T.R.
GEBZE TECHNICAL UNIVERSITY
GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES

SITE-DIRECTED-MUTAGENESIS OF METALLO-BETA-LACTAMASE-LIKE GENE OF SOLIBACTER USITATUS

GÖZDE GÖRGÜLÜ
A THESIS SUBMITTED FOR THE DEGREE OF
MASTER OF SCIENCE
DEPARTMENT OF MOLECULAR BIOLOGY AND GENETICS

GEBZE
2016

T.R.

GEBZE TECHNICAL UNIVERSITY

GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES

SITE-DIRECTED-MUTAGENESIS OF METALLO-BETA-LACTAMASE-LIKE GENE OF *SOLIBACTER USITATUS*

GÖZDE GÖRGÜLÜ

A THESIS SUBMITTED FOR THE DEGREE OF

MASTER OF SCIENCE

DEPARTMENT OF MOLECULAR BIOLOGY AND GENETICS

THESIS SUPERVISOR

PROF. DR. SEDEF TUNCA GEDİK

GEBZE

2016

T.C.
GEBZE TEKNİK ÜNİVERSİTESİ
FEN BİLİMLERİ ENSTİTÜSÜ

SOLIBACTER USITATUS' un METALLO-BETA-LAKTAMAZ BENZERİ GENİNİN YÖNLENDİRİLMİŞ MUTAGENEZİ

GÖZDE GÖRGÜLÜ

YÜKSEK LİSANS TEZİ

MOLEKÜLER BİYOLOJİ VE GENETİK ANABİLİM DALI

DANIŞMANI
PROF. DR. SEDEF TUNCA GEDİK

GEBZE
201

GEBZE TEKNİK ÜNİVERSİTESİ	**YÜKSEK LİSANS JÜRİ ONAY FORMU**

GTÜ Fen Bilimleri Enstitüsü Yönetim Kurulu'nun 27/06/2016 tarih ve 2016/43 sayılı kararıyla oluşturulan jüri tarafından 20/07/2016 tarihinde tez savunma sınavı yapılan Gözde GÖRGÜLÜ'nün tez çalışması Moleküler Biyoloji ve Genetik Anabilim Dalında YÜKSEK LİSANS tezi olarak kabul edilmiştir.

JÜRİ

ÜYE (TEZ DANIŞMANI)	: Prof. Dr. Sedef TUNCA GEDİK
ÜYE	: Prof. Dr. Halil KAVAKLI
ÜYE	: Yrd. Doç. Dr. F. İnci ÖZDEMİR

ONAY

Gebze Teknik Üniversitesi Fen Bilimleri Enstitüsü Yönetim Kurulu'nun

...../...../..... tarih ve ...../..... sayılı kararı.

İMZA/MÜHÜR

SUMMARY

Beta-lactam (β-lactam) antibiotics, which are the most widely used antibiotic group in medicine, bind specifically to penicillin-binding proteins and inhibit bacterial cell wall synthesis. The most important resistance development against β-lactams is the production of β-lactamase enzymes by resistant bacteria.

In a previous work carried out in "University of Tor Vergata (Italia)", metallo-β-lactamase-like (MβL) proteins from seven environmental bacteria were identified and putative genes were cloned, however no β-lactamase activity was obtained from any of them. *Solibacter usitatus* MβL-like ORF (Sol_65) has been chosen for further analysis to find an answer for the lack of β-lactamase activity, since its amino acid sequence was highly homologous to the functional MβL of *Fluoribacter gormanii* (FEZ).

Overlapping of the two protein's 3D structures had highlighted the structural differences between Sol_65 and FEZ proteins and it was predicted that amino acids at 223^{rd} ($Asn_{223:}$ N_{223}) and 266^{th} (Tyr_{266}: Y_{266}) positions in the active center of Sol_65 could be responsible for the possible steric hindrance that prevent the entry of the β-lactam ring in the active site. It was decided to change those amino acids with the once present in FEZ at the same sites (223^{rd} and 226^{th}) and vice versa by site directed mutagenesis.

Present study covers experimental results of site directed mutagenesis of 223^{rd} amino acid in both Sol_65 ($N_{223}\rightarrow G$) and FEZ ($G_{223}\rightarrow N$) proteins. First, a template plasmid to be used in site directed mutagenesis was prepared (pR_pelBSol65) to introduce selected mutation into Sol_65. Template plasmid for FEZ (pET24Fez) was prepared in a previous study. Although, restriction enzyme digestion and DNA sequencing prove that the mutation was successful, the result of the antibiotic test showed that recombinant strain has very poor β-lactamase activity.

Key Words: β-lactamase, Metallo-β-lactamase, Site-directed-mutagenesis, *Solibacter usitatus, Fluoribacter gormanii,* FEZ protein.

ÖZET

Tıp alanında en sık kullanılan antibiyotik grubu olan β-laktam antibiyotikleri, penisilin bağlayıcı proteinlere spesifik olarak bağlanarak bakteriyel hücre duvarı sentezini inhibe ederler. β-laktam antibiyotiklerine karşı en önemli direnç gelişimi dirençli bakteriler tarafından β-laktamaz enzimlerinin üretimidir.

Tor Vergata Üniversitesi'nde (İtalya) yürütülen bir çalışmada, yedi ayrı bakteriden metallo-β-laktamaz (MβL) benzeri proteinler tespit edilmiş, olası genler klonlanmış, ancak hiç birinde β-laktamaz aktivitesi gözlenmemiştir. β-laktamaz aktivitesinin görülmeyişine cevap bulabilmek amacıyla, *Fluoribacter gormanii*'nin fonksiyonel MβL'si (FEZ) ile yüksek homoloji gösteren *Solibacter usitatus* metallo-β-laktamaz benzeri proteini (Sol_65) daha detaylı analiz için seçilmiştir.

Sol_65 and FEZ proteinlerin üç boyutlu yapılarının birbiriyle örtüştürülmesi sonucunda iki proteinin yapısal farklılıkları ortaya çıkmıştır. Buna göre Sol_65'in aktif bölgesinde yer alan 223. (Asn_{223}: N_{223}) ve 266. (Tyr_{266}: Y_{266}) amino asitlerin yarattığı olası bir sterik engellemenin, β-laktam halkasının aktif bölgeye girişini engelleyebileceği düşünülmüştür. Bunun üzerine, yönlendirilmiş mutagenez ile Sol_65'in 223. ve 266. amino asitlerinin FEZ proteininin aynı pozisyonlarında (223. ve 226.) bulunan amino asitler ile değiştirilmesi ve aynı işlemin FEZ için de yapılması kararlaştırılmıştır.

Bu çalışma, hem Sol_65 (N_{223}→G) hem de FEZ (G_{223}→N) proteinlerinin 223. amino asitinin yönlendirilmiş mutasyonu ile ilgili deney sonuçlarını kapsamaktadır. Öncelikle, amaçlanan mutasyonun Sol_65 proteinine yerleştirilmesi için kalıp bir plazmit (pR_pelBSol65) oluşturulmuştur. FEZ mutasyonu için, daha önce oluşturulmuş olan kalıp plasmid (pET24Fez) kullanılmıştır. Restriksiyon enzim kesimleri ve DNA sekansı ile mutasyonun doğruluğu kanıtlanmış, ancak antibiyotik test sonuçlarına göre rekombinant suşun çok zayıf β-laktamaz aktivitesi gösterdiği bulunmuştur.

Anahtar Kelimeler: β-laktamaz, Metallo-β-laktamaz, Yönlendirilmiş mutagenez, *Solibacter usitatus, Fluoribacter gormanii,* FEZ proteini.

ACKNOWLEDGEMENTS

For my big family Nazli GORGULU, Esra GORGULU, and Suat GORGULU with all my love and with all my soul…

I wish to express my thanks to all my colleagues from Department of Microbiology at the University of Rome ‘’Tor Vergata’’;

Firstly, I would like to express my profound thanks to Prof. Dr. Maria Cristina Thaller; for accepting me to her laboratory, project and team as a graduate student. Grateful thanks for scientific advices and for reviewing the thesis. I am so grateful to know her and also to work with her.

Secondly, I wish to express my thanks to Dr. Gustavo Di Lallo; for accepting me to his laboratory as a graduate student. It was really a big opportunity for me to work with him.

Also, I am really obliged to Ilaria Di Meo; for showing me all the methods with great patience and also for kind cooperations.

Special thanks to my big family at Department of Molecular Biology and Genetics at ‘’Gebze Technical University’’;

Firstly, I would like to express my thanks to my first supervisor, Prof. Dr. I. Yavuz SEZEN for his guidance, supervision and endless patience throughout the study.

