ISTANBUL TECHNICAL UNIVERSITY ★ GRADUATE SCHOOL OF SCIENCE ENGINEERING AND TECHNOLOGY

IDENTIFICATION OF BACILYSIN PRODUCER CELLS DURING BIOFILM FORMATION IN *Bacillus subtilis*

M.Sc. THESIS

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ISTANBUL TEKNİK ÜNİVERSİTESİ ★ FEN BİLİMLERİ ENSTİTÜSÜ

Bacillus subtilis'DE OLGUNLAŞMIŞ BİYOFİLM YAPISINDA BASİLİSİN ÜRETEN HÜCRELERİN BELİRLENMESİ

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To my family,

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ABBREVIATIONS

µg	**:** Microgram
µl	**:** Microliter
Amp	**:** Ampicillin
BLAST	**:** Basic Local Alignment Search Tool
bp	**:** Base pair
DNA	**:** Deoxyribonucleicacid
dNTP	**:** Deoxynucleoside triphosphate
EDTA	**:** Ethylene diamine tetra-acetic acid
LB	**:** Luria-Bertani
MFI	**:** Median fluorescence intensity
ml	**:** Milliliter
mM	**:** Millimolar
NCBI	**:** National Center for Biotechnology Information Database
ng	**:** Nanogram
PCR	**:** Polymerase chain reaction
RNA	**:** Ribonucleicacid
rpm	**:** Rounds per minute
TAE	**:** Tris-Acetic acid-EDTA
Tm	**:** Melting temperature
U	**:** Units
UV	**:** Ultraviolet
QS	**:** Quorum-sensing

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IDENTIFICATION OF BACILYSIN PRODUCER CELLS DURING BIOFILM FORMATION IN *Bacillus subtilis*

SUMMARY

Biofilm is defined as clusters of microbial cells that are surrounded by extracellular polymeric substances (EPS), and irreversibly attached on a surface or to each other. *Bacillus subtilis* NCIB 3610 undomesticated strains form complex, thick biofilms with wrinkled structures. *B. subtilis* as a model organism is preferred in biofilm studies, because it's a well-known and non-pathogenic organism.

Biofilm formation initiates with the expression of matrix genes in response to some external signals. At the beginning, cells are short, motile rods but as the biofilm develops, they form long chains of non-motile cells that adhere to each other and to the surface by secreting an extracellular matrix. During biofilm maturation process, genetically identical *Bacillus subtilis* cells express different genes and specialized into cell types that carry out different functions within the population. Therefore, mature biofilms contain multiple coexisting cell types which are spatially organized: long chains of nonmotile cells that are held together in bundles which produce an extracellular matrix, small rod shape flagellated motile cells and spores. Differenciation of *B. subtilis* population into various cell-types enhances by combination of Quorum Sensing (QS) signals and heterogeneity within the QS signaling networks.

Bacilysin produced by *Bacillus* species is one of the simplest peptide antibiotics that composed of an L-alanine residue at the N-terminus and a non-proteinogenic L-anticapsin at the C-terminus. The polycistronic *bacABCDEF* operon (formerly *ywfBCDEFG*), the monocistronic *bacG* (*ywfH*) gene are responsible for bacilysin biosynthesis. The production of bacilysin is controlled by ComQ/ComX, PhrC (CSF), ComP/ComA mediated by quorum sensing system and also by Spo0K (Opp). The comparative transcriptome analysis of *B. Subtilis* PY79 and its bacilysin non-producer derivative strain in bacilysin production medium performed by our research group strongly suggested that bacilysin plays important roles in sporulation, biofilm formation, competence development. Under normal condition, NCIB 3610 strain cannot form biofilm in LB medium. But, it was observed that 3610 strain can produce a thin layer biofilm in LB medium upon supplementing with bacilysin. *B. subtilis* QS molecule ComX and the lipopeptide antibiotic surfactin encoding by *srfA* operon in *B. subtilis* were shown to be required for bacilysin biosynthesis by our research group. Recently, surfactin was shown to be another QS molecule triggering a subpopulation of *B. subtilis* cells to become extracellular matrix producers. Interestingly, ComX QS molecule triggers only a subpopulation of cells to produce surfactin, in turn, surfactin causes another subpopulation of cells to produce an extracellular matrix. All of those findings not only strengthened the regulatory role of

bacilysin in the biofilm formation process, but also strongly suggested that bacilysin, like surfactin, could be another QS molecule triggering cells to differentiate into distinct cell-type within a developing biofilm. To obtain initial evidences about this possibility, the present study was designed to elucidate whether bacilysin is produced by subpopulation of cells. For this, a transcriptional fusion with the open reading region encoding the green fluorescent protein (GFP) and the promoter region (P_{bac}) of the *bacABCDEF* operon was constructed in 3610 to identify individual cells producing bacilysin in biofilm structure. The resulting recombinant strain, *amy*E::P_{bac}::*gfp* 3610 was, then grown on biofilm-inducing minimal medium MSgg agar for 24, 48, 72 h. Samples were taken from 3 different regions in the biofilm structure on MSgg agar dependent on spatiotemporal organization, and observed under bright-field and fluorescence microscope comparatively to detect cells expressing P_{bac}-*gfp*. But, distinct fluorescent cells could not identified in the *amyE*::P_{bac}::*gfp* 3610 cells with direct fluorescence microscopy, most probably, due to very faint expression level of P_{bac}-*gfp* in 3610 cells. In order to check the expression level of bacilysin operon, β-galactosidase assay was performed with *B. subtilis* 3610 strain containing a transcriptional P_{bac}-*lacZ* fusion at *bacA* locus (*bacA::erm::lacZ*). It was found that, expression level of bacilysin operon was about 8 fold reduced in 3610 cells compared to the domesticated laboratory strain PY79 cells. Furthermore, flow cytometry analysis performed with the cells harvested after 24 h and 72 h growth on biofilm-inducing medium showed a subpopulation of 8.2% and 2.22% of the cells expressing the reporter for bacilysin operon at 24 h and 72 h of growth, respectively.

B. subtilis PY79 strain keeps the ability to proliferate into heterogeneous population existing in morphologically distinct cell-types like 3610 even it lost the ability to produce extracellular matrix. Therefore, *amyE*::P_{bac}::*gfp* PY79 recombinant strain was immediately contructed and grown on MSgg agar to identify individual cells producing bacilysin under biofilm condition. The samples were collected and analysed as described before. Flow cytometry analysis demonstrated that like 3610 cells, distribution of PY79 cells expressing fluorescence was bimodal with most cells exhibiting little or no expression of the P_{bac}-directed *gfp* and a subpopulation of %50.3 and %63.8 of the cells exhibiting fluorescence at 24 h and 72 h growth, respectively. Direct microscopic observation of the PY79 cells supported the flow cytometry results. Small rod shape motile cells and single individiul cells exhibited little or no fluorescence, but stronger fluorescence was detected only in the cells aggregated and clumped together and in long chains of nonmotile cells that formed bundles which constitute matrix producing cells in a biofilm community, revealing that bacilysin could be produced by a small subset of matrix producing cells in *B. subtilis* biofilm structure.

Bacillus subtilis'DE OLGUNLAŞMIŞ BİYOFİLM YAPISINDA BASİLİSİN ÜRETEN HÜCRELERİN BELİRLENMESİ

ÖZET

Gram pozitif bakteriler arasında yer alan *Bacillus subtilis* çubuksu yapıya sahip, patojen olmayan, mezofilik, özellikle toprak, bitki kökleri veya hayvanların gasterointestinal sistemlerinde yaşayan bir bakteri türüdür. *B. subtilis* besin kıtlığı veya çevresel stresler gibi ortamından aldığı sinyallere ve büyüme koşullarına göre fizyolojik durumunu değiştirebilen, yüksek adaptasyon kabiliyetine sahip bir bakteridir ve bu değişimler morfolojik ve fenotipik olarak da gözlemlenebilmektedir. Fizyolojik durum değişikliğini tetikleyen sebepler arasında çevredeki besin kaynaklarının ulaşılabilirliği, kullanılabilirliği ve onlara karşı olan rekabet, ortamın pH değeri, sıcaklığı ve oksijen miktarı gösterilebilir. Bu fizyolojik durum değişikliklerine bakterinin verdiği adaptif cevaplar arasında da sporulasyon, kemotaksis, genetik kompetans gelişimi ve antibiyotik üretimi sayılabilir ve tüm bunlar hücre yoğunluğunun algılanmasına bağlı oluşan sinyalizasyon ağı (quorum sensing) tarafından düzenlenmektedir.

Eğer logaritmik büyüme evresi sonunda, *B. subtilis* hücreleri sınırlı besin kaynağı sinyali alırsa, bu durumdan kaçınabilmek için kemotaksis (kimyasala yönelim) ya da hareketlilik (motilite) özelliklerini geliştirmektedir. Bu besinsel kıtlık durağan büyüme evresinde hala devam ediyorsa, *B. subtilis* çevredeki alternatif gıda kaynaklarını parçalayacak proteazlar gibi ya da ortamda üstünlük elde etmesini sağlayacak antibiyotikler gibi ikincil metabolitler üreterek dış ortama gönderebilmektedir. Daha ileri safhalarda ise, hücreler kompetans geliştirip dışarıdaki DNA parçalarını içine alarak genetik çeşitliliğini arttırmaktadır. Tüm bu adaptif tepkilerin yetersiz kaldığı durumda ise *B. subtilis* son çare olarak hayati fonksiyonlarını durdurarak spor oluşturmaktadır.

Biyofilm, bakteriler tarafından üretilen hücredışı polimerik maddelerle (EPS) çevrili, bakterilerin geri dönüşümsüz olarak bir yüzeye veya birbirlerine yapıştıkları mikrobiyal hücre kümeleri olarak tanımlanmaktadır. Biyofilm besin kıtlığı gibi çevresel değişimlere karşı geliştirilen bir adaptasyon olarak, bakteriler arasındaki iletişime dayanan gen ekspresyonunun düzenlenmesi sonucu oluşur ve topluluk içindeki bakterilere antibiyotiklerden veya dezenfektanlardan korunma gibi çeşitli avantajlar sağlar. *B. subtilis* NCIB 3610 yaban suşu kompleks, damar benzeri görünüme sahip, kalın biyofilm yapıları oluştururken PY79 gibi evcileştirilmiş laboratuvar suşları belirgin şekilde zayıflatılmış, ince, kırılgan, ayırt edici bir makroskopik yapıya sahip olmayan hücre toplulukları oluşturmaktadır. *B. subtilis* genomunun tamamı dizilenmiş, iyi bilinen ve patojen olmayan bir model organizma olması sebebiyle biyofilm çalışmalarında tercih edilmektedir. Biyofilm oluşumu, bazı dış sinyallere cevap olarak matris genlerinin ifadesiyle başlar. Başlangıçta,

hücreler kısa, hareketli, çubuk şeklindedir ancak biyofilm geliştikçe, hücre dışı bir matris salgılayıp birbirlerine ve yüzeye yapışarak hareketli olmayan uzun hücre zincirlerini oluştururlar. Biyofilm olgunlaşma sürecinde, genetik olarak aynı *B. subtilis* hücreleri, farklı genleri ifade eder ve popülasyon içinde farklı fonksiyonları yerine getiren hücre tiplerine özelleşirler. Olgun biyofilmler, mekansal olarak organize olmuş çok sayıda ve bir arada var olan hücre tipini içerir. Bunlar uzun zincirler oluşturup birbirlerine ürettikleri hücre dışı matris ile tutunan hareketsiz hücreler, hareketli kısa çubuk şeklindeki hücreler ve sporlanan hücrelerdir. *B. subtilis* popülasyonunun çeşitli hücre tiplerine farklılaşması, hücre yoğunluğunun algılanmasına bağlı oluşan sinyalizasyon ağı (quorum sensing) ve onu kontrol eden sinyalleşme ağlarındaki heterojenliğin bir araya gelmesiyle artar.

Bacillus suşları tarafından üretilen basilisin çeşitli bakterilere ve bazı mantarlara karşı antimikrobiyal etki gösteren, L-alanin ve protein kökenli olmayan L-anticapsin'den oluşan basit yapılı bir dipeptit antibiyotiktir. Basilisinin antimikrobiyal aktivitesi yapısındaki L-anticapsinden kaynaklanmaktadır. Polisistronik *bacABCDEF* (eski ismiyle *ywfBCDEFG*) operonunun, monosistronik *bacG* (*ywfH*) geni ile birlikte basilisin biyosentezinden sorumlu olduğu bilinmektedir. *bacABCDF* ve *bacG* basilisinin L-anticapsin oluşumu ve L-alanin ile ligasyonu gibi üretim aşamalarında görev alırken, *bacE* antibiyotiğin dışarı sentezlenerek üretici organizmanın korunmasından sorumludur. Basilisin biyosentezi ComQ/ComX, PhrC (CSF), ComP/ComA etkisinde hücre yoğunluğuna bağlı sinyalizasyon ağı tarafından kontrol edilir ve Spo0K (Opp)'ya bağlı bir şekilde gerçekleşir. İkincil metabolit olan antibiyotiklerin, üretici organizmaya kazandırdıkları antimikrobiyal aktivite dışında tür içi yada türler arası iletişimde kullanılan anahtar sinyal molekülü gibi farklı görevleri olup olmadığı hep merak konusu olmakta ve en popüler çalışma konularını oluşturmaktadır. Basilisinin özellikle araştırma grubumuzda yürütülen çalışmalarla *B. Subtilis*'de çoklu etkiye sahip bir regülatör sinyal peptiti (feromon) olabileceği gösterilmiştir. Bu bulgunun temeli laboratuvar grubumuz tarafından yapılmış olan basilisin üreten *B. subtilis* PY79 ve onun basilisin üretmeyen mutant türevi ile basilisin üretim besiyeri olan PA ortamında karşılaştırmalı transkriptom analizlerine dayanmaktadır. Bu analizler basilisinin kompetans gelişimi, sporulasyon ve biyofilm oluşumu gibi birçok adaptif cevap oluşumunda düzenleyici etkisi olduğunu işaret etmektedir. Bu çalışmalara ek olarak *B. Subtilis* NCIB 3610 suşunun normal şartlarda biyofilm oluşturamadığı kompleks bir besi yeri olan Luria Broth (LB) içerisine basilisin ekstraktı eklendiğinde yüzeyde kırılgan yapıda ince bir biyofilm oluşturduğu gözlemlenmiştir. En önemli çalışma ise, *srfA* operonu tarafından ifade edilen sürfaktin antibiyotiğinin *B. Subtilis*'de biyofilm oluşumunu tetikleyen bir hücre yoğunluğunun algılanmasına bağlı oluşan sinyalizasyon ağı molekülü olduğunun bulunmasıdır. Biyofilm yapısındaki hücrelerin çoğunluğu ComX üretebilirken, yalnızca küçük bir alt populasyon ComX'e yanıt olarak sürfaktin üretebildiği ve sürfaktin ise başka bir hücre kümesini matriks üretici hücrelere dönüştürdüğü bulunmuştur. Daha önceki çalışmamızda, *srfA* operonun inaktive edilmesi sonucunda basilisinin biyosentezinin durması ile sonuçlanması, aynı zamanda sürfaktin biyosentezinde olduğu gibi basilisin biyosentezinin de hücre yoğunluğuna bağlı sinyalizasyon ağı molekülü olan ComX tarafından tetiklenmesi, sürfaktin gibi basilisinin de biyofilm oluşumu sürecinde düzenleyici rolü olabileceği ihtimaline işaret etmektedir. Tüm bu bilgilerin ışığında, bu proje kapsamında, basilisinin de sürfaktin gibi hücre yoğunluğuna bağlı sinyalizasyon ağı molekülü olup olmadığı bilgisine ulaşmamızı sağlayacak çalışmaların başlangıç bulgusunu oluşturacak, biyofilm yapısı içerisinde bulunan

farklılaşmış hücre toplulukları arasında basilisin biyosentezinin özelleşmiş belirli hücre tipleri tarafından üretilip üretilmediği ihtimalinin araştırılması hedeflenmiştir. Bu amaçla öncelikle basilisin biyosentezinden sorumlu *bacABCDEF* operonunun promotör bölgesi (P_{bac}) ve yeşil floresan proteinini (GFP) kodlayan açık okuma bölgesi ile transkripsiyonel bir füzyon oluşturulmuştur. Oluşturulan P_{bac}::*gfp* vektörü biyofilm oluşturabilen yabanıl bir suş olan *B. subtilis* NCBI 3610 transforme edilerek *amy*E::P_{bac}::*gfp* 3610 rekombinant suşu elde edilmiştir. *B. subtilis* NCBI 3610 rekombinant suşu biofilm oluşturma besiyeri olan MSgg katı besiyerinde 24, 48, 72 saat büyütülmüş, *bacABCDEF* operonunun ifade edildiği hücreleri tespit etmek amacıyla floresan mikroskobu altında incelenmişlerdir. Fakat floresan mikroskobu altında *amy*E::P_{bac}::*gfp* 3610 suşunun oluşturduğu biyofilm yapısından farklı zamanlarda ve farklı bölgelerden alınan örneklerde muhtemelen çok düşük P_{bac}-*gfp* ekspresyon seviyesi sebebiyle, P_{bac}-*gfp* füzyonunu ifade eden tekli hücreler tanımlanamamıştır. Bu ihtimalin doğrulanması için, *bacA* lokusunda (*bacA*::erm::*lacZ*) transkripsiyonel P_{bac}-*lacZ* füzyonu içeren *B. subtilis* 3610 suşu kullanılarak, basilisin operonunun ifade seviyesi β-galaktosidaz testi ile ölçülmüş ve tahmin edildiği gibi, laboratuar suşu PY79'a kıyasla, yabanıl suş 3610'da basilisin operonunun ifade seviyesinin yaklaşık 8 kat az olduğu bulunmuştur. Diğer taraftan, 24 ve 72 saat MSgg katı besiyerinde büyütülmüş hücrelere uygulanan akış sitometrisi analizinde olumlu sonuçlar elde edilmiş, floresan ifadesi görülen hücrelerin sırasıyla populasyonun %8.2 ve %2.22 oluşturduğu belirlenmiştir. Bu durumdan, 3610 suşunun biyofilm yapısında basilisin üretiminin küçük bir alt grup tarafından gerçekleştirildiği yani bimodal ekspresyon paterni gösterdiği anlaşılmıştır.

