novel Microcin B17/Streptolysin S-Like Natural Product from *Bacillus amyloliquefaciens FZB42*. *J. Bacteriol*. 193 :215-224.

Seed, P.C., Passador, L., & Iglewski, B.H. (1995). Activation of the Pseudomonas aeruginosa lasI gene by LasR and the Pseudomonas autoinducer PAI: An autoinduction regulatory hierarchy. *J Bacteriol*.177: 654 – 659.

Serror, P., & Sonenshein, A. L. (1996). CodY is required for nutritional repression of Bacillus subtilis genetic competence. *Journal of bacteriology*, *178*(20), 5910-5915.

Shivers, R. P., & Sonenshein, A. L. (2004). Activation of the Bacillus subtilis global regulator CodY by direct interaction with branched-chain amino acids. *Molecular microbiology*, *53*(2), 599-611.

Sieber, S. A., & Marahiel, M. A. (2003). Learning from nature's drug factories: nonribosomal synthesis of macrocyclic peptides. *Journal of bacteriology*, *185*(24), 7036-7043.

Silkin, L., Hamza, S., Kaufman, S., Cobb, S.L., & Vederas, J.C. (2008). Spermicidal bacteriocins: lacticin 3147 and subtilosin A. *Bioorganic & Medicinal Chemistry Letters.* 18(10):3103-6.

Sinderen, D., Berge, T., Hayema, A., Hamoen, B.J.L., & Venema, G. (1994). Molecular cloning and sequence of *comK*, a gene required for genetic competence in *Bacillus subtilis*. *Mol Microbiol*. 11: 695–703.

Slack, F., Serror, P., Joyce E., & Sonenshein, A.L. (1995). A gene required for nutritional repression of the *Bacillus subtilis* dipeptide permease operon. *Mol Microbiol*.;15:689–702.

Snyder, L., Peters, J. E., Henkin, T. M., & Champness, W. (2013). *Molecular genetics of bacteria*. American Society of Microbiology.

Sonenshein A.L, Hoch J., & Losick R. (1993). Bacillus subtilis and Other Gram-Positive Bacteria: Biochemistry, Physiology, and Molecular Genetics. *ASM Press*, Washington, DC. pp.3–16. doe: 10.1128/9781555818388.ch1.

Spizizen, J. (1958). Transformation of biochemically deficient strains of *Bacillus subtilis* by deoxyribonucleate. *Proceedings of the National Academy of Sciences of the United States of America*, 44(10), 1072–1078.

Srivatsan, A. (2008). High-precision, whole-genome sequencing of laboratory strains facilitates genetic studies. *PLoS Genet*. 4, e1000139.

Stein, T., Borchert, S., Conrad, B., Feesche, J., Hofemeister, B., Hofemeister J., & Entian, K.D. (2002). Two different lantibiotic-like peptides originate from the ericin gene clus- ter of *Bacillus subtilis A1/3*. *J. Bacteriol.* 184: 1703–1711.

Stein, T., Heinzmann, S., Kiesau, P., Himmel, B., & Entian, K.D. (2003b) The spa-box for transcriptional activation of subtilin biosynthesis and immunity in Bacillus subtilis. *Mol Microbiol.* 47: 1627–1636.

Stein, T., Heinzmann, S., Solovieva, I., & Entian, K.D. (2003a) Function of Lactococcus lactis nisin immunity genes nisI and nisFEG after coordinated expression in the surrogate host *Bacillus subtilis*. *J Biol Chem*. 278: 89–94.

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

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

Stevens, K.A., Sheldon B.W, Klapes N.A., & Klaenhammer T.R. (1991). Nisin treatment for inactivation of Salmonella species and other Gram-negative bacteria. *Appl. Environ. Microbiol*. 57: 3613- 3615.

Strauch, M., Webb, V., Spiegelman, G., & Hoch, J.A. (1990). The SpoOA protein of *Bacillus subtilis* is a repressor of the abrB gene. *Proc Natl Acad Sci USA*; 87:1801–1805.

Tabata, K., Ikeda, H., & Hashimoto, S. (2005). ywfE in *Bacillus subtilis* codes for a novel enzyme, l-amino acid ligase. *J. Bacteriol*. 187:5195-5202.

Takashi, I., Guojun, W., & Kozo, O. (2009). ScoC Regulates Bacilysin Production at the Transcription Level in *Bacillus subtilis*. *J. Bacteriol*. vol. 191 no. 23 7367-7371.