Secondly, I wish to express my thanks to my supervisor Prof. Dr. Sedef TUNCA GEDIK for her tolerance and understanding and, of course to accept me as a graduate student.

You should know that I am really obliged to Dr. Mine GUL SEKER to teach me all she knows with great patience and love.

Endless thanks to my Italian family; Anna Garipoli, Augusto Rossi, Daniele Rossi and Marco Rossi and also to all my friends who constantly motivating me to finish writing my thesis erewhile; Burak Nizamoglu, Sara Altuntas, Fatma Sumer Kart, Ayse Turkan, Zeynep Girgin Ersoy, Diego Laurenti and lastly to Episome Biotech family.

TABLE of CONTENTS

LIST of ABBREVIATIONS and ACRONYMS

Abbreviations and Acronyms		Explanations
α	:	Alpha
β	:	Beta
γ	:	Gamma
~	:	Tilde
∞	:	Infinity
Asn	:	Asparagine
bp	:	Base pair
cm	:	Centimetre
Fw	:	Forward
Gr	:	Gram
G	:	Glycine
Gly	:	Glycine
His	:	Histidine
kb	:	Kilo base
KCl	:	Potassium Chloride
Kv	:	Kilovolt
L	:	Litter
M	:	Methionine
mg	:	Milligram
$MgCl_2$	:	Magnesium Chloride
Met	:	Methionine
µg	:	Microgram
µl	:	Microliter
µM	:	Micro molar
min.	:	Minute
ml	:	Millilitres
M	:	Molar
mM	:	Millimolar
N	:	Asparagine

NaCl	:	Sodium Chloride
pM	:	Picomolar
Rpm	:	Revolutions per minute
Rev	:	Reverse
Ta	:	Annealing Time
Tm	:	Melting Time
Tyr	:	Tyrosine
Vf	:	Final Volume
V	:	Volt
Y	:	Tyrosine
Zn	:	Zinc
aDNA	:	Ancient DNA
aDNA	:	Ancient DNA
Amp	:	Ampicillin
AMR	:	Antimicrobial Resistance
ATP	:	Adenosine triphosphate
*BamH*I	:	*Bacillus amyloliquefaciens* endonuclease I
BHI	:	Brain Heart Infusion
BLAST	:	Basic Local Alignment Search Tool
BSA	:	Bovine Serum Albumin
CFU	:	Colony-forming unit
Cys	:	Cysteine
dH_2O	:	Distilled water
DNA	:	Deoxyribonucleic acid
*Dpn*I	:	*Diplococcus pneumoniae* endonuclease I
DTT	:	Dithiothreitol
*Eco*RI	:	*Escherichia coli* endonuclease I
EDTA	:	Ethylene diamine tetra acetic acid
ESBLs	:	Extended-spectrum enzymes
EtBr	:	Ethidium Bromide
EUCAST	:	European Committee on Antimicrobial Susceptibility Testing
IPTG	:	Isopropyl β-D-1 thiogalactopyranoside
*Kpn*I	:	*Klebsiella pneumoniae* endonuclease I

LacZ	:	Lactose Operon Z
LB	:	Luria Bertani
LD	:	Loading Dye
MβL	:	Metallo-beta-lactamase
MHA	:	Muller Hinton Agar
MIC	:	Minimal Inhibitory Concentration
NAG	:	N-acetyl glucosamine
NAM	:	N-acetyl muramic acid
*Nco*I	:	*Nocardia coralline* endonuclease I
*Nde*I	:	*Neisseria denitrificans* endonuclease I
OD	:	Optical Density
ORF	:	Open Reading Frame
PABA	:	Para-amino benzoic acid
PBP	:	Penicillin Binding Protein
PCR	:	Polymerase Chain Reaction
Pfam	:	Protein families
PG	:	Peptidoglycan
*Pst*I	:	*Providencia stuartii* endonuclease I
RBS	:	Ribosomal Binding Site
RNA	:	Ribonucleic acid
*Sac*II	:	*Streptomyces achromogenes*
SDM	:	Site-directed-mutagenesis
*Sma*I	:	*Serratia marcescens* endonuclease I
SOC	:	Super Optimal Broth with Catabolite Repression
SOB	:	Super Optimal Broth
TAE	:	Tris-Acetate-EDTA
TE	:	Tris-EDTA
Tn	:	Transposon

LIST of FIGURES

LIST of TABLES

1.INTRODUCTION

Antibiotics are one of the important type of antimicrobial drug used either to kill bacteria or to inhibit their growth. In the late 19th century, the search for convenient preventative and therapy regimens was stimulated with the discovery of antimicrobial agents; however, a half century later, successful treatment was progressed with the discovery and development of antibiotics [1].

Figure 1.1. [1] shows the history of antibiotic discovery and concomitant development of antibiotic resistance;

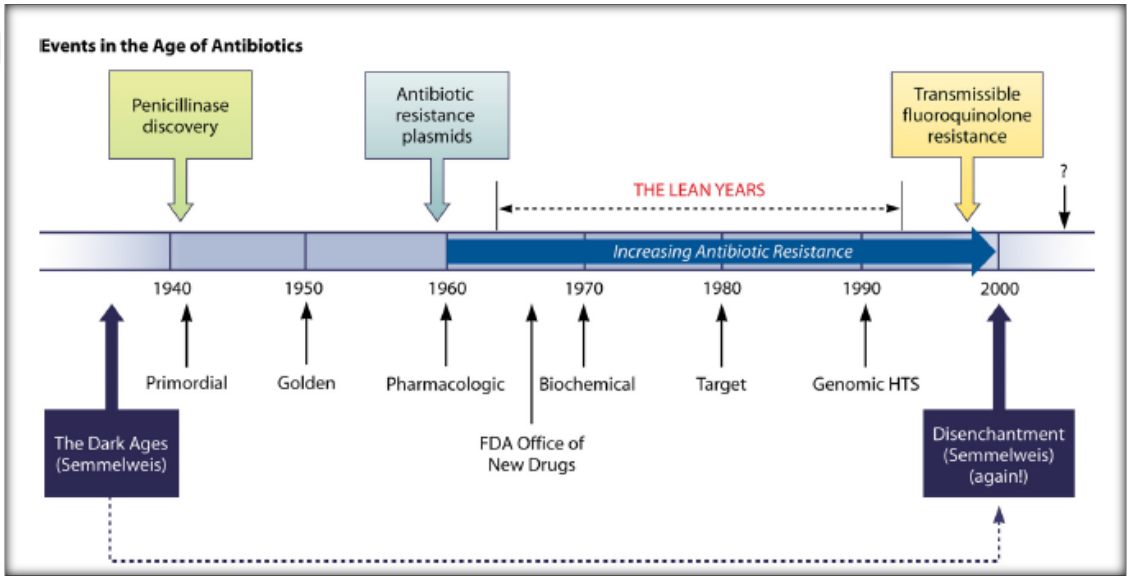


Figure 1.1: History of antibiotic discovery and concomitant development of antibiotic resistance.

Chemical substances, called as ‘’Chemotherapeutic’’, has started to be used in the treatment of infectious diseases from the 17th century. The biggest breakthrough of microbiology was made in the second half of the 19th century by Pasteur and Joubert. They first envisioned that microorganisms could have a potential benefit in treatment of infectious diseases. In 1935, Domagk started to modern chemotherapy of infectious diseases with sulphonamides and, because his work on Prontosil, won the Nobel Prize in 1938. Sulphonamides were used extensively as the most effective drugs of antibacterial chemotherapy until 1942 when penicillin was the first clinically tested. Penicillin is first discovered by Fleming in 1929 and isolated from *Penicillium notatum* by Chain and Flarey in 1940. After penicillin and

sulphonamides, new antibiotics were isolated in 30-60 years. However, as the new antibiotic is discovered, it is almost the same rate developing resistance against that antibiotic. The rapid emergence of resistant bacteria is occurring worldwide. β-lactamases are the most important enzymes that cause development of antibiotic resistance. These enzymes inactivate β-lactam antibiotics (penicillins, cephalosporins etc.) by hydrolysing β-lactam bond in their structure. In the present study, metallo-β-lactamase-like (MβL) gene of an environmental bacterium, *Solibacter usitatus*, was mutated by "site directed mutagenesis" to obtain higher similarity to functional MβL of *Fluoribacter gormanii*.

1.1. Objective Of The Study

In a previous study carried out in "University of Tor Vergata, Department of Biology (Italia)", metallo-β-lactamase-like (MβL) proteins from seven environmental bacteria were identified on the basis of sequence homology with 13 different functional enzymes by BLAST analysis. Putative metallo-β-lactamase genes from these seven bacteria were cloned into pGEMT vector in *E. coli*, however no β-lactamase activity was obtained. One of the clones (containing ORF *Acid_6874* of *Solibacter usitatus*) have been chosen for further analysis to find an answer for the lack of β-lactamase activity. *S. usitatus* MβL-like ORF (Sol_65) has been chosen since its amino acid sequence was highly homologous to the functional MβL of *Fluoribacter gormanii* (FEZ).