Floresan mikroskobu altında biyofilm yapısındaki hangi hücre grubunda; hareketli, kısa çubuk şeklinde olan hücrelerde mi yoksa matriks üreticisi hareketsiz uzun hücre zincirlerinde mi basilisin üretiminin gerçekleştirildiğinin belirlenmesi için, matriks üretme özelliğini kaybetse bile 3610 gibi farklı hücre tiplerine farklılaşarak, heterojen populasyon oluşturabilen PY79 suşunda *amyE*::P_{bac}::*gfp* PY79 rekombinant suşu oluşturulmuştur. Daha önce tanımlandığı şekilde MSgg katı besiyerinde büyütülmüş, örnekler toplanıp analiz edilmiştir. 3610'da olduğu gibi akış sitometrisi analizi, PY79 suşunda da P_{bac}-*gfp* füzyonunu ifade eden hücrelerin bimodal dağılım gösterdiğini ortaya koymuştur. 24 ve 72 saat büyütülmüş hücrelerde floresan raportör ifadesi görülenler sırasıyla popülasyonun % 50.3 ve % 63.8'ini oluşturduğunu göstermiş, mikroskobik gözlemlerde bu sonucu desteklemiştir. Küçük çubuk şekilli hareketli hücrelerde ışıma düşük olmasına ya da hiç görülmemesine rağmen hücre toplulukları ve uzun zincirler oluşturup birbirlerine tutunan hareketsiz hücrelerde daha güçlü ışıma gözlemlenmiştir. Bu durum, basilisinin biyofilm yapısında matris oluşturan hücreler tarafından üretiliyor olabileceğini ortaya çıkarmıştır.

1. INTRODUCTION

1.1 Biofilm

Bacteria are generally found in nature either as free-floating planktonic cells or as sessile aggregates (biofilm). Microorganisms were thought to be exist as freely suspended cells until discovery of surface-attached organisms (‘“animalcule”) on human tooth by Antoni van Leeuwenhoek using his simple microscope in the 17thcentury (Donlan, 2002; Garrett et al., 2008). Later, number of bacteria (99% of wild population) observed on surfaces either as solid or air-liquid interface of aqueous environments was examined much higher than in bulk medium such as seawater (Zobell, 1943; Dalton and March,1998). The term ‘Biofilm’ was put forth by Bill Costerton in 1978 (Chandki et al., 2011). Recently, usage of CLSM to monitor biofilm structure and study on genes associated with biofilm formation have improved understanding of biofilms.

Biofilms are defined as aggregates of microbial cells that are surrounded with self-produced extracellular polymeric substances (EPS) and irreversibly attached to a surface (adhesion) and/or each other (cohesion). Substratums that biofilm forms can be both living such as tissues or nonliving things such as industrial water system piping (Figure 1.1) (Donlan, 2002; Flemming et al., 2016).

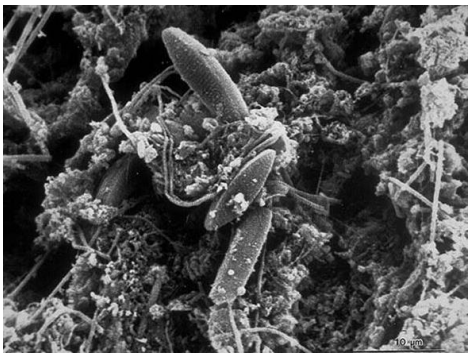

Figure 1.1 : Scanning electron microscope image of biofilm formation on a mild steel surface in an industrial water system (Donlan, 2002).

Advantages of biofilm formation for bacteria are various such as protection from antibiotics, disinfectants. Also, regulation of gene expression based on

communication between biofilm forming cells induces temporal adaptation to changing environment such as nutrient deficiency (Garrett et al., 2008).

1.1.1 Biofilm development

Biofilms comprise of micro colonies of bacterial cells (15% by volume) separated by water channels and of extra polymeric substances (85%) which provide essential nutrients, facilitate intracellular communication and genetic transfer, and offer suitable environment to grow (Kokare et al., 2008; Vasudevan, 2011). The matrix is mainly composed of water (97%) containing polysaccharides along with metal ions, DNA, protein, lipids (Flemming et al., 2016).

Biofilm development process has various step in general: primary contact with the surface; reversible adhesion; irreversible adhesion; biofilm maturation and dispersal (Figure 1.2). Firstly, Brownian movement or force of gravity enables bacterial cells to contact with the surface. Although some gram negative pathogens such as *E.coli* or *Salmonella* use their flagella to facilitate motility and overcome repulsive forces, nonmotile bacteria develop biofilms equally due to surface adhesion molecules called curli. After initial contact to surface, bacteria in planktonic phase reversibly attach to the surface in consequences of physiochemical and electrostatic interactions, thereby environmental conditions such as temperature, pH affects this attachment. Then, the reversibly attached cells becomes immobilized, and followed by irreversible adhession biofilm matures with speciliazed cells and enclosed matrix (Vasudevan, 2011; Garrett et al., 2008).

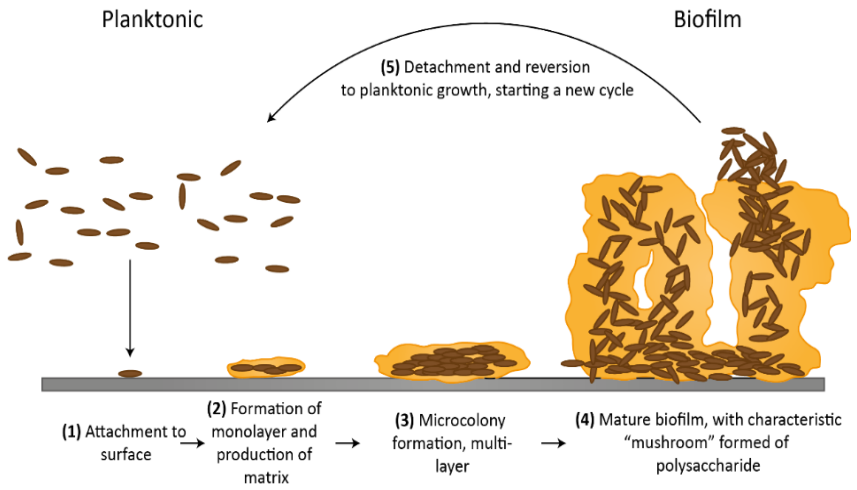

Figure 1.2 : Diagrammatic representation of biofilm development.

1.1.2 *Bacillus subtilis* as a biofilm forming bacterium

It is important to understand biofilm development mechanisms due to govern detrimental biofilm formation and promote beneficial biofilm, and because of rigorous requirements to control biofilm of pathogenic bacteria such as *E.coli*, *P. aeruginosa*, *V. cholerae*, *Candida* sp., *Staphylococcus* sp., using suitable bacterium has been more preferable (Morikawa, 2005). *B. subtilis* is non-pathogenic, motile, rod-shaped, spore-forming, Gram-positive bacterium and also a robust model organism for studying the mechanism of biofilm development. *B. subtilis* is the best-known member of the Gram-positive bacteria, and its whole genome sequence was finished in 1997. Its genome consists of 4,214,810 base pairs related to about 4,100 protein-coding genes (Kunst et al., 1997). Besides *B. subtilis* might grow in a medium composed of only carbon, nitrogen and salts, the bacterium achieves to survive in limiting environment by its sporulation and competence features (Vlakamis et al., 2013).

B. subtilis undomesticated, ancestral strain called NCIB 3610 is broadly studied as a model under laboratory conditions for biofilm formation on different areas including air-agar interface as colony biofilm, or air-liquid interface as floating biofilm (pellicle) (Vlakamis et al., 2013, Cairns et al., 2014). Wrinkled structures form during growth of the cells in consequences of localized cell deaths along with rigidity of extracellular matrix. The matrix of *B. Subtilis* comprises of EPS synthesized by *epsA-epsO* operon, and proteins like TasA, TapA (TasA anchoring/assembly protein, formely YqxM), and coat protein called BslA (formerly YuaB) (Cairns et al., 2014; Vlakamis et al., 2013; Branda et al., 2006).

1.1.3 Regulation of biofilm development in *Bacillus subtilis*

During biofilm formation of *Bacillus subtilis*, individual cells within isogenic population demonstrate cellular differentiation leading to heterogenous population composed of motile, matrix-producing, and sporulating cells within distinct region of the biofilm (Figure 1.3) (Vlakamis et al., 2008).

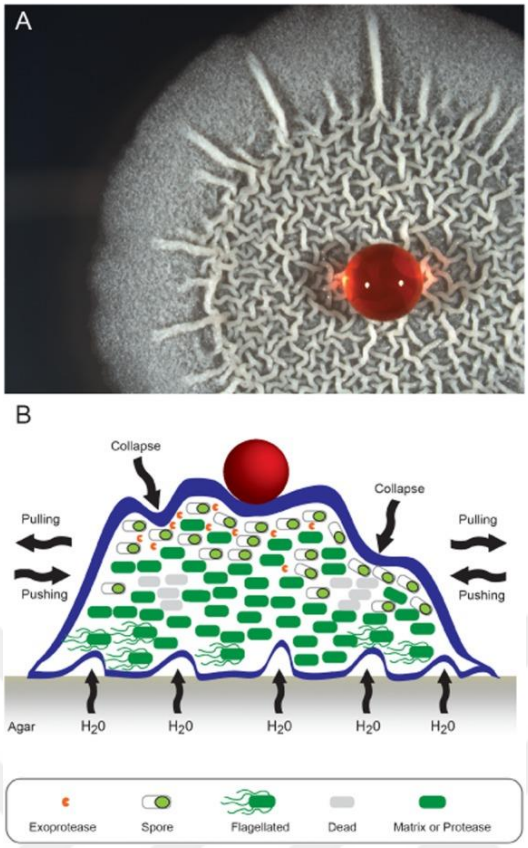

Figure 1.3 : (A) The mature biofilm **(B)** Schematic representation in cross-section of figure A. Dark blue layer: BsIA coat; Red ball: a water droplet representing the hydrophobicity produced by the structure.

Phosphorlyation of transcriptional regulator Spo0A is essential for initiation of biofilm formation, and the concentration of active form of Spo0A (Spo0A~P) determined by four kinases (KinA, KinB, KinC, KinD) facilitates cellular differantiation. Low or intermediate levels of Spo0A~P result in the induction of the matrix genes expression by triggering removal of two repressor (AbrB and SinR) by two antirepressor (AbbA and SinI), while higher levels of Spo0A~P induce sporulation genes as the biofilm matures. Also, action of SlrR on SinR occurs in two ways: SlrR prevents SinR from repressing expression of matrix genes and forms SinR-SlrR complex due to turn into a repressor of autolysin and motility genes resulting in cell chains. Another signaling cascade is triggered by inhibition of flagellar rotation. The level of phosphorylation of DegU (DegU~P) determines cell faith. At the unphosphorylated level, DegU activates ComK expression leading to competence development. Intermediate level of DegU~P triggers formation of coat protein BslA leading to biofilm development, however higher level of DegU~P

causes inadequate transcription from the *tapA* and *eps* operons resulting in inhibition of biofilm formation (Vlamakis et al., 2013; Cairns et al., 2014; Kobayashi, 2008).

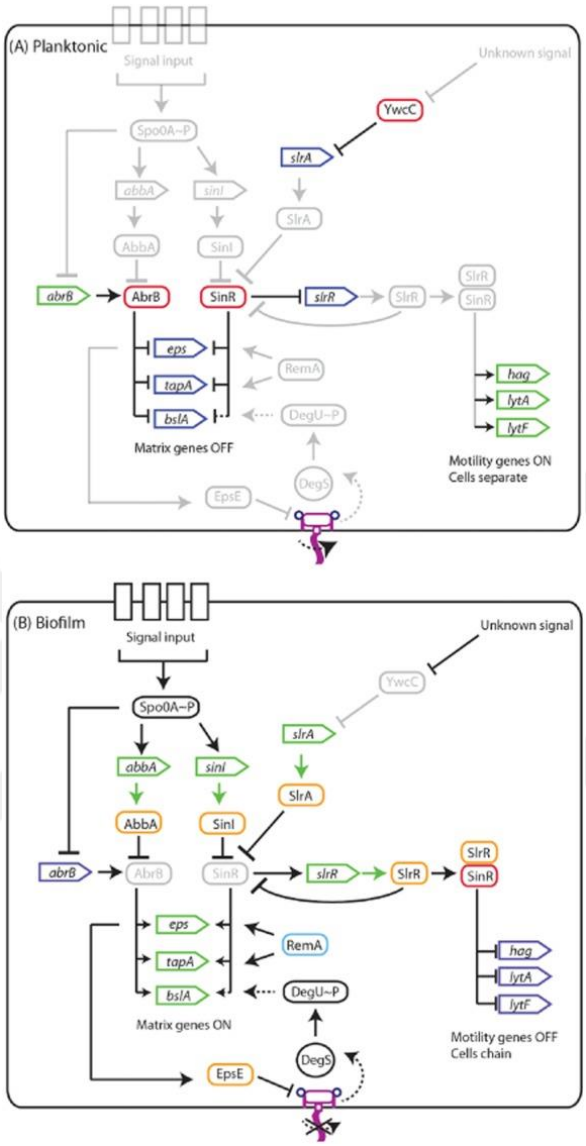

Figure 1.4 : Schematic representation of regulatory networks of **(A)** planktonic growth and **(B)** biofilm formation. Rounded rectangles: proteins; Triangles: ORFs; Arrows: activation; T-bars: repression; Dashed arrows or T-bars: indirect activation and repression respectively; Green: active gene transcription; Green arrow: translation; Dark blue: absence of gene transcription; Red: a transcriptional repressor; Orange: a protein–protein interaction; Light blue: a protein that is able to bind to DNA to activate transcription; Faded shading: parts of the pathway that are inactive. Pink structure: a flagellum, with the curved arrow indicating rotation and the cross indicating inhibition of flagellar rotation. Vertical rectangles labelled with "signal input" : sensor kinases for the Spo0A pathway (Adopted from Vlamakis et al., 2013; Cairns et al., 2014).

1.2 Quorum Sensing

Quorum sensing (QS) is cell-cell communication system, which occurs both within and between bacterial species, by regulating gene expression in response to alteration in cell-population density. Bacteria release signal molecules termed as autoinducers to increase in concentration until threshold value as a function of cell density leading to change in gene expression and then physological activities in cells. Competence development, antibiotic production, sporulation, biofilm formation, motility, chemotaxis, and symbiosis can be controlled by this system in both Gram-negative or Gram-positive bacteria (Miller & Bassler, 2001). Quorum sensing system is thought to lead the development of multicellularity.

Several kind of QS signals exist, but in general there are two types of the system: Gram-negative LuxIR circuits and Gram-positive oligopeptide two-component circuits (Taga et al., 2003). In Gram-negative quorum sensing, bacteria commonly have protein homologous to LuxI and LuxR proteins of *Vibrio fischeri* ,bioluminescent marine bacterium, which is the first discovered QS system (Nealson & Hastings, 1979; Taga et al., 2003). The LuxR-type proteins, cytoplasmic autoinducers, bind a specific acylated homoserine lactone (AHL) autoinducer, whose formation catalyzed by the LuxI-type proteins, autoinducer synthase, when the concentration of AHL reaches a threshold level out of the cell. Then, the LuxR–AHL complexes binds luciferase operon (*luxICDABE*) required for light production (Figure 1.5) (Engebrecht et al. 1983; Engebrecht & Silverman, 1987; Taga et al., 2003; Water & Bassler, 2005).

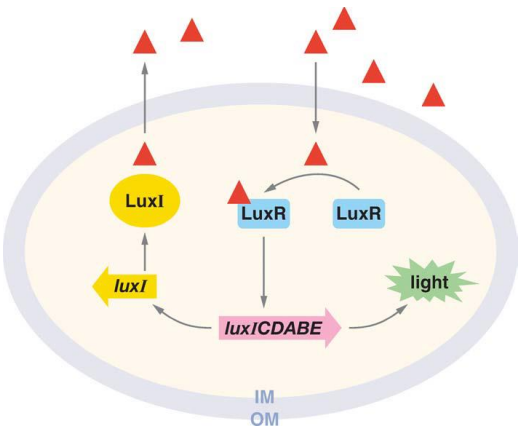

Figure 1.5 : Quorum sensing in Gram-negative bacteria. Red triangles: the autoinducer produced by LuxI. OM, outer membrane; IM, inner membrane (Water & Bassler, 2005).

Unlike gram-negative bacteria, in gram-positive bacteria QS system, secreted peptides throughout an ATP Binding Cassette (ABC) transporter are used as autoinducers. These autoinducers are detected by two component adapter response proteins, which are receptor histidine kinases and a DNA-binding response regulator, through phophorylation/dephosphorylation cascade. Firstly, autoinducer signal peptide is translated and then processed by pheromone precursor locus, then secreted out of the cell. When the autoinducers reach a threshold level, they are detected by sensor kinase, and the interaction between them trigger autophosphorylation of histidine residue on the kinase. After that, the phosphoryl group is moved to aspartate residue on response regulator, and transcription of the target gene is altered (Figure 1.6) (Miller & Bassler, 2001; Water & Bassler, 2005; Vijayalakshmi, M., 2013).

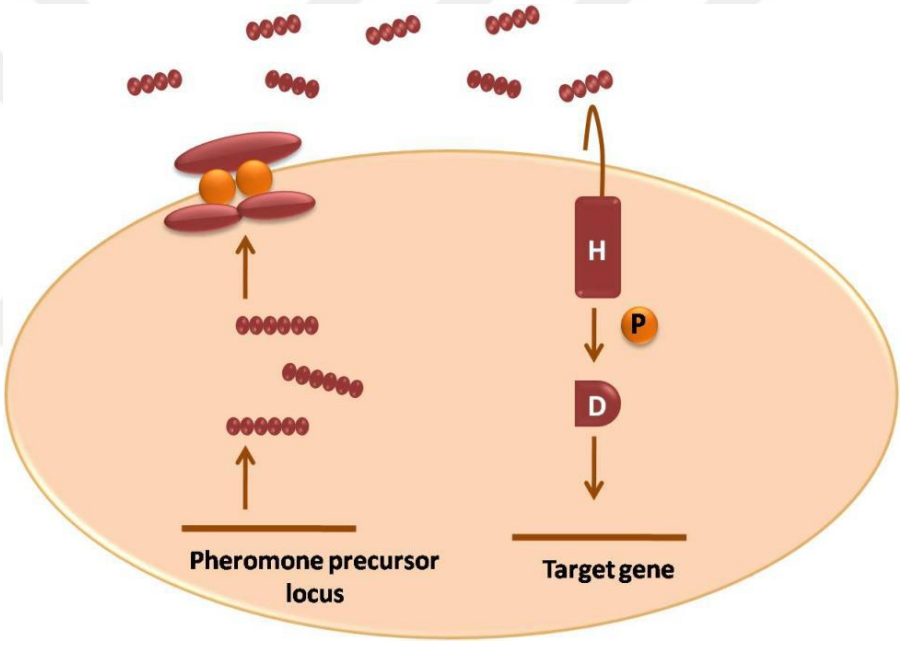

Figure 1.6 : Quorum sensing in Gram-positive bacteria. Sensor kinase, histidine residue (H); Response regulator, aspartate residue (D); phosphorylation cascade (P) (Vijayalakshmi, M., 2013).

