ISTANBUL TECHNICAL UNIVERSITY ★ GRADUATE SCHOOL

IMPROVEMENT OF BACILYSIN BIOSYNTHESIS IN *BACILLUS SUBTILIS* PY79 VIA CRISPR/CAS9 TECHNOLOGY

Ph.D. THESIS

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ISTANBUL TEKNİK ÜNİVERSİTESİ ★ LİSANSÜSTÜ EĞİTİM ENSTİTÜSÜ

BACİLLUS SUBTİLİS PY79 SUŞUNDA BASİLİSİN BİYOSENTEZİNİN CRISPR/CAS9 TEKNOLOJİSİ İLE İYİLEŞTİRİLMESİ

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EYLÜL 2023

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To my mother, Doç. Dr. Sawsan A. Zaidan,

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Hadeel WALEED ABDULMALEK
(Genetic Engineering and Biotechnology M.Sc.)

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ABBREVIATIONS

A : Adenine, a purine nucleobase.
AHL : Acyl-Homoserine Lactones.
AI : Autoinducer.
AIP : Autoinducer Peptide.
Amp (Amp^R) : Ampicillin (Ampicillin resistant).
bp : Base pair
C : Cytosine, a pyrimidine nucleobase.
Cas : CRISPR association.
CIAP : Calf intestinal Alkaline Phosphate.
CRISPR : Clustered regularly Interspaced Short Palindromic repeats.
crRNA : CRISPR ribonucleic acid.
CSF : Competence and sporulation factor.
CSM : Counter-selectable marker.
Cq : Quantitative cycle.
Cpf1 : CRISPR protein from *Francisella novicida*.
Csp : Cold shock protein.
dH$_2$O : Distilled Water.
DNA : Deoxyribonucleic Acid.
DSB : Double-strand breaks.
DSM : Difco sporulation medium.
G : Guanine, a purine nucleobase.
GFP : Green Fluorescent protein.
GlcN6P : Glucosamine-6-phosphate synthase.
GRAS : Generally Regarded as Safe.
HA : Hyaluronic acid.
HDR : Homology-directed repair.
HPLC : High-pressure liquid chromatography.
hr. : An Hour.
IPTG : Isopropyl-beta-D-thioglactoside.
Km (Km^R) : Kanamycin (Kanamycin Resistance).

LB : Loria broth media.

LPS : Lipopolysaccharide.

MBF : Modified basalt fiber.

M-CGH : Microarray-based comparative genomic hybridization.

min : Minute.

mRNA : Messenger Ribonucleic acid.

nt : Nucleotide.

Op or Oop : Oligopeptide permease.

ORF : Open reading frame.

PAM : Protospacer Adjacent Motif.

PA- medium : Perry and Abraham Medium.

PGPR : Plant growth-promoting rhizobacterium

PI : Interacting part.

QS : Quorum Sensing.

RBS : Ribosomal Binding Site.

RNA : Ribonucleic Acid.

RT-PCR : Reverse transcription polymerase chain reaction.

Sdp : Sporulation delaying protein.

SD-seq : Shine Dalgarno Sequence.

SKF : Sporulation killing factor.

sgRNA : Singl guide ribonucleic acid.

TLC : Thin layer chromatography.

T4PNK : T4 polynucleotide kinase.

UPLC-MS : Ultra-performance liquid chromatography.

**5`UTR** : 5` Untranslated region.

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IMPROVEMENT OF BACILYCIN BIOSYNTHESIS IN *BACILLUS SUBTILIS* PY79 VIA CRISPR/CAS9 TECHNOLOGY

SUMMARY

Bacillus subtilis, a common Gram-Positive soil bacterium, has received significant attention from scientists and researchers in various fields such as agriculture, industry, medicine, and pharmacology. It is widely regarded as an excellent host model due to its highly adaptable metabolic system, which allows it to withstand nutrient scarcity by forming highly resistant endospores. Furthermore, *B. subtilis* is capable of producing biofilms in response to cell density. From an environmental perspective, *B. subtilis* is a crucial probiotic bacterium that helps plant, animal, and human health. Whole genome analysis reveals its low G+C content and over 4000 functional protein-coding genes, making it an attractive genetic tool extensively utilized in genetic engineering and biotechnology. Moreover, this rod-shaped aerobic (facultatively anaerobic) prokaryote exhibits a remarkable secretion capacity for a wide range of valuable proteins, enzymes, and significant secondary metabolites with antimicrobial properties.

Bacilysin, also known as bacillin or tetaine, is a non-ribosomal synthesized dipeptide antibiotic made up of two amino acids: The N-terminal L-alanine and the C-terminal non-proteinogenic amino acid L-anticapsin. It is produced by various species of *Bacillus*, including *Bacillus subtilis*, *Bacillus amyloliquefaciens*, *Bacillus pumilis*, and *Bacillus velenzensis*. Despite its simple structure, bacilysin has a wide range of antimicrobial activity against bacteria and fungi. The antimicrobial activity of bacilysin is due to its anticapsin component, which is released when it is transported into the cell and undergoes proteolysis. This anticapsin then inhibits glucosamine-6-phosphate synthase, preventing the biosynthesis of mannoprotein in fungi and peptidoglycan in bacteria. As a result, the microbial cells undergo lysis. Recently, bacilysin was found to be effective in controlling fire blight disease in orchard trees and rice diseases caused by *Xanthomonas* sp. It also exhibited anticyanobacterial activity against harmful algal species. Bacilysin is highly heat-stable and remains active over a wide pH. These characteristics make it a promising candidate for various industries, including pharmaceuticals, food, and agriculture. However, the low production by the native producer hinders its use in industrial applications. Therefore, in this thesis study, we aimed to improve the bacilysin biosynthesis in its native producer, *B. subtilis* PY79.

In bacteria, translation initiation is often the rate-limiting step of translation and a major factor in determining gene expression levels. Translation initiation occurs at the ribosome binding site (RBS) within the 5′ untranslated region (5′UTR) of an mRNA. In bacteria, the RBS contains the Shine Dalgarno (SD) sequence, the start codon, and a short spacer sequence. The SD sequence plays a crucial role in translation initiation by recruiting the 30S ribosomal subunit to the start codon through base-pairing with the anti-SD sequence in the 16S rRNA. The effectiveness of the SD sequence depends on its base pairing potential and its spacing from the start codon. The canonical SD

sequence for most bacteria is UAAGGAGG, which complements well with the anti-SD sequence of 16S rRNA. Studies have shown that increasing the SD-anti-SD interaction improves protein production and overall translation levels. The spacing between the SD sequence and start codon also affects translation initiation efficiency. If the spacing is too close or too far, initiation levels decrease. In bacterial genomes, the spacing of the SD sequence generally varies from 5 to 13 nt, with 8 to 10 nt being ideal for *E. coli* genes. Similarly, the ideal spacing between the canonical SD sequence and the AUG start codon is 7-9 nt for various *B. subtilis* genes.

In *B. subtilis* 168, the *bacABCDEF* operon and the monocistronic gene *bacG* encode enzymes responsible for the synthesis of anticapsin and bacilysin. The sequence around the *bacA* transcriptional start site shows a strong consensus signature for the *B. subtilis* sigma factor σ^A, but there is no obvious strong SD-like sequence surrounding the translation start codon AUG. This suggests that improving the RBS region could enhance bacilysin production. Therefore, in this study, by using a CRISPR/cas9 single plasmid system (pJOE9958.1), the putative RBS region of *bac*A, as the first gene of the bacilysin biosynthetic operon, was edited by introducing the conanical SD sequence (TAAGGAGG) at 8 nt optimum spacing from the *bac*A translation start codon (AUG). To achieve this, a 932 bp homology template was initially generated through overlapped-extension PCR. This template contained a strong ribosomal binding site (5'-GCTTAAGGAGGACAAACTC-3') (*bacA*$_{\text{strongRBS}}$), which was then cloned into the *Sfi*I cutting site of pJOE9958. A 20 nt spacer sequence from the upstream region was selected, specifically at positions -28 and -8 relative to the *bacA* start codon, by using the software CCTop. This sequence was synthesized into two complementary oligonucleotides and inserted it between the *Bsa*I sites of pJOE9958. The resulting plasmid was named pJOE9958.1.*bacAstrong*RBS.sgRNA, and it was used for the transformation of *B. subtilis* PY79-competent cells. Transformants were selected on LB agar plates with Km and 0.2% mannose at 30^{o}C to induce *cas9* expression. Within two days, 21 colonies appeared, and they were streaked again on LB plates with Km and incubated at 30^{o}C. Before the plasmid curing step, colonies resistant to Km were tested for their ability to produce bacilysin, eliminating any mutants that were unable to do so. Only three colonies exhibited antibacterial activity against *S. aureus*, indicating that the efficiency of homology-directed repair (HDR) was insufficient to repair Cas9-induced double-strand breaks (DSBs), and most of the colonies in our study likely arose from the NHEJ pathway. Subsequently, only colonies resistant to Km and capable of producing bacilysin were plated on LB plates without Km and incubated at 50^{o}C overnight, which prevents plasmid replication. They were then restreaked on LB plates at 42^{o}C to generate plasmid-cured cells, and approximately 42% of the examined colonies were found to be plasmid-cured. In the final step, 11 randomly selected Km-sensitive (plasmid-cured) transformants were screened with colony PCR to identify mutants with the substitution of the *bacA*$_{\text{strongRBS}}$ sequence with the *bacA*$_{\text{nativeRBS}}$ sequence. Out of the 11 transformants, 10 displayed the expected PCR fragment of approximately 440 bp. DNA sequencing of one randomly chosen PCR-positive colony confirmed the successful replacement of the *bacA*$_{\text{nativeRBS}}$ sequence with the *bacA*$_{\text{nativeRBS}}$. To assess the impact of the strong RBS insertion, eight mutants, including the strong RBS mutant confirmed by sequence analysis and the seven randomly selected mutants (from the PCR-positive colonies), were cultured in PA media for 16-18 hours. The level of bacilysin in their culture fluids was then determined using a paper disk-diffusion assay. Compared to the PY79 strain, each of the examined mutants produced more bacilysin, and there were no significant differences in their increased bacilysin levels. These results demonstrated that the

strong RBS substitution led to enhanced bacilysin production. Finally, one of the mutants with the *bacA*$_{strongRBS}$ sequence was chosen to represent the strong RBS-carrying mutants and designated as *B. subtilis* HWA for further investigation.

To further investigate the impact of the strong RBS substitution on bacilysin overproduction, HWA and the parental strain PY79 were grown in PA medium at 37°C with shaking for 24 hours, and their growth and bacilysin production levels were monitored at regular intervals. no significant differences in the growth patterns or the accumulation of bacilysin in HWA compared to PY79. In both strains, the highest bacilysin activity was detected during the early stage of the stationary phase, which occurred around 16-18 hours of cultivation. However, the most notable finding was that HWA consistently exhibited overproduction of bacilysin at all growth stages, with the peak occurring at 18 hours of incubation. The bacilysin titer in HWA was 2.87 times higher than that of the wild-type strain. This significant increase in bacilysin activity was further confirmed by UPLC-MS analysis, which revealed a higher relative abundance of the peak m/z of 271 $(M + H)^{+,}$ corresponding to the mass of bacilysin, in HWA compared to PY79 during the 16-18 hours culture period.

In bacteria, the processes of transcription and translation are closely linked. Modifying the nucleotides in the 5' untranslated region (5'UTR) can have an impact on both transcription and translation. Therefore, to demonstrate the effect of modifying the 5'UTR through a strong ribosome binding site (RBS) substitution on intracellular mRNA levels, the relative amounts of transcripts from the *bac* operon were examined in two bacterial strains, HWA and PY79, by using the RT-qPCR technique. These measurements were performed during the early and late stationary phases of growth, which correspond to 18 hours and 24 hours of growth, respectively. In HWA, the mRNA levels of the *bac* operon showed a significant change. Specifically, during the early stationary phase, the transcript levels were three times higher compared to PY79. Additionally, the transcript level in HWA was detected at 25 quantitative cycles (Cq) during the early stationary phase (18 hours) and 21 Cq during the late stationary phase (24 hours). In contrast, PY79 exhibited a Cq value of 29 at 24 hours. These findings indicated that the high transcript level of the *bac* operon in HWA is maintained throughout the stationary phase. In conclusion, these results provide evidence that the strong RBS substitution also improves the stability of the *bac* operon mRNA.

In summary, in this study, a CRISPR/Cas9 single-plasmid system was used to edit the putative RBS region of the *bac* operon. The canonical SD sequence (TAAGGAGG) at an optimal spacing of 8 nt from the *bacA* start codon (AUG) was introduced. Because the upstream region of the *bac* operon did not have an ideal core SD sequence with a spacing of 7-9 nt. Therefore, editing the bacA-RBS most likely has a positive impact on bacilysin production in its native producer. As expected, modifying the 5'UTR through strong RBS substitution led to a significant increase in bacilysin production. Additionally, the transcript level of the *bac* operon was significantly increased, especially in the late stationary phase. This suggests that a strong RBS substitution in the *bac* operon can improve translation initiation efficiency and mRNA stability. Overall, these findings indicate that extensive RBS engineering could be a promising approach to enhancing bacilysin production in native producers. This study is the first to employ RBS editing to improve bacilysin production in *B. subtilis* PY79, and our results will contribute to further enhancing bacilysin production in native strains.

BACİLLUS SUBTİLİS PY79 SUŞUNDA BASİLİSİN BİYOSENTEZİNİN CRISPR/CAS9 TEKNOLOJİSİ İLE İYİLEŞTİRİLMESİ

ÖZET

Gram-pozitif bir toprak bakterisi olan *Bacillus subtilis*; tarım, tıp ve farmakoloji gibi çeşitli alanlarda çalışan biliminsanları ve araştırmacıların büyük ilgisini çekmektedir. Hücre yoğunluğuna bağlı biyofilm ve dirençli endosporlar oluşturarak besin eksikliğine ve kötü şartlara dayanabilmesini sağlayan, yüksek adaptasyona sahip metabolik sistemi sayesinde, özellikle hücre farklılaşma çalışmaları için, üstün bir model organizma olarak kabul görmektedir. Ek olarak, *B. subtilis* bitki, hayvan ve insan sağlığına katkıda bulunan önemli bir probiyotik bakteri olarakda oldukça değer görmektedir. *B. subtilis*'in tam genom analiz sonuçları, onun düşük G+C yüzdesine sahip olmasının yanı sıra, 4000'den fazla fonksiyonel protein kodlama genine sahip olduğunu ortaya çıkarmıştır. Çubuk şekline sahip, oksijenli ve oksijensiz ortamda büyüyebilen bir bakteri olan *B. subtilis*, aynı zamanda pekçok değerli proteinleri, enzimleri ve antimikrobiyal aktiviteye sahip pek çok ikincil metabolitleri üretip dışarıya salabilmektedir. Tüm bu özellikler, *B. subtilis* bakterisini, genetik mühendislik ve biyoteknoloji alanında yaygın olarak kullanılan çekici bir bakteriyel araç yapmıştır.

Basilisin, ribozom dışı sentezlenen, L-alanin ve protein dışı bir aminoasit olan L-antikapsinden oluşan ikili peptit yapıda bir antibiyotiktir. *B. subtilis* başta olmak üzere, *Bacillus amyloliquefaciens*, *Bacillus pumilis* ve *Bacillus velenzensis* gibi pek çok farklı *Bacillus* türleri tarafından üretilmektedir. Küçük yapısına rağmen, bakterilerden küflere kadar etki edebilen çok geniş spektrumlu bir antibiyotik olması ile dikkat çekmektedir. Basilisin, antimikrobiyal etkisini yapısında bulunan antikapsine borçludur. Basilisin hücre içine taşındığında, peptidazlar tarafından kesilerek açığa çıkan antikapsin, glukozamin-6-fosfat sentetaz enzimini inhibe etmektedir. Bu nedenle, basilisin, küflerde mannoprotein yapılı hücre duvarı sentezini, bakterilerde ise petidoglikan hücre duvarı sentezinin durdurarak hücrelerin ölümüne neden olmaktadır. Son zamanlarda yapılan çalışmalar basilisinin elma, armut, ayva vb. pek çok bahçe ağacında ateş yanığı hastalığına neden olan *Erwinia amylovora* bakterisine ve pirinç bitkisinde hastalığa neden olan *Xanthomonas* türlerine karşı etkili olduğunu göstermiştir. Ek olarak, patojen alg türlerine karşıda antimikrobiyal etkiye sahip olduğu gösterilmiştir. Basilisin aynı zamanda yüksek sıcaklıkta ve çok geniş pH değişiminde aktivitesini koruyabilen oldukça dayanıklı bir yapıya sahipdir. Tüm bu özellikler, basilisini, ilaçdan, gıdaya ve tarıma çok farklı sektördeki uygulamalar için oldukça cazip, potansiyel bir aday yapmaktadır. Ancak, *B. subtilis* gibi doğal üretici organizmalarda, basilisin üretim seviyelerinin çok düşük olması, endüstriyel uygulamada yaygın kullanımını oldukça kısıtlamaktadır. Bu nedenle, bu tez çalışmasında basilisinin doğal üreticisi, *B. subtilis* PY79 suşunda basilisin üretim seviyesinin iyileştirilmesi amaçlanmıştır.

Bakterilerde, translasyon başlangıç hızı, gen ifade düzeyini belirleyen, en önemli sınırlayıcı etkendir. Translasyonun başlatılması, bir mRNA'nın 5′ UTR bölgesi içinde

yer alan ribozom bağlanma bölgesinde (RBS) meydana gelir. Bakterilerde, RBS bölgesi, "Shine-Dalgarno" (SD) dizisi olarak adlandırılan bilinen korunmuş bir DNA dizisi, başlangıç kodonu ve ikisi arasında kalan kısa bir uzantı dizisinden oluşur. SD dizisi, 30S ribozomal alt biriminde bulunan 16S rRNA'daki anti-SD dizisi arasında temel baz eşleşmesi yoluyla, 30S ribozomal alt biriminin başlangıç kodununu içine alacak şekilde mRNA 5'UTR bölgesine yeleştirilmesini sağlamaktadır. Bu nedenle translasyon başlangıcında anahtar rol oynar. SD dizisinin etkinliği, hem 16S rRNA'nın anti-SD dizisiyle olan baz eşleşme potansiyeline hemde başlangıç kodonuna olan uzaklığına bağlıdır. Çoğu bakteri için kanonik SD dizisi, 16S rRNA'nın anti-SD dizisiyle en uyumlu olan "UAAGGAGG"dizisidir. Yapılan çalışmalarda, SD-anti-SD etkileşiminin artırılması ile gen ifade düzeyinin yükiseltildiği ve böylece protein üretiminin iyileştirildiği gösterilmiştir. Yukarıda belirtildiği gibi, SD dizisi ve başlangıç kodonu arasındaki mesafe, translasyon başlangıç hızını etkilmektedir. Eğer mesafe çok yakın veya çok uzak ise, başlangıç verimliliği azalmaktadır. Bakteriyel genomlarda, SD dizisi ve başlangıç kodonu arasındaki mesafe aralığı genellikle 5 nt ila 13 nt arasında değişmektedir. Ancak 8 nt ila 10 nt aralığındaki mesafenin *E. coli* genleri için ideal olduğu gösterilmiştir. Benzer şekilde, pek çok *B. subtilis* geni içinde kanonik SD dizisi ile AUG başlangıç kodonu arasındaki ideal mesafe 7-9 nt olarak bulunmuştur.

B. subtilis 168'de, *bacABCDEF* operonu ve *bacG* geni, anticapsin ve basilisin sentezinden sorumlu enzimleri kodlamaktadır. *bacABCDEF* operonunun ilk geni olan *bacA* transkripsiyon başlangıç bölgesi incelendiğinde, *B. subtilis* sigma faktörü σ^A için güçlü bir promotor dizisi bulunurken, translasyon başlatma kodonu AUG'yi çevreleyen, belirgin güçlü bir SD-dizisinin bulunmadığı görülmüştür. Bu eksiklik, RBS bölgesinin iyileştirilmesi ile basilisin üretiminin artırılabileceğine kuvvetle işaret etmiştir. Bu öngörü ile, bu çalışmada, CRISPR/Cas9 tek plazmid sistemi (pJOE9958.1) kullanılarak, *bacA*'nın varsayılan RBS bölgesinin, *bacA* AUG başlangıç kodonuna mesafesi 8 nt olacak şekilde dizayn edilmiş kanonik SD dizisi "TAAGGAGG" içeren "GCTTAAGGAGGACAAACTC-3" (*bacA*.$_{\text{güçlüRBS}}$) dizisi ile yer değiştirilmesi hedeflenmiştir. Bu amaçla öncelikle, "*bacA*.$_{\text{güçlüRBS}}$ dizisini içeren 932 bç DNA homoloji şablonu, eşlenik uzatma PZR (overlap extension PCR) yaklaşımı ile oluşturulmuş ve pJOE9958'nin *SfiI* kesim bölgesine klonlanmıştır. CCTop yazılımı kullanılarak, -28 ve -8 pozisyonlarında *bacA* başlangıç kodonuna göre -28 nt ve -8 nt pozisyonunda yer alan 20 nt lik bölge, klavuz RNA (gRNA) dizisi olarak seçilmiştir. İki ucunda *Bsa*I kesimi dizisi olacak şekilde çift DNA dizisi olarak sentezlettirilen klavuz RNA dizisi, pJOE9958'in *Bsa*I kesim bölgesine klonlanmıştır. Oluşturulan plazmit pJOE9958.1.$_{\text{bacAgüçlüRBS.sgRNA}}$ olarak adlandırılmış ve hedef değişikliğin gerçekleştirilmesi için, *B. subtilis* hücrelerine aktarılmıştır. İlgili transformantlar, 30°C sıcaklıkda, Km ve % 0.2 mannoz içeren LB agar plakalar üzerinde seçilmiştir. İki günun sonunda ortaya çıkan 21 koloni toplanarak, Km içeren LB plakalara aktarılmış ve 30^{o}C sıcaklıkda bir gece inkübe edilmiştir. Plazmid giderimi işleminden önce, olası homolog olmayan uç birleştirme (non-homologous end joining) (NHEJ) onarımı nedeni ile basilisin üretim kabiliyetini yitiren kolonilerin elenmesi için, seçilen Km dirençli koloniler, basilisin üretme kabiliyeti yönünden disk difüzyon testi ile taranmıştır. Sadece üç koloni, test organizma olarak kullanılan *S. aureus*'a karşı antibakteriyel aktivite göstermiştir. Bu sonuç, bizim çalışmamızda, Cas9 kaynaklı DNA çift zincir kırığının (DSB'ler) onarılması için, homoloji-yönlendirilmiş onarım (homology-directed repair) (HDR) yolu verimliliğinin düşük kaldığı, çalışmamızdaki kolonilerin çoğunun muhtemelen NHEJ yolundan kaynaklandığına işaret etmiştir. Km dayanıklı ve basilisin üretebilen üç koloni ile

plazmid giderimi basamağına devam edilmiştir. Bunun için, koloniler Km içermeyen LB plakalara taşınmış ve gece boyunca 50^{o}C'de inkübe edilmiştir. Ertesi gün, Km içermeyen LB plaklara tek koloni düşecek şekilde ekim yapılarak, 42°C'de tekrar bir gece inkübe edilmiştir. Test edilen koloniler içinde yaklaşık % 42'sinin pJOE9958.1.$_{bacAgüçlüRBS.sgRNA}$ plazmidini kaybettiği tespit edilmiştir. Akabinde, rastgele seçilen onbir adet Km-duyarlı plazmidi giderilmiş koloni, *bacA*.$_{güçlüRBS}$ dizisi varlığı açısından, koloni PZR yöntemi ile taranmıştır. Yapılan taramada yaklaşık on kolonide, 440 bp'lik beklenen PZR ürünü elde edilmiştir. PZR-pozitif koloniler arsından rastgele bir tanesi seçilerek DNA dizileme analizine gönderilmiştir. Yapılan DNA dizi analizi, *bacA*$_{güçlüRBS}$ dizisinin *bacA*$_{doğalRBS}$ dizisi ile değiştirildiğini doğrulamıştır.

Güçlü RBS değişiminim etkisinin değerlendirilmesi için, sekans analizi ile doğrulanmış güçlü-RBS mutantı ile birlikte rastgele seçilmiş yedi mutant (PZR-pozitif kolonilerden) 16-18 saat boyunca PA ortamında kültive edilmiş ve kültür sıvılarında bulunan basilisin seviyesi disk difüzyon testi ile belirlenmiştir. PY79 ile karşılaştırıldığında, incelenen mutantların her birinin yüksek düzeyde basilisin ürettiği ve kendi aralarında artan basilisin seviyelerinde önemli bir farklılık olmadığı görülmüştür. Bu sonuç, güçlü RBS değişiminin basilisin üretiminin artırılmasına yol açtığını göstermiştir. Test edilen bu mutantlar arasından, *bacA*$_{güçlüRBS}$ değişimini temsil etmek üzere rastgele bir mutant seçilmiş ve daha ileri analizlerde kullanılmak üzere *B. subtilis* HWA olarak adlandırılarak -80^{o}C de stoklanmıştır.

Bir ileri analiz olarak, HWA ve PY79, 24 saat boyunca 37°C'de PA besi yerinde çalkalamalı olarak kültive edilmiş, ve belirli aralıklarla örnekler alınarak, büyüme profilleri ve basilisin üretim düzeyleri takip edilmiştir. PY79 suşuna kıyasla, HWA'da hem büyüme hemde basilisin birikim profillerinde bir değişiklik bulunmamıştır. Her ikiside en yüksek basilisin aktivitesini, 16-18 saatlik kültivasyon sürecine denk gelen durgunluk aşamasının erken aşamasında ulaşmaktadır. Ancak, en dikkat çekici bulgu, HWA'nın tüm büyüme aşamalarında, basilisin düzeyinin PY79 suşuna kıyasla hep yüksek seyretmesidir. Her ikisinin maksimum seviyede seyrettiği 18 saatlik kültür sıvılarındaki basilisin düzeyleri karşılaştırıldığında, HWA suşundaki basilisin miktarının, 2.87 kat daha yüksek olduğu belirlenmiştir. Bu önemli artış, Yüksek Performanslı Sıvı Kromatografisi-Kütle Spektrometresi (UPLC-MS) ile de doğrulanmıştır. UPLC-MS analizinde, HWA suşunun 16-18 saatlik PA kültür ekstresi, PY79 ile karşılaştırıldığında, basilisinin kütlesine karşılık gelen m/z 271 $(M + H)^{+}$ pik düzeyi çok daha yüksek bir oranda bulunmuştur.

Bakterilerde, transkripsiyon ve translasyon basamakları birbirleri ile içiçe olduğu olduğu için, 5' UTR yapılan bir değişiklik hem transkripsiyon hemde translayon düzeyini etkileyebilir. Bu nedenle, çalışmamızda gerşekleştirilen güçlü RBS değişiminin *bac* operon transkripsiyon düzeyine olası etkisi, Ters Transkriptaz-Kantitatif Polimeraz Zincir Reaksiyonu (RT-qPZR) ile araştırılmıştır. Bu analiz için, büyümenin erken ve geç durağan fazına denk gelen 18 saatlik ve 24 saatlik HWA ve PY79 RNA örnekleri kullanılmıştır. HWA suşunda, *bac* operon-mRNA seviyesi, PY79 suşuna kıyasla, erken durağan fazda 3 kat daha yüksek bulunmuştur. Ayrıca, geç durağan fazda PY79 suşunda tespit edilen 29 kantitatif döngünün (Cq) aksine, HWA suşunda *bac* operon-mRNA seviyesinin, erken durağan fazda 25 ve geç durağan fazda 21 kantitatif döngü olarak tespit edilmesi, HWA daki *bac* operon-yüksek mRNA seviyesinin durağan faz boyunca korunduğunu göstermiştir. Tüm bu sonuçlar, güçlü RBS değişiminin *bac* operon-mRNA stabilitesini artırdığına işaret etmiştir.

Özet olarak, bu çalışmada, basilisin üretim düzeyinin iyileştirilmesi amacı ile, basilisin biyosentezinden sorumlu *bac* operonunun RBS bölgesinin iyileştirilmesi için CRISPR/Cas9 tek-plasmid sistemi kullanılarak, standart SD dizisinin (TAAGGAGG) *bacA b*aşlangıç kodununa (AUG) 8 nt'lik en uygun mesafe ile yerleşimi gerçekleştirilmiştir. Beklenildiği gibi, güçlü RBS değişimi, basilisin üretim düzeyinde önemli bir artış ile sonuçlanmıştır. Ayrıca, *bac* operonunun transkripsiyon seviyesinin özellikle geç durağan fazda PY79 suşuna kıyasla yüksek düzeyde korunduğu görülmüştür. Elde edilen bu veriler, güçlü bir RBS değişiminin, hem translasyon verimliliğini hemde mRNA stabiletesini iyileştirdiğini göstermiştir. Sonuç olarak, bu çalışma geniş kapsamlı bir RBS mühendisliğinin, doğal üretici suşlarda basilisin üretimini artırmak için uygulanabilcek umut verici bir yaklaşım olabileceğinide ortaya çıkarmıştır.

1. INTRODUCTION

1.1 *Bacillus subtilis*

1.1.1 Description and genetic aspects

Bacillus subtilis is a remarkable Gram-positive model system with a ubiquity appearance capable of colonization and adaptation within the abundant biosphere of diverse settings due to its ability to form highly resistant endospores in response to harsh environmental conditions and their metabolic diversity [1-5]. Thus, those adaptable bacterium species can be isolated from soil, where spores accumulate, rivers, estuarine water, and associated plants [6,7]. They can also be found in the gastrointestinal (G1) tract of animals that consume plants [8-10]. In addition, this ubiquitous bacterial species was categorized as an epiphyte [11] and, in some cases, an endophyte [12], a plant growth-promoting rhizobacterium (PGPR) organism. Therefore, they enhanced soil fertility as a biofertilizer instead of chemicals [13, 14]. *B. subtilis*, originally named *Vibrio subtilis*, was first isolated from fresh hay in 1872 and characterized by Ferdin and Cohn, who described their life cycle involving the formulation of spores and their germination [15, 16]. Unlike *Escherichia coli*, the well-known Gram-negative prokaryote, *B. subtilis* has only one cell membrane that leads to facilitating the secreted proteins directly (in their active form) into the extracellular medium, thus simplifying their purification in easy and low-cost downstream processing. Moreover, in contrast to *E. coli*, *B. subtilis* is considered a GRAS organism (Generally Recognized as Safe); as a probiotic, it is safe and non-toxic, making it highly favorable in the food industry over using *E. coli,* which has an endotoxin lipopolysaccharide (LPS) in its outer cell membrane [17,18]. Furthermore, *B. subtilis* has the most uncomplicated cellular differentiation activity due to sporulation and endospores. B. subtilis has the best fermentation capacity (the fermentation cycle of *B. subtilis* around 48 h is shorter than *Saccharomyces cerevisiae* which has a fermentation cycle of 180 h) and produces numerous heterologous proteins [19, 20]. This rod-shaped bacterium became an exceptional model used to study motility, chromosome segregation, competence host system for bacteriophage, transcription regulation,

biofilm formation, and producing many valuable enzymes such as protease and amylase, secondary metabolites with antimicrobial properties, etc. 60% of the industrial enzymes was produced commercially by *Bacillus subtilis* [21-25].

The whole genome sequence of *B. subtilis* was a significant event in microbial ecology, and it was the first published genome sequence for soil bacterial species [7]. It was completed in June 1997, which identified that *B. subtilis* 168 is a tryptophane auxotroph [26 -28], and it is NOT pathogenic bacteria due to no virulence coding genes found [29]. These nonpathogenic bacteria can reproduce aerobically; however, the putative respiratory nitrate reductase-encoding genes found in the *B. subtilis* genome suggested that *B. subtilis* should be capable of propagating anaerobically by using nitrate instead of oxygen as an energy source. The proliferation within animals' mostly anaerobic G1 tract supported this idea [30, 31]. The whole sequenced genome of *B. subtilis* holds more than four million base pairs in length that possess more than four thousand functional protein-coding genes [7]. For instance, cold shock proteins (Csp) are expressed by one of the heat shock inducible genes, which synthesize in response to a sudden temperature downshift. These proteins facilitate the initiation stage of translation via binding to RNA co-operatively in case of dramatically decreasing temperature [32,33]. At least four percent of the *B. subtilis* whole genome is dedicated to sporulation, propagation, and germination [34]. Approximately four to five percent of this bacterial genome is committed to antibiotics and antibiotic-like compounds biosynthesis, including three coding regions for multifunctional polyketide synthetase responsible for peptide syntheses like surfactin and fengycin, the antifungal antibiotic [35].

A recent study published in August 2022 by Thiruvengadam and his colleagues has defined a complete genome sequencing of *Bacillus subtilis* Bbv57 that revealed a close genetic / sequence similarity to other *B. subtilis* strains with only minimal variation in the genetic content between the *B. subtilis* Bbv57 and different *B. subtilis* strains [36]. It was found that the whole genome sequence of *B. subtilis* Bbv57 consists of 4,302,465 bp, of which the total number of genes is 4363 genes, and 4281 of these genes are functional protein-coding genes. In addition, it has 44.5% G+C content, five copies of rRNA operon (16S, 23S, and 5SRNA), and 76 tRNA genes. Most of these functional genes involved amino acid, transport, metabolism, and transcription, followed by metabolism and carbohydrate transport. Only sixty-six of those genes

contributed to defense mechanisms. Moreover, this interesting study shows the presence of >100 CAZymes, which play a crucial role in carbohydrate metabolism as well as nine gene groups included in the biosynthesis of several secondary metabolites with antimicrobial activity, exhibited a vital role in *B. subtilis* antagonistic properties against phytopathogens infections [36].

The M-CGH analysis (Microarray-based comparative Genomic Hybridization) revealed that *B. subtilis* 168 could be competent naturally to accept an extra-cellular DNA and recombine it into its genome by transformation [4]. Extracellular signals are required to initiate the competence machinery; these signals are synthesized, processed, and recognized by a three-gene *comPQX* operon in a population-density-depending manner [37-42].

1.1.2 Quorum Sensing in *Bacillus subtilis*

Bacillus subtilis isolates can monitor and respond to each other in the environment by secreting and sensing oligopeptide molecules called autoinducer peptides (AIP) that play a crucial role in creating an adaptational attitude based upon the rising cell population through a mechanism called "Quorum Sensing" (QS) [43, 44]. The QS technique is a central mechanism to regulate genetic competence, sporulation, biofilm construction, and antibiotic production in the *B. subtilis* strains [45-47]. Generally, there are at least three different manners of QS in bacteria: the first pathway (LuxI/ LuxR- style) uses N-acyl-homoserine lactones (AHLs) as autoinducer signals in gram-negative bacteria (Figure 1.1), the second one is Two-component system that uses small peptides as signal molecules in gram-positive bacteria. In contrast, the third type of QS system is luxS-encoded autoinducer 2 (AI-2) in Gram-Negative and Gram-Positive microorganisms [48-53]. Specifically, the quorum sensing mechanism in *Bacillus subtilis* comprises two receptor-signal systems: ComXPAQ signaling peptides and Rap- Phr (CSF) protein system [43] [54, 55]. In the first ComXPAQ sensing pathway, ComX is an autoinducer oligopeptide consisting of ten amino acid residues [56]: Extracellularly, when the bacterial cell density reaches the quorum, ComX pheromone bonds to the ComP sensor protein kinase (figure 1.2). The activated ComP, in turn, transports the phosphorylation indicative to the ComA transcription factor that regulates the expression of genes involved in competence development, antibiotic production, biofilm formation, and sporulation [56-59].

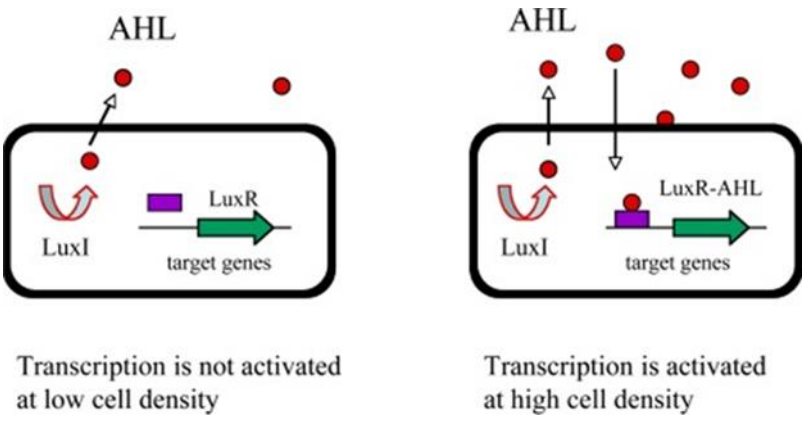

Figure 1.1: QS-type LuxI / luxR in Gram-Negative microbes. The autoinducer synthase LuxI like-protein stimulates the creation of AHLs (Acyl-homoserine lactone), which diffuse out of the cell. The transcriptional regulator LuxR binds to these AHLs at high cell density, activating its target genes' expression [54].

At the same time, ComQ is the receiver domain responsible for exporting, processing, and modulation of ComX and producing the grown QS signals [52][60]. Intracellularly, the phosphorylated form of ComA directly controls the expression of genes responsible for Surfactin secretion [61], a cyclic lipopeptide antibiotic and bacilysin production [62], while indirectly leading to regulating other public production [63-64]. For instance, the ComA~P sets the degQ reproduction; sequentially, DegQ modifies DegU phosphorylation leading to the production and the secret of important exoenzymes like exoprotease, which digest complex nutrient molecules [65]. Also, the excretion of Surfactin provides an indirect route to phosphorylate SpoOA and facilitates the production and secretion of the extracellular biofilm matrix (Figure 1.3) [66-68]. In addition to the ComXPAQ QS system, another QS system found in *B. subtilis* is Rap-Phr sensing proteins chain (Rap-Phr pairs). Unlike the ComXPAQ chain, which has a single receptor-signal system encoded by the chromosomal genome, each *B. subtilis* isolate contains multiple Rap-Phr regulation processes. These processes exhibit significant strain specificity, as evidenced by the encoded Rap-Phr pairs [69]. For example, *B. subtilis* 168 has eight Rap-Phr pairs (Rap-Phr A, C, E, F, G, H, I, K) and three orphan receptors (Rap B, D, and J). The QS systems in *B. subtilis* seem to converge and regulate the same physiological responses. This is accomplished as several different Rap proteins repress the activity of ComA, the response regulator of the ComQXPA system [69-72]. Unlike *Vibrio Harveyi*, a Gram-Negative bacterium, the two-different signaling QS pathways of *B. subtilis* depend more on autoinducer peptides concentration than their simultaneous presence.

For example, CSF (Competence and sporulation factor) encoded by *phrC* is a linear pentapeptide that has a crucial role, directly or indirectly, in regulating the genes involved in competence, sporulation, surfactin, and biofilm formation in a cell density manner in which CSF-quorum concentration decides which physiological strategies should be chosen by the *B. subtilis* population (Figure 1.3) [55, 56] [73- 76].

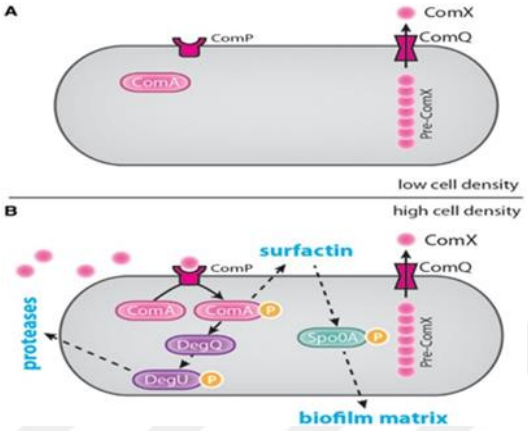

Figure 1.2: First QS pathway ComXPAQ in Bacillus subtilis, has a crucial role, directly and indirectly, in regulating the expression of genes involved in surfactin, proteases, and biofilm matrix synthesis [55].

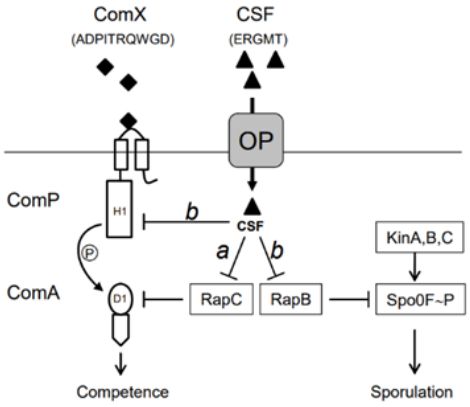

Figure 1.3: Function of CSF pheromone with ComX autoinducer, cooperatively, in directing the cell to choose the appropriate physical activity in a cell density-dependent manner. (a) at low cell density, CSF pheromone boosts the competence by repressing the phosphatase RapC responsible for dephosphorylating ComA and blocking the competence. (b) at high cell density, CSF enhances sporulation cell activity by inhibiting ComP/ ComA phosphorylation cascade and phosphatase RapB that represses SpoOF~p and sporulation subsequently. (OP) is an oligopeptide permease that exports CSF into the cell [56].

In the Rap-Phr Qs system, Rap proteins are phosphatases that have a double action with the ComXPAQ cycle once when the bacterial cell population is at low concentrations; Rap proteins act conversely to ComXPAQ by de-activate ComA~P via

dephosphorylating and inhibiting the process [77]. The other action appears when the bacterial cell is at a high concentration (reaches the quorum); the function of these phosphatases is repressed by binding of Phr peptides to their corresponding Rap receptors. Subsequently, the inhibition of the ComXPAQ system is relieved [69][77]. In addition, Rap protein controls the DegU regulator that regulates other mutual processes like competence, exoprotease excretion, and biofilm construction [79,80]. Moreover, these proteins organize the SpoOF regulator, part of the SpoOA phosphor-relay process [79,80], and control the biofilm matrix expression by adjusting the phosphorylated SpoOA level [81] (Figure 1.4).

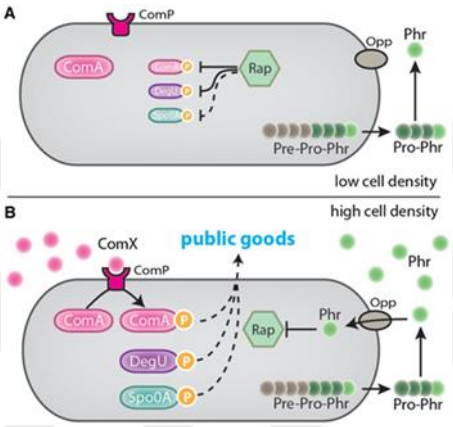

Figure 1.4: Function of Rap-Phr proteins in promoting or repressing the ComXPAQ signaling cascade dependent on the bacterial cell population. (A) At low cell density, Rap proteins block the ComXPAQ cycle. (B) At high cell density, Phr peptides enhance the ComXPAQ signaling cascade by inhibiting the activity of these Raps by binding when exported into the cell by the oligopeptide permease (Opp). Thus, it helps the cell to produce different public goods like surfactin, exoprotease, and biofilm matrix [55] [78] [82, 83].

1.1.3 Sporulation in *Bacillus subtilis*

Spore-formation is one of many strategies derived by the bacteria to cope with harsh environmental conditions such as starvation and sudden changes in temperature like coldness etc. The bacteria enter an inactive, dormant form enabling itself to survive until the environmental conditions become favorable for growing and germinating again [84]. Thus, spores are metabolically un-active dormant cells. *B. subtilis* can form both biofilms and spores [85]. Strong links are present between biofilm formation and sporulation in *B. subtilis*, and they are related processes regulated by the same transcriptional regulatory proteins, such as the SpoO family (SpoOA, SpoOB, SpoOF, SpoOH, SpoOK) [86-88]. The transcriptional master regulator SpoOA plays a central

role through phosphorylation in both processes; the intracellular level of SpoOA~P is critical. A high concentration of phosphorylated SpoOA~P (Its active form) inside the cell stimulates the genes involved in spore-formation. In contrast, the low intracellular level of phosphorylated SpoOA~P induces the formation of the Matrix required for biofilm formation [89]. Spores can be formed within biofilms where the bacterial cells ultimately sporulate for further protection from disinfecting agents and deleterious chemicals [90]. Cannibalistic behavior is the first and foremost step in the spore-formation process [91]. When the bacterial cells undergo nutritional scarcity, a subpopulation of *B. subtilis*, defined as cannibals, is developed. These cannibalistic cells kill and lyse neighboring sensible cells by producing and secreting two toxins, Skf (sporulation killing factor) and Sdp (sporulation-delaying protein), in a process known as "cannibalism." The dead cells are consumed by other living cells as a nutrient to delay the initiation of sporulation. Interestingly, these specialized cannibalistic toxins, Skf and Sdp, kill and lyse all susceptible cells except the cannibalistic toxins producer cells because they have immunity against these specialized toxins [92, 93]. In addition, the competent cells pick up the DNA released from degraded cells. However, these competent cells are also immune to cannibalistic proteins because they undergo the semi-dormant state (K-state), making them resistant to antibiotics and toxins [92] [94-96]. The genes encoding these toxins are regulated positively when the phosphorylated SpoOA (SpoOA~P) exists in a low intracellular concentration [89] [97-99].

Many genes become activated and expressed in correspondence to different levels of phosphorylated SpoOA (SpoOA ~P). The SpoOA regulator is central and involved in various feedback loops; it directly controls the transcription of one hundred and twenty-one genes and indirectly regulates genes involved in the asymmetric division of cells, and genes play a role in the intervention of sigma factor cascades like σ^E and σ^F. The master regulator SpoOA is activated by histidine sensor kinases (A, B, C, D, and E) [100-102]. Kinase sensor has three PAS-PAC sensory domains, PAS -A, PAS-B, and PAS-C; through these KinA domains, it can regulate several critical factors and metabolites intracellularly as well as phosphorylates the central regulator SpoOA when required via the activation of sigma factors cascade [103, 104]. At harsh environmental conditions, by sensing energy potential or redox state, the sensor kinase KinA is activated, and kinE activates the SpoOF regulator through the phosphorylation

process. Subsequently, the phosphate group is shifted from SpoOF~P to SpoOA ~P through SpoOB, triggering the transcription regulatory mechanism responsible for sporulation (Figure 1.5) [100].

The central phosphorylated regulator SpoOA~P inhibits the *abrB* gene from initiating sporulation. The phosphorylated regulator SpoOA~P also activates the *spoIIA* operon encoding sigma factor F (σ^F), the *spoIIG* operon encoding sigma factor E (σ^E), and the *spoIIE* gene, which is necessary for activation of sigma F in the forespore [105]. Interestingly, the regulator SpoOE can dephosphorylate the SpoOA~P and repress the initiation of the sporulation pathway when the environmental conditions become favorable for bacterial growth [106]. When the DNA is damaged, or DNA replication is impaired, the Sda protein is activated and stops the sporulation pathway by preventing phospho- transfer from KinA to SpoOF and delaying sporulation [107,108].

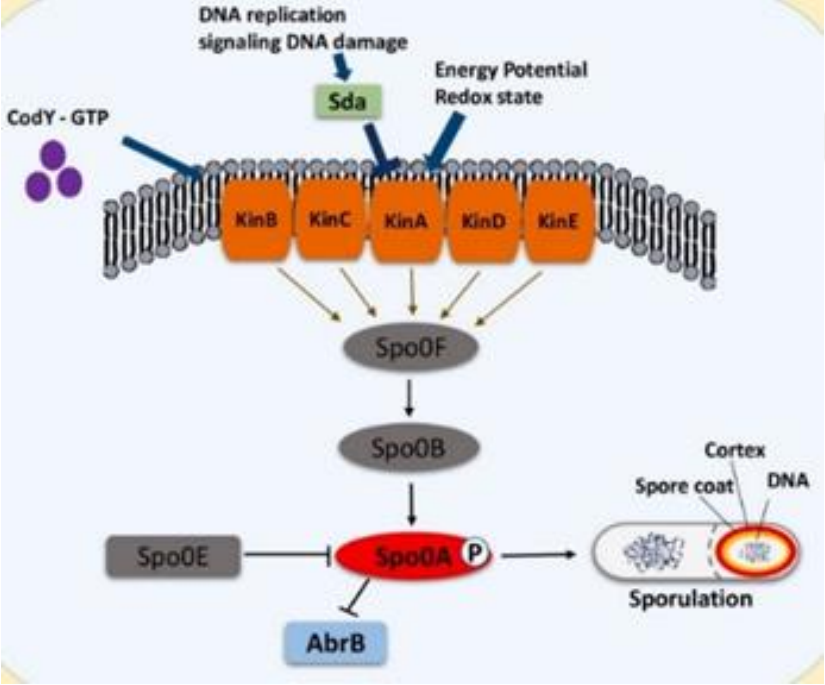

Figure 1.5: Development of sporulation in *Bacillus subtilis* as a response to nutrient scarcity [100].

Moreover, the extracellular production of surfactin as a signaling molecule triggers the expression and regulation of cannibalism. Indirectly, surfactin production stimulates KinC that phosphorylates the master regulator SpoOA, SpoOA~P, in turn, enhances the expression of σ^H that promotes the expression of genes required for expression and phosphorylation of the master transcription regulator SpoOA and the sensor kinase

KinA, involving in sporulation [109, 110]. *B. subtilis* cells divide into asymmetric cells with two distinct segments; one belongs to the mother cell, and the other refers to the forespore. This unidentical division triggers a cascade of sigma factors, δ^F is activated in the forespore and later in the mother cell leading to activate σ^E, and both σ^F and σ^E factors regulate genes involved in the engulfment of the forespore. After engulfment, σ^G is activated in the forespore leading to active σ^K in the mother cell. Finally, the spore coat and cortex are formed, completing the maturation spore (Figure 1.6) [111].

1.1.4 Applications

A ubiquitous Gram-Positive non-pathogenic bacterium, *Bacillus subtilis*, is widely used as an attractive host model, universal cell productive, and effective genetic tool for medicine, industry, agriculture, and biomaterial application in part because of its easy-to-grow and genetically manipulated in the laboratory and more because of its significance in producing a broad range of valuable secondary metabolites, which have antimetabolic and pharmacological properties, and non-toxic bioproducts like enzymes and heterologous proteins. *Bacillus* species, including *B. Subtilis*, generate %60 commercially beneficial enzymes [112-114] such as carbohydrase, amylase, and protease due to their magnificent fermentation qualities (around 20-25 g/L product yields) [115]. In addition, *B. subtilis* can form biofilm and spores, which are beneficial for humans and the environment through producing value-additives used in different sectors of healthcare, the food industry, the chemical industry, the petrol industry, and animal feedstock [85] [116-118].

B. subtilis spores and biofilms are more attractive among all common spore-forming prokaryotes, such as *Bacillus*, *Clostridium*, *Anoxy bacillus*, *Geobacillus*, and *Sporolactobacillus*, due to *B. subtilis* forms spores that possess low G+C content, and suitable cell wall structure; moreover, this non- pathogenic prokaryote biofilm and spores are GRAS microorganisms (Generally regarded as safe) [119].

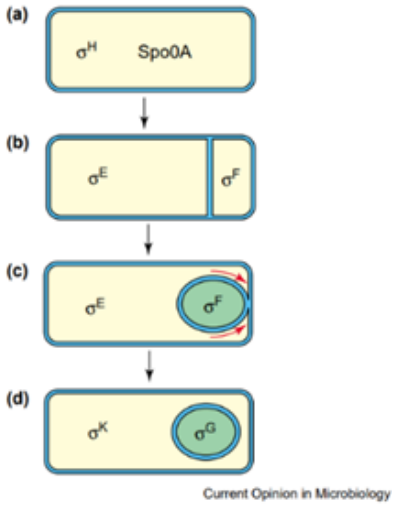

Figure 1.6: Gene regulation and morphogenesis of sporulation in *Bacillus subtilis*. a) Asymmetric division via the activated SpoOA and σ^H. b) The pre-spore with activated σ^F and the mother cell with activated σ^E. c) The engulfment stage, the degradation of the asymmetric septum in the mother cell, and the membrane migration around the pre-spore. d) The final stage of sporulation, when the pre-spore is released as a protoplast inside the mother cell and activated σ^G in the pre-spore and σ^K in the mother cell, enhances the transcription of genes involved in building the structural compounds of the spore, which are responsible for its resistance property [111].

B. subtilis has improved in the last decades and has become the fundamental microbe and the first choice for producing several industrial bioproducts, including enzymes [120] such as alpha-amylase, Xylanases, Lichenase, β-galactosidase [121], Alkaline serine proteases [122], and cellulases [123]. These enzymes are essential in several industrial sectors, like detergent, leather, textile, paper, pharmaceutical, feed, and food industries. For instance, The GRAS *B. subtilis* is used to prepare soybean hydrolysate, milk coagulation, meat tenderization, and food waste treatment [124]. Moreover, metabolically engineering *B. subtilis* produces valuable vitamins like vitamin K family, vitamins B1, B5, B6, and B7 [125], and various significant chemicals and pharmaceuticals crucial in different fields. Hyaluronic acid (HA) is a highly significant glycosaminoglycan in cosmetic, pharmaceutical, and biomedical technology. Recently, Li et al. (2019) found a genetically constructed *B. subtilis* capable of biosynthesis of hyaluronic acid with different titers and molecular weights at different temperatures. They reported that increasing the biomass decreases the molecular weight of the produced HA; however, the titer rises [126]. *B. subtilis* is also improved to synthesize new chemicals, such as N-acetylglucosamine (GLcNAc), essential in repairing and maintaining cartilage and joint tissue function. Liu et al. (2014) applied

modular pathway engineering to increase the production of GLcNAc in *B. subtilis* from 1.85 to 31.65 g/L [127]. Furthermore, a biofilm derived from genetically modified B. subtilis was applied for bioremediating pollutants from wastewater in recent applications. Due to their strong adhesion to the inorganic modified basalt fiber (MBF) and their ability to instantly form quickly, *B. subtilis* acts as an excellent redox mediator that biodegrades the contamination from wastewater [128]. In the enzyme industry, B. subtilis spores are more efficient and favorable than the vegetative cells due to their non-toxicity, grown without adding nutrients, and high stability at high temperatures and pressure. Additionally, the immobilized enzymes displayed on the spore surface are produced at a low economic cost. Furthermore, they can be re-purified and recycled easily. The current development of genetic manipulation of *B. subtilis* biofilms and spores has participated in the progression of bioremediation, biocatalysis, and biomaterial engineering techniques that improved human research and industrial application (Figure 1.7) [85].

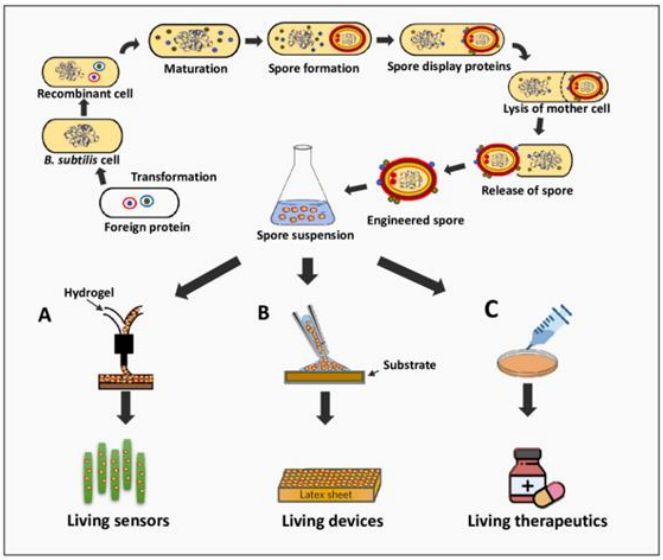

Figure 1.7: Applications of genetically modified *B. subtilis* spores as a biomaterial [85].

B. subtilis has a high recombination rate and efficient, simple genetic tools. The three common genetic engineering techniques used for manipulating *B. subtilis* are methods that rely on counter-selectable markers (CSM), such as the jellyfish-derived Green Fluorescent protein (GFP) [129, 130], site-specific recombination technique, such as higher recombination efficiency *Cre/loxP* [131], and *FLP/FRT* [132] techniques, and the recently promoted CRISPR/ Cas9 the excellent genome engineering toolbox [133].

1.2 CRISPR/ Cas9 Technology

1.2.1 Description and history

CRISPR is a short abbreviated for **C**lustered **R**egularly **I**nterspaced **S**hort **P**alindromic **R**epeats loci. At the same time, Cas is also an acronym for **C**RISPR-**as**sociated genes which encode proteins and enzymes with helicase and nucleases activity. (Figure 1.8) CRISPR/ Cas system is derived from the immune system in prokaryotes against viruses and bacteriophages [134, 135]. This was first discovered in 1987 by Nakata and colleagues, who observed officious sequence repeats and non-repeats polynucleotide downstream of the *iap* gene under investigation [136].

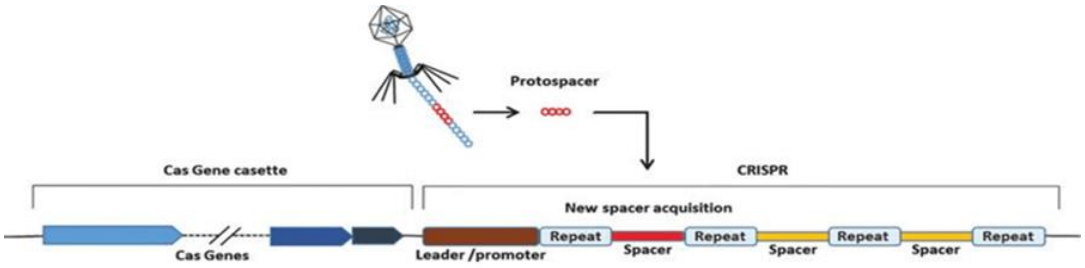

Figure 1.8: A graphic of CRISPR locus [137].

Later, in 2005, Mojica and colleagues revealed that these non-repetitive DNA sequences (spacers) units belong to bacteriophages [138]. Shortly after that, the function and location of the Cas genes were clarified by Bolotin *et al*., which are located near CRISPR loci and encode DNA endonucleases. Indicating that the CRISPR/ Cas primary function robustly is digesting any forging DNA entering the bacterial cell [139]. A few years later, this theory was confirmed by discovering a motif within the CRISPR structure located upstream of the protospacer called "PAM" (**P**rotospacer **A**djacent **M**otif). The primary function of PAM with sequence NGG (N refers to any nucleotide A, T, C, G) is its specificity to Cas protein and protospacer homology. The protospacer adjacent motif, specifically differentiated by Cas proteins, allows the CRISPR/ Cas system to distinguish between self and foreign DNA to target and cut [140- 143].

Consequently, by the early 2000s, the CRISPR/ Cas system is identified in prokaryotes as a defense approach. By 2010, three classes of CRISPR/ Cas protein were demonstrated in bacteria: class I, class II, and class III; among all of them, class II is the most applicable system due to its simplicity, then adapted for genetic editing in all mammalian cells [144]. In addition, there are many Cas proteins with helicase and

nuclease activity, some of them universally existing in all classes of CRISPR systems, like Cas1 and Cas2. In contrast, the others are involved differently in other CRISPR/Cas technology types. For instance, Cas9 endonuclease is the essential protein, in CRISPR/ Cas class II, for interference and DNA cleavage by making double-stranded breaks (DSB) in a sequence-specific manner [145]. Generally, based on CRISPR/ Cas9 strategy, immunity in bacteria passes through immunization and immunity (Figure 1.9) [146]. First, at the immunization phase, when a virus infects a bacterial cell, it is chopped up, and a section of its DNA is integrated into the bacterial chromosome, as a spacer-repeat unit within the CRISPR loci, by associating Cas1 or Cas2 (in case of RNA viral infection) [147]. Once the same virus re-infects the bacteria again, at the immunity stage, the spacer-repeat units in the CRISPR region are translated into CRISPR ribonuclear acid(crRNA), which binds to Cas9 protein and guides it to the complementary targets by assisting with trans-activating-CRISPR RNA (tracrRNA- crRNA) also is called guide RNA (gRNA). The Cas9 complex (tracrRNA-crRNA-Cas9 protein or gRNA-Cas9) altogether destroys the viral. As a result, the bacterial cell can NOT be infected by the same virus twice [148]. Cas9 is a large protein comprised of six domains (Figure 1.10). Only two domains, HNH and RuvC, have the nuclease ability to cleave the double-strand DNA (DSB), whereas Rec1 is the gRNA binding domain and PAM interacting part (PI) is the DNA-specific binding initiator domain. Consequently, Cas9 endonuclease plays a vital role in the CRISPR technique. Once the Cas9 complex is delivered to the target DNA sequence by gRNA, Cas9 starts scanning the DNA, looking for PAM to cut the DNA at this site [149-153].

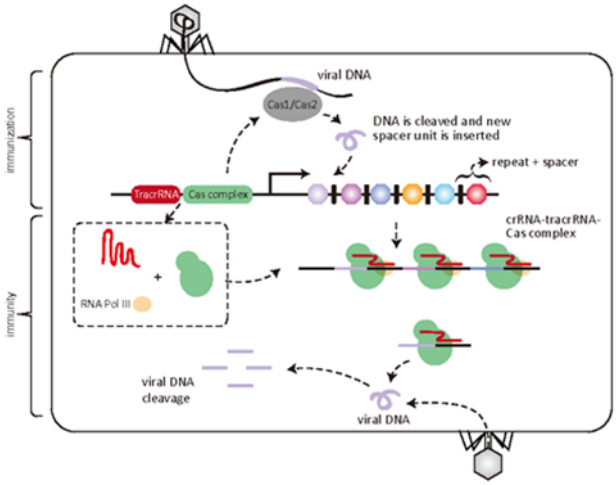

Figure 1.9: A diagram illustrates the CRISPR-based immunity mechanism in Prokaryote [148].

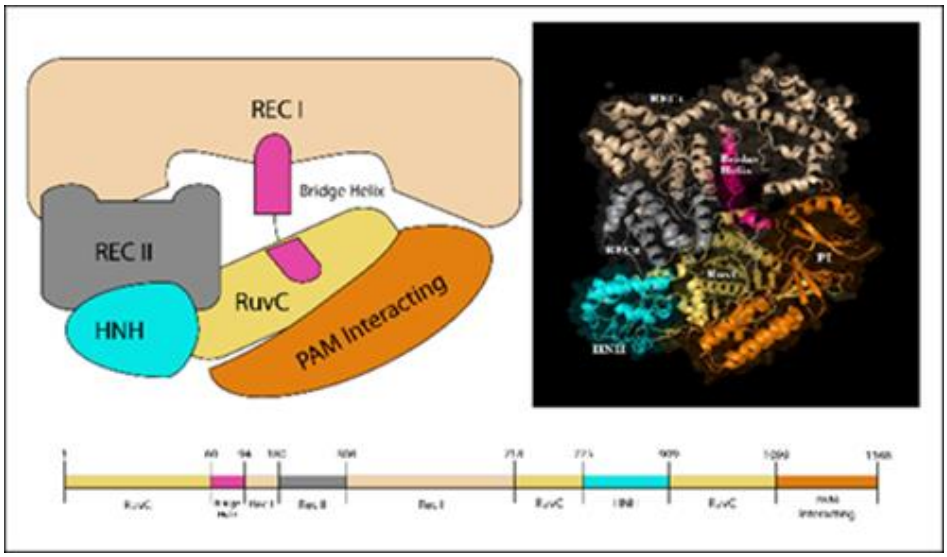

Figure 1.10: 2D and 3D Cas9 protein structure [149].

Accordingly, the CRISPR/Cas technique in the immunity of bacteria inspired scientists to use it as a crucial tool for genome editing technology in prokaryotes [153, 154], higher eukaryotes [155- 158], and yeast [159, 160]. By 2013, the CRISPR/Cas technique was used, for the first time, as a genome editing agent [161, 162].

1.2.2 CRISPR/ Cas9 Technology in *Bacillus subtilis*

Recently, three strategies of CRISPR/Cas9 technique have been widely applied in *B. subtilis* as a genome editing tool; The single plasmid-based system, the first strategy, where the Cas9, DNA homology template, and sgRNA (single guide RNA) and some other elements all together are assembled in the single plasmid DNA system in which Cas9 protein and sgRNA are expressed by inducible or strong promoters. Altinbuchner, in 2016, developed a single-plasmid structure named pJOE8999 for efficiently utilizing in genome editing in *B. subtilis* [163]. That holds the *cas9* gene transcribed via the mannose-inducible promoter P_{manp} and a single guide RNA (sgRNA), located between *Bsa*I sites, under the control of a strong promoter. Additionally, pJOE8999 has the temperature-sensitive replication origin of plasmid pE194 for *B. subtilis,* pUC minimal origin of replication for *Escherichia coli,* and a Kanamycin resistance gene expressing in both organisms (Figure 1.11) [163].

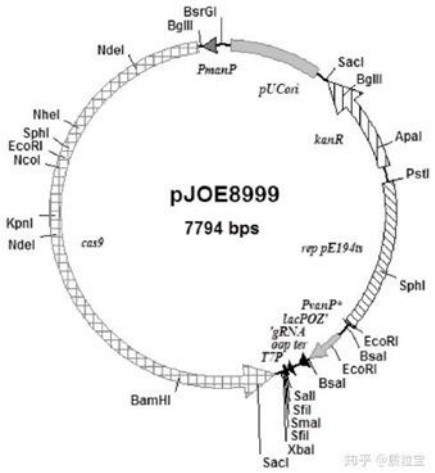

Figure 1.11: Genetic map of the pJOE8999 vector [163].

The second strategy is the two-plasmid model wherein Cas9, gRNA, and the DNA homology template are gathered on two shuttle vectors. The third technique is more effective and stable than the previous two strategies. The chromosomally integrated approach requires genetically modified strains in which Cas9 is integrated into the *B. subtilis* genome, and a multi-gRNA transmission vector was constructed using the ara E/R initiation subsystem [164]. In the metabolic engineering sector, CRISPR interference (CRISPRi) is another toolkit applied for transcriptional-level regulation in *B. subtilis*. In this technique, deactivated Cas9 (dCas9) protein and gRNA guided the dCas9 to any target gene on the genome to repress its transcription without stimulating a double-strand break [165]. Liu et al. [2019] developed a precise CRISPR/Cas9n-based multiplex genome editing tool in *B. subtilis,* further improving the efficiency of large genomic deletions and multiplex gene editing by regulating the nicks repair mechanism. This Cas9n-mediated multiplex editing technique was applied in *B. subtilis* to enhance the capacity of riboflavin production by generating an RBS modulation library of riboflavin operon, wherein three genetic loci were synchronously modulated [166].

In 2023, Ferrando J. et al. [2023] present, for the first time, a novel, simple, and rapid CRISPR/ Cas9 technology for one-step multiplex gene insertion (up to three genes) in *B. subtilis* [167]. This technique combines a single CRISPR/Cas9-mediated plasmid with the development high- a throughput White/ yellow colorimetric screening technique to establish a plasmid-less, marker-free, and high-expression stable *B. subtilis* producer strain by introducing *crtMN* operon (up to three copies), driven from *Staphylococcus aureus,* encoding a yellow pigment integrated at three ectopic sites

within *B. subtilis* genome, rendering the engineered bacteria capable of form yellow colonies. This novel technology expands genome editing in *B. subtilis* and significantly boosts the development of this distinctive strain as a framework in the synthetic biology field.

CRISPR/Cpf1 Technology is the most potent tool in *B. subtilis* for higher efficiency of multiple gene insertion compared to CRISPR/Cas9 technology. Cpf1, a protein from *Francisella novicida,* an array of novel Cas proteins derived from CRISPR system type V, which has been engineered as a genome editing system in *Clostridium difficile* [168, 169] *Corynebacterium glutamicum* [170] and rice [171]. Cpf1 is more effective than Cas9 protein. However, it differs from Cas9 in its structure and function. Cpf1-dependent CRISPR technology does not require tracrRNA because Cpf1 can process pre-crRNA to mature crRNA. Thus, Cpf1 simplified multiplex genome editing mechanisms in *B. subtilis* [169, 172]. The Cpf- mediated editing technique is the most efficient technique, particularly in *B. subtilis*, for many reasons; Cpf1 cleaves the target DNA efficiently by utilizing a T-rich PAM sequence, unlike Cas9, which uses a G-rich PAM sequence. Moreover, Cpf1 establishes a staggered end with 5`-overhang DNA cleaving fragments, facilitating the DNA repairing system by non-homologous end joining (NHEJ) or homology-directed repair [169, 170]. Cpf1 is a non-toxic protein compared to Cas9, like *Sp*Cas9 [170]. Furthermore, Cpf1 is a small protein with only 1300 amino acids and only a single well-identified nucleases domain, unlike Cas9, which has two nuclease domains, making it more suitable for the sgRNA complex. In conclusion, Cpf1- mediated editing technique is an excellent candidate Cas protein for genetic manipulation in *B. subtilis* strains [173].

1.3 Peptide Antibiotics Produced by *Bacillus subtilis*

Antibiotics are secondary metabolites mainly secreted by soil microorganisms. Among all these soil bacteria, *B. subtilis* is the best gram-positive, rod-shaped endospore-forming bacteria, which has a high capacity of non–toxic antibiotic secretions into the cell's exterior [174]. *B. subtilis* strains produce dozens of high-quality antimicrobial compounds with various structures. Approximately four to five percent of *B. subtilis* genomes are dedicated to antibiotic biosynthesis [175]. They produced and secreted antibiotics as a particular attribute to compete favorably with other microorganisms within the environment [176]. These antimicrobial peptides are synthesized by *B.*

subtilis, either ribosomal and post-translationary modified, such as Lantibiotics and lantibiotic-like peptides or non-ribosomal. Furthermore, they produced a couple of non-peptides, phospholipids, and polyketides. The biosynthesis of ribosomal peptide antibiotics appeared during the bacterium growth. In contrast, non-ribosomal antibiotics are synthesized after the end of cellular growth [177, 178]. The non-ribosomal peptide antibiotics participate in biofilm and swarming development. The Lantibiotics function as an autoinducer (pheromone) in quorum sensing and a killing factor that affects programmed cell- death in sister cells [178].

Surfactin, one of the non-ribosomal antibiotics, is a circular polypeptide with a fatty acid chain in its structure responsible for its antimicrobial activity. Other non-ribosomal antibiotics include bacilysin, turin, and fengycin [179, 180]. Most peptide antibiotics secreted by *B. subtilis* have antimicrobial activity against Gram-Positive bacteria; however, polycolistine and circulin exhibit antimicrobial activity against Gram-Negative bacteria exclusively. While in contrast, bacillomycin and mycobacillin display antifungal activity against molds [181]. Tumborski and Denkova present a study revealing that *B. subtilis* ATCC6633 exhibits high antagonist activity against the fungi *Aspergillus flavus*, Alternaria sp., *Penicillium chryogenum,* and *Sclerotinia sclerotiorum.* In addition, *B. subtilis* ATCC6633 displayed moderate antagonist activity against *Aspergillus niger* and *Aspergillus awamoria*. In contrast, the antimicrobial activity of this strain against *Aspergillus oryzae*, *Fusarium oxysporum,* and *Beauveria bassiana* was low. In comparison, no antimicrobial activity was against *Fusarium moniliforme* [182]. In 2022, Egbuloun et al. discovered a new peptide antibiotic identified as “Bacilysocine” extracted from the filtered crude extraction, which was obtained at the late exponential growth of *B. subtilis* soil-isolated strains and purified using high-pressure liquid chromatography HPLC and biochemical analysis was performed on it that reported its molecular weight (less than 2.5 KDa) by Zymogram analysis and TLC [183].

1.4 Bacilysin

1.4.1 Description and Significance

Bacilysin, the simplest dipeptide antibiotic investigated with a small molecular weight of 270.28 g/mol and a molecular formula $C_{12}H_{18}N_2O_5$, is secreted and synthesized by *Bacillus subtilis* Marburg 168 strain [184- 186], as well as *Bacillus amyloliquefaciens*

[187], *Bacillus pumili*, and *Bacillus velenzensis* [188]. The chemical structure of this dipeptide antibiotic consists of an exceptional non-proteinogenic L-anticapsin residue at its C-terminus and L-alanine amino acid at the N-terminus. (Figure 1.12) [189-192].

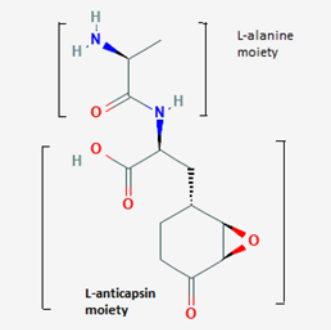

Figure 1.12: Chemical structure of the bacilysin dipeptide antibiotic [189].

Bacilysin has a broad spectrum of antimicrobial activity versus fungi, like *Candida albicans,* and bacteria. Bacilysin, excreted by *Bacillus veleuzensis,* has antibacterial activity against Gram-Negative *Escherichia coli* and *Salmonella enterica*, foodborne pathogens [188]. In contrast, *Bacillus amyloliqefaciens* has anti-cyanobacterial characteristics against harmful algal species like *M. aeruginosa*, *A. flos-aquae*, *Nostoc* sp., and *Anabaena* sp. due to the secretion of bacilysin [193]. Furthermore, the plant-associated *Bacillus amyloliquefaciens* subsp. *Plantarum* strain FZB42 can prevent the growth of *Erwinia amylovora*, causing fire blight disease in Orchard trees and *Xanthomo oryzae* pv. *oryzae* and X. *oryzicola* responsible for rice diseases via bacilysin secretion [187][194]. Although bacilysin is a great antimicrobial agent, it does NOT have antimicrobial activity alone; the antimicrobial activity belongs to its L-anticapsin moiety. Studies have shown that anticapsin blocks the biosynthesis of glucosamine-6-phosphate (GlcN6P) synthase, the enzyme responsible for N-acetylglucosamine GlcN6P or glucosamine biosynthesis, leading to repress the peptidoglycan formation of the microbial cell wall and lysis the microbial cell, subsequently (Figure 1.13) [195, 196]. Thus, intracellularly, bacilysin needs to be hydrolyzed by peptidase to release anticapsin and become active enzymatically since bacilysin can transport into the microbial cell easier than anticapsin. Recently, it was shown that anticapsin binds to the amino acid Cystein1 (CyS1) of GlcN6P synthase; in contrast, bacilysin as a dipeptide molecule can NOT bond to this enzyme directly [197].

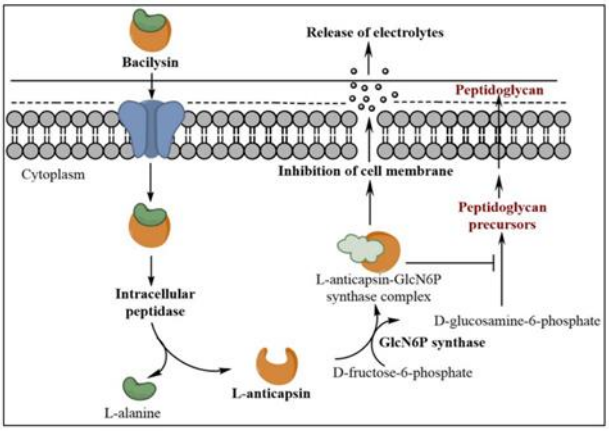

Figure 1.13: Antimicrobial action of bacilysin that blocks the microbial cell wall biosynthesis [198].

Bacillus subtilis produces bacilysin actively when the bacterial cells are grown in a **P**erry and **A**braham (PA) medium [199]. However, the biosynthesis of bacilysin is repressed by casamino acid and glucose [200- 202]. Furthermore, bacilysin is a white amorphous powder, freely soluble in water and %80 Alcohol, heat stable dipeptide (15 min/100C^{o}), and antimicrobial activity stays functional in a broad range of pH (1.4-12) [201].

1.4.2 Biosynthesis and molecular regulation

The polycistronic operon *bacABCDEF (formerly ywfBCDEFG)* and the *bacG (formerly ywfH)* monocistronic gene are mainly responsible for the biosynthesis of bacilysin in *Bacillus subtilis* [203]. The *bacABCFG* genes carry the bacilysin biosynthetic core action, latterly renamed *bacABCDE* [186]. The *bacABCFG* genes encode for anticapsin production, whereas *bacDE* genes are critical for bacilysin self-immunity and the function of amino acid ligation [184] [203,192].

To date, studies on this certain antibiotic have revealed that bacilysin biosynthesis is regulated by quorum sensing regulatory mechanism (QS) via the activity of ComQ/ComX, ComP/ComA, Phr peptides C, F, K, and in SpoOK (Opp)-dependent manner [204- 206]. In 2003, Karataş et al. reported that the *srfA* operon, a gene responsible for the surfactin lipopeptide antibiotic biosynthesis, functions positively in bacilysin production [204]. In 2011, Türkan et al. exhibited that multiple regulatory factors regulate bacilysin biosynthesis by directly affecting the *bac* operon expression, which includes four master regulators Com A, SpoOA, AbrB, and CodY in *B. subtilis*. The two transcriptional regulators, AbrB and CodY, directly suppress the expression of the *bac* operon, while ComA and SpoOA act as positive transcriptional regulators

that activate and induce the expression of the *bac* operon. In addition, PhrC, PhrF, PhrG, and PhrK are required for full-level activation of ComA, thereby they indirectly positively affect the transcription of the *bac* operon (Figure 1.14) [206]. In addition, two other regulators, LutR (formerly known as YvfI) and ScoC, play a significant role in controlling bacilysin synthesis. LutR acts as a positive regulator, while ScoC acts as a negative regulator [207, 208]. The regulation of the *bac* operon is also influenced by the levels of GTP in the cell [203]. When GTP levels decrease, it triggers an increase in the expression of the *bac* operon. This response is controlled by the CodY repression system [203]. Moreover, the DegS/DegU two-component signal transduction system positively regulates the *bac* operon and *bacG* in *B. amyloliquefaciens* [209].

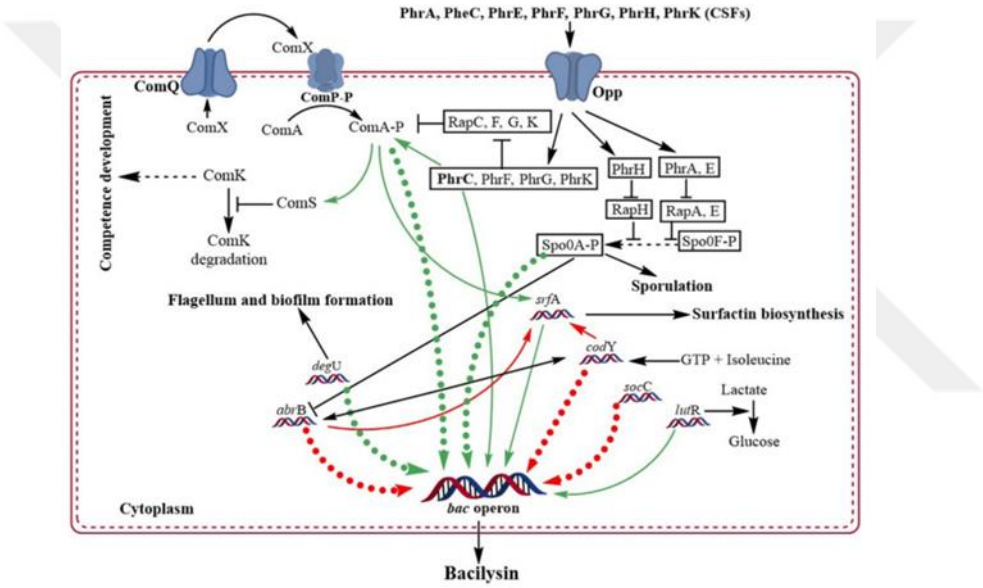

Figure 1.14: All transcription regulators involved in bacilysin biosynthesis in *Bacillus subtilis.* The red dots denote the regulators that bind to the *bac* promoter directly and negatively regulate bacilysin production. The green dots elucidate the regulators which bind to bac promoter and positively regulate bacilysin production. Other red and green arrows elucidate transcription regulators engaged in indirect positive and negative regulation [198].

1.5 Aim of The Current Study

Bacilysin is gaining attention in industrial agriculture and pharmaceutical industries because of its powerful ability to combat bacterial, fungal, and algal pathogens. However, its use in industrial applications is limited due to low production levels in the native producer. Achieving high-level expression of a gene requires more than just high-level transcription. The mRNA produced during transcription must be efficiently translated into the corresponding protein. In bacteria, translation initiation is often the

rate-limiting step of translation and a major factor in determining the final expression level of a gene [210]. Translation initiation occurs at the ribosome binding site (RBS), located within an mRNA's 5' untranslated region (5'UTR). In bacteria, the RBS contains the Shine Dalgarno (SD) sequence, the start codon of the target gene, and a short spacer sequence in between.

The SD sequence plays a crucial role in translation initiation by recruiting the 30S small ribosomal subunit to the start codon through base-pairing with the anti-SD sequence located on the 3' terminus of the 16S rRNA [208]. The effectiveness of an SD sequence is determined by its base-pairing potential with the anti-SD sequence and its spacing from the translation initiation codon [210-212]. The canonical SD sequence, 5-UAAGGAGG-3', found in most bacterial species, including *B. subtilis*, exhibits the highest complementarity with the anti-SD sequence of 16S rRNA, making it a "strong" SD sequence. It was shown that the SD sequence-UAAGGAGG triggers translation four times more efficiently than the shorter AAGGA sequence [212]. The spacing between the SD sequence and the start codon is another critical factor affecting translation initiation efficiency. If the spacing is too close (less than four nucleotides) or too far (more than 14 nucleotides) from the start codon, it can result in lower levels of initiation. In bacterial genomes, the spacing of the SD sequence generally ranges from 5 to 13 nucleotides, with about 8 to 10 nucleotides being ideal for *E. coli* genes. Similarly, the perfect length of separation between the canonical SD sequence, 5-UAAGGAGG-3', and the AUG start codon has been determined to be 7-9 nucleotides [212-214]. The biosynthesis of bacilysin primarily relies on the *bacABCDEF* operon, and the transcription of the bacilysin operon has been found to initiate at T residues located 29 bases upstream of the translational start codon [208].

Analysis of the sequence around the *bacA* transcriptional start site has revealed a strong consensus signature for the *B. subtilis* sigma factor σ^A (TTGACA (-35) and TAAAAT (-10) with a 17-base pair spacer) [215] (Figure 1.15). However, there is no obvious strong SD-like sequence surrounding its translation start codon AUG, suggesting that improving the RBS region could be a promising option for enhancing bacilysin production. Therefore, in this thesis study, it was aimed to investigate the effect of a strong ribosome binding region containing the canonical Shine-Dalgarno (SD) sequence (TAAGGAGG) with an 8-nucleotide spacing from the AUG start codon on bacilysin production in *B. subtilis* PY79. A CRISPR/Cas9 technique was utilized to

modify the *bac* operon's 5' untranslated region (5'UTR). Subsequently, the impact of a strong ribosome binding site (RBS) on the bacilysin production levels was examined. Additionally, it was evaluated whether this modification influenced the mRNA levels of the *bac* operon.

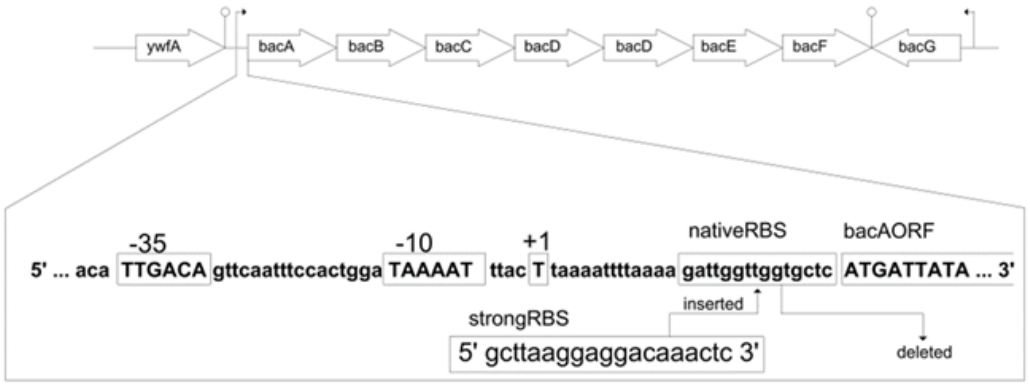

Figure 1.15: Genomic organization of the *bacABCDEF* operon and the upstream DNA sequences of the *bacA* [216].

2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Bacterial strains

A prototrophic derivative of *Bacillus subtilis 168* strain, *B. subtilis PY79,* was used as the bacilysin producer strain. *Escherichia coli* DH5α was used as a host for cloning throughout the study. *recA*- proficient strain *E. coli* BL-21 (DE3) pLysS strain was used to propagate the plasmid DNA to transform *B. subtilis. Staphylococcus aureus* ATCC 9144 strain is used for bacilysin detection as the bioassay organism. All strains used in this study are listed with their genotype in Table 2.1.

2.1.2 Plasmids

All PCR products and genetically manipulated genes constructed throughout this study are cloned into pGEM®- T Easy vector (Promega) (Figure 2.1).

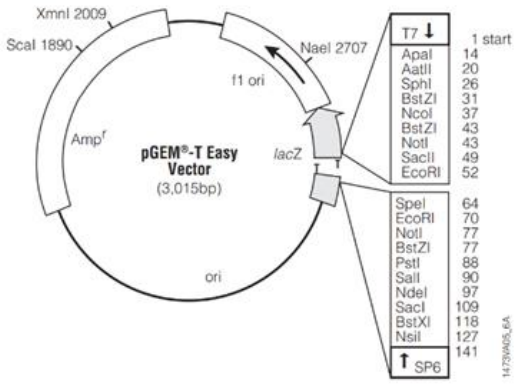

Figure 2.1: Genetic map of pGEM®-T Easy vector and sequence reference points from Promega.

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

Strain	Genotype	Source
Bacillus subtilis PY79	BSP cured prototrophic derivative of *B. subtilis 168*	Lab Collection
E. coli DH5α	F⁻ φ80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F)U169 *rec*A1 *end*A1 *hsd*R17(r_K^-, m_K^+) *pho*A *sup*E44 λ⁻*thi*-1 *gyr*A96 *rel*A1	Lab Collection
E. coli BL-21 (DE3) pLys	*F⁻ompT hsdS$_B$ (r_B^-, m_B^-) gal dcm (DE3) pLysS(Cam^R)*	Lab Collection
Staphylococcus aureus	ATCC 9144	Lab Collection
E. coli JM109 / 9958.1	pJOE9958.1/ Km^R	J. Altenbuchner
E. coli DH5α.RBS-pGMT11	pGMT.*bacAstrong*RBS/ Amp^R	This study
E. coli DH5α.RBS58	pJOE9958.1.*bacAstrong*RBS/ Km^R	This study
E. coli DH5α.bacsg58	pJOE9958.1.*bacAstrong*RBS.sgRNA/ Km^R	This study
E. coli DH5α .582005	pGMT.$_{sgRNA}$ (200bp) Amp^R	This study
E. coli BL21.A5821	pJOE9958.1.*bacAstrong*RBS.sgRNA/ Km^R *recA*- proficient	This Study
E. coli DH5α. bacsg4$^-$ Control Negative (- ve)	pJOE9958.1.*bacA*.$_{sgRNA}$ / Km^R	This Study
E. coli BL21.4213 Control Negative (- ve)	pJOE9958.1.*bacA*.$_{sgRNA}$ / Km^R *recA*- proficient	This Study
E. coli BL-21. 58211 Control Positive (+ ve)	pJOE9958.1.*bacAstrong*RBS.sgRNA/ Km^R *recA*- proficient	This Study
E. coli DH5α. pGMT20-1 (Used for sequencing)	pGMT.*bacAstrong*RBS (400bp)/ Amp^R	This Study
Bacillus subtilis PY79 HWA (Genetically engineered)	P*bacAstrong*RBS	This Study

The pJOE9958.1 plasmid is a CRISPR/Cas9 (Fig.2.2) derived from the shuttle plasmid pJOE8999.1. It is a single-plasmid system based on the *cas9 gene* controlled by the mannose-inducible promoter P_{manP} of *B. subtilis* and a single guide RNA (sgRNA) located between *Bsa*I sites under the control of a strong promoter. Additionally, like pJOE8999.1, it has the temperature-sensitive replication origin of plasmid pE194 for *B. subtilis,* pUC minimal origin of replication for *E. coli,* and a Kanamycin resistance gene expressing in both organisms. Unlike the pJOE8999.1 vector, the pJOE9958.1 plasmid possesses the *ccdB* located between the two *Sfi* cutting sites, which is used as a positive-selection marker that kills the background of cells without DNA insertion, thus only cells owning a recombinant plasmid giving rise to viable clones in the F^- *E. coli* cells [156].

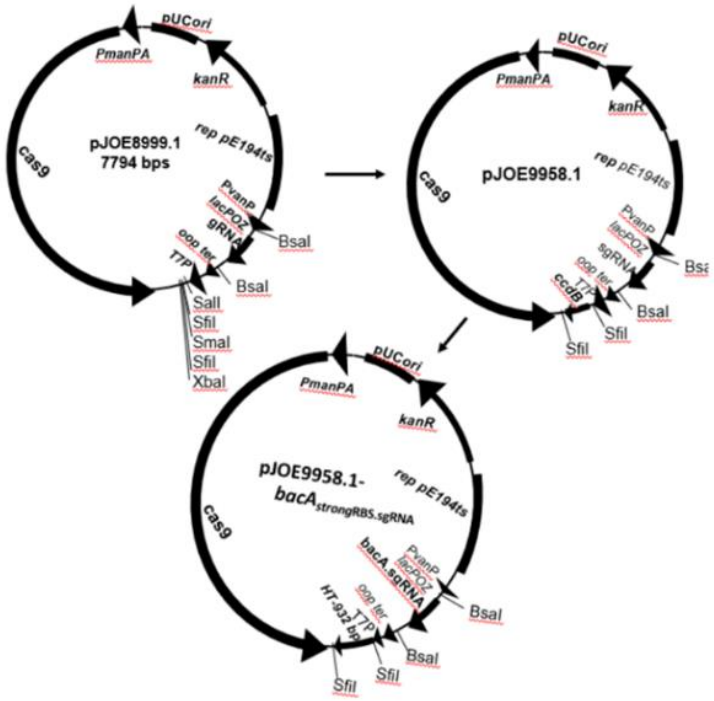

Figure 2.2: Maps of pJOE8999.1, pJOE9958.1 and pJOE9958.1-*bacA*$_{strong}$RBS.sgRNA constructed in this study [216].

2.1.3 Bacterial culture and media.

The preparation techniques and components of all bacterial culture media used in this study are listed in Appendix B.

2.1.4 Solutions and buffers.

The preparation of solutions and buffers with compounds are given in Appendix C.

2.1.5 Enzymes.

The enzymes that are used in this thesis are listed in Appendix D.

2.1.6 Laboratory equipment.

All equipment that is used in this study is listed in Appendix E.

2.1.7 Cultures and maintenance of bacterial strains.

E. coli and *B. subtilis* strains were cultured in Luria- Bertani (LB) liquid medium and stored at 4ºC on LB agar plates at most for one week. However, DSM medium was mainly used for storing *B. subtilis* strains for one month at 4ºC. 10% glycerol/ LB stock was prepared for long-term storage, and all bacterial strains were stocked at – 80 ºC. The Perry and Abraham medium (PA-medium) was used for bacilysin production. While the paper disc-agar diffusion assay was applied to determine the bacilysin bioactivity on Bioassay media agar plates by employing *S. aureus* ATCC9144 as the assay organism.

2.2 Methods

2.2.1 Constructing the DNA homology template

2.2.1.1 Chromosomal DNA isolation

The *B. subtilis* PY79 chromosomal DNA was extracted and purified according to Cutting and Horn's standard method designed explicitly for *Bacillus* species [217]. 1.5 mL of overnight culture of *B. subtilis* was harvested by centrifugation at 13000 rpm for 5 min. The supernatant was discarded, and the pellet was resuspended in 567 µL of TE buffer (Appendix C). 10 µL of proteinase K (20 mg/mL), 6 µL of RNase (10 mg/mL), 24 µL of lysozyme (100 mg/mL) and 30 µL of 10% SDS were added to the cell mixture. The homogenized mixture was incubated for 1 hour at 37°C. Following the incubation, 100 µL of 5M NaCl solution and 80 µL of CTAB/NaCl (Appendix B) (65ºC) solution were added, and the sample was incubated for 10 min. at 65ºC. Freshly prepared phenol/chloroform/isoamyl alcohol (25:24:1) was added to the mixture in equal volume. It was centrifuged at 13000 rpm for 10 min. After centrifugation, the upper phase was carefully transferred to a new 1.5 mL microfuge tube, and 0.7 volume isopropanol was added to precipitate the DNA. After a gentle mix, the sample was centrifuged at 13000 rpm for 15 min. The supernatant was removed, and the pellet was washed with 1 mL 70% ethanol and centrifuged at 13000 rpm for 5 min. Subsequently,

ethanol residues evaporated at 37°C for 1 hour, and isolated DNA was dissolved in 10 µL of TE buffer and stored at 4ºC.

2.2.1.2 Agarose gel electrophoresis

Electrophoresis was applied in a 1% (w/v) agarose gel system prepared with 1X TAE buffer (Appendix C) and RedSafe™ Nucleic Acid Staining Solution (iNtRON) at 4V/cm on a horizontal submarine electrophoresis apparatus. Before uploading the DNA samples into an agarose gel, 6X loading dye was mixed gently with the DNA sample (1:6 v/v) to get 1X diluted dye in the sample. Electrophoresis was performed at 70-100 volts for 30 min. DNA bands were visualized on a shortwave UV transilluminator (UVP).

2.2.1.3 Polymerase chain reaction (PCR)

All primers used in this study with their sequences and melting temperature (TM) are listed in Appendix A. For Polymerase Chain Reaction (PCR), all compounds and reaction conditions generally utilized in this study are organized in Table 2.1 and 2.2, respectively.

Table 2.2: Compounds used for establishing a PCR reaction. Supplied by Thermo Scientific^{Tm}.

Component	Volume/ µl (50 µl in total)
10X Dream Taq polymerase**	5
dNTPs	5
Forward Primer (10 µM)	1
Reverse Primer (10 µM)	1
DNA Template	1
Dream Taq DNA Polymerase (Unite sini yaz) *	1
DNase/ RNase- Free Distilled Water	31

* In constructing the homology template stage, *pfu* DNA polymerase was used (500U).

**10X *pfu* buffer + 1.25 µl $MgSO_4$ in case of *pfu* DNA polymerase.

Table 2.3: General Conditions of Polymerase Chain Reaction Used in this Study.

Step	Temperature / ºC	Time/min	Cycle
Initiation	95	10	
Denaturation			
Denaturation	94	0.30	
Annealing	Based on Primer Tm	0.30	30
Extension	72	0.30	
Final Extension	72	10	

2.2.1.4 Gel extraction and PCR products

All PCR products and desired DNA samples extracted from agarose gel were cleaned and purified using Macherey-Nagel™ NucleoSpin™ Gel and PCR Clean-up Kit from Fisher Scientific, a Thermo Fisher Scientific, following the manufacturer's protocol. All cleaned DNA samples were stored in -20 C°**.**

2.2.1.5 Construction of the homology template via overlap extension PCR

As the first step in this study, the homology template was prepared to bear the new strong RBS (GCTTAAGGAGGACAAACTC) to be ready for repairing the double-strand break caused by the endonuclease Cas9, subsequently providing the replacement of the old ribosome binding region of *bacA* gene. For this purpose, six primers are designed to establish an overlap PCR reaction. A DNA fragment is amplified at the beginning, which stands between 400 bp upstream of the *bacA* open reading frame (ORF) and 80 bp downstream, respectively, using the bac.template forward and the bac.template reverse primers (Appendix A). After the cleanup of the PCR product by using Macherey-Nagel™ NucleoSpin™ Gel and PCR Clean-up System from Thermo Fisher Scientific, it was used as a template for the overlap extension PCR reactions. The DNA fragment obtained from the last step was used as a DNA template for establishing two main PCR reactions: the first reaction (R1) was performed with primers P1 and P2 (Appendix A), and the second reaction (R2) was performed with the primers P3 and P4 by using Pfu polymerase. The PCR products From R1 and R2 were cleaned up and overlapped together with the third PCR reaction by performing 15 cycles WITHOUT using any primers and then additional 25-30 PCR cycles with the primers P5 and P6 that resulted in 932 bp homology template (Figure 2.3) [216].

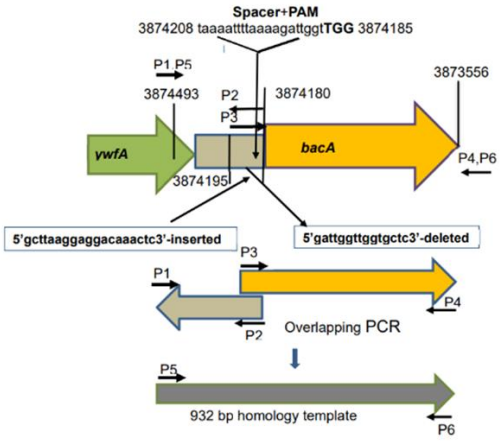

Figure 2.3: Schematic representation of the strong RBS substitution region of the B. subtilis genome and the homology template's construction via overlapping extension PCR [216].

2.2.1.6 Verification stage

Sequence analysis was applied using vector-specific pUC/M13 forward and reverse primers by Medsantek Ltd. Co. to verify the constructed homology template. For this, the following steps were performed.

Cloning of the constructed homology template into pGEM®- T easy cloning vector

For sequence analysis, the constructed 932 bp homology template was cloned into pGEM®- T Easy vector (Figure 2.1). Before the insertion of the homology template into the pGEM-T vector, the homology template is incubated with dream Taq polymerase for around one cycle at 72ºC for 30 min in the PCR instrument for Adenine (A) tailing; the PCR reaction of incubation with Taq polymerase is shown in Table 2.3. Then the resulting product was uploaded into agarose gel electrophoresis. After extracted and cleaned up from the gel, it was ligated into pGEM®-T easy cloning vector. The ligation reaction to pGEM®-T easy cloning vector is shown in Table 2.4. The ligation mixture (Table 2.5) was incubated at 22ºC for 10 min and then at 4ºC overnight. The T4 ligase was deactivated by incubating the ligation mixture at 65°C for 10 minutes. The resulting cloned pGEM®-T was named **RBS- pGEM11 (pGMT.*bacA*$_{strong}$RBS)** and stored at – 20 º C for further research.

Table 2.4: Component of the PCR reaction used for incubating the constructed homology template with Taq polymerase before cloning in pGEM®-T easy cloning vector.

Component	Volume (µl)/ 10 µl
Dream Taq polymerase 10X Buffer	1
dNTPs	0.2
Dream Taq Polymerase	1
Constructed DNA Homology Template	6
DNase/ RNase- Free Distilled Water (dH_2O)	1.8

Table 2.5: Component of ligation reaction into pGEM®-T easy cloning vector.

Component	Volume (µl)/ up to 10 µl
2X Rapid Ligation Buffer	5
pGEM®-T Easy Vector (50ng/µl)	1
Inserted DNA Homology Template	3
T4 DNA Ligase (3 Weiss units/µl)	1

2.1.6.2 Transformation into *E. coli*

Preparation of chemically competent *E. coli* DH5α cells

An overnight *E. coli* DH5α growth culture was prepared by inoculating a single colony into (3 ml) of 2XYT medium and incubating at 37°C with vigorous shaking overnight. The next day, 1 ml of the growing culture was inoculated into 50 ml 2XYT medium and incubated at 37 C° at 180 rpm. When the optical density at 600 nm (OD_{600}) reaches 0.4 - 0.5, 20 ml of the culture aliquots were placed into screwcap centrifuge tubes (50 ml volume tube) and incubated on ice for 10 min. and then centrifuged at 5000 rpm for 10 min. at 4°C. After discarding the supernatant, the pellet was resuspended in 10 ml of 100 mM $CaCl_2$ solution and incubated on ice for 30 min. In the last step, the sample was centrifuged once again at 5000 rpm for 10 min at 4°C, and the supernatant was removed. The pellet was resuspended in 1 ml of 100 mM $CaCl_2$ solution with Glycerol (110 µl), split into 1.5 ml Eppendorf tubes, and stored at -80°C.

Transformation of chemically competent *E. coli* DH5α cells

Pre-prepared competent *E. coli* cells were melted on ice. 5-10 µl of the ligation product was added to the cells and incubated on ice for 30 min. Then, heat shock was applied at 42 ^{o}C for (2 minutes). The tube was put back on the ice to reduce cell damage for about 5 min. Next, 1 ml of LB medium was added to the cells. After incubation at 37^{o}C for 1 hr with vigorous shaking at 180 rpm, cells were harvested by centrifugation at 13000 rpm for 5 min. and resuspended in 200 µl of physiological saline solution. Finally, 100 µl of the resuspended cells were spread on an LB plate with selective antibiotics (Amp) and incubated at 37^{o}C overnight. Consequently, the transformants containing RBS- pGEM11 (pGMT.$bacA_{strong\text{RBS}}$) that bears the strong RBS of the *bacA* gene were selected and screened on X-gal/IPTG LB plate with ampicillin (100 µg/ml). The resulting transformant strain, established in this stage, was named *E. coli* RBS-pGMT11, which contains pGMT.$bacA_{strong\text{RBS}}$. It was cultured in %10 glycerol LB liquid media with ampicillin (100 µg/ml) and stored at -80 ^{o}C for further use.

Verification stage of RBS- pGEM11 (pGMT.$bacA_{strong\text{RBS}}$)

Plasmid DNA isolation

The RBS- pGEM11 plasmid was extracted following the Plasmid DNA Extraction general protocol from a fresh bacterial growth streaked on a selective LB agar plate. For this, a single colony was inoculated into 3 ml of LB broth medium with an appropriate antibiotic Amp (100 µg / ml) for RBS- pGEM11, then incubated overnight at 37 ^{o}C with vigorous shaking. The next day, the growing cells were harvested by centrifugation at 13000 rpm for 5 min. After centrifugation, the supernatant was discarded, and the pellet was resuspended in 330 µl of B1 buffer (Appendix C). Next, 330 µl of B2 buffer was added to the cell suspension, gently inverting the mixture 4-7 times, and then incubated for 5 min. at room temp. After that, 330 µl of chilled B3 buffer was added to the mixture, immediately inverting the tube 4-7 times and incubating on ice. After incubating for 15 min. on ice, centrifugation at 13000 rpm for 15 min. was applied, and then the supernatant was transferred to a new sterile tube. For 0.7 volume of supernatant, isopropanol was added into the tube. The plasmid DNA was precipitated by centrifugation at 13000 rpm for 30 min. After removing the supernatant, the pellet (Plasmid DNA) was washed with 1ml of %70 ethanol by centrifugation at 13000 rpm for 5 min. After the ethanol was evaporated completely,

plasmid DNA was dissolved in 20-25 µl elution buffer (Appendix C) and stored at -20 until used.

Verification of the cloned vector via PCR and restriction enzymes

The cloning pGEM-T vector that bears the strong RBS of *bacA* (DNA homology template) was checked using specific primers like *bacA* forward and *bacA* reverse, and *bacA* forward with P6. As well as it was digested with specific restriction enzymes like *Sfi*I, *Not*I, and *Eco*R1 following the general restriction enzyme reaction shown in Table 2.6 below. Enzyme digestion reactions were incubated at 37^{o}C for 1 to 5 hours.

Table 2.6: General restriction enzymes digestion reaction.

Component	Volume (µl)/ Total Volume 10 µl
10X Buffer	1 µl
DNA (0.5-1 µg/µL)	1 µl
Enzyme	1 µl
Nuclease-free water	7 µ

2.2.2 Ligation of desired homology template into pJOE9958.1

2.2.2.1 Cutting with *Sfi*I restriction enzyme

Cutting pJOE9958.1 plasmid with *Sfi*I restriction enzyme

The cutting with *Sfi*I was performed as shown in Table 2.7. The digestion reaction was done by incubating the pJOE9958.1 with the *Sfi*I restriction enzyme for 2 hr at 37 ^{o}C. After adding 1µl of Calf Intestinal Alkaline Phosphates (CIAP), the digestion reaction was incubated for 1 hr. at 37 ^{o}C, and CIAP was deactivated by incubating at 65 ^{o}C for 15 min. The digested pJOE9958.1 vector was uploaded on agarose gel electrophoresis. It was extracted from the gel and cleaned using PCR Clean-up Kit (Macherey-Nagel) according to the manufacturer's protocol.

Table 2.7: Digestion reaction of pJOE9958.1 plasmid with *Sfi*I restriction enzyme.

Reaction components	Volume (20 µl total reaction volume)
10X Buffer	2 µL
DNA (18-19 µg/µL)	4 µL
*Sfi*I Restriction Enzyme	2 µL
Nuclease-free water	12 µL

Digestion of RBS- pGEM11 (pGMT.*bacA*$_{strong\text{RBS}}$) vector with *Sfi*I restriction enzyme.

RBS- pGEM11 vector that contains desired homology template constructed before was cut with *Sfi*I, as shown in Table 2.8. The digestion reaction was incubated at 37ºC for 2 hr; the digestion mixture was then applied on agarose gel electrophoresis. 932 bp DNA insert (the homology template) was extracted from the agarose gel and cleaned with PCR Clean-up Kit (Macherey-Nagel).

Table 2.8: Digestion reaction of pGEMT.*bacA*$_{strong\text{RBS}}$ vector with *Sfi*I restriction enzyme.

Reaction components	Volume (30 µl total reaction volume)
10X *Sfi*I Buffer	3 µl
DNA (40.3 µg/µL)	5 µl
*Sfi*I Restriction Enzyme	2 µl
Nuclease-free distilled water	20 µl

2.2.2.2 The ligation of homology template into pJOE9958.1 expression vector.

After cutting with the *Sfi*I restriction enzyme, the homology template containing the new strong RBS of the *bacA* gene was ligated into the pJOE9958.1 vector using T4 DNA ligase. The Ligation reaction mixture is given in Table 2.9. The reaction mixture was incubated at 22ºC for 10 minutes and then at 4ºC overnight.

Table 2.9: Ligation reaction of homology template into the pJOE9958.1 vector.

Reaction Components	Volume (10 µl total reaction volume)
2X Rapid Ligation Buffer	1 µl
pJOE9958.1 (19 ng / µl)	5 µl
DNA insert:homology template (40.3 ng/ µl)	1 µl
T4 DNA Ligase (3 Weiss units/µl)	1 µl
Nuclease-free distilled water	2 µl

2.2.3. The preparation of the sgRNA for targeting of *bacA*$_{RBS}$.

sgRNA for targeting of *bacA* RBS site is designed with the CRISPR/Cas9 online prediction tool CCTop [218]. *Bacillus subtilis* subsp. subtilis 168 genome sequence (Accession CP010052) was selected as the reference. Target site and core lengths were adjusted to 20 bp and 12 bp, respectively. A total of five mismatches were allowed. This sequence was synthesized into two complementary oligonucleotides with custom 5′ overhangs (TACG and AAAC) to produce compatible ends to the *Bsa*I-treated vector pJOE9958.1.*bacA*$_{strong}$RBS and inserted into the *Bsa*I-cut RBS58 (pJOE9958.1.*bacA*$_{strong}$RBS) to get **pJOE9958.1.*bacA*$_{strong}$RBS.sgRNA** (Figure 2.2). The insertion of the sgRNA into the *Bsa*I-cut pJOE9958.1.*bacA* was achieved as the following; firstly, synthesized sgRNA was phosphorylated by using T4 polynucleotide Kinase (PNK) as shown in Table 2.11. The reaction was incubated at 37 ºC for 30 min. then the PNK was deactivated by incubation at 65ºC for 20 min.

Table 2.10: Phosphorylation reaction of the sgRNA- of-*bacA*$_{RBS}$.

Component	Volume µl
T4 DNA ligase buffer	1
T4 polynucleotide Kinase (PNK)	1
sgRNA-of- BacA RBS Diluted sample up to 10^{-4} from the main stock.	20

Subsequently, the phosphorylated sgRNA product was ligated into the *Bsa*I-cut-dephosphorylated pJOE9958.1.*bacA*$_{strong}$RBS vector to get pJOE9958.1.*bacA*$_{strong}$RBS.sgRNA which was done according to Table 2.12.

Table 2.11: Reaction of ligation of the sgRNA-of-*bacA*$_{RBS}$ encoding sequence into the *Bsa*I-cut RBS58 (pJOE9958.1.*bacA*$_{strong RBS}$).

Components	Volume (10 µl total reaction volume)
2X Rapid Ligation Buffer	1 µl
Digested RBS58 by Bsa1 (18 ng/µl)	1 µl
sgRNA insertion fragment (0.147 ng/ µl)	5 µl
T4 DNA Ligase	1 µl
Nuclease-free distilled water dH2O	2 µl

2.2.4 Transformation of *Bacillus subtilis* PY79 competent cells with the pJOE9958.1. *bacA*$_{strong}$RBS.sgRNA plasmid DNA

The plasmid DNA used to transform *B. subtilis* was first propagated in the recA-proficient strain *E. coli* BL21(DE3) pLysS to form the multimeric plasmid DNA, thereby increasing the efficiency of *B. subtilis* transformation. The transformations of the *B. subtilis* PY79 competent cells were achieved, as represented by Klein et al. (1992). To prepare the *B. subtilis* competent cells, HS and LS media were utilized (Appendix B). One day before, a loop full of fresh *B. subtilis* mass grown on an LB agar plate was inoculated into 3 ml of HS medium and incubated at 37^{o}C overnight by shaking at 180 rpm. The next day, 500 µl of the overnight culture was inoculated into 20 ml of freshly prepared LS medium in a 250 ml sterile flask and incubated at 30 ^{o}C with gently shaking at 100 rpm until OD_{600} value reached 0.53- 0.55. Then, 5 µl of the multimerized pJOE9958.1. *bacA*$_{strong}$RBS.sgRNA plasmid DNA was added into 1 ml of the competent cells in the 2 mL Eppendorf tube and incubation at 37^{o} C for 2 hr with shaking at 250 rpm. After centrifugation at 5000 rpm for 15 min, the supernatant was discarded, and the pelleted cells were resuspended in 100 µl of physiological saline solution. Then, the cell resuspension was spread on an LB agar plate with Kanamycin (Km) (50 µg/ml) and 0.2% mannose for induction of cas9 under the control of P$_{manP}$ and incubated at 30^{o}C. Km^{R} colonies appearing within two days were streaked on LB plates containing Km and 0.5% glucose and incubated at 30^{o}C overnight [219].

2.2.5 The Plasmid-Curing Step.

Before plasmid curing, Km^R colonies were first screened for bacilysin phenotype to eliminate the potential bacilysin nonproducers (Bac^-) since they are likely to have arisen from cells repaired through the non-homologous end-joining (NHEJ) pathway. Km^R colonies were cultured on a bioassay plate containing *Staphylococcus aureus* ATCC9144 as the assay organism using the pick and patching technique at 37°C overnight. The colonies arising an inhibition zone (Km^R and Bac^+) were selected and placed on the LB agar plate WITHOUT antibiotic to single colonies at 50°C overnight as a nonpermissive temperature for plasmid replication and then restreaked to single colonies on LB plates at 42°C. In the last step, all colonies were checked for plasmid-lack by cultivating on an LB agar plate with and without Km at 42°C overnight. Km^S (plasmid-cured) colonies were selected and kept for further studies.

2.2.6 Bacilysin Production.

Fresh culture of *B. subtilis* strains grown on LB agar was used to inoculate 10 ml of PA medium and cultivated overnight at 37°C by shaking at 200 rpm. These overnight cultures were used to inoculate 100 ml of fresh PA medium to an initial OD_{600} value of 0.1. The cultures were incubated at 37°C for 24 h by shaking at 200 rpm. The growth of cultures was monitored at $OD_{600,}$ and bacilysin activity in culture fluids was determined by the paper disc-agar diffusion assay using *S. aureus* ATCC 9144 as the assay organism [200]. For the disk diffusion method, 6.0 mm paper disks were sterilized by Autoclave (15 İb/in^2,15 min). The antibiotic disks were first treated with 20 µl of acetone and left for 5-10 min. After the acetone was wholly evaporated, 20 µl of culture supernatant was embedded into a 6.0-mm paper disk, which was then transferred onto bioassay plates containing assay organisms, prepared as described in Appendix B and incubated for 16 hr at 37°C.

2.2.7 Colony PCR for *Bacillus subtilis*.

a single colony of *B. subtilis* from an agar plate and put into 10 µl of Tris buffer (5 mM Tris/HCL, pH8.5). The sample was incubated on ice for 5 min, then incubated for 1 min in the microwave for full power. Next, the sample was incubated in an ice bath for 30 sec. After that, the previous process was repeated twice, three times in the microwave, then incubated in an ice bath for five min. Finally, 1 µl of the sample was directly used for a PCR reaction as a template DNA. At the end of PCR, to prevent the

degradation of the PCR product, EDTA was added in a final concentration of 50 mM since *B. subtilis* has thermostable DNAse and run it immediately**.**

2.2.8 Ultra-Performance Liquid Chromatography-Mass Spectrometry (UPLC-MS) Analysis.

To extract 200 µl of culture supernatants, one-fourth of the volume of butanol was utilized. The resulting mixture was then lyophilized to dryness. Samples were analyzed by UPLC-MS (Waters Acquity UPLC H-Class and Waters Synapt G2-Si HDMS) in the positive detection mode. After dissolving in 10 µl of a solvent mixture (H_2O: 5% CH3CN + 1% HCOOH), 7.5 µl of the sample was loaded onto a Waters Acquity Peptide BEH C18 column (2.1 mm × 100 mm) that had been equilibrated in a solution mixture (1% CH_3CN + 0.1% HCOOH + H_2O) with a flow rate of 0.2 ml/ min. The temperature in the column was kept constant at 65°C. The following gradient was used to elute bound compounds: 0.1% HCOOH: H_2O (Solution A) and CH_3CN (Solution B): 1% Solution B for 1 min, 1–40% of Solution B over 10 min, 40–80% Solution B over 1 min and kept for 2 min, 80–1% of Solution B over 50 sec, and re-equilibration with 1% Solution B for 10 min before loading the following sample.

2.2.9 RT-qPCR Analyses

Fresh cultures of strains produced on LB agar were used to inoculate 10 mL of PA medium and cultured overnight at 37ºC by shaking at 200 rpm. This overnight culture was used to inoculate 100 mL of fresh PA medium to an initial OD600 of 0.1 and grown for 24 hr at 37ºC by shaking at 200 rpm. Total RNA extractions were performed on samples obtained at the early and late stationary phases of growth (corresponding to 18 and 24 hr of growth, respectively). The Qiagen RNeasy Mini Kit extracted total RNA from the samples. The Nanodrop Lite spectrophotometer (Thermo Fisher Scientific, USA) was used to determine the quality and quantity of RNA. 2 µg of total RNAs were reverse transcribed using a Transcriptor cDNA Synthesis Kit (Roche) and random hexamer primers (60 M) as directed by the manufacturer. The SYBR Green Master Mix Kit (Roche) and a Light Cycler 480 (Roche) instrument were used for qPCR amplification and detection. Then, in a 20 µl real-time PCR mixture, 2 µl of generated cDNAs were employed as the template. Preheating at 95°C for 5 min was followed by 45 cycles of 95°C for 5 seconds, 52°C for 20 seconds, and 72°C for 30 seconds. The specificity of the reaction was monitored via melting curve analysis. The

relative transcription level of the target gene was calculated by the $2^{-\Delta\Delta CT}$ method [220], with the *sigA* gene serving as an internal reference. Two independent qPCR assays were carried out, each with three biological and two technical replicates. The t-test was used for statistical analysis, and statistical significance was stated as ns (no significance, $p > 0.05$), *($p < 0.05$), and **($p< 0.01$).

3. RESULTS AND DISCUSSION

3.1 Designing of Strong RBS Sequence

In this study, the canonical strong SD sequence "5`-TAAGGAGG-3`" combined with "ACAAACTC" eight-nucleotide spacer sequence, thus resulting in the 5`-*GCTTAAGGAGGACAAACTCATG*-3` RBS sequence was named $bacA_{strongRBS}$. In this sequence, "*ACAAAC*" was selected as an optimized spacer sequence typically used within the *B. subtilis* IPTG inducible P $_{Phyperspank}$. Additionally, it was also used as the standard spacer by Guiziou et al. [221]. However, the eight-nucleotide spacer sequence was the optimum sequence in previous studies [207][211][222]. Therefore, the spacer sequence "*ACAAAC*" was boosted with the original "TC" nucleotides upstream of the start codon ATG. Subsequently, the RBS calculator (https://www.denovodna.com/software) [223], which is vastly used to estimate the translation initiation rate of RBS sequence, was also used to predict the expected translation initiation rate of $bacA_{strongRBS}$ and $bacA_{nativeRBS}$ *(5'-GATTGGTTGGTGCTCATG-3')* in advance before in vivo analyzing. As shown in Figure 3.1, the predicted translation initiation rate of $bacA_{strongRBS}$ was 219113,65 au, which was approximately 93-fold higher than that of the native RBS sequence from *bacA* (2347,58 au). These results verified our hypothesis about the weak performance of the native RBS region of the *bac* operon along with the positive impact of a strong RBS.

Figure 3.1: Expected translation initiation rates of bacAstrongRBS **(A)** and bacAnativeRBS **(B)** predicted by using the RBS calculator [216].

3.2 *bacA*-5'UTR Editing

3.2.1 Construction of the homology template possessing the strong RBS-sequence

The delivery of gRNA and the repair template using CRISPR/Cas9 single-plasmid systems were effectively utilized for genome editing in *B. subtilis* (Altenbuchner J. 2016). This approach was also successfully applied for gene-operon deletion and mutation repair in *B. subtilis* [156] [167]. Therefore, for editing *bacA*-RBS, we opted to employ a CRISPR/Cas9 single-plasmid system called pJOE9958.1, based on the wild-type cas9 gene controlled by the mannose-inducible promoter P_{manP}. To enable CRISPR/Cas9-based genome editing on the *bac*A-5'UTR, a 932 bp homology template consisting of 19 nt $bacA_{strongRBS}$ flanking by the -298 bp upstream and 615 bp

downstream of the *bacA* start codon was established by performing an overlap-extension PCR approach as shown in Figure 3.2. For this, as a first step, one thousand two hundred base pair (1200 bp) DNA in length, which stands between four hundred base pair (400 bp) upstream and eighty base pair (80 bp) downstream of the *bacA* ORF, was amplified by PCR using chromosomal DNA of *B. subtilis* PY79 wild type strain as a template and the bac.template forward and the bac.template reverse primers (Appendix A) Figure 3.3. As the second step, the resulting amplicon (~1200 bp) was used as a DNA template for the two overlapping extension PCR reactions (R1) and (R2) after verification with PCR using the *bacA* gene-specific forward and reverse primers (Figure 3.4) The first overlap extension PCR reaction (R1) performed with the primers P1 and P2 resulted in ~400 bp PCR product (Figure 3.5) and the second overlap PCR reaction (R2) performed with the primers P3 and P4 ~600 bp PCR product (R2) as expected (Figure 3.6). Subsequently, R1 and R2 PCR products were overlapped together with the third PCR reaction by performing 15 cycles WITHOUT using any primers and then additional 25-30 PCR cycles with the primers P5 and P6 (the fourth PCR reaction), which yielded 932 bp homology template (Figure 3.7).

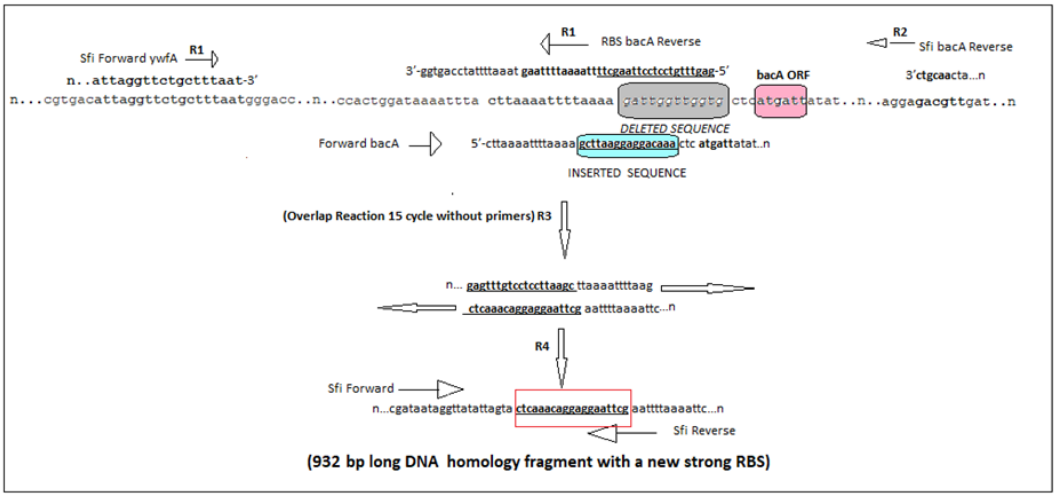

Figure 3.2: The construction of a 932bp Homology Template using the splicing by overlap extension PCR. The genetic manipulation appeared near the pink shadow of *the bacA ORF* (n…atgatt...n). The deletion sequence is shadowed in gray (n…*gattggttggt*ctcatg…n), and the insertion sequence is light blue (n…*gcttaaggaggacaaa*ctcatg…n). The PCR fourth reaction (R4) was amplified by the primers P5 and P6.; as a result, the desired 932bp Homology Template holding a new strong RBS sequence (inside the red box) was established and became ready to be cloned.

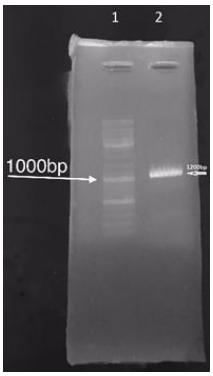

Figure 3.3: ~ 1200 bp DNA template for the overlap extension PCR reactions. Line 1: GeneRuler^{TM} DNA Ladder Mix. line 2: the 1200bp PCR product.

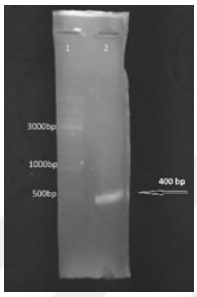

Figure 3.4: Verification of the 1200 bp DNA template using *bacA*-specific primers. Line 1: GeneRuler^{TM} DNA Ladder Mix. Line 2: ~400 bp PCR product.

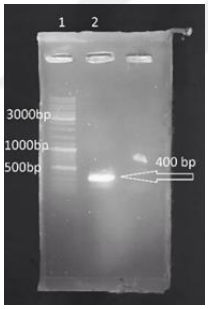

Figure 3.5: First PCR reaction (R1) product performed with the primers P1 and P2, Line 1: GeneRuler^{TM} DNA Ladder Mix, Line 2: 400 bp PCR product.

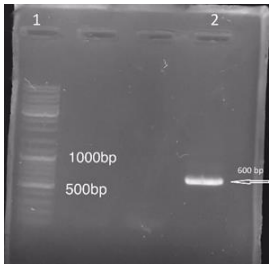

Figure 3.6: Second PCR reaction (R2) product performed with the primers P3 and P4, Line 1: GeneRuler^{TM} DNA Ladder Mix, Line 2: 600 bp PCR product.

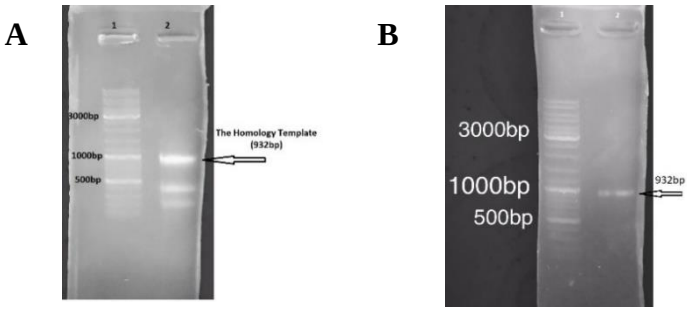

Figure 3.7: Overlap extension PCR product amplified by the primers P5 and P6, **(A)** line 1: GeneRulerTM DNA Ladder Mix. Line 2: 932 bp homology template **(B)** 932 bp homology template after extraction from agarose gel and purification (932 bp).

The overlap extension PCR, also known as the splicing by overhang extension polymerase chain reaction, is one of the polymerase chain reaction techniques that is used to create new sequences that do not exist in the original DNA template by splicing two smaller DNA sequences or more together into one large DNA fragment without dependence on ligase or restriction sites and used for genetic engineering purposes [224– 230]. Unlike routine PCRs, it is critical that utilize Phusion proofreading DNA polymerase (*Pfu* DNA polymerase) instead of *Taq* DNA polymerase in overlap extension PCR techniques [224][231] since *Pfu* polymerase possesses 3` to 5` exonuclease proofreading activity, which makes this enzyme highly accurate with a low error rate, about one error in one million three hundred thousand base pair, during the DNA amplification and results in blunt-ended amplicons [226- 237]. Taq DNA polymerase, in contrast, has 5` to 3` exonuclease activity due to an inactive proofreading domine in its enzymatic structure [238-240]. The accuracy and efficiency of *Taq* polymerase particularly decreases with amplicons sequences longer than one kilobase. Furthermore, it has a high error rate of around one error in nine thousand nucleotides and the ability to add adenine at the 3` end of PCR amplicons that may create unwanted mutations in the constructed DNA amplicons [241, 242]. All these reasons make *Taq* polymerase problematic for DNA cloning and sequencing applications. Moreover, *Pfu* proofreading DNA polymerase acts slower than *Taq* DNA polymerase, so the PCR extension time at 72 ^{0}C is increased to 1 minute for each cycle [225].

3.2.2 Verification of the homology template DNA

Following the construction stage, the 932 bp homology template was cloned into the pGEM®-T Easy Vector, forming pGEMT.*bacA*$_{strong}$RBS, and transformed into *E. coli*

DH5α competent cells, giving *E. coli* RBS pGEMT11 for maintenance and easy sequencing. To verify the cloning of the homology template DNA, plasmid DNAs isolated from the transformants were separately digested with *Not*I and *Eco*RI. *Not*I digestions resulted in a 3015 bp pGEMT vector and a 932 bp DNA insert (Figure 3.8) due to the two *Not*I cutting sites in the pGEMT Easy vector. *EcoR*I, digestion yielded a 3015 bp pGEMT vector and an approximately 700-800 bp DNA insert (Figure 3.9) since there are three *Eco*RI cutting sites, two of them in the pGEMT easy vector itself while the other is inside cloned homology template DNA (see Figure 2.1 in Chapter 2). The plasmid DNAs isolated from the transformants were also screened via PCR by using the *bac*A gene-specific primers, which produced a 400 PCR fragment as expected (Figure 3.10), and using the primers *bacA*-forward and P6, resulting in a 700 bp PCR fragment as expected (Figure 3.11).

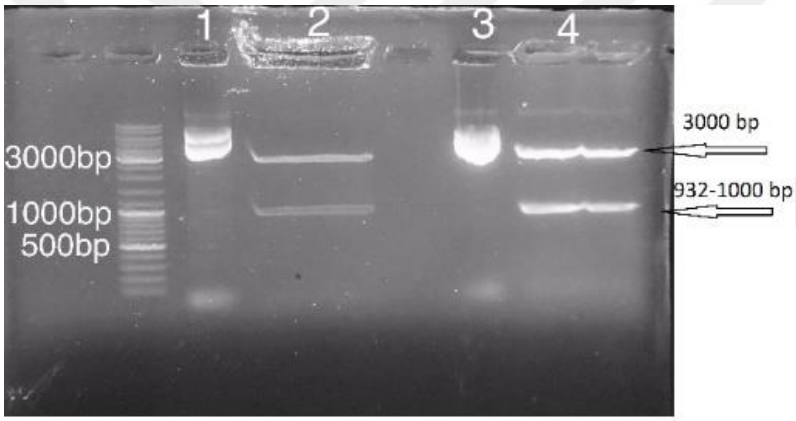

Figure 3.8: *Not*I digestion of the plasmid DNAs isolated from the selected transformants. Line 1 represents an undigested vector of transformants 1; Line 2 represents the digested vector of transformant 1. Line 3 and 4 represent undigested and diegected vectors of transformant 2, respectively. Marker: GeneRulerTM DNA Ladder Mix.

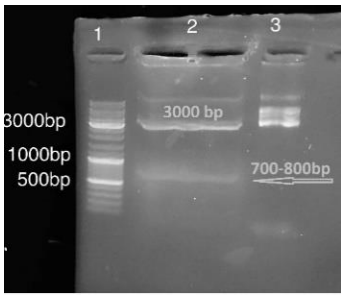

Figure 3.9: *EcoR*I digestion of plasmid DNA isolated from a selected transformant. Line 1 represents GeneRuler^{TM} DNA Ladder Mix. Line 2 represents a digested vector of a transformant. Line 3 represents the undigested vector.

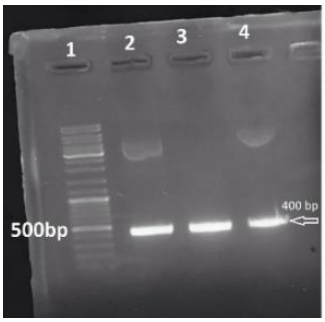

Figure 3.10: Screening of the plasmid DNAs isolated from the transformants via PCR using *bac*A gene-specific primers. Line 1 represents GeneRuler^{TM} DNA Ladder Mix. Line 2-4 400 bp PCR products of the plasmid DNAs isolated from transformants.

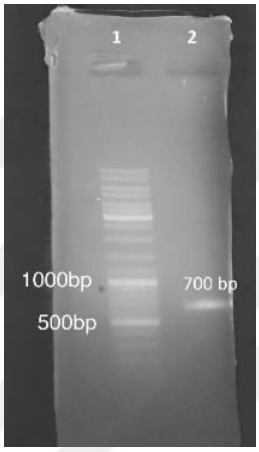

Figure 3.11: Amplification of the pGEMT.*bacA*$_{strong}$RBS vector with *bacA* forward and *Sfi*I bac A reverse primers. Line 1 represents GeneRuler^{TM} DNA Ladder Mix. Line 2 represents the PCR product of recombinant; their size is 700 bp.

After verification, the pGEMT.*bacA*$_{strong\ RBS}$ plasmid was submitted for sequencing using pUC/M13 Forward Sequencing [5′-d(CGCCAGGGTTTTCCCAGTCACGAC)-3′]. As shown in Figure 3.12, the outcomes of the sequence analysis verified the whole sequence of the DNA homology template bearing the new strong RBS of the *bacA* gene(5`-*GCT**TAAGGAGG**ACAAACTC**ATG***-3`).

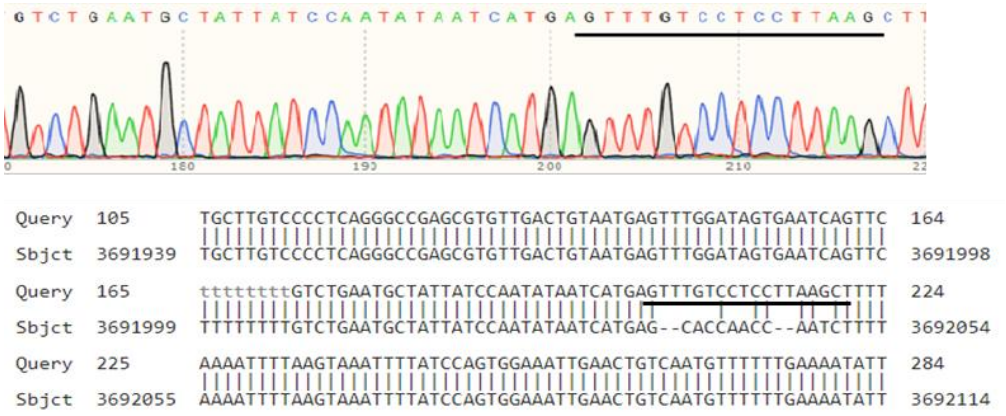

Figure 3.12: Sequence analysis of the constructed homology template bearing the new ribosome binding region. The reverse complement sequence of the introduced *bacA*$_{strong}$RBS sequence "GCTTAAGGAGGACAAA" is highlighted in its DNA sequencing chromatogram and its BLAST analysis result (https:// blast.ncbi.nlm.nih.gov/Blast.cgi). The Blast analysis used *B. subtilis* PY79 (taxid:1415167) as the reference genome Its sequence is the reverse complement.

3.2.3 Cloning of the homology template into the pJOE9958.1 CRISPR/Cas9 single plasmid Vector

To enable CRISPR/Cas9-based genome editing on the *bac*A-5'UTR, the 932 bp homology template DNA possessing the 19 nt *bacA*$_{strongRBS}$ was cloned between the two *Sfi*I sites in the plasmid pJOE9958.1. For this, the *Sfi*I digestion reactions were performed in two stages; the first stage was the *Sfi*I digestion of pJOE9958.1 which yielded two DNA fragments. One was the 7294 bp digested vector purified from the 1% agarose gel, and the second was a 500 bp DNA fragment eliminated (Figure 3.13). The digested vector was then dephosphorylated with Calf Intestinal Alkaline Phosphatase (CAIP from Invitrogen^{TM} by Thermo Fisher Scientific), which is also known as Alkaline Phosphomonoesters by catalyzing the hydrolysis of 5`- phosphate termini from the linear vector to avoid re-circularization of the linear vector. This increases the ligation reaction efficiency and minimizes the number of colonies containing un-cloned vectors [243, 244]. Mammalian Alkaline Phosphatase was used in this study because it is 20-30-fold more active than bacterial alkaline phosphatase (BAP) due to the differences in the active-site sequences between the two enzymes. The Histidine (His) substitutions at the 153 and 328 positions in this mammalian enzyme's active site leads to a change in their properties [245].

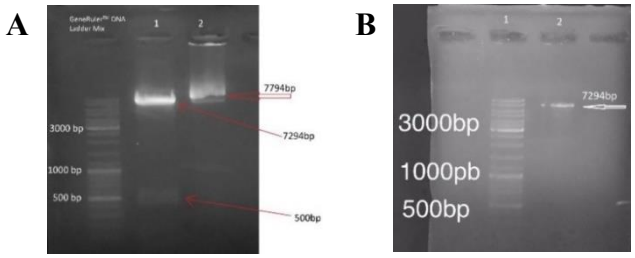

Figure 3.13: The *Sfi*I digestion of the pJOE9958.1 vector. **(A)** Line 1 represents the un-digested vector; Line 2 represents the digested vector. **(B)** Line 1 represents the GeneRulerTM DNA Ladder Mix; Line 2 represents the linear vector (7294 bp) after purifying from % 1 agarose gel.

The second stage of the *Sfi*I digestion reaction was the *Sfi*I digestion of the pGEMT.$bacA_{strongRBS}$ vector. Before the *Sfi*I digestion, the 932 bp homology template on pGEMT.$bacA_{strong}$RBS was verified by PCR using the *bacA* forward and the P6 primers, which resulted in the 600-700bp PCR fragment, as expected (Figure 3.14A). The *Sfi*I digested 932 bp homology template (Figure 3.15B) was purified from the 1% agarose gel (Figure 3.14C).

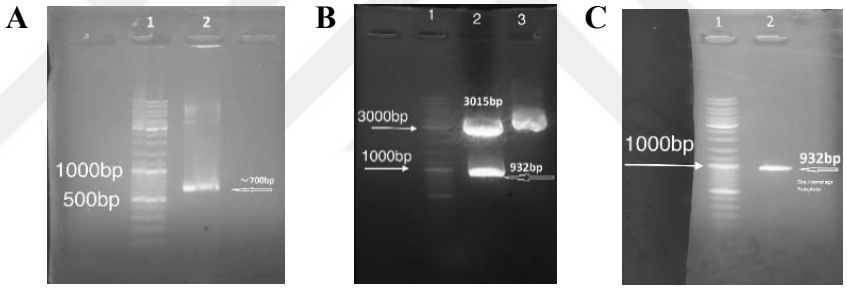

Figure 3.14: The *Sfi*I digestion stages of the pGEMT.$bacA_{strong}$RBS vector. **(A)** The amplification of construct 932bp homology template with the bacA Forward and P6 primers; Line 1 defines the GeneRuler^{TM} DNA Ladder Mix; Line 2 defines the 700bp PCR product. **(B)** The *Sfi*I digestion of the pGEMT.$bacA_{strong}$RBS vector; Line 1 represents GeneRuler^{TM} DNA Ladder Mix; Line 2 defines the digested vector (3015 bp) and the 932 bp homology template; Line 3 means the un-digested vector (3947bp). **(C)** The digested 932bp homology template after purifying from the %1 agarose gel.

The 932 bp homology template was then ligated into the linear pJOE9958.1 vector (7294 bp) using T4 DNA ligase to get a new vector to get the pJOE9958.1.$bacA_{strong}$RBS plasmid (8226bp in size). The ligation mixture was transformed into *E. coli*DH5α strains and the resulting transformants were re-named *E. coli*DH5αRBS58. Subsequently, the plasmid DNAs were extracted from seven selected transformants (Figure 3.15) and screened via PCR using *bacA*-specific primers and *EcoR*I and *Sfi*I

digestion (Figure 3.16) *EcoR*I has four cutting sites; three of them in the pJOE9958.1 itself and one inside the cloned DNA fragment (see Figure 1.11 in chapter one) thus three DNA fragments were observed; ~3200 bp, 4500 bp, and 1100 bp respectively, *Sfi*I digestion resulted in two DNA fragments with 7294 bp and 932 bp length, respectively, as expected.

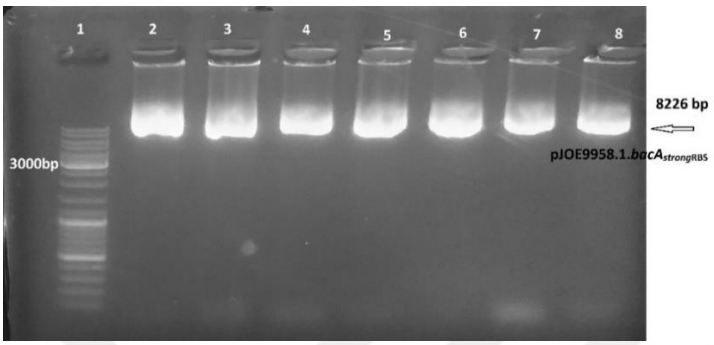

Figure 3.15: Plasmid DNAs isolated from seven selected transformants in % 1 agarose gel (Lines 2-8). Line 1 represents GeneRuler^{TM} DNA Ladder Mix.

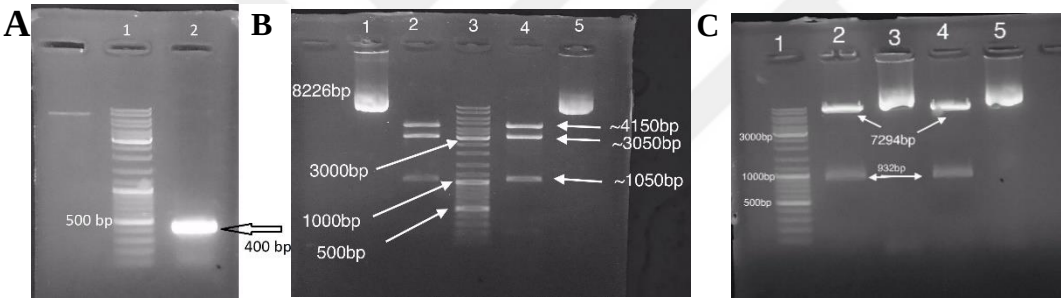

Figure 3.16: Verification of pJOE9958.1.*bacA*$_{strong}$RBS. **(A)** PCR amplification of pJOE9958.1.*bacA*$_{strong}$RBS by *bacA*-specific genes. Line 1 stands for the GeneRuler^{TM} DNA Ladder Mix; line 2 stands for the 400 bp PCR product. **(B)** The *EcoRI* digestion of pJOE9958.1.*bacA*$_{strong}$RBS, lines 1 and 5 refer to un-digested vectors from two selected transformants; lines 2 and 4 refer to the *Eco*RI digested vectors, and line 3 represents the GeneRuler^{TM} DNA Ladder Mix. **(C)** *Sfi*I digestion of pJOE9958.1.*bacA*$_{strong}$RBS, line 1 stands for the GeneRuler^{TM} DNA Ladder Mix; lines 2 and 4 refer to the *Sfi*I digested vectors from two selected transformants; lines 3 and 5 refer to the undigested vectors.

The shuttle vector pJOE9958.1 *bacA*$_{strong}$RBS was also modified to carry a 20 nt spacer sequence that guides the Cas9 to the upstream of the *bacA* region. The small guide RNA (gRNA) sequence was selected from the upstream laying at -28 and -8 bp position from the *bacA* start codon (ATG) (see Figure 1.15 in Chapter One and Figure 2.3 in Chapter Two) by using the software CCTop [215] due to its no off-target score. The designed sgRNA was synthesized into a duplex oligonucleotide (24 mer) to be fitted between the *Bas*I sites of the pJOE9958.1.*bacA*$_{strong}$RBS instead of *lacZ*α DNA

fragment (see Figure 2.2 in Chapter 2), which was found as a selectable marker for rapid and easy exchange of the spacer sequence[163]. Consequently, the *BasI* digested the linear vector pJOE9958.1.*bacA*$_{strong}$RBS and was treated with CIAP to prevent self-ligation (Figure 3.17). The duplex sgRNA oligonucleotide was treated with T4 polynucleotide Kinase (T4PNK) to phosphorylate the 5`-hydroxy termini of the sgRNA oligonucleotide to allow subsequent ligation into the linear pJOE9958.1.*bacA*$_{Strong}$RBS by T4 DNA ligase [246-248]. Finally, the phosphorylated sgRNA was ligated with the dephosphorylated *Bsa*I linearized pJOE9958.1.*bacA*$_{strong}$RBS and transferred into *E. coli* DH5α competent cells, resulting in Km^{R} transformants named *E. coli* DH5αbacasg58. The selected *E. coli* DH5αbacasg58 transformants bearing the 20 nt sgRNA encoding sequence were easily selected on XGAL-IPTG Km LB agar plates by forming white colonies due to the substitution of the cloned sgRNA with the *lacZ*α coding sequence [249] (Figure 3.18). Further verification, the plasmid DNAs isolated from the white colonies were digested with *EcoR*I restriction enzyme, which yielded three DNA fragments as expected sizes (Figure 3.19). Moreover, a PCR reaction was also performed with the primers P5 and P6 to confirm the constructed pJOE9958.1.*bacA*$_{Strong}$RBS.sgRNA, which resulted in the 932 bp homology template as expected (Figure 3.20).

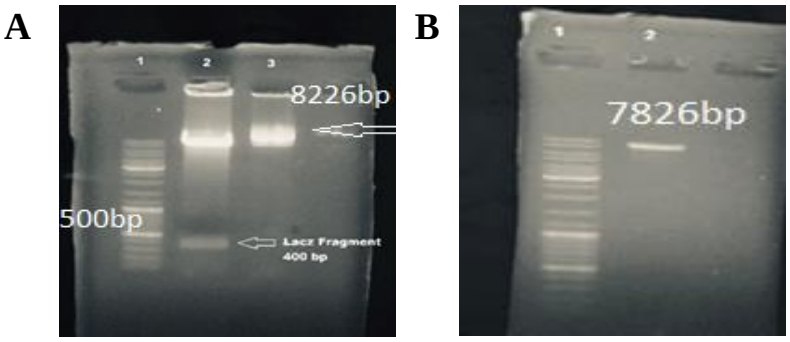

Figure 3.17: *Bsa*I digestion of pJOE9958.1.*bacA*$_{strong}$RBS. (A) line 1 represents the GeneRuler^{TM} DNA Ladder Mix; line 2 refers to the *Bsa*I digested vectors; line 3 refers to un- digested vector. (B) line 1 represents the GeneRuler^{TM} DNA Ladder Mix; Line 2 refers to the *Bsa*I digested vector after being purified from 1% agarose gel.

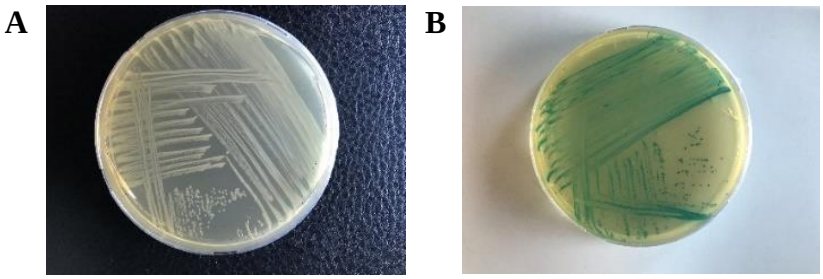

Figure 3.18: Verification of the selected transformant *E.coli* DH5αbacsg58 possessing the cloned vector pJOE9958.1.*bacA*$_{Strong}$RBS.sgRNA on XGAL-Km LB agar plate. (A) The *E.coli* DH5αbacsg58 growth culture with white color colonies is due to removing the *lacZα* coding sequence from the constructed pJOE9958.1.*bacA*$_{Strong}$RBS.sgRNA. (B) Blue colonies were observed in the *E.coli* DH5αRBS58 growth culture due to the *lacZα* coding sequence in the pJOE9958.1.*bacA*$_{Strong}$RBS vector that encodes the β-galactosidase enzyme.

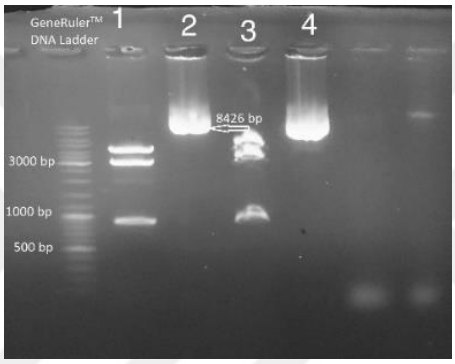

Figure 3.19: *Eco*RI digestion of the pJOE9958.1.*bacA*$_{Strong}$RBS.sgRNA; Lines 1 and 3 represent the digested vector from two selected transformants. Lines two and four represent the un-digested vector from the same chosen transformants, respectively.

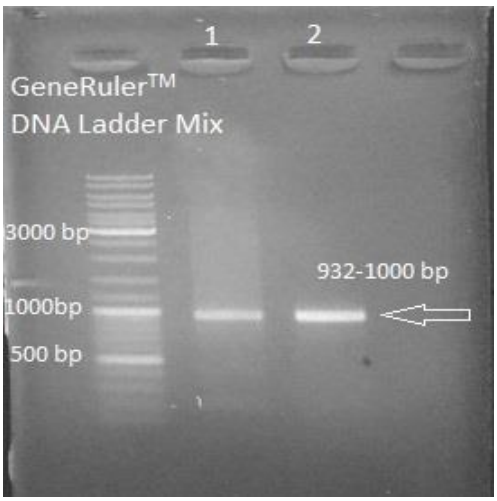

Figure 3.20: Amplification of the 932 bp homology template from pJOE9958.1.*bacA*$_{Strong}$RBS.sgRNA with P1 and P4 primers; Lines 1 and 2 refer to selected transformants and show positive results, a DNA band (932-1000 bp) was observed as expected.

3.2.4 Transformation of the *Bacillus subtilis* PY79 with pJOE9958.1.*bacA*$_{Strong}$RBS.sgRNA

After verification of the constructed the pJOE9958.1.*bacA*$_{Strong}$RBS.sgRNA plasmid, it was first propagated in the *rec*A-proficient strain *E. coli* BL21(DE3) pLysS to form the multimeric plasmid DNA to increase the efficiency of *B. subtilis* transformation and then used to transform the *B. subtilis* PY79 competent cells. Transformants were selected on the LB agar plates containing 50 μg ml^{-1} kanamycin and 0.2% mannose for induction of *cas9* under the control of P_{manP} and incubated at 30°C overnight. Twenty-one colonies were obtained within two days, re-streaked on an LB plate agar containing only Km, and incubated at 30°C. Before plasmid curing, Km^R colonies were screened for bacilysin phenotype to eliminate the potential bacilysin nonproducers (Bac$^-$) since Bac$^-$ candidates are likely to have arisen from cells repaired through the non-homologous end-joining (NHEJ) pathway. NHEJ and the homology-directed repair (HDR) pathways are critical for repairing double-strand breaks (DSBs) in genomic DNA. Therefore, high throughput bacilysin screening was employed by placing the resulting colonies with toothpicks on bioassay plates containing *S. aureus* ATCC9144, which were then incubated overnight at 30°C. As shown in Figure 3.21, of the twenty-one Km^R selected colonies, only three colonies exhibited significant antibacterial activity against *S. aureus*, suggesting that the efficiency of HDR remained too low for repairing Cas9-mediated-DSBs and that most of the colonies in this study were likely derived from the NHEJ pathway. This finding contradicted prior studies that found the NHEJ pathway in *Bacillus* species being inefficient, inactive in growing cells, and generally more active in the stationary phase or during sporulation [250-252].

For that reason, a test was achieved to prove whether the error-prone NHEJ pathway plays any role in DSB in *B. subtilis* PY79 by using pJOE9958.1.*bacA*$_{sgRNA}$, which only contains a 20 nt spacer sequence directed against *bacA* and no homology template, allowing surviving colonies to be generated only through the NHEJ pathway. This plasmid was quickly created by removing the homology template from the control plasmid, pJOE9958.1.*bacA*$_{strong}$RBS.sgRNA (Figure 3.22). The linear pJOE9958.1.*bacA*$_{sgRNA}$ vector was extracted from the agarose gel, re-ligated using T4 ligase, and then used to transform *E. coli* DH5α cells after the propagation of

pJOE9958.*bacA*$_{sgRNA}$ in *E. coli* BL21 cells. It was used in parallel with the control plasmid pJOE9958.1.*bacA*$_{strong}$RBS.sgRNA for *B. subtilis* PY79 transformation. Compared to the control plasmid, which produced only 46 ± 18 transformants with a transformation efficiency of 5.5 ± 2.1 transformants/µg DNA, pJOE9958.*bacA*.$_{sgRNA}$ yielded 147 ± 13 transformants with a transformation efficiency of 19.6 ± 1.7 transformants/µg DNA. These results confirm that Cas9-caused DSB was primarily repaired by the error-prone NHEJ rather than HDR in this work.

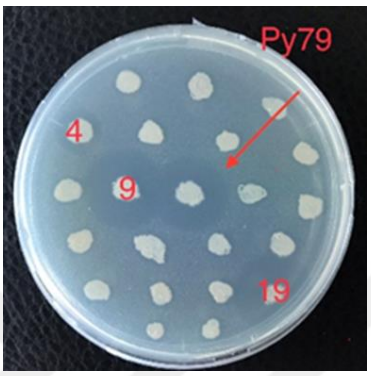

Figure 3.21: High throughput screening of Km^R transformants containing pJOE9958.1.*bacA*$_{strong}$RBS.sgRNA on bioassay plates containing *S. aureus* ATCC 9144 for bacilysin activity. Colonies were directly transferred on bioassay plates with toothpicks and incubated for 16 hrs at 37°C. The wild-type bacilysin producer *B. subtilis* PY79 was used as the positive control [219].

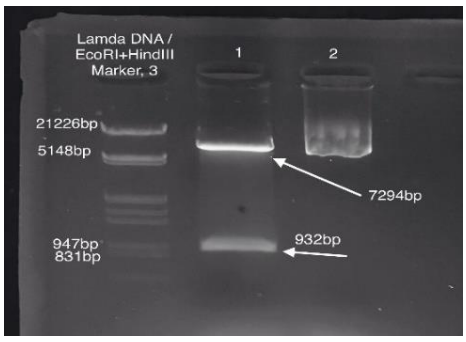

Figure 3.22: Construction of the pJOE9958.1.*bacA*.$_{sgRNA}$ plasmid from the control plasmid pJOE9958.1.*bacA*$_{Strong}$RBS.sgRNA. Line 1 refers to the *SfiI* digested pJOE9958.1.*bacA*$_{Strong}$RBS.sgRNA. , 7294 bp DNA fragment, linearized pJOE9958.1.*bacA*.$_{sgRNA}$ plasmid, was purified from the agarose gel and re-ligated with T4-ligase to obtain pJOE9958.1.*bacA*.$_{sgRNA}$.

As a further step, to generate the plasmid-cured cells, only Km^R and Bac$^+$ colonies were plated on LB plates without Km to single colonies at 50°C overnight as a nonpermissive temperature for plasmid replication and then restreaked to single colonies on LB plates at 42°C. The colonies were then screened for the loss of the Km^R phenotype, and roughly 42% of all colonies examined had lost the plasmids. This

efficiency was lower than expected for pJOE8999 derivatives in *B. subtilis* strains since highly efficient plasmid curing rates of 83-90% have been reported previously for those derivatives [163]. However, the curing rate of the pJOE9958.1 derivative used in this study was still adequate for generating plasmid-cured cells with the desired modification, as seen below. In the last stage, to find mutant candidates possessing the substitution of the $bacA_{strong}$RBS. sequence with the $bacA_{native}$RBS sequence, 11 randomly selected Km^S (plasmid-cured) transformants were tested by colony PCR by using the oligonucleotide 5'-AGCTTAAGGAGGAACAACTC-3' (specific to substituted strong RBS sequence) as the forward primer and the oligonucleotide 5'-GGTAAAATATTTTATTAAA-3 (reverse complement to the 402- 420 nt region of *bacA*) as the reverse primer. As a result, ten transformants clearly exhibited the expected PCR fragment of about 440 bp (Figure 3.23A). DNA sequencing results of the one candidate, selected randomly among the PCR^+ colonies, indicated that the $bacA_{strong}$RBS sequence was successfully replaced with the $bacA_{native}$RBS sequence as expected (Figure 3.23B).

Figure 3.23: (A) Colony PCR analysis performed with KmS and Bac+ colonies. 5'-AGCTTAAGGAGGAACAACTC-3'(bacAstrongRBS sequence) was used as the forward primer, and 5'-GGTAAAATATTTTATTAAA-`3 (reverse complement to the 402-420 nt region of bacA) was used as the reverse primer. M: Marker (*EcoR*I and *Hind*III digested Lamda DNA). **(B)** DNA sequence analysis of a KmS and Bac+ mutant. The introduced bacAstrongRBS sequence "GCTTAAGGAGGACAAA" was underlined in its DNA sequencing chromatogram and indicated with arrows in the BLAST analysis result (https:// blast.ncbi.nlm.nih.gov/Blast.cgi). The Blast analysis used *B. subtilis* PY79 (taxid:1415167) as the reference genome [216].

3.3 Screening of bacilysin activities of *bacA*$_{strong}$RBS candidates

To analyze the effect of the strong RBS insertion, the eight candidates comprising the strong RBS mutant verified by sequence analysis described above, together with the other seven candidates (randomly selected among the PCR^{+} colonies), were cultured in PA medium for 16-18 h, and the bacilysin level in their culture fluids was detected by paper disk-diffusion assay. Compared to PY79, each of the examined mutants produced more bacilysin, in which there were no considerable changes in their increased bacilysin titers (Figure 3.24), demonstrating that the strong RBS substitution resulted in increased bacilysin production. Antibacterial activity exhibited by mutants was entirely due to bacilysin. Since their antibacterial activities were completely suppressed by supplementing the bioassay plate with N-acetylglucosamine, a particular antagonist of bacilysin activity (Figure 3.25) [253]. Finally, one of the *bacA*$_{strong}$RBS mutants (numbered 5 in Figure 3.24) was chosen to represent strong RBS-carrying mutants and named *B. subtilis* HWA for further research.

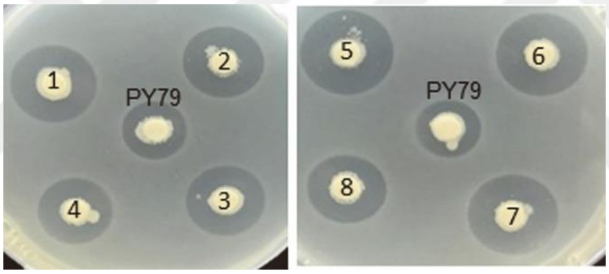

Figure 3.24: Bacilysin titers in mutants harboring strong RBS and the parental strain (PY79). Strains were cultivated in PA medium for 16-18 h at 37o C via shaking at 200 rpm, and bacilysin levels in their culture fluids were detected by paper disc diffusion assay using *S.aureus* ATCC 9144 as the assay organ [216].

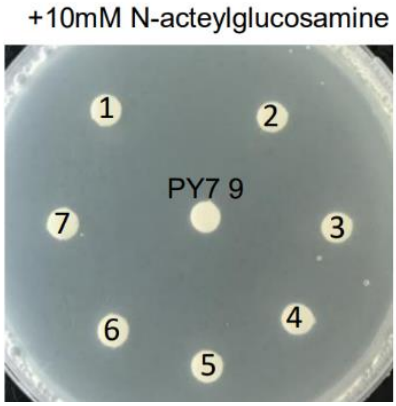

Figure 3.25: Culture fluids of transformants bearing strong RBS and the parental strain (PY79) screened on the bioassay medium containing *S. aureus* ATCC 9144-, and 10-mM N-acetylglucosamine [216].

3.4 Comparison of the intracellular mRNA level of the *bac* operon in HWA with PY79

In bacteria, transcription and translation are usually linked together. Changing the nucleotides at 5'UTR can impact both transcriptional and translational processes [254, 255]. Research conducted by Berg et al. [255] has shown that point mutations within the 5′UTR not only affect translation rates but also influence the abundance of transcripts. Similarly, a study by Eriksen et al. [254] suggests that the strength of the RBS can also affect protein synthesis by potentially influencing the stability of mRNA. This can lead to increased ribosome occupancy, which protects the mRNA from degradation by exo- and endonucleases, resulting in higher mRNA levels.

To demonstrate the effect of modifying the 5'UTR through a strong RBS substitution on intracellular mRNA levels, we measured the relative transcript amounts of the *bac* operon in HWA and PY79 using RT-qPCR during the early and late stationary phases of growth (corresponding to 18 h and 24 h of growth, respectively). Figure 3. 26 illustrates the comparison of relative transcript amounts of the *bac* operon in HWA and PY79 samples at 18 h of growth in which the maximum bacilysin activity was detected in both strains (Figure 3. 27). HWA, with the strong RBS, exhibited a significant increase in the *bac* operon mRNA levels, with a 3-fold higher level during the early stationary phase. Additionally, the transcript level in HWA was measured at 25 quantitative cycles (Cq) during the early stationary phase (18 h) and 21 Cq during the late stationary phase (24 h), while PY79 showed a measurement of 29 Cq at 24 h (Figure 3. 27). This indicates that the high transcript level of the *bac* operon in HWA appears to be maintained throughout the stationary phase. In conclusion, these results demonstrate that a strong RBS substitution also improves the mRNA stability of the *bac* operon.

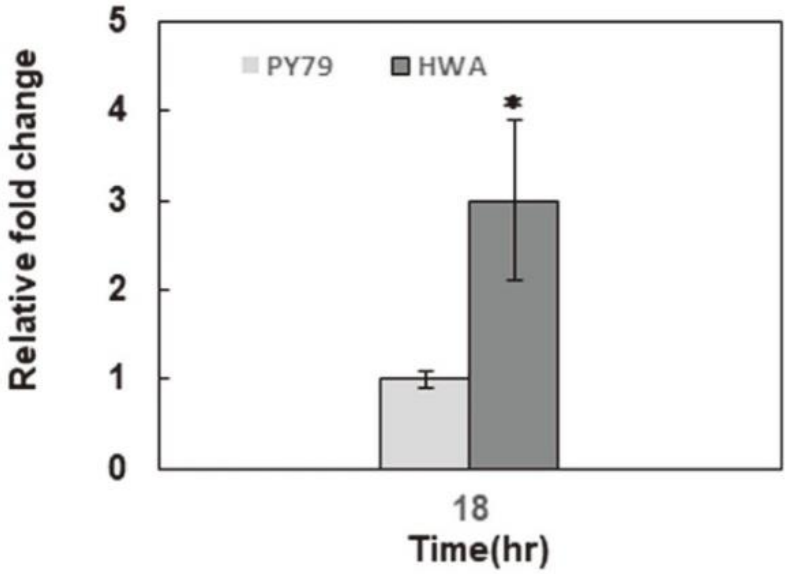

Figure 3.26: Transcriptional analysis of the *bac* operon in HWA and PY79. The relative transcript level of *bacB* as the second gene in the bac operon was detected in HWA and PY79 cells via RT-qPCR at the early stationary phase of growth (corresponding to 18 h). Error bars indicate the standard errors of the means of the two independent qPCR experiments performed with three biological and two technical replicates. Asterisk (*) indicates statistically significant differences from the parental strain PY79 with ($p < 0.05$) according to paired Student's t-test [216].

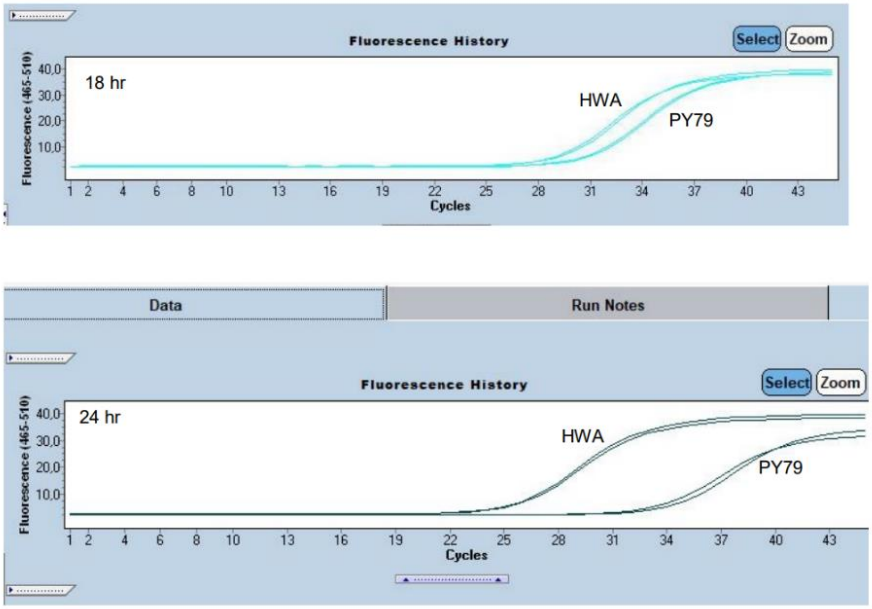

Figure 3.27: RT-qPCR quantitative Cycle (Cq) values of *bacB* detected in HWA and PY79 cells at the end of 18 hrs and 24 hrs of growth [216].

3.5 Comparison of bacilysin production performance of HWA with PY79

To further verify the interaction between the strong RBS substitution and over-production of bacilysin, HWA and the parental strain PY79 were cultivated in PA medium at 37ºC with shaking for 24 h; their growth and bacilysin production levels were measured at intervals throughout their cultivation periods. As shown in (Figure 3.28), there was no considerable change in the growth and the cell density dependent-bacilysin accumulation profiles of HWA in comparison to PY79 [206], in which the maximum bacilysin activity was still detected at the early stage of the stationary phase (corresponding to 16-18 h of cultivation). The most striking finding was that HWA exhibited over-production of bacilysin at all growth stages by peaking at 18 h of incubation. The bacilysin titer of HWA was 2.87-fold higher than the wild-type strain, with bacilysin activity rising from 27.9 ± 4.5 U/ml (in parental strain) to 80.39.1 U/ml (in HWA) at the end of 18 h of cultivation. UPLC-MS analysis further confirmed the significant change in the bacilysin activity of HWA in the 16-18 h culture. The result was higher relative abundance levels of the peak m/z of 271 $(M + H)^+$, corresponding to the mass of bacilysin, relative to that of PY79 (Figure 3.29). All these findings are highly significant as the results of the first study on 5'UTR editing employed to improve bacilysin production in its producer strain have revealed that extensive RBS engineering seems to be a promising strategy for enhancing bacilysin production.

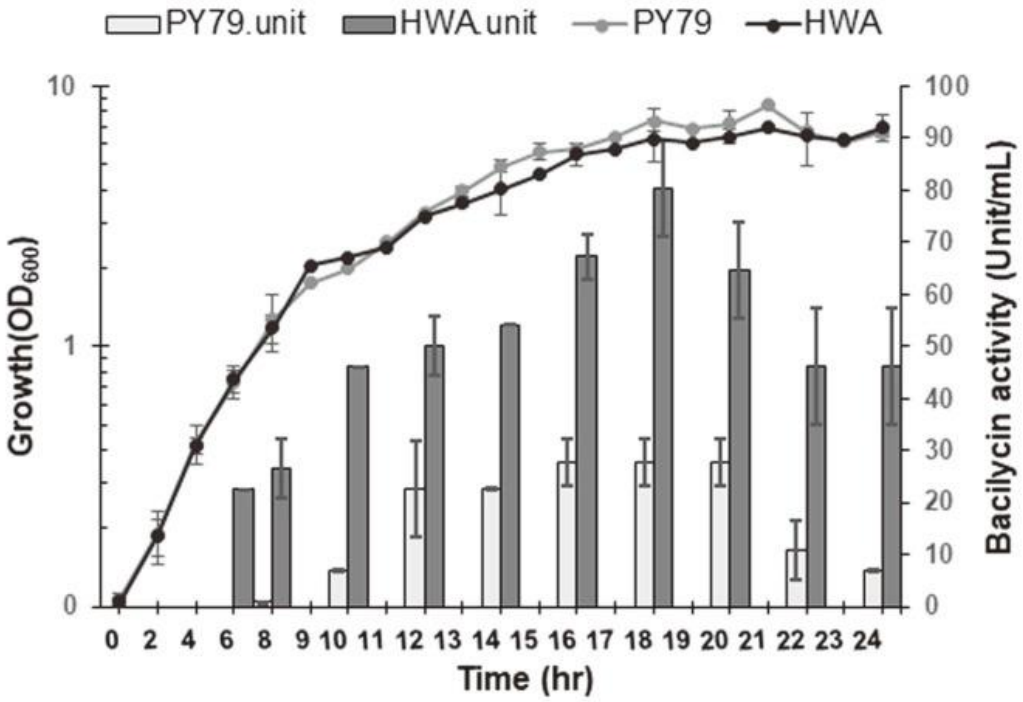

Figure 3.28: Growth and bacilysin activity profiles of HWA and PY79. Strains were grown in PA medium for 24 h at 37ºC via shaking at 200 ×g, and bacilysin activity in their culture fluids was detected by paper disk-diffusion assay. Error bars represent the standard deviation of the means of three independent experiments [216].

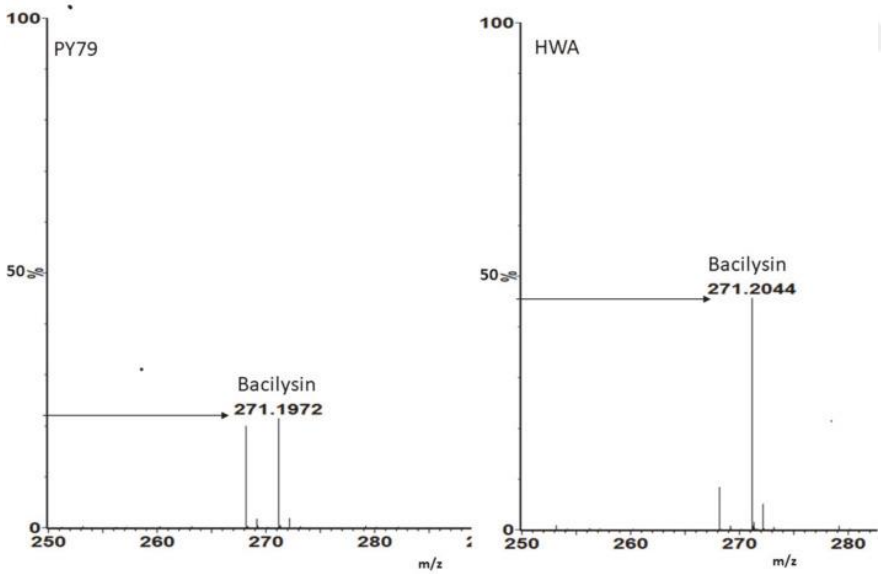

Figure 3.29: UPLC-MS spectrum of HWA and PY79 culture extracts. UPLC-MS analyses were performed in positive detection mode. Bacilysin $[M + H]^+$ at m/z 271 was detected in their culture extracts [216].

In this study, we focused on testing a single strong RBS sequence. However, it's important to conduct additional investigations to optimize the nucleotide compositions around the nucleotide sequences upstream and downstream from the SD site. Because

these regions can also affect translation efficiency by forming mRNA secondary structures, which might hinder the RBS and limit translation initiation [223] [256]. Additionally, in our study, we used an 8 nt spacer length as the optimal length based on previous reports [207][211][222]. These reports indicated that spacer lengths ranging from 7 to 9 nt were among the most effective, consistent with their computer-based predictions. However, it's worth noting that the optimal spacer length can vary depending on the specific gene of interest. A particular RBS may be strong when used with one gene but weak when used with another gene [221] [222, 223] [257]. Moreover, accurately predicting the optimal strength of RBSs for genes in a biosynthetic pathway or operon is challenging. Therefore, RBS engineering, which involves creating libraries with different strengths and sequences, is commonly used to precisely adjust gene expression at the translational level [258, 259]. For example, Jin et al. [259] successfully engineered *B. subtilis* 168 to produce specific low-molecular-weight hyaluronan (LMW-HA) from sucrose. They used a series of RBS variants with different translation efficiencies to optimize the level of leech hyaluronidase (LHAase), enabling the production of LMW-HAs with a molecular weight range of $2.20 \times 10^3 - 1.42 \times 10^6$ Da. Furthermore, Guiziou et al. [221] developed a genetic toolbox that includes libraries of promoters, ribosome binding sites (RBS), and protein degradation tags to precisely regulate gene expression in *B. subtilis*. This study further supported the effectiveness of promoter and RBS libraries in fine-tuning gene expression in *B. subtilis*. In a recent study, the recombinant production of TypeI L-asparaginase from *Bacillus licheniformis* Z-1 (BlAase) in *B. subtilis* RIK 1285 was significantly enhanced by combining library-based promoter and RBS engineering strategies. In sum, leveraging a library-based RBS optimization approach could be highly effective in further enhancing and fine-tuning the bacilysin biosynthetic pathway in *B. subtilis*.

4. CONCLUSION

In this study, a CRISPR/Cas9 single-plasmid system was used to change the putative RBS region of the *bac* operon (bacABCDEF). The standard SD sequence (TAAGGAGG) was inserted about 8 nt away from the start codon (AUG) of *bac*A. Since, the inspection of the upstream region of the bac operon did not reveal a core SD sequence with an ideal spacing (7-9 nt). This suggested that editing the *bacA*-RBS region is likely to have a positive impact on the production of bacilysin in its native producer. As we expected, changing the 5'UTR by substituting a strong RBS resulted in a significant increase in bacilysin production. Moreover, the amounts of transcripts for the *bac* operon were noticeably higher, especially during the late stationary phase. This indicates that a strong RBS substitution in the *bac* operon could improve not only the efficiency of translation initiation but also the stability of its mRNA. To sum up, our findings show that extensive RBS engineering has great potential for enhancing bacilysin production in natural producers. This study is the first successful application of RBS editing to improve bacilysin production in its natural producer, *B. subtilis* PY79. Furthermore, our results will contribute to further progress in bacilysin production in natural strains.

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APPENDICES

Appendix A: List the primers designed

Appendix B: Composition and Preparation of Culture Media**.**

Appendix C: Solutions and buffers**.**

Appendix D: Enzymes.

Appendix E: Equipment.

APPENDIX A : List the primers designed

Table A.1: List the primers designed for this study with their oligonucleotide sequences and melting temperatures.

Oligonucleotide (Primer)	Sequence	Melting Temperature (Tm)/ C°
***bac*.template Forward**	5`- AGT GAC TGC TCA GAG CGT CAT -3`	61.3
***bac*.template Reverse**	5`- GGT TGA ATA CTG CCT CAC TCC - 3`	61.3
(P1)	5`- AAG GCC AAC GAG GCC ATT AGG TTC TGC TTT AAT -3`	72.7
(P2)	5`- GAG TTT GTC CTC CTT AAG CTT TTA AAA TTT TAA GTA AATTTT ATC CAG TGG - 3`	74.9
(P3)	5`- CTT AAA ATT TTA AAA GCT TAA GGA CAA ACT CAT GAT TAT ATT GGA TAA TAG C -3`	75.2
(P4)	5`- AAG GCC TTA TTG GCC TTA GTT TTC ATC ATC AAC GTC- 3`	73.2
(P5)	5'-AAGGCCAACGAGGCC ATT-3'	72.7
(P6)	5'-AAGGCCTTATTGGCCTTAGTTTTCATC-3'	73.2
sgRNA_of_BacA.RBS_1 (Duplex)	(sgRNA_of_BacA.RBS_1_F) 5`- TAC GTA AAA TTT TAA AAG ATT GGT - 3`	53.3
	(sgRNA_of_BacA.RBS_1_R) 5`- AAA CAC CAA TCT TTT AAA ATT TTA - 3`	51.6
P8999 FORWARD	5`- GAC GAG CGT TCA CCG ACA AAC- 3`	61.8
P8999 REVERSE	5`- GAA TTC TTG ACC GTG ATT AGA GAA TTG AGT-3`	62.7
Hedil.Tarama. Forward	5`- AGC TTA AGG AGG GAC AAA CTC -3`	57.9
***bacA* Forward**	5` -GCCAAGCTTATGATTATATTGGATAAT -3`	52
***bacA* Reverse**	5` -GCGGATCCGGTAAAATATTTTATTAAA -3`	53

APPENDIX B: Composition and Preparation of Culture Media

Luria Bertani (LB) Medium (1000 ml)

Tryptone …….10 g

Yeast Extract…….. 5 g

NaCl……5 g

Distilled water was added 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……...15g (read the instruction on the agar vial)

Distilled water was added to 1000 ml and then autoclaved at 121ºC for 15 minutes. 25 ml of the LB media is poured into sterilized plates and waited for the medium to harden, then ready for use.

DSM- Medium (Difco's Sporulation Medium)

DSM Stocks

- 1M NaOH
- 1M Ca $(NO_3)_4$
- 0.01M $MnCl_2.2H_2O$
- 1mM $FeSO_4.7H_2O$

These solutions are sterilized using Autoclave (15 İb/in^2,15 min) and stored at 4 C^o.

DSM preparation method

This medium contains per Litre (1000ml)

- Bacto- nutrient broth ..... 8 g.
- 10% (w/v) KCl .....10 ml.
- 1.2% (w/v) $MgSO_4.7H_2O$ ….10 ml.
- 1M NaOH .....0.5 ml.

Autoclave (15 İb/in^2,15 min), allow to cool to 50 C^o and then add the following sterile solutions: -

- 1M $Ca(NO_3)_4$.....1.0 ml.
- 0.01M $MnCl_2.2H_2O$.....1.0 ml.
- 1mM $FeSO_4.7H_2O$.....1.0 ml.

This medium is solidified with 1.5% (w/v) agar.

PA medium

PA Stocks and Buffers

- **Glutamate Stock**

Dissolve (10 g) of L - Glutamate acid Sodium Salt monohydrate (Glutamate Na. H_2O) in 30 ml of dH_2O, then sterile by filtration and store at 4 C°.

- **Ferric Citrate Stock**

Dissolve (1.5 g) in 100 ml of dH_2O, then sterile by filtration and store at 4 C°.

- **Trace Metals Stock**

Dissolve the following elements in 100 ml of dH2O, then sterile by filtration and store at 4 C°.

- ZnSO4.7H2O ..... 0.1 mg/ml. (0.01 g / 100 ml)
- COCl2 .6H2O .....0.1 mg/ml. (0.01 g / 100 ml)
- Ammonium Molybdate ….0.1 mg/ml. (0.01 g / 100 ml)
- NaCl2 .4H2O .....1.0 mg/ml. (0.1 g / 100 ml)
- CuSO4.5H2O 0.01mg/ml. (0.001 g/100ml)

PA - Medium Preparation Method

To prepare 1 liter of PA medium, do the following steps: -

- Prepare KH_2PO_4/ KCl solution by adding (1 g) of KH_2PO_4 and (0.5 g) of KCl to

(850 ml) of dH_2O, then adjust the PH to 7.5 and complete the volume to (900 ml) in a 1000 ml scroll glass bottle. Autoclave (15 İb/in^2,15 min).

- Prepare $MgSO_4$/Sucrose solution by adding (10 g) of Sucrose and (0.5 g) of $MgSO_4$ to (77.0 ml) of dH_2O, then Autoclave (15 İb/in^2,15 min).
- After autoclave, Mix $MgSO_4$/Sucrose solution with KH_2PO_4/ KCl solution in the same 1000 ml glass Scroll glass bottle, then add the following stocks to complete the volume to 1000 ml (1 liter) of PA medium: -

  - Add (12 ml) of Glutamate Stock.
  - Add (10 ml) of Ferric Citrate Stock.
  - Add (1 ml) of Trace Metal Stock.

Then store at 4 C°.

Bioassay Medium

Bioassay Medium Stocks and Buffers

- **$FeSO_4.7H_2O$ Stock**

Dissolve (0.2 g) in (40 ml) of dH_2O, then sterile by filtration and store at 4 C°.

- **The Amino Acid Stocks**

Thirteen amino acids are used in this study (Arginine, Cysteine, Glycine, Histidine, Leucine, Methionine, Phenyl Alanine, Proline, Threonine, Tryptophan, Tyrosine, Valine, and Alanine). Each amino acid is prepared by dissolving (0.25 g) in (40 ml) dH2O, then sterilized by filtration and stored at 4 C°.

- **Glutamate Stock**

Dissolve (10 g) of L - Glutamate acid Sodium Salt monohydrate (Glutamate Na. H_2O) in 30 ml of dH_2O, then sterile by filtration and store at 4 C^o.

- **Yeast Extract Stock**

Dissolve (1 g) of Yeast extract into (40 ml) of dH2O, then sterile by filtration and store at 4 C^o.

Bioassay Preparation Method

For 1 liter of Bioassay medium, prepare the following solutions: -

- Glucose / $MgSO_4.7H_2O$ solution, which is prepared by adding (10 g) of L-glucose and (0.7 g) of $MgSO_4.7H_2O$ to (40 ml) of dH_2O. Autoclave (15 İb/in^2,15 min).
- Na_2HPO_4(3.3 g) / KH_2PO_4(1 g) / NaCl (1 g) dissolves all in (700 ml) dH_2O, adjust the PH to 7.1, then complete the volume to (720 ml) .....Autoclave (15 İb/in^2,15 min).
- Na_3.Citrate .$2H_2O$ solution, dissolve (0.5 g) of Na3.Citrate .$2H_2O$ into (46 ml) dH_2O. Autoclave (15 İb/in^2,15 min).
- Dissolve (20 g) of Agar -Agar into (160 ml) dH_2O in a 1000 ml scroll glass bottle containing a magnetic bar, then autoclave (15 İb/in^2,15 min).
- After the autoclave is finished, mix all the solutions above in the 1 Litre stroll glass bottle, which contains sterile Agar with a magnetic bar (step No. 4), then add: -

(2 ml) of $FeSO_4.7H_2O$ Stock, (2 ml) of each amino acid stock solution, (7.2 ml) of Glutamate Stock, and (2 ml) of yeast extract stock.

- Mix well using the magnetic sterile and heater until the agar completely dissolves.
- Inculcate 0.2 ml of an overnight broth culture of S. Aureus, used as a bioassay microorganism tester, into 9.8 ml of fresh broth in a 100 ml flask.
- İncubate by shaking (200 rpm) for 1.5 hr. at 37 C^o.
- Inculcate (1%) of *S. Aureus* culture growth into assay medium.
- Mix well and pour (25 ml) aliquots into each plate.
- **The preparation of broth culture in PA media for Disk Diffusion assay**

1. *B. subtilis* Py79 strain is İnoculated into 15 ml of PA – medium in a 100 ml flask, then shaken overnight (16-18 hr.) at 37 C^o.
2. (0.5 ml) of overnight bacterial broth culture is inoculated into fresh (20 ml) of PA- Medium, then Shaken (200 rpm) overnight for 16-18 hr. at 37 C^o; it's centrifuged at 1300 rpm for 5 min.
3. Culture broth supernatant stores at -20 C^o.

HS Medium (20 ml)

10X-S Base ……2 ml

%50 glucose……200 µl

%10 yeast extract……. …..200 µl

%2 casein hydrolysate……. ……..200 µl

%8 Arginine + %0,4 Histidine…… …….2 ml

%0,5 tryptophan…….....200 µl

%0,3 phenylalanine……30 µl

dH_2O ……..15,170 ml

All buffers and distilled water(dH_2O) listed above are sterilized by autoclaving (15 İb/in^2,15 min).

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

10X-S Base Stock (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 cooling down to 50 C^o (1 ml) of

sterile 1 M $MgSO_4$ (sterilized by filtration) was added to the solution.

LS Medium (30 ml)

10X-S Base…….3 ml

%50 glucose……300 µl

%10 yeast extract……300 µl

%2 casein hydrolysate……150 µl

%0,5 tryptophane……30 µl

%0,3 phenylalanine……45 µl

50 mM spermidine……..300 µl

1 M $MgCl_2$……..75 µl

NOTE: LS medium must be prepared freshly. All buffers and solutions are sterilized by autoclaving, except “50 mM Spermidine,” which is sterilized by filtration.

APPENDIX C: The preparation of solutions and buffers

The preparation of solutions and buffers with all components:

B1 Buffer 1000 ml (pH 8)

Tris-base ……6.06 g

EDTA.2H2O……...3.72 g

RNase A……..100 mg/L

Mix Tris-base and EDTA in 800 ml distilled water (dH_2O), mix well, and adjust the PH to 8 by Hydrochloric acid (HCl), then complete the buffer volume to 1000 ml by dH_2O in a 1000 ml volumetric flask. Add 100 mg of RNase A to 1 liter of buffer B1.

B2 Buffer (1000 ml)

NaOH……..8 g

SDS solution (% 20 W/V) …….50 mL

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

B3 Buffer (500 ml)/ pH 5.5

Potassium acetate…….....294.5 g

Dissolve the potassium acetate in 450 mL dH2O. Adjust the pH to 5.5 by acetic acid (CH_3COOH), then complete the buffer volume to 500 ml with dH_2O using a 500 ml volumetric flask.

TE Buffer (100 ml) / pH 8

Tris-base (10 mM) …….0.12g

EDTA (1 mM)…….0.03g

Add Tris-base and EDTA to 80 ml dH_2O and mix well—adjusted pH to 8 with Hydrochloric acid HCl. Then complete the volume to 100 ml with dH_2O using a 100 ml volumetric flask.

50X TAE Buffer (1000 ml)/ PH 8

Tris- base ……...242 g

EDTA (0.5 M) ……....14.615 g

Glacial acetic acid ……..57.1 ml

Dissolve Tris-base in 942.9 ml dH2O completely, then add the EDTA, mix well, and add 57.1 ml of glacial acetic acid. Adjust the PH to 8 and autoclave the buffer.

APPENDIX D: Enzymes

Enzyme	**Supplier**
*EcoR*I	Thermo scientific.
*Not*I	BioLabs.
*Sfi*I	BioLabs.
*Bsa*I	BioLabs.
CIAP	BioLabs.
T4 ligase	Thermo Scientific.
T4 polynucluotide kinase	BioLabs.
Q3 High. Fidelity DNA polymerase	BioLabs and Thermo Scientific.
Maximo Taq DNA polymerase	GeneOne and ThermoScientific.

APPENDIX E: Equipments

Equipment	**Supplier**
Autoclave:	TOMY SX-700E/high-pressure steam sterilizer.
Balance:	Precisa XB 620C.
Balances:	Precisa 125 A SCS.
Centrifuge:	Eppendorf/ 5424.
Electrophoresis power supply:	Thermo Electron Corporation EC1000-90 / Biogene.
Ice machine:	AF 10, Scotsman (UK).
Incubators:	Nüve EN400, Nüve EN500.
Light Cycler® 480:	Roche.
Magnatic Stirrer:	Stable Temp Cole- Pormer.
Nanodropmetere 2000:	Thermo Scientific.
Orbital shaker incubator:	Thermo 430 and Sartorius Certomat SII.
pH meter:	Hanna Instruments.
Pipettes:	Eppendorf research 2,5 µL 10 µL, 20 µL, 200 µL,1000 µL.
Pure water systems:	USF Elga UHQ-PS-MK3, Elga Labwater.
Thermal cycler:	Multigene gradient TC9600-G-230V.
Thermomixer:	Eppendorf, 1.5 ml thermomixer comfort.
Transilluminator:	BIOLAB Laboratory Equipments.
UV-Visible Spectrophotometers:	Shimadzu UV-Pharmaspec 1700 (Japan).
UV-Visible Spectrophotometers:	BIO RAD.
Vortex mixer:	Heidolph Reax top.
Waters Acquity UPLC H-Class and Waters Synapt G2-Si HDMS:	Waters.
Water bath:	Memmert wb-22.

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