ISTANBUL TECHNICAL UNIVERSITY ★ GRADUATE SCHOOL OF SCIENCE ENGINEERING AND TECHNOLOGY

THE EFFECT OF BACILYSIN ON *Bacillus subtilis* WILD TYPE 3610 STRAIN

M.Sc. THESIS

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İSTANBUL TEKNİK ÜNİVERSİTESİ ★ FEN BİLİMLERİ ENSTİTÜSÜ

BASİLİSİNİN *Bacillus subtilis* YABAN SUŞU 3610 ÜZERİNE ETKİSİ

YÜKSEK LİSANS TEZİ

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İleri Teknolojiler Anabilim Dalı

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Betim Karahoda, a **M.Sc.** student of ITU **Graduate School of Science Engineering and Technology** student ID 521101118, successfully defended the **thesis** entitled "**THE EFFECT OF BACILYSIN ON *Bacillus subtilis* WILD TYPE 3610 STRAIN**", which he prepared after fulfilling the requirements specified in the associated legislations, before the jury whose signatures are below.

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To Melisa Kaylan,

FOREWORD

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June 2013 Betim KARAHODA

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ABBREVIATIONS

bp	**:** Base pair
dH_2O	**:** Distilled water
DNA	**:** Deoxyribonucleic acid
Erm	**:** Erythromycin
EtBr	**:** Ethidium bromide
kb	**:** Kilobase
LB broth	**:** Luria Bertani broth
Ln	**:** Lyncomycin
OD	**:** Optical density
PA	**:** Perry and Abraham Medium
PCR	**:** Polymerase Chain Reaction
RNA	**:** Ribonucleic acid
RT-qPCR	**:** Reverse transcription quantitative PCR
QS	**:** Quorum Sensing
TAE	**:** Tris acetate EDTA
AHL	**:** N-achylhomoserine Lactone
CSF	**:** Competence and Sporulation Factor

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THE EFFECT OF BACILYSIN ON *Bacillus subtilis* 3610 STRAIN

SUMMARY

Unlike laboratory strains such as *Bacillus subtilis* PY79 that is not able to perform swarming motility and biofilm formation, *B. subtilis* NCBI 3610 is a well-known undomesticated wild type strain for its ability to form robust biofilms called as pellicles at non-agitated liquid and solid surfaces. The formation of biofilm matrix can be induced as a response to external signals such as lipopeptide antibiotic surfactin in *B. subtilis*. Initially, biofilm cells are motile and while the maturation of biofilm proceeds, cells become sessile (non-motile) adherent to each other. The biofilm matrix has a polymeric structure that is encoded by *epsA-O* operon and it is mainly composed of polyssacharides. Additionally, proteins, lipids and other biopolymers are present inside the matrix. Although most of the factors that are crusial for the formation of biofilm are revealed, signals required for the initiation of mutlicellularity is still mysterious.

It is known that antibiotics not only act as an antimicrobial agent but it may also act as a signaling molecule by regulating the expression of specific genes as a response to environmental changes. Although it was shown that some antimicrobial agents such as subtilisin can act as a signaling molecule by recognizing cell density, the effects of many others remain still unknown. Bacilysin is a dipeptide antibiotic, synthesizd by *Bacillus subtilis*, composed of L-alanine and non-ribosomally synthesized L-anticapsin aminoacid that is active against wide-range of bacteria and some fungi. It was recently investigated that the biosynthesis of bacilysin depends on polycistronic operon *ywfBCDEFG* (renamed as *bacABCDEF*) and an adjacent monocistronic gene *ywfH*. Bacilysin biosynthesis is under the control of a quorum sensing regulation system through the action of ComQ/ComX, PhrC (CSF), ComP/ ComA and in a Spo0K (Opp)-dependent manner in *B. subtilis*. Furthermore, it can be positively regulated by global regulatory factor Spo0A and negatively regulated by transition state regulators CodY and AbrB. In addition, bacilysin biosynthesis is under synergistic regulation of guanosine 5'-diphosphate 3'-diphosphate (ppGpp).

Despite having known the synthesis mechanism and regulation of bacilysin biosynthesis quite well, much less is known about the role of bacilysin in the host organism. The present study mainly focused on the effects of bacilysin on *B. subtilis* wild type 3610 strain by analyzing the expression level of several regulatory genes related to extracellular matrix component of biofilm, chemotaxis, swarming motility on bacilysin deficient *bac*$^{-}$ 3610 mutant strain under biofilm forming condition. For this purpose, both wild type and its bacilysin deficient derivative were grown under MSgg agar medium in order to promote biofilm formation, cells were collected and RNAs were extracted at 24th, 48th and 72nd growth phases to determine the change in expression levels of several genes. Relative gene expression analysis was conducted

by using reverse transcription quantitative PCR (RT-qPCR). For the relative quantification, $2^{-\Delta\Delta Cp}$ method was used and then log2 transformed. Our results from the RT-qPCR analysis indicates that in the absence of bacilysin, expression of many genes related to chemotaxis, motility, extracellular alkaline protease synthesis and biofilm formation were significantly down regulated. These data were further supported by swarming motility and biofilm (pellicle and colony architecture) assays. *bac*$^{-}$ 3610 strain was unable to exhibit swarming motility and was unable to form biofilm under non-agitated standing culture biofilm promoting MSgg medium. Consistently, when compared with undomesticated 3610 strain that is able to form complex colony architecture, mutant strain forms fragile flat surface growth in the solid MSgg agar medium suggesting that bacilysin disruption impaired biofilm formation. These results were compatible with obtained RT-qPCR data where most of the genes related to swarming and extracellular matrix component were shown to be up-regulated by bacilysin in most of the growth phases.

Briefly, this study revealed that, *bacABCDEF* operon is responsible for the regulation of many adaptive responses of *B. subtilis* including biofilm formation, chemotaxis, swarming motility, competence development and stress response. Moreover, bacilysin, as a signaling molecule, might be involved in the induction of surface growth that is preliminary step of biofilm formation.

BASİLİSİNİN *Bacillus subtilis* YABAN SUŞU 3610 ÜZERİNE ETKİLERİ

ÖZET

Bacillus subtilis sporlanabilen, patojen olmayan, çubuksu yapıya sahip bir bakteri türüdür. Ayrıca Gram pozitif bakteri türü olup, doğada özellikle toprakda yaygın olarak bulunurlar. Kemotaksis, katı yüzeyde kayma hareketliliği, kompetans gelişimi, antibiyotik üretimi ve sporlanma gibi, değisken çevre kosullarına karşı birden fazla şekilde tepki verebilme özelliğine sahiptirler. Hücre dışına pekçok parçalayıcı enzim salgılayabildikleri gibi, rakipleri ile mücadele edebilmek icin *B. subtilis* hücreleri 12 den fazla farklı antibiyotik sentezleyebilirler.

B. subtilis PY79 gibi evcilleşmiş laboratuar suşları biyofilm yapma özelliğini yitirmişselerde, evcilleştirilmemiş *Bacillus subtilis* NCBI 3610 suşu sıvı veya katı yüzeylerde biyofilm yapabilme özelliği taşımaktadır. Bir yüzeyde koloniler halinde tutunarak yaşayabilen mikroorganizmaların oluşturdukları her tabaka biyofilm yapısının özelliklerini taşımamaktadır. Biyofilm, bakteriler tarafından oluşturulan, herhangi bir yüzeye, yapışmalarını sağlayan ve büyüme oranları ve gen transkripsiyonuna bağlı olarak farklı fenotip gösterebilen ve oluşturan mikroorganizmanın içinde gömülü olarak bulunduğu hücredışı polimerik maddeden (EPS) oluşmuş matriks olarak tanımlanmıştır. Biyofilm oluşum süreci hücre dışı sinyalin algılanması ile başlar. Bu hücre dışı sinyallere örnek olarak, mesela *B. subtilis* de üretilen, lipopeptid antibiyotik sürfaktin, biyofilm matriks bileşen genlerinin ekspresyonunu indükler. İlk başta, biyofilm oluşturacak hücreler hareketli yapıdadır (hücrelerde hareket özelliğinin, biyofilm oluşumu için ilk adım olduğu düşünülmektedir). Biyofilm matriksi EPS olarak da bilinen hücredışı polimerik maddelerden oluşmaktadır ve biyofilm içerisinde yaşayan mikroorganizmalar tarafından sentezlenen polisakkaridler biyofilmin ana komponentini oluştururlar. Ayrıca bu matriksin içinde proteinler, lipidler ve diğer biyopolimerler gibi makromoleküller de bulunmaktadır. Bu matriksi oluşturan faktörler iyi bilinse de, bu sürecin başlaması icin gerekli sinyaller cok az bilinmektedir.

Antibiyotiklerin sadece antimikrobial aracı olarak hareket etmediği, belirli genlerin ifade edilmesini düzenleyerek molekülleri uyardığı uzun zaman önce keşfedildi. Subtilisin gibi bazi antimikrobiyal ajanlarin hücre yoğunluğu algılama sinyal molekülü olduğu ispatlanmış olsa da, cok sayıda antibiyotiğin antimikrobiyal etkileri dışında ki rolleri bilinmemektedir. Basilisin, geniş bir bakteri yelpazesi ve bazı mantarlara karşı aktif bir antibakteriyel ajan olan, L-anticapsin ile L-alanin'den oluşan, belirli *Bacillus* suşlari tarafından sentezlenen bir dipeptid antibiyotiktir. Basilisinin antimikrobiyal etkisi protein kökenli olmayan bir amino asit olan L-antikapsin tarafindan gerçekleştirilir. Basilisin biyosentezinin polisistronik operon *ywfBCDEFG* (yeni ismiyle *bacABCDEF*) 'a bağlı olduğu ve monosistronik geni *ywfH* ile çok yakın olduğu kısa bir zaman önce keşfedildi. Bunu takiben, bu genler

arasından, BacA, BacB, YwfG ve YwfH proteinleri antikapsin üretiminde görev aldığı, BacD proteininin, L-alanin ve L-antikapsinin ligasyonundan sorumlu olduğu, bir hücre transport proteini olan BacE proteininin ise, basilisin immünitesinden sorumlu olduğu gösterilmiştir. *Bacillus subtilis*'de basilisin biyosentezi; degredatif enzim üretimi, kompetens gelişimi, diğer *Bacillus* antibiyotiklerinin üretimi, sporlanma prosesi ile birlikte global hücre yoğunluğu sinyal regulasyon ağı tarafından düzenlendiği bulunmuştur. Bunun yanı, *B. subtilis* de sporlanma prosesinin başlatılmasında anahtar rolü oynayan global regülatör Spo0A basilisin biyosentezini pozitif yönde regüle ederken, global geçiş fazı düzenleyicileri AbrB ve CodY tarafından, logaritmik fazda baskılandığı ortaya çıkarılmıştır. Aynı zamanda basilisin biyosentezinin besinsel yönden fakir bir ortamda bakterilerin kıtlık ortamlarına adaptasyonunu sağlayan ve dört-beş fosforlu guanin nukleotid (ppGpp) moleküllerinin kontrolununu içeren "stringent" global kontrol sisteminin etkisi altında olduğuda gösterilmiştir.

Her ne kadar basilisin biyosentezinin sentezleme mekanizması ve düzenlenmesi iyi bilinse de host organizmada basilisin'in rolü çok daha az bilinmektedir. Bu çalışmada öncelikle, biofilm oluşum koşullarında, basilisin üretemeyen mutant suşda, biyofilm matriks genleri, kemotaksis ve kayma hareketliligi genleri, ve bu prosesleri düzenleyen regülatör genlerin ifade etme seviyeleri incelenerek, basilisinim *B. subtilis* NCBI 3610 suş üzerindeki etkilerinin aydınlatılması hedeflenmiştir.Bu amaç doğrultusunda, öncelikle, hem ana suş *B. subtilis* NCBI 3610 hem de basilisin üretemeyen Bac$^-$ *B. subtilis* NCBI 3610, biyofilm oluşturma besi yeri olan MSgg katı besi yerinde 3 gün boyunca büyütülerek, 24, 48 ve 72 saat aralıkları ile hücreler toplanmış ve bu hücre örneklerinden total RNA izole edilmiştir. Gen ifade miktarlarını belirlemek çin, ters transkripsiyon kuantitatif polimeraz zincir reaksiyonu (RT-qPZR) yöntemi uygulanmıştır. Reaksiyon spesifitesi, erime eğrisi analizi aypılarak takip edilmiştir. $2^{-\Delta\Delta Cp}$ method kullanılarak, relatif gen ekspresyon miktarları hesaplanmış ve Log2 değerleri alınmıştır. Sonuç olarak, hareketlilik ve kemotaksis genlerinin ekspresyonundan sorumlu alternatif sigma faktörü SigmaD(σ^D) kodlayan *sigD* geni, bununla uyumlu olarak kemotaksis genleri *cheY* ve *cheB* gen ekspresyonlarının basilisin yokluğunda bir hayli düştüğü bulunmuştur. Bu sonuçlarla uyumlu olarak biyofilm ve kayma hareketliliği için gerekli olduğu gösterilen surfaktin biyosentezinden sorumlu *srfA* operonunda gen ifadesinin, basilisin yokluğunda bir hayli düştüğü bulunmuştur. Biyofilm matriksini oluşturan yapısal genleri kodlayan *eps* operonu içinde yer alan *epsD*(*yveN*), *epsO* (*yveO*) ve *epsK*(*yvfA*) genlerinin de basilisin yokluğunda ifadelerinin azaldığı gösterilmiştir. Benzer şekilde, *B. subtilis'*de geçiş fazında yada besin kıtlığında, hücre dışı parçalayıcı enzim üretimini indükleyen, kompetans gelişimi ve biyofilm oluşumu için gerekli ana regülatörler arasında bulunan DegU proteinini kodlayan *degU* gen ifadesinin ve DegU regülonu içerisinde yer alan hücredışı alkalin serin proteaz kodlayan *aprE* genininde gen ifadesinin bir hayli düştüğü tespit edilmiştir. Elde edilen bu gen ekspresyonu verilerinin yanında ayrıca, basilisin üretemeyen mutant suş ile ana suş 3610 nun biyofilm oluşturma ve kayma hareketliliği özellikleri, katı besi yerinde analiz edildiğinde, basilisin üretemeyen mutant suşun biyofilm oluşturma ve kayma hareketlilik özelliğini tamamen kaybettiği gözlenmiştir. Biyofilm oluşturma kapasiteleri sıvı besi yerinde kıyaslandığında, basilisin üretemeyen mutant suşun hiç bir şekilde yüzeyde biyofilm oluşturamadığı ayrcı sabit sıvı ortamında, ana suş gibi tüm alana dağılarak homojen büyüme yerine, minimal bir besi yeri olan MSgg besi yerinde tabanda kitleler oluşturarak toplu toplu

büyüyebildiği gözlenmiştir. Bu durum basilisin yokluğunun herhangi bir okzotrofiye neden olabileceği olasılığını akla getirmekle birlikte, Basilisin üretemeyen Bac$^-$ *B. subtilis* NCBI 3610 suşunun denenen kompleks, minimal ve sıvı biyofilm oluşturma besi yerinde çalkalamalı ortamda rahatlıkla büyüdüğü, ana suş ile aynı büyüme hızını gösterdiği, herhangi bir okzotrofik bozukluğunun bulunmadığı anlaşılmıştır. Tüm bu sonuçlar basilisinin ilgili üretici suşta, kemotaksis, kayma harketlilik ve biyofilm oluşum mekanizmalarını pozitif yönde kontrol edilmesini sağlayan bir sinyal molekülü olduğuna işaret etmektedir.

Bu çalışmada aynı zamanda, biyofilm oluşturma katı ve sıvı besi yerinde glikoz, galaktoz ve ksiloz gibi faklı karbon kaynaklarının, basilisin üretemeyen mutant ve ana *B. subtilis* NCBI 3610 suşlarında, biyofilm oluşumunu nasıl etkilediğide incelenmiştir. Glikozun ana suşda sıvı ve katı besi yerinde hem büyümeyi hemde biyofilm oluşumunu desteklediği gözlenirken, ksilozda katı besi yerinde oluşturulan koloni büyüklüğünün glikoza göre belirgin şekilde küçüldüğü fakat kompleks koloni morfolojisinin yada diğer deyişle biyofilm oluşumunun gerçekleştiği görülmüştür, ayrıca sıvı besi yerinde glikoza kıyasla daha az yüzey büyümesi gözüksede 72. saat sonunda belirgin bir yüzey biyofilmi oluştuğu görülmüştür. Galaktoz içeren sıvı besi yerinde, glikoza ve ksiloza göre daha az bir yüzey büyümesi oluşmuş fakat ksiloz gibi 72. saat sonunda gene belirgin bir yüzey büyümesi oluşturulurken, galaktoz içeren katı besi yerinde, büyümenin bir hayli kısıtlandığı, biyofilm oluşumu olmadığına işaret eden, küçük pürüzsüz bir koloni oluşumu gözlenmiştir. Basilisin üretemeyen mutant suşda ise glikoz, ksiloz ve galaktoz içeren sıvı besi yerinde belirgin bir büyüme gözlenmezken, glikoz, ksiloz ve galaktoz içeren katı besi yerlerinde bir hayli küçük pürüzsüz koloni oluşturabildiği gözlenmiştir. Özellikle mutant suşda açığa çıkan bu büyüme kısıtlaması mutant suşun oksijen çözünürlüğünün kısıtlı bulunduğu sabit ortamda glikoz, ksiloz ve galaktozu kullanmada sorun yaşadığı olasılığına işaret etmektedir. Nitekim, glikoz, ksiloz ve galaktoz içeren aynı besi yerine ekilen fakat çalkalanmıs ortamda büyütülen mutant suşun, glikoz ve ksiloz içeren sıvı besi yerinde rahatlıkla büyüdüğü ve hatta ana suşla aynı spesifik büyüme hızına sahip olduğu ve durağan faza ana suşa kıyasla daha geç girdiği gözlemlenmiştir. Diğer taraftan, galaktoz içeren çalkalamalı ortamda, spesifik büyüme hızının ana suşa göre biraz daha yavaşladığı ve durağan faza daha erken girdiği gözlenmiştir.

Kompleks bir besi yeri olan Luria sıvı besi yerinde *B. subtilis* NCBI 3610 suşunun biyofilm oluşturamadığı bilinmektedir. Bu çalışmada son olarak, böyle bir ortamda basilisinin sinyal molekülü gibi davranıp biyofilm oluşumunu tetikleyebilme olasılığı da araştırılmıştır. Bu amaçla basilisin üretici suş *B. subtilis* PY79 basilisin üretim besi yeri PA sıvı besi yerinde 16 saat büyütülerek kültür sıvısından basilisin konsantresi hazırlanmış, artan konsantrasyonlarda, sabit LB besi yerine eklenerek, ekilen *B. subtilis* NCBI 3610 kültürlerinin yüzey büyümeleri üç gün boyunca izlenmiştir. Basilisinin tam anlamı ile biyofilm oluşumunu tetiklemesede, biyofilm oluşumunun ilk basamağı sayılan yüzey büyümesini belirgin bir şekilde tetiklediği gösterilmiştir.