1.2.1 Quorum sensing system in *Bacillus subtilis*

Bacillus subtilis quorum sensing system coordinates genetic competence development, sporulation, degradative enzyme production, biofilm formation, secretion of numerous molecules such as antibiotics by ComQXPA pathway, which is major QS pathway (Figure 1.7) (Bendori et al.,2015; Köroglu et al., 2011). Competence and sporulation processes of *Bacillus subtilis* interact mutually. After processing and modification by the the ComQ isoprenyl transferase, QS molecule

ComX is secreted into extracellular medium. Upon reaching the threshold concentration, ComX binds to histidine kinase ComP and induce its autophosphorylation. Phosphorylated ComP, transfer its phosphorly group to response regulator ComA. Thus, phosphorylated and activated the response regulator ComA elicits QS response. The ComQXPA QS system controls the expression of various genes including the *srfA* operon encoding lipopeptide antibiotic surfactin and the *comS* gene, which regulates the competence development and is embedded within the *srfA* operon. On the other hand, competence and sporulation factor (CSF) secreted out of the cell is back transported into the cell by oligopeptide permase Opp (Spo0A). When internal concentration of CSF is low, CSF binds to RapC protein and prevents the dephosphorylation of ComA by RapC, thus stimulating the competence development. When concentration of CSF is high, CSF reduces the phosphorylated level of ComA in an unknown manner, thus decreasing the competence development, while it directly prevents the binding of the serine phosphotase protein RapB on Spo0F~P, thereby directly promoting the sporulation, since Spo0F, in its phosphorylated form, indirectly induces the genes required for sporulation (Water & Bassler, 2005).

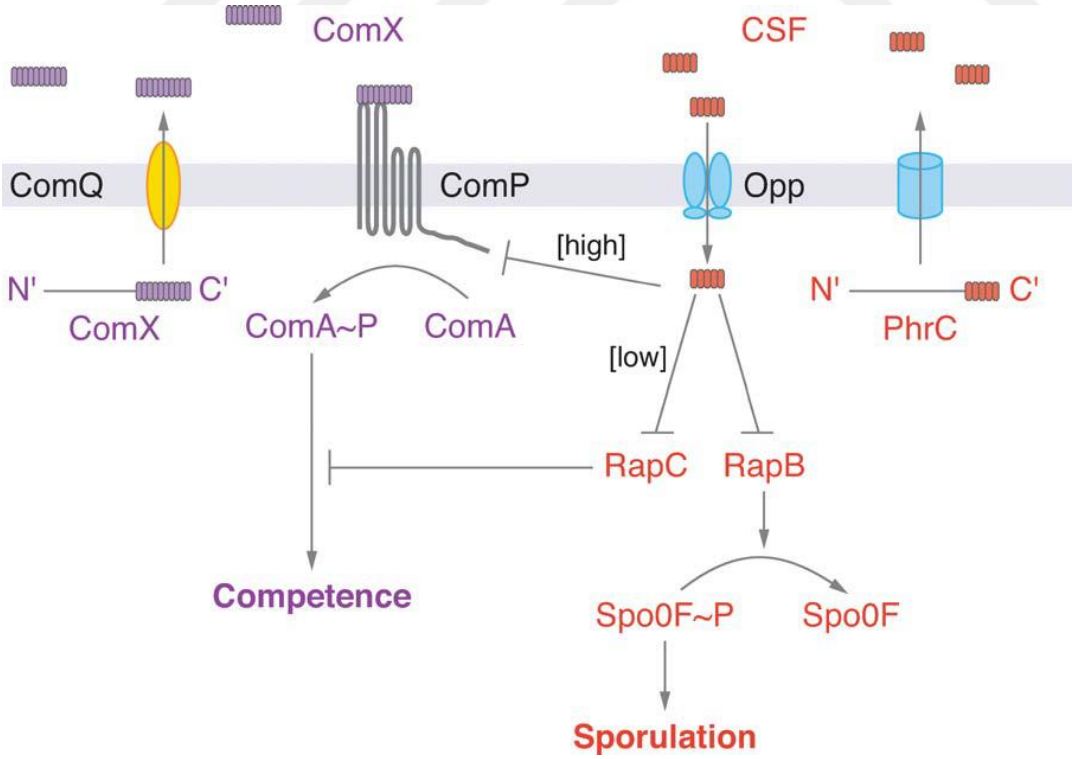

Figure 1.7 : Generel scheme of major quorum sensing pathway in *Bacillus subtilis* (Water & Bassler, 2005).

1.3 Bacilysin

Firstly, bacilysin antibiotic was named after discovered in synthesis by isolates of a strain of *Bacillus subtilis* (NCTC 7197) from the soil at Oxford in the 1940s (Florey et al., 1949). This dipeptide bacilysin, 270 kDa, (Figure 1.8) is one of the simplest peptide antibiotics known that it is composed of two aminoacids: an L-alanine residue at the N-terminus and a non-proteinogenic L-anticapsin at the C-terminus (Walker & Abraham, 1970).

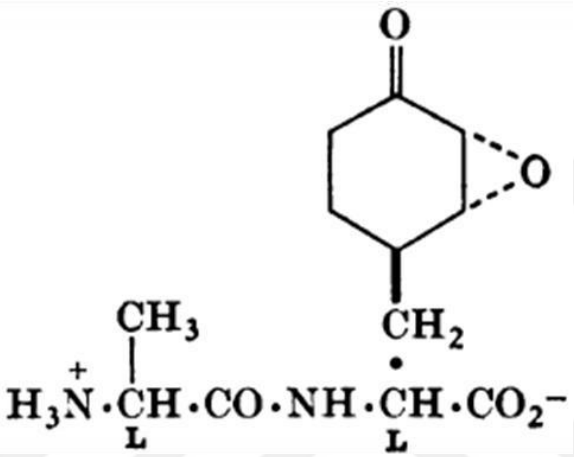

Figure 1.8 : The structure of bacilysin (Walker & Abraham, 1970).

As a species trait of *Bacillus subtilis*, bacilysin production was also found in some other *Bacillus* species like strains of *B. amyloliquefaciens*, *B. licheniformis* and *B. pumilus* (Loeffler et al., 1986). Its antibiotic activity is against to variety of bacteria and some fungi such as *Candida albicans* (Kenig & Abraham, 1976). Uptake of bacilysin by sensitive cells through a peptide transport system and releasing of L-anticapsin moiety of bacilysin by peptidases gives antimicrobial activity to bacilysin (Perry & Abraham, 1979; Kenig & Abraham, 1976 ; Chmara *et al*., 1982). Since, L-anticapsin inhibits glucosamine synthesis necessary for the synthesis of microbial cell wall, thus leading to cell protoplasting and lysis. (Kenig & Abraham, 1976). Thereby, due to its metabolic target, antimicrobial action of bacilysin is antagonized by glucosamine or N-acetlyglucosamine (Kenig & Abraham, 1976). L-alanine ligation to L-anticapsin accomplishes the protection of bacilysin producer cells from inactivation of their own glucosamine synthase.

The polycistronic *bacABCDEF* operon (previously *ywfBCDEFG*), and the monocistronic *bacG* (*ywfH*) gene are found to be responsible for the production of

bacilysin (Figure 1.9) (Inaoka et al., 2003; Steinborn et al., 2005; Köroğlu et al., 2008). *Bac* cluster organization is same in distinct species of *Bacillus* with the sequence identities about 72.6–88.6% (Steinborn et al., 2005). The *bacABCDF* operon and the *bacG* gene are necessary for the flux from prephenate to bacilysin while *bacE* is associated with acquiring self-resistance to the antibiotic by effluxing bacilysin out of the cell (Steinborn *et al*., 2005; Parker and Walsh, 2013). In general *bacA*, *bacB*, *bacC*, *bacD*, *bacE*, *bacF*, *bacG* encode prephenate decarboxylase, H_2HPP isomerase, bacilysin biosynthesis oxidoreductase, bacilysin synthetase, putative bacilysin exporter, transaminase, NADPH-dependent reductase respectively (Özcengiz and Öğülür, 2015; Parker and Walsh, 2013; Steinborn et al., 2005).

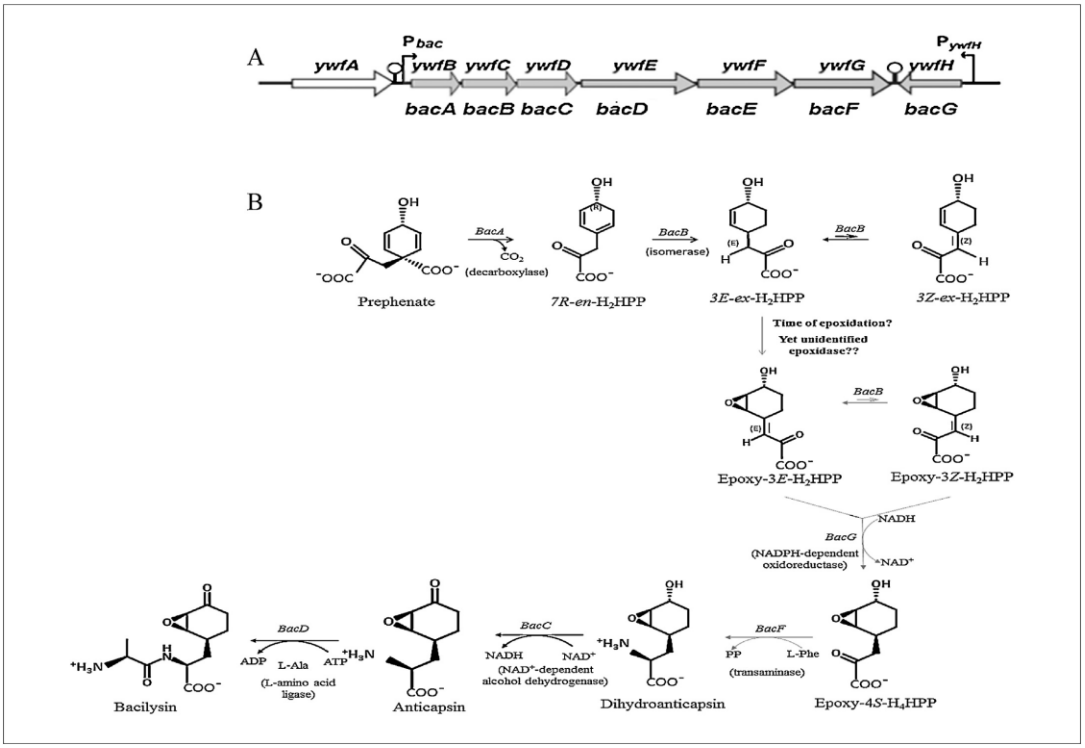

Figure 1.9 : (A) The bacilysin operon *bacABCDEF* in *Bacillus subtilis* 168 **(B)** The biosynthetic pathway from prephenate to bacilysin (Özcengiz and Öğülür, 2015).

Bacilysin production is dependent on the culture composition and physilogical factors such as that bacilysin production is repressed in rich medium or the presence of ammonium and casamino acid, besides, temperature above 30°C (Kenig & Abraham, 1976; Ozcengiz et al., 1990; Ozcengiz & Alaeddinoglu, 1991). Bacilysin synthesis is shown to be under stringent response, feedback regulation, and global quorum sensing system in *B. subtilis* (Inaoka et al., 2003; Özcengiz and Alaeddinoglu, 1991; Yazgan et al., 2001; Karatas et al., 2003). Stringent response

represses stable RNA synthesis and expression of various genes for translational factors, ribosomal proteins while it expresses certain genes for such as aminoacid biosythesis. In general, stringent response based on short-term increase in guanosine 5'-diphosphate 3'-diphosphate (ppGpp) in respond to uncharged tRNA entering to A site of ribosome. Bacilysin production is under control of this dual regulation system of ppGpp as a positive regulator and CodY-mediated GTP as a negative regulator (Inaoka et al., 2003).

Karatas et al. (2003) showed that bacilysin biosynthesis is also mediated by QS system by the action of ComQ/ComX, PhrC (CSF), ComP/ComA in *Bacillus subtilis*. In addition to these systems, products of *srfA, spo0A, spo0H* and *abrB* genes play crucial role in regulation of bacilysin biosynthesis (Figure 1.10) (Karatas et al., 2003). ComX and CSF, cell-derived extracellular pheromnes, trigger initiation of the pathway. After binding of ComX to membrane-bound receptor kinase (ComP), ComP is activated by autophosphorlylation and gives a phosphate group to response regulator ComA. Then, ComA~P stimulates transcription of *bacABCDEF* operon and *srfA* operon which is required for surfactin biosynthesis. Surfactin, which is a bacterial cyclic lipopeptide antibiotic produced by *Bacillus* sp., is recognized as one of the most effective surfactants, so it reduces surface tension and permits surface spreading (Heryani and Putra, 2017; Patrick and Kearns, 2009). Moreover, surfactin is found be a signaling molecule that stimulates the activity of KinC leading to phosphorylation of Spo0A. Then, Spo0A~P triggers transcription of the genes required for matrix production (Lopez et al., 2009). Disruption of *srfA* operon causes bacilysin negative phenotype, so *srfA* has a role in the production of bacilysin (Karatas et al., 2003). Both *srfA* and *bacABCDEF* operons are triggered by ComA. Besides, ComA is negatively regulated by RapC. Rap proteins has three targets: Spo0F, ComA and DegU. On the other hand, CSF encoded by *phr*C gene and PhrF,PhrK peptide pheromones return to the cell by Opp (Spo0K). CSF, PhrF, and PhrK inhibite the activity of Rap proteins RapC, RapF and RapK respectively resulting in stimulation of ComA (Omer Bendori et al., 2015; Auchtung et al., 2006). Another positive regulator of *bac* operon , Spo0A is activated by phosphorelay mechanism comprising of KinA, KinB, KinC and Spo0F phosphorylation. Activated Spo0A (Spo0A~P) initiates transcription of sporulation genes while represses AbrB production, which negatively regulates transcriptipn of bacilysin operon (Karatas et

al., 2003; Köroğlu et al., 2011).. In summary, ComA and Spo0A positively regulate transcription of the *bac* operon , while AbrB and CodY proteins negatively regulate the *bac* operon by binding to the promoter region of bacilysin operon (P_{bac}) (Köroğlu et al., 2011; Ozcengiz and Ogulur, 2015). To sum up, bacilysin biosynthesis is regulated by many factors included in other physiological processes in *B.subtilis*.

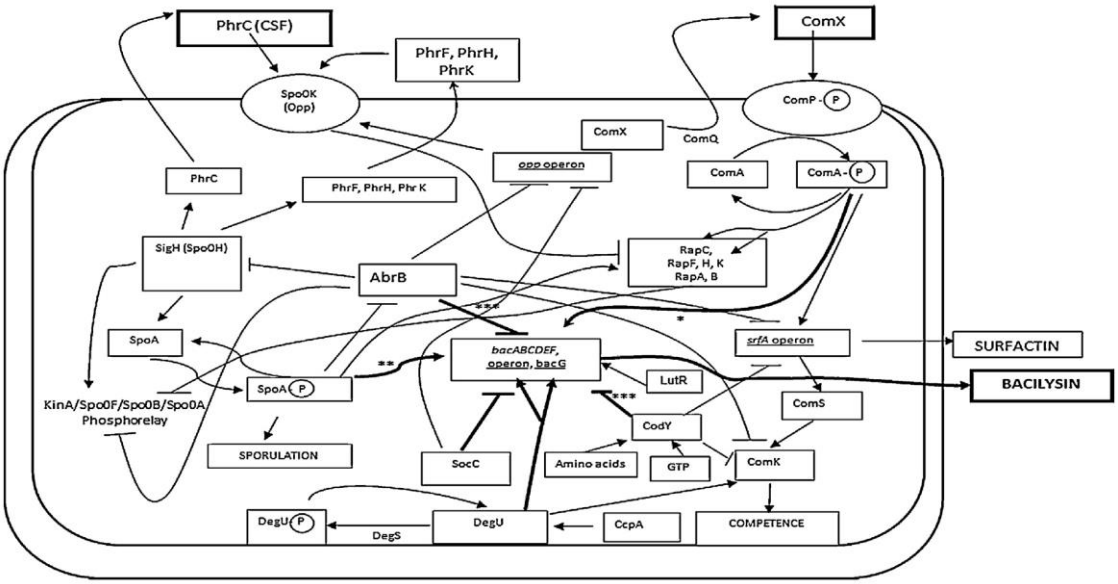

Figure 1.10 : Regulation pathways of bacilysin. Arrows: positive regulation; T-bars: negative regulation; Bold lines: direct interaction with *bac* promoter (Özcengiz and Öğülür, 2015).

1.4 Aim of the present study

The comparative transcriptome analysis of *B. subtilis* PY79 and its bacilysin non-producer derivative strain in bacilysin production medium performed by our research group strongly suggested that bacilysin plays important roles in sporulation, biofilm formation, competence development. Under normal condition, NCIB 3610 strain cannot form biofilm in LB medium. But, it was observed that 3610 strain can produce a thin layer biofilm in LB medium upon supplementing with bacilysin. *B. subtilis* QS molecule ComX and the lipopeptide antibiotic surfactin encoding by *srfA* operon in *B. subtilis* were shown to be required for bacilysin biosynthesis by our research group. Recently, surfactin was shown to be another QS molecule triggering a subpopulation of *B. subtilis* cells to become extracellular matrix producers. Interestingly, ComX QS molecule triggers only a subpopulation of cells to produce surfactin, in turn, surfactin causes another subpopulation of cells to produce an

extracellular matrix. All of those findings not only strengthened the regulatory role of bacilysin in the biofilm formation process, but also strongly suggested that bacilysin, in case of surfactin, could be another QS molecule triggering cells to differentiate into distinct cell-type within a developing biofilm. To obtain initial evidences about this possibility, the present study focused on whether bacilysin is produced by subpopulation of cells and to identify individual cells producing bacilysin within a developing biofilm. For this purpose, a transcriptional fusion with the open reading region encoding the green fluorescent protein (GFP) and the promoter region (P_{bac}) of the *bacABCDEF* operon was constructed. The P_{bac}-*gfp* contruct was transformed into wild type NCIB 3610 and laboratory PY79 strain, and then the recombinant strains were grown on biofilm-inducing MSgg medium. The samples were taken from different regions on biofilm structure and observed under bright-field and fluorescence microscope comparatively. Also, flow cytometry analysis was performed to both 3610 and PY79 strains harboring the reporter to assay the distribution of fluorescence throughout a population.

2. MATERIAL AND METHODS

2.1 Materials

2.1.1 Bacterial strains and plasmids

Throughout this study, *E.coli* Top10F was used as host strain for cloning bacilysin operon promoter region, and *B. subtilis* 3610 and PY79 strains were used as host for the construction of *amyE*::P_{bac}::*gfp*, Cm^R recombinant strains. The strains and their genotypes used in this project are listed in Table 2.1. As plasmids, pGEM®-T Easy Vector (Figure 2.1) was used for cloning of PCR products, and phy-GFP (Figure 2.2) for construction of green fluorescent protein and bacilysin operon promoter. pGEM®-T Easy Vector and phy-GFP were supplied by Promega and A.T.Kovacs respectively.

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

Strain	Genotype	Source
E.coli Top10F'	[*lac*Iq Tn10(Tet^r)], *mcrA* Δ *(mrr-hsdRMS-mcrBC), f80lacZΔM15* Δ*lacX74, deoR, recA1, araD139* Δ*(ara-leu)7697,galU, galK, rpsL (Strr), endA1, nupG*	M.A.Marahiel
B. subtilis 3610	Wild type, Δ*ComI*	A.T.Kovacs
P_{bac}::*gfp* fusion 3610	*amy*E::P_{bac}::*gfp*, Cm^R	This study
B. subtilis PY79	Laboratory strain, BSP cured prototrophic derivative of *B. subtilis* 168	P. Youngman et. Al, 1984
P_{bac}::*gfp* fusion PY79	*amy*E::P_{bac}::*gfp*, Cm^R	This study
P_{bac}::*lacZ* fusion PY79	*bacA::lacZ::erm*	İ.Öğülür M.Sc. Thesis
P_{bac}::*lacZ* fusion 3610	*bacA::lacZ::erm*	D. Ergün M.Sc. Thesis (not published yet)

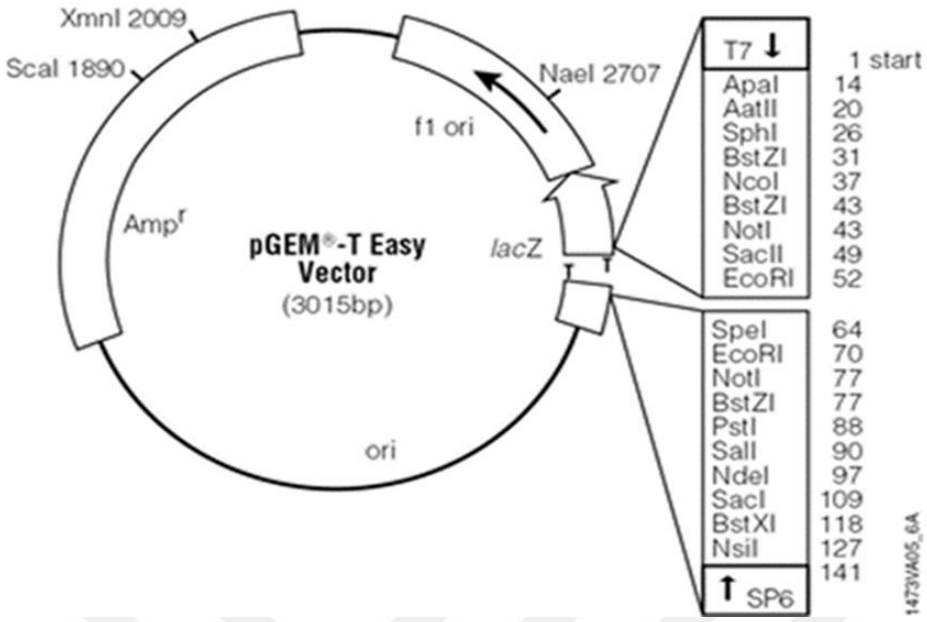

Figure 2.1 : Genomic map of pGEM®-T Easy vector (Promega)

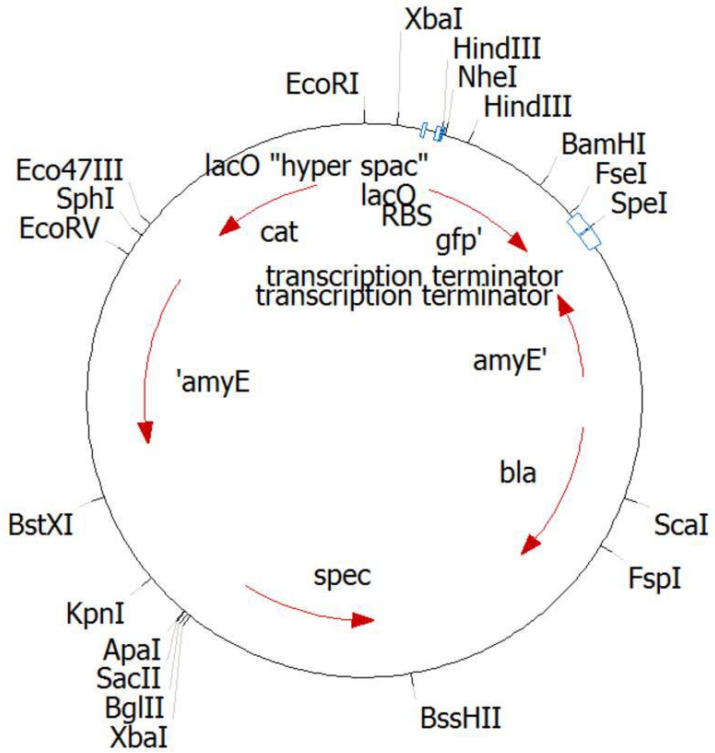

Figure 2.2 : Genomic map of phy-GFP with the size of 8,387 bp (van Gestel et al., 2014)

2.1.2 Culture media

Compositions and preparation of culture media are given in Appendix A.

2.1.3 Buffers and solutions

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

2.1.4 Chemicals and enyzmes

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

2.1.5 Laboratory equipment

The laboratory equipments that were used are given in Appendix D.

2.1.6. Maintenance of bacterial strains

B. subtilis and *E. coli* strains were grown in Luria-Bertani (LB) liquid medium and kept on Luria-Bertani (LB) agar or Nutrient agar plates at +4ºC. For long term storage, 10% LB glycerol stock for *E.coli* and 15% LB glycerol stock for *B. subtilis* were prepared and these stocks were kept at -80ºC.

2.2 Methods

2.2.1 DNA techniques and manipulation

2.2.1.1 Chromosomal DNA isolation

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

1,5 ml of overnight culture was harvested by centrifugation at 13000 rpm for 5 minutes. Supernatant was discarded, and pellet was resuspended in 567 µl of TE buffer (Appendix B) by repeated vortex or pipetting. Then, 10 µl of proteinase K (20 mg/ml), 6 µl of RNase (10 mg/ml), 24 µl of lysozyme (100 mg/ml) were added to the resuspended cells via vortexing, and then 30 µL of 10% SDS were added into to the cell mixture. After homogenized mixture was incubated for 1 hour at 37°C, 100 µl of 5M NaCl solution was added, and the sample was mixed by inverting the tubes until the mucosal white substance becomes visible. Next, 80 µl of CTAB/NaCl solution (Appendix B) (prewarmed at 65°C by virtue of viscosity) was added into the mixture, and the mixture was incubated in 65°C waterbath for 10 minutes. Subsequently, the sample was extracted with same volume (820 µl) of freshly prepared phenol/chloroform/isoamly alcohol (25:24:1) solution by mixing by

inverting the tube and gentile vortexing. After centrifugation at 13000 rpm for 10 minutes, the upper phase (~ 800 µl) was transferred to a new 1.5 ml microcentrifuge tube and 0.7 volume isopropanol was added. After mixing shortly, the sample was centrifuged at 13000 rpm for 15 minutes. The pellet was removed, and the supernatant was washed with 1 ml of 70% ethanol via centrifugation at 13000 rpm for 5 minutes. Later, the pellet was dried at 37°C for about 1 hour and dissolved in 10 µl TE buffer. Finally, isolated chromosomal DNA was stored at 4°C after checking with agarose gel electrophoresis.

2.2.1.2 Agarose gel electrophoresis

Electrophoresis was applied on a horizontal submarine electrophoresis apparatus and in a gel system composed of 1% (w/v) agarose gel prepared with 1X TAE buffer (Appendix B) and RedSafe™ Nucleic Acid Staining Solution (iNtRON) at 4V/cm. DNA bands were visualized on a shortwave UV transilluminator (UVP).

2.2.1.3 Polymerase chain reaction (PCR)

Taq polymerase enzyme is supplied by Thermo Fisher Scientific Inc.. The sequences of the primers, components and amount of PCR reaction, and PCR conditions used in the study are given in Table 2.2, Table 2.3, Table 2.4 respectively.

Table 2.2 : Oligonucleotide sequences of primers.

Primer	**Oligonucleotide sequence**	**Tm (ºC)**
bacA-gfp Forward	5'-CACGGAATTCTAGGTTCTGCTTTAATGGGAC-3'	67
bacA-gfp Reverse	5'-CGACGCTAGCCATGAGCACCAACCAATCTTTTAA-3'	69
Cm Forward	5'-TAGTGACAAGGGTGATAAACTCAA-3'	58
Cm Reverse	5'-AGGCCTATCTGACAATTCCTGAATAGAGTTC-3'	66

Table 2.3 : Components and amount of PCR reaction.

Components	**Amount** (50 µl total reaction volume)
10X Buffer	5 µl
$MgCl_2$	5 µl
dNTP	5 µl
Template	1 µl
Forward primer	1 µl
Reverse primer	1 µl
i-Taq polymerase	1 µl
dH_2O	31 µl

Table 2.4 : PCR conditions.

	Temperature (°C)	Time	Cycle
Initial Denaturation	95	2 min	
Denaturation	95	30 sec	
Annealing	(based on primers)	30 sec	30 cycles
Extension	72	1 min	
Final Extension	72	10 min	

2.2.1.4 Ligation of the PCR products into the pGEM®-T Easy cloning vector

pGEM®-T Easy vector is supplied by Promega. Ligation reaction mixture is given in Table 2.5. The reaction mixture was incubated at 22°C for 1 hour, then at 4°C overnight.

Table 2.5 : Components and amount of ligastion reaction mixture.

Reaction components	Amount (10 µl total reaction volume)
2X Rapid Ligation Buffer	5 µl
pGEM®-T Easy Vector (50ng/µl)	1 µl
Insert DNA (PCR product)	2 µl
T4 DNA Ligase (3 Weiss units/µl)	1 µl
dH_2O	1 µl

Before transformation, T4 ligase enzyme activity was inhibited by incubating the ligation mixture at 65°C for 10 minutes. After that, the ligation product was transformed into chemically competent *E.coli* Top10F' cells.

2.2.1.5 Plasmid isolation

A single colony was picked from a freshly streaked selective plate and inoculated into 2,5 ml of LB medium containing the appropriate selective antibiotic as a starter culture. The LB medium containing a bacterial colony was incubated at 37ºC for overnight with vigorous shaking. After that, bacterial cells were harvested by centrifugation at 13000 rpm for 5 minutes. The bacterial pellet was completely resuspended in 330 µl P1 buffer. Then, 330 µl of P2 buffer was added into the cell suspension. The cell suspension was mixed gently by inverting the tubes 4-6 times and incubated at room temperature for 5 minutes. Next, 330 µl of chilled P3 buffer was added into the suspension, and the suspension was mix immediately by inverting the tubes 4-6 times and incubated on ice for 15 minutes. After centrifugation of the tubes for 15 minutes at 13000 rpm, supernatant was transferred into new tube, and

isopropanol was added into the tube as 0.7 volume of supernatant. Plasmid DNA was precipitated by centrifugation at 13000 rpm for 30 minutes. Then, supernatant was discarded and plasmid DNA was washed with 1 ml of 70% ethanol by centrifugation for 5 minutes at 13000 rpm. After ethanol evaporation, plasmid DNA was dissolved in 20 µl elution buffer. Isolated plasmid DNA was stored at -20ºC.

2.2.1.6 Restriction enyzme digestion

Restriction enzyme digestion reactions were carried out as recommended in the manufacturer's protocols (Thermo Scientific). Components and amounts of single enzyme digestion and sequential double digestion reactions performed in the study are given in Table 2.6, Table 2.7 respectively. Enzyme digestion reactions were incubated at 37ºC for 1 to 5 hours. *Nhe*I and *Eco*RI was used in sequential double digestion reaction and the reaction was incubated at 37ºC for 2 hours for each reaction.

Table 2.6 : Components and amounts of standard single restriction enzyme digestion reaction.

Reaction components	**Amount (10 µl total reaction volume)**
10X Buffer	1 µl
DNA (0.5-1 µg/µL)	1 µl
Enzyme	1 µl
Nuclease-free water	7 µl

Table 2.7 : Components and amounts of *Nhe*I-*Eco*RI sequential double digestion reaction.

Reaction components	**Amount (first total reaction volume: 30 µl; second total reaction volume: 34,75 µl)**
10X Tango Buffer	3 µl
DNA	10-15 µl
*Nhe*I	1
Nuclease-free water	11-16 µl
Incubation time, temperature	2 hours, 37ºC
10X Tango Buffer	3,75 µl
*Eco*RI	1 µl
Incubation time, temperature	2 hours, 37ºC

2.2.1.7 Ligation of digested insert into GFP vector

Ligation mixture of *bac* operon promoter and GFP vector which were double digested with *Nhe*I and *Eco*RI restriction enzymes are given in Table 2.8. The

mixture was incubated at 22℃ for 10 minutes, then 4℃ for overnight before transformation into competent *E.coli* Top10F' cells.

Table 2.8 : Compenents and amounts of ligation of bac promoter and phy-GFP vector.

Reaction components	Amount (µl)
10X T4 DNA Ligase Buffer	1
Insert DNA	2
Vector	3
Thermo Scientific T4 DNA Ligase (5 U/µl)	1
Water, nuclease-free	3

2.2.1.8 Gel extraction and PCR clean-up

Purification of PCR products and extraction of digested DNA fragments with restriction enzymes from agarose gel were applied by using NucleoSpin® Gel and PCR Clean-up Kit (Macherey-Nagel) according to the manufacturer's protocol.

2.2.2 Transformation

2.2.2.1 Preparation of chemically competent *Escherichia coli* Top10F' cells

A single colony of *E.coli* Top10F' was inoculate into 3 ml of 2XYT medium containing 20 µg/mL tetracyclin and incubated at 37 ºC overnight with vigorous shaking. Then, 1 ml of overnight culture was inoculate to 50 ml of 2XYT medium, and the culture was incubated at 37ºC at 180 rpm until OD_{600} value reached 0,4-0,5. After that, 20 ml of the culture aliquotes were placed into 50 ml screwcap centrifuge tubes and incubated on ice for 10 minutes. Supernatant was discarded after centrifugation at 5000 rpm for 10 minutes at 4℃. Pellet was resuspended in 10 ml of 100 mM $CaCl_2$ solution and incubated on ice for 30 minutes. After centrifugation at 5000 rpm for 10 minutes at 4℃, supernatant was discarded, and pellet was resuspended in 1 ml of 100 mM $CaCl_2$ solution. After addition of 110 µl of glycerol into the tube, 111 µl of the competent cells was split into 1.5 ml cryotubes and stored at -80℃.

2.2.2.2 Transformation of chemically competent *Escherichia coli* Top10 F' cells

For transformation, pre-prepared competent *E.coli* Top10F' cells were melted on ice. 5-10 µl of ligation product was added into the cells and incubated on ice for 30 minutes. Then, heat shock was applied at 42℃ for 2 minutes. The tube was put back

on ice to reduce damage to cells for about 5 minutes. Next, 1 ml of LB medium was added onto cells. After incubation at 37℃ for 1 hour with vigorous shaking at 180 rpm, cells were harvested by centrifugation at 13000 rpm for 5 minutes, and resuspended in 100-200 µl of physiological saline solution. Finally, 100 µl of the resuspended cells was spreaded on LB plate with selective antibiotics, and incubated at 37℃ overnight. In this study, pGEM®-T Easy vector containing the promoter of bacilysin operon and GFP vector containing the promoter were transformed into *E.coli* cells. Transformants containing pGEM®-T with the promoter were selected and screened on X-gal/IPTG LB plate with 100 µg/ml ampicillin, while transformants containing GFP vector with the promoter were selected on LB plate with 50 µg/ml ampicillin.

2.2.2.3 Transformation of *Bacillus subtilis* 3610 and PY79

Transformation of *B. subtilis* 3610 and PY79 competent cells were performed as described by Klein et al. (1992). HS and LS medium (Appendix A) were used for preparation of competent *B.subtilis* cells. Firstly, plenty of freshly grown *B.subtilis* cells on LB plate was inoculated into 3 ml HS medium and incubated overnight at 37℃. 500 µl of overnight culture was inoculated into 20 ml of LS medium in 250 ml sterile flask and incubated at 30℃ with shaking at 100 rpm until OD_{600} value reached 0,55-0,59. Next, 1 ml culture was transformed with about 10 µl of plasmid containing insert DNA and incubated for 2 hours at 37℃ with shaking at 250 rpm. After centrifugation at 5000 rpm for 15 minutes, the cell pellet was resuspended in 100 µl of physiological saline solution, and spreaded on LB selective plate. In this study, GFP vector containing the promoter of bacilysin operon was transformed into *Bacillus subtilis* 3610 and PY79 cells, and transformants were selected on LB plates with 5 µg/ml chloramphenicol. After that, starch hydrolysis test was applied to confirm insertion of GFP vector to chromosomal DNA of *B.subtilis*.

2.2.2.4 The Starch Hydrolysis Test

Bacillus subtilis 3610 and PY79 cells as a control and their candidate transformants were inoculated as a single streak line or a single dot on starch agar plate (Appendix A), and incubated at 37℃ overnight. Then, iodine was applied to the starch plate surface, and excess dye was discarded. After 5 minutes incubation at room temperature, reaction of iodine with starch was observed.

2.2.3 DNA Sequencing

pGEM®-T Easy vector containing the promoter of bacilysion operon was sequenced using vector specific pUC/M13 forward and reverse primers by Medsantek Ltd. Co..

2.2.4 Biofilm

2.2.4.1 Pellicle formation assay

B. subtilis strains were inoculated into 3 ml LB medium and incubated overnight at 37℃ with shaking. Overnight culture was inoculated into fresh LB medium with 1:100 dilution and incubated at 37℃ at 200 rpm until OD_{600} value has reached 1.0 which is mid-exponential phase. 2,5 µl of starting culture was added into 2,5 ml of MSgg medium contained within a well of 24-well microplate, and incubated at 30℃ for 24, 48, 72 hours without agitation.

2.2.4.2 Colony architecture assay

Freshly prepared MSgg agar plates were dried at 30 ℃ overnight. *B. subtilis* strains were inoculated into 3 ml LB medium and incubated at 37℃ with shaking. Overnight culture was inoculated into fresh LB medium with 1:100 dilution and incubated at 37℃ at 200 rpm until OD_{600} value has reached 1.0. 3 µl of culture was spotted onto middle of MSgg agar plates, and the plates were incubated for 24, 48, 72 hours at 30℃.

2.2.5 Microscopy

2.2.5.1 Sonication

Sample of biofilm on MSgg agar surface were removed using toothpick, and placed into 500 µl of PBS buffer in 1.5 ml microcentrifuge tubes. Sonication was applied with 50% amplitude and pulsation with 0,7 sec on/3,0 sec off for 45 sec on ice to disperse biofilm.

2.2.5.2 Preparation of slides

In a purpose of fixation of dispersed biofilm sample, Poly-L-lysine Solution (Sigma) was applied onto slides according to manufacturer’s protocol.