Tamehiro, N., Okamoto-Hosoya, Y., Okamoto, S., Ubukata, M., Hamada, M., Naganawa, H., & Ochi, K. (2002). Bacilysocin, a novel phospholipid antibiotic produced by *Bacillus subtilis 168*. *Antimicrob Agents Chemother*. 46: 315–320.

Tan, Z., Lin, B., & Zhang, R. (2013). A novel antifungal protein of *Bacillus subtilis B25*. *SpringerPlus*. 2:543.

Tortosa, P., Albano, M., & Dubnau, D. (2000). Characterization of *ylbF*, a new gene involved in competence development and sporulation in *Bacillus subtilis. Mol Microbiol.* 35: 1110–1119.

Turgay, K., Hahn, J., Burghoorn, J., & Dubnau, D. (1998). Competence in *Bacillus subtilis* is controlled by regulated proteolysis of a transcription factor. *EMBO J* .17: 6730–6738.

Van-Sinderen, D., & Venema, G. (1994). comK acts as an autoregulatory control switch in the signal transduction route to competence in Bacillus subtilis. *Journal of bacteriology*, *176*(18), 5762-5770.

Vanittanakom, N., Loeffler, W., Koch, U., & Jung, G. (1986). Fengycin a novel antifungal lipopeptide antibiotic produced by *Bacillus subtilis F-29-3*. *J. Antibiot*. 39, 888–901. 10.7164/antibiotics.39.888.

Veening, J.-W., Murray, H., & Errington, J. (2009). A mechanism for cell cycle regulation of sporulation initiation in *Bacillus subtilis. Genes & Development*, 23(16), 1959–1970. http://doi.org/10.1101/gad.528209.

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

Walsh, C.T. (2004). Polyketide and nonribosomal peptide antibiotics: modularity and versatility. *Science.*303(5665):1805-10.

Walton, R. B., & Rickes, E. L. (1962). Reversal of the antibiotic, bacillin, by N-acetylglucosamine. *Journal of bacteriology*, *84*(6), 1148-1151.

Wang, W., Chen, L.N., Wu, H., Zang, H., Gao, S., Yang, Y., Xie, S. & Gao, X. (2013). Comparative proteomic analysis of rice seedlings in response to inoculation with *Bacillus cereus*. *Applied Microbiology*. Volume 56: 208–215.

Wanga, H., David, P. Fewera, L.H., Leo, R., & Kaarina, S. (2014). Atlas of nonribosomal peptide and polyketide biosynthetic pathways reveals common occurrence of nonmodular enzymes. *PNAS. 111*: 9259–9264.

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

Williams, P., & Cámara, M. (2009). Quorum sensing and environmental adaptation in Pseudomonas aeruginosa: a tale of regulatory networks and multifunctional signal molecules. *Current opinion in microbiology*, *12*(2), 182-191.

Wilson, G. A., & Young, F. E. (1972). Intergenotic transformation of the Bacillus subtilis genospecies. *Journal of bacteriology*, *111*(3), 705-716.

Xie, Z., Johansen, L.K., Gustafson, A.M., Kasschau, K.D., & Lellis, A.D. (2004) Genetic and Functional Diversification of Small RNA Pathways in Plants. *PLoS Biol*. 2(5): e104. doi: 10.1371/journal.pbio.0020104.

Yasuhito, S., Emi, H., Hajime, I., Akihiro, S., Yuichi, T., Jun-ichi, S., Hirofumi, K., Naoki, S., Yoshiyuki, Y., & Yoshiki, H. (2012). Structural and enzymatic characterization of BacD, an L-amino acid dipeptide ligase from *Bacillus subtilis. Protein Science.* 21:707—716.

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

Zheng, G., Hehn, R., & Zuber, P. (2000). Mutational analysis of the sbo-alb locus of *Bacillus subtilis*: identification of genes required for subtilosin production and immunity. *J. Bacteriol.* 182:3266-3273.

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

Zhongzhong, L., Anto, B., Pengfei, W., Hui, S., Juan, F., Rainer, B., Keqin Z., & Xiaowei, H. (2013). The highly modified microcin peptide plantazolicin is associated with nematicidal activity of *Bacillus amyloliquefaciens FZB42. Applied Microbiology and Biotechnology.* 97:10081-10090.