1. INTRODUCTION

1.1 Biofilm: A general overview

Microorganisms exist in nature either as planktonic (free floating single cells) or as a biofilm (sessile form). The latter is thought to correspond to more than 99% of all living microorganisms on earth though this ratio was expected much less before. In fact, planktonic living form of microorganisms is now well understood that it is rarely found in nature. All began with the investigation of microbial aggregates in the form of dental plaques and tartar present in a tooth surfaces by Antonie van Leeuwenhoek in 1683 with his very simple microscope (Tal, 1980). In 1940, Heukelekian and Heller observed that the number of bacteria attached is directly proportional with the increase in the surface area. Using glass beads in the medium, they observed many bacteria remaining attached onto the beads after the medium was poured off following the elution of those beads with fresh sterile water. As a result, bacterial attachment to any surface resulted to the increase of bacterial growth and activity (Heukelekian & Heller, 1940). Another important observation throughout history was the discovery of the number of bacteria being much higher in the surfaces of medium instead of the surrounding medium (Zobell, 1943). Kusnetzowa (1937) previously claimed that all seawater bacteria are able to attach to glass surfaces. Until the development of high resolution microscopes, there were vast amount of questions waiting to be answered. Although many of the questions related to regulation still unclear, recent visualization and staining techniques have led us know much more about the morphological structure and properties of a biofilm matrix. First observation on matrix component was made by Jones and colleagues in 1969. They used previously known staining method specific for polysaccharides with simple modification. Ruthenium red and fixative agent osmium tetroxide was combined and after the material (slime layer) was stained, the sample was visualized under electron microscope which showed that the surrounding material around bacterial cells was polysaccharide-like structure (Jones *et al*., 1969).

1.2 Definition of Biofilm

A biofilm is an aggregation of microbial communities irreversibly attached to a liquid or solid surface and encased in the tight self-produced matrix structure mainly composed of polysaccharide material. The matrix may be comprised by one or more bacterial species. It can form on a living (biotic) or non-living (abiotic) surfaces such as living tissues, indwelling medical devices, industrial or potable water system piping, natural aquatic systems (Donlan & Costerton, 2002; O'Toole et al., 2000). Biofilm usually contains more than one type of species but there are numerous cases where only single microbial species can correspond to single biofilm and these mostly are infectious microbial species. *Pseudomonas aeruginosa* is a well-studied as a single species biofilm forming bacterium following *Pseudomonas fluorescens, Escherichia coli, Vibrio cholerae* among gram-negative and *Staphylococcus epidermidis, Staphylococcus aureus*, enterococci and *Bacillus subtilis* among the gram-positive counterparts (O'Toole *et al*., 2000; Makin & Beveridge, 1996; Vlamakis *et al*., 2013). Biofilm is a well-studied subject due to being very problematic in medical, clinical and industrial issues (Donlan & Costerton, 2002; Dror-Ehle *et. al*., 2010). On the other hand, there are some beneficial aspects of biofilm forming bacteria in the cases of wastewater treatments and energy source in the form of microbial fuel cells (Logan & Regan 2006; Nicolella *et al*., 2000).

1.3 *Bacillus subtilis*: a model organism as a biofilm forming bacterium

B. subtilis is a non-pathogenic, motile, gram-positive, rod-shaped bacterium which is predominantly studied organism in the field of molecular basis of biofilm formation. As a chemoorganotroph, this bacterium is able to survive in a very simple growth conditions such as medium containing only salt, carbon and nitrogen with simple aeration (Nicholson *et. al*., 1990). Furthermore, *B. subtilis* is able to survive and adapt to various limiting environment by activating its competence and sporulation responsible genes (Vlamakis *et al*., 2013; Lemon *et al*., 2008; Grossman, 1995). This bacterium is capable of producing extracellular macromolecule degrading enzymes in response to starvation in a limited nutrient resource. In order to avoid competitors, Bacillus species secrete various antibiotics where some of them are peptide antibiotics such as bacilysin. Induction of motility and chemotaxis are another two important features of Bacillus species as a response to limited nutrient source

(Msadek, 1999; Kenig & Abraham, 1976). *B. subtilis* whole genome sequence was completed and published in 1997 coordinated by 33 different laboratories around the world (Kunst *et al*., 1997). *B. subtilis* genome size is around 4.2Mb corresponding to 275 genes (25 of unknown function), 17 sigma factors, around 250 DNA binding transcriptional regulators, 4106 protein coding genes, 86 tRNA, 30 rRNA and 3 small stable RNA genes (Kobayashi & Ogasawara, 2002). *B. subtilis* is able to synthesize many different types of antibiotics and peptides both ribosomally and post-translationally modified (lantibiotics & lantibiotic-like peptides) or non-ribosomally against wide-range bacteria and fungi (Stein, 2005). Interestingly, other than being an alternative source of antibiotics, *Bacillus subtilis* is also used for a novel prophylactic, therapeutic and as a growth-promoting agent in many different studies (Hong *et al*., 2005; Williams, 2007; Gobbetti *et al*., 2010)

1.4 The Life Cycle of a *Bacillus subtilis* Biofilm Formation & Regulation and Swarming Motility

Undomesticated strains of *Bacillus subtilis* (NCIB 3610) are able to form robust biofilms called as pellicles both at non-agitated liquid and solid surfaces (Branda *et al*., 2001). Biofilm formation begins following to an external signal such as lipopeptide surfactin which induces the expression of matrix components genes. Initially, biofilm cells are motile (meanwhile swarming for most bacteria is believed to be the preliminary step while forming biofilm) but as biofilm structure develops most cells become non-motile adhered to both each other and surface while extracellular matrix builds up. Unlike biofilm cells in the matrix, motile cells and spores are spatially and differently organized inside maturing biofilm matrix but yet swarming and biofilm share many common features (Figure 1.1). Biofilm matrix is mainly composed of so called EPS referring to “Extracellular Polymeric Substances” including macromolecules such as polysaccharides, proteins, nucleic acids, lipids and other biopolymers (Vlamakis *et al*., 2013; Branda *et al*., 2005; Wingender *et al*., 1999).

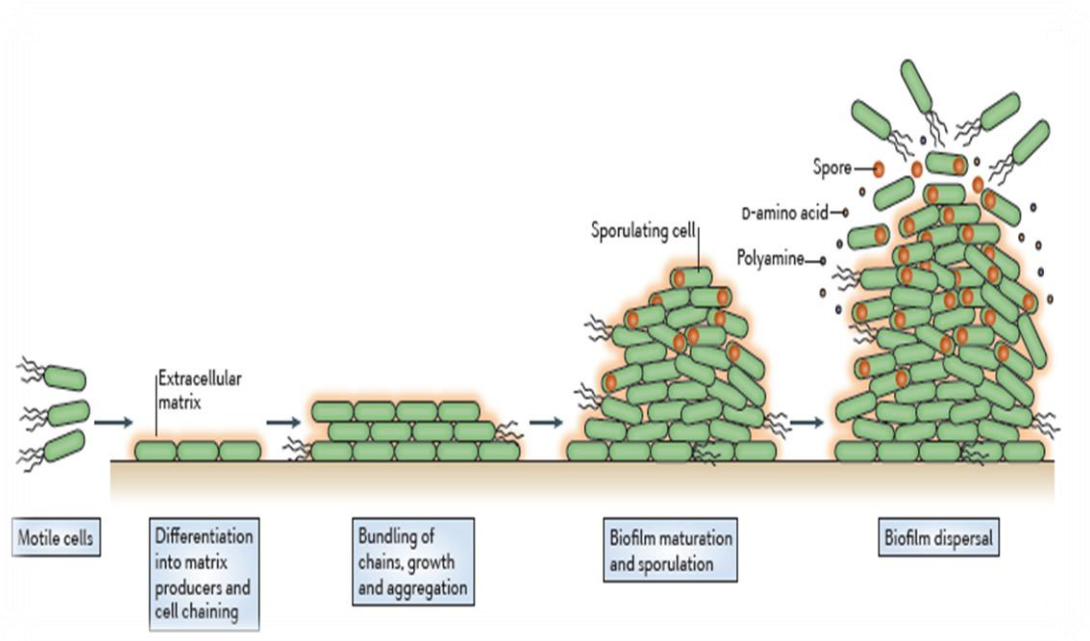

Figure 1.1: *Bacillus subtilis* biofilm formation pathway. The formation of a biofilm occurs in many stages; motile cells differentiate into matrix producers and cell chaining where motile cells become sessile, chains, growth and aggregation are bundled and biofilm maturation and sporulation at some sites take place. Finally, biofilm dispersal may occur at different sites (Vlamakis *et al*., 2013).

Bacillus subtilis NCIB 3610 mutants that lack mature biofilm structure showed that the biofilm architecture can be affected by many factors such as hydrodynamic conditions, nutrient concentration, bacterial motility and intracellular signaling, exopolysaccharides and proteins (Flemming & Wingender, 2010). Secreted protein TasA is vital for the structural integrity and formation of a biofilm in the form of fruiting body like structure as described by Branda *et al*. (2006). It is clear that sporulation pathway regulators Spo0A and sigma factor σ^H together are responsible for the regulation of the operon *epsA-O*. *yhxM* and *epsA-O* (15 gene long) are the genes that govern the synthesis of the enzymes necessary for EPS synthesis during biofilm formation in *B. subtilis* (Branda *et al*., 2004; Hamon & Lazazzera, 2011). Moreover, genes within *yqxM-sipW-tasA* operon encode other proteins that are related to extracellular matrix of biofilm architecture. It was also shown that sipW which is a type I signal peptidase necessary for the products namely TapA (formerly known as yqxM) and tasA in the *tapA-sipW-tasA* operon may also be required for the structural quality of biofilm formation. To simplify, the product of *sipW* converts the pre-proteins TapA and TasA into their mature forms. TapA controls the polymerization of TasA and anchors it in the cell wall (Romero *et al*., 2011). Another

role of SipW is that when *B. subtilis* forms biofilm on solid surface SipW acts as an inducer in the expression of the *tapA-sipW-tasA* and *epsA-O* operons (Terra, 2012). Both *epsA-O* and *tapA-sipW-tasA* operons are repressed by SinR which is known as the master regulator of biofilm formation in *B. subtilis* as shown in figure 1.2. SinR promotes cell motility and separation and that is why it acts as a switch between planktonic and biofilm form of the *B. subtilis* lifestyle (Kearns *et al*., 2005). During the promotion of biofilm formation, Spo0A induces the expression of *sinI* whose product SinI is an antagonist of the repressor SinR by protein-protein interaction (Shafikhani *et al*., 2002; Bai *et al*., 1993). Another regulatory protein Slr, which is negatively controlled by SinR, induces the transcription of *tapA-sipW-tasA* operon (Chu *et al*., 2008). Several other important genes have been discovered for proper biofilm development. For instance, *pgcA* (formerly *yhxB*) mutant strain resulted in impaired biofilm formation which suggests that the gene is responsible for the transcription of the enzyme that catalyzes the production of nucleotide sugars necessary for the biosynthesis of EPS (Lazarevic *et al*., 2005). In addition, genes required for the activation of *spo0A* namely *spo0B*, *spo0F* and *kinC-E* were also proved that they have curial role on biofilm formation. *yveQ* and *yveR* were shown to be essential for the production of an exopolysaccharide component of extracellular matrix of the biofilm. Surfactin mediated genes *srfAA* and *sfp* are required for proper biofilm formation (Branda *et al*., 2004; Hamon & Lazazzera, 2011). *ecsB*, gene that encodes transmembrane subunit of ABC trasporter, mutant strain resulted in smooth pellicles. Inactivation of *yqeK* (gene encoding phosphatase) was shown to be responsible for community development during biofilm formation. Two genes of unknown functions *ymcA* and *ylbF* mutant 3610 strain accounted for flat and undifferentiated colonies (Branda *et al*., 2004).

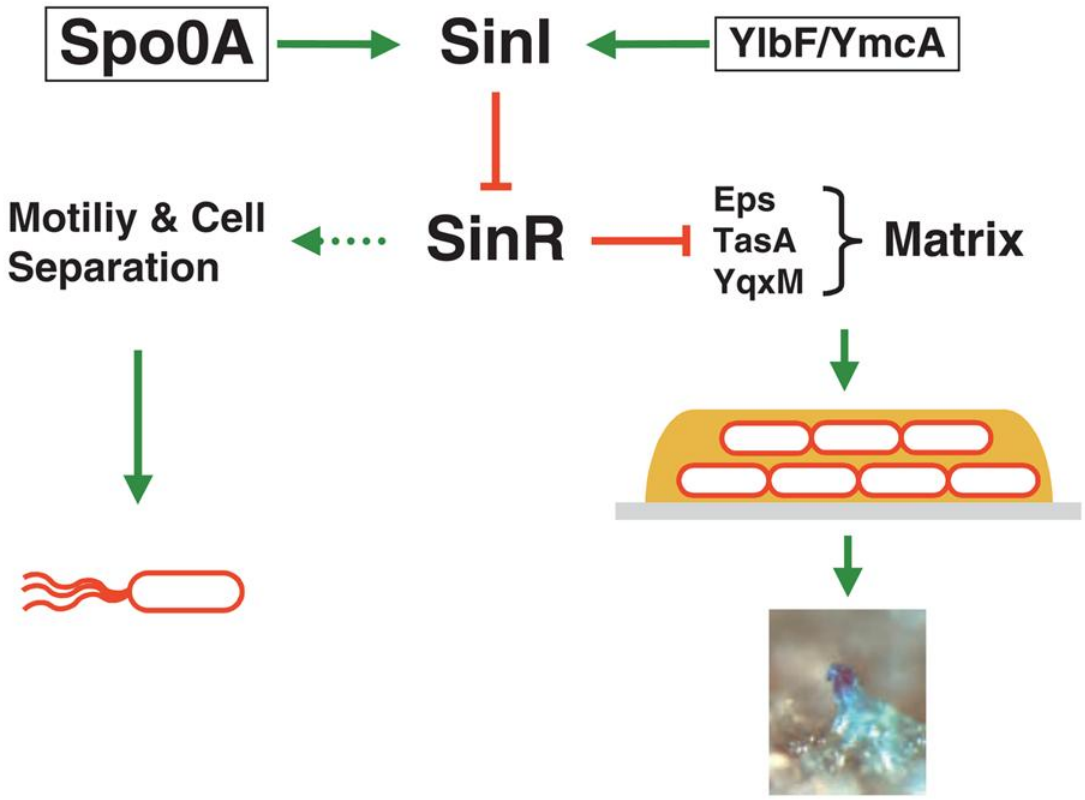

Figure 1.2: Regulation of *B. subtilis* biofilm formation. SinI is induced by Spo0A, Ylb and YmcA. SinI allowing *B. subtilis* to switch to sessile form of lifestyle represses SinR, which is a repressor of matrix components Eps, TasA and YqxM and an inducer of cellular motility and separation (Lemon *et al*., 2008).

1.5 Quorum Sensing

It is now evident that bacteria use different classes of more than one type chemical signal to coordinate and communicate both within and between species. This hierarchical regulatory circuit ensures the bacterial survival and interaction in their natural habits. Such special monitoring and regulatory system is known as quorum sensing (QS) and signal molecules are termed as "autoinducers" (Miller & Bassler, 2001; Daniels, 2004). The mechanism is responsible for the regulation of motility, chemotaxis, degradative enzyme secretion, antibiotic synthesis, competence development and sporulation during the survival of *Bacillus subtilis* as a response to changes in population density (Msadek, 1999). As the population density increases, the number of autoinducer secreted increases until threshold value has reached which may result to any response mentioned above. In this case, the gene expression is altered and bacterial community begins to show different adaptation behaviors. Consequently, QS allows bacteria to respond as a multicellular organism (Schauder &

Bassler, 2001). Quorum sensing system differs among gram-positive and gram-negative bacteria. Gram-negative bacteria use AHL (acyl-homoserine lactone) and peptide-based signaling system. On the other hand, gram-positive bacteria use AI2 (autoinducer 2) system (Bassler, 2002). Autoinducer is synthesized inside the cell and then secreted outside of the membrane. Then, it is recognized by specific receptors such as ComX pheromone (competence development regulator) in *B. subtilis* (Winzer, 2002). Secondary metabolites, molecules such as toxic metabolites and by-products are secreted to the extracellular matrix and these metabolites may influence the expression of specific genes. Furthermore, antibiotics also may play role in QS machinery and it might be considered as QS molecule. For instance, an antibiotic subtilisin produced by *B. subtilis* was previously discovered as QS molecule. Yet, many of others remain unknown (Yim *et al*, 2006; Kleerebezem, 2004). In gram-negative bacteria LuxI is responsible for the production of acyl homoserine lactones (AHLs) which are detected by LuxR-type protein. After the diffusion of AHLs into the extracellular space, AHL bounded LuxR is activated and *lux* operon (*luxICDABEG*) is transcribed. Figure 1.3 represents simplified scheme of the mechanism of QS process in gram-negative bacteria (Miller & Bassler, 2001)

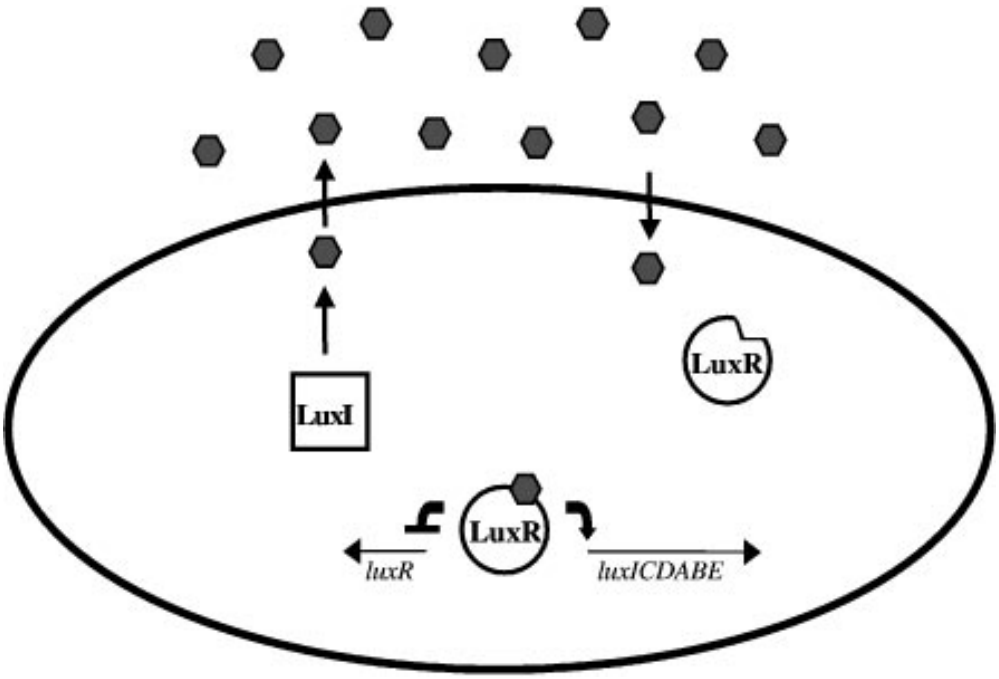

Figure 1.3: QS in gram-negative bacteria (Miller & Bassler, 2001).

Luciferase is an enzyme complex which shows bioluminescence when its presence is achieved to the specific level. Transcription of lux operon leads to the activation of

luciferase gene in *V. fischeri* thus showing bioluminescence and representing the high cell density (Miller & Bassler, 2001). It is well known fact that there are many types of AHLs that are utilized by gram-negative bacteria used as a regulatory mechanism in order to adapt to the environmental changes. Moreover, it is evident that most bacteria can produce more than one type of AHL that are characterized by acyl chains of variable length, saturation level and oxidation state (Withers *et al*., 2001).