2.2.5.3 Fluorescence microscopy

The cells from collected samples were imaged using Zeiss Axio Vert.A1 inverted microscope equipped with FITC filter and AxioCam ICc 1 camera. Expression time and gain was set to 4.09 seconds and 20 dB, respectively.

2.2.6 Flow cytometry

The biofilm formed on the MSgg agar surface was removed using a toothpick and placed into 3 ml of PBS buffer. The biofilm was dispersed by mild sonication (50% amplitude, pulsation with 1 sec on/3 sec off for 48 sec), and the efficiency of cell dispersion was confirmed by ligh microscopy. After that, 300 µl of the cell suspension was resuspended in 1 ml of 4% paraformaldehyde solution, and incubated at room temperature for exactly 7 minutes. The sample was diluted 1:100 in PBS buffer before flow cytometry analysis. 500 µl of the diluted sample was directly measured on BD FACSCalibur flow cytometry. For each sample, 50,000 events were analyzed. Data were captured using FACS Diva software (BD Biosciences) and further analyzed using FlowJo 10.

3. RESULTS AND DISCUSSION

3.1 Construction of P_{bac}-*gfp* Vector

The previous studies in our laboratory depend on a genome-wide comparative transcriptome analysis performed between bacilysin producer PY79 strain and its bacilysin non-producer mutant strain (*bacA::erm::lacZ*) strongly suggested that bacilysin can be a pleiotropic regulatory factor involved in various physiological and adaptive cellular processes such as competence development, sporulation and biofilm formation (Köroğlu, 2013). Also, under normal condition, wild type NCIB 3610 strain cannot form biofilm in LB medium. However, it was observed that 3610 strain can produce a thin layer biofilm in LB medium supplementing with bacilysin (Karahoda, 2003). Moreover, *B. subtilis* QS molecule ComX and the lipopeptide antibiotic surfactin encoding by *srfA* operon in *B. subtilis* were shown to be required for bacilysin biosynthesis by our research group (Karatas et al., 2003). Recently, surfactin was shown to be another QS molecule triggering a subpopulation of *B. subtilis* cells to become extracellular matrix producers. Interestingly, ComX QS molecule, which is produced by almost all of biofilm cells (unimodal), triggers only a subpopulation of cells to produce surfactin (bimodal), in turn, surfactin causes another subpopulation of cells to produce an extracellular matrix. These matrix-producer cells can not respond to ComX, and become surfactin producers (unidirectional signal) (Lopez et al., 2009). All of these common findings suggests that bacilysin could be a signaling molecule that functions in the formation and maintenance of different cell types that control the physiological change of other cells during biofilm development. To identify bacilysin producing cells within a biofilm, *amy*E:P_{bac}:*gfp B. subtilis* recombinant strains was contructed. For this purpose, firstly, the promoter region (P_{bac}) of bacilysin operon was amplified by PCR with primers *bacA-gfp* F and *bacA-gfp* R (Table 2.2) using chromosomal DNA of *B. subtilis* 3610 as a template (Figure 3.1). After that, the purified 335 bp long of P_{bac} PCR fragment containing the extra residues including *Nhe*I-*Eco*RI restriction sites

and start codon was ligated into PGEM-T® Easy cloning vector, and then the recombinant plasmid was transformed into chemically competent *E.coli* Top10F' cells. White colonies containing recombinant plasmid was selected on X-gal/IPTG/Amp-containing LB agar plates. After that, PGEM-T® vector carrying insert DNA was isolated from the selected recombinant colony and digested with *Eco*RI to check insertion by releasing the fragment (Figure 3.2).

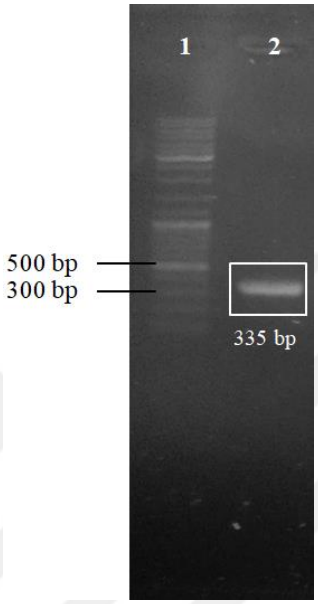

Figure 3.1 : Promoter region of bacilysin operon (P_{bac}) PCR product obtained with primers *bacA-gfp* F and *bacA-gfp* R from *B. subtilis* 3610 chromosomal DNA. Lane 1: GeneRuler™ DNA Ladder Mix, Lane 2: P_{bac} PCR fragment.

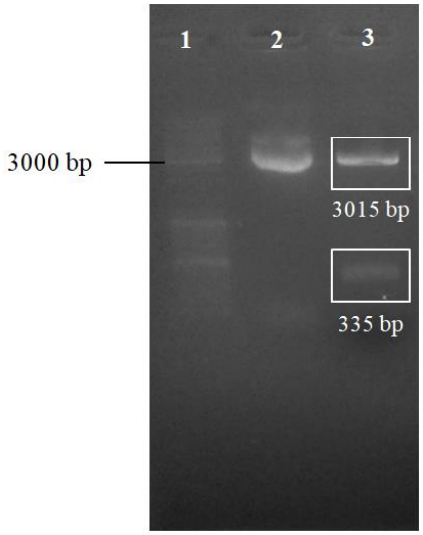

Figure 3.2 : PGEM-T® Easy vector carrying P_{bac} PCR fragment from selected transformant. Lane 1: GeneRuler™ DNA Ladder Mix, Lane 2: Recombinant plasmid, Lane 2: *Eco*RI digestion of recombinant PGEM-T® plasmid.

The ligation of the P_{bac} PCR fragment into PGEM-T® Easy vector was also confirmed by sequencing the recombinant plasmid by M13 F and M13 R primers (Figure 3.3).

5'...CACGGAATTCTAGGTTCTGCTTTAATGGGACCCGTAT
ATGTGGCATACGGCTATGCAGCTGTCGGATTGATTTGCG
CCGCCATCACAGTCCTCGGATTTGTGCTCAGTGTATTCGC
ATATAAAAAGTACGGCAAACTTGAGCAGAAGGCCGATC
AGTCCTTATCTCAATAAACAAAGTTTCTAAATTCCTATA
ATTAAAAACCCTTTTTAAAGGGTTTTTTTGTTTGTATGGG
AATAATTATCAATATTTTCAAAAAACATTGACAGTTCAA
TTTCCACTGGATAAAATTTACTTAAAATTTTAAAAGATT
GGTTGGTGCTCATGGCTAGCGTCG...3'

Figure 3.3 : The confirmed sequence of the P_{bac} region from recombinant PGEM-T® Easy vector. *bac*A F and *bac*A R primers are highlighted with green and yellow colours respectively.

To construct P_{bac}-*gfp* vector, phy-gfp vector and amplified the P_{bac} fragment from the recombinant PGEM-T® vector by PCR (Figure 3.4) were double digested with *Nhe*I and *Eco*RI restriction enzymes (Figure 3.5). 386 bp long hyper spank promoter in phy-gfp vector was released to replace with the P_{bac} region. After linearized 8000 bp long gfp vector fragment and digested the P_{bac} PCR fragment were extracted from the agarose gel (Figure 3.6), these two fragments were ligated and the construct were transformed into chemically competent *E.coli* Top10F' cells. Transformants were selected on ampicillin-containing LB agar plates.

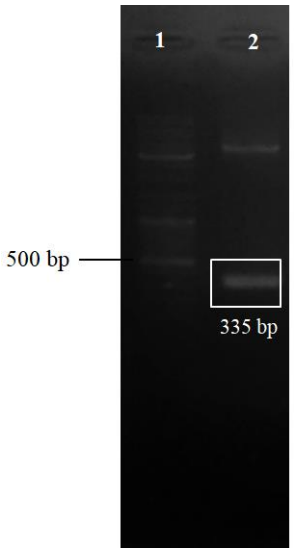

Figure 3.4 : PCR product of P_{bac} region obtained with with primers *bacA-gfp* F and *bacA-gfp* R using recombinant PGEM-T® Easy vector as a template. Lane 1: GeneRuler™ DNA Ladder Mix, Lane 2: PCR product.

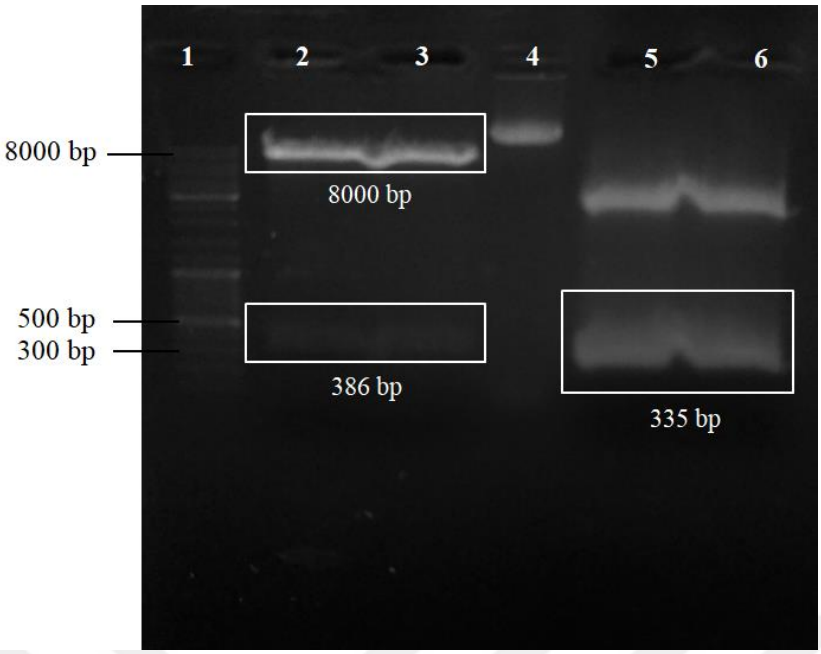

Figure 3.5 : *Nhe*I and *Eco*RI double restriction enzyme digestion of phy-gfp plasmid and PCR product of P_{bac}. Lane 1: GeneRuler^{TM} DNA Ladder Mix, Lane 2-3: digested phy-gfp plasmid, Lane 3: undigested phy-gfp plasmid, Lane 5-6: digested P_{bac} region.

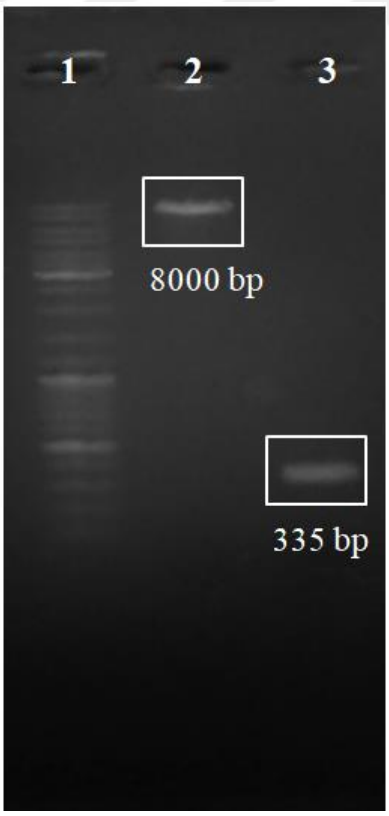

Figure 3.6 : Gel extraction products of the digested phy-gfp plasmid and the digested P_{bac} PCR fragment. Lane 1: GeneRuler^{TM} DNA Ladder Mix, Lane 2: digested phy-gfp, Lane 3: digested P_{bac} PCR region..

P_{bac}-*gfp* vector was isolated from selected *E.coli* transformant and digested with *Xba*I and *Pvu*II separately to verify releasing of hyper spank and insertion of the P_{bac} region into gfp vector (Figure 3.7). *Xba*I restriction site is present on both hyper spank promoter and the opposite site of gfp vector (Figure 2.2), thereby the construct digested with *Xba*I gave linear 8000 bp fragment, confirming the removal of hyper spank promoter region. On the other hand, *Pvu*II restriction site is present only in the P_{bac} region, thereby the linearized contruct digested with PvuII verified the insertion of the P_{bac} region into gfp vector. Also, P_{bac} amplification by PCR with *bacA-gfp* F and *bacA-gfp* R primers using P_{bac}-gfp vector as a template confirmed the proper contruction.

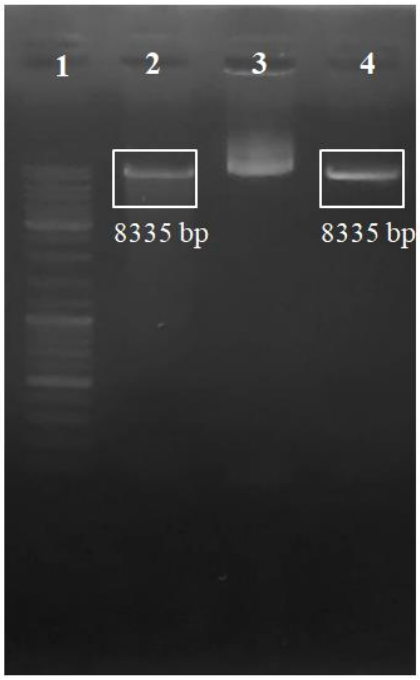

Figure 3.7 : Confirmation of P_{bac}-*gfp* plasmid construct. Lane 1: GeneRuler^{TM} DNA Ladder Mix, Lane 2: *Pvu*II digestion of the plasmid, Lane 3: undigested P_{bac}-*gfp* plasmid from selected transformant, Lane 4: *Xba*I digestion of the plasmid.

3.2 Construction of *amy*E::P_{bac}::*gfp* Recombinant Strain

P_{bac}-*gfp* vector was transformed into competent *B. subtilis* 3610 and PY79 cells. Transformants were selected on chloroamphenicol-containing LB agar plates. After that, selected colonies were incubated on starch agar plate because the P_{bac}::*gfp*::*cm* must be inserted into α-amylase gene on chromosome of *B. subtilis* by homologous recombination with *amy*E loci on gfp vector (Figure 2.2) and the recombinant strain

cannot produce α-amylase enzyme and hydrolyze starch, thereby iodine test was applied and confirmed the recombination (Figure 3.8). While clear zone was formed around *B. subtilis* 3610 wild-type and PY79 strain, iodine reacted with starch around *amy*E::P_{bac}::*gfp* recombinant strains producing blue/black color. After that, chromosomal DNA of the recombinant strain was isolated, and screened with PCR analysis by using *Cm* R primer and *bacA-gfp* R primer verifying proper integration (Figure 3.9).

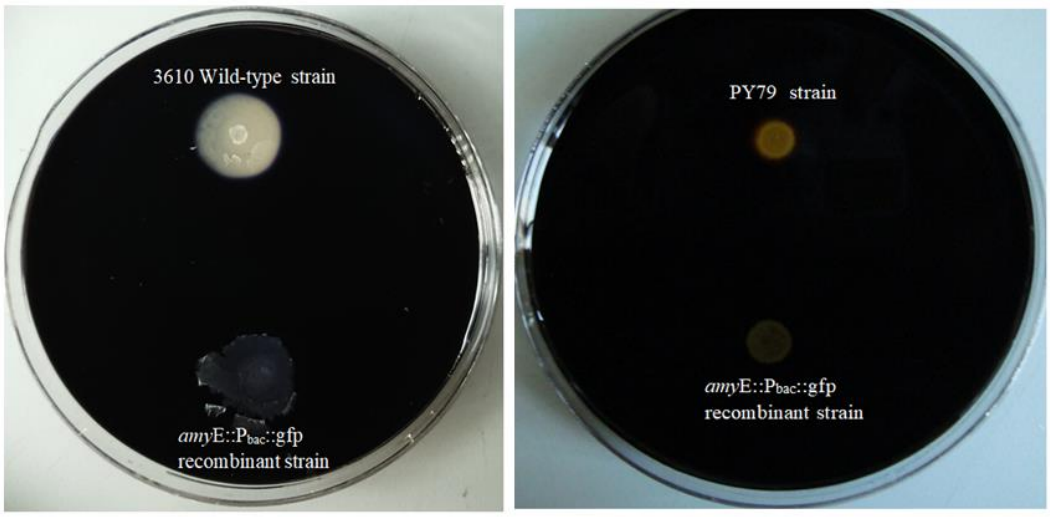

Figure 3.8 : Iodine test for starch due to verify *amy*E locus insertion of the selected transformant. Right view: *B. subtilis* 3610 strain and its recombinant strain; Left view: *B. subtilis* PY79 strain and its recombinant strain.

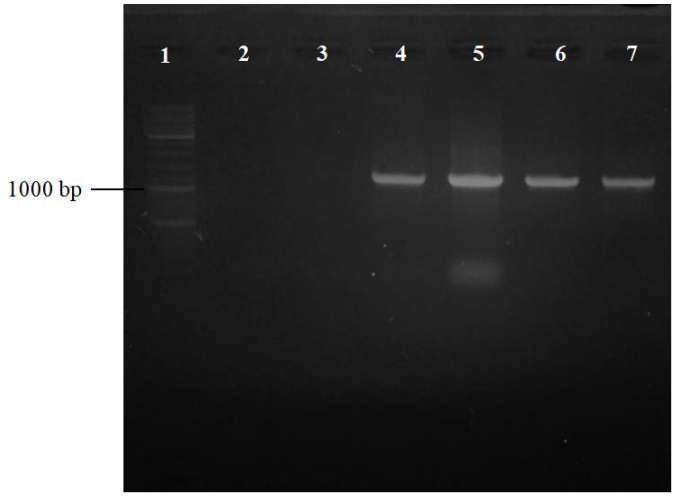

Figure 3.9 : Confirmation of construction of *amy*E::P_{bac}::*gfp* recombinant 3610 strain with PCR amplification using *bac*A-*gfp* R and *Cm* R primers. Lane 1: GeneRuler^{TM} DNA Ladder Mix, Lane 2: PCR reaction using *B. subtilis* 3610 chromosomal DNA as a template, Lane 3: PCR reaction using sterile dH_2O as a template, Lane 4-5-6: PCR fragments using chromosomal DNAs of selected transformants as a template, Lane 7: PCR fragment using P_{bac}-*gfp* construct as a template.

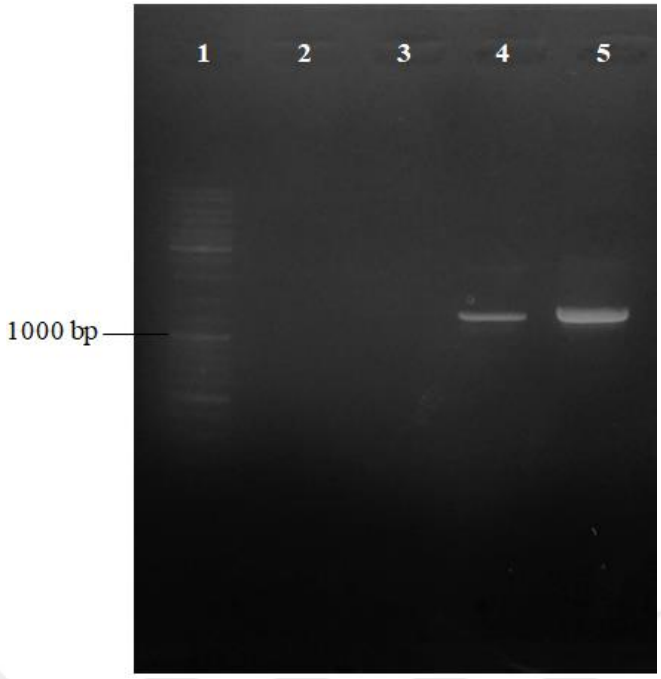

Figure 3.10 : Confirmation of construction of *amy*E::P_{bac}::*gfp* recombinant PY79 strain with PCR amplification using *bac*A-*gfp* R and *Cm* R primers. Lane 1: GeneRuler^{TM} DNA Ladder Mix, Lane 2: PCR reaction using *B. subtilis* PY79 chromosomal DNA as a template, Lane 3: PCR reaction using sterile dH_2O as a template, Lane 4: PCR fragments using chromosomal DNAs of selected transformants as a template, Lane 5: PCR fragment using P_{bac}-*gfp* construct as a template.

3.3 Identification of *B. subtilis* 3610 Biofilm Cells Producing Bacilysin

To investigate the possibility of bacilysin biosynthesis by differentiated cell types within the biofilm structure under fluorescence microscope, *amy*E::P_{bac}::*gfp* recombinant *B.subtilis* 3610 strain was grown on biofilm-inducing minimal medium MSgg agar and broth. *B. subtilis* cells grow as a multicellular commumity with an extraordinary spatiotemporal organization forming complex colony architecture (Veening et al., 2006). Aerial projections are observed within mature biofilm structure called as 'fruiting bodies' where preferential sites for sporulating cells. (Veening et al., 2006; Branda et al., 2001). Biofilm and pellicle formation of *amy*E::P_{bac}::*gfp* recombinant *B. subtilis* 3610 strain was observed for 24, 48, 72 h (Figure 3.11, Figure 3.12). Colony biofilm on MSgg agar propagated outwards radially forming ring-shaped structure. At 16^{th} h, fruiting-bodies were not observed on MSgg agar, and at 24^{th} hour the wrinkled structures began to form at the center of the biofilm, and to spread out radially since 24^{th} h to 72^{nd} hour (Figure 3.11). High population density is an important to promote bundle formation (Veening et al., 2006).

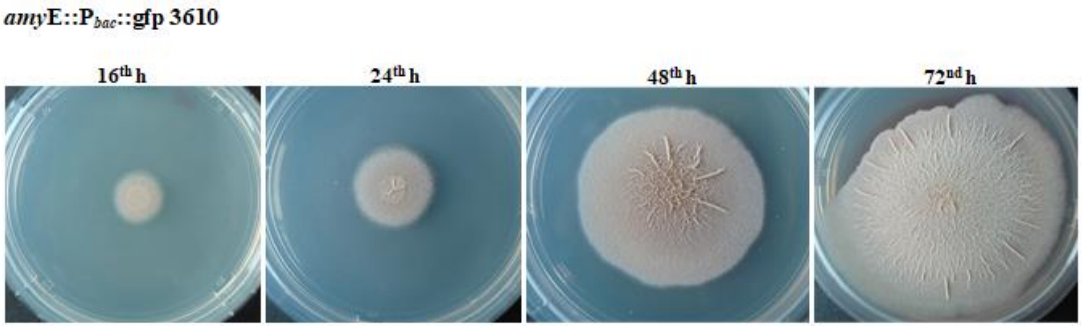

Figure 3.11 : Colony architecture of *amy*E::P$_{bac}$::*gfp* recombinant *B. subtilis* 3610 strain on MSgg agar.

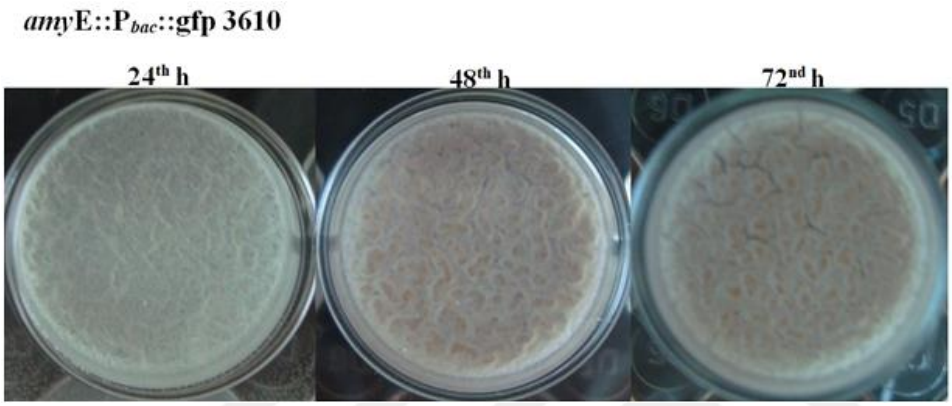

Figure 3.12 : Pellicle formation assay for *amy*E::P$_{bac}$::*gfp* recombinant *B. subtilis* 3610 strain in MSgg medium.

After *amy*E::P$_{bac}$::*gfp* recombinant *B.subtilis* 3610 strain was incubated on MSgg agar for 24 h, 48 h, and 72 h, samples were taken from 3 different regions in biofilm structure on MSgg agar for each hour to differentiate morphologically distinct cell-types, and then to observe under light and fluorescence microscope (Figure 3.13-3.15). These regions were the center (1th region) where cells are spotted, the middle (2nd region) where wrinkled structures propogate outwards, and the edge (3th region) of the biofilm structure. Vlakamis et al. (2006) show population dynamics of *B. subtilis* during biofilm formation as a function of time by using cell-type-specific promoters. During biofilm formation, predominant unicellular motile cells differentiate to a mixture of cell types. At least 3 different cell types form which are small rod-shaped motile cells, long chains of nonmotile cells as matrix-producing cells, and sporulating cells. High proportion of cells are motile in the early stages of biofilm formation. At 24th h, cells from first and second region are observed as small rod-shaped motile cells (Figure 3.13). However, majority of these motile cells differentiates into matrix-producing cells, and then matrix-producing cells localize to the periphery (Srinivasan et al., 2017; Vlakamis et al., 2006). When Spo0A~P level

is low; SlrR production is induced, and it binds to SinR, resulting with repression of motility and autolysin genes which are required for planktonic cell separation (Chen et al., 2009; Kobayashi, 2007, Vlamakis *et al*., 2013). Moreover, SinR become antagonized by SinI allowing transcription of operons responsible for matrix production. Thereby cells lose their motility, form cell chains, and produce matrix (Branda et al., 2001; Branda et al., 2006; Fujita and Losick, 2005). Cells from edge of the biofilm at all hours were observed cell chains as expected (Figure 3.13-3.15). At late stages of biofilm formation (After 24 h) Spo0A~P level increases, and sporulation begins especially in aerial projections. Major proportion of sporulating-cells are derived from matrix-producing cells (Vlakamis et al., 2006). At 48th and especially 72nd h, cells from first and second regions were observed to begin sporulating and forming free endospores as expected to be seen in aerial projections (Figure 3.14, Figure 3.15). During all formation processes of biofilm in a time-dependent manner, these 3 cell types are present with different proportions at different locations.

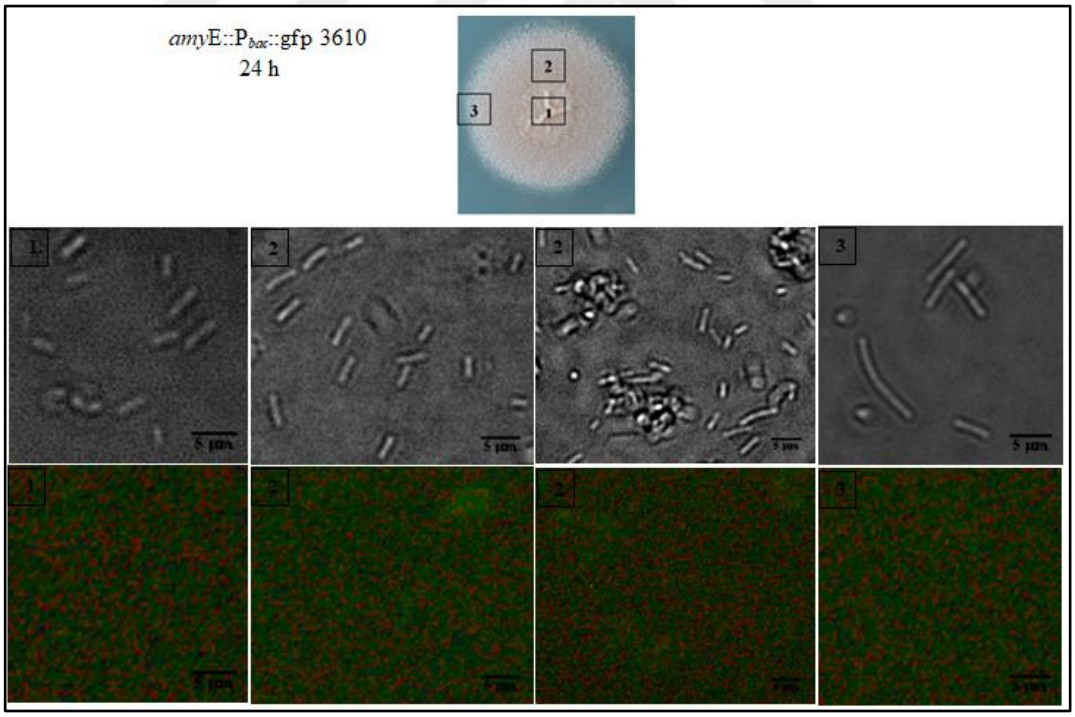

Figure 3.13 : Bright-field and fluorescence microscope images of *amy*E::P$_{bac}$::*gfp* recombinant *B.subtilis* 3610 strain incubated on MSgg agar for 24 h. Top view: The regions from which samples are taken are marked as 1 (center), 2 (middle), 3 (edge).

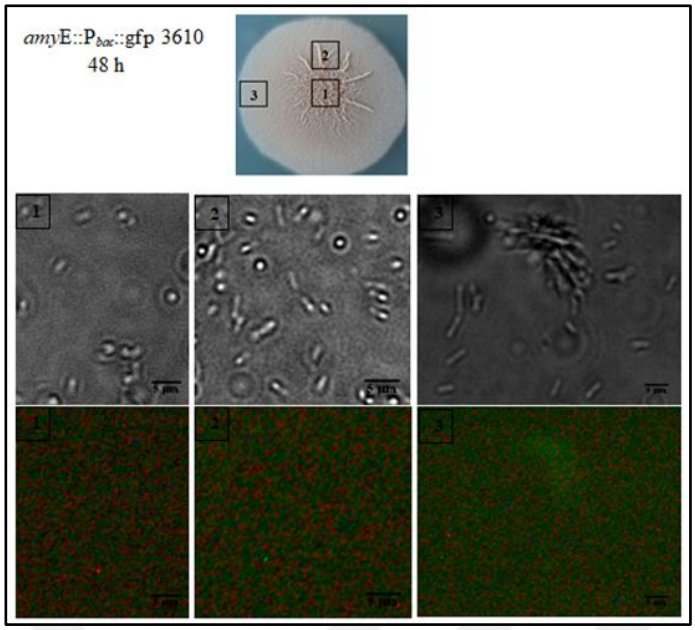

Figure 3.14 : Bright-field and fluorescence microscope images of *amy*E::P_{bac}::*gfp* recombinant *B.subtilis* 3610 strain incubated on MSgg agar for 48 h. Top view: The regions from which samples are taken are marked as 1 (center), 2 (middle), 3 (edge).

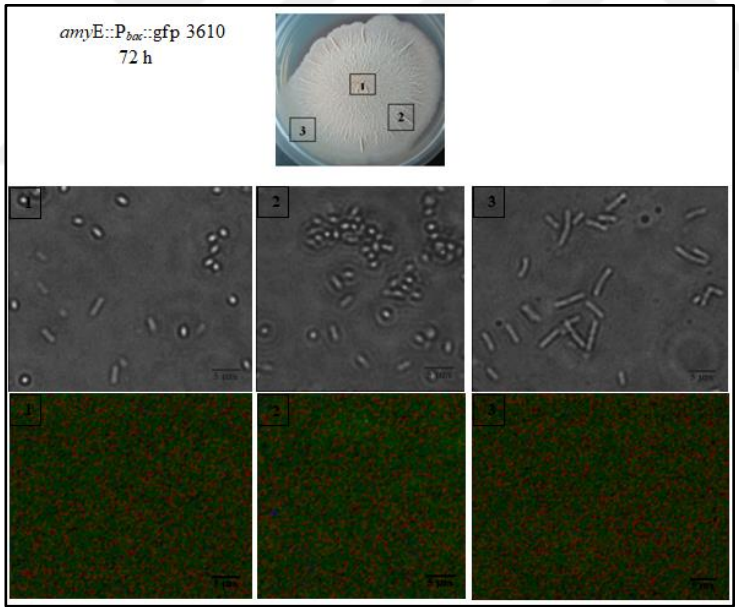

Figure 3.15 : Bright-field and fluorescence microscope images of *amy*E::P_{bac}::*gfp* recombinant *B.subtilis* 3610 strain incubated on MSgg agar for 72 h. Top view: The regions from which samples are taken are marked as 1 (center), 2 (middle), 3 (edge).

These morphologically distinct cell types were observed under fluorescence microscope to identify bacilysin producer cells (Figure 3.13-3.15). However, distinct fluorescent cells could not identified under fluorescence microscope on any regions of the biofilm formed by *amy*E::P_{bac}::*gfp* recombinant *B.subtilis* 3610 cells although

exposure time and detector sensitivity (gain) was set to highest level (4.09 s and 20 dB respectively). Nonetheless, hardly visible cell clumps expressing P_{bac}-*gfp* reporter was observed in the biofilm structure suggesting that bacilysin production in 3610 strain during biofilm formation could be exist but in very faint level (Figure 3.13; Figure 3.14; Figure 3.15). After that, flow cytometry analysis was performed to *amy*E::P_{bac}::*gfp* recombinant 3610 strain after growth on MSgg agar for 24 h and 72 h to quantify single cells (Figure 3.16, 3.17). 8.20% of cells was observed to express P_{bac}-*gfp* reporter at 24th hour, and then amount of the cells expresing the reporter was reduced to 2.22% at 72nd hour (Figure 3.16). To understand whether these cells appearing to express the reporter form a different population, zebra plot and dot plot were drawn (Figure 3.17). Protrusion of cells expressing P_{bac}-*gfp* reporter from background GFP fluorescence level compared to the control cells was observed in the colonies indicating a small subset of biofilm cells producing bacilysin. However, median fluorescence intensity (MFI) of total cells at 24th h was obtained as 3.79 AU (MFI of the cells expressing the reporter: 11.8 AU at 24th h), while MFI of total cells at 72nd h was 2.29 AU (MFI of the cells expressing the reporter: 9.82 AU at 72nd h) which those are very faint expression level.

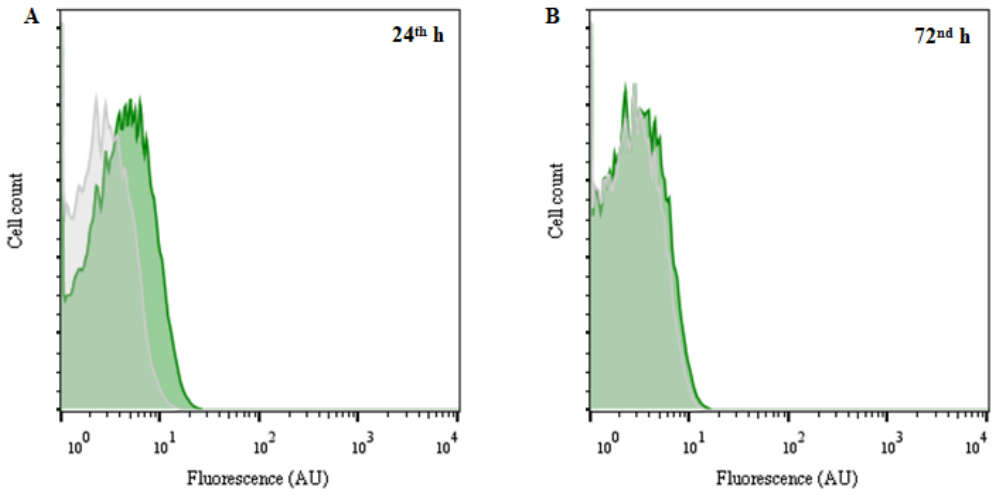

Figure 3.16 : Flow cytometry monitoring the expression of the reporter P_{bac}-*gfp* from *amyE*::P_{bac}::*gfp* recombinant 3610 strain after growth on MSgg agar for 24 h (A) and 72 h (B). Cells with no reporter contruct were used as negative control (gray shading). Cells labeled with P_{bac}-*gfp* reporter are shown as green shading. The Y-axis represents cell counts (50,000 ungated cells were counted), while the X-axis is arbitrary unit (AU) of fluorescence in a logaritmic scale.

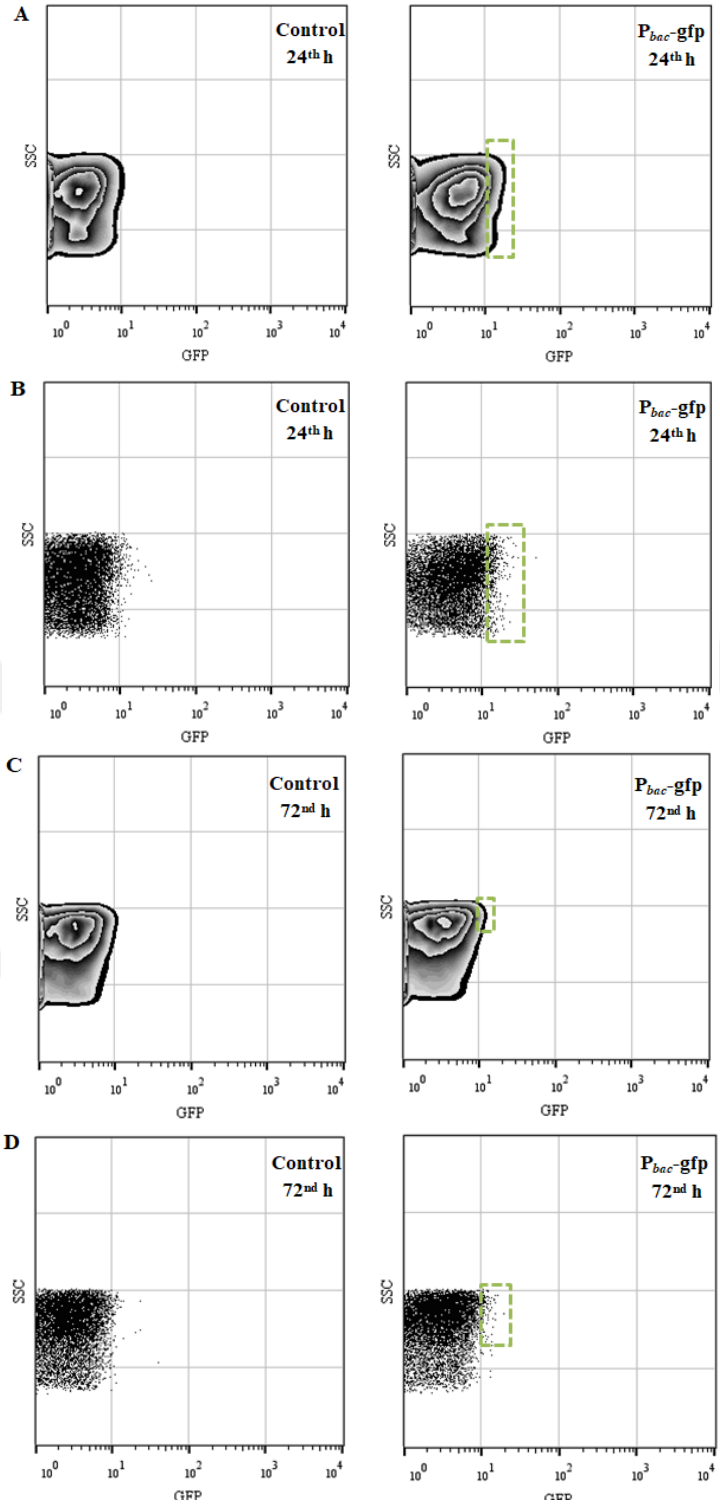

Figure 3.17 : Flow cytometry analysis of *amyE*::P_{bac}::*gfp* recombinant 3610 strain after growth on MSgg agar for 24 h and 72 h time points represented as zebra plot (A, C) and dot plot (B, D). The Y-axis shows SSC while the X-axis represents GFP fluorescence. The control of background fluorescence for GFP in a strain harboring no fluorescent protein genes was shown on left panels. The strain harboring P_{bac}-*gfp* (right panels) lowly expresses the reporter (framed in green). The number of cells is represented by isolines on the Z-axis coming out of the plane of the paper in zebra plots.

Then, in order to localize and compare activity of P_{bac} in NCIB 3610 and PY79 strains during biofilm formation, *B. subtilis* 3610 and PY79 strains containing the transcriptional P_{bac}-*lacZ* fusion at *bacA* locus were grown on MSgg/X-gal agar. If *bacA*-directed β-galactosidase is secreted, X-gal is hydrolyzed and produces insoluble blue pigment. Blue colour was observed in PY79 cells unlike 3610 cells (Figure 3.18) indicating that PY79 exhibits P_{bac} activity in detectable level during biofilm-inducing medium but P_{bac} activity of 3610 cells is undetectably low. Moreover, for more precise evidence of differences of bacilysin operon expression level between 3610 and PY79 strain, P_{bac}-*lacZ* fusion 3610 strain was grown in PA medium, which induces bacilysin production, and β-galactosidase activity of 3610 cells was measured at 24 h growth. Expression of P_{bac}-*lacZ* fusion was detected as 8,3 Miller unit at 24th h, while according to the previous study, expression of P_{bac}-*lacZ* fusion PY79 strain was measured as about 65 Miller units at 24th h (Öğülür, 2008). Expression level of bacilysin operon was approximately 8 fold reduced in the wild type 3610 strain compared to bacilysin producer PY79 strain. It was confirmed that P_{bac} activity is very low in 3610 strain compared to PY79 strain even in PA medium. Therefore, to investigate which cell-types: small rod shape motile cells or long chains of nonmotile matrix producing cells in a biofilm produce bacilysin with direct fluorescence microscopy, all of the studies described above were repeated with PY79 strain.

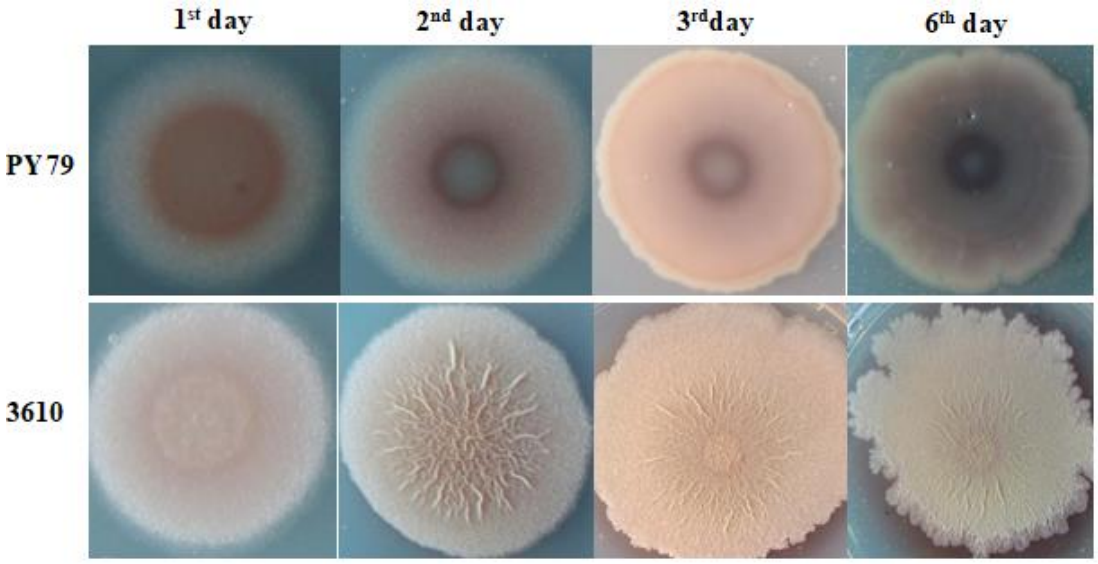

Figure 3.18 : Blue pigment formation of P_{bac}-*lacZ* fusion strains on MSgg/X-gal agar plate.

3.4 Identification of the Bacilysin Producer Cells within Heterogeneous Population of *B. subtilis* PY79

PY79 strain can proliferate into heterogeneous population existing in morphologically distinct cell-types like NCIB 3610 strain even it lost the ability to produce extracellular matrix. Differently, PY79 strain produces high proportion of cell chains compared to 3610 strain because of a mutation in *swrA* gene which leads to lower sigma D factor activity (Kearns and Losick, 2005).

After *amy*E::P_{bac}::gfp recombinant PY79 strain was constructed immedeately it was grown on biofilm-inducing medium MSgg agar for 16, 24, 48, 72 h. The samples were collected and analysed as described before due to observe P_{bac}::*gfp* expression under fluorescence microscope. Recombinant PY79 cells could not form architecturally complex biofilm structure (Figure 3.19) but proliferated into long chains of nonmotile cells which formed bundles as expected (Figure 3.22 and 3.23).

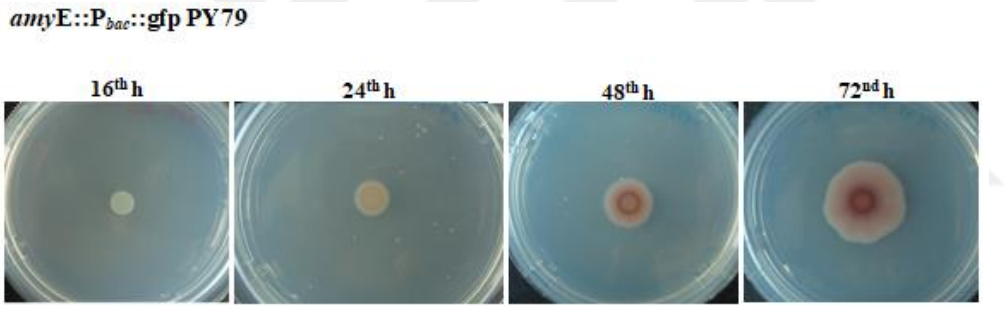

Figure 3.19 : Colony architecture of *amy*E::P_{bac}::*gfp* recombinant *B.subtilis* PY79 on MSgg agar.

Fluorescent cells became visible only when exposure time was set to longest level. The exposure time and detector sensitivity were constant in all fluorescence microscope images (4.09 s and 20 dB respectively) to compare the intensity of the signal from fluorescent cells. But the use of longest exposure time caused to the detection of basal level of GFP expression in cells, thus, majority of the cells regardless of spatiotemporal organization exhibited fluorescence. Although this situation made actual population expressing P_{bac}-gfp reporter hard to differenciate, fluorescence intensity detected in cells aggregated and clumped together, which constituates the initial stage of switching from being single motile cells to growing in long-chain of nonmotile cells is stronger than of single cells or single cell chains (Figure 3.20). Strikingly, another stronger GFP fluorescence was detected in the long

chains of nonmotile cells forming bundles which constitute matrix producer cells in biofilm community (Figure 3.22 and 3.23).

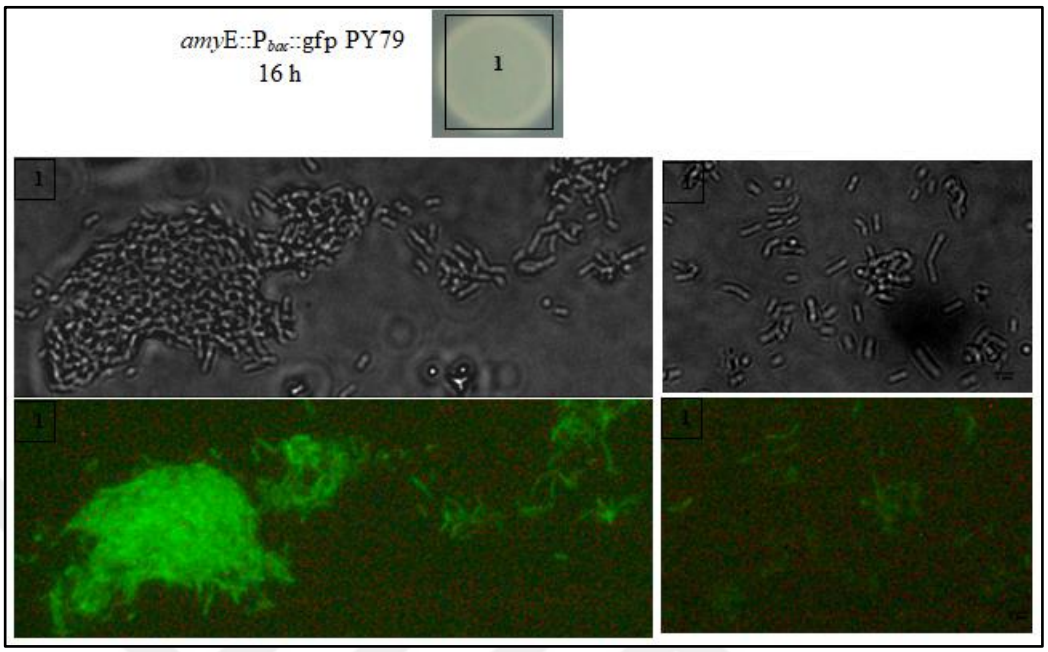

Figure 3.20 : Bright-field and fluorescence microscope images of *amy*E::P$_{bac}$::*gfp* recombinant *B.subtilis* PY79 strain incubated on MSgg agar for 16 h. Top view: The region from where sample is taken.

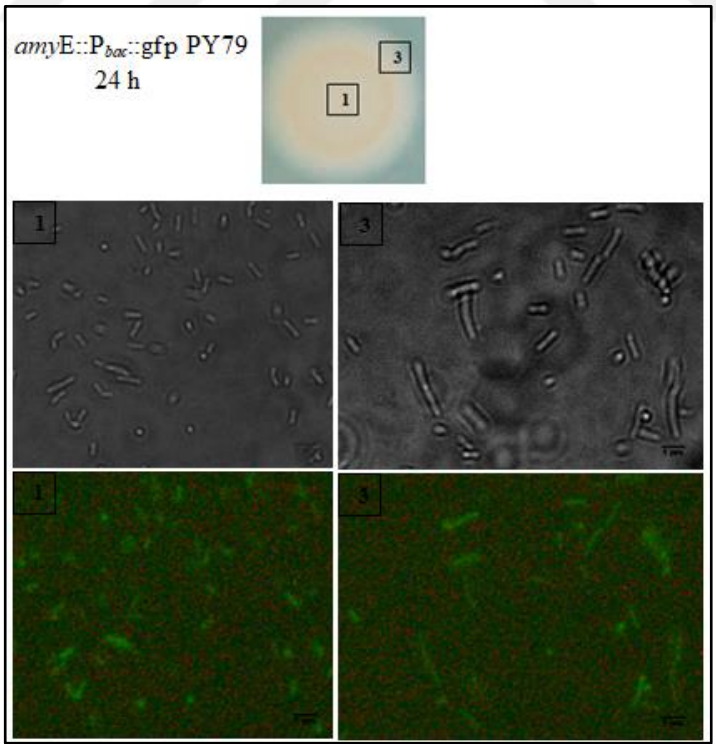

Figure 3.21 : Bright-field and fluorescence microscope images of *amy*E::P$_{bac}$::*gfp* recombinant *B.subtilis* PY79 strain incubated on MSgg agar for 24 h. Top view: The regions from which samples are taken are marked as 1 (center), 3 (edge).

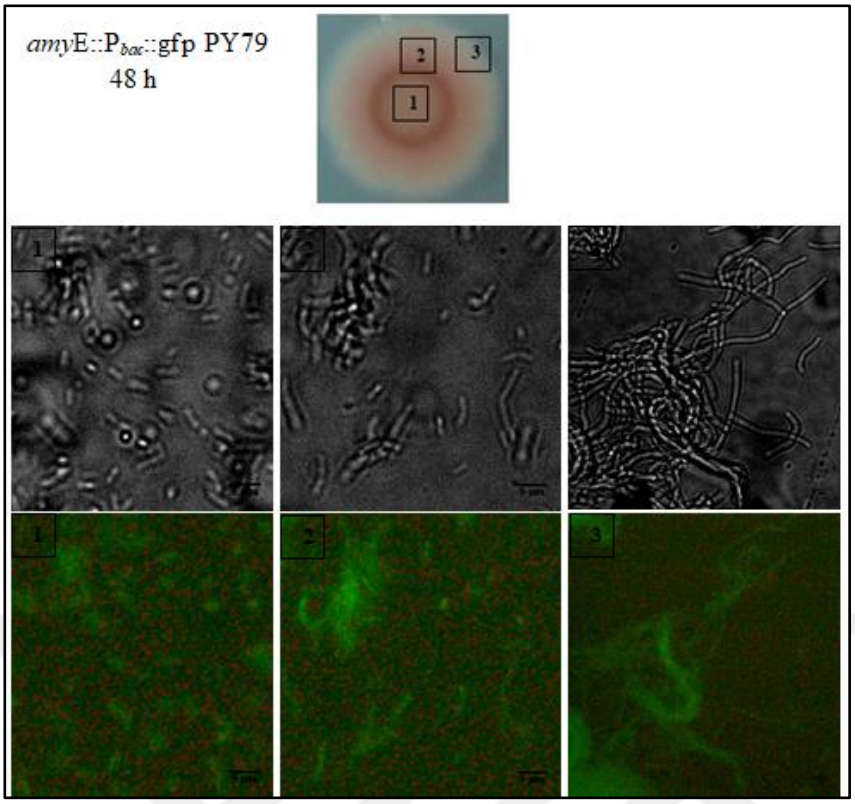

Figure 3.22 : Bright-field and fluorescence microscope images of *amy*E::P_{bac}::*gfp* recombinant *B.subtilis* PY79 strain incubated on MSgg agar for 48 h. Top view: The regions from which samples are taken are marked as 1 (center), 2 (middle), 3 (edge).

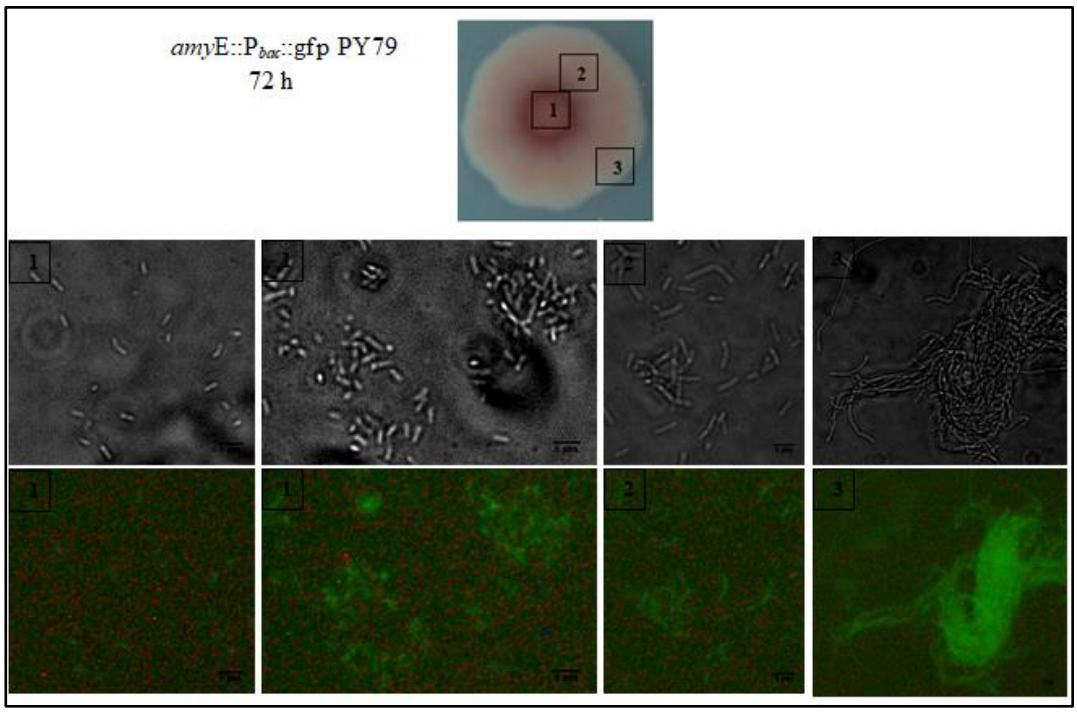

Figure 3.23 : Bright-field and fluorescence microscope images of *amy*E::P_{bac}::*gfp* recombinant *B.subtilis* PY79 strain incubated on MSgg agar for 72 h. Top view: The regions from which samples are taken are marked as 1 (center), 2 (middle), 3 (edge).