Zweers, J. C., Barák, I., Becher, D., Driessen, A. J., Hecker, M., Kontinen, V. P., & Saller, M. J. (2008). Towards the development of *Bacillus subtilis* as a cell factory for membrane proteins and protein complexes. *Microb. Cell Fact.* 7, 10.

APPENDICES

APPENDIX A: Composition and Preparation of Buffers and Solutions.

APPENDIX B: Buffers and Solutions.

APPENDIX C: Laboratory Equipment

APPENDIX D: Markers.

APPENDIX E: Chemicals

APPENDIX A

Compositions and Preparation of Culture Media

Perry and Abraham (PA) Medium (pH 7.4)

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

*Autoclave separately

**Filter sterilization

Luria Bertani (LB) Medium (1000ml)

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

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

Luria Bertani (LB) Agar Medium (1000 ml)

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

10X-S-base

$(NH_4)_2SO_4$	20 g/L
$K_2HPO_4.3H_2O$	140 g/L
KH_2PO_4	60 g/L

Na_3.citrate.$2H_2O$ 10 g/L

Autoclave together and cool down to 50^0C and supplement with 1 ml sterile 1 M $MgSO_4$.

HS medium (30 ml)

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

Complete up to 30 ml with sterile distilled H_2O and store at cold room (+4°C) up to one week at most.

LS Medium (20 ml)

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

Freshly prepare and complete up to 20 ml with sterile distilled H_2O.

APPENDIX B

Buffers and Solutions

P1 Buffer (pH 8)

Tris-base	6.06 gr
EDTA.2H2O	3.72 gr

Dissolve Tris-base and EDTA with 800 mL dH2O. Adjust pH to 8 with HCl. Adjust volume to 1 lt dH2O. Add 100 mg RNase A per liter of P1.

P2 Buffer

NaOH	8 gr
SDS solution (20%)	50 mL

Dissolve NaOH in 950 mL dH2O.

Add 50 mL SDS solution.

P3 Buffer (pH 5.5)

Potassium acetate	294.5 gr

Dissolve in 500 mL dH2O.

Adjust pH.

TE Buffer (pH 7)

Tris base	10 mM
EDTA	1 mM

Adjusted pH 7 with HCl.

TAE Buffer (50X)

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

Add Distilled H_2O up to 1L and adjust pH to 8 by HCl.

TGE Buffer (10X)

Tris base (2 moles) 30,3 g

Glycine 142 g

EDTA (100mL 0.5M) 37,2 g

Add distilled H_2O up to 1L and adjust pH to 8,4 by HCL

Low Melting Agarose Gel (1%)

Agarose 0.5 g

TAE buffer (1X) 50 mL

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

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

4.1 g of NaCl was dissolved in 80 mL of 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.

Buffers for Purification Under Native Conditions

Lysis buffer (1 liter)

NaH2PO4 6.90 g

NaCl 17.54 g

Imidazole (10 mM) 0.68 g

Dissolve in 800 ml dH2O and adjust pH to 8.0 using NaOH. Adjust volume to 1 lt dH2O.

Wash buffer (1 litre):

NaH2PO4 6.90 g

NaCl 17.54 g

Imidazole (20 mM) 1.36 g

Dissolve in 800 ml dH2O and adjust pH to 8.0 using NaOH. Adjust volume to 1 lt dH2O.

Elution buffer (1 liter):

NaH2PO4 6.90 g

NaCl 17.54 g

Imidazole (250 mM) 17.00 g

Dissolve in 800 ml dH2O and adjust pH to 8.0 using NaOH. Adjust volume to 1 lt dH2O.

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 dH2O and stored at 40C in the dark.

Separating gel buffer for SDS-PAGE

Tris base (1.5 M) 18.2 g

Dissolve in 80 ml dH2O and adjust pH to 8.8. Add distilled H2O up to 100 ml.

Stacking gel buffer for SDS-PAGE

Tris-HCl (1 M) 12.1 g

Dissolve in 80 ml dH2O and adjust pH to 6.8. Add distilled H2O 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)

10x Binding Buffer Stock Solution B for EMSA

KCl 372 mg

MgCl2 102 mg

EDTA 3.7 mg

Tris base 8 ml (100 mM, pH 8.0)

Final volume was made up to 10 mL with dH2O.

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 H2O up to 1ml.

Loading (Sample) buffer for EMSA

0.25x Native gel buffer 60%
Glycerol 40%

Bromophenol blue 0.2% (w/v).

APPENDIX C

LABORATORY EQUIPMENT

Autoclave: Tuttnauer Systec Autoclave (2540 mL)

Balances: Precisa 620C SCS

Precisa 125 A SCS

Centrifuge: Beckman Coulter, Microfuge 18

Centrifuge rotor: F241.5P

Deep freezes and refrigerators: -80°C Heto Ultrafreeze 4410

-20°C Arçelik 209lt

+4°C Arçelik

Electrophoresis equipments: E – C mini cell primo EC320

Gel documentation system: UVI PHotoMW Version 99.05 for Windows

Incubators: Nüve EN400

Nüve EN500

Orbital shaker incubators: Sertomat S – 2

Thermo 430

Pipettes: Gilson pipetteman 10 µL, 20 µL, 200 µL, 1000 µL

Volumate Mettler Toledo 10 µL, 20 µL, 200 µL, 1000 µL

Eppendorf research 2,5 µL 10 µL, 20 µL, 200 µL, 1000 µL

pH meter: Mettler Toledo MP220

Spectrophotometer: PerkinElmer Lambda25 UV/VIS Spectrometer

Thermomixer: Eppendorf thermomixer comfort (1.5 mL)

Transillumunator: Biorad UV transilluminator 2000

Vortexing machine: Heidolph Raax top

Waterbaths: Memmert wb-22

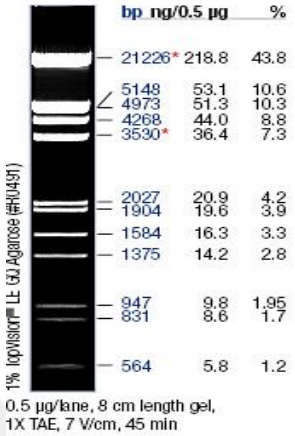
Ultrafiltration tube: VIVASPIN

Power supply: Bio-Rad

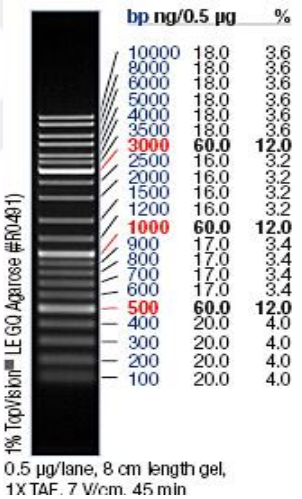
ChemiDoc™ XRS+ System: Bio-Rad

APPENDIX D

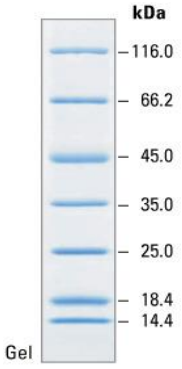
Marker	**Supplier**
Lambda DNA/*Eco*RI+ *Hind*III Marker (Marker 3)	Fermentas



GeneRuler™ DNA Ladder mix, ready-to-use	Fermentas



Protein molecular mass marker	Fermentas



APPENDIX E

Chemicals	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
MgCl2.6H2O	Carlo Erba
NaCl	Merck
NaOH	Carlo Erba
NaH2PO4	Merk
Phenol-chloroform-isoamylalcohol	Fluka
SDS	Merck
TEMED	Carlo Erba
Tris-base	Merck
Yeast Extract	Acumedia

Enzymes

Lysozyme	AppliChem
Proteinase K	Sigma
RNAse A	Sigma
Taq DNA Polymerase	Intron

CURRICULUM VITAE

Name Surname **:** Ali Mohammed Nasser HAIDER

Place and Date of Birth **:** Yareem-Ibb. Republic of Yemen

E-Mail **:** a_haider3@yahoo.com

EDUCATION:

B.Sc. **:** Agricultural Science – Sana'a University.

PROFESSIONAL EXPERIENCE:

Assistant Researcher for Agricultural Research and Extension Authority (AREA).

Training Courses:

- Biotechnology and Molecular Biology - Plant Tissue Culture.
- Research Projects Formulation and Statistical Analysis.
- Experiments Design and Statistical Analysis using GENSTAT programme.
- Scientific Writing of Reports and Research Articles.
- Integrated Pest Management (IPM).
- Seeds Production and Handling.
- Biotechnology and its Uses in Agriculture Purposes.
- Introductory Course on Bioinformatics.
- Introduction to Personal Computers and Windows.
- Introduction to Microsoft Word.
- Introduction to MS Excel.
- Introduction to Power Point.