Gram-positive bacteria use different QS molecule than gram-negative bacteria. LuxI/R system or N-acyl homoserine lactone-like signal is not involved in gram-positive bacterial quorum sensing system. Rather, small peptides are used as signaling molecules that are modified oligopeptides that are synthesized and secreted to the extracellular matrix. Here, two-component signal transduction proteins namely sensor histidine kinase and a response regulator play major role by detecting fluctuations in the environmental stimuli and trigger signal transductions by using phosphorylation to transmit information. As a result, transcription of QS genes is controlled directly or indirectly by phosphorylation of the response regulators that either repress or induce the DNA-binding activity of the regulator (Henke & Bassler, 2004; Bassler, 1999).

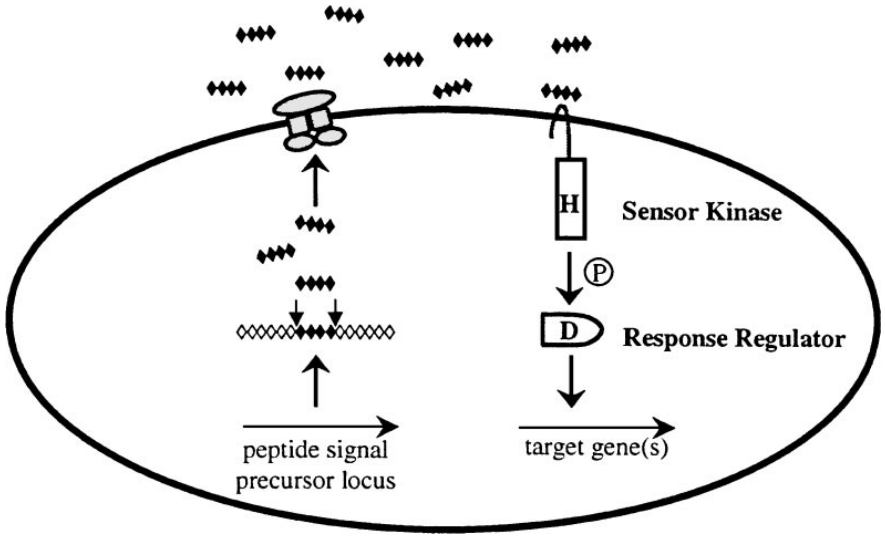

Figure 1.4: An overview of the gram-positive oligopeptide two-component system. Firstly, the peptide signal is exported outside of a cell by ABC transporter system. After the amount of signal is reached to the level desired, sensor kinase can detect it so that response regulator is phosphorylated on a conserved aspartate residue. Consequently, it is the phosphorylated form of the response regulator that can activate the target gene(s) (Miller & Bassler, 2011).

Interestingly, it was discovered that *Vibrio harveyi*, which is a gram-negative bacterium, is able to secrete the molecules belonging to both gram-negative and gram-positive bacteria (Figure 1.5). *V. harveyi* secretes and responds to AHL type signal (autoinducer 1). Unlike gram-negative bacteria, the signal detection consists of two component proteins as it is in gram-positive bacteria. Furthermore, it was found that Al-2 (autoinducer 2) is present in *V. harveyi* and together with Al-1 they play major role in the regulation of luciferase operon (Henke & Bassler, 2004).

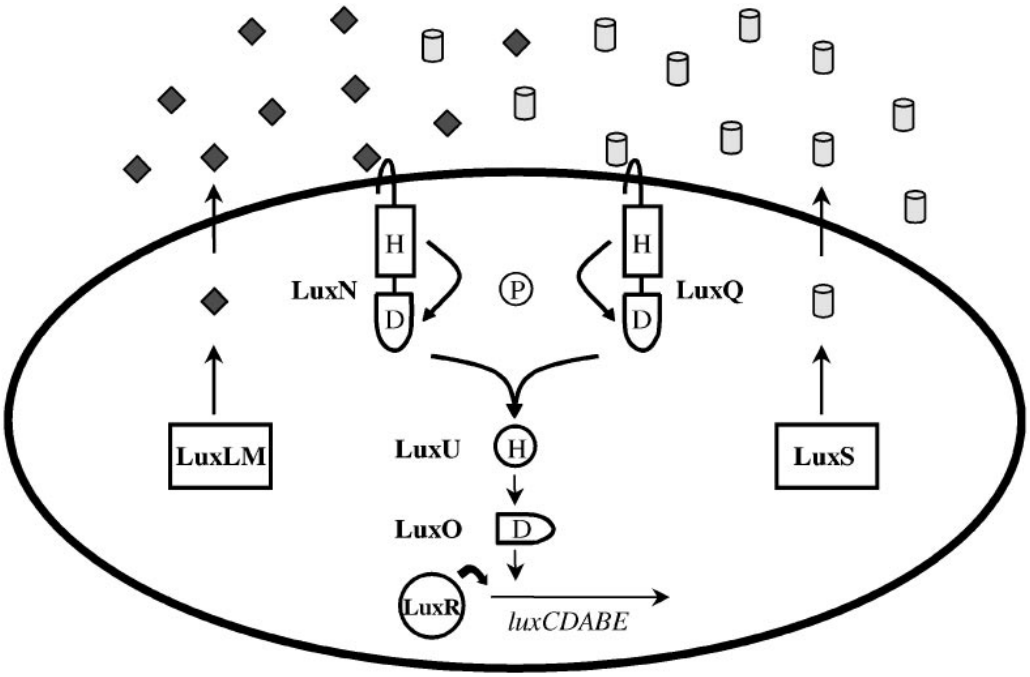

Figure 1.5: The combination of two QS systems in a *V. harveyi* which alone can perform the regulation of density-dependent light production using both AI-1 and AI-2 systems. Diamonds stand for AI-1 which is *N*-(3-hydroxybutanoyl)-homoserine lactone and cylinders refer to AI-2 with unknown structure. As it can be seen, proteins LuxLM and LuxS are vital components for the production of AI-1 and AI-2 autoinducers. LuxN is responsible for the detection of AI-1, whereas LuxQ for AI-2. LuxU is an integrated phosphotransferase protein which conveys the sensory information to response regulator protein LuxO. LuxO negatively controls the luciferase structural operon *luxCDABE*. LuxR which is a transcriptional activator is also needed for the expression of luciferase *luxCDABE* operon. Under low cell density, LuxN and LuxQ autophosphorylate and transfers phosphate via LuxU to LuxO where in this case phosphorylated form of LuxO inactivates LuxR resulting to the indirect repression of *luxCDABE*. On the contrary, under high cell density LuxN and LuxQ changes their autoinducer ligands from kinases to phosphatases. Therefore, LuxU removes the phosphate from LuxO which in that case cannot repress LuxR. LuxR alone is able to bind to *luxCDABE* promoter resulting to the expression of the operon and light production (Miller & Bassler, 2001).

In *B. subtilis*, QS mechanism is responsible for several behaviors such as sporulation, the production of degradative enzymes and exopolysacharides, secretion of antibiotics, motility and genetic competence development. Two important signaling peptides ComX pheromone and CSF (competence and sporulation factor) are important for the regulation of ComA which is an activator of many genes such as for the development of genetic competence (Schneider *et al*., 2002; Solomon & Grossman, 1996; Lazazzera *et al*., 1997). ComX pheromone is encoded by *comX* that is located in *comQXPA*. Genes controlling competence development in Bacilli are classified into two groups; early and late competence genes. Early competence genes are required for the transfer of the information to the late genes that carry out the development of competence apparatus requiring *comC, comE, comF*, *comG* and *nucA*. ComX pheromone plays vital role in the controlling of competence development whereas CSF as the name implies is able to direct both competence development and sporulation in *B. subtilis*. Both ComX pheromone and CSF move together to activate response regulator and transcription factor ComA. Additionally, ComA is regulated by several other density-dependent signaling pathways. Four pentapeptides namely PhrC, PhrF, PhrH, and PhrK are secreted to the milieu and then transported back into the cell via oligopeptide permease. Pentapeptides inside bind and inhibits the activity of their cognate Rap proteins (RapC, RapF, RapH, RapK). Rap proteins RapC, RapF, RapH and RapK then inhibit the activity of ComA by binding its target site (Griffith & Grossman, 2008). *comQ* is important for the maturation and activation of ComX pheromone (Magnuson, 1994; Schneider *et al*., 2002). In a high cell density, the expression of *comX* limits the production of ComX suggesting that pheromone available in the environment optimizes the schedule of the expression of QS genes such as *srfA* which is a surfactin biosynthetic operon (Schneider *et al*., 2002). In *B. subtilis*, two-component transduction system involves ComP-ComA regulatory proteins. They are composed of many characteristic domains such as a histidine kinase/sensor and a response regulator which can be phosphorylated on both histidine and aspartate residues. Sensory domain of histidine kinase detects an external signal and activates the enzyme that catalyzes an ATP-dependent trans-autophosphorylation reaction. The transfer of phosphoryl group from histidine residue to aspartate determines the activation of the effector domain resulting to specific output response. Then, phosphorylated ComA activates the expression of several genes including *comS*. ComS dissociate ComK from ClpC

(chaperon-like protein) and MecA resulting to the released active ComK. ComK, then, binds to its own promoter and consequently increasing ComK promotes the transcription of late competence genes that build up the DNA-uptake machinery (Alex & Simon, 1994; Susanna *et al*., 2006; Turgay *et al*., 1997). In addition, factors necessary for the synthesis of degredative enzymes, response regulator DegU and histidine protein kinase DegS are involved in the initiation steps of competence development. Phosphorylated DegU activates the production of degredative enzymes while DegU alone binds to *comK* promoter and stands as a competence development factor (Hamoen *et al*., 2000; Hamoen *et al*., 2003). However, beside DegU and ComK itself, transcription factors AbrB, CodY and Rok are also involved in the direct control of *comK* promoter. AbrB simply binds to the *comK* transcription initiation site so that RNA polymerase cannot interact with promoter region and transcription is repressed. AbrB inhibits premature expression of *comK* and has a stimulatory effect on competence development. Rok protein encoded by *rok* is a repressor of *comK*. However, with the increase in concentration of ComK, *rok* expression is inhibited (Hamoen *et al*., 2003).

Second important signaling peptide involved in the *B. subtilis* density-dependent competence development control is competence sporulation factor CSF (Figure 1.6). CSF is a penta-peptide encoded by *phrC*. *phrC* transcription is regulated by RNA polymerase σ^H. During the exponential growth, σ^H concentration decreases while in the stationary phase the concentration is high which promotes the induction of *phrC* transcription. In this case, internal or external concentration level of CSF determines whether the cell goes to competence development or sporulation. When CSF concentration in milieu is in a critical level, it is transported back into the cell by an ATP-binding cassette transporter oligopeptide permease (Opp). High σ^H levels means high extracellular CSF concentration which corresponds to the concentration of peptide signal that promotes sporulation by interacting with histidine-protein kinase ComP. On the other hand, low extracellular CSF concentration results in a low internal CSF level suggesting that the cell then goes to competence development by the inhibition of the activity of aspartylphosphate phosphatase namely RapC which is a negative regulator of *srfA*. In addition, the inhibition of RapB, aspartyl-phosphate phosphatase, by CSF is required for the induction of sporulation (Lazazzera *et al*., 1997; Lazazzera & Grossman, 1998; Perego, 1997; Pottathil *et al.*, 2008; Solomon *et*

al., 1996). Initiation of sporulation requires master regulator Spo0A which is positively regulated by PhrA. RapA and RapB repress this regulation by dephosphorylating Spo0F. In order to overcome the inhibition of sporulation, CSF together with PhrA blocks the phosphatase activity of these two phosphatases, RapB and RapA respectively. Phosphorylated Spo0A directs sporulation. The mechanism involves a complex phosphorelay system and a signal cascade involving different histidine kinases. First, Spo0F is phosphorylated by KinA, KinB, KinC, KinD and KinE. Phospho-Spo0F transfers its phosphate to Spo0B. Spo0B is a phosphotransferase which phosphorylates the transcription factor Spo0A (Grossman, 1995; Perego *et al*., 1994). Thus, accumulation of phosphorylated Spo0A directs the transcription of at least seven genes corresponding sporulation by binding to their promoters (Baldus *et al*., 1994).

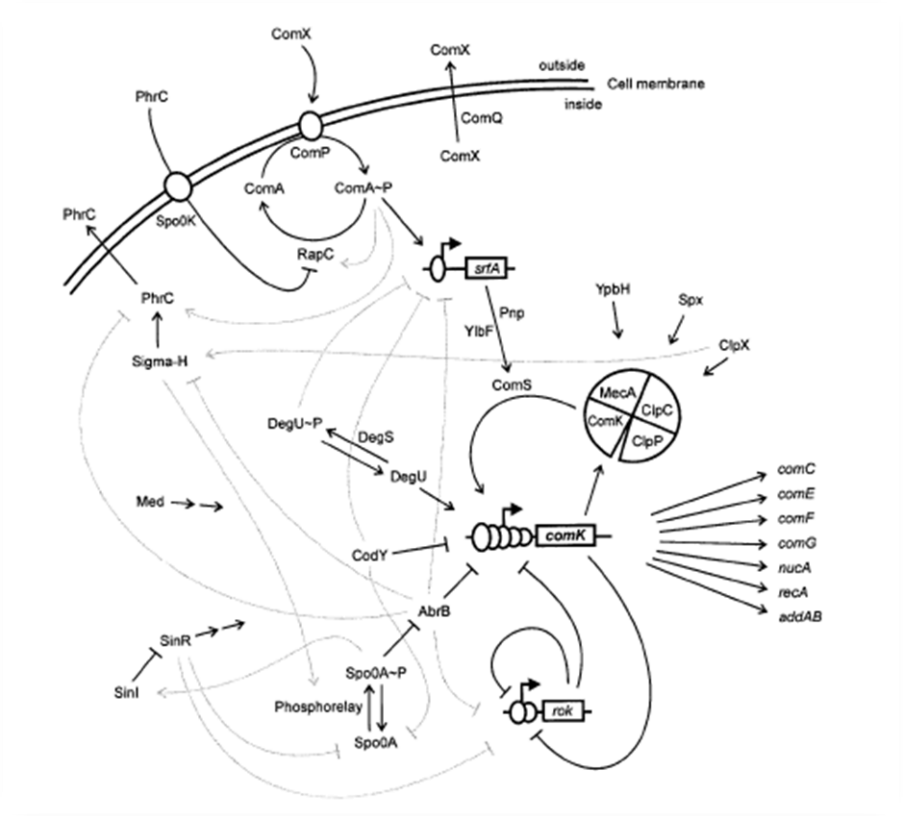

Figure 1.6: Various competence development regulation pathways of *B. subtilis* (Hamoen *et al*., 2003).

1.6 Dipeptide Antibiotic Bacilysin

Bacilysin (Figure 1.7) is a dipeptide antibiotic composed of L-alanine residue at the N-terminus and non-ribosomally synthesized L-anticapsin aminoacid at the C-

terminus (Walker & Abraham, 1970). Bacilysin synthetase is an enzyme responsible for the non-ribosomal catalysis of peptide formation with L-alanine (Sakajoh *et al*., 1987). Baciylsin displays antimicrobial activity against many bacteria and some fungi.

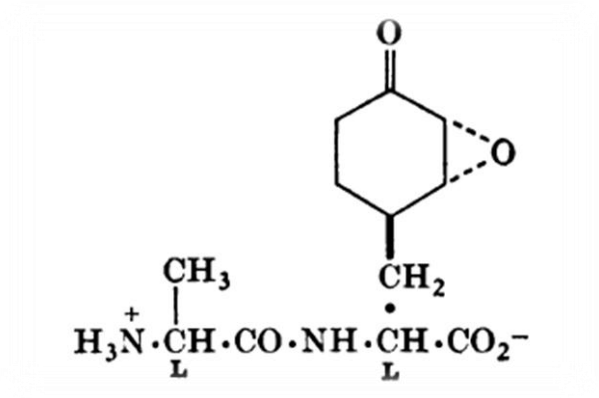

Figure 1.7: Chemical structure of bacilysin (Walker & Abraham, 1970).

Bacilysin antimicrobial activity depends on the release of L-anticapsin that is carried out by certain peptidases (Steinborn *et al*., 2005). After the uptake of bacilysin by susceptible cells through their peptide permease system, it is hydrolyzed to L-alanine and L-anticapsin where the latter inhibits glucosamine synthetase activity necessary for bacterial peptidoglycan and fungal mannoprotein biosynthesis and such cascade-like pathway results to the cell protoplasting and lysis (Kenig & Abraham, 1976; Perry & Abraham, 1979; Chmara *et al.*, 1981; Milewski, 1993). It was reported that, bacilysin L-anticapsin activity is antagonized by glucosamine, N-acetyl glucosamine and wide range of dipeptides (Kenig & Abraham, 1976). *Bacillus subtilis* does not produce bacilysin especially in the rich growth conditions such as LB medium and in the presence of glucose or casamino acid. Physiological factors and temperature might also influence the production of bacilysin (Ozcengiz *et al*., 1990). Under synthetic or minimal media, however, bacilysin production is induced suggesting that its synthesis is under stringent response (Inaoka *et al*., 2003) and feedback regulation (Ozcengiz and Alaeddinoglu, 1991). Oligosporogenous (Osp) strains are temperature sensitive strains in a way that their spores cannot tolerate to high temperatures, as do their parental wild-type strains successfully (Coote, 1972). NG79, which is bacilysin-deficient strain, was found to be oligosporogenous. Spore quality was remarkably improved by the external addition of bacilysin to the cultured cells for approximately 4-7 hours that suggests that the time of its addition settles the extent of acquired

resistance. Extracellular fluid of aged cultures did not show any presence of bacilysin activity implying that the dipeptide can be directly cleaved by the alkaline serine protease produced by *Bacillus subtilis*. As a result, together with the induction of spore quality by the addition of bacilysin and later the hydrolysis of peptide showed that bacilysin is a component of the quorum-sensing control system (Ozcengiz & Alaeddinoglu, 1991). Furthermore, it was shown that bacilysin is under the control of a quorum sensing regulation system through the action of ComQ/ComX, PhrC (CSF), ComP/ ComA and in a Spo0K (Opp)-dependent manner in *B. subtilis* (Yazgan *et al*., 2001; Yazgan-Karatas, *et al*., 2003). The *bacABCDEF* (previously *ywfBCDEFG)* genes are responsible for the bacilysin biosynthesis. Moreover, monocistronic *ywfH* gene and *yvfI* were found to be vital for the biosynthesis of bacilysin (Inaoka *et al*., 2003; Köroğlu *et al.*, 2008). *bacABC* (*ywfBCD*) encode proteins which perform the biosynthesis of anticapsin, *bacD* (*ywfE*) in the amino acid ligation of anticapsin to alanine and lastly *bacE* (*ywfF*) is important for the acquiring bacilysin resistance. *ywfB* and *ywfG* encode a prephenate dehydratase and an aminotransferase, respectively, which are allocated in the anticapsin production from prephenate of the 12 aromatic amino acid pathway (Hilton *et al.*, 1988a; Inaoka *et al.*, 2003; Steinborn *et al*., 2005). On the other hand, the fuction of *ywfH* gene is still unknown but it might be involved in the alanine-anticapsin ligation (Inaoka *et al.*, 2003).

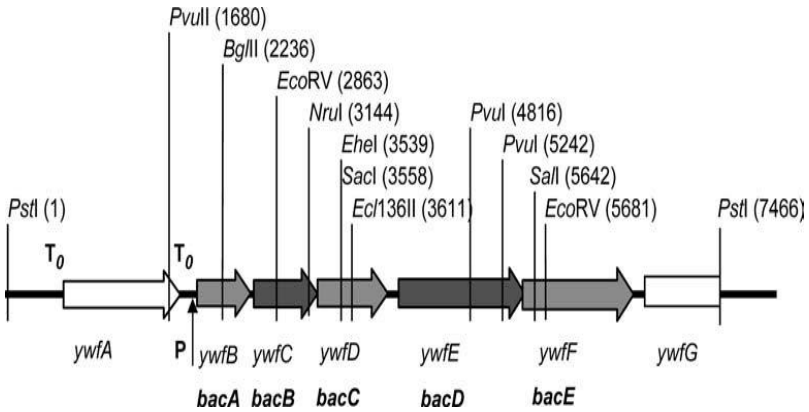

Figure 1.8: Bacilysin biosynthetic operon of *Bacillus subtilis* 168 (Steinborn *et al*., 2005).

The regulation of bacilysin production can take place in various levels. It can be negatively controlled by GTP through transcriptional regulator CodY and AbrB or positively regulated by guanosine 5'-diphosphate 3'-diphosphate (ppGpp) and peptide pheromone PhrC (Inaoka *et al*., 2003; Karatas *et al*., 2003). Therefore,

bacilysin biosynthesis is under synergistic regulation of guanosine 5'-diphosphate 3'-diphosphate (ppGpp) and guanosine triphosphate (GTP) affecting *bacABCDE* operon. Figure 9 shows general view of different control levels of bacilysin and some other peptides biosynthesis.

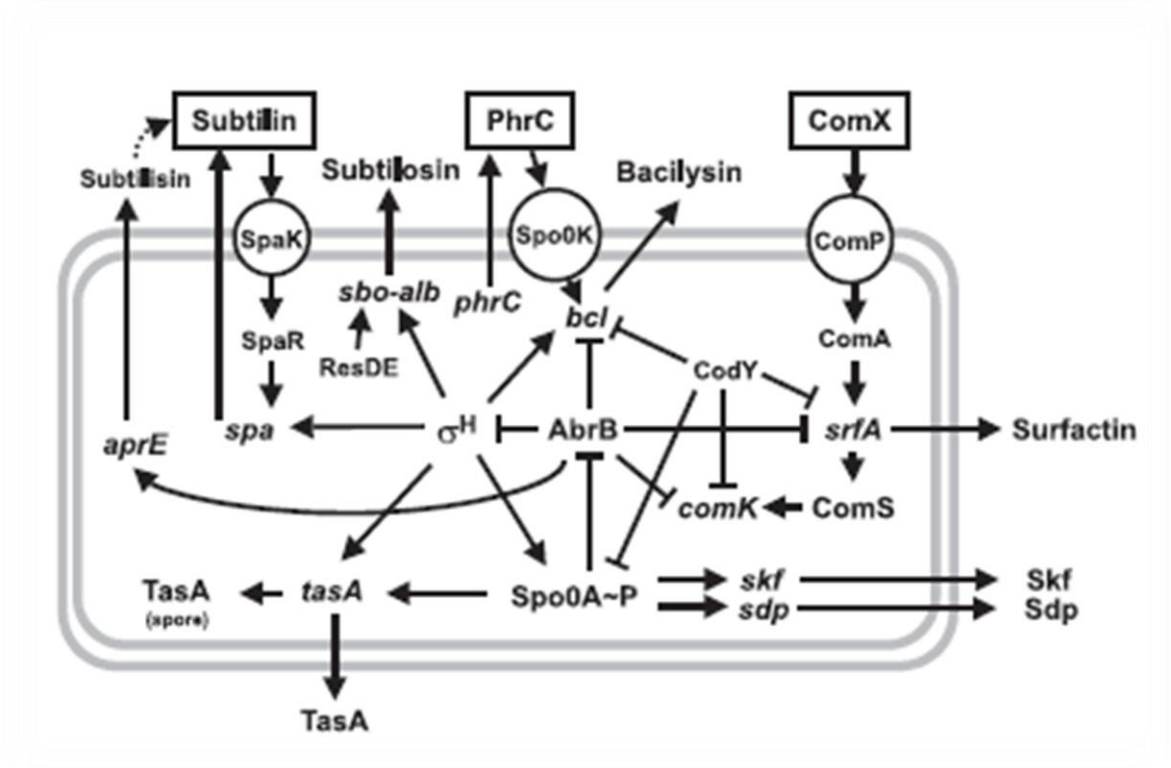

Figure 9: Regulation pathways of antibiotics such as subtilin, subtilosin, bacilysin, surfactin, the killing factor Skf and the spore-associated anti-microbial polypeptide TasA in *B. subtilis* (Stein, 2005).

1.7 Aim of the Present Study

The present study mainly focused on the effects of bacilysin on *B. subtilis* wild type 3610 strain by analyzing its swarming behavior, morphological features such as pellicle and colony architectures. The expression level of several regulatory genes related to swarming motility, chemotaxis, competence development and biofilm formation relative to bacilysin deficient *bac*$^{-}$ 3610 mutant strain during biofilm forming condition were also studied in this project. For this purpose, both wild type and mutant strains were grown in MSgg agar medium in order to promote biofilm formation, the cells were collected, and total RNAs were extracted from different growth phases to determine the change in expression levels of genes. Relative gene expression analysis were conducted by using reverse transcription quantitative PCR

(RT-qPCR). At the end of analysis, the relative gene expression data were analyzed by using $2^{(-\Delta\Delta Cp)}$ formula. Another purpose of this project is to understand the effect of bacilysin on surface growth formation that is a preliminary step for pellicle (biofilm) formation.

2. MATERIALS AND METHODS

Bacillus subtilis PY79, a prototrophic derivative of *B. subtilis* 168, was used as bacilysin producer in this study. The strains and their genotypes that were used in the study are listed in Table 2.1. *Staphylococcus aureus* ATCC 9144 was used as the assay organism for bacilysin determinations. Bacilysin deficient *bac*$^-$ 3610 was constructed previously by the transformation of natural biofilm producing wild type strain NCIB 3610 with the *bac*$^-$ OGU1 chromosomal DNA. Resulting bacilysin deficient *bac*$^-$ 3610 strain was used throughout this study.

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

Strain	Relevant Genotype, phenotype, and/or characteristics	Construction, source or reference
B. subtilis 3610	Wild type	O. Kuipers
B. subtilis PY79	Laboratory strain, BSP cured prototrophic derivative of *B.subtilis* 168	P.Youngman *et al*, 1984
OGU1	*bacA::lacZ::erm*	İ.Öğülür M.Sc. Thesis
bac$^-$ 3610	*bacA::lacZ::erm*	T. E. Koroglu PhD. Thesis

2.1 Bacterial Culture Media

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

2.2 Buffers and Solutions

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

2.3 Chemicals and Enzymes

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

2.4 Laboratory Equipment

The laboratory equipment and primers used during the project are listed in Appendix D.

2.5 Maintenance of Bacterial Strains

B. subtilis and other strains were grown in Luria-Bertani (LB) liquid medium and kept on Luria-Bertani (LB) agar plates at +4 °C. All strains were sub-cultured repeatedly. 10% LB glycerol stock was prepared for long time storage of strains at -80°C.

2.6 Chromosomal DNA isolation

Chromosomal DNA of *B. subtilis* OGU1 strain was isolated by using a standart procedure devised for *Bacillus* species (Cutting and Horn, 1990).

1,5 ml of overnight culture was centrifuged at 13000 rpm for 5 min. The pelleted cells were resuspended in 567 µl of TE (Appendix B) by repeated vortexing. Then, 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 were added and the mixture was incubated for 1 hour at 37°C. After addition of 100 µl of 5M NaCl solution, the sample was mixed without vortexing until the mucosal white substance becomes visible. 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. At a later stage, the upper phase was transferred to a new 1.5 ml microfuge tube and 0.7 volume isopropanol was added. After mixing shortly, the sample was centrifuged at 13000 rpm for 15 min. The pellet was washed with 1ml of 70% ethanol centrifuged at 13000 rpm for 5 min. Subsequently, the pellet was left 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.

2.7. Agarose Gel Electrophoresis

Electrophoresis was carried out on a horizontal submarine electrophoresis apparatus and in a gel system composed of ~1% agarose gel containing 1xTAE buffer (Appendix B) and ethidium bromide of a 0.2 µg/mL final concentration. 6X Loading dye was added into the samples. Electrophoresis was performed at 90-120 Volts for 20-30 minutes. The DNA bands were visualized on a shortwave UV transilluminator (UVP) and photographed by using Gel Imaging System. *Eco*RI+*HindIII* digested DNA marker and Middle range DNA Marker (Appendix D) was used to determine the molecular weights of DNA bands for desired purposes.

2.8 Preparation of *B. subtilis* Component Cells and Transformation

Preparation of *B. subtilis* competent cells and transformation were performed as described by Klein *et al* (1992). HS and LS (Appendix A) mediums were used for the preparation of *B. subtilis* competent cells. 3 mL of overnight culture was firstly prepared in the HS medium by incubation at 37°C and shaking at 250 rpm. Later, 0,5 mL of this overnight inoculum was transformed into 20 mL of LS medium and incubated at 30°C with shaking at 100 rpm until OD600 of cultures reached to 0,55. 1 mL of competent cells was transferred into 2 mL Eppendorf tube and 1 µL of DNA was added. Cells were then incubated at 37°C for 2 h with shaking at 250 rpm and were harvested by centrifugation at 5000 rpm for 15 minutes. Cells were resuspended in 100 µL of sterile saline and spread out on selective LB agar plates and incubated at 37°C for 16 h.

2.9 Preparation of the Bacilysin Extratcs

B. subtilis PY79, OGU1 and NG79 strains were grown overnight at 37°C on Luria-Bertani (LB) agar plates (Appendix A). Then, they were used to inoculate 10 mL of PA medium (Perry and Abraham, 1979) (pH 7.4) contained in 50 mL falcon tubes and the cultures were grown at 37°C (200 rpm) for 24 h. These cultures were then used to

inoculate 100 mL of PA medium to an initial optical density of about 0.1 at 595 nm (OD_{595}). After inoculation, the cultures were incubated at 37°C (200 rpm) for 16 h. Finally, culture was centrifuged and supernatant was collected for further extraction through freeze-dryer. Culture supernatants of strains were extracted 3 times with one-fourth volume of butanol by vortexing for 20 s followed by 2 min centrifugation at 13 000 rpm to separate the organic phase. Collected butanol layer was evaporated to dryness under vacuum. The residue was dissolved in dH_2O and analyzed.

2.11 Bioassay of Bacilysin

Bacilysin was extracted with butanol from cells grown for 24 h in PA medium. The bioassay was performed with *S. aureus* ATCC 9144 as the test organism. The inhibition zones were visible after 6-8 h of incubation at 37°C.

2.12 Bacilysin Digestion and Inhibition of Bacilysin/Anticapsin Activity

The digestion of bacilysin was performed by peptidase R (Amano Enzymes Co. Nagoya, Japan) in the culture fluid (10 mg/ml peptidase) for ~5 h at 40°C. Bioassay medium were supplemented with 10mM N-acetylglucosamine in order to inhibit the activity of bacilysin over *S. aureus* bioassay medium.

2.13 Pellicle Formation Assay

B. subtilis wild type 3610 strain was inoculated in LB and incubated with agitation at 37°C overnight. Overnight culture were diluted with 100-dilution factor and incubated at 37°C until the mid-exponential phase. 2.5 µL of starting culture were added to 2.4 mL of MSgg or LB broth contained within a well of a 24-well microtiter dish and the dish was incubated without agitation at 30°C for 24-72 hours.

2.14 Crystal Violet Assay

Before applying crystal violet, the liquid was taken by the help of glass Pasteur pipet. Then, previously prepared 1000µl of 1% crystal violet was added to each well and stored at 25°C for 15-20 min. At this moment, the remaining unbound crystal violet in the wells was removed again via Pasteur pipet and washed several times with

dH_2O. After washing step, the plates were allowed to dry in a room temperature. 1mL of 33% acetic acid solution was added to each plate in order to solubilize biofilm associated crystal violet following vortex. Samples were diluted 1000 times in order to be quantified under OD_{567} spectroscopy. Obtained absorbances were used in the calibration curve equation to calculate final biomass values.

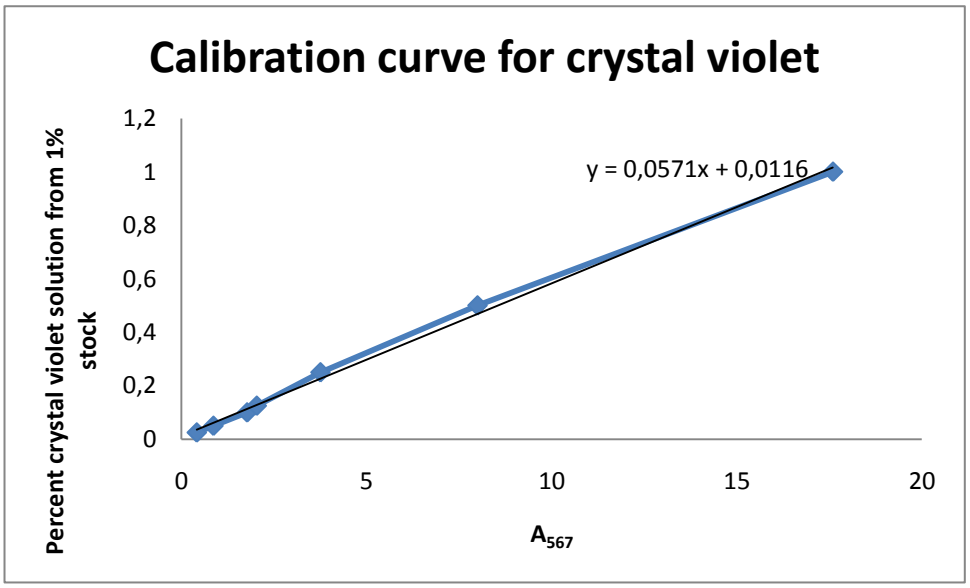

Figure 2.1: Calibration curve for crystal violet. In order to calculate total biomass formation from crystal violet assay, calibration curve was generated and the equation of the line was used as a formula for biomass calculation.

2.15 Colony Architecture and Swarming Motility Assay

In order to analyze the colony architecture and to collect samples for RNA isolation, fresh MSgg agar medium was prepared and allowed to dry for 16hours at room temperature before use. Overnight cultures of both wild type and mutant strains were inoculated in fresh LB broth with 1:100 dilution and cells were grown at 37°C with agitation until OD_{600} reached at 1, which represents the mid-log growth phase. 3 µL of these cultures were spotted onto MSgg agar and the plates were incubated at 30°C for 24 h, 48 h and 72 h, respectively. For the swarming motility assay at OD_{600}=1 moment, cells were concentrated to OD_{600}=10. 10 µL of the concentrated cells were plated on 0,7% LB agar plates.

2.16 RNA Isolation

Bacillus subtilis cells grown in MSgg+Agar medium were washed three times with 0,085% saline solution (NaCl) and collected biomass was stored at -80°C before isolation. Cells were pelleted by centrifugation at 10000xg for 5 minutes and total RNA was isolated using "Qiagen RNeasy Midi Kit". Procedure was conducted at room temperature. In order to prevent RNA degradation, RNAse free environment was provided. Lysis buffer RLC containing guanidine hydrochloride, was supplemented with 10 µl β-Mercaptoethanol (β-ME), just before use as indicated in the kit manual. Additionally, on column DNase I digestion with RNase free DNase set (Qiagen) was applied fot the prevention of possible DNA contamination. Isolated RNA then was divided into aliquots and stored at -80°C.

2.17 Formaldehyde denaturing gel

The integrity of purified total RNA was checked by denaturing agarose gel electrophoresis. Gel was run at 5–7 V/cm in 1x FA gel running buffer.

2.18 Quantitative PCR Analysis

Total RNA isolation procedure was performed using the RNeasy Mini RNA Isolation Kit (Qiagen). Equal amounts (2 µg) of total RNAs were reversely transcribed by using a "Transcriptor cDNA Synthesis Kit" (Roche) with random hexamer primers (60µM) supplied with the kit. The obtained cDNA was used as template for the reverse transcription quantitative PCR. Amplification and detection of PCR products were performed with the "SYBR Green Master Mix Kit" (Roche) and Light Cycler 480 (Roche) instrument. As recomended by the constructors, 2 µls of the cDNA synthesis reaction mixture was directly used as template in a 20 µl of reverse transcription PCR mixture with 10 pmols of gene specific primers. Reverse transcription PCRs were run at 52^{o}C annealing temperature, as all the primers used in this study were designed to work at this temperature to obtain the comparable data. Melting curve analysis was used to monitor the specificity of the reaction. The $2^{-\Delta\Delta Cp}$ method was used to calculate relative gene expressions (Pfaffl, 2004). The

expression levels of the investigated genes were determined relative to the wild type *Bacillus subtilis* PY79 sample. The ratios ($2^{-\Delta\Delta Cp}$) were calculated and log2 transformed. Experiments were performed as replicates and minimum three independent experiments were run. The *sigA* and *veg* genes were used as internal controls, since the expression of those genes were constant under both control and mutant conditions in both microarray and real-time PCR experiments.

3. RESULTS AND DISCUSSION

3.1 Relative gene expression analysis between wild type 3610 and bacilysin deficient *bac*$^{-}$ 3610 strain via reverse transcription qPCR

One of the purposes of this study was to understand the effect of bacilysin biosynthetic operon on biofilm forming undomesticated 3610 strain. For this purpose, total RNA was isolated from 3610 and its bacilysin deficient derivative *bac*$^{-}$ 3610 strain grown in biofilm promoting agar medium MSgg previously used by Branda *et al* (2001). RNAs were isolated from different incubation periods, 24th, 48th and 72nd hours respectively. Following total RNA isolation step, "Transcriptor High Fidelity cDNA Synthesis Kit" (Roche) was used to reversebly transcribe of an aliquot of 2 μg (our optimized concentration) of these total RNAs into cDNA using random hexamer primers. Those cDNAs served as templates for Real Time-Quantitative-PCR (RT-qPCR). 2 μl of the observed cDNA reaction was directly used for RT-qPCR on "the LightCycler® 480 Instrument" with a reaction volume of 15 μl using the LightCycler® 480 SYBR Green I Master (Roche) and gene specific primers to each gene (Forward and reverse; 20 μm each). Primers that were used in this experiment are listed in Appendix D. Relative quantification that compares the expression of a target gene versus a reference gene and the expression of same gene in target sample versus reference samples, was used in this study for the comparison of wild and mutant strains. Relative quantification determines the change in steady-state mRNA levels of a gene across multiple samples and expresses it relative to the levels of an internal control RNA (Pfaffl, 2004). In this study, *sigA* and *veg* genes were selected to be reference genes relying on their constitutive expression where they were expressed at same levels both in wild and mutant strains. As reference values, geometric mean of the Cp values of those two given genes were used. Relative expression ratio for each gene was calculated using a calculation method which uses the results of each sample that was expressed in N-fold changes in the mutant strain target gene copies compared to wild type strain target gene, normalized to reference genes relative to the copy number of the target gene: $2^{-\Delta\Delta Cp}$, ($2^{-[\Delta Cp\ sample}$

$^{-\Delta Cp\ control]}$). The results obtained from this study indicates that sigma factor D (σ^D) that is a transcription factor necessary for the transcription of swarming motility and chemotaxis genes was significantly repressed in the absence of bacilysin. Chemotactic genes *cheY*, *cheB* and *cheV* were also down-regulated in the bacilysin deficient background. *srfAA* (surfactin synthetase), that is crusial for the development of swarming motility and biofilm formation, was shown to be strongly repressed in the absence of bacilysin. *yvzD* (*swrAA*), that is required for the synthesis of protein for hyperflagellation, had a variable transcription profile. *yvzD* was induced in 24th and 72nd hours and repressed in 48th hour in the absence of bacilysin(Table 3.1).

Table 3.1: Bacilysin effect on genes related to chemotaxis and swarming motility

Gene	qPCR[1] (24Hr)	qPCR[1] (48Hr)	qPCR[1] (72Hr)	Function	Transcriptional Organisation[2]
cheY	-0,75(0,10)	1,69(0,26)	6,19(0,08)	Response regulator containing CheY-like receiver, AAA-type ATPase, and DNA-binding domains	$\overrightarrow{flgB}\ \overrightarrow{flgC}\ \overrightarrow{fliE}\ \overrightarrow{fliF}\ \overrightarrow{fliG}\ \overrightarrow{fliH}$ $\overrightarrow{fliI}\ \overrightarrow{fliJ}\ \overrightarrow{ylxF}\ \overrightarrow{fliK}\ \overrightarrow{ylxG}$ $\overrightarrow{flgE}\ \overrightarrow{fliL}$
cheB	-0,48(0,25)	4,43(0,23)	5,06(0,08)	chemotaxis-specific methylesterase	$\overrightarrow{fliM}\ \overrightarrow{fliY}\ \overrightarrow{\mathbf{cheY}}\ \overrightarrow{fliZ}\ \overrightarrow{fliP}\ \overrightarrow{fliQ}$ $\overrightarrow{fliR}\ \overrightarrow{flhB}\ \overrightarrow{flhA}\ \overrightarrow{flhF}\ \overrightarrow{ylxH}$ $\overrightarrow{\mathbf{cheB}}\ \overrightarrow{cheA}\ \overrightarrow{cheW}\ \overrightarrow{cheC}\ \overrightarrow{cheD}$
sigD	0,16(0,09)	2,99(0,13)	1,58(0,27)	RNA polymerase sigma factor	$\overrightarrow{\mathbf{sigD}}\ \overrightarrow{ylxL}$
cheV	0,91(0,30)	1,54(0,22)	-0,99(0,21)	coupling protein and response regulator for CheA activity	$\overrightarrow{\mathbf{cheV}}$
yvzD	-1,70(0,10)	0,69(0,03)	-0,81(0,07)	swarming motility protein	$\overrightarrow{\mathbf{yvzD}}\ \overrightarrow{yvJD}$
srfAA	9,10(0,07)	5,68(0,29)	6,88(0,07)	surfactin synthetase / competence	$\overrightarrow{\mathbf{srfAA}}\ \overrightarrow{srfAB}\ \overrightarrow{srfAC}\ \overrightarrow{srfAD}$

[1] Numbers indicate the log2 transformed expression ratio.

[2] Transcriptional organization retrieved from http://genolist.pasteur.fr/SubtiList/, "ǂ" indicates the termination sites (The mean of minimum three independent replicate experiments is given and standart deviation of the mean is shown in paranthesis).

The *eps* genes required for the biosynthesis of the structural matrix components of biofilm architecture were shown to be affected by bacilysin. *epsD*, *epsE* and *epsK* were shown to be down-regulated in the bacilysin deficient background (Table 3.2). Transition state degredative enzyme synthesis regulator *degU* was strongly repressed in the absence of bacilysin (Table 3.2). The *aprE* gene, precursor of antimicrobial peptide subtilin, is responsible for the expression of the extracellular protease subtilisin and has been used as a model to understand the complex regulatory network that controls gene expression during the transition stage and the stationary phase in *B. subtilis* (Corvey *et al*., 2003; Kunst *et al*., 1974; Ferrari *et al*., 1985;). The expression of *aprE* is controlled by several regulators such as DegU/DegS, AbrB,

ScoC and SinR (Henner *et al*., 1988; Strauch *et al*., 1989; Kallio *et al*., 1991; Gaur *et al*., 1991). In this study, it was investigated that *aprE* was upregulated by bacilysin. The *aprE* expression was repressed in 24th and 72nd hour growth phases and there was no significant fold change in the 48th growth phase in bacilysin deficient background (Table 3.2).

Table 3.2: Bacilysin effect on genes related to biofilm formation, competence development and protease synthesis

Gene	qPCR[1] (24Hr)	qPCR[1] (48Hr)	qPCR[1] (72Hr)	Function	Transcriptional Organisation[2]
aprE	0,81(0,04)	0,05(0.30)	1,37(0.08)	extracellular alkaline serine protease (subtilisin E)	$\neq\overleftarrow{yhfM}\ \overrightarrow{yhfN}\neq\overleftarrow{\boldsymbol{aprE}}\ \overrightarrow{yhfO}$ $\overrightarrow{yhfP}\ \neq$
degU	-0,81(0,06)	1,45(0,18)	7,75(0,21)	two-component response regulator involved in degradative enzyme and competence regulation	$\neq\overleftarrow{yvıA}\ \overleftarrow{\#\boldsymbol{degU}}\ \overleftarrow{degS}\ \overrightarrow{yvyE}$ $\overrightarrow{yvhJ\#}$
yveN(epsD)	0,88(0,18)	2,80(0,25)	5,08(0,22)	extracellular matrix biosynthesis enzyme	$\neq\overrightarrow{sıgL}\ \overrightarrow{yvfG}\ \overleftarrow{yvfF}\ \overleftarrow{yvfE}$
yveO(epsE)	1,95(0,16)	0,18(0,01)	-1,08(0,21)	Glycosyltransferase	$\overleftarrow{yvfD}\ \overleftarrow{yvfC}\ \overleftarrow{yvfB}\ \overleftarrow{\boldsymbol{yvfA}}\ \overleftarrow{yveT}$ $\overleftarrow{yveS}\ \overleftarrow{yveR}\ \overleftarrow{yveQ}\ \overleftarrow{yveP}\ \overleftarrow{\boldsymbol{yveO}}$
yvfA(epsK)	1,45(0,13)	2,88(0,09)	0,25(0,01)	extracellular matrix component exporter	$\overleftarrow{\boldsymbol{yveN}}\ \overleftarrow{yveM}\ \overleftarrow{yveL}\ \overleftarrow{yveK}\ \overrightarrow{slr}$

[1] Numbers indicate the log2 transformed expression ratio.

[2] Transcriptional organization retrieved from http://genolist.pasteur.fr/SubtiList/, "ǂ" indicates the termination sites (The mean of minimum three independent replicate experiments is given and standart deviation of the mean is shown in paranthesis).

3.2 The effect of bacilysin in swarming motility and chemotaxis

It is a well-known fact that swarming is a pre-requisite step required for biofilm formation. Initially, biofilm cells are motile but as biofilm structure develop most cells become non-motile adhered to both each other and to the surface while extracellular matrix builds up. Yet, swarming and biofilm share many common features. Most bacteria show swarming motility which requires the formation of functioning flagella and the production of lipopeptide surfactant namely surfactin (Kearns and Losick, 2003). For instance, laboratory domesticated *B. subtilis* PY79 and 168 strains are unable to show swarming behavior due to the presence of a mutation in surfactin biosynthetic *srfA* operon. On the other hand, undomesticated wild type strains of *B. subtilis* are capable of performing swarming motility. Thus, we first checked the swarming behavior of bacilysin deficient *bac*$^{-}$ 3610 strain, by performing swarm agar assay. For this, 0.7% agar LB plates were centrally inoculated and incubated at 37°C. After one day of incubation, none of the laboratory

and mutant strains showed proper swarming motility except undomesticated wild type 3610 strain (Figure 3.1).

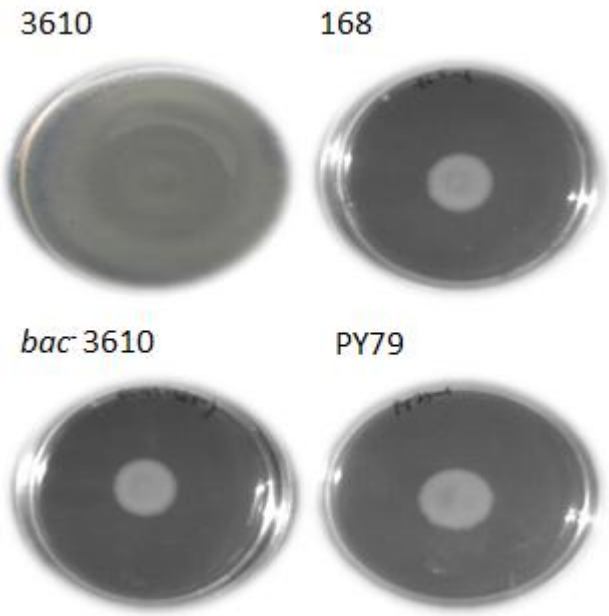

Figure 3.1: Swarming motility assay of laboratory strains 168, PY79, bacilysin deficient 3610 derivative *bac*- 3610 and wild type undomesticated 3610 strain.

B. subtilis undomesticated 3610 strain is able to construct a floating biofilm known as pellicle in a standing liquid minimal culture. The process begins through dramatic switch in its growth process. Single free-floating cell moves to the air-liquid interface and proliferate as long chains of filamentous non-motile cells and biofilm architecture is formed by those differentiated bundled filamentous cells (Branda *et al*., 2001; Branda *et al*., 2006). Pellicle architecture of 3610 strain grown under MSgg broth is present in Figure 3.2. Pellicle structure is more visible after 24 hours and continues growing in following days. However, bacilysin deficient 3610 derivative strain was unable not only to form pellicle but also to grow properly under standing liquid culture (Figure 3.2).

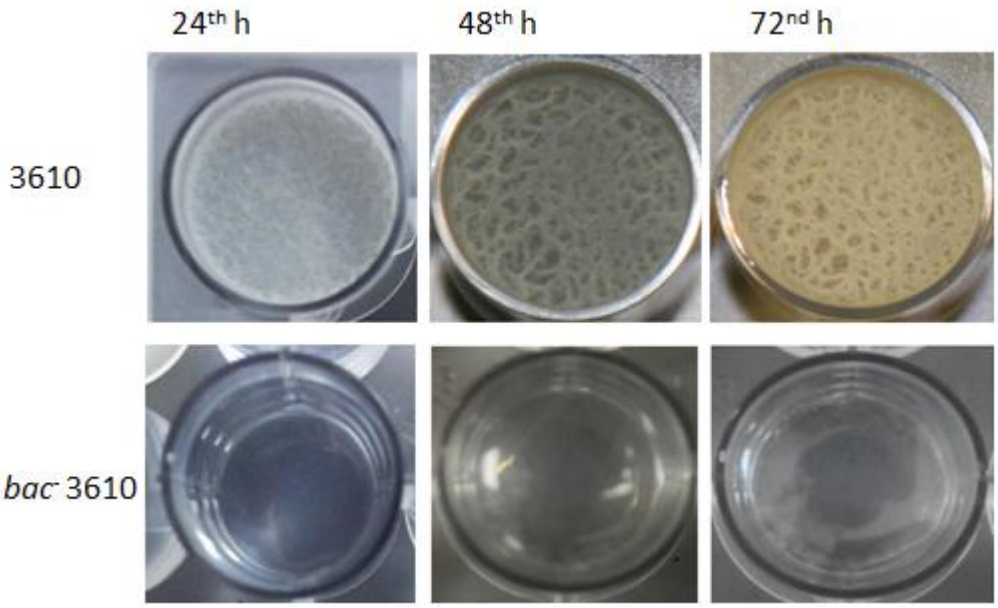

Figure 3.2: Pellicle formation assay for *B. subtilis* wild type 3610 strain and its bacilysin deficient derivative *bac*⁻ 3610 strain in MSgg medium.

As previously described, wild 3610 strains of *B. subtilis* form floating biofilm (pellicle) and this formation generates larger structures known as fruiting bodies in liquid medium. In solid medium, however, some features that are not involved in pellicles such as concentric rings occur (Branda *et al*., 2004). Colony architectures of wild type 3610 and its bacilysin deficient mutant strain in MSgg medium are shown in Figure 3.3. Results of colony architecture assays revealed that *bac*⁻ 3610 strain biofilm formation was impaired. Phenotype of mutant 3610 did not show the mature fruiting body-like biofilm structure even after 72nd hour (Figure 3.3).

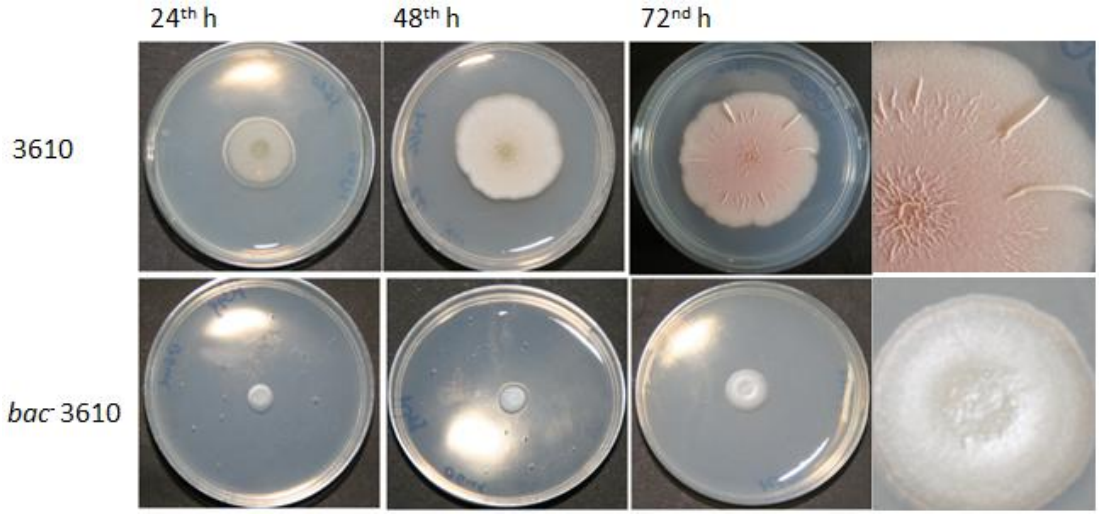

Figure 3.3 : Colony architectures of wild type *B. subtilis* 3610 strain and its bacilysin deficient derivative *bac*⁻ 3610 strain in MSgg medium.

In a previous study, it was shown that *srfAA* gene, that mediates synthesis of surfactin, was needed for the maturation of fruiting body (Branda *et al*., 2004). Surfactin, which takes role as a surfactant in the biofilm matrix, is also a signalling molecule that stimulates the activity of the kinase KinC, which, in turn, leads to the activation by phosphorylation of the response regulator Spo0A (López *et al*., 2009). As a result, Spo0A~P triggers a regulatory cascade that leads to matrix production and cell filamentation (Branda *et al*., 2001; Kobayashi, 2007; Chu *et al*.,2008). In addition, surfactin is required for the bacterial motility (Kearns and Losick, 2003). Relative quantification of wild type 3610 and its mutant derivative through RT-qPCR analysis showed that bacilysin positively regulates *srfAA* that may answer why bacilysin deficient strain is not able to swarm properly (Figure 3.1) and form fruiting body structure (Figures 3.2, 3.3). In all three cases, 24^{th}, 48^{th} and 72^{nd} hours respectively, the expression profiles of *srfAA* were strongly reduced in bacilysin deficient 3610 strain background (Table 3.1). As previously mentioned, *sigD* encodes sigma factor D necessary for flagellin, chemotaxis and motility genes. Sigma factor D was shown to be directly responsible for transcription of flagellin structural gene together with motility genes *motA* and *motB* and the *flgM* regulatory genes (Helmann *et al*., 1988; Marquez *et al*., 1990; Mirel and Chamberlin, 1989; Mirel *et al*., 1992; Mirel *et al*., 1994). According to our RT-qPCR analysis, *sigD* was upregulated by bacilysin in all three cases. During 24^{th} hour, up regulation factor was lower when comparing with 48^{th} and 72^{nd} hours. The highest fold difference was obtained in 48^{th} hour phase. Chemotactic genes such as *cheV, cheB* and *cheY* were also shown to be affected by bacilysin. Previous studies show that phosphorylated CheY directs flagellar motility (Bourret *et al*., 1990; Welch *et al*., 1993). *cheV*, which is required for optimal chemotaxis, encodes protein CheV similar to CheY. It has been observed that inactivation of *cheV* results in the loss of swarming motility (Rosario *et al*., 1994). Phosphorylated CheB regulates adaptation by demethylating methyl-accepting chemotaxis proteins (MCPs) (Hess *et al*., 1988). According to the results obtained in this study, bacilysin was responsible for the up-regulation of *cheV, cheB* and *cheY* in most of the cases namely 24^{th}, 48^{th} and 72^{nd} hours of growth phases. *cheV* was only downregulated by bacilysin in 72^{nd} hour growth phase. Whereas, *cheB* was slightly downregulated by bacilysin with a factor of 0.48 fold change only in 24^{th} growth phase. On the other hand, *cheY* was shown to be up-regulated by bacilysin in both 48^{th} and 72^{nd} growth phases, giving the highest fold

change in 72nd hour which is a factor of 6.19 in the expression of *cheY* (Table 3.1). Swarming motility protein *yvzD* plays vital role in regulating the degree of cell flagellation as described in Calvio *et al*. (*yvzD* was renamed as *swrAA* in this study, 2005). However, in the same study, they proposed that *yvzD* alone is insufficient to enable surface-adhering cells to differentiate into hyperflagellated and elongated swarm cells. It was demonstrated that *swrAA* (*yvzD*) defective cells lack flagella when grown over solid surfaces and do not exhibit swarming behavior. In our study, we observed that *yvzD* either was up or down regulated by bacilysin depending to its growth phase suggesting that bacilysin plays crusial role in the development of swarming motility in *B. subtilis*. Additionaly, genes required for the synthesis of extracellular matrix components namely *yveN* (*epsD*), *yveO* (*epsE*) and *yvfA* (*epsK*) were shown to be upregulated by bacilysin since the relative quantification results indicated that these three genes were downregulated in the mutant 3610 strain except that *yveO* was upregulated at 72nd hour quantification (Table 3.2). The *yveO* (*epsE*) directly grasps the flagella rotor to inhibit motility (Guttenplan *et al*., 2010) which is compatible with our swarming behaviour result of bacilysin deficient strain and may explain the reason of being unable to swarm. DegU, a DNA-binding protein, is responsible for the control of many genes and biological processes such as genetic competence, extracellular degrading enzyme and regulation of motility by binding to *fla-che* operon (Msadek *et al*., 1995; Amati *et al*., 2004). According to the study done by Kobayashi (2007), the phenotype of the *degU* mutant suggested that DegU might activate the transcription of flagellar genes and genes required for biofilm formation. It was shown that high levels of DegU is responsible for decrease in the half-life of the phosphorylated form of DegU, this in turn decrease the protease level and increase the flagellar synthesis (Msadek *et al*., 1990; Amati *et al*., 2004). The *degU* mutant strain was neither able to perform swarming nor pellicle in SGG culture medium (Kobayashi, 2007). In our qPCR data, the expression of *degU* was induced in 24th hour growth phase whereas the expression was dramatically repressed in 48th and 72nd hour growth phases in bacilysin mutant 3610 background (Table 3.2). The results obtained from qPCR data seemed to be compatible with swarming (Figure 3.1) and biofilm assays (Figures 3.2, 3.3) in this study. These data strongly emphasizes the importance of bacilysin that might be a crusial control element of *B. subtilis* 3610 in swarming, chemotaxis and biofilm development.

Mutant strains that are unable to grow and form pellicle in MSgg minimal medium, may form pellicle in rich medium 2xSG (SGG). Since mutant strains might have defects either directly or indirectly on nutrient requirements and thus may not be able to grow and form pellicle in minimal medium, mutation may result in auxotrophic phenotype (Kobayashi *et al*., 2007). Therefore, in order to see if bacilysin deficiency caused auxotrophy in 3610 strain, the same protocol was applied to SGG medium both in liquid and solid medium and then, the formation of pellicle and colony architectures of both *bac*$^{-}$ 3610 and 3610 were observed. As shown in Figure 3.4, *bac*$^{-}$ 3610 was able to grow; however, it was not able to form any surface growth or pellicle. On the contrary, wild type 3610 strain was able to form clear visible pellicle only at 24th hour of incubation that is faster than it can form in MSgg minimal medium. Colony architecture of bacilysin deficient strain showed higher growth in SGG medium but still it was unable to form complex colony architexture whereas wild type 3610 showed mature biofilm at 24th hour of incubation that is faster than it can form in MSgg agar medium (Figure 3.5). While wild type 3610 and its bacilysin deficient derivative were subjected to grow on shaking rich (LB, Figure 3.6), minimal (MMS, Figure 3.7) and biofilm (MSgg, Figure 3.8) media, it was shown that bacilysin deficient strain was able to grow on a shaking culture and it had a similar growth curve profile as wild type 3610 strain. These results suggest that bacilysin deficiency does not cause any auxothrophy but it clearly causes disruption to organism that results in *B. subtilis* 3610 being unable to form biofilm.

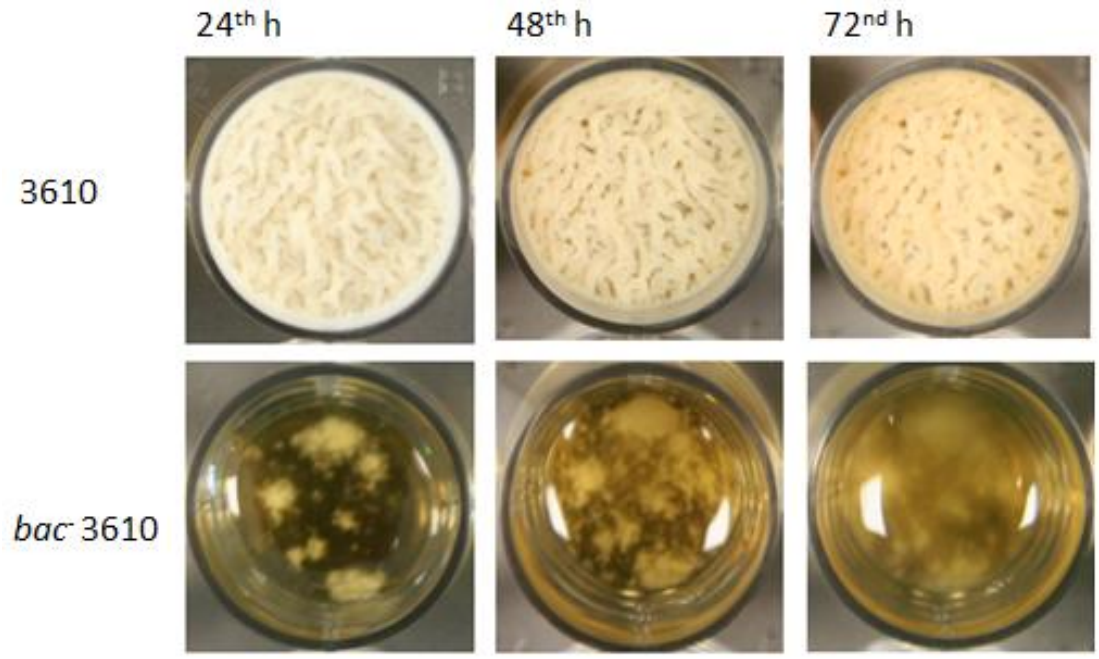

Figure 3.4: Pellicle formation assay for *B. subtilis* wild type 3610 strain and its bacilysin deficient derivative *bac*⁻ 3610 strain in SGG medium. Cells were grown in 2xSG (SGG) medium incubated at 30°C in a standing culture.

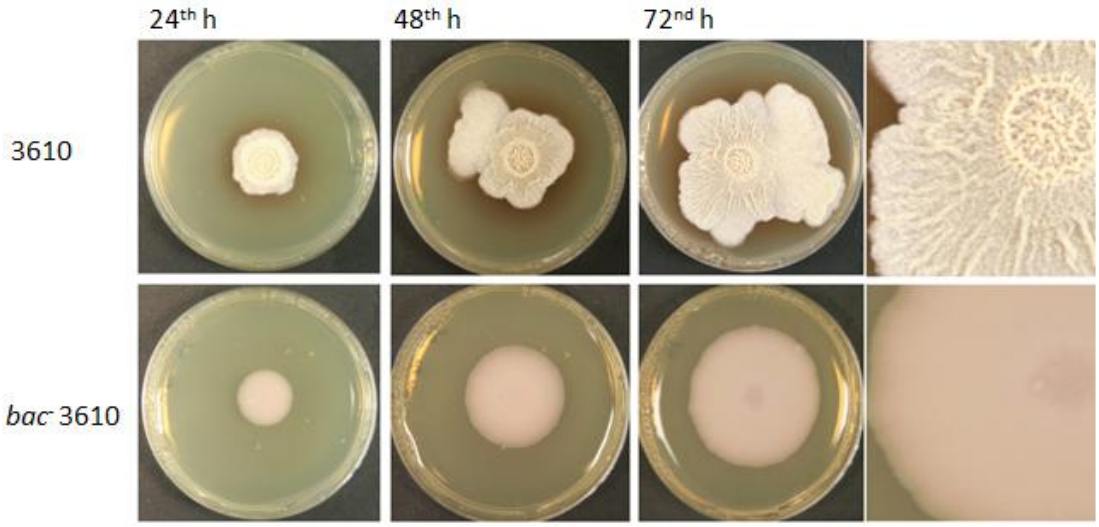

Figure 3.5 : Colony architecture formation assay for *B. subtilis* wild type 3610 strain and its bacilysin deficient derivative *bac*⁻ 3610 strain in SGG agar medium. Cells were grown in 2xSG (SGG) medium incubated at 30°C in a standing culture.

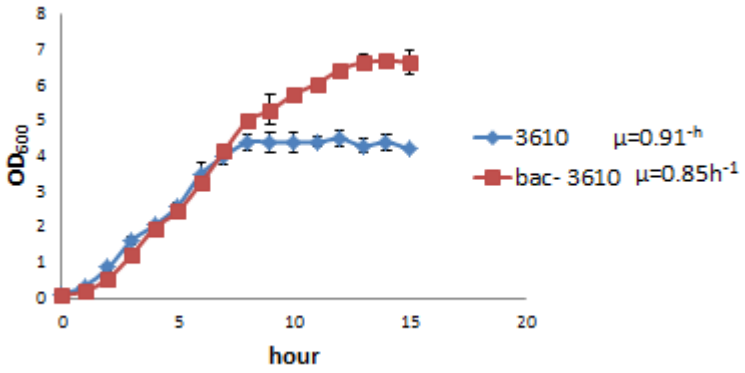

Figure 3.6: Growth curves of undomesticated wild strain *B. subtilis* 3610 and its bacilysin deficient derivative strain. Cells were grown in LB medium at 37°C with 225 rpm agitation.

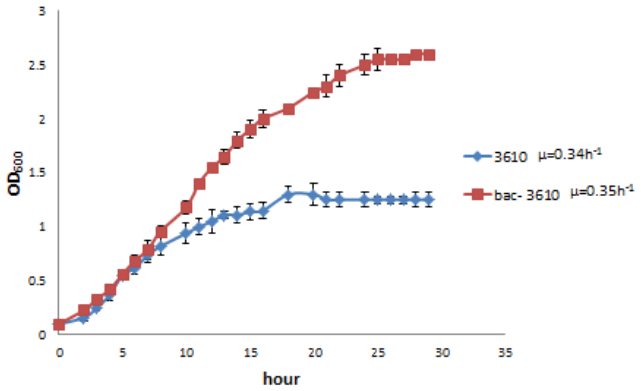

Figure 3.7: Growth curves of undomesticated wild strain *B. subtilis* 3610 and its bacilysin deficient derivative strain. Cells were grown in minimal medium at 37°C with 225 rpm agitation.

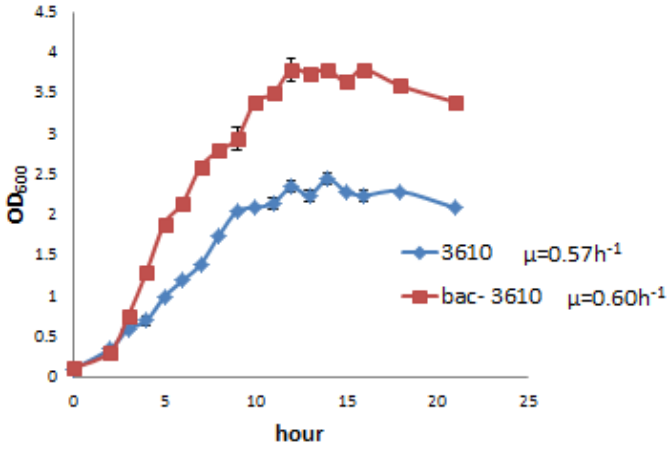

Figure 3.8: Growth curves of undomesticated wild strain *B. subtilis* 3610 and its bacilysin deficient derivative strain. Cells were grown in MSgg medium, containing glycerol as a carbon source, at 37°C with 225 rpm agitation. Data shown in each graph are representative of three independent experiments.

Bacillus subtilis is able to grow in different carbon sources such as glucose, xylose and galactose and it can utilize all these sugars as carbon sources. In a previous dissertation done by Turkan Ebru Koroglu, it was investigated that bacilysin has an effect on genes related to carbohydrate and nitrogen metabolism in *B. subtilis*. Genes related to the utilization of carbohydrates were shown to be affected by bacilysin. Under the light of above information, one of our aims was to understand the effects of various carbon sources on biofilm formation, particularly glucose, xylose and galactose, on wild type 3610 and bacilysin deficient *bac*$^{-}$ 3610 strains. For this purpose, carbohydrate source of MSgg media (glycerol) was replaced with glucose, xylose and galactose and tested separately both in standing and shaking liquid culture. According to our observations, glucose, as a carbohydrate source, highly promotes growth and biofilm formation in undomesticated 3610 strain. Complex colony architecture (Figure 3.10) and pellicle (Figure 3.9) structures are visible in all three growth phases, 24th, 48th and 72nd hours respectively. On the other hand, xylose as a carbon source showed smaller colony size with respect to glucose but biofilm formation was present (Figure 3.12) and pellicle formation (Figure 3.11) was visible at 72nd growth phase in 3610 strain. Galactose, as a carbon source, highly affected biofilm formation in wild type 3610 strain. Colony size was highly reduced when compared with glucose and xylose and biofilm was not formed but only small sized colony and flat growth was observed (Figure 3.14). In a liquid culture (Figure 3.13), surface growth was observed only at 72nd growth phase as in the xylose pellicle assay. Bacilysin deficient strain did not show any significant growth under liquid media where glucose, xylose and galactose were used as carbon sources (Figures 3.9, 3.11, 3.13). Furthermore, *bac*$^{-}$ 3610 strain showed small and smooth colony formation in solid media containing glucose, xylose and galactose as carbon sources (Figures 3.10, 3.12, 3.14). These obtained results may imply that bacilysin deficient strain (*bac*$^{-}$ 3610) might have difficulty in utilizing these sugars in a standing culture where oxygen levels are quite low. Subsequently, while the same carbon sources were used in the shaking culture, bacilysin deficient strain did not show any growth deficiency. In fact, specific growth rates (μ) were almost the same between wild type 3610 and its bacilysin deficient derivative (Figures 3.15, 3.16). In addition, it was shown that the entry of bacilysin deficient strain to the stationary phase was prolonged compared with wild type 3610 strain in glucose and xylose media (Figures 3.15, 3.16). However, in a galactose medium, bacilysin deficient strain showed less

specific growth rate and the entry to the stationary growth phase was earlier when compared with 3610 strain (Figure 3.17).

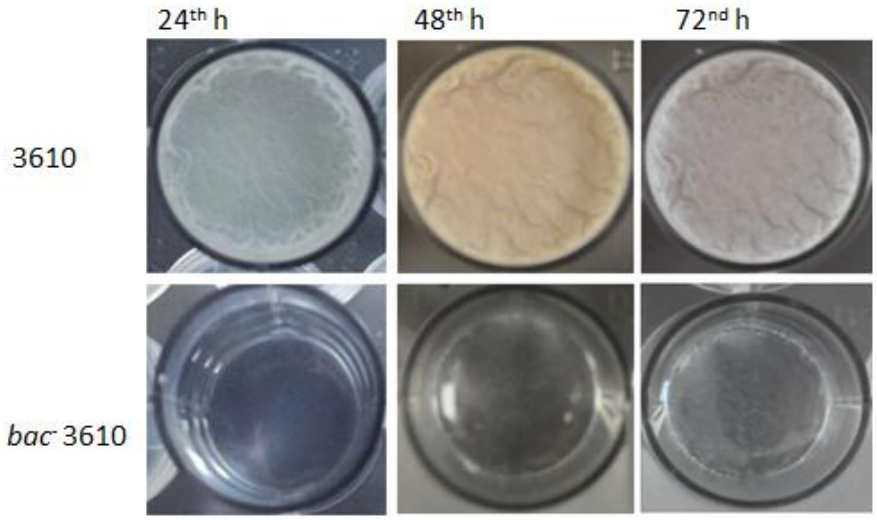

Figure 3.9: Pellicle formation assay for *B. subtilis* wild type 3610 strain and its bacilysin deficient derivative *bac*$^{-}$ 3610 strain. Cells were grown in MSgg medium but glycerol was replaced with glucose as a carbon source and incubated at 30°C in a standing culture.

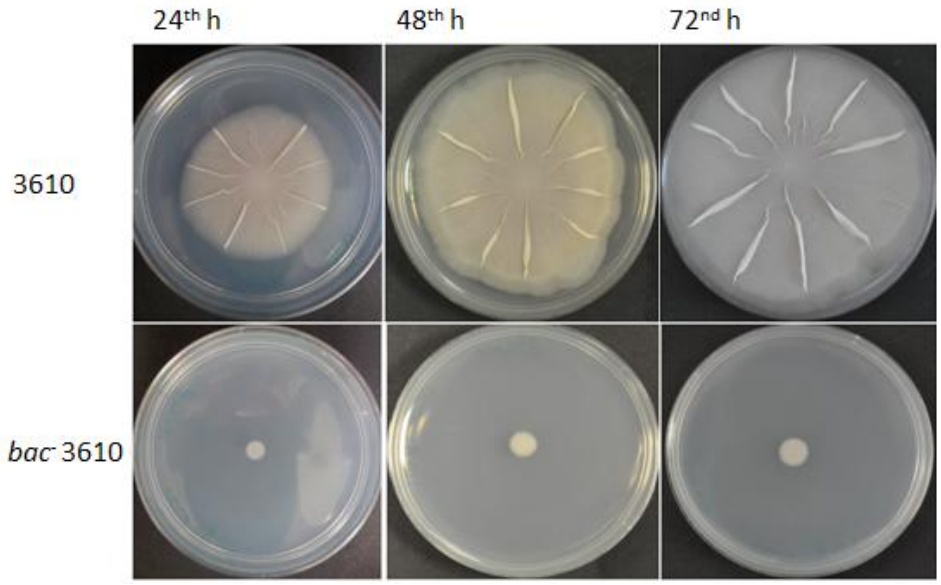

Figure 3.10 : Colony architectures of wild type *B. subtilis* 3610 strain and its bacilysin deficient derivative *bac*$^{-}$ 3610 strain. Cells were grown in MSgg+agar medium but glycerol was replaced with glucose as a carbon source and plates were incubated at 30°C.

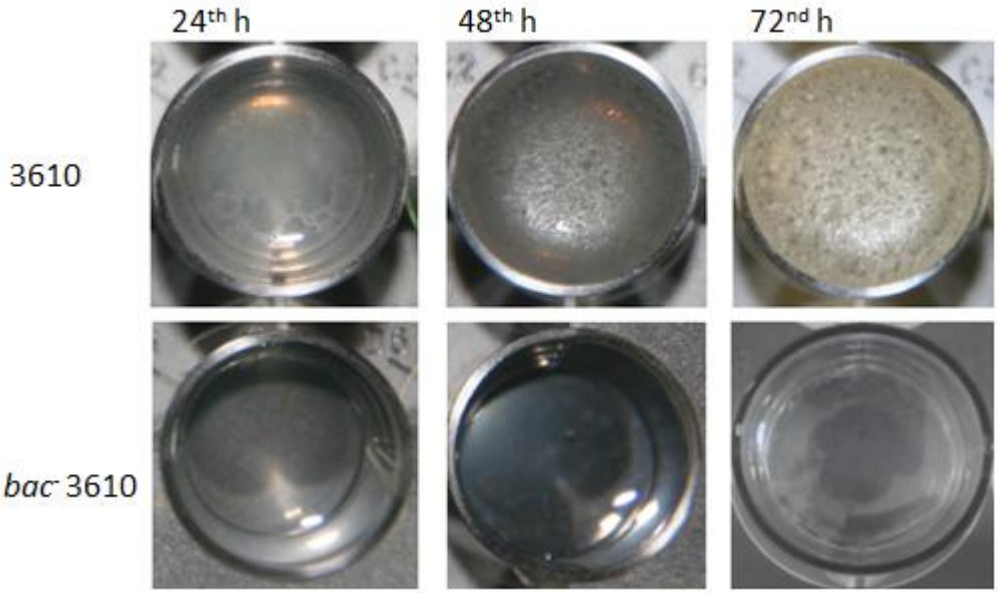

Figure 3.11: Pellicle formation assay for *B. subtilis* wild type 3610 strain and its bacilysin deficient derivative *bac*$^{-}$ 3610 strain. Cells were grown in MSgg medium but glycerol was replaced with xylose as a carbon source and incubated at 30°C in a standing culture.

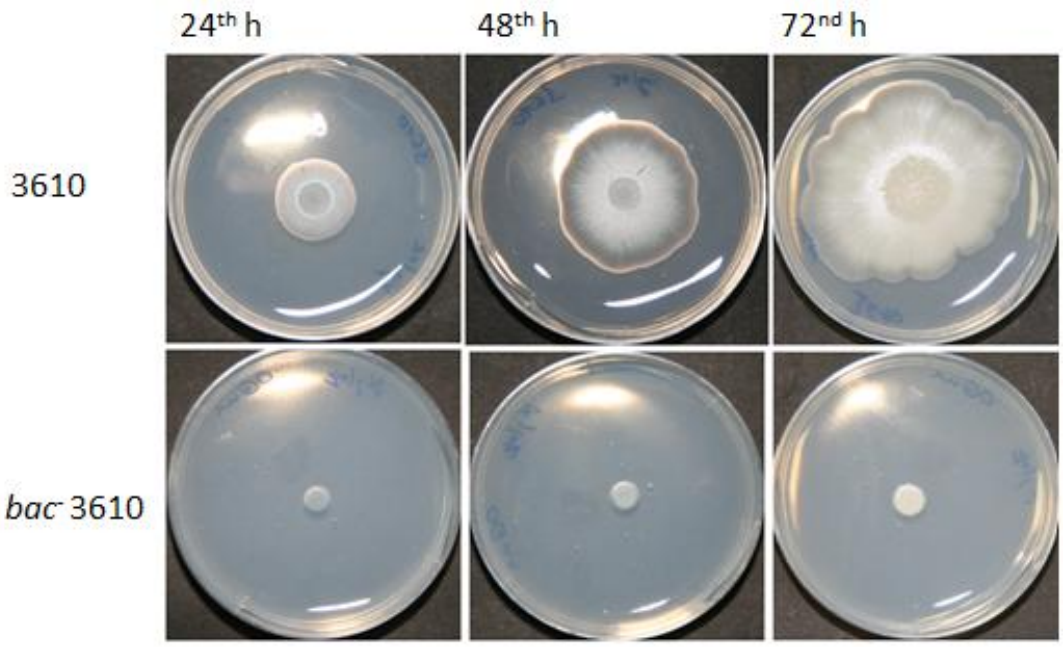

Figure 3.12: Colony architectures of wild type *B. subtilis* 3610 strain and its bacilysin deficient derivative *bac*$^{-}$ 3610 strain. Cells were grown in MSgg+agar medium but glycerol was replaced with xylose as a carbon source and plates were incubated at 30°C.

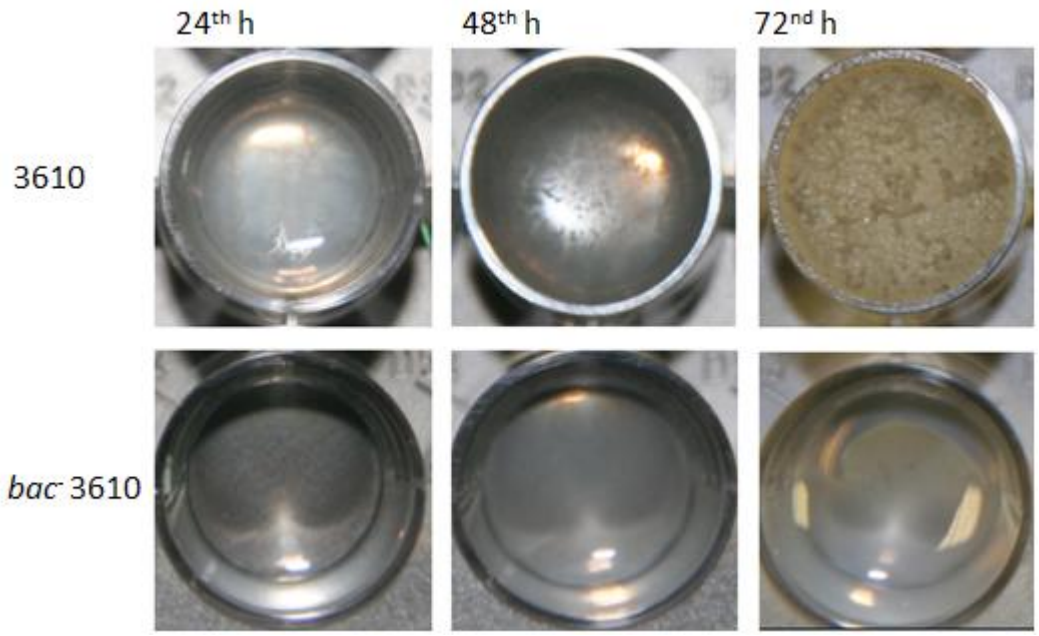

Figure 3.13: Pellicle formation assay for *B. subtilis* wild type 3610 strain and its bacilysin deficient derivative *bac*$^{-}$ 3610 strain. Cells were grown in MSgg medium but glycerol was replaced with galactose as a carbon source and incubated at 30°C in a standing culture.

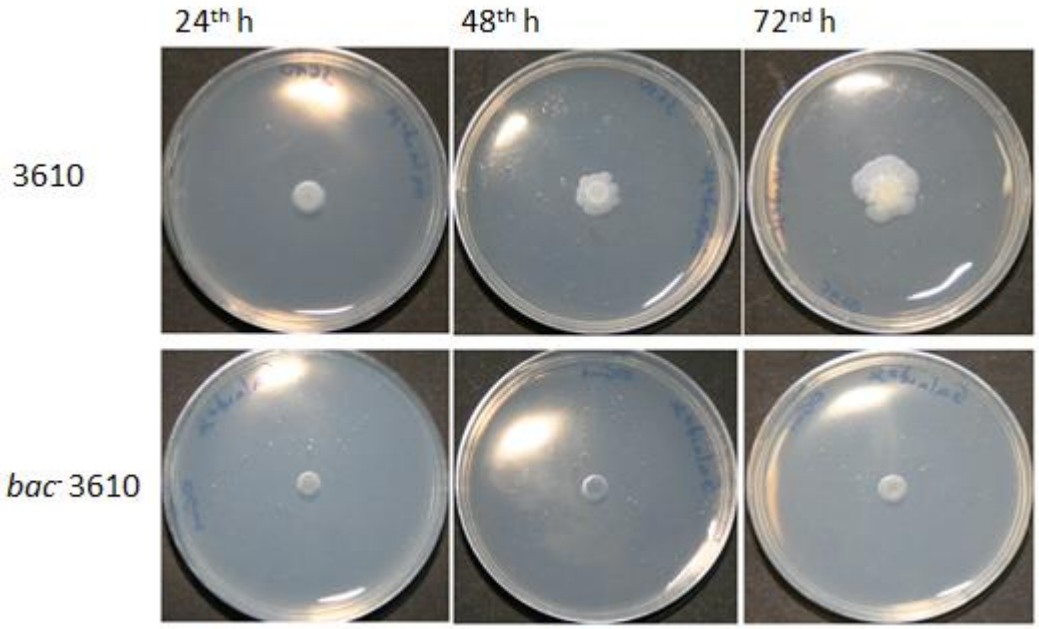

Figure 3.14: Colony architectures of wild type *B. subtilis* 3610 strain and its bacilysin deficient derivative *bac*$^{-}$ 3610 strain. Cells were grown in MSgg+agar medium but glycerol was replaced with galactose as a carbon source and plates were incubated at 30°C.

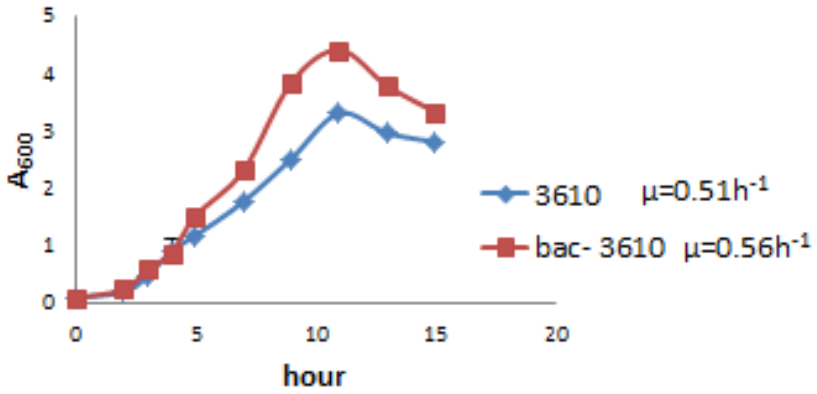

Figure 3.15: Growth curves of undomesticated wild strain *B. subtilis* 3610 and its bacilysin deficient derivative strain. Cells were grown in MSgg medium, containing D-glucose as a carbon source, at 37°C with 225 rpm agitation. Data shown are representative of three independent experiments.

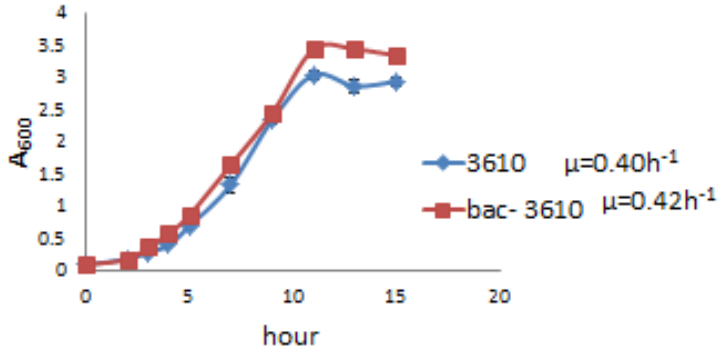

Figure 3.16: Growth curves of undomesticated wild strain *B. subtilis* 3610 and its bacilysin deficient derivative strain. Cells were grown in MSgg medium, containing xylose as a carbon source, at 37°C with 225 rpm agitation. Data shown are representative of three independent experiments.

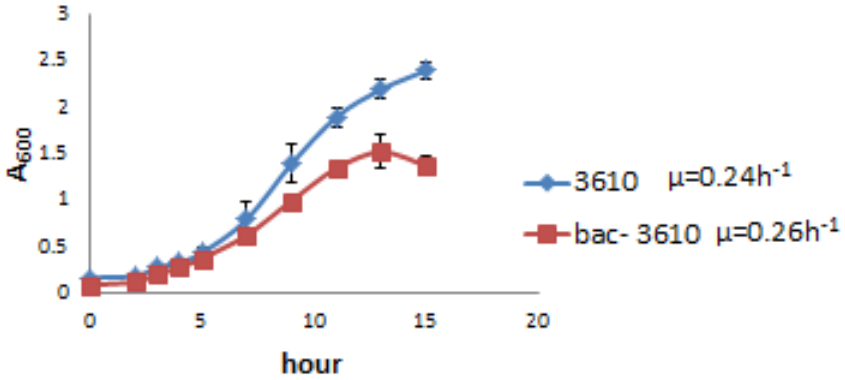

Figure 3.17: Growth curves of undomesticated wild strain *B. subtilis* 3610 and its bacilysin deficient derivative strain. Cells were grown in MSgg medium, containing galactose as a carbon source, at 37°C with 225 rpm agitation. Data shown are representative of three independent experiments.

3.3 The Effect of Bacilysin on Cellular Morphology

To investigate cellular morphologies of 3610 and its bacilysin deficient *bac*$^-$ 3610 mutant strain, they were grown in LB medium with agitation until mid-log phase (~4h). According to previous studies, undomesticated 3610 strain synthesizes high level of σ^D and strain is biased toward motile cells during exponential growth phase. On the other hand, laboratory strain PY79 due to a mutation in *swrA* gene, shows lower σ^D activity and produces a high proportion of chains (Kearns and Losick, 2005). Under the light of above information, we tested the effect of bacilysin on cell chaining during exponential-growth phase. Our result indicates that bacilysin deficiency generates relatively high proportion of cell chains. Figure 3.19 shows undomesticated 3610 motile cells on the left and cell chains of *bac*$^-$ 3610 on the right. This result is also compatible with our RT-qPCR analysis where the expression of σ^D was repressed in all 24th, 48th and 72nd hour growth phases.

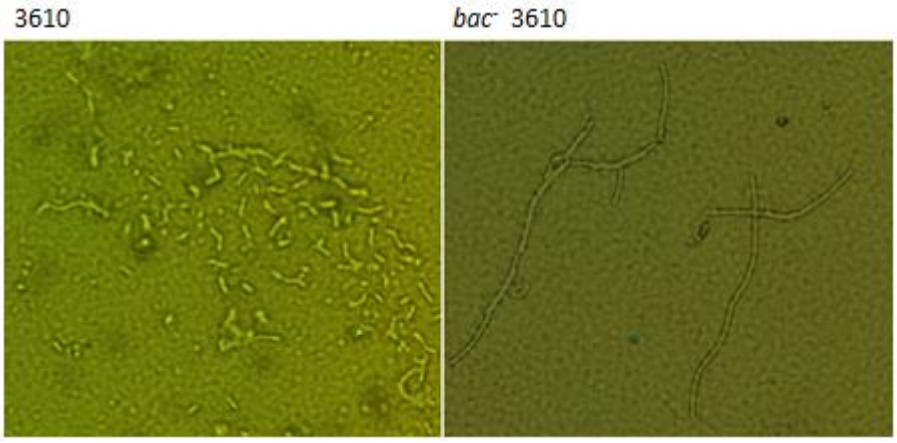

Figure 3.18: Cellular morphologies of 3610 and *bac*$^-$ 3610. Cells of the mid-exponential phase of growth were observed under light microscopy, 100X magnification.

3.4 Peptides as Signalling Molecules

Microbial communities are highly communicative so as they are highly competitive. Microbes use different types of signalling molecules for cell—cell interactions such as small efector molecules. Antibiotics have been used as a therapeutic agent since long time before. Since then, another possible function of antibiotics has been disregarded. Recent studies have shown that small molecules produced by microbes may display a variety of bioactivities other than their antimicrobial properties.

Antibiotics at sub-inhibitory concentrations result in up or down regulation of a huge number of transcripts in different bacteria (Yim *et al*., 2007). For a molecule to be defined as cell-cell signaling molecule, it should meet many criteria that are already characteristic features of many antibiotics. For instance, a signaling molecule generates a cellular response, it is accumulated extracellularly and is recognized by specific receptor, its biosynthesis occur during specific stages of growth and under different physiological conditions (Winzer *et al*., 2002). In bacteria autoinducer, quorum sensing molecule, such as N-(3-oxododecanoyl) homoserine lactone produced by *Pseudomona aeruginosa* may have antibiotic activity (Kaufmann *et al*., 2005). According to previous studies, biofilm formation is stimulated by a variety of small molecules produced by bacteria. For instance, in *B. subtilis*, non-ribosomally synthesized peptide surfactin and nystatin were found to be inducers for biofilm formation (Lopez *et al*., 2009). In order to test the possible effect of bacilysin as a signaling molecule, pellicle formation assay was performed. Normally, undomesticated 3610 *B. subtilis* strain is unable to form biofilm in LB medium. It needs certain condition such as minimal MSgg medium, which is used as biofilm medium (Branda *et al*., 2001). In addition, *B. subtilis* can not produce bailysin in rich medium (Ozcegniz *et al*., 1990). Therefore, in order to perform the assay and to promote bacilysin biosynthesis, PY79 firstly was grown on PA medium and then bacilysin was extracted as described at section 2.9. Before the use of exctract, it was tested on *S. aureus* bioassay medium. Inhibition zone of bacilysin is clearly shown in Figure 3.19. Since the extract does not purely contain bacilysin, the extracts of non-bacilysin producer strains were also isolated in order to see if other factors might influence biofilm formation during pellicle formation assay. Therefore, together with bacilysin extract, bacilysin non-producing extracts were also applied in 24-well microtitter wells. For this purpose, bacilysin deficient OGU1 strain was used. Bioassay of OGU1 extract did not show any inhibition zone over *S. aureus* as expected since OGU1 strain do not produce bacilysin and its extract do not contain bacilysin (Figure 3.19). In addition, N-acetylglucosamine antagonizes L-anticapsin of bacilysin that makes it ineffective against organisms (Kenig & Abraham, 1976). Under the light of this information, 10mM N-acetylglucosamine was applied to bacilysin extract and bioassay of the product did not show any inhibition zone over *S. aureus* which is shown in Figure 3.19. Furthermore, bacilysin digestive enzyme peptidase R was used for the release of L-anticapsin as described in Steinborn *et al*

(2005). After enzymatic reaction, peptidase R treated bacilysin did not show any inhibition zone (Figure 3.19).

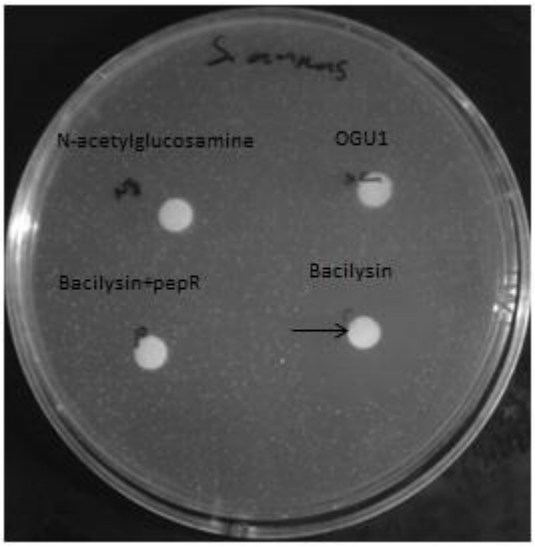

Figure 3.19: Bioassay of N-acetylglucosamine treated bacilysin, OGU1, peptidase R treated bacilysin and bacilysin extract over *S. aureus*. Since OGU1 strain is bacilysin deficient, it is unable to produce bacilysin and no zone formation is seen. The same result was seen in peptidase R treated and N-acetylglucosamine treated bacilysin tests. Before treatment, bacilysin was digested by peptidase R for 4 hr at 40°C. On the other hand, bacilysin extract itself inhibited the growth of *S. aureus* and inhibition zone was clearly present.

At first look, as the concentration of bacilysin extract applied to microtiter dish increase, the biomass formed on the surface increases, as it is shown in the pellicle formation assay (Figure 3.20). Additionally, applied crystal violet assay (Figure 3.21) revealed that there was increase in surface growth biomass when the volume of extract was further increased. Surprisingly, when peptidase R treated bacilysin extract was applied to wells, the surface growth was induced. This result might suggest that L-anticapsin region of bacilysin is an active site for the promotion of surface growth in *B. subtilis* 3610 strain. N-acetylglucosamine treated bacilysin extract and non-bacilysin producer strains PY79 and OGU1 exctracts were used as negative control since their extracts do not contain bacilysin. As expected, surface growth was not present in those assays performed. Therefore, the only factor that might promote and induce surface growth was the presence of bacilysin. As a result, it was shown that bacilysin plays vital role in the induction of surface biomass, which is preliminary step of biofilm formation.

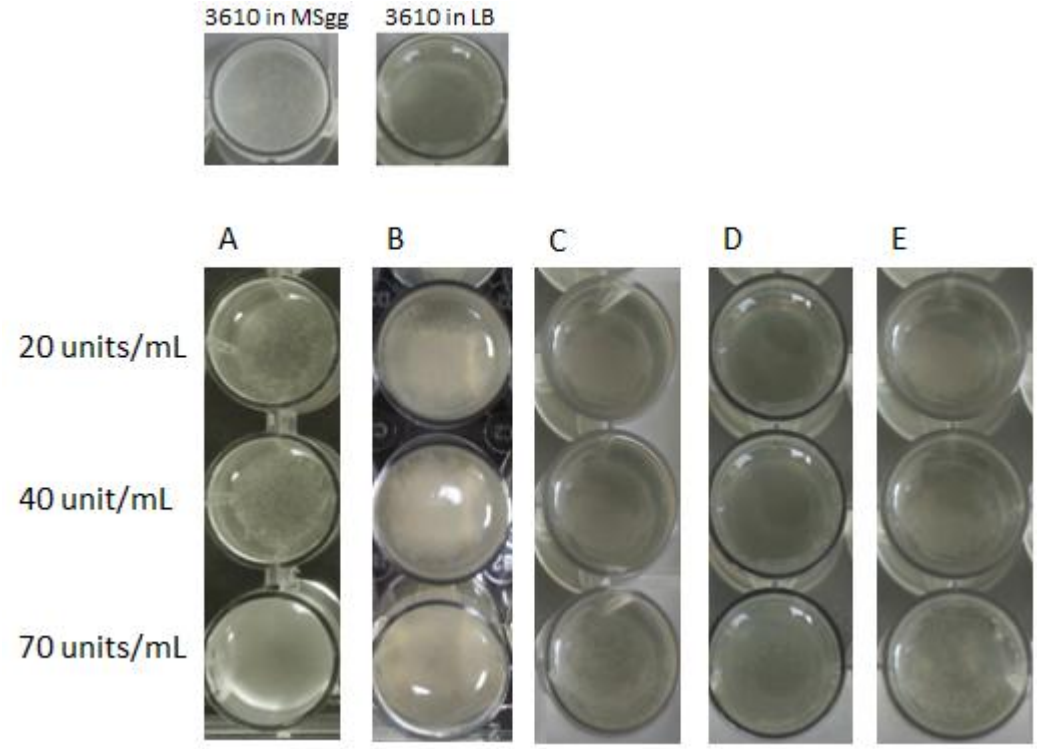

Figure 3.20: Pellicle formation assay for the effect of bacilysin on 3610 in LB. Negative control did not have any treatment. Positive control cells were grown on MSgg biofilm medium. Volumes of the extracts were applied in the given order. (A) The effect of bacilysin on surface growth. The bottom well shows the greatest surface growth. (B) The effect of peptidase treated bacilysin on surface growth. Bacilysin was digested by peptidase R before it was applied. (C) The effect of bacilysin deficient OGU1 strain's extract on surface growth. (D) The effect of Peptidase R treated bacilysin deficient OGU1 strain's extract on surface growth. (E) The effect of bacilysin in surface growth supplemented with 10mM N-acetylglucosamine as an antagonist for L-anticapsin.

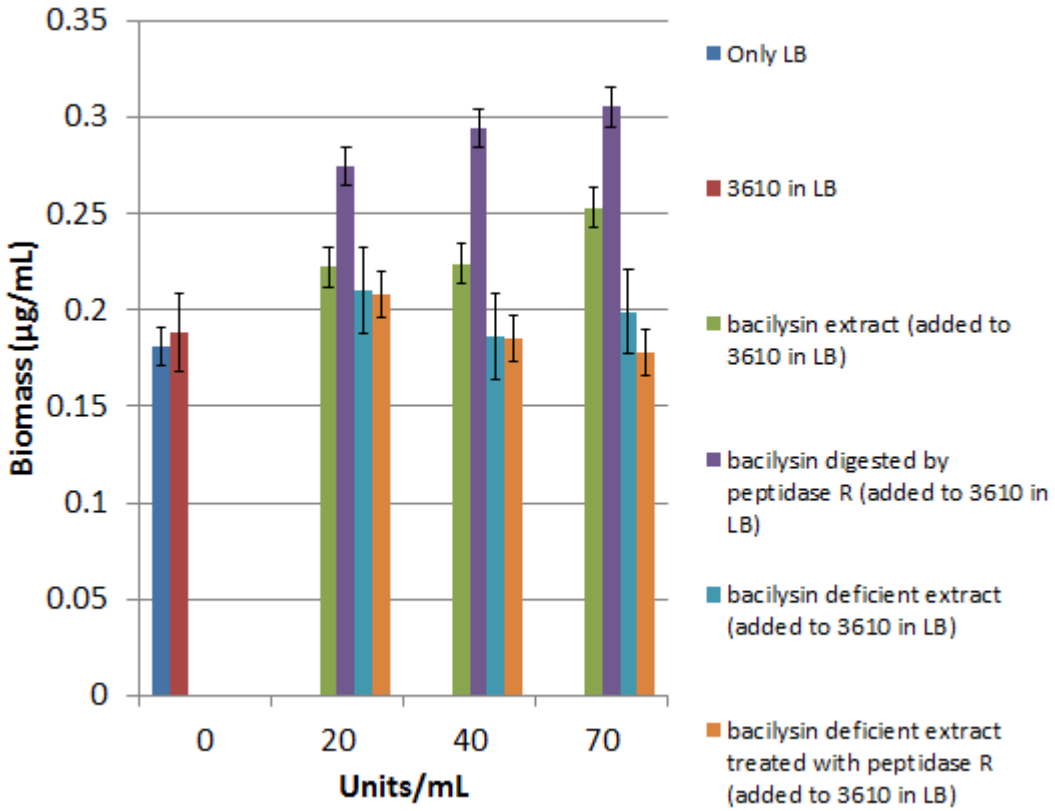

Figure 3.21: Surface growth quantification with crystal violet staining assay. Dark blue color refers to the quantification of sample only containing LB medium. Since not all crystal violet are washed from the wall of microtitter well, it is necessary to consider dye that is remained after washing step on the wall. Red color refers to the sample containing 3610 culture in LB medium. Green color refers to the sample containing 3610 in LB medium in which bacilysin extract was added. Purple color refers to the sample containing 3610 culture in LB medium in which bacilysin extract digested by peptidase R was added. Light blue color refers to the sample containing 3610 in LB medium in which the extract of bacilysin deficient strain was added. Orange color refers to the sample containing 3610 in LB medium in which the extract of bacilysin deficient strain that was digested by peptidase was added.

As it can be derived from Figure 3.21, bacilysin extract treated with peptidase gave the highest surface growth biomass while it was quantified with crystal violet assay. Secondly, bacilysin extract also induced biomass formation that was verified with crystal violet quantification. The results obtained in this experiment give strong evidence that bacilysin might be involved as a signaling molecule to regulate the level and timing of the distinct biological responses that enable *B. subtilis* to adapt to changing environment.

4. CONCLUSIONS AND RECOMMENDATIONS

Despite having extensive information on the synthesis mechanism and regulation of bacilysin biosynthesis, less is known about the role of bacilysin in the host organism. In order to understand the possible effect of bacilysin to host cell, undomesticated wild type *Bacillus subtilis* 3610 strain and its bacilysin deficient derivative *bac*$^{-}$ 3610 was used throughout this study. For the quantification analysis of various genes related to *B. subtilis* adaptation responses such as swarming motility, chemotaxis and biofilm, 3610 and its bacilysin deficient derivative strains were subjected to grow on biofilm promoting minimal agar medium known as MSgg that was previously used by Branda *et al*. Cells were collected from different time periods of incubation, 24th, 48th and 72nd hour growth phases respectively and their total RNA was isolated prior to quantification by RT-qPCR. Relative quantification calculations was done by $2^{-\Delta\Delta Cp}$ method and the value was then log2 transformed. Results from the qPCR data strongly indicates that bacilysin is responsible for the up-regulation of gene related to surfactin synthetase, *srfAA* that is crusial element for surfactin biosynthesis and is responsible for the proper motility and biofilm formation in *B. subtilis* 3610 strain. In addition, genes related to chemotaxis, motility, extracellular alkaline protease synthesis and biofilm formation such as *sigD, degU, epsD, epsK, cheB, cheV, cheY* and *aprE* were shown to be upregulated by bacilysin. Others such as *swrAA* and *epsE* either were up or down regulated by bacilysin depending on growth phases. These data were further supported by swarming motility and biofilm assays. *bac*$^{-}$ 3610 strain was unable to perform motility in the swarming motility assay. In addition, when compared with undomesticated 3610 strain that is able to form complex colony architecture, mutant strain forms fragile smooth surface growth in the MSgg agar medium. These results were compatible with our RT-qPCR analysis. According to the previous studies, bacilysin was responsible for the up or down regulation of genes related to carbohydrate metabolism in PY79 laboratory strain. Since bacilysin deficiency showed weak growth under minimal media, this strain was subjected to grow under rich standing culture and agitated minimal and rich cultures in order to

see if bacilysin deficiency may cause to auxotrophy. However, it was clearly observed that *bac*$^-$ 3610 strain was able to grow in shaking minimal and rich media. Moreover, *bac*$^-$ 3610 strain was also able to properly grow under standing rich media (SGG) as wild type 3610 strain, indicating that bacilysin deficiency did not cause in auxotrophic phenotype. In this study, bacilysin deficient 3610 and undomesticated wild type 3610 strains did not have any significant difference when carbon sources were changed under shaking condition suggesting that bacilysin deficient strain is able to utilize these sugars as wild type 3610 strain when oxygen concentration is in optimum level.

While cells are subjected to grow in a standing culture, the preliminary step for pellicle (floating biofilm) formation is surface attachment and thus surface growth in a defined or minimal liquid media. In order to see the effect of bacilysin on surface growth, *B. subtilis* 3610 strain was cultured in LB medium where undomesticated cells of *B. subtilis* 3610 normally does not form surface growth or pellicle in LB medium. Based on the pellicle formation assays, samples supplied with bacilysin extract showed significant increase in surface growth. In order to see if L-anticapsin domain is involved in the induction of surface growth, bacilysin was cleaved with peptidase R to its L-anticapsin and L-alanine moieties. Bacilysin extracts that were digested by peptidase R prior to the addition to 3610 LB culture, showed increase in surface growth that may suggest that seperation of L-anticapsin from L-alanine by cleavage with peptidase R might trigger surface growth in 3610 strain and the L-anticapsin domain of bacilysin might be involved in the induction of surface adhesion. In order to see if any other factor rather than bacilysin is involved in induction of surface growth, extracts that were collected from bacilysin deficient strains and applied to 3610 in LB as a negative control. This time there was no any surface growth. To see if peptidase R is responsible for triggering surface growth, bacilysin deficient extract was subjected to peptidase R treatment before it was applied. The result was the same as the result obtained from bacilysin deficient extract treatment to 3610 in LB where there was no any surface growth. These results were further proved by crystal violet biomass count assay that showed that as the amount of bacilysin extract applied to samples increased, the surface growth were proportionally increased. Results of this study strongly indicate that bacilysin might be involved in the promotion of surface growth that is preliminary step for biofilm

formation in undomesticated *B. subtilis* 3610 strain and strongly signifies the importance of bacilysin to the host organism.

To sum up, the study revealed the impact of bacilysin deficiency that caused many significant changes to host organism. *bacABCDEF* operon is responsible for the regulation of many adaptive responses of *B. subtilis* including biofilm formation, chemotaxis, swarming motility, competence development and stress response. Moreover, bacilysin, as a signaling molecule, might be involved in the induction of surface growth that is preliminary step of biofilm formation.

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APPENDICES

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

MSgg Medium (pH 7.0)

MOPS	20.93g/L (200mM, pH=7.0)
$CaCl_2$ $2H_2O$	0.103g/L (700µM)
$MnCl_2$ $2H_2O$	0.0081g/L (50µM)
Fe(III)Cl_3 $6H_2O$	0.0135g/L (50µM)
$MgCl_2$	0.190g/L (2mM)
$ZnCl_4$	0.00000136g/L (1µM)

Glycerol	0.5%
L-Phenylalanine	0.05g/L
L-Tryptophan	0.05g/L
Naglutamate	0.5g/L
Thiamine	0.00602g/L

5mM Potassium phosphate (pH 7)*

* 1M Potassium phosphate buffer is prepared accordingly;

6,15mL of 1M K_2HPO_4 with 3,85mL of 1M KH_2PO_4 were mixed and added directly to the medium with a final concentration of 5mM potassium phosphate.

1.5 % Agar was added if necessary before

Luria Bertani (LB) Medium (1000ml)

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

Luria Bertani (LB) Agar Medium (1000 ml)

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

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

Bioassay Medium (pH 7.1)

$Na_2HPO_4.2H_2O$	3.3 g/L
KH_2PO_4	1 g/L
NaCl	1 g/L
Glucose*	10 g/L
$MgSO_4.7H_2O$*	0.7 g/L
Na_3.citrate.$2H_2O$	0.5 g/L
Glutamic acid.Na.H_2O**	2.4 g/L
12 amino acid***	0.025 g/L (each)
$FeSO_4.7H_2O$**	0.01 g/L
Agar****	

*Autoclave glucose and $MgSO_4.7H_2O$ together, separately

**Filter Sterilization

***Arginine, cystidine , glycine, histidine, leucine, methionine, phenylalanine, proline, threonine, tryptophane, tyrosine, valine, alanine (all in L-form)

****Autoclave separately

SMS/MM (Spipizen's Minimal Salts/Medium)

$(NH_4)_2SO_4$	2 g/L
K_2HPO_4	14 g/L
KH_2PO_4	6 g/L
Na_3.citrate.$2H_2O$	1 g/L
$MgSO_4.7H_2O$	0.2 g/L

Autoclave, cool down to 50^0C, add the following sterile solutions to one liter of SMS:

50% (w/v) D-Glucose*	10 ml
L-tryptohan (5 mg/ml)*	10 ml

*Filter sterilization

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.

SGG Medium (1000mL)

Nutrient Broth	16 g
KCl	2 g
$MgSO_4$ $7H_2O$	0.5 g
$Ca(NO_3)_2$	1 mM
$MnCl_2$ $4H_2O$	0.1 mM
$FeSO_4$	1 µM
Glucose	0.1 %
Glycerol	1 %

APPENDIX B

Compositions of Buffers and Solutions

TE Buffer (pH 8)

Tris base (2 moles)	10 mM
EDTA	1 mM

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

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

$NaCl_2$ 8.5 g

Dissolve in 1000 ml distilled water and autoclave.

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

Tris (hydromethyl)aminomethane 3.03 g

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

APPENDIX C

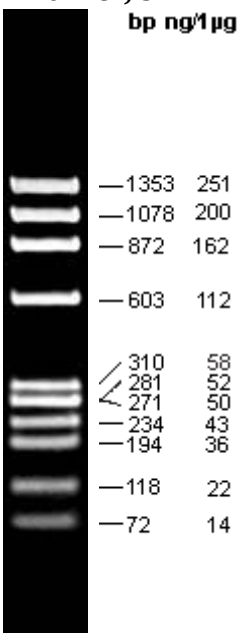
ENZYMES AND CHEMICALS

Enzyme	**Supplier**
Taq polymerase	Fermantas
T4 DNA Ligase	Fermentas
Chemical	**Supplier**
Agar	Sigma
Agarose	Sigma
Calcium chlorid ($CaCl_2$)	Merck
D(+)-Glucose monohydrate	Merck
Iron(III) sulfate – 7 – hydrate ($FeSO_4.7H_2O$)	Riedel-de Haën
L- Amino acids	Merck
Lysozyme	Sigma
Magnesium sulphate ($MgSO_4.7H_2O$)	Riedel-de Haën
Natruim hydroxid (NaOH)	Riedel-de Haën
Natrium sulfate (Na_2SO_4)	Riedel-de Haën
Polyethyleneglycol ($HO(C_2H_4O)_nH$)	Merck
Potassium chloride (KCl)	Riedel-de Haën
Potassium di hydrogen phosphate (KH_2PO_4)	Riedel-de Haën
di Potassium hydrogen phosphate (K_2HPO_4)	Riedel-de Haën
Sodium chloride (NaCl)	Riedel-de Haën
Sodium hydrogen phosphate($Na_2HPO_4.7H_2O$)	Merck
Tris (hydrocymethyl) aminomethane	Merck
Triton-X100	Sigma
Tryptone	Sigma
Yeast Extract	Sigma

APPENDIX D

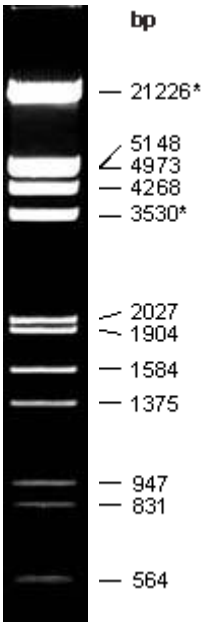
MARKERS……Fermentas

Marker 1 : PhiX174 DNA / *Bsu*RI (*Hae*III) Marker, 9



1.7% agarose
0.5µg/lane,
8cm length gel,
1X TBE, 12V/cm

Marker 3: Lambda DNA / *Eco*RI+*Hind*III Marker, 3



1.0% agarose
0.5µg/lane,
8cm length gel,
1X TAE, 17V/cm

LABORATORY EQUIPMENT

Autoclave: Tuttnauer Systec Autoclave (2540 ml)

Balances: Precisa 620C SCS

Precisa 125 A SCS

Centrifuge: Beckman Coulter, Microfuge 18

Centrifuge rotor: F241.5P

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

-20°C Arçelik 209lt

+4°C Arçelik

Electrophoresis equipments: E – C mini cell primo EC320

Gel documentation system: UVI PHotoMW Version 99.05 for Windows

Incubators: Nüve EN400

Nüve EN500

Orbital shaker incubators: Sertomat S – 2

Thermo 430

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

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

Eppendorf research 10 µl, 20 µl, 200 µl, 1000 µl

pH meter: Mettler Toledo MP220

Spectrophotometer: PerkinElmer Lambda25 UV/VIS Spectrometer

Thermocycler: Techne FTGENE 5D

Thermomixer: Eppendorf thermomixer comfort (1.5ml)

Transillumunator: Biorad UV transilluminator 2000

Vortexing machine: Heidolph Raax top

Waterbaths: Memmert wb-22

LightCycler 480: Roche

List of primers used in this study

yveN forward 5'- aag ctg ccg tat gtg gat gag-3'
yveN reverse 5'- gtg cgc tgt gtc cag cac ctt-3'
yveO Forward 5'-tgc gat gat gcg tca aca-3'
yveO Reverse 5'-aac cac ctg ata gtg tcg gtg-3'
yvfA forward 5' ctc acg gct ttt ctc ttg tct 3'
yvfA reverse 5' ata cgc att tgc ttt ctc ccg 3'
aprE Forward 5' gcg ttc agc aac atg tct 3'
aprE Reverse 5'cac ata tgc aac gct cgg 3'
cheY Forward 5' gaa gtt gtg gcg gaa gct
cheY Reverse 5' gaa atc ctt agc
cheV Forward 5' gtc ggc tct gtt tct caa
cheV Reverse 5' atc ctg caa gag gcg cat
cheB Forward 5' ttg ccg gtc att atg gtg
cheB Reverse 5' ccg aac tgt ctg gga aac
sigD Forward 5' ccc agc cgg gac tta aaa
sigD Reverse 5' tgt tga cac gac atc ctg
degU Forward 5' gcg gct cgt att gtt gag
degU Reverse 5' gta aga tcc gcc ctc agc
yvzD Forward 5' ctg ttc agc aga tat ttg gtc
yvzD Reverse 5' cag cgt gac att cga tag tga
srfAA Forward 5' tat ttg tac agg gtc agc ggc
srfAA Reverse 5' aag cag ctt ctc ttt ctc cgc

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PUBLICATIONS/PRESENTATIONS ON THE THESIS

▪ **Betim Karahoda**, T. Ebru Köroğlu, Ayten Yazgan-Karataş, 2011: The Effects of Bacilysin on the *eps* Operon of *Bacillus subtilis*. 15th *EUROPEAN CONGRESS on BIOTECHNOLOGY of the European Federation of Biotechnology* - ECB15, September 33-26, 2011 Istanbul Turkey