ISTANBUL TECHNICAL UNIVERSITY ★ GRADUATE SCHOOL

INVESTIGATION OF THE EFFECTS OF ABRB AND CODY DELETIONS ON THE BACILYSIN OVER PRODUCER B. SUBTILIS HWA STRAIN

M.Sc. THESIS

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JULY 2024

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İSTANBUL TEKNİK ÜVİVERSİTESİ ★ LİSANSÜSTÜ EĞİTİM ENSTİTÜSÜ

ABRB VE CODY YOKLUĞUNUN BASİLİSİN YÜKSEK ÜRETİCİSİ B. SUBTİLİS HWA SUŞUNDAKİ ETKİLERİNİN İNCELENMESİ

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TEMMUZ 2024

Göksu TARTAR, a M.Sc. student of ITU Graduate School of Science Engineering and Technology 521211106 successfully defended the thesis entitled “INVESTIGATION OF THE EFFECTS OF ABRB AND CODY DELETIONS ON THE BACILYSIN OVER PRODUCER B. SUBTİLİS HWA STRAIN”, which she prepared after fulfilling the requirements specified in the associated legislations, before the jury whose signatures are below.

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Date of Submission : 24 May 2024
Date of Defense : 12 July 2024

To my beloved family,

FOREWORD

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MAY 2024

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(Molecular Biologist)

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ABBREVIATIONS

µg :	Microgram
ng:	Nanogram
µl :	Microliter
bp :	Base pair
DNA :	Deoxyribonucleic acid
dNTP :	Deoxy nucleoside triphosphate
LB :	Luria-Bertani
ml :	Milliliter
M:	Molar
GRAS:	Generally recognized as safe
RBS:	Ribosome binding site
SD:	Shine Dalgarno
5'UTR:	5' Untranslated region
CRISPR:	Clustered regularly interspaced short palindromic repeats
SDS:	Sodium dodecyl sulphate
EB:	Elution buffer
SMM:	Spizizen's minimal medium
PBS:	Phosphate buffered saline
PCR :	Polymerase chain reaction
RNA :	Ribonucleic acid
rpm :	Rounds per minute
TAE :	Tris-Acetic acid-EDTA
Tm :	Melting temperature
U :	Units
UV :	Ultraviolet
QS :	Quorum-sensing

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INVESTIGATION OF THE EFFECTS OF ABRB AND CODY DELETIONS ON THE BACILYSIN OVERPRODUCER *B. SUBTILIS* HWA STRAIN

SUMMARY

Bacillus subtilis is a gram-positive, rod-shaped bacterium and a highly studied model organism. It has a highly adaptable metabolism and diverse physiological states regulated according to environmental conditions. *B. subtilis* species go through sporulation to form endospores that can survive harsh conditions such as high temperatures, and UV radiation. They can also form biofilms, attach to plant roots or fungal hyphae, take up extracellular DNA by its natural competence, show surface motility, produce and secrete secondary metabolites. Approximately 4-5% of the *B. subtilis* genome codes for secondary metabolites, including antibiotic bacilysin. Bacilysin, the main focus of this study, is a non-ribosomal dipeptide by linking non-proteinogenic amino acid anticapsin and L-alanine. Bacilysin causes selective cell wall disruption against bacteria, fungi, and algae species, some of which are pathogenic. Bacilysin is a pleiotropic signaling molecule for *B. subtilis* cells as it affects diverse cellular functions such as sporulation, germination and outgrowth. Previous studies have shown that the absence of bacilysin can have negative effects on spore quality and germination. A bacilysin non-producer strain was more sensitive to heat, chemicals and lysozyme. Additionally, comparative transcriptome analysis of *B. subtilis* PY79 and a bacilysin non-producer strain revealed that some genes related to competence development and biofilm formation are also affected by bacilysin.

In *B. subtilis*, bacilysin biosynthesis relies on the expression of the *bacABCDEF* operon and a monocistronic gene *bacG*. Bacilysin production is regulated according to both external and internal factors. It has been established that growth conditions such as medium contents, temperature, and pH affect bacilysin production level. At the transcriptional level, bacilysin biosynthesis is regulated mainly via two mechanisms: quorum sensing pathway and stringent response that occur through the direct-action of the positive transcriptional regulators, including ComA~P, Spo0A~P, and LutR as well as negative transcriptional regulators AbrB, CodY, and ScoC.

Bacilysin has broad-range activity against bacteria, with heat stability up to 15 min at 100°C, and activity within the pH range of 1.4 to 12.0. These characteristics give bacilysin significant clinical importance and make it an effective alternative to traditional drugs and biocontrol agents. However, bacilysin is produced at low levels, cannot be extracted with organic solvents, and has a low isolation yield. In our group, the bacilysin production level was increased at 2.87- fold via editing the 5' untranslated region (5'UTR) of the *bac* operon using the CRISPR/Cas9 approach, thereby obtaining the bacilysin overproducing strain *B. subtilis* HWA. Subsequently, to further boost the bacilysin production level in the over-producing strain *B. subtilis* HWA, the aim of this study was to examine how production levels are affected by eliminating the global regulators AbrB and CodY. To achieve this, the mutant strains *B. subtilis* PY79-GT0A (Δ*abrB*::*cat*) and *B. subtilis* PY79-GT0C (*unkU*::*spc* Δ*codY*) were constructed by transforming competent PY79 cells with chromosomal DNA from the *abrB* deleted

mutant strain *B. subtilis* BAL 373 (*trpC2 pheA1* Δ*abrB*::*cat*) and the *codY* deleted mutant strain *B. subtilis* TMH 307 (*trpC2 unkU*::*spc* Δ*codY*), respectively. Similarly, the mutant strains *B. subtilis* HWA HWA-GTA (Δ*abrB*::*cat*) and *B. subtilis* HWA-GTC (*unkU*::*spc* Δ*codY*) were constructed by transforming competent HWA cells. Furthermore, the *codY-abrB* double mutant strains *B. subtilis* PY79-GT0AC (Δ*abrB*::*cat unkU*::*spc* Δ*codY*) and *B. subtilis* HWA HWA-GTAC (Δ*abrB*::*cat unkU*::*spc* Δ*codY*) were constructed by transforming competent cells of GT0A and GTA with chromosomal DNA from *B. subtilis* TMH 307. Potential tryptophan and/or phenylalanine auxotrophic mutants were eliminated by restreaking on solid Spizizen Minimal Media. Bacilysin phenotypes of the selected mutants were first detected by transferring colonies via toothpicks onto bioassay plates using *Staphylococcus aureus* ATCC 9144. Subsequently, mutants were grown in PA media for 16-18 hours and the bacilysin levels in their culture fluids were detected via paper disk-diffusion bioassay. Results showed that *abrB* disruption in HWA and PY79 cells significantly increased bacilysin production, though each strain was affected differently based on its baseline bacilysin production level. The bacilysin level in HWA-GTA increased by 7.7% relative to parental HWA strain, while PY79-GT0A displayed a 21.8% bacilysin level relative to its parent PY79. Interestingly, while *codY* mutation alone did not significantly affect bacilysin activity in HWA or PY79, adding *codY* mutation to AbrB mutants of both strains caused further increases of 22.1% and 13.4% relative to HWA and HWA-GTA, respectively, and 25.7% and 3.2% relative to PY79 and PY79-GT0A, respectively. In a final attempt to further enhance bacilysin production, the *scoC* mutation as an additional negative regulator of the *bac* operon was combined with the *abrB-codY* double mutation strain. However, the constructed triple mutant strain could not grow in liquid media, demonstrating that simultaneous disruption of these three global regulators severely compromised growth abilities of *B. subtilis* cells. In summary, the findings of this thesis study are important to revealing that concurrent inactivation of AbrB and CodY, two key negative regulators of bacilysin biosynthesis, provides the potential for improving bacilysin production levels further, even in the high-producing strain HWA.

YOKLUĞUNUN BASİLİSİN YÜKSEK ÜRETİCİSİ *B. SUBTİLİS* HWA SUŞUNDAKİ ETKİLERİNİN İNCELENMESİ

ÖZET

Bacillus subtilis gram pozitif, çubuk şeklinde bir toprak bakterisi ve çokça çalışılmış bir model organizmadır. Çevresel koşullara göre düzenlenen adapte olabilir bir metabolizmaya ve çeşitli fizyolojik hallere sahiptir. *B. subtilis* türleri sporülasyon ile yüksek sıcaklık ve UV radyasyonu gibi ağır çevresel koşullarda hayatta kalabilen endosporlar oluştururlar. Ayrıca biyofilm oluşturma, bitki köklerine veya fungal hiflere tutunma, doğal kompetentlikleri sayesinde dışarıdan DNA alma, yüzey motilitesi ve ikincil metabolitleri üretme ve salgılama özelliklerine sahiptirler. *B. subtilis* genomunun yaklaşık %4-5'i ikincil metabolitleri kodlamaktadır. *B. subtilis* tarafından üretilen ikincil metabolitler ilaç, tarım, hayvancılık ve tekstil endüstrilerinde kullanılmaktadır. Bu ikincil metabolitlerden bir tanesi, bu çalışmanın ana odağı olan basilisin molekülüdür.

Basilisin ribozom dışı sentezlenen, protein kökenli olmayan amino asit L-antikapsin ve L-alaninin birleşmesiyle oluşturulan bir dipeptit antibiyotiktir. Basilisin geniş spektrumlu bir antibiyotik olmasına ek olarak küçük yapısıyla da ilgi çekmektedir. Basilisin bazıları patojenik olan çeşitli bakteri, mantar ve alg türlerinde seçici hücre duvarı yıkımına sebep olmaktadır. Basilisini oluşturan amino asitlerden L-alanin basilisinin hücre içiresine transferini kolaylaştırırken L-antikapsin hücre duvarı yıkımına sebep olmaktadır. Hedef hücrelerin içerisine alınmasının ardından basilisinin çeşitli peptidazlar tarafından hidroliz edilmesiyle yapısındaki antikapsin serbest kalarak glukozamin-6-fosfat sentetaz enzimini inhibe etmektedir. Glukozamin-6-fosfat sentetaz enziminin inhibasyonu hücre duvarı sentezini durdurarak hücre ölümüne sebep olmaktadır. Bu özelliği sayesinde basilisin ve basilisin üreten *B. subtilis* suşları birer biyokontrol ajanı adayı haline gelmiştir. Basilisin, *B. subtilis* hücrelerinde sporülasyon, germinasyon ve vejetatif hücre formuna geçiş süreçlerinde etkili pleitropik bir sinyal molekülüdür. Yapılan çalışmalar basilisin yokluğunun spor kalitesi ve germinasyon üzerinde negatif etkileri olduğunu göstermiştir. Basilisin üretemeyen suşun sıcaklığa, kimyasallara ve lizozime karşı daha yüksek hassasiyet gösterdiği gözlemlenmiş, ek olarak *B. subtilis* PY79 ve bacilysin üretmeyen suş ile yapılan karşılaştırılmalı transkriptom analizi kompetent gelişimi ve biyofilm oluşumu ile ilgili pek çok genin de basilisin yokluğundan etkilendiği belirlenmiştir.

B. subtilis'te *bacABCDEF* operonu ve komşu tekli gen *bacG* basilisin biyosentezinden sorumludur. *bacABCDFG* genleri L-antikapsinin sentezlenmesi ve L-alanin ile ligasyonunda görev alarak basilisin biyosentezini gerçekleştirirken *bacE* geni biyosentezi tamamlanan basilisinin hücre dışına transferini sağlayarak üretici olan *B. subtilis* hücrelerinin korunmasından sorumludur. Basilisin üretimi hem iç hem dış faktörlere göre düzenlenmektedir. Yapılan çalışmalar besi yeri içeriği, sıcaklık, pH gibi büyüme koşullarının basilisin üretimi seviyesini etkilediğini göstermiştir. Transkripsiyonel seviyede basilisin biyosentezi iki ana mekanizma ile düzenlenmektedir. Bu mekanizmalar quorum sensing yolağı ve stringent response

olarak adlandırılmaktadır. Basilisin biyosentezinin kontrolü pozitif düzenleyiciler olan ComA~P, Spo0A~P, ve LutR molekülleri ve negatif düzenleyiciler olan AbrB, CodY ve ScoC moleküllerinin direkt görev aldığı gösterilmiştir. Bu düzenleme mekanizmalarına ek olarak basilisinin kendi biyosentezini geribildirim (feedback) mekanizması ile kontrol ettiği gösterilmiştir. Yapılan çalışmada besi ortamına basilin ilavesinin basilisin üretimi üzerinde baskılayıcı etki gösterdiği, basilisin ilavesinin daha erken fazda eklenmesinin daha yüksek baskılayıcı etkiye sebep olduğu belirlenmiştir.

Basilisinin geniş spektrumlu bir antibiyotik olması, 100°C de 15 dakikaya kadar stabil kalabilmesi ve pH 1.4 ve 12.0 aralığında aktif olması gibi özellikleri basilisinin klinik açıdan önemini arttırmakta ve geleneksel ilaçlar ve biyokontrol ajanları yerine kullanılabilecek etkili bir alternatif haline getirmektedir. Fakat basilisinin düşük seviyelerde üretimi, organik çözücüler kullanılarak izole edilebilmesi ve izolasyon veriminin düşüklüğü basilisinin ticari bir ürün haline gelmesine engel olmaktadır. Daha önce laboratuvarımızda basilisin biyosentezini artırmak amacı ile 5' UTR bölgesine CRISPR/Cas9 yaklaşımı ile uyguladığımız genom düzenleme uygulamaları neticesinde, 2.87 kat daha fazla basilisin üreten, yüksek basilisin üretici suşu HWA elde edilmiştir. Bu çalışmayı takiben, bu tez çalışmasında *B. subtilis* HWA suşunda basilisin üretiminin daha da arttırılması hedeflenerek, global düzenleyiciler AbrB ve CodY yokluğunun basilisin üretimi üzerindeki etkilerinin incelenmesi amaçlanmıştır. Bu amaca ulaşmak için *B. subtilis* PY79-GT0A (Δ*abrB*::*cat*) ve *B. subtilis* PY79-GT0C (*unkU*::*spc* Δ*codY*) mutant suşları; kompetent PY79 hücrelerinin sırasıyla *abrB* silinmiş *B. subtilis* BAL 373 (*trpC2 pheA1* Δ*abrB*::*cat*) mutant suş ve *codY* silinmiş *B. subtilis* TMH 307 (*trpC2 unkU*::*spc* Δ*codY*) mutant suşundan izole edilmiş kromozomal DNA ile transformasyonu sonucu oluşturulmuştur. Benzer şekilde *B. subtilis* HWA-GTA (Δ*abrB*::*cat*) ve *B. subtilis* HWA-GTC (*unkU*::*spc* Δ*codY*) mutant suşları kompetent HWA hücrelerinin transformasyonu ile oluşturulmuştur. Sonrasında *codY-abrB* çifte mutant suşları *B. subtilis* PY79-GT0AC (Δ*abrB*::*cat unkU*::*spc* Δ*codY*) ve *B. subtilis* HWA-GTAC (Δ*abrB*::*cat unkU*::*spc* Δ*codY*); kompetent HWA-GTA ve PY79-GT0A hücrelerinin sırasıyla HWA-GTC ve PY79-GT0C mutant suşlarından izole edilmiş kromozomal DNA ile transformasyonu sonucu oluşturulmuştur. Olası triptofan ve/veya fenilalanin oksotrofisine sahip mutantlar, Spizizen Minimal besiyeri üzerine çizilerek elenmiştir. Seçilen mutantların basilisin fenotipi öncelikle *Staphylococcus aureus* ATCC 9144 içeren katı besi yerine çizilerek tespit edilmiş, sonrasında mutantlar PA besi yerinde 16-18 saat büyütülüp kültür sıvısınlarındaki basilisin seviyeleri disk difüzyon yduyarlıöntemiyle belirlenmiştir.

Bu çalışmanın sonuçları HWA ve PY79 hücrelerinde *abrB* yokluğunun basilisin üretimini önemli ölçüde arttırdığını, fakat bu artışın iki suş için farklı oranlarda olduğunu göstermiştir. Basilisin üretimi HWA-GTA suşunda atası olan HWA suşundaki basilisin üretimine kıyasla %7.7 oranında artış gösterirken, PY79-GT0A suşunda atası olan PY79 suşundaki basilisin üretimine kıyasla %21.8 oranında artış göstermiştir. İlginçtir ki AbrB mutantı olan suşlara *codY* mutasyonunun eklenmesi her iki suşta da basilisin üretimini HWA ve HWA-GTA suşlarına kıyasla sırasıyla %22.1 ve %13.4 oranlarında, PY79 ve PY79-GT0A suşlarına kıyasla sırasıyla %25.7 ve %3.2 oranlarında daha da arttırırken, *codY* mutasyonu basilisin üretiminde tek başına önemli ölçüde bir etkiye sahip olamamıştır. Son olarak basilisin üretimini daha da arttırmak amacıyla *bac* operonunun bir diğer negatif düzenleyicisi olan *scoC* mutasyonu *abrB-codY* çifte mutant suşlara eklenmiştir. Ancak elde edilen üçlü mutant suş sıvı besi yerinde büyüyememiş ve bu üç global düzenleyici molekülün aynı anda ortadan

kaldırılmasının büyüme becerisini ciddi derecede etkilediğini göstermiştir. Özetle, basilisin biyosentezinin iki önemli negatif düzenleyicisi olan AbrB ve CodY moleküllerinin eş zamanlı inaktivasyonunun basilisin üretimini, basilisin yüksek üreticisi olan HWA suşunda bile, daha da arttırabileceği gösterilmiştir.

1. INTRODUCTION

1.1 Introduction to *B. subtilis*

Bacillus subtilis is a gram positive, rod shaped bacterium and a highly studied model organism (Kovács, 2019; Nannan et al., 2021; Su et al., 2020). The *B. subtilis* species were found in various environments, both soil and marine habitats thanks to its highly adaptable metabolism and diverse physiological states according to environmental conditions (Earl et al., 2008; Kovács, 2019). *B. subtilis* species go through sporulation to form endospores that can survive harsh conditions, such as high temperatures, UV and radiation, form biofilms, attaches to plant roots or fungal hyphae, take up extracellular DNA by its natural competence, show surface motility, produce and secrete secondary metabolites. Behaviors of cytoplasm exchange via intercellular nanotubes, extracellular vesicle release, and kin-discrimination can be also studied in *B. subtilis* (Earl et al., 2008; Kovács, 2019). The behaviors presented by *B. subtilis* given in Figure 1.1.

B. subtilis strain widely used in industrial and biotechnological applications because of its combined properties. *B. subtilis* is classified as GRAS (generally recognized as safe) and in addition to its highly adaptable metabolism, *B. subtilis* has efficient protein expression and secretion systems (Nannan et al., 2021; Su et al., 2020; Tolibia et al., 2022). These properties make *B. subtilis* easy to cultivate, and simplifies the downstream processing. Genetic stability of the specie, its ability to take up and recombine extracellular DNA to its genome naturally by competence and not having a strong codon preference allow for genetic manipulations to produce or enhance the production amount of the desired secondary metabolites and enzymes that used in baking, animal feed, agriculture, textile, laundry industry, therapeutics and medicine (Kaspar et al., 2019; Nannan et al., 2021; Su et al., 2020). Such applications of *B. subtilis* given in Figure 1.2.

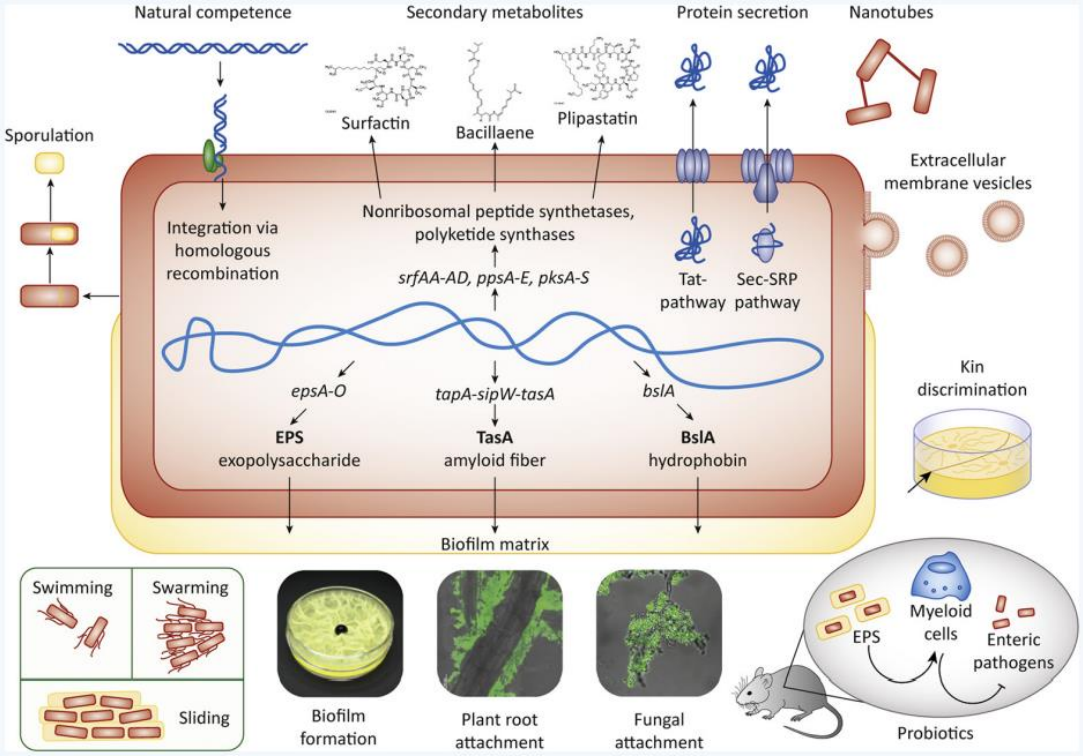

Figure 1.1: Schematic representation of behaviors presented by *B. subtilis* (Kovács, 2019).

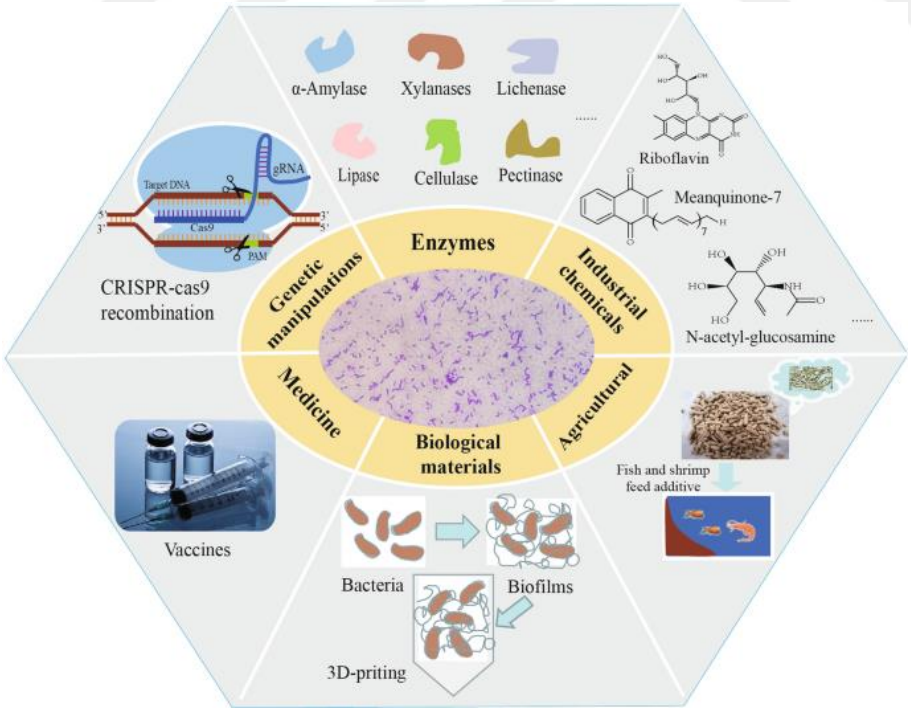

Figure 1.2: Biotechnological applications of *B. subtilis*, such as genetic manipulation, enzyme and industrial chemical production, agricultural, medicine and biological material production (Su et al., 2020).

~4-5% of *B. subtilis* genome code for secondary metabolites such as extracellular proteases, antibacterial, antifungal, surfactants, antiviral, herbicidal, anti-inflammatory gastroprotective, antiulcer, anticancer, hypocholesterolemic, bio simulant, heterologous proteins and vitamins that can be used in many industrial applications and medicine (Iqbal et al., 2023; Nagórska et al., 2007; Nannan et al., 2021; Su et al., 2020). Bioactive secondary metabolites of *B. subtilis* have wide spectrum of action, resistant to hydrolysis by peptidases and proteases, and also resistant to high temperatures and wide range of pH (Iqbal et al., 2023). They are abundant, have diverse variety and structures.

These metabolites classified into five different categories, which are polyketides, volatile metabolites, ribosomal peptides, non-ribosomal peptides, hybrid non-ribosomal peptide/polyketides as shown in Figure 1.3 (Iqbal et al., 2023; Kaspar et al., 2019; Nagórska et al., 2007). Some of the bioactive secondary metabolites can be listed as surfactin with its surfactant activity, iturin A with its antifungal activity, bacilosarcins with its herbicidal activity, macrolactin A with its cytotoxic activity and bacilysin which is the main focus of this study with its broad-range activity against bacteria, fungi and algae species (Iqbal et al., 2023; Nagórska et al., 2007; Nannan et al., 2021).

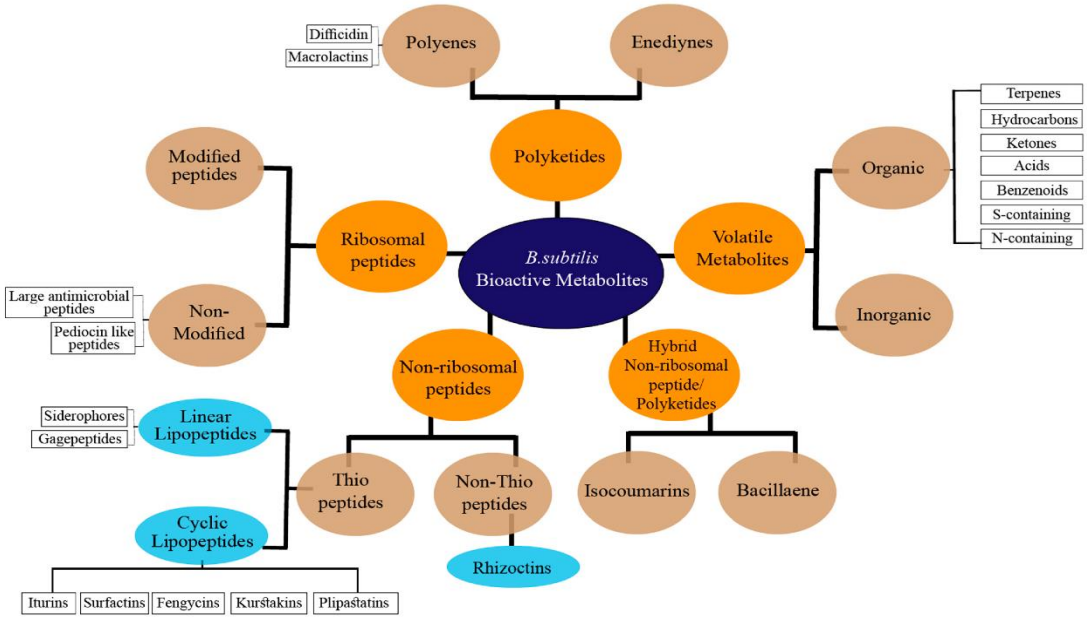

Figure 1.3: Bioactive secondary metabolites produced by *B. subtilis* and their categorization (Iqbal et al., 2023)

Bacilysin is a non-ribosomal peptide that synthesized by non-thio-template mechanism (Iqbal et al., 2023). Bacilysin cause selective cell wall disruption against pathogenic species such as *Candida albicans, Saccharomyces cerevisiae,* rice pathogen *Xanthomonas oryzae, Aspergillus fumigatus, Escherichia coli, Aphanizomenon flos-aquae, Nostoc sp., Anabaena sp.,* cyanobacterium *Microcystis aeruginosa* and *Staphylococcus aureus.* which makes bacilysin and bacilysin producing *B. subtilis* strains a candidate biocontrol agent (Iqbal et al., 2023; Islam et al., 2022; Nannan et al., 2021).

1.2 Bacilysin: Structure, Function, and Biosynthesis

Bacilysin, also known as tetain and bacillin, first discovered in 1946 by Abraham and his colleagues (Abraham et al., 1946; Özcengiz & Öğülür, 2015). It is a dipeptide antibiotic produced by Bacillus species. Bacilysin produced non-ribosomally by linking non-proteinogenic amino acid anticapsin and L-alanine together and has its molecular weight at 270 Da (Islam et al., 2022; Özcengiz & Öğülür, 2015; Stein, 2005). Structure of bacilysin given in Figure 1.4. Its broad-range activity against bacteria, fungi and algae species, heat stability (15 min at 100℃) and showing activity in the range of 1.4 to 12.0 pH give bacilysin a significant clinical importance and make it an effective alternative to traditional drugs and biocontrol agents (Özcengiz & Alaeddinoglu, 1991;Islam et al., 2022). However, bacilysin produced in low levels, it is unextractable with organic solvents, and isolation yield is low (Islam et al., 2022; Özcengiz & Öğülür, 2015).

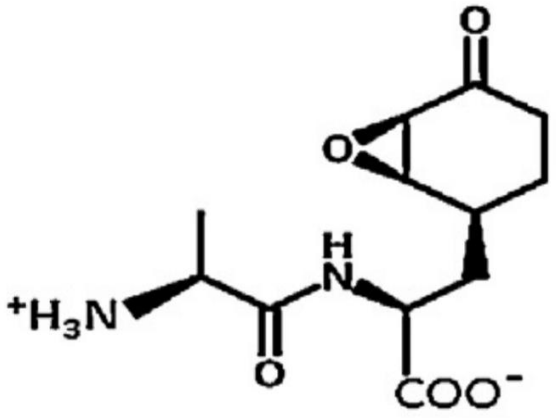

Figure 1.4: Structure of bacilysin (L-alanyl-b-(2,3-epoxycyclohexanonyl)-L-alanine, $C_{12}H_{18}N_2O_5$)(Adapted from Özcengiz & Öğülür, 2015).

Bacilysin function as a broad-spectrum antimicrobial agent by disrupting the cell walls of bacterial, fungal and algal species such as; *Candida albicans, Saccharomyces cerevisiae,* rice pathogen *Xanthomonas oryzae, Aspergillus fumigatus, Escherichia coli, Aphanizomenon flos-aquae, Nostoc sp., Anabaena sp.,* cyanobacterium *Microcystis aeruginosa* and *Staphylococcus aureus* (Islam et al., 2022; Özcengiz & Öğülür, 2015). Bacilysin function as an antimicrobial after its transportation into the target cell. When bacilysin hydrolyzed intracellularly produced anticapsin act as glutamine analogue and inhibit glucose amine 6-phosphate synthase by covalently binding. Cells of growing cultures, being unable to renew their cell walls, experience partial lysis. Antimicrobial activity of bacilysin can be reversed by addition of glucose amine and N-acetyl glucose amine (Islam et al., 2022; Özcengiz & Öğülür, 2015). Since bacilysin transport into the cell is greater than anticapsin transport, bacilysin can be a novel antifungal candidate (Islam et al., 2022).

One of the functions of bacilysin is being a pleiotropic signaling molecule for *B. subtilis* cells as it affects diverse cellular functions (Ertekin et al., 2020). These cellular functions include sporulation, germination and outgrowth. Studies showed that absence of bacilysin had negative effects on spore quality and germination, while bacilysin non-producer strain was more sensitive to heat, chemicals and lysozyme. In addition, comparative transcriptome analysis of *B. subtilis* PY79 and non-producer strain OGU1 revealed that competence development and biofilm formation also effected by bacilysin expression. Based on the research it is speculated that bacilysin might be a signaling molecule that plays role in regulation of biofilm formation and cell differentiation (Ertekin et al., 2020; Özcengiz & Alaeddinoglu, 1991a; Özcengiz & Öğülür, 2015).

In *B. subtilis* cells bacilysin biosynthesis rely on the expression of *bacABCDEFG* genes, encoded by *bac* operon. The *bac* cluster organization found to be conserved among different *Bacillus* species, such as *B. velenzensis, B. amyloliquefaciens, and B. subtilis*, with 72.6 to 88.6% sequence identity (Islam et al., 2022; Ertekin et al., 2020). Bacilysin biosynthesis machinery consist of prephenate decarboxylase, H_2HPP isomerase bacilysin biosynthesis oxidoreductase, bacilysin synthase, putative bacilysin exporter, transaminase, NADPH-dependent reductase coded by the genes *bacA, bacB, bacC, bacD, bacE, bacF* and *bacG* respectively. While *bacABCDFG* responsible from the reactions required to synthesize bacilysin from prephenate, *bacE*

provide putative bacilysin exporter that needed for pumping the produced bacilysin out from the producer to ensure immunity against effects of bacilysin (Ertekin et al., 2020; Islam et al., 2022; Özcengiz & Öğülür, 2015). Biosynthesis pathway of bacilysin given in Figure 1.5.

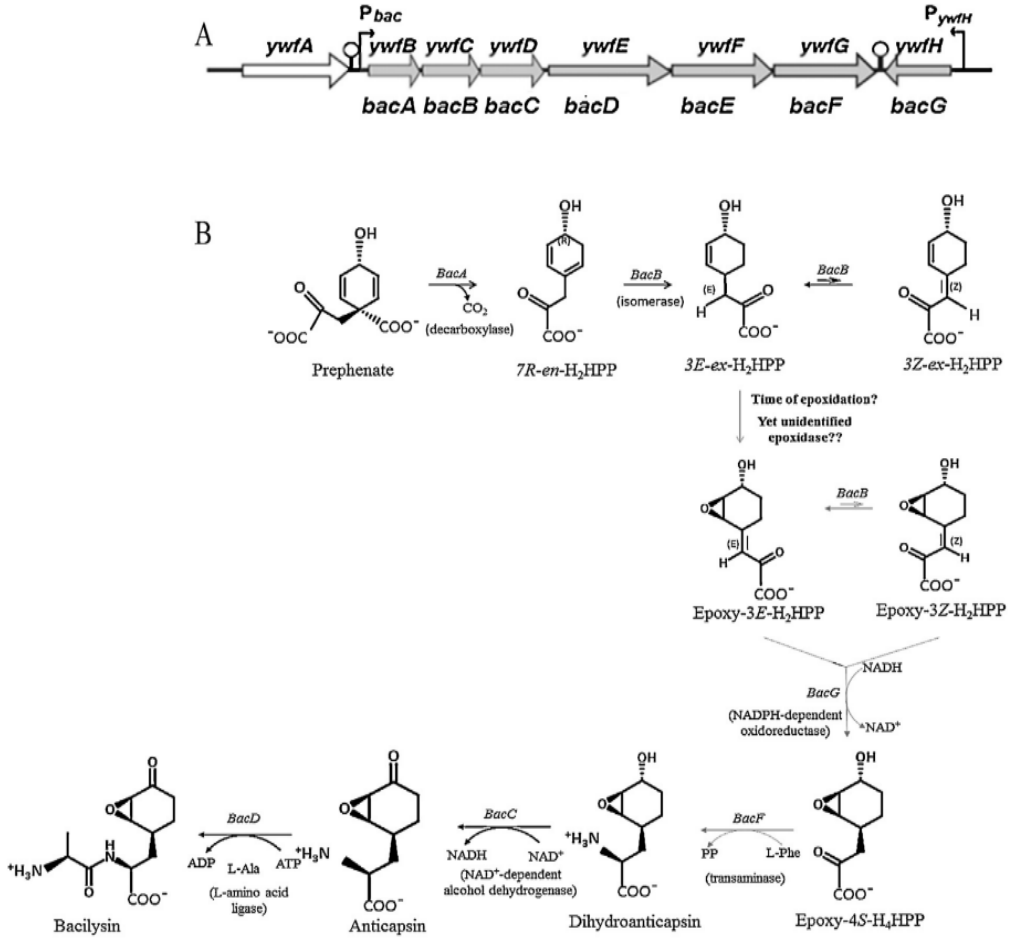

Figure 1.5: *bac* operon (a) and biosynthetic pathway of bacilysin (b) (Özcengiz & Öğülür, 2015).

1.3 Regulation of Bacilysin Biosynthesis

Bacilysin production is regulated according to both external and internal factors. It has been established that the growth medium and conditions effect the bacilysin production. Bacilysin production showed to be higher with the mediums containing sucrose compared to other carbon sources, while using ammonium salts as sole nitrogen source, adding casamino acid in a medium containing glutamate, glucose and temperatures above 30°C suppressed the production (Ozcengiz et al., 1990; Özcengiz & Alaeddinoglu, 1991b). Bacilysin biosynthesis regulated by three mechanisms,

which are quorum sensing pathway, stringent response, and feedback regulation by bacilysin of its own synthesis (Ertekin et al., 2020; Inaoka et al., 2009; Islam et al., 2022; Karataş et al., 2003; Köroğlu et al., 2011; Özcengiz & Alaeddinoglu, 1991b). Regulatory molecules effective on bac operon given in Figure 1.6.

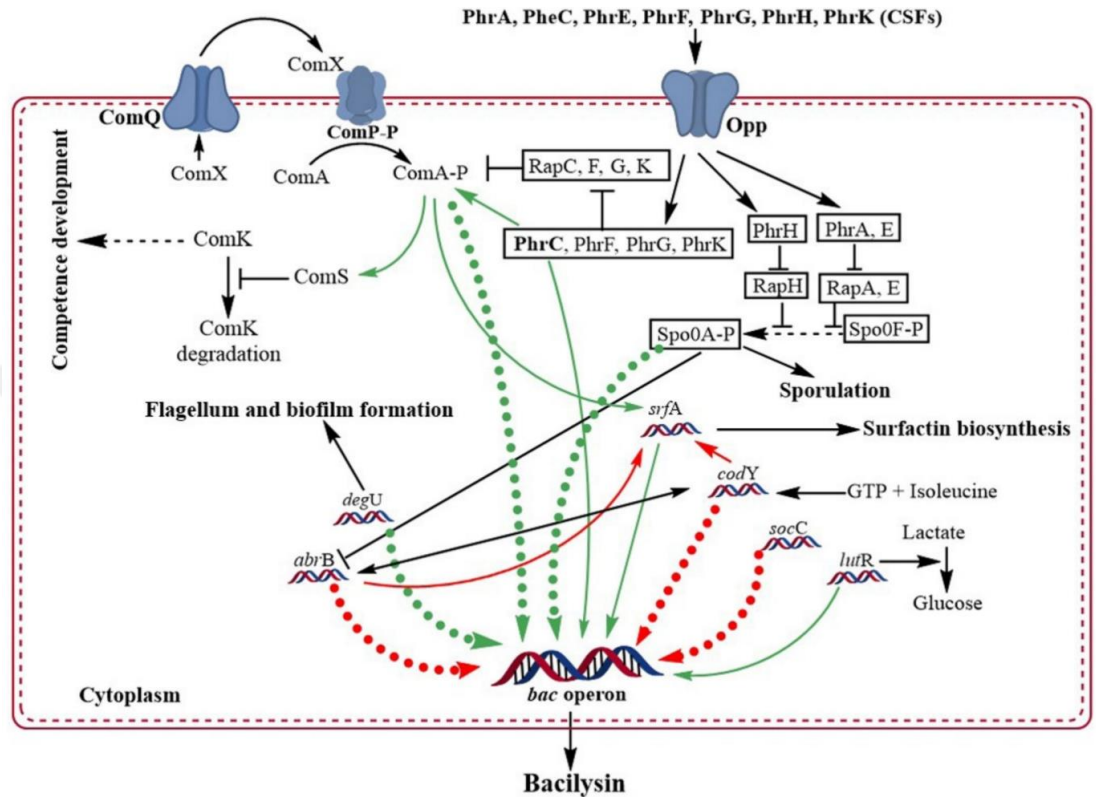

Figure 1.6: Schematic representation of transcriptional regulation of bacilysin biosynthesis. Colors of arrows indicate positive or negative regulation, which indicated by green and red respectively. While dotted arrows show direct binding to bac operon, and regular arrows show indirect regulators. (Islam et al., 2022).

1.3.1 Quorum sensing

Quorum sensing (QS) first described in 1979 by Nealson and Hastings's work on Vibrio fischeri and Vibrio harveyi. They discovered that the expression of luminescence genes was depended on cell density of the colony. The expression was regulated by secreted molecules named autoinducers. Depending on the concentration of autoinducers cells were changing their expression patterns (Nealson & Hastings, 1979). Throughout the time many more species were discovered that utilizes cell density dependent mechanisms for cell-cell signaling. It was discovered that the microbial cells were coordinating their behavior according to secreted autoinducers by the microbial community that they belong. This mechanism allows cells to efficiently regulate their gene expressions according to cellular density changes (Miller &

Bassler, 2001; Qu & Liu, 2024). Microbial communities used QS system to regulate expression of genes that participate in competence, sporulation, biofilm formation, antibiotic production, fruiting body formation, virulence factors, fratricide, bioluminescence emission, and symbiosis (Li & Tian, 2012; Pena et al., 2019). Different type of autoinducers were employed by the different species' QS systems. The QS systems divided into three groups which are LuxI/LuxR-type quorum sensing employed by gram-negative bacteria, peptide mediated quorum sensing employed by gram-positive bacteria and interspecies communication (Miller & Bassler, 2001; Qu & Liu, 2024).

1.3.1.1 LUXI/LUXR-type quorum sensing

Gram-negative bacteria mediate QS systems that employs homologues of LuxI and LuxR proteins found in V. fischeri. The autoinducers called acylated homoserine lactone signaling molecule (HSL or AHL) produced by the activity of LuxI (or LuxI homologues). The autoinducers secreted to extracellular medium. Increase of cellular density cause increase of concentration of autoinducers in extracellular medium. When the autoinducer concentration reach a certain level called minimum threshold concentration, autoinducer LuxR (or LuxR homologue) binding occur, given in Figure 1.7. Autoinducer-LuxR complex formation triggers cellular cascades that regulated by the QS mechanism (Miller & Bassler, 2001; Waters & Bassler, 2005; Kumar & Rawat, 2020; Vashistha et al., 2023).

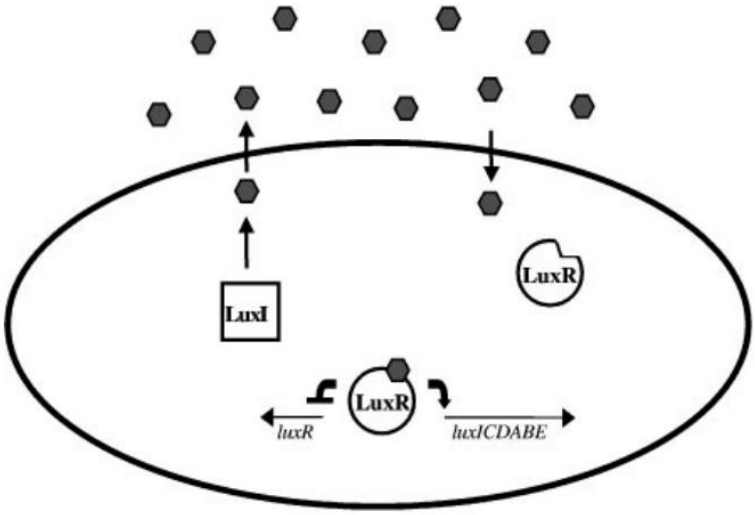

Figure 1.7: Schematic representation of Quorum Sensing Mechanism LuxI/LuxR system utilized by Gram negative bacteria (Miller & Bassler, 2001).

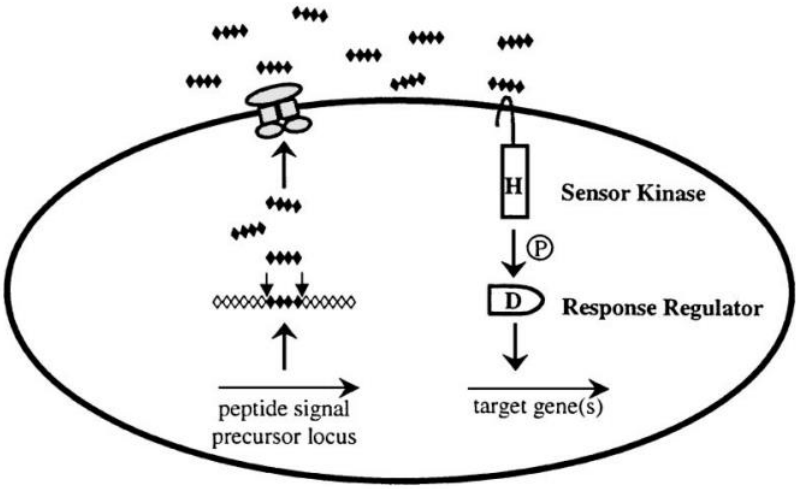

Figure 1.8: Schematic representation of Quorum Sensing Mechanism Oligopeptide System utilized by Gram positive bacteria (Miller & Bassler, 2001).

1.3.1.2 Peptide mediated quorum sensing

Gram-positive bacteria utilize peptides as autoinducers as a part of their QS mechanism. They use ATP-binding cassette (ABC) transporters to secrete the peptides to extracellular medium. As it stated previously depending on cellular density, the concentration of autoinducers reaches to a certain level in extracellular medium. The peptides interact with two-component sensor kinases after reaching to minimum threshold concentration. The interactions cause a phosphorylation cascade inside the cells which result in phosphorylation of response regulator protein (transcription factor). The phosphorylated transcription factor proceeds to bind to the DNA and regulate the transcription of the cellular response controlled by QS, given in Figure 1.8 (Miller & Bassler, 2001; Waters & Bassler, 2005; Kumar & Rawat, 2020).

1.3.1.3 Interspecies communication

It is stated that there are possible mechanisms for interspecies communication that involves LuxS gene which is responsible for the synthesis of Autoinducer-2 (AI-2) (Bridges & Bassler, 2019; Li & Tian, 2012; Miller & Bassler, 2001; Schauder et al., 2001). The steps of synthesis of AI-2 was found to be same in Escherichia coli, Salmonella typhimurium, V. harveyi, Vibrio cholerae and Enterococcus faecalis species (Schauder et al., 2001). Also, it is found that LuxS homologues are present in many species . This founding speculated as AI-2 being the interspecies communication signaling molecule (Schauder et al., 2001).

1.3.2 Quorum sensing systems in *B. subtilis*

Regulation of complex physiological processes; such as competence development, sporulation, biofilm formation, antibiotic and proteolytic enzyme synthesis depends on quorum sensing mechanisms in *B. subtilis* cells. There are two identified QS mechanisms in the *B. subtilis* which are ComQXPA and Rap-Phr Systems. ComQXPA QS mechanism employs ComX peptide as autoinducer of the system. ComX processing, modification and export require ComQ activity. Once the ComX concentration reaches the critical threshold in extracellular medium, ComX binds to receiver domain of ComP. ComP is a sensor protein kinase, which activates ComA by phosphorylation after ComX-ComP binding. Phosphorylated ComA upregulates ComS, surfactin and DegQ production (Kalamara et al., 2018; Miller & Bassler, 2001; Vlamakis et al., 2013; Spacapan et al., 2020). Surfactin secretion leads to Spo0A phosphorylation while DegQ production leads to secretion of exoprotease and other extracellular enzymes by DegU phosphorylation (Kalamara et al., 2018; Vlamakis et al., 2013; Spacapan et al., 2020). ComS production prevent degradation of ComK, which is a transcriptional activator responsible for expression of the genes required for competence development, given in Figure 1.9 (Rai et al., 2015).

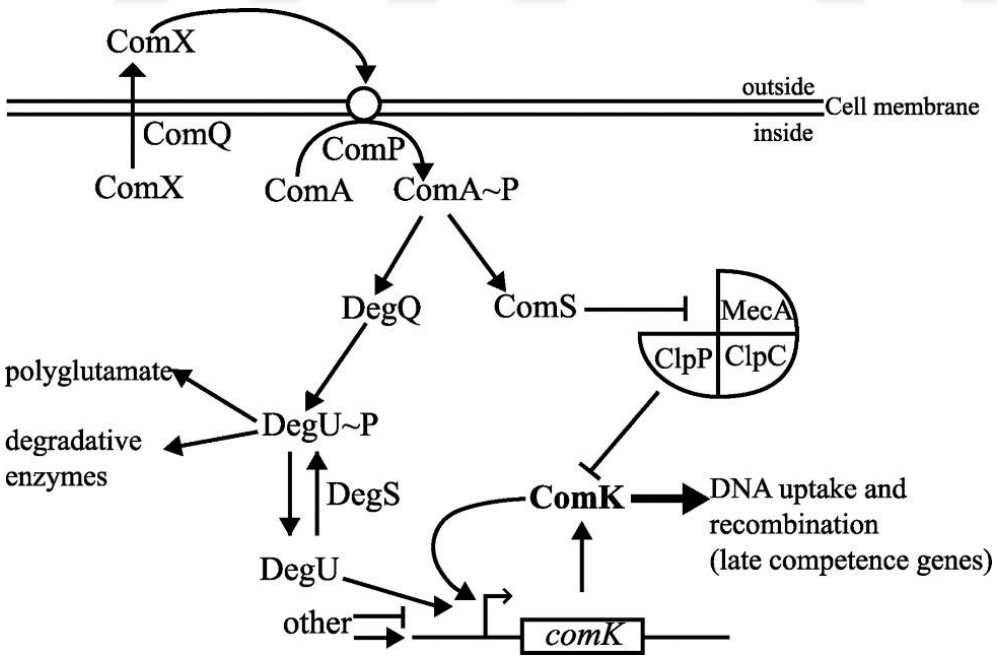

Figure 1.9: Schematic representation of ComQXPA pathway. Activation represented as arrows; repression represented as t-bars (Hoffmann et al., 2010).

There are multiple Rap-Phr systems identified in *B. subtilis* strains. The system acts as antagonist of ComQXPA system. In low cellular concentrations the Rap proteins inhibits ComA~P activity and downregulates DegU and Spo0F activities. The inhibition reversed by binding of Phr peptides to Rap receptors (Kalamara et al., 2018; Gallegos-Monterrosa et al., 2021). The Pro-Phr secreted to the extracellular medium by cleavage of pre-pro-Phr that synthesized in the cytoplasm. Modifications of Pro-Phr done in extracellular matrix and Phr produced. Extracellular Phr concentration increased by the increase of cellular density. When the concentration of Phr reaches to critical threshold, ABC type oligopeptide transporter Opp system used for entrance of Phr molecules into the cells. Phr molecules in the cytoplasm inactivates the Rap activity, given in Figure 1.10 (Kalamara et al., 2018).

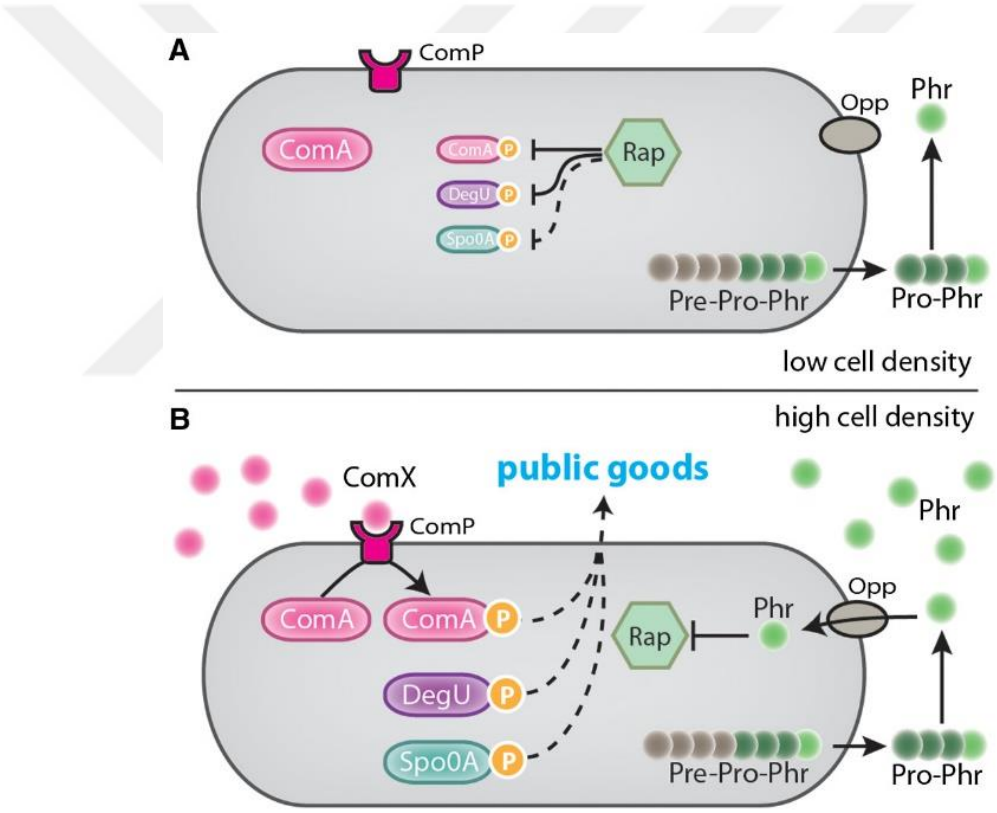

Figure 1.10: Schematic representation of Rap-Phr pathway. Activation represented as arrows; repression represented as t-bars; indirect activation and repression represented as dashed arrows or t-bars respectively (Kalamara et al., 2018).

One of the precursor Phr molecules PhrC, cleaved to form CSF (competence and sporulation factor). When low levels of CSF imported into cells, it binds to RapC and cause its inhibition. RapC inhibition results in phosphorylated ComA increase in the cells, which leads to competence development. High concentration of CSF in the cells inhibits competence through inhibition of ComS production due to inhibition of ComA

phosphorylation by binding to ComP. Inhibited ComS production result in degradation of ComK hence inhibition of competence occurs (Rai et al., 2015; Gallegos-Monterrosa et al., 2021). High concentration of CSF lead to inhibition of RapB, which dephosphorylates phosphorylated Spo0F. Thus, would activate sporulation pathway by increasing phosphorylated Spo0A levels, given in Figure 1.11 (Kalamara et al., 2018; Miller & Bassler, 2001; Rai et al., 2015; Waters & Bassler, 2005; Gallegos-Monterrosa et al., 2021).

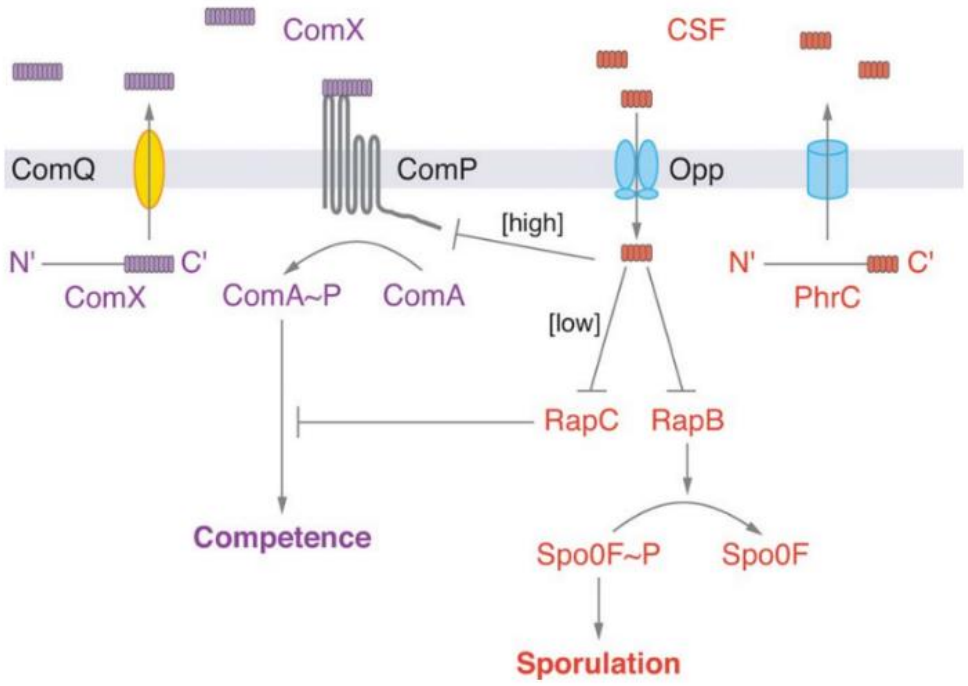

Figure 1.11: Schematic representation of quorum sensing pathway in *B. subtilis*. activation represented as arrows; repression represented as t-bars; indirect activation and repression represented as dashed arrows or t-bars respectively (Waters & Bassler, 2005).

Studies showed that bacilysin biosynthesis regulated by QS systems in *B. subtilis* (Karataş et al., 2003; Yazgan et al., 2001; Ertekin et al., 2020). It was revealed that *comA, comP* and *comQ* mutant strains lost cell density dependent induction of *bac* operon. Results showed that *comA*, and *comP* mutants were able to only showed 2 and 5% of the expression showed by parental strain respectively and ComA directly interacts with bac operon, which makes ComA essential for bacilysin biosynthesis (Köroğlu et al., 2011). It is stated that PhrC, PhrF and PhrK are necessary to achieve maximum ComA level (Auchtung et al., 2006), while OppA and Spo0H is required

for import of Phr molecules back into cell and PhrC maturation respectively. Thus, disruption of PhrC, PhrF, PhrK, OppA and Spo0H coding genes result in decreased or no bacilysin activity (Perego, 1997; Lazazzera et al., 1999). In addition, disruption of ComA dependent srfA promoter inhibited the expression of bac operon and global regulator AbrB, which negatively regulates the transcription of srfA shown to negatively regulates the transcription of bac as well (Karataş et al., 2003; Robertson et al., 1989). AbrB is a transition state regulator in *B. subtilis*, which is active during early exponential and transition phases. AbrB controls transcription of almost 270 genes that related to antibiotic synthesis, biofilm formation, motility, virulence, extracellular enzyme production, sporulation and use of alternative metabolic pathways. Activity of AbrB allow cellular functions to be coordinated for adaptation, cell survival and proliferation. AbrB influence transcription of the genes by recognizing and binding to its target sequences. Spo0A~P inhibits *abrB* transcription, thus depresses all genes repressed by AbrB. Studies showed that deletion of AbrB resulted in increased biosynthesis of certain secondary metabolites such as alkaline protease AprE and antibiotics. (Islam et al., 2022; Karataş et al., 2003; Köroğlu et al., 2011; Robertson et al., 1989; Tolibia et al., 2022) As it shown by Karataş et al., negative regulation of AbrB relieved by Spo0A, while both molecules affect bac operon by binding (Karataş et al., 2003).

1.3.3 Stringent response

Resources of a cell carefully monitored while making decisions about physiological changes in the cell to ensure survival. In the case of bacilysin biosynthesis, ppGpp (guanosine 5'-diphosphate 3'-diphosphate) is an important factor for transcription of the *bac* operon and its amount in the cell depends on intracellular GTP level. The regulation based on the intracellular GTP level maintained by GTP-sensing transcriptional pleotropic repressors coded by *codY*. Studies showed that increase in intracellular GTP level or deletion of *codY* increases transcription of *bac* operon (Islam et al., 2022). CodY is a modulator of cellular nutritional status and energy availability in *B. subtilis*, which is active during exponential and transition phases. CodY acts according to cellular levels of guanosine triphosphate (GTP) and branched chain amino acids (BCAAs), which in high levels act as cofactors of CodY binding to recognized DNA sequences of the genes that required to be expressed when there is nutritional stress. Low levels of GTP and BCAAs remove CodY from DNA, thus

releases its inhibitory effects. CodY also interact with the genes that are involved in nitrogen and carbon metabolism. In *B. subtilis* 188 genes that are involved in diverse functions in the cell such as, competence, surfactant production, dipeptide transportation regulated by CodY (Köroğlu et al., 2011; Tolibia et al., 2022).

1.3.4 Feedback regulation by bacilysin

Bacteria have many mechanisms to regulate gene expression tightly to ensure survival on limited resources. These regulations can be on transcriptional, post-transcriptional, translational, and post-translational levels. One of the post translational regulation mechanism is the feedback regulation (Bervoets & Charlier, 2019). It is found that bacilysin inhibits its own biosynthesis by feedback mechanism. However, results revealed that inhibition is not exerted by inactivation of bacilysin synthase, which is last enzyme on bacilysin biosynthetic pathway. Since earlier addition of bacilysin resulted in greater feedback response (Özcengiz & Alaeddinoglu, 1991b).

1.4 *B. subtilis* HWA Strain

B. subtilis HWA strain constructed by our group to improve bacilysin production in *B. subtilis*. As high-level expression of a gene depends on translation of transcribed mRNAs as much as high-level transcription, translation initiation is one of the major rate limiting factors of gene expression level in bacteria (Vellanoweth & Rabinowitz, 1992). In bacteria translation initiation occurs at 5′ untranslated region (5′UTR) of an mRNA which contain the ribosome binding site (RBS). Within the RBS there is a sequence in bacteria named Shine-Dalgarno (SD) sequence, the start codon of the target gene, and a short spacer sequence in between. SD sequence have a great importance in translation initiation as it base pairs with anti-SD sequence found on 3' terminus of 16Sr RNA of 30S small ribosomal subunit (Shine & Dalgarno, 1974) This base pairing lead to enhanced ribosome recruitment to the start codon of the gene. Based on the SD sequence ribosome recruitment to the start codon of the gene differ in effectiveness thus lead to categorization of SD sequences as weak or strong (Ringquist et al., 1992; Ma et al., 2002). Investigation done on bac operon and its upstream DNA sequences showed that there was no strong SD sequence surrounding start codon of the operon as it shown in Figure 1.13. Then the study proceeds to improve RBS region of the bac operon by adding strong SD sequence with

CRISPR/Cas9 mediated editing system to increase bacilysin production in *B. subtilis* PY79 and its effects on bacilysin titer and mRNA investigated as it shown in Figure 1.14. The results of the study showed that introduction of the strong RBS increased bacilysin biosynthesis 2.87-fold compared to *B. subtilis* PY79 and improved mRNA stability of bac operon, without effecting the growth (Abdulmalek & Yazgan-Karataş, 2022).

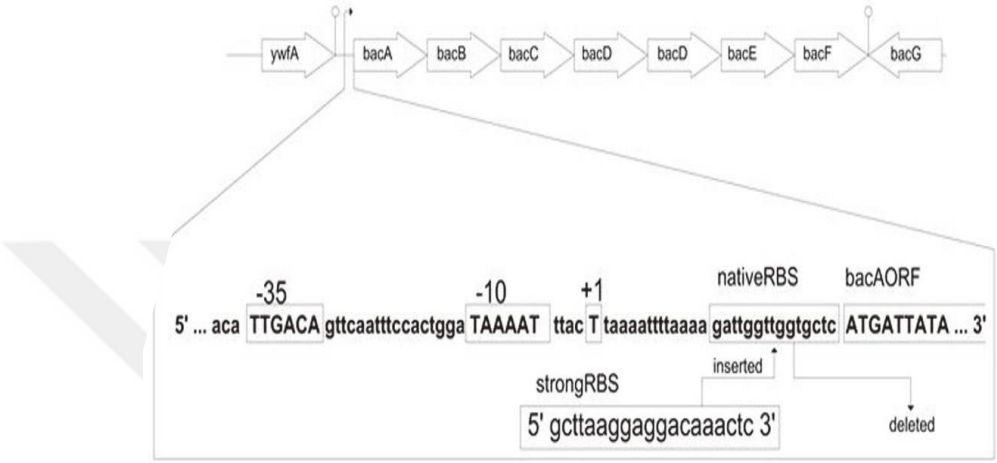

Figure 1.12: Genomic organization of the bacABCDEF operon and the upstream DNA sequences (Adapted from Abdulmalek & Yazgan-Karataş, 2022).

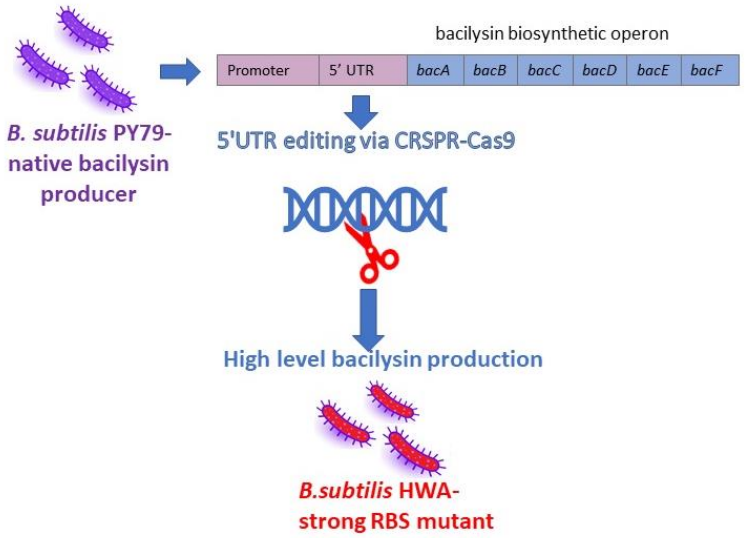

Figure 1.13: Schematic representation of study named "Improvement of Bacilysin Production in Bacillus subtilis by CRISPR/Cas9-Mediated Editing of the 5'-Untranslated Region of the bac Operon" (Abdulmalek & Yazgan-Karataş, 2022).

1.5 Aim of The Present Study

In this project, the aim was to further increase the production of bacilysin in our bacilysin over-producer strain *B. subtilis* HWA. For this purpose, it will be examined how the production level of bacilysin is affected as a result of the elimination of the AbrB and CodY global regulators, which are known to have a suppressive effect on the production of bacilysin.

2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Bacterial strains

B. subtilis strains used in this study and their genotypes were given in Table 2.1.

Table 2.1 : Bacterial strains and their genotypes

Strain	Genotype	Source
***B. subtilis* BAL 373**	*trpC2 pheA1ΔabrB::cat*	A.D. Grossman
***B. subtilis* TMH 307**	*trpC2 unkU::spc ΔcodY*	A.D. Grossman
***B. subtilis* PY79**	Laboratory strain, BSP cured prototrophic derivative of *B. subtilis* 168	P. Youngman
***B. subtilis* HWA**	Bacilysin over-producer strain, derivative of *B. subtilis* PY79	Our laboratory
***B. subtilis* PY79-GT0A**	Δ*abrB::cat* derivative of *B. subtilis* PY79	This study
***B. subtilis* PY79-GT0C**	*unkU::spc* Δ*codY* derivative of *B. subtilis* PY79	This study
***B. subtilis* PY79-GT0AC**	Δ*abrB::cat unkU::spc* Δ*codY* derivative of *B. subtilis* PY79	This study
***B. subtilis* HWA-GTA**	Δ*abrB::cat* derivative of *B. subtilis* HWA	This study
***B. subtilis* HWA-GTC**	*unkU::spc* Δ*codY* derivative of *B. subtilis* HWA	This study
***B. subtilis* HWA-GTAC**	Δ*abrB::cat unkU::spc* Δ*codY* derivative of *B. subtilis* HWA	This study

2.1.2 Culture media

The compositions and preparation of culture media are given in Appendix A.

2.1.3 Buffers and solutions

The compositions and preparation of buffers and solutions are given in Appendix B.

2.1.4 Chemicals and enzymes

The chemicals and enzymes used and their suppliers are given in Appendix C.

2.1.5 Laboratory equipment

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

2.1.6 Maintenance of bacterial strains

Luria-Bertani (LB) liquid medium was used as growing medium for *B. subtilis* strains. For short term storage strains were kept on Luria-Bertani (LB) agar at +4ºC. 15% LB glycerol stock for *B. subtilis* strains were prepared and stored at -80ºC for long term storage.

2.2 Methods

2.2.1 DNA techniques and manipulation

2.2.1.1 Chromosomal DNA isolation

Target strains, inoculated into 2ml of LB broth and incubated overnight at 37°C. Overnight culture centrifuged at 13000 rpm for 5 minutes, supernatant discarded. The pellet resuspended with 567µl of P1 buffer (Appendix B) by pipetting. Then, 10µl of proteinase K (20mg/ml) and 24µl of lysozyme (100mg/ml) added into tube and mixed by vortex. 30µl of 10% SDS added into tubes, then the tubes incubated at 37°C for 1hr. After incubation 100µl of 5M NaCl mixed into tubes by up-down motion until the white precipitation is visible. 80µl of CTAB/NaCl (Appendix B) added into tubes and the tubes incubated at 65°C for 10 minutes. After incubation 820µl of phenol-chloroform-isoamyl alcohol (25:24:1) mixed into tubes by up-down motion, then centrifuged at 13000 rpm for 10 minutes. Upper phase that formed after centrifugation transferred into a fresh tube and 0.7 volume of collected upper phase of isopropanol added into the tube. Tubes centrifuged at 13000 rpm for 15 minutes. Supernatant removed and precipitation washed by addition of 1 ml of 70% ethanol and centrifugation at 13000 rpm for 5 minutes. After centrifugation supernatant discarded and residual ethanol removed by incubating the tubes at 37°C until they are completely dry. Then pellet dissolved in 10µl of EB buffer (Qiagen) by pipetting and incubated at 37°C for 1-2 hrs. Isolated DNA samples stored at 4°C after confirmatory agarose gel electrophoresis.

2.2.1.2 Agarose gel electrophoresis

The DNA samples were visualized by electrophoresis with neutral agarose gel system. 1X TAE buffer (Appendix B) used to prepare the 1% (w/V) agarose gel of the system

and to run gel at 4V/cm. Visualization under UV transillumination achieved with RedSafe™ Nucleic Acid Staining Solution (iNtRON).

2.2.1.3 Polymerase chain reaction (PCR)

PCR reaction was set with Taq DNA Polymerase (Thermo Scientific) using the primers given in Table 2.2. The used PCR mixture and PCR conditions are given in Table 2.3 and 2.4 respectively.

Table 2.2 : Sequences of primers used in PCR.

Primer	Sequence
***abrB* Forward**	5'- ATTCCTATCGAACTGCGT -3'
***abrB* Reverse**	5'- CAATTTACCGCCTGCAAG -3'

Table 2.3: Reaction mixture for confirmation PCR

Reaction Component	Volume (µl)
PCR-Grade Water	37
dNTP	1
10X DreamTaq Buffer	5
$MgCl_2$	2
Template	1
***abrB* Forward Primer**	1
***abrB* Reverse Primer**	1
DreamTaq DNA Polymerase	
Total	50

Table 2.4: Reaction conditions for confirmation PCR

Condition	Temperature (℃)	Duration	Number of Cycles
Initial Denaturation	95	2 min	x1
Denaturation	95	30 sec	x30
Annealing	54	30 sec	
Extension	72	1 min	
Final Extension	72	1 min	x1

2.2.1.4 Preparation of *B. subtilis* competent cells and transformations

To prepare *B. subtilis* competent cells, the protocol described by described by Klein et al. (1992) was used. Host cells were thawed from -80℃ stock and inoculated onto LB plates that contained appropriate selective conditions. Plates incubated overnight at 37℃. Loopful of host cells were inoculated into 3ml of HS media (Appendix A) and incubated overnight at 37℃ on a shaker set to 180 rpm. 500µl of HS overnight culture

inoculated into 20ml of LS media (Appendix A). The culture incubated at 37℃ on a shaker set to 100 rpm until the absorbance at OD_{600} was 0,50 to 0,55. Subsequently 1 ml of LS culture added into Eppendorf tubes that contained 35-45ng/ml chromosomal DNA. The tubes incubated at 37℃ on a shaker set to 200 rpm for 2hrs. After incubation the tubes centrifuged at 5000 rpm for 15 minutes. The supernatant discarded and cells resuspended with 100µl PBS (Appendix A). Resuspended cells spread onto LB plates that contained appropriate selective conditions and incubated overnight at 37℃. Strains with Δ*abrB* were selected with chloramphenicol (5µg/ml), strains with Δ*codY* were selected with spectinomycin (100µg/ml), and strains with both Δ*abrB* and Δ*codY* were selected with both chloramphenicol (5µg/ml) and spectinomycin (100µg/ml).

2.2.2 Screening and selection of mutants

2.2.2.1 Elimination of auxotrophic mutants

To eliminate auxotrophic mutants, transformants colonies were restreaked on SMM plates (Appendix A) and incubated overnight at 37°C. Colonies unable to grown on SMM plates were discarded from further analysis.

2.2.2.2 Screening of bacilysin activity on bioassay plates

The transformant colonies were first screened on bioassay plates containing *Staphylococcus aureus* ATCC 9144 as assay organism (Appendix A). For this, transformants were inoculated onto LB plates with selective antibiotics, as described previously, and incubated overnight at 37°C. Then colonies were transferred onto bioassay plates with toothpicks and incubated overnight at 37°C. *B. subtilis* HWA and PY79 strains were used as control groups. Based on the zone formation, bacilysin producer colonies were selected for further analysis

2.2.2.3 Screening of bacilysin production performance of transformants in PA liquid medium

The selected colonies were inoculated onto LB plates with according selective antibiotics, as described previously, and incubated overnight at 37°C. The colonies were then inoculated into 15 ml of PA medium developed by Perry and Abraham (1979) for bacilysin production (Appendix A) and incubated overnight at 37°C on shaker set to 180 rpm. 500µl of the overnight culture was inoculated into 20 ml of PA

medium and incubated at 37℃ for 16-18hrs on shaker set to 180 rpm. 1 ml of the culture was centrifuged at 5000 rpm for 10 minutes and supernatant collected and stored at -20℃. High throughput screening was also performed by using 96-well plates by downsizing the experiment to 150 µl from 15 ml PA for the first overnight incubation and 200 µl from 20ml PA and for the second overnight incubation. 5 µl of first overnight culture inoculated into 195 µl of PA medium for the second overnight incubation. Incubations were performed at the same conditions as described above.

To perform the paper disc-agar diffusion assay, sterilized 6.0 mm paper discs were first, treated with 20 µl of acetone and left to dry at room temperature until all acetone was evaporated, approximately for 10 minutes and 20 µl of culture supernatant added onto discs and left to dry for 5 minutes. Then the discs were placed onto bioassay plates and incubated overnight at 37℃. Bacilysin activity was estimated as previously described (Özcengiz et al., 1990).

2.2.3 Statistical analysis

Student's T-test was used for statistical analysis, and statistical significance was stated as ns (no significance, $p > 0.05$), *($p < 0.05$), **($p < 0.01$) and ***($p<0.001$).

3. RESULT AND DISCUSSION

3.1 Construction of *abrB, codY* and *abrB-codY* Double Mutants and Their Effects on Bacilysin Production

3.1.1 Chromosomal DNA isolation

Chromosomal DNAs from *abrB* mutant *B. subtilis* BAL 373 (*trpC2 pheA1* Δ*abrB::cat*) and *codY* mutant *B. subtilis* TMH 307 (*trpC2 unkU::spc* Δ*codY*) were isolated successfully and visualized using 1% (w/V) agarose gel as shown in Fig. 3.1. 5µl of isolation product loaded into the agarose gel with 1 µl of 6X loading dye (Thermo Scientific). Electrophoresis was made under 120V for 30 minutes. The amounts of isolated chromosomal DNA from *B. subtilis* BAL 373 and *B. subtilis* TMH 307 were also measured as 355.1 ng/µl and 394,9 ng/µl respectively by using NanoDrop (Thermo Scientific). Since chromosomal DNA is very large it is not unexpected to see smears and the bands at the top of the gel.

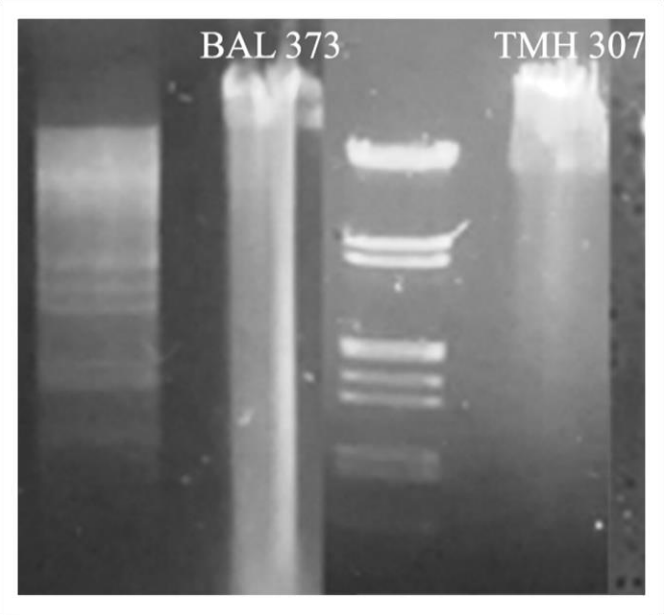

Figure 3.1: Chromosomal DNA isolation from *B. subtilis* BAL 373, *B. subtilis* TMH 307 strains. Marker: Fermentas λ DNA/EcoRI+HindIII Ladder Mix (Thermo Scientific)

3.1.2 Constructions of a*brB, codY* and *abrB-codY* double mutants

To analyze the effect of transition state global regulators AbrB and CodY on bacilysin production, *abrB* and *codY* genes disrupted separately via homologous recombination by transforming the HWA and PY79 competent cells by using the chromosomal DNA from *abrB* mutant *B. subtilis* BAL 373 ((*trpC2 pheA1*Δ*abrB::cat*) and *codY* mutant *B. subtilis* TMH 307 (*trpC2 unkU::spc* Δ*codY*), thereby *abrB* mutant *B. subtilis* PY79-GT0A (Δ*abrB::cat*), *codY* mutant *B. subtilis* PY79-GT0C (*unkU::spc* Δ*codY*), *abrB* mutant *B. subtilis* HWA-GTA (Δ*abrB::cat*)and *codY* mutant *B. subtilis* HWA-GTC) strains were generated. Moreover *abrB-codY* double mutant *B. subtilis* PY79-GT0AC and *B. subtilis* HWA-GTAC strains were also constructed by transforming the PY79-GT0A and HWA-GTA competent cells with the chromosomal DNAs from PY79-GT0C and HWA-GTC, respectively.

Since *abrB* mutant BAL 373 was tryptophan and phenylalanine auxotroph due to *trpC2 and pheA1* mutations and *codY* mutant TMH 307 was also tryptophan auxotroph due to *trpC2* mutation, the respective auxotrophic mutants would also be generated beside to *abrB* and *codY* mutations. Therefore, auxotrophic mutants among resulting transformants were first eliminated and only prototrophic *abrB* and *codY* mutants were analyzed for bacilysin activity. At the end of each transformation process, approximately 250 transformants were restreaked on SMM plates and non-growing transformants on SMM plates were eliminated. Approximately 100 prototrophic transformants growing on SMM plates were selected and stored for the analyses of bacilysin phenotypes (Fig. 3.2).

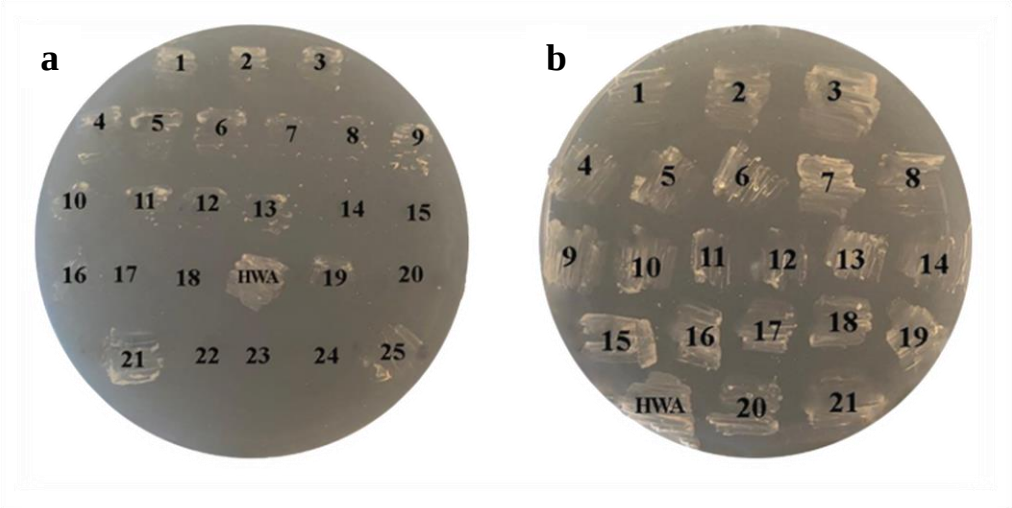

Figure 3.2: Screening of transformants against auxotrophy on SMM plates. Auxotrophic transformants non-growing on SMM plates were eliminated and only prototrophic transformants growing on SMM plates were selected and stored for bacilysin phenotypes analyses.

3.1.3 Bacilysin activity screening on bioassay plates

Approximately 100 transformants were screened rapidly by inoculating onto bioassay plates and incubated overnight at 37°C to exclude any spontaneous mutants that lost their ability to produce bacilysin from further analysis. The bacilysin production can be observed on the bioassay plates containing *S. aureus* ATCC 9144 as assay organism. Since growth of *S. aureus* inhibited by bacilysin, clear zones are formed around the colonies on the bioassay plates. As it shown in Figure 3.3 and 3.4, most of the transformants exhibited higher bacilysin activity compared to parental strains, a few transformants completely or partially lost their ability to produce bacilysin, which were excluded from this study. Results showed that *B. subtilis* HWA and its mutant strains formed bigger zones compared to *B. subtilis* PY79 and its mutant strains, which were expected results since HWA strain is bacilysin over-producer strain descended from PY79.

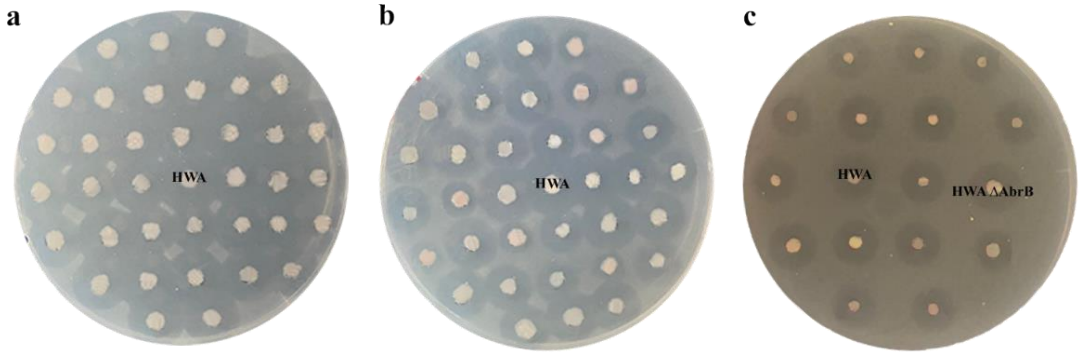

Figure 3.3: Screening of transformants on bioassay plates. (a) *abrB* mutants *B. subtilis* HWA transformants, (b) *codY* mutants *B. subtilis* HWA transformants, (c) *abrB-codY* double mutants *B. subtilis* HWA transformants HWA-GTAC (c) mutants were grown on bioassay plates. Clear zone formed around colonies indicates bacilysin activity. HWA strain was used as positive control.

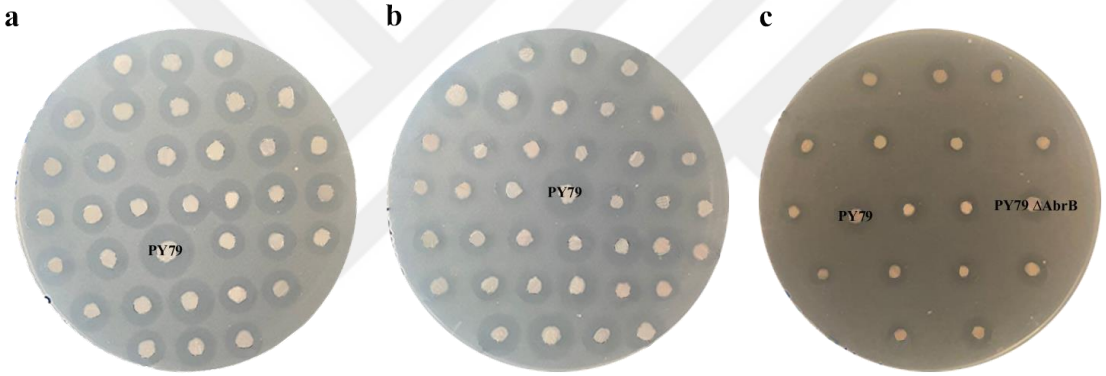

Figure 3.4: Screening of transformants on bioassay plates. (a) *abrB* mutants *B. subtilis* PY79 transformants, (b) *codY* mutants *B. subtilis* PY79 transformants, (c) *abrB-codY* double mutants *B. subtilis* PY79 transformants were grown on bioassay plates. Clear zone formed around colonies indicates bacilysin activity. PY79 strain was used as positive control.

While this method of testing is not reliable to measure or compare exact levels of bacilysin production, it is efficient enough to eliminate undesirable colonies and select promising bacilysin producer candidates for further analysis. Unreliability of this method roots from the non-standardized inoculated cell amount. Inoculation size vary among colonies thus affects the resulted zone formation. However, this technique provides fast screening of high number of colonies.

3.1.4 Bacilysin activity screening in PA liquid medium

Initial screening was performed in a 96-well-plate to enhance as much as possible transformants that could be screened whether the mutations caused a significant difference in the expression of bacilysin compared to parental strains as well as a significant number of cells were affected by each mutation. Approximately 50 transformants selected on bioassay plates for each individual mutation, that were further analyzed for bacilysin production by cultivating in PA medium via paper disc diffusion assay. Results of this initial screening (Fig. 3.5 and 3.6.) revealed that the bacilysin production levels were substantially impacted across nearly all transformants screened for each mutation, thereby validating that the observed alterations in bacilysin activity were driven by the induced mutations

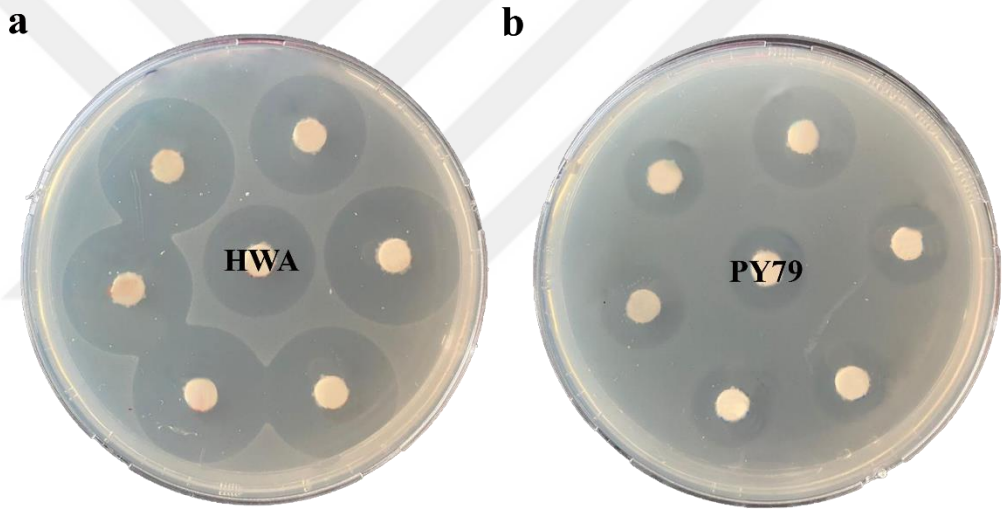

Figure 3.5: Paper disc diffusion assay results of initial screening of *abrB* mutants of (a) HWA transformants and (b) PY79 transformants. HWA and PY79 strain used as control.

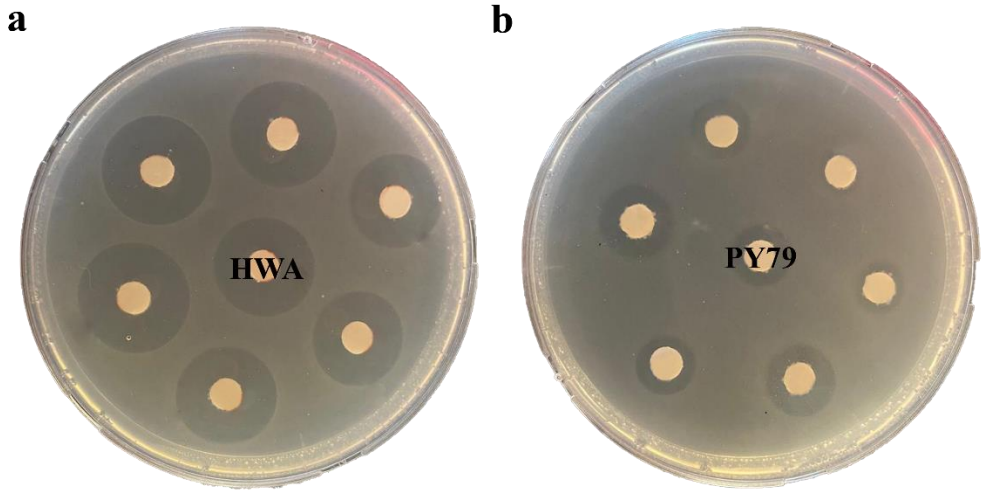

Figure 3.6: Paper disc diffusion assay results of initial screening of *codY* mutants of (a) HWA transformants and (b) PY79 transformants. HWA and PY79 strain used as control.

For final screening step, three candidate mutants for each individual mutation were selected and named as: HWA-GTA1, HWA-GTA2 HWA-GTA3 for the *abrB* mutants *B. subtilis* HWA strains; HWA-GTC1, HWA-GTC2, HWA-GTC3for the *codY* mutants *B. subtilis* HWA strains; HWA-GTAC1, HWA-GTAC2, HWA-GTAC3 for the *abrB-codY* double mutants *B. subtilis* HWA strains; PY79-GT0A1, PY79-GT0A2 PY79-GT0A3 for the *abrB* mutants *B. subtilis* PY79 strains; PY79-GT0C1, PY79-GT0C2, PY79-GT0C3 for the *codY* mutants *B. subtilis* PY79 strains; PY79-GT0AC1, PY79-GT0AC2, PY79-GT0AC3 for the *abrB-codY* double mutants *B. subtilis* PY79 strains. Before going to final screening step explained below, the generated mutation was confirmed via PCR analysis.

3.1.5 Confirmation of the mutations in the generated strains via PCR

To confirm selected strains had the introduced mutations, PCR analysis was performed with the forward and reverse primers specific to the *abrB* gene and the chromosomal DNA from HWA, HWA-GTA, HWA-GTAC, PY79, PY79-GT0A and PY79-GT0AC as templates (Fig. 3.7). The PCR products were visualized using 1% (w/V) agarose gel. 2.5 µl of PCR product loaded into the agarose gel with 0.5 µl of 6X loading dye (Thermo Scientific). Electrophoresis was made under 120V for 30 minutes. Results of the agarose gel electrophoresis are given in Figure 3.8. PCR fragment size was expected to be 166 bp, indicating the presence of *abrB.* This band was successfully amplified only from in HWA and PY79 genomes, no any PCR products was resulted

from not from the genomes of the HWA-GTA, HWA-GTAC, PY79-GT0A and PY79-GT0AC strains. These results confirmed the mutations in the generated strains in this study.

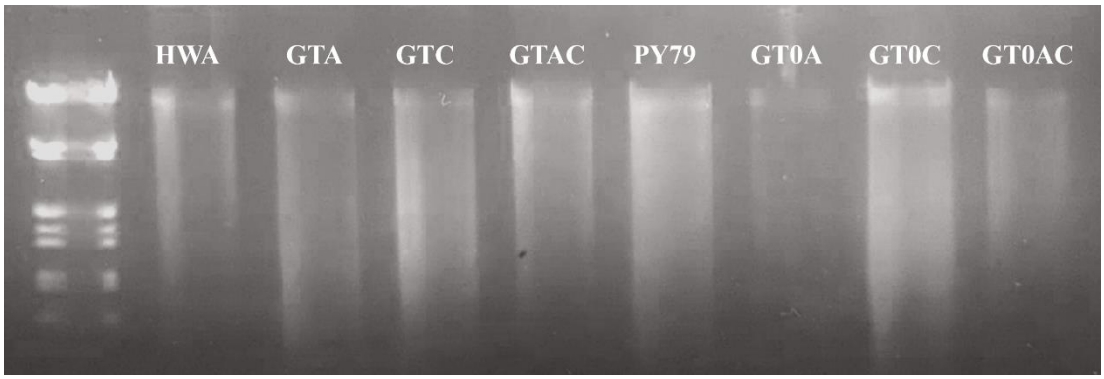

Figure 3.7: Chromosomal DNA isolation from HWA, HWA-GTA, HWA-GTAC, HWA-GTC, PY79, PY79-GT0A, PY79-GT0AC, PY79-GT0C strains. Marker: Fermentas λ DNA/EcoRI+HindIII Ladder Mix (Thermo Scientific)

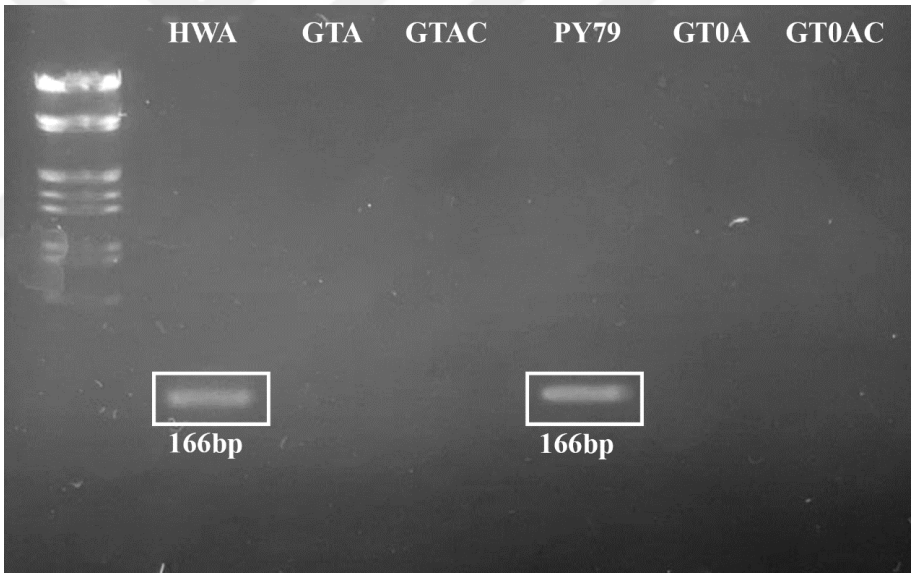

Figure 3.8: PCR analysis performed with the forward and reverse primers specific to the *abrB* gene and the chromosomal DNA from HWA, HWA-GTA, HWA-GTAC, PY79, PY79-GT0A strains Marker: Fermentas λ DNA/EcoRI+HindIII Ladder Mix (Thermo Scientific).

3.1.6 Final screening step performed in 15 ml PA medium

The mutants selected from the initial screening step, HWA-GTA1, HWA-GTA2, HWA-GTA3, HWA-GTC1, HWA-GTC2, HWA-GTC3,HWA-GTAC1, HWA-GTAC2, HWA-GTAC3, PY79-GT0A1, PY79-GT0A2 PY79-GT0A3, PY79-GT0C1, PY79-GT0C2, PY79-GT0C3, PY79-GT0AC1, PY79-GT0AC2, PY79-GT0AC3, were grown in 15 mL PA medium in duplicate manner and the bacilysin level in their

culture fluids were determined via paper-disc bioassay. Zone of diameter around the disc were measured and bacilysin level were estimated as unit/mL as described before (Özcengiz et al., 1990) . The final screening step was repeated at least three times for each mutant and Student's t-tests were performed to analyze whether the mutations caused a significant difference in the expression of bacilysin compared to parental strains. One of the paper disc diffusion assay results belonging HWA-GTA, PY79-GT0A, HWA-GTAC, and PY79-GT0AC candidates were given in Fig. 3.9, Fig. 3.10, Fig. 3.11, and Fig. 3.12, respectively, as an example.

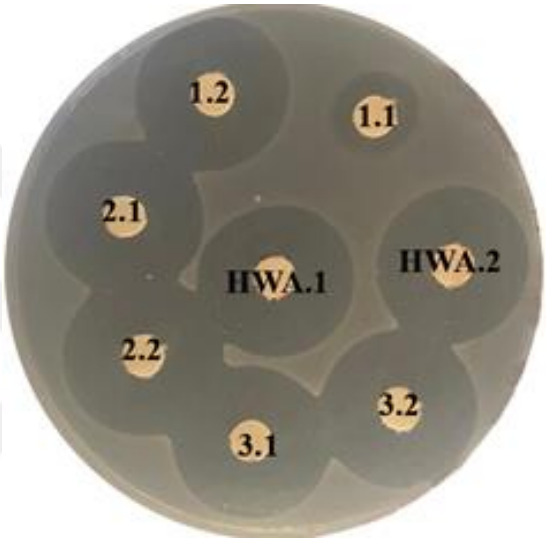

Figure 3.9: Paper disc diffusion assay result of HWA-GTA1 (1.1 and 1.2) , HWA-GTA2 (2.1 and 2.2) . HWA-GTA3 (3.1 and 3.2). HWA strain used as control group.

Figure 3.10: Paper disc diffusion assay results of PY79-GT0A1 (1.1 and 1.2) , PY79-GT0A2 (2.1 and 2.2) . PY79-GT0A3 (3.1 and 3.2). PY79 strain used as control group.

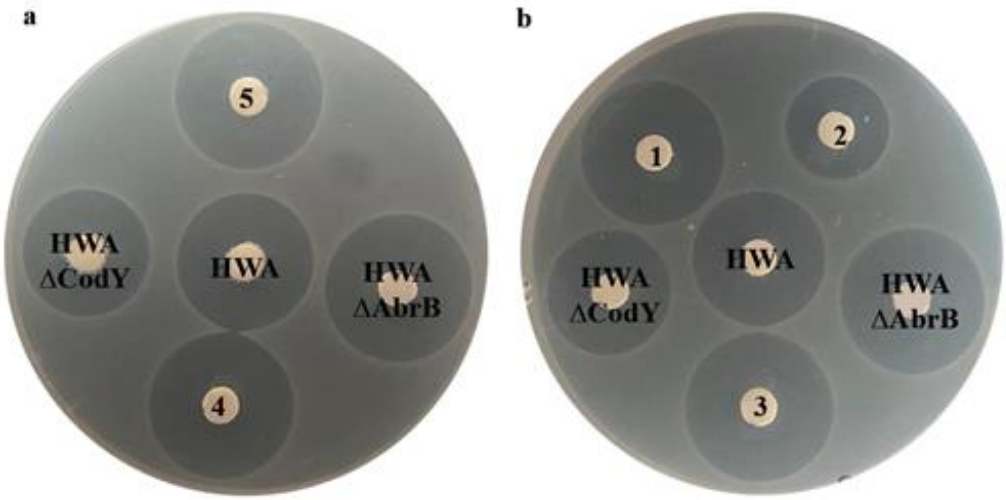

Figure 3.11: Paper disc diffusion assay results of *codY-abrB* double mutants HWA-GTAC strains HWA, HWA-GTA and HWA-GTC strains used as control groups.

Figure 3.12: Paper disc diffusion assay results of PY79-GT0AC candidates PY79, PY79-GT0A and PY79-GT0C strains used as control groups.

As shown in Figure 3.13, the results of the final screening steps revealed that *abrB* mutants of both HWA and PY79 had significantly higher bacilysin activity compare to their parental strain ($p<0.01$ for HWA and $p< 0.05$ for PY79). Bacilysin activity of HWA-GTA was 95.0 ± 7.9 unit/ml , which is 7.7% higher than the bacilysin activity of HWA, which was 88.2 ± 7.5 unit/ml on average. Bacilysin activity of PY79-GT0A was 63.7 ± 13.4 unit/ml on average, which is 21.8% higher than the bacilysin activity

of PY79, which was 52.3 ± 12.6 unit/ml on average. Deletion of *abrB* seems to be more effective in PY79 in order to increase bacilysin activity, as increase in HWA was 7.7% while it was 21.8% in PY79. As seen, from *abrB* mutation, each strain was affected differently based on their production level since the bacilysin production level in HWA was increased at 2.87-fold relative to PY79 and there might be a limitation to increase of bacilysin activity above a certain level. This limitation, most probably, arises from the feedback inhibition exerted by bacilysin itself. Since the study conducted by Özcengiz and Alaeddinoğlu revealed that bacilysin addition to culture represses the bacilysin biosynthesis with greater feedback effect the earlier it was added (Özcengiz & Alaeddinoğlu, 1991b).

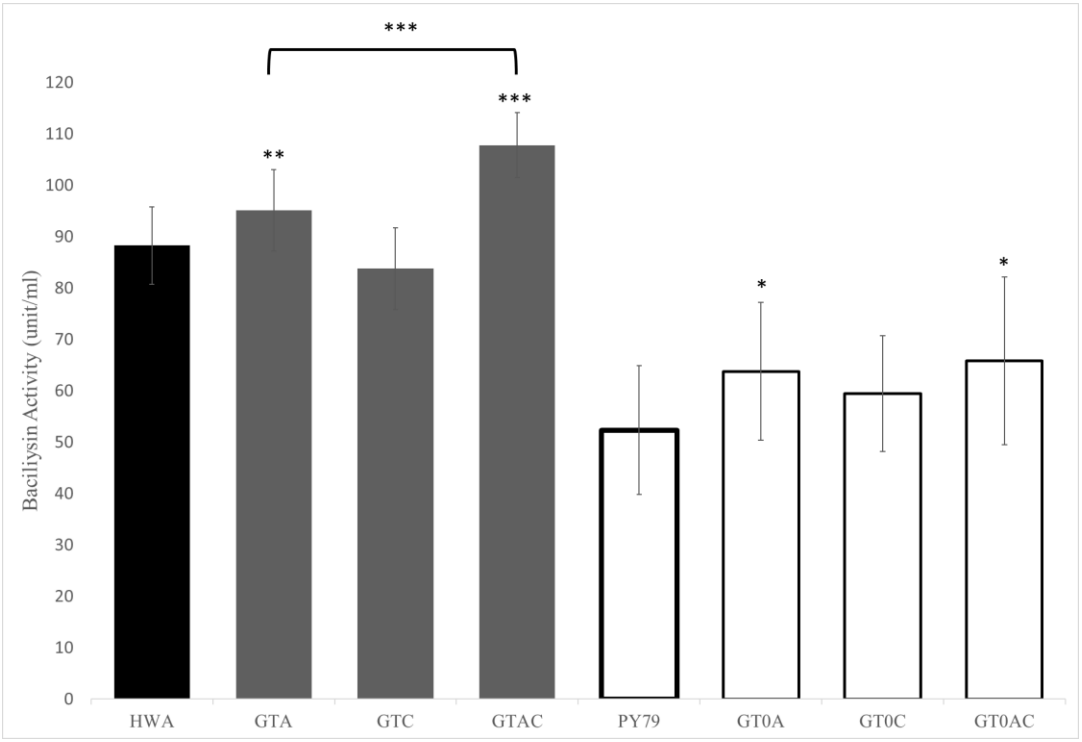

Figure 3.13: Average bacilysin activity of HWA, HWA-GTA, HWA-GTC, HWA-GTAC, PY79, PY79-GT0A, PY79-GT0C and PY79-GT0AC strains detected in their cell free culture via paper-disc diffusion assays. According to paired Student's t-test, asterisks (*), (**),and (***) indicate statistically significant differences from the parental strains with $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively and ns indicate statistically no significance ($p > 0.05$).

As seen in Figure 3.13, for *codY* mutation alone, the change in bacilysin level compared to parental strain PY79 or HWA was statistically insignificant ($p > 0.05$). On the other hand, interestingly, as in the case of *abrB* mutation, *codY* mutation leaded to different outcomes in HWA and PY79 strain. c*odY* mutants of HWA had lower

bacilysin activity than HWA. Bacilysin activity of HWA-GTC was 83.7 ± 7.9 unit/ml, which is 5.1% lower than the bacilysin activity of HWA, which was 88.2 ± 7.5 unit/ml. Whereas, in PY79, the deletion of *codY* seemed to be more effective in order to increase bacilysin activity compare to HWA bacilysin activity of PY79-GT0C was 59.4 ± 11.3 unit/ml on average, which is 13.6% higher than the bacilysin activity of PY79, which was 52.3 ± 12.6 unit/ml on average. The possible reason for the different outcome arises from *codY* mutations in HWA and PY79 strains might be related to the editing of the 5' untranslated region (5'UTR) of the *bac* operon in HWA strain. AbrB and CodY regulate the expression of the *bac* operon via direct binding (Köroğlu, 2013). Studies showed that CodY binding motifs found close to upstream of −35 promoter region or downstream of the transcription start point or overlap the transcription start point (Belitsky & Sonenshein, 2008). Due to lack of its consensus sequence AbrB hypothesized to be recognizing the three-dimensional structures while binding DNA (Chumsakul et al., 2010). In silico analysis done by our group revealed the putative binding sequences of AbrB and CodY as 5' CGCATATAAAAA 3' and 5' AAAAAACATTGACA 3 were detected upstream of bac operon, respectively (Köroğlu et al., 2013, 2013). As seen in Figure 3.12, CodY binding site to the *bac* operon is right on -10 position which is very close to the editing region of the *bac* operon in HWA strain. Therefore, the binding affinity of the CodY on the bacilysin operon in HWA most likely is affected and leads to different outcomes in HWA and PY79.

In this study, the most significant results were obtained from the *codY* and *abrB* double mutations in HWA and PY79 strains. As seen in Fig. 3.13, *codY-abrB* double mutants HWA strains (HWA-GTAC) had significantly higher bacilysin activity than the HWA strain ($p < 0.001$). Bacilysin activity of HWA-GTAC, which was 107,8 ± 6.3 unit/ml, observed to be significantly higher than HWA strain by 22.1%. Results showed that HWA-GTAC had significantly higher bacilysin activity than the HWA-GTA, and HWA-GTC strains by 13.4% ($p < 0.001$) and 28.7% ($p < 0.001$) respectively. While *codY-abrB* double mutant PY79 strains (PY79-GT0AC) had significantly higher bacilysin activity than the PY79 strain ($p<0.05$). PY79-GT0AC showed 65.8 ± 16.3 unit/ml bacilysin activity. Bacilysin activity of PY79-GT0AC observed to be significantly higher than PY79 strain by 25.7%. Bacilysin activity of PY79-GT0AC

was higher than the activity of PY79-GT0A by 3.2% and PY79-GT0C by 10.7% but the increases were not significant.

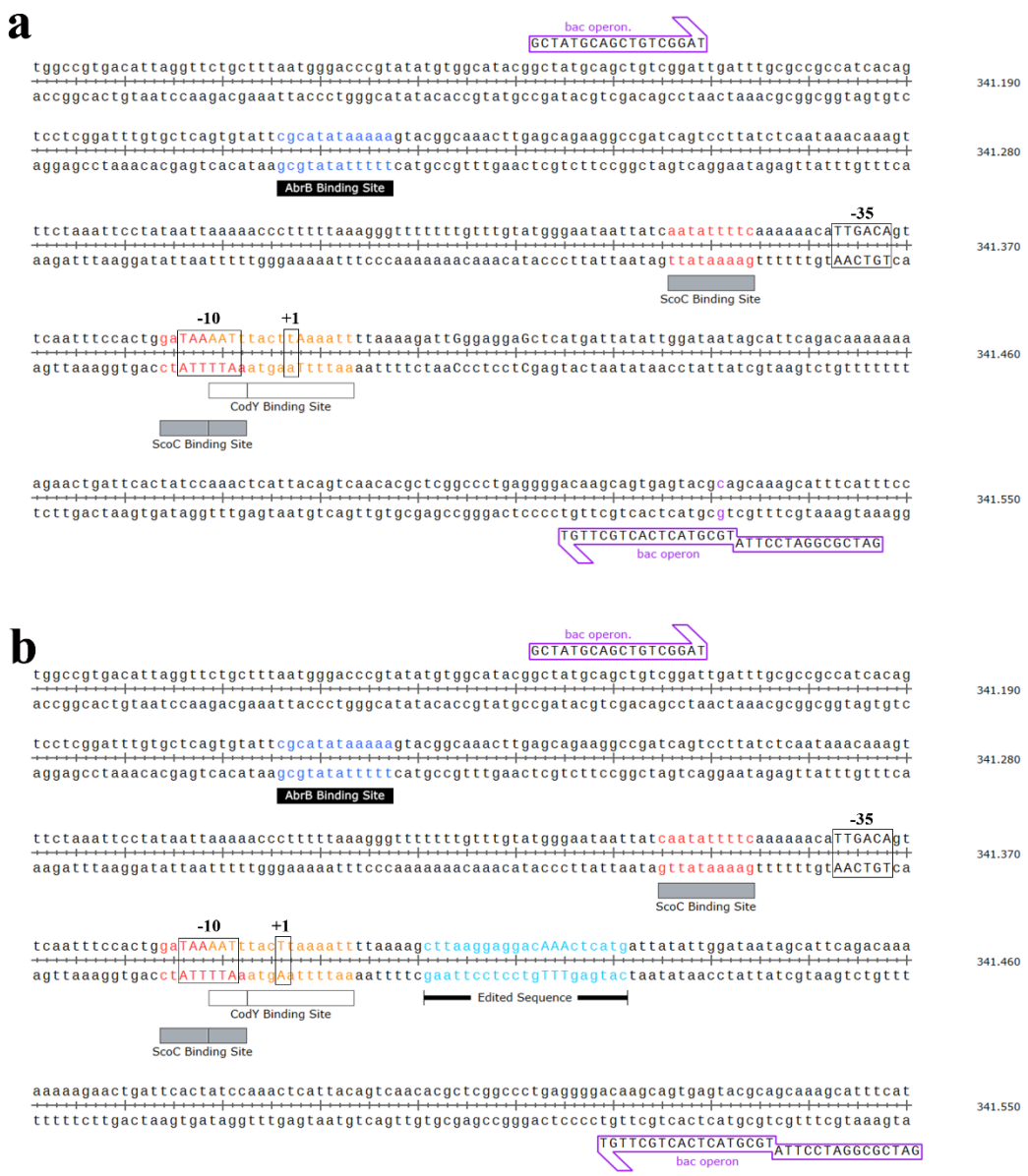

Figure 3.14: Mapping of *bacA* promoter in PY79 (a) and HWA (b) strains. AbrB binding site indicated with blue, CodY binding site indicated with orange, ScoC binding site indicated with red. CRISPR/Cas9 mediated edited parts indicated with cyan.

3.1.7 *abrB-codY-scoC* triple mutations

ScoC is another global regulator of *B. subtilis*, which represses bac operon. As previously shown that absence of one negative regulator might increase another negative regulator effect and elimination of CodY binding leads *codY* mutants

appeared with decreased bacilysin activity, while the opposite is expected when the simple logic followed (Köroğlu et al., 2013, Islam et al., 2022; Tolibia et al., 2022). To investigate if deletion of another inhibitor would increase bacilysin activity by removing another repressor of the *bac* operon, construction of triple mutant strains with the addition of *scoC* deletion to *abrB-codY* double mutants HWA-GTAC and PY79-GT0AC strains were planned and carried out by using the *scoC* disruption plasmid constructed in our group previously (Gün, 2011). Since there was no growth on the plates after overnight incubation, plates incubated for 48 hrs at 37°C. In total three colonies from PY79-GT0AC and two colonies from HWA-GTAC strains were observed on the selective LB plate with kanamycin (50µg/ml), which given in Fig. 3.15.

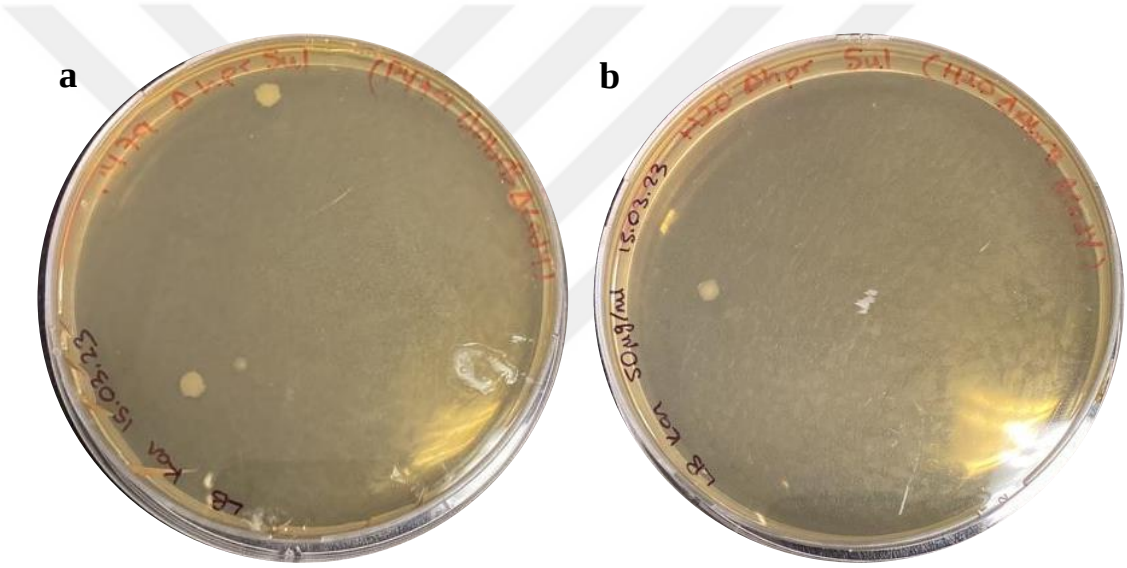

Figure 3.15: Triple mutant strains constructed by ScoC deletion from PY79-GT0AC (a) and HWA-GTAC (b).

Observed colonies appeared wider than the usual *B. subtilis* colonies. To confirm that these colonies belong to *B. subtilis* cells they were observed under the microscope. As seen in Fig. 3.16, only typical rod-shape *B. subtilis* cells were observed, indicating that they were not contaminants. However, when the colonies were inoculated into LB broth for further studies, there was no growth observed. Transformation process to obtain the triple mutants were repeated several times, even colonies appeared, they could not be cultivated for further analysis. This finding points out that simultaneous disruption of these three global regulators severely compromised the growth of *B. subtilis* cells.

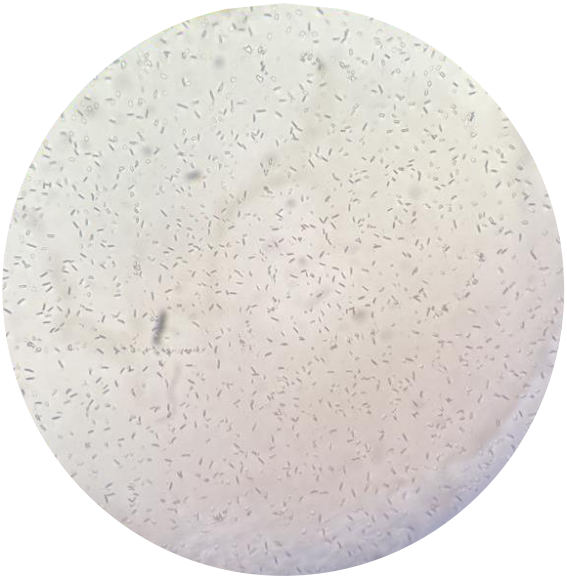

Figure 3.16: Microscopic image of *abrB-codY-scoC* triple mutant cells generated from HWA-GTAC strain under the total magnification of 400X+=.

4. CONCLUSION

In this study, the aim was to further increase the production level of bacilysin in our bacilysin over-producer strain *B. subtilis* HWA, and examining how the production level of bacilysin is affected as a result of the elimination of the AbrB and CodY global regulators. To achieve this purpose the *abrB* mutant HWA-GTA, *the codY* mutant HWA-GTC, the *abrB-codY* double mutant HWA-GTAC, the *abrB* mutant PY79-GT0A, *the codY* mutant PY79-GT0C, and the *abrB-codY* double mutant PY79-GT0AC strains were constructed and their bacilysin activities were analyzed via paper disc diffusion assays.

abrB mutants of both HWA and PY79 exhibited significantly higher bacilysin activity compare to their parental strain. Bacilysin level of HWA-GTA was 7.7% higher than the bacilysin level of HWA. Bacilysin level of PY79-GT0A was 21.8% higher than the bacilysin level of PY79. Deletion of *abrB* seems to be more effective in PY79 in order to increase bacilysin activity, revealing that from *abrB* mutation, each strain was affected differently based on their production level and there might be a limitation to increase of bacilysin activity above a certain level. This limitation likely arises from the feedback inhibition exerted by bacilysin itself.

The effect of *codY* mutation on bacilysin production in both HWA and PY79 was found as statistically insignificant and resulting in different outcomes for each strain. It caused a 5.1% decreased in the bacilysin activity in HWA while leading to an 13.6% increase in PY79. This difference likely arises from the editing 5'UTR region of the *bac* operon in HWA strain since that region is very close to the putative binding sites of CodY detected on -10 position of the *bac* operon, thereby likely changing the binding affinity of CodY on the *bac* operon in HWA.

In this study, the most significant results were obtained from the *codY- abrB* double mutations in HWA and PY79 strains. Bacilysin activity of *codY-abrB* double mutant HWA-GTAC was significantly higher than HWA and HWA-GTA by 22.1% and 13.4%, respectively. Similarly, bacilysin level of *codY-abrB* double mutant PY79-GT0AC was significantly higher than PY79 by 25.7%. Briefly, the concurrent

inactivation of AbrB and CodY, two key negative regulators of bacilysin biosynthesis, demonstrates the potential for further improving bacilysin production levels, even in the high-producing strain HWA. Finally, it was planned to investigate the effects of *abrB-codY-scoC* triple mutation on the bacilysin activity by introducing ScoC deletion mutation to the *abrB-codY* double mutant strains. However, the resulting colonies were unable to cultivated for further analysis. These results point out that simultaneous disruption of these three global regulators together severely compromised the growth of *B. subtilis* cells. As a further study, the effect of only scoC mutation, *scoC-abrB* double mutation, and *scoC-codY* double mutation on the bacilysin activity should be investigated to get deep inside the complex regulation exerted by these three global regulators.

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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

Bioassay Medium (1000 ml)

1	Glucose	10g
2	$MgSO_4.7H_2O$	0.7g
3	KH_2PO_4	1g
4	NaCl	1g
5	$Na_2HPO_4.2H_2O$	3.3g
6	Na_3Citrate. $2H_2O$	0.5g
7	Agar	20g
8	$FeSO_4.7H_2O$ (0,5%(w/v))	2ml
9	Glutamate NaH_2O (33,3%(w/v))	7.2ml
10	Yeast Extract (2,5%(w/v))	2ml
11	Arginine (0,625%(w/v))	2ml
12	Cysteine (0,625%(w/v))	2ml
13	Glycine (0,625%(w/v))	2ml
14	Leucine (0,625%(w/v))	2ml
15	Methionine (0,625%(w/v))	2ml
16	Phenylalanine (0,625%(w/v))	2ml
17	Proline (0,625%(w/v))	2ml
18	Threonine (0,625%(w/v))	2ml
19	Tryptophan (0,625%(w/v))	2ml
20	Tyrosine (0,625%(w/v))	2ml
21	Valine (0,625%(w/v))	2ml
22	Alanine (0,625%(w/v))	2ml
23	Histidine (0,625%(w/v))	2ml

Listed ingredients 1 and 2 dissolved in 40ml distilled water; 3, 4, and 5 dissolved in 720ml distilled water at pH 7.1; 6 dissolved in 46ml distilled water; 7 dissolved in 160ml distilled water then autoclaved at 121℃ for 15 minutes separately. The rest of the listed ingredients filter sterilized. Ingredients mixed together after agar cooled down to 50-60℃. *Staphylococcus aureus* colony inoculated into 5 ml of LB and incubated at 37℃ overnight at 180rpm. Then upscaled by adding 200µl of culture into 9.8ml of LB broth and incubated at 37℃ for 1.5hrs at 200rpm. *S. aureus* culture mixed with bioassay medium by 1%(v/v). The medium then poured into plates and stored at 4℃.

Luria Bertani (LB) Medium (1000 ml)

Tryptone	10g
Yeast Extract	5g
NaCl	5g

Listed ingredients dissolved in distilled water, then volume adjusted to 1000ml with distilled water. The mixture autoclaved at 121℃ for 15 minutes.

Luria Bertani (LB) Agar Medium (1000 ml)

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

Listed ingredients dissolved in distilled water, then volume adjusted to 1000ml with distilled water. The mixture autoclaved at 121℃ for 15 minutes.

LS Medium (30ml)

dH_2O	25.8ml
10X Base	3ml
Glucose (50%(w/v))	300µl
Yeast Extract (10%(w/v))	300µl
Casein Hydrolysate (2%(w/v))	150µl
50mM spermidine	300µl
Tryptophan (0.5%(w/v))	30µl
Phenylalanine (0.3%(w/v))	45µl
1M $MgCl_2$	75µl

Listed solutions autoclaved at 121℃ for 15 minutes separately. Spermidine filter sterilized.

HS Medium (20ml)

dH_2O	15.17ml
10X Base	2ml
Glucose (50%(w/v))	200µl
Yeast Extract (10%(w/v))	200µl
Casein Hydrolysate (2%(w/v))	200µl
Arginine (8%(w/v)) and Histidine (0.4%(w/v)) Solution	2ml
Tryptophan (0.5%(w/v))	200µl
Phenylalanine (0.3%(w/v))	30µl

Listed solutions autoclaved at 121℃ for 15 minutes separately.

Spizizen's Minimal Medium (SMM) (1000ml)

$(NH_4)_2SO_4$	2g
KH_2HPO_4	6g
K_2HPO_4	14g
$Na_3Citrate.2H_2O$	1g
MgSO4.7H2O	0.2g
Glucose (50%(w/v))	40ml
Tryptophan (5mg/ml)	40ml
Agar	15g

Listed ingredients autoclaved at 121℃ for 15 minutes separately.

PA Medium (50ml)

1	Sucrose	5g
2	$MgSO_4.7H_2O$	0.25g
3	KH_2PO_4	0.5g
4	KCl	0.25g
5	Glutamate NaH_2O (33,3%(w/v))	6ml
6	Ferric Citrate (1.5%(w/v))	5ml
7	Trace Element Solution	0.5ml

Listed ingredients 1 and 2 dissolved in 38.5ml distilled water; 3 and 4 dissolved in 445ml distilled water at pH 7.5 then completed to 450ml then autoclaved at 121℃ for 15 minutes separately. The rest of the listed ingredients filter sterilized. Ingredients mixed together after agar cooled down to 50-60℃.

APPENDIX B

TAE Buffer (50X): 242 g Tris Base, 57.1 ml Glacial acetic acid, and 100 ml EDTA (0.5M, pH 8.0) dissolved in distilled water. Mixture volume adjusted to 1L with distilled water and mixture pH adjusted to 8.0 by adding HCl.

Agarose Gel (1%): 0.5 g Agarose was melted in 50 ml TAE Buffer (1X), following to cooling down the gel, 2.5 µl of 20000X RedSafe™ was added. The gel was transferred into the tray.

P1 Buffer: 6.06 g Tris-base and 3.72 g EDTA.$2H_2O$ dissolved in distilled water. Mixture volume adjusted to 1L with distilled water and mixture pH adjusted to 8.0 by adding HCl. 100 mg RNase A was added for 1 L buffer.

10X Base (50ml)

$(NH_4)_2SO_4$	1g
KH_2HPO_4	3g
K_2HPO_4	5.35g
$Na_3Citrate.2H_2O$	0.5g

Ingredients mixed and autoclaved at 121℃ for 15 minutes. 1ml of filter sterilized 1M $MgSO_4$ added into solution after it cooled down to 50℃.

CTAB/NaCl: 4.1g NaCl dissolved in 80ml dH2O then 10g CTAB added into slowly while heating and stirring. At the 65℃ the ingredients completely dissolved. Volume adjusted to 100ml by adding dH_2O. Stored at room temperature.

Trace Element Solution (100ml)

$ZnSO_4.7H_2O$	0.01g
$CoCl_2.6H_2O$	0.01g
Ammonium molybdate	0.01g
$MnCl_2.4H_2O$	0.1g
$CuSO_4.5H_2O$	0.001g

Ingredients mixed and filter sterilized.

APPENDIX C

Chemicals	Supplier
Acetic acid	Merck (Germany)
Agar	Biolife (Italy)
Agarose	VWR (Austria)
Ammonium peroxosulphate	Merck
Ammonium sulfate	Riedel-de Haën (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 Haën
D-glucose anhydrous	Merck
Ethylene Diamine Tetraacetic Acid (EDTA)	MP
Glycerol	Carlo-Erba (France)
Hydrochloric acid	Merck
Iron(III) chloride - 6 hydrate	Riedel-de Haën
L- Aminoacids	Merck
LB Broth	Sigma
Lysozyme	Merck
Magnesium chloride 6-hydrate	Applichem
Magnesium sulfate heptahydrate	Merck
Manganese(II) chloride dihydrate	Merck
Natruim hydroxide	Merck
2-Nitrophyl _-D-galactopyranoside (ONPG)	Sigma
Potassium di-hydrogen phosphate	Riedel-de Haën
Proteinase K	Applichem
Spermine	Fluka
Sodium acetate	J.T. Baker (USA).
Sodium azide	Merck
Sodium chloride (NaCl)	Riedel-de Haën
Sodium dihydrogen phosphate	Riedel-de Haën
Sodium dodecyl sulfate	Sigma-Aldrich
Sodium hydrogen phosphate(Na2HPO4.7H2O)	Merck
Sodium hydroxide	Riedel-de Haën
Sodium L-glutamate monohydrate	Merck
Starch	Merck
Thiamine Hydrochloride	Sigma

Tris (hydroxymethyl) aminomethane	Merck
Triton-X100	Sigma
Tryptone	Sigma
Yeast Extract	Sigma
Zinc Chloride	BDH

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

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

Microplate reader: (CLARIOstar-430-0667)

NanoDrop: (Thermo Scientific)

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).

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

CURRICULUM VITAE

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• B.Sc. **:** 2021, Istanbul Technical University, Faculty of Science and Letters, Department of Molecular Biology and Genetics

PUBLICATIONS, PRESENTATIONS AND PATENTS ON THE THESIS:

• **Tartar G.,** Yazgan-Karataş A. 2023: Investigation of the Effects of AbrB and CodY Deletions in Bacilysin Ove-Producer *B. subtilis* HWA Strain, International Graduate Research Symposium – IGRS`23, May 2-5, 2023, İstanbul, Turkey.