After that, flow cytometry analysis was performed to *amy*E::P_{bac}::*gfp* recombinant PY79 strain after growth on MSgg agar for 24 h and 72 h to assay the distribution of fluorescence throughout a population and complement qualitative data from fluorescence microscopy. Analysis of the cells harboring the P_{bac}-*gfp* reporter demonstrated that the bimodal distribution of the reporter expression which bifurcates into two distinct subpopulation: first group that overlaps the autofluorescence levels, and second group that expresses P_{bac} (50.3%) exhibiting low fluorescence (MFI of total cells: 11.7 AU; MFI of the cells expressing the reporter: 22.1 AU) at 24th h (Figure 3.24a). The amount of cells expressing bacilysin-specific promoter was increased to 63.8% at 72nd h as maintaining a low level of expression (MFI of total cells: 12.9 AU; MFI of the cells expressing the reporter: 18.4 AU) (Figure 3.24b). Also, zebra and dot plots were drawn in order to differentiate potential subpopulations clearly (Figure 3.25). Protrusion of cells expressing P_{bac}-*gfp* reporter from background GFP fluorescence level was observed in 24 h and 72 h growth indicating subpopulation of bacilysin-producing cells.

These findings establish the initial signs of heterogeneous expression of bacilysin within a developing biofilm, and reveal that bacilysin could be produced by a subset of matrix producing cells in *B. subtilis* biofilm structure.

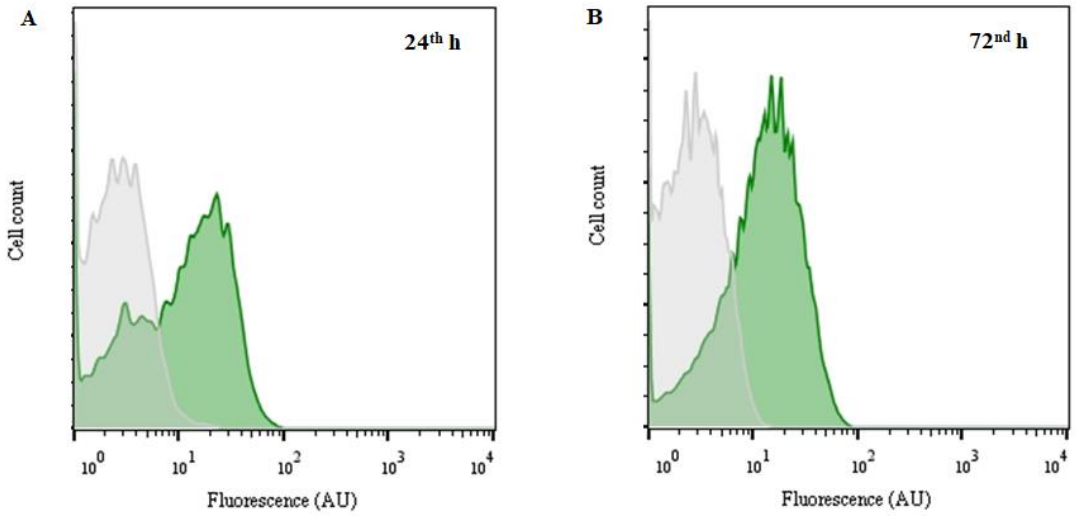

Figure 3.24 : Flow cytometry monitoring the expression of the reporter P_{bac}-gfp from *amyE*::P_{bac}::*gfp* recombinant PY79 strain after growth on MSgg agar for 24 h (A) and 72 h (B). Cells with no reporter contruct were used as negative control (gray shading). Cells labeled with P_{bacA}-gfp reporter are shown as green shading. The Y-axis represents cell counts (50,000 ungated cells were counted), while the X-axis is arbitrary unit (AU) of fluorescence in a logaritmic scale.

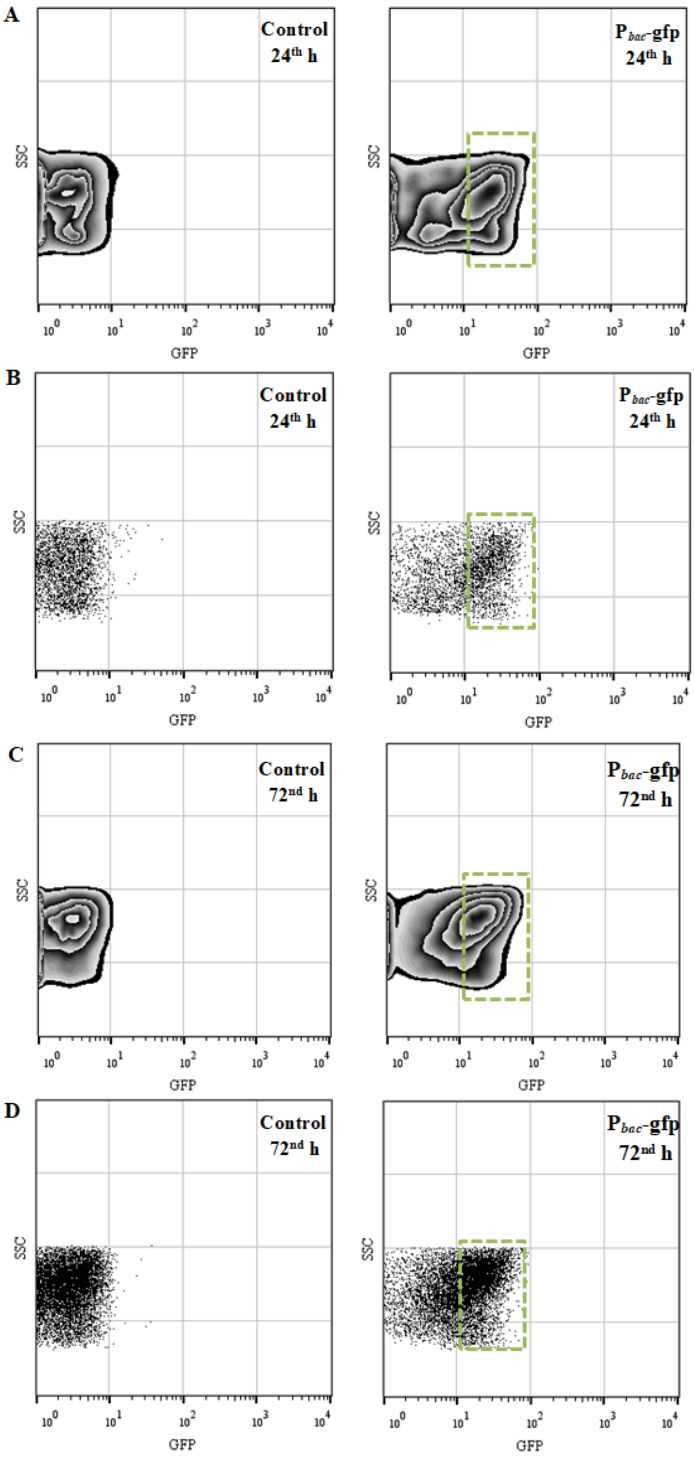

Figure 3.25 : Flow cytometry analysis of *amyE*::P_{bac}::*gfp* recombinant PY79 strain after growth on MSgg agar for 24 h and 72 h time points represented as zebra plot (A, C) and dot plot (B, D). The Y-axis shows SSC while the X-axis represents GFP fluorescence.The control of background fluorescence for GFP in a strain harboring no fluorescent protein genes was shown on left panels. The strain harboring P_{bac}-*gfp* (right panels) lowly expresses the reporter (framed in green). The number of cells is represented by isolines on the Z-axis coming out of the plane of the paper in zebra plots.

4. CONCLUSION

In this study, to identify individual bacilysin producing cells within a developing biofilm structure, a transcriptional fusion with the open reading region encoding GFP and the promoter region of the *bacABCDEF* operon was contructed in undomesticated *Bacillus subtilis* strain NCIB 3610. The obtained recombinant strain, *amy*E::P_{bac}::*gfp* 3610 was, then, grown on biofilm-inducing medium MSgg agar for 24 h, 48 h and 72 h. Samples were taken from different regions of the biofilm to identify the cells expressing P_{bac}-*gfp* reporter based on spatiotemporal organization under fluorescence microscope. Distinct fluorescent cells were not detected within the biofilm structure of *amy*E::P_{bac}::*gfp* 3610 strain even exposure time was set to 4.09 seconds, however some cell clumps were observed to give weak GFP signal. Then, analysis of cells harboring P_{bac}-*gfp* fusion by flow cytometry demonstrated a subpopulation of % 8.20 and 2.22% of the cells expressing the reporter at 24 h and 72 h growth, respectively. These findings reveal that only a small subset of biofilm cells produces bacilysin with faint expression level. After that, in order to localize and compare P_{bac} activity between 3610 and PY79 strains, 3610 and PY79 strains containing P_{bac}-*lacZ* fusion were grown on MSgg/X-gal agar to observe blue pigment formation. Also P_{bac}-*lacZ* 3610 cells were grown in bacilysin-inducing PA medium to quantify the expression level of bacilysin operon with β-galactosidase assay. These experiments confirmed that the expression level of bacilysin operon in 3610 cells is very low, approximately 8 fold is reduced in 3610 compared to PY79. Therefore *amy*E::P_{bac}::*gfp* PY79 strain was constructed and used in ongoing studies to investigate which cell types within a biofilm produce bacilysin. Since, PY79 strains maintain its ability to proliferate into heterogeneous population existing in morphologically distinct cell-types like 3610 strain.

Firstly, *amy*E::P_{bac}::*gfp* PY79 recombinant strain were grown on MSgg agar, and samples were collected and analysed as described before. Flow cytometry analysis demonstrated that distribution of PY79 cells expressing fluorescence was bimodal

with a subpopulation of % 50.3 and % 63.8 of the cells exhibiting fluorescence at 24 h and 72 h of growth, respectively. Direct microscopic observation of the PY79 cell supported the flow cytometry results. Small rod shape motile cells and single individual cells exhibited weak fluorescence, however stronger fluorescence was detected only in the cells aggregated and clumped together and in long chains of nonmotile cells that formed bundles which constitute matrix producer cells in biofilm community. Thereby, these results indicate that bacilysin could be produced by a small subset of matrix producing cells, and strengthen the possibility that bacilycin is a QS molecule taken a role in cell differentiation processes during *B. subtilis* biofilm formation.

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APPENDICES

APPENDIX A: Culture media composition

APPENDIX B: Buffers and solutions

APPENDIX C: Chemicals and enzymes

APPENDIX D: Laboratory equipment

APPENDIX A

Luria Bertani (LB) Medium (1000 ml)

Tryptone	10 g
Yeast Extract	5 g
NaCl	5 g

Distilled water was added up to 1000 ml and then autoclaved at 121ºC for 15 minutes.

Luria Bertani (LB) Agar Medium (1000 ml)

Tryptone	10 g
Yeast Extract	5 g
NaCl	5 g
Agar	15 g

2X MSgg Medium (100 ml)

MOPS	4,186 g
50 mM $MnCl_2$	200 µl
$MgCl_2.6H_2O$	0,081 g
1 mM $ZnCl_2$	200 µl
Glycerol	1 ml
Phenlyalanine	0,01 g
Tryptophan	0,01 g
Sodium L-Glutamate	1 gr
1 M Potassium phosphate*	1 ml
1 mM Thiamine Hydrochloride	200 µl

*6,15 ml of 1M K_2HPO_4 and 3,85 ml of 1M KH_2PO_4 were mixed to prepare 1M potassium phosphate (pH 7.0).

Firstly, 2X MSgg solution without the calcium chloride and iron(III) chloride hexahydrate was prepared and steriled by 0,22 µm filter. Then, 200 µl of sterile 700 mM the $CaCl_2$ was added into 2X MSgg solution. Before use, 2X MSgg solution was diluted 1X MSgg with sterile distilled water or %1.5 agar solution. After that, sterile $Fe(III)Cl_3.6H_2O$ solution was added into 1X MSgg as 50 µM final concentration. 2X Msgg solution was stored at 4ºC.

For MSgg/X-gal agar plate, final concentration of X-gal was 300 µg/ml.

Starch Agar (1000 ml)

Peptone from casein	5 g
Yeast extract	2,5 g
Glucose	1 g
Starch	5 g
Agar	15 g

Perry and Abraham (PA) Medium (500 ml)

KH_2PO_4-KCl*	450 ml
Sucrose-$MgSO_4$**	38,5 ml
Glutamate.Na.H_2O (10g/30ml)***	6 ml
Ferric citrate (1,5g/100ml)***	5 ml
Trace element solution***	0,5 ml

*

KH_2PO_4	0,5 g
KCl	0,25 g

Distilled water was added up to 450 ml. pH was adjusted to 7.5, then the solution was autoclaved.

**

Sucrose	5 g
$MgSO_4$	0,25 g

Distilled water was added up to 38,5 ml, then the solution was autoclaved.

***Filter sterilized

Trace element solution:

$ZnSO_4.7H_2O$	0,1 mg/ml
$CoCl_2.6H_2O$	0,1 mg/ml
Ammonium molybdate	0,1 mg/ml
$MnCl_2.4H_2O$	1 mg/ml
$CuSO_4.5H_2O$	0,01 mg/ml

10X-S Base (50 ml)

$(NH_4)_2SO_4$	1 g
K_2HPO_4	5,35 g
KH_2PO_4	3 g
Na_3Citrate,$2H_2O$	0,5 g

These components were autoclaved together, and after cool down to 50ºC 1 ml of sterile 1 M $MgSO_4$ was added into the solution.

HS Medium (20 ml)

10X-S Base	2 ml
%50 glucose	200 µl
%10 yeast extract	200 µl
%2 caseinhydrolysate	200 µl
%8 arg+%0,4 his	2 ml
%0,5 tryptophone	200 µl
%0,3 phenlyalanine	30 µl
dH_2O	15,170 ml

HS medium was stored at 4ºC for one week at most.

LS Medium (30 ml)

10X-S Base	3 ml
%50 glucose	300 µl
%10 yeast extract	300 µl
%2 caseinhydrolysate	150 µl
%0,5 tryptophone	30 µl
%0,3 phenlyalanine	45 µl
50 mM spermidine	300 µl
1 M $MgCl_2$	75 µl

LS medium must be prepared freshly.

APPENDIX B

CTAB/NaCl Solution: 4,1 g NaCl dissolved in 80 ml distilled water. 10 g CTAB was added slowly while heating to 65 ℃. Distilled water was added up to 100 ml.

TE Buffer (pH 8): 0,12 g Tris Base and 0,03 g EDTA were mixed respectively. Distilled water was added up to 100 ml.

50X TAE Buffer: 242 g Tris Base, 57.1 ml Glacial acetic acid, and 100 ml EDTA (0.5M, pH 8.0) were mixed respectively. Distilled water was added up to 1 L and pH was adjusted to 8.0 by adding HCl.

P1 Buffer : 6.06 g Tris-base, 3.72 g EDTA.$2H_2O$ were mixed. pH adjusted to 8.0 with HCl and distilled water was added up to 1 L. 100 mg RNase A was added for 1 L buffer.

P2 Buffer: 8 g NaOH, 50 ml SDS (20% w/v) dissolved in 1000 ml distilled water

P3 Buffer: 294.5 g potassium acetate dissolved in 500 ml distilled water. pH was adjusted to 5.5 by adding acetic acid.

Physiological Sodium Chloride Solution (0,85%): 0,85 g NaCl dissolved in 100 ml distilled water**.**

APPENDIX C

Chemicals	**Supplier**
Acetic acid	Merck (Germany)
Agar	Biolife (Italy)
Agarose	VWR (Austria)
Ammonium peroxosulphate	Merck
Ammonium sulfate	Riedel-deHaen (Germany)
Calcium chloride	Merck
Casein Hydrolysate	Fluka
Copper sulfate pentahydrate	Merck
Di-Potassium hydrogen phosphate	Riedel-de Haën
Di-potassium hydrogen phosphate	Merck
Di-sodium hydrogen phosphate	Riedel-de Haen
D-glucose anhydrous	Merck
Ethylene Diamine Tetraacetic Acid (EDTA)	MP
Glycerol	Carlo-Erba (France)
Hydrochloric acid	Merck
Iron(III) chloride - 6 hydrate	Riedel-de Haen
L- Aminoacids	Merck
LB Broth	Sigma
Lysozyme	Merck
Magnesium chloride 6-hydrate	Applichem
Magnesium sulfate heptahydrate	Merck
Manganase(II) chloride dihydrate	Merck
Natruim hydroxid	Merck
Potassium di-hydrogen phosphate	Riedel-de Haën
Proteinase K	Applichem
Spermine	Fluka
Sodium acetate	J.T. Baker (USA).
Sodium chloride (NaCl)	Riedel-de Haën
Sodium dihydrogen phosphate	Riedel-de Haën
Sodium dodecyl sulphate	Sigma-Aldrich
Sodium hydrogen phosphate($Na_2HPO_4.7H_2O$)	Merck
Sodium hydroxide	Riedel-de Haen
Sodium L-glutamate monohydrate	Merck
Starch	Merck
Thiamine Hydrochloride	Sigma
Tris (hydrocymethyl) aminomethane	Merck
Tryptone	Sigma
Yeast Extract	Sigma
Zinc Chloride	BDH

Enzymes	**Supplier**
*Eco*RI	Fermentas
*Nhe*I	Fermentas
*Xba*I	Fermentas
*Pvu*II	Fermentas
Taq polymerase	Fermantas
T4 DNA Ligase	Fermentas

Kits	Supplier
NucleoSpin® Gel and PCR Clean-up Kit	Macherey-Nagel
pGEM®-T and pGEM®-T Easy Vector Systems	Promega

APPENDIX D

Autoclave: TOMY SX-700E high pressure steam sterilizer

Balance: Precisa XB 620C

Centrifuge: Eppendorf, centrifuge 5424

Deep freeze: -80°C New Brunswick Scientific ultra-low temperature freezer, -20°C Bosch, -20°C Beko

Electrophoresis equipment: E - C mini cell primo EC320, The Mini Protean III

Electrophoresis power supply: Thermo Electron Corporation EC1000-90

Flow cytometry : BD FACSCalibur

Fluoresence Microscope: ZEISS Axio Vert.A1

Ice machine: AF 10, Scotsman (UK)

Incubators: Nüve EN400, Nüve EN500

Laminar flow cabinet: Biolab Faster BH-EN2003 (Italy)

Microbiological Safety Cabinets: Faster BH-EN 2003 class-II

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

Orbital shaker: Sartorius Certomat SII

pH meter: Hanna Instruments, HI 221 Microprocessor pH meter

Pure water systems: USF Elga UHQ-PS-MK3, Elga Labwater

Refrigerator: Bosch +4°C

System Bio-Rad: (USA).

Sequencing instrument: ABI PRISM® 3100 Genetic Analyzer

Thermal cycler: Labnet International, Multigene gradient TC9600-G-230V

Thermomixer: Eppendorf, 1.5 ml thermomixer comfort

Transilluminator: BIOLAB Laboratory Equipments

UV-Visible Spectrophotometers: Shimadzu UV-Pharmaspec 1700 (Japan)

Vortex mixer: Heidolph Reax top

Water bath: Memmert wb-22

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