Overlapping of the two protein's 3D structures had highlighted the structural differences between Sol_65 and FEZ proteins and it was predicted that amino acids at 223^{rd} (Asn_{223} : N_{223}) and 266^{th} (Tyr_{266} : Y_{266}) positions in the active center of Sol_65 could be responsible for the possible steric hindrance that prevent the entry of the β-lactam ring in the active site. It was decided to change those amino acids with the once present in FEZ at the same sites (223^{rd} and 226^{th}) and vice versa by site directed mutagenesis.

The aim of the present study is to change 223^{rd} amino acid in both Sol_65 ($N_{223}\rightarrow G$) and FEZ ($G_{223}\rightarrow N$) proteins by site directed mutagenesis and see the effect of these mutations on metallo-β-lactamase activity.

2. BACKGROUND ASPECTS

2.1. Antibiotic types and action mechanisms

On the basis of their effects antibiotics are divided mainly into two categories:

- *Bacteriostatic;* they prevent the development or growth of bacteria (for example: tetracyclines, macrolides and lincosamides).
- *Bactericidal;* they are able to kill microorganisms (for example: penicillins, cephalosporins, rifamycins).

Figure 2.1 [3] shows bacterial target sites of different antibiotics.

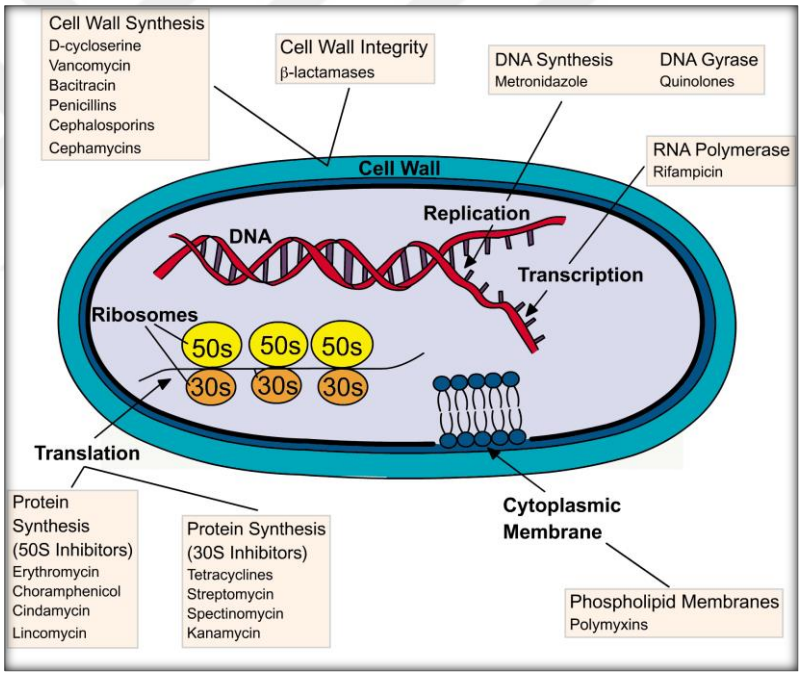


Figure 2.1: Main target sites of antibiotics in bacteria.

All bacteria except mycoplasma have peptidoglycan layer in their cell walls. Peptidoglycan layer, which is also known as “murein” or “mucopeptide”, is a polysaccharide composed of repeating N-acetyl glucosamine (NAG) and N-acetyl muramic acid (NAM) units. The formation of peptidoglycan synthesis involves three enzyme systems; *transpeptidase*, *carboxypeptidase*, *endopeptidase* and β-lactam antibiotics act by blocking the activity of *transpeptidase* enzyme (Figure 2.2) [4].

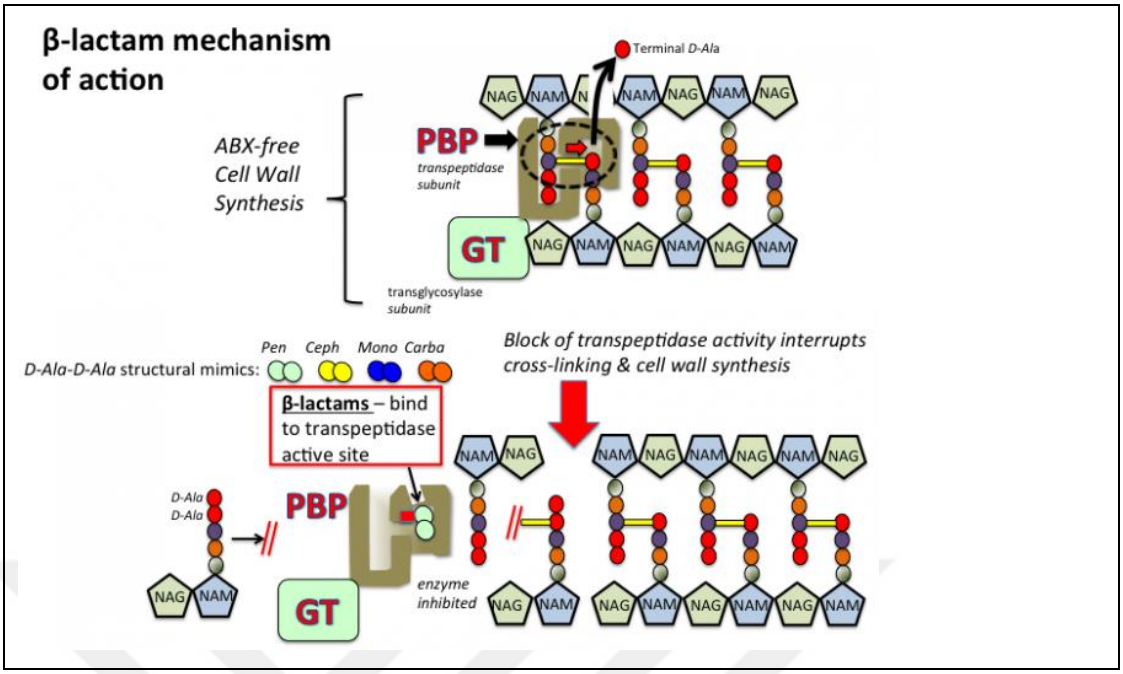


Figure 2.2: Action mechanism of β-lactam antibiotics.

The sulphonamides have a similar structure to that of para-amino benzoic acid (PABA) from which folic acid is synthesized. Sulphonamides inhibits the synthesis of folic acid due to this chemical similarity with PABA. DNA synthesis is inhibited indirectly by this group of antibiotic [41].

Rifampicin, instead, inhibits beta subunit of DNA-dependent RNA polymerase enzyme and inhibit transcription.

Quinolones, which are a family of synthetic broad-spectrum antibiotics, acts by inhibiting the formation of DNA. Quinolones bind to the topoisomerase IV and topoisomerase II and prevent bacterial DNA unwinding and duplicating, thus, causing fragmentation of the chromosome and cell death.

Polymyxins are another group of antibiotics, act by disrupting the structure of the bacterial cell membrane. The polymyxins have a cyclic peptide with a long hydrophobic tail and act as surfactants on membrane phospholipids [41].

Aminoglycosides and tetracyclines inhibit protein synthesis by binding to 30S ribosome units.

Chloramphenicol, macrolides, azalides, and lincosamides inhibit protein synthesis by binding to the 50S ribosome units. These antibiotics, therefore, show bacteriostatic effect [41].

2.2. β-Lactams

This category represents the largest group of antibiotics known and most widely used for therapeutic purposes. Even though the β-lactam compounds were discovered in filamentous fungi, different types of β-lactams are also produced by actinomycetes and gram-negative bacteria [8].

The target of β-lactam antibiotics is penicillin-binding protein (PBP), which catalyses the transpeptidation phase of the cell wall synthesis. In fact, these drugs resemble the dimer D-alanyl-D-alanine (natural substrate of PBP) and therefore are incorrectly recognized as true substrates by enzymes. β-lactam antibiotics linked by these enzymes and inhibit the enzyme binding to its substrate, thus, it inhibits bacterial cell wall synthesis and cause lysis [42, 43]

β-lactam resistance occur in three ways. First, targets which are penicillin-binding proteins (PBP) can change. It can occur as a result of expression of novel PBP with lower affinity to β-lactam antibiotics, reduction in the number of PBP or any change that decrease affinity of β-lactam antibiotics to PBS. Second, prevention of drug entrance into the cell via alterations in the outer membrane proteins. The last one is inactivation of the antibiotics with β-lactamase enzymes. β-lactamases of gram negative bacteria are located in the periplasmic space between the cytoplasmic membrane and the outer membrane and they are synthesized from the chromosome or plasmid [42, 43]

2.2.1. Subtypes of β-lactam antibiotics

The β-lactam antibiotics include five different structural subtypes: penicillins, cephalosporins, carbapenems, monobactams and finally, inhibitors of β-lactamases.

- Penicillin

In addition to a powerful bactericidal effect, penicillins have relatively low toxicity, which are commonly used, natural and semi-synthetic antibiotics. First, the natural penicillin discovered by Fleming in 1929 and later on semi-synthetic penicillins, correcting various drawbacks of natural penicillins has been developed.

- The natural penicillins

Their molecular structures are simpler than other penicillin types. These types of penicillin are showing the highest activity to Gram positive bacteria. The main representative is penicillin G or benzyl-penicillin. Currently, penicillin G is produced by fermentation of *Penicillium crysogenum* which ensures high yields. Penicillin V (or phenoxymethyl-penicillin), which is orally administrable being gastro-resistant, has similar activity with Penicillin G against Gram positive bacteria [42, 43, 44].

- Semi-synthetic penicillins

The antibiotics belonging to this class are derived from 6-aminopenicillanic acid and have improved characteristics compared to penicillin G [42, 43, 44].

- Cephalosporin

In terms of chemical structure and antibacterial mechanism they are similar to penicillin. Cephem derivative of 7-amino cephalosporanic acid creates the core of cephalosporins. Cephalosporins show their bactericidal effect by inhibiting PBP as penicillin. Resistance to cephalosporins is evolving with three main mechanisms. Preventing the passage of the drug from the peptidoglycan layer, reduction of drug affinity to PBPs, or degradation of the drug with β-lactamase enzyme [43, 44, 45].

Cephalosporins are distinguished as: I°, II°, III°, IV° generation cephalosporins.

- First generation

This group cephalosporins (cephalexin, cefadroxil, cefazolin, cephalothin) are highly effective on Gram positive cocci. Oral forms of these antibiotics can be safely used in the treatment of community-acquired Gram positive infections, especially *Staphylococcus* and *Streptococcus* infections [43, 44, 45].

- Second generation

Gram-negative activity of second generation cephalosporins (cefaclor, cefuroxime, cefotetan, cefoxitin) are greater than the first generation ones [43, 44, 45].

- Third generation

Although gram-negative activity of this group antibiotic (cefotaxime, ceftazidime, ceftriaxone, cefixime) is higher compared to first and second generation drugs, gram positive activity is low. Half-live of third generation cephalosporins is long and their effect is bactericidal [43, 44, 45].

- Fourth generation

They are particularly effective against gram-negative bacteria. There is also more potent gram-positive antibacterial activity compared to the third generation cephalosporins. They are resistant to β -lactamases. Cefepime is an example for this group [43, 44, 45]

- Carbapenems

Carbapenems include β-lactam ring in the structure like other β-lactam members. However, unlike the other β-lactams, carbon atom at position 1 is replaced by sulphur (Figure 2.3) [4]. As in all β-lactam antibiotics, carbapenems connected to PBPs and act by inhibiting the synthesis of bacterial peptidoglycan. Two members that are used for clinical application are imipenem and meropenem. In particular imipenem is a thienamycin derivative and it has a broad spectrum activity. Meropenem, however, has greater stability than thienamycin and imipenem [43, 44, 45].

- Monobactams

They are β-lactam antibiotics that differ from other β-lactams because of the monocyclic structure in the core content (Figure 2.3) [4]. The best known example is aztreonam, obtained from *Chromobacterium violaceum*.

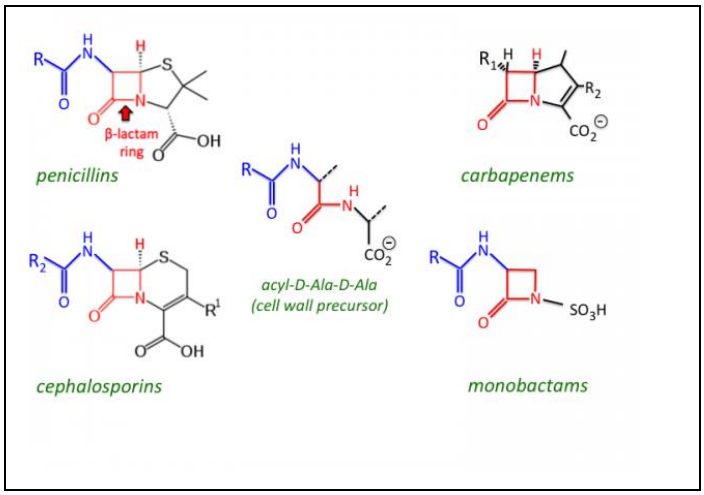


Figure 2.3: Core structure of β-lactam antibiotics.

2.3. Antimicrobial Resistance

Antimicrobial resistance (AMR) is resistance of a microorganism to an antimicrobial medicine to which was originally sensitive. Resistant organisms such as bacteria, fungi, viruses and some parasites are able to resist to attacks by antimicrobial medicines (antibiotics, antifungals, antivirals and antimalarials), so that standard treatments become ineffective [5]. Antibiotic resistance is accelerated with the misuse of antimicrobial medicines and the spread of AMR is encouraged with poor infection control practices [5].

Intrinsic (natural) resistance is naturally present in the bacteria, resistance gene is on the chromosomal DNA. On the other hand, in the acquired resistance, resistance to antibiotics are acquired by various mechanisms developed by bacteria. Either existing genetic material is changing by mutations or new genetic material is acquired from different sources. Chromosome resistance depends on the formation of spontaneous mutations on chromosome. Other resistance mechanisms include, decrease in drug permeability or change in the drug targets. Resistance via plasmids is usually caused by enzymes which inactivates the antibiotics. Presence of resistance genes on transposons can also generate resistance against antibiotics. Horizontal gene transfer mechanisms such as conjugation, transformation or phage transduction are the main routes for the entry of resistance genes [6].

Figure 2.4. [7] summarize main antibiotic resistance mechanisms. Efflux pumps are transport proteins located in the cell membrane that eliminate the toxic

substances out of the cell. Activation of this mechanism occurs as a result of environmental stimuli or mutations in the structure of the regulatory gene. Efflux pumps requires energy which is provided via proton motive force or via ATP. Some specific enzymes are able to degrade antibiotics, such as β-lactamases are able to degrade β-lactam antibiotics.

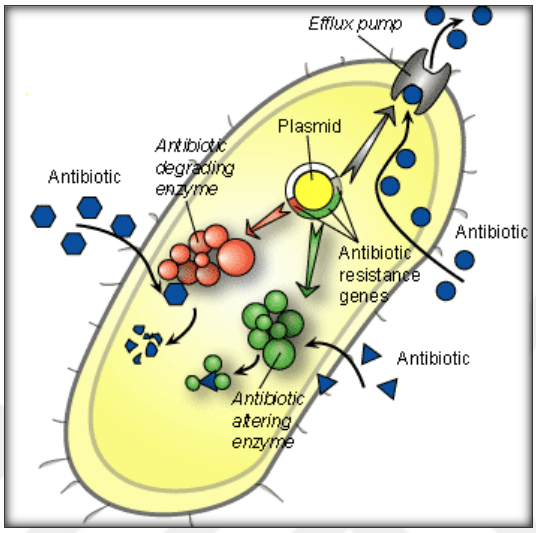


Figure 2.4: Mechanisms of antibiotic resistance in bacteria.

2.4. β-lactamases

β-lactamase enzymes inactivate β-lactam antibiotics (penicillins, cephalosporins and other β-lactam antibiotics) by hydrolysing β-lactam bond in their structure (Figure 2.5) [4]. β-lactamases are the most important factor that cause development of antibiotic resistance. These enzymes have attracted great attention because of their ecological and evolutionary interest and their clinical importance [9].

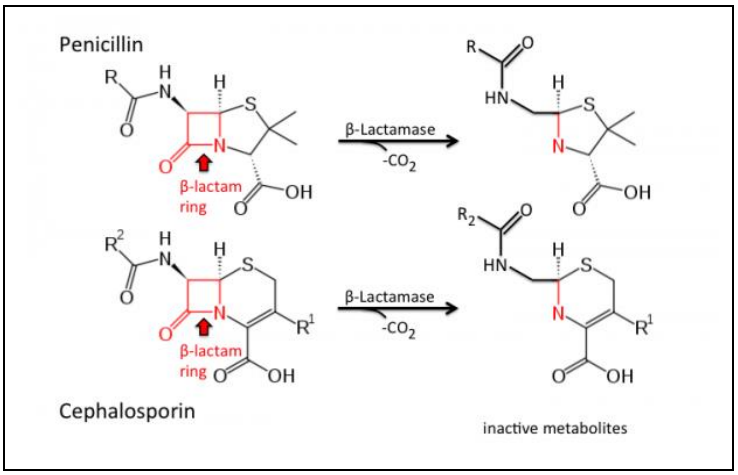


Figure 2.5: Hydrolysis of penicillin and cephalosporin core structures by β-lactamase.

2.4.1. Classification of β-Lactamases

Classification of β-lactamases made by Richmand and Sykes for the first time in 1973 and it has been extended by Sykes and Matthew in 1976. β-lactamase was initially identified by their ability to hydrolyse penicillin. After that with the usage of each new β-lactam antibiotics new β-lactamases have been added to this table.

Ambler (1980) classified β-lactamases based on their amino-acid sequences. Classes A, C and D cover evolutionarily distinct groups of serine enzymes, and class B include zinc-dependent ("EDTA-inhibited") enzymes (Table 2.1) [10]. According to the classification made by Bush, Jacoby, and Medeiros [21] β-lactamases are divided into three groups according to their biochemical properties and substrate profile (Table 2.1) [10].

Table 2.1: Classification of β-lactamases.

Bush-Jacoby-Medeiros (Integron) System	**Ambler System**	**Enzyme Activity-Examples**
Group 1 Cephalosporinases	C	Chromosomal AmpC resistance (usual) to β-lactams, except carbapenems; not inhibited by clavulanic acid (e.g., *Enterobacteriaceae*, except *Salmonella* and *Klebsiella*)
Group 2 Penicillinases	D	Staphylococcal penicillinases; TEM-1, TEM-2, SHV-1(broadspectrum); TEM and SHV variants (e.g., ESBLs, 1cephalosporinase inhibited by clavulanic acid; carbapenemases inhibited by clavulanic acid)
	A	Oxacillin-hydrolyzing, OXA; cloxacilin; ESBL cephalosporins; weak activity for carbapenems (e.g., *A.baumannii*)
Group 3 Metallolo-β-lactamase	B	Zinc-dependent carbapenemases; resistant to inactivation by clavulanic acid, sulbactam, tazobactam, some to aztreonam; (bla_{VM-1}, bla_{VM-2}) *Pseudomonas aeruginosa* … IMP (e.g., *Pseudomonas putida*, *Serratia marcescens*, *A baumannii*, *K pneumoniae*, *Klebsiella oxytoca*, et al. NDM-1 in *E. coli*, *K. pneumoniae*, *Enterobacter cloacae*)

2.4.1.1. Chromosomal-Borne β-Lactamases

Chromosomal-borne β-lactamases found in all Gram-negative bacteria. Among them the most important enzymes are in Group 1 of Bush-Jacoby-Medeiros classification and they exhibit cephalosporinase activity. The most important feature of this group is that they are inducible enzymes.

2.4.1.2. Plasmid Born β-lactamases

TEM-1, TEM-2, and SHV-1 enzymes are the most important plasmid-encoded β-lactamases found in Gram-negative bacteria. These enzymes are referred to as Extended-spectrum enzymes (ESBLs). This group is inhibited by clavulanic acid and are resistance to ampicillin, carbenicillin, ticarcillin, cephalothin and cefamandole [43].

- Group I (Ambler Class C) β-lactamases (AmpC enzymes)

They are mainly encoded by chromosomal genes mostly found in Gram-negative bacteria. These β-lactamases are responsible for cephalosporin resistance. They are not inhibited by β-lactamase inhibitors such as clavulanic acid, sulbactam and tazobactam. Although found in most of the Gram-negative bacteria the main bacteria containing AmpC-type enzyme are; *Enterobacter spp, C. freundii, Serratia spp, P. stuartii, P.rettger, Pseudomonas spp.* and *Hafnium alvei.*

The producers of Group I β-lactamases are resistant to β-lactam/β-lactamase inhibitor combinations, penicillins, cephamycins, as well as 1st, 2nd and 3rd generation cephalosporins. They are sensitive to cefepime and carbapenems [46].

- Group II (Ambler Class A and Class D) β-lactamases

This group can be encoded by chromosomal or plasmid DNA. They are also called Extended-spectrum β-lactamases (ESBLs) due to their effect on oxyimino-β-lactam antibiotics. ESBLs are effective on broad-spectrum penicillins, 1st, 2nd and 3rd generation cephalosporins and partially effective on cefepime; they are ineffective to carbapenems, cephamycins and β-lactam inhibitors. TEM-1 is the most common ESBL encoded by Gram negative bacteria and this enzyme is responsible for resistance occurred in 90% of the ampicillin-resistant *E. coli*. Due to a mutation in the structure of TEM-1 (Lysine replaced Glutamine at position 39), TEM-2 occurs. In terms of the spectrum of action, TEM-1 and TEM-2 are similar.

SHV-1 enzyme, another ESBL, is responsible for about 20% of the plasmid-mediated ampicillin resistance in *Klebsiella pneumoniae*. The first derivative of SHV type enzyme was found in 1983 and has been identified as SHV-2. The number of SHV-1 derived enzyme and changes in amino acid positions is fewer when compared to TEM group of ESBLs. Apart from *K. pneumoniae,* SHV group enzymes have been reported in *Citrobacter diversus, E. coli* and *P. aeuroginosa*.

OXA type β-lactamases are able to hydrolyse oxacillin. Despite their presence mostly in *P. aeruginosa* they are also determined in other Gram negative bacteria. OXA-1 β-lactamase is the most common one and 1-10% of *E. coli* strains have this enzyme.

- Group III (Ambler Class B) enzymes

The Group III enzymes are metallo-enzymes and they are capable of destroying carbapenems [46]. Carbapenemases which are located in Ambler class B or Bush group 3 are known as metallo-β-lactamase (MBL) enzymes and they are clinically most important carbapenemases. These enzymes are frequently found in *P. aeruginosa*, *Bacteroides fragilis* and *Stenotrophomonas maltophilia*.

2.5. Metallo-Beta-Lactamases

Metallo-β-lactamases (MBLs) are enzymes with Zn^2 ion in the active center. Although they are not affected by the classical β-lactamase inhibitors, they are inactivated with metallo chelators, such as mercapto compounds or ethylene diamine tetra acetic acid (EDTA). The first of these enzymes was identified in *Bacillus cereus* in 1960 and then it was shown also in *Stenotrophomonas maltophilia* in the early 1980's.

The origin of MβL is still unknown. However, it is certain that these enzymes are belong to the large superfamily of enzymes known as protein Pfam00753. In 2001, the Pfam00753 members were classified into 17 families based on the biological functions, however most of them need to be properly characterized [34].

Metallo-β-lactamases, encoded by genes carried on mobile DNA elements of Gram negative pathogens (*Enterobacteriaceae, P. aeruginosa*, and *Acinetobacter* species) make the situation more important. Many metallo-β-lactamase genes are present in environmental species which constitute reservoirs for β-lactam resistance genes [11].

2.5.1. Classification of Metallo-β-Lactamases

Based on their structural analogies, MβLs are classified as: B1, B2 and B3 and each subgroup is characterized by a specific conserved motif (Table 2.2) [11]. The most frequently encountered enzymes in the clinical setting (B1) show a very large activity spectrum and is composed of enzymes that containing three histidine and a cysteine amino acid. B2 has a narrow spectrum. The substantial difference among B1, B2 and B3 is rely on amino acid residue, at position 116: In the subclass B2, the

histidine residue is replaced by an asparagine residue, while in B1 and B3 groups, it is replaced by a glutamine residue. B3 MβLs have a broad spectrum activity and they do cephalosporin hydrolysis with high specificity and mainly found in environmental or opportunistic bacterial species [2, 11].

Table 2.2: Classification of Metallo-β-lactamases.

Subclass	Enzyme	Strain	Discovery In	GenBank accession no.	Accession no.(protein)	Structure in
B1	BCII	*B.cereus*	1966	M11189	AAA22276	Mono-zinc,
	CcrA	*B.fragalis*				Di-zinc,
	BlaB	*E.meningosep*				Apo-form
	IND-1	*tica*	1990	M63556	AAA22904	Di-zinc
	EBR-1	*Chryseobacte*	1998	AF189298	O08498	Di-zinc
	SFB-1	*rium*	1999	EF394437	ABO21411	
	SLB-1	*indologenes*	2002			
		E.brevis	2005	AF416700	AAN32638	
		Shewanella		AY590119	AAT90847	
Acquired	IMP-1	*frigidimarina*	2005			
-B1			1994	AY590118	AAT90846	
	VIM-1	*Shewanella*	1999			
		livingstonensi	2000			
	VIM-2	*s*	2000	S71932,	AAB30289,	Di-zinc
		S.marcescens,	2002	AY168635	AAN87168	
	IMP-2	*P.aerugosa*	2003	Y18050	CAE46717	
		P.aeruginosa,	2004		AAK26253	
	SPM-1	*A.baumannii*	2005	AF191564		Mono-zinc
	VIM-4	*P.aeruginosa,*	1991		BAD26594	1KO2
		A.baumannii	1996	AB182996		Di-zinc
		A.baumannii,	2003		AAR15341	1KO3
	GIM-1	*S.marcescens*	1991	AY341249		
	SIM-1	*P.aeruginosa*	2000	AY135661	AAR22402	
		P.aeruginosa,	2000		CAF05098	Mono-zinc
B2	CphA	*K.pneumonia*	2001			
	ImiS	*e,*	2001	AJ620678		
	Sfh-1	*Enterobacter*	2002	AY887066	AAX76774	
		cloacae			P26918	
B3	L1	*P.aeruginosa*	2006	X57102		
		A.baumannii		Y01415	CAA71411	
	GOB-1	*A.hydrophila*		AF197943	AAF09244	Mono-zinc
	FEZ-1	*Aeromonas*		AB294542	CAB75346	
	THIN-B	*veronii*		AF090141	ABO21417	
	MbL1b	*S.fonticola*		Y17896	CAB96921	
	CAU-1	*Stenotrophom*		AJ250876	CAC33832	Di-zinc,
		onas		AJ315850	CAC48262	Mono-zinc
	BJP-1	*maltophilia*		AJ308331	CAC87665	2H6A
		E.meningosep			NP_772870	Apo-form
		tica				2FU6
		L.gormanii				Di-zinc
		J.lividum				Di-zinc
		C.crescentus				2GMN
		Caulobacter				
		vibrioides				
		B.japonicum				

2.5.2. Structure of Metallo-β-lactamase

The MβLs are monomeric enzymes consisting of a polypeptide chain of 25-28 kDa [2]. The only exception is the L1 of *Stenotrophomonas maltophila*, which has tetrameric structure [35].

They have two potential Zn^{2+} binding sites (Figure 2.6) [11]. However, all members of the Pfam00753 exhibit a conserved motif, indispensable for the hydrolytic activity, which consists of histidine residues responsible for binding to zinc ion. In the case of B1 enzymes, one zinc ion has a tetrahedral coordination sphere and is composed of His_{116}, His_{118}, His_{196} and a water molecule or OH^- ion. The other metallo ion has a trigonal-pyramidal coordination sphere which involves Asp_{120}, Cys_{221}, His_{263} and two water molecules. One water/hydroxide molecule serves as a ligand for both metallo ions. The two binding sites are named as ‘’histidine’’ and ‘’cysteine’’ sites, respectively [11].

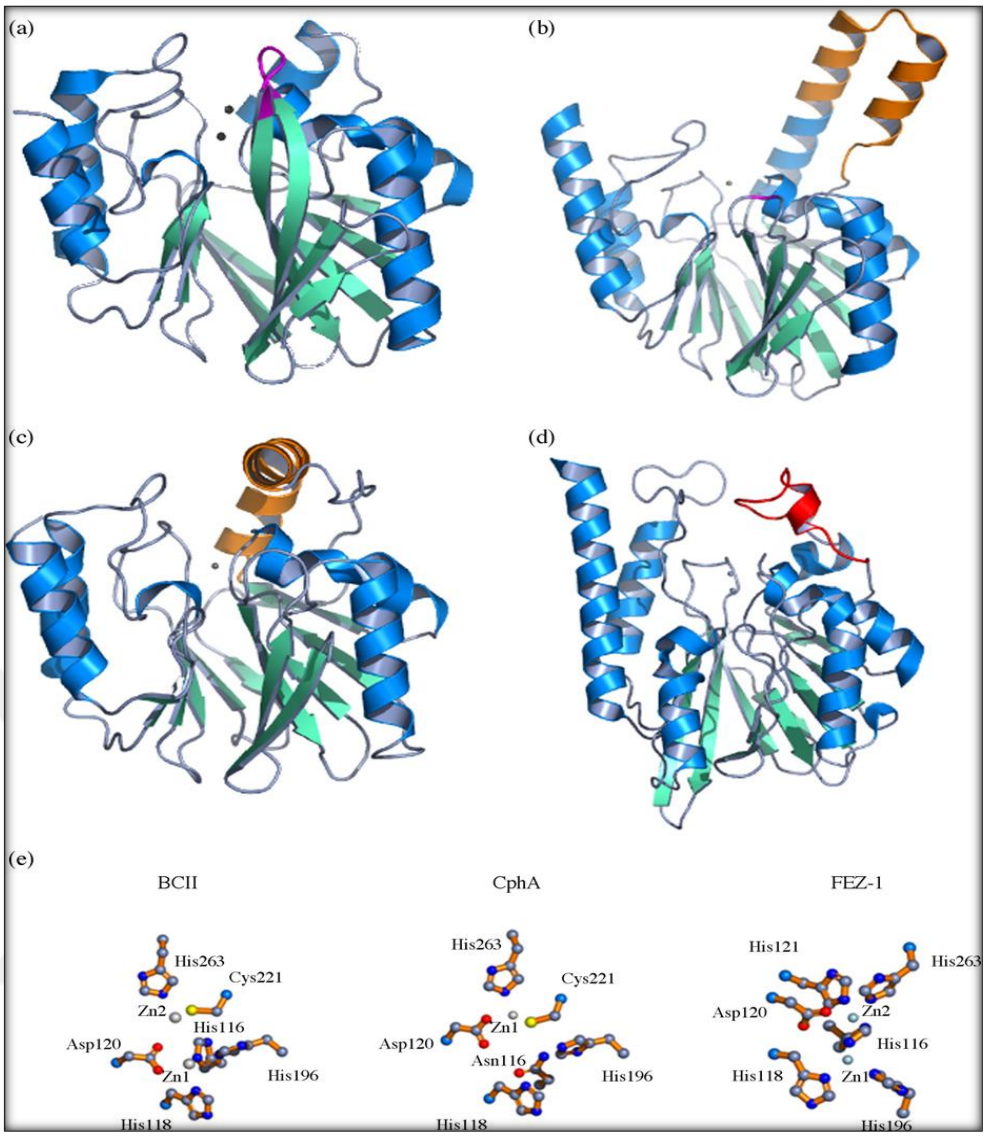


Figure 2.6: Structure of metallo-β-lactamases. The helices are represented in blue, strands in green and loops in grey. a) Subclass B1 BcII enzyme with the mobile 61–65 loop in magenta. b) Subclass B1 SPM-1 enzyme with residues 61–65 in magenta and the extended α3-α4 region in orange. c) Subclass B2 CphA enzyme with the elongated α3 helix in orange. d)Subclass B3 FEZ-1 enzyme with the 151–166 loop in red. e) Representation of the zinc binding sites of subclass B1 (BcII), B2 (CphA), and B3 (Fez-1) β-lactamases.

3. MATERIALS AND METHODS

3.1. Materials

3.1.1. Chemicals

Agarose (Merck), Ethidium Bromide (Sigma Aldrich), DNA Ladder (MBI Fermentas), Absolute EtOH (Merck), SDS (Sigma Aldrich), NaOH (Merck), EmeraldAmp GT PCR Master Mix (Takara Bio Inc.), Glycerol (Sigma Aldrich), DMSO (Thermo Fisher Scientific), Glucose (Merck), Tris (Merck), EDTA (Merck), Tris-HCl (Merck), Deoxynucleotide (dNTP) Solution Mix (New England Biolabs), $MgCl_2.6H_2O$ (Merck), Tryptone Broth (Himedia), HiVeg (Himedia), Yeast Extract (Himedia), Agar Powder (Merck), NaCl (Merck), KCl (Merck), 7,5M Ammonium Acetate (Sigma Aldrich), Bovine Serum Albumin (BSA 10X) (New England Biolabs), Glacial Acetic Acid (Sigma Aldrich), Mueller-Hinton Agar (Liofilchem), Ampicillin (Sigma Aldrich), Chloramphenicol (Sigma Aldrich), Brain Heart Infusion Broth (BHI) (Sigma Aldrich).

3.1.2. Enzymes

*Nco*I restriction endonuclease (Promega), *Pst*I restriction endonuclease (Fermentas), *T4 DNA Ligase* (Promega), *EcoR*I restriction endonuclease (New England Biolabs), *Nde*I restriction endonuclease (New England Biolabs), *Kpn*I restriction endonuclease (New England Biolabs), *Dpn*I restriction endonuclease (New England Biolabs), *Sma*I restriction endonuclease (New England Biolabs), *Taq DNA polymerase* (Thermo Fisher Scientific), *Sac*II restriction endonuclease (New England Biolabs).

Enzyme reactions were performed with buffer recommended by the producing companies.

3.1.3. Kits and Molecular DNA Markers

- Two different kits were used to purify of the DNA fragments from gel in this experiment: Wizard®SV Gel and PCR Clean-up System (Promega), and Gel/PCR DNA Fragments Extraction Kit DF/100/DF300 (Geneaid).
- Isolation of plasmid DNA from bacterial culture was performed with Euro Gold Plasmid MiniPrep Kit (EuroClone).
- Purification of the PCR products has made use of the following kits: Microelute Cycle-Pure Kit (OMEGA bio-tec).

In this study 1kb molecular-weight size marker (MBI Fermentas) used in gel electrophoresis (Figure 3.1).

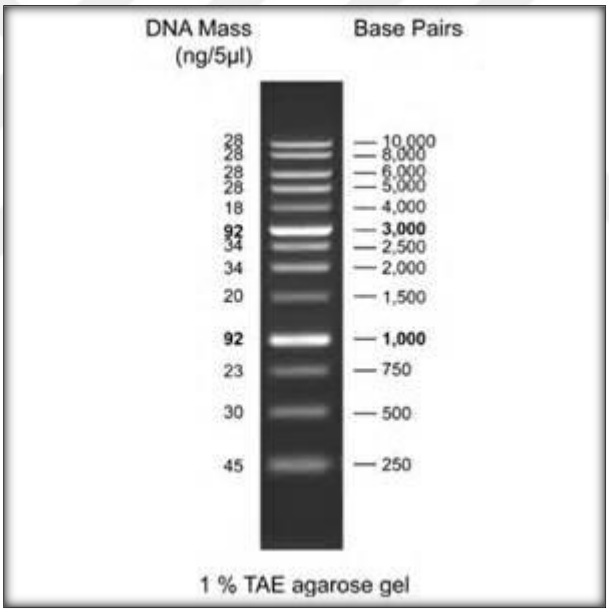


Figure 3.1: 1 kb DNA Ladder (MBI Fermentas).

3.1.4. Plasmids, Bacterial Strains and Growth Conditions

Bacterial strains and plasmids used in this study were shown in Table 3.1. and Table 3.2. All bacterial strains used in this study were grown at 37°C in aerobic conditions at 180rpm. Ampicillin (30 µg/mL) and chloramphenicol (68 µg/ml) was used when necessary.

Table 3.1: Bacterial strains used in this study.

Bacterial Strains	**Reference**
E. coli DH5α	Hanahan, 1983
Candidatus Solibacter usitatus (Ellin6076)	Ward et al. 2009
Fluoribacter gormanii	ATCC 33297^{T}
Sol65	This study
Fez	This study

Table 3.2: Plasmids used in this study.

Plasmids	**Resistance gene**	**Resistance**	**Size**	**Reference**
pGEM®-T Easy	*Amp*	Ampicillin	3015bp	Promega
pBluescript SK	*Amp*	Ampicillin	2958bp	Stratagene
pBCSK	*Cat*	Chloramphenicol	3400bp	Di Meo, 2012
pLBII	*Cat*	Chloramphenicol	3362bp	Di Meo, 2012
pR_Nco	*Amp*	Ampicillin	3580bp	This study
pRistu	*Amp*	Ampicillin	4518bp	This study
pComb-III	*Amp*	Ampicillin	4029bp	Creative Biogene
pR pelBSol65	*Amp*	Ampicillin	3822bp	This study
pR pelBSing65	*Amp*	Ampicillin	3822bp	This study
pBC SING65	*Cat*	Chloramphenicol	4261bp	This study

3.1.5. Media and Solutions

- LB (Luria Broth)

  -10 gr/L Trypthone

  -5 gr/L Yeast Extract

  -10 gr/L NaCl

  It has been prepared with dH_2O and autoclaved at 121° C for 20 minutes.

- LBA (Luria Agar)

  -10 gr/L Trypthone

-5 gr/L Yeast Extract

-10 gr/L NaCl

-1.5% Agar Powder

It has been prepared with dH_2O and autoclaved at 121° C for 20 minutes.

- SOB

  -% 2 Trypthone

  -% 0.5 Yeast Extract

  -10 mM NaCl

  -10 mM KCl

  -10mM $MgSO_4$

  It has been prepared with dH_2O and autoclaved at 121° C for 20 minutes.

- SOC

  -To make 1 L of media:

  -970 mL SOB

  -20 mL Glucose %20

  -10 mL $MgCl_2$

All solutions have been prepared in sterile conditions and after they have been mixed into a sterile tube under sterile conditions.

- %20 Glyserole Brain Heart Infusion Broth

  -For 1 liter:

  -37 gr BHI

  -800 ml dH_2O

  -200 ml Glycerol

  It has been autoclaved at 121° C for 15 minutes.

- MHA (Mueller-Hinton Agar)

It has been prepared according to the supplier's instructions, 36g of powder was dissolved in 1 liter of deionised water and autoclaved at 121° C for 15 minutes.

- Tris-EDTA (TE) Buffer

-10mM Tris-HCl (pH 8.0)

-1mM EDTA (pH 8.0)

- Tris-Acetate-EDTA (TAE) Buffer (50X)

For 1 liter :

-242 gr Tris

-57,1 ml Glacial Acetic Acid

-100 ml 0,5M EDTA (pH 8.0)

- Loading Dye

-88 µl MilliQ

-12 µl Ladder

-20 µl Buffer

3.1.6. Equipments

+4°C Refrigerator (Ariston), -20°C Deep-Freezer (Ariston), -80°C Deep-Freezer (Ariston), Ice Maker (BAR-LINE BF85), Benchtop Centrifuge (Eppendorf), Mini Centrifuge (Eppendorf), High Speed Refrigerator Centrifuge (Eppendorf), Autoclave (Hirayama, Hiclave HVE-50), pH Meter (Lab-Kits), Sterile Cabin (Heraeus, HP48), Fume Hood (Biraghi), Incubator (Binder, 9010-0078), Shaking Incubator (Edmund Bühler, KS-15), Vortex mixer (Scilogex MX-S), Electronic Laboratory Balance / Bench-top (KERN), Hot Water Bath (TECHNE,TE-10A Tempette), UV Transilluminator (Wealtec, MD-25/HD-25), Microwave Oven (Siemens), PCR (Thermo Fisher, GeneAmp® PCR System 9700), Spectrophotometer (BIO-RAD, Hercules, California), Electroporation Device (BIO-RAD, Hercules, California, U.S.A.).

3.2. Methods

3.2.1. Information About Plasmids

- pGEM®-T Easy (Promega)

Commercial vector pGEM-T Easy (Figure 3.2) was used for the cloning of PCR products.

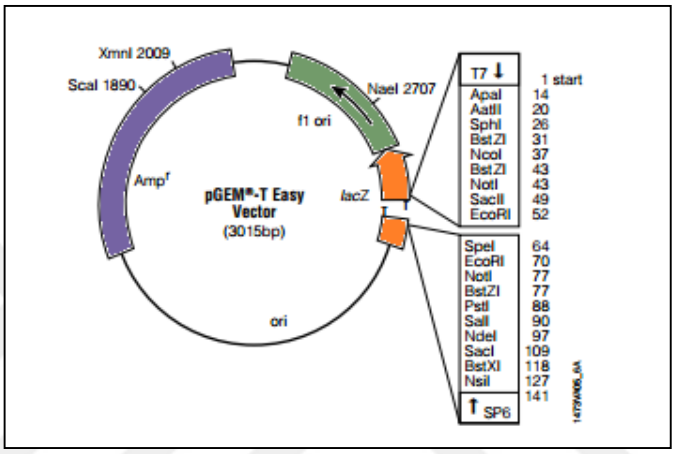


Figure 3.2: pGEM®-T Easy Vector Map (Promega).

- Plasmids pBCSK and pLBII

pBCSK (Figure 3.3a) is derived from pBluescript II SK phagemid, with a chloramphenicol resistance gene and commonly used cloning and sequencing procedures such as site-specific mutagenesis. Vector pLBII (Figure 3.3b) has been used to place the cloned ORFs, under the pLac promoter, with a selection resistance different from the ampicillin one.

pLBII is 3362 bp plasmid which was obtained by changing the polylinker site of pBCSK. With this vector directional cloning is possible into *Nde*I and *Bam*HI sites.

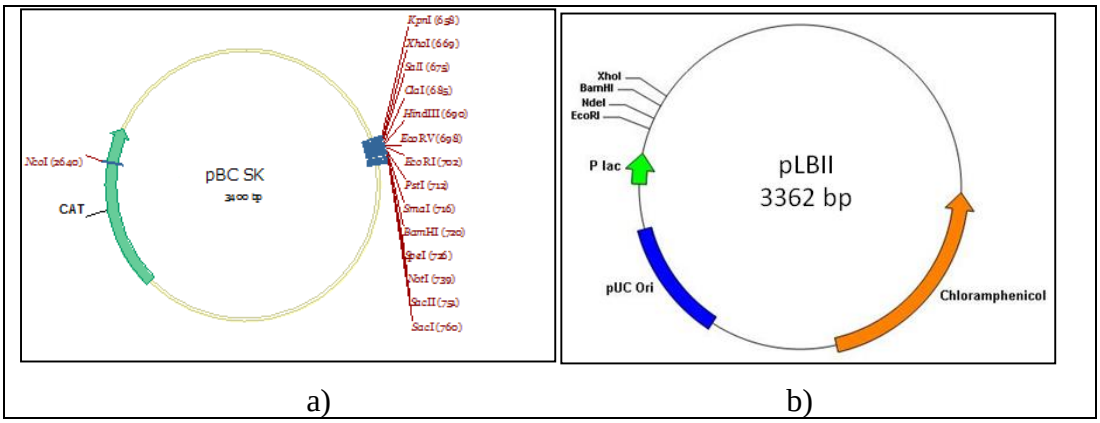


Figure 3.3: Circular map of pBCSK a) and pLBII b).

- Plasmid pR_Nco

This plasmid was used to obtain fusion on the studied ORFs with the *pelB* signal secretion sequence. It was obtained by digesting a pCombIII vector with *Nco*I and self-ligating it. pCombIII vector (Figure 3.4.) is a phagemid vector which was designed for phage display of Fabs also other proteins like zinc fingers, peptides and cDNA fragments [36].

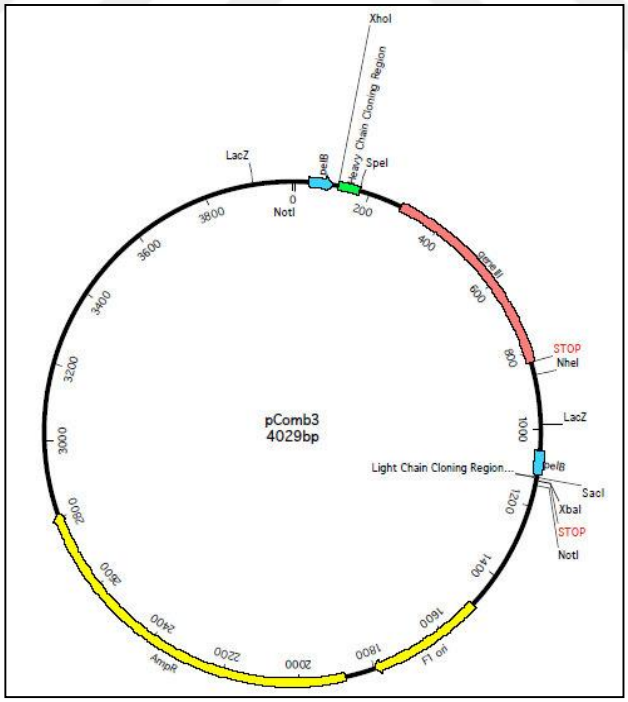


Figure 3.4: pCombIII vector map (Creative Biogene).

- Plasmid pRistu

The pR_Nco vector lacks of *BamH*I and *Eco*RI sites, both present in the reverse primer originally to amplify this ORF and therefore necessary to ligate it to the vector. To circumvent this problem, a stuffer plasmid was prepared. One of the other previously cloned ORFs (Sol_51) also had *BamH*I and *Eco*RI sites at the 3' end. It was excised from pGEM using the *Nco*I and *Pst*I restriction sites located on the pGEM vector itself, and ligated to pR_Nco, digested with the same enzymes. In this way, the desired restriction sites were introduced and, at the same time, a useful tool to check the restriction reactions was provided. The double *Nco*I-*BamH*I or *Nco*I-*Eco*RI restrictions, indeed, detache about 800 bp from this new plasmid, which was named pRistu (Figure 3.5.).

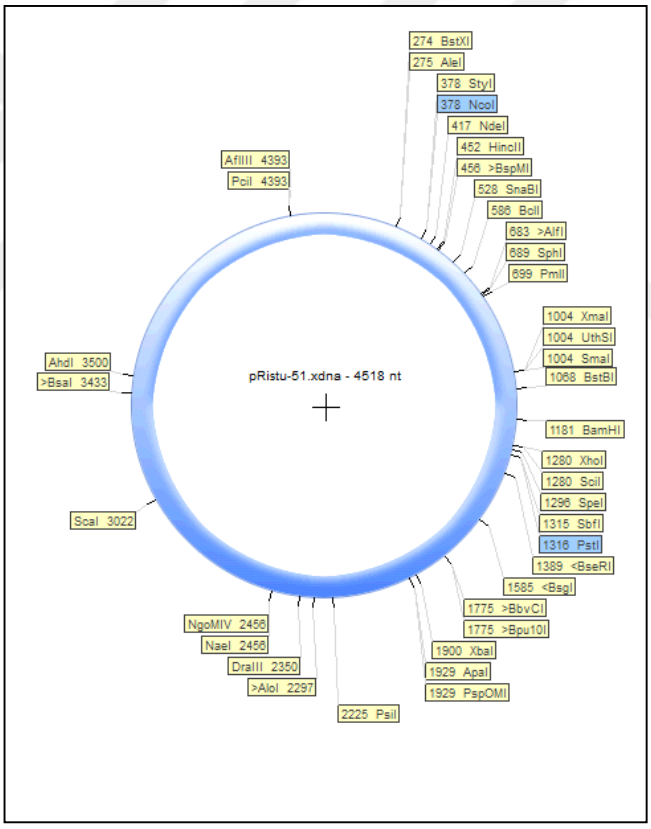


Figure 3.5: Circular map of plasmid pRistu.

3.2.2. Genomic DNA Isolation

Genomic DNA isolation was carried out according to Sambrook et.al. (1989). Approximately 10^{-8} bacteria were suspended in 100 µl of sterile bi-distilled water and

incubated for 5 minutes at 95°C, then few minutes in ice. Subsequently, the samples were centrifuged for 5 minutes at 14000 rpm at room temperature. Supernatant which contain DNA was then used as template for PCR.

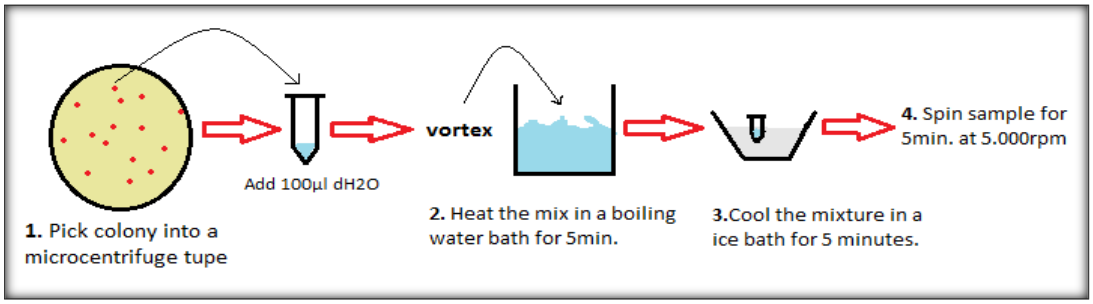


Figure 3.6: Schematic overview of the protocol for extraction of genomic DNA.

3.2.3. Plasmid DNA Isolation

Plasmid DNAs from overnight cultures were extracted by using "Isolation of Plasmid DNA from Bacterial Culture with Euro Gold Plasmid MiniPrep Kit", according to the directions of manufacturer's.

3.2.4. Purification of the PCR Products

PCR products and DNA from agarose gels were purified by using Microelute Cycle-Pure Kit (OMEGA bio-tec), Wizard®SV Gel and PCR Clean-up System (Promega) and Gel/PCR DNA Fragments Extraction Kit DF/100/DF300 (Genaid) according to the directions of manufacturer's.

3.2.5. Ammonium Acetate Precipitation of DNA

This technique was applied in case of sequential enzymatic digestion according to Crouse et.al. (1987). The product of the first digestion was purified to allow proceeding with the second. First of all, a solution was prepared of ammonium acetate 7.5 M, to which was added 0.5 volumes to the sample to be purified. Immediately after 3 volumes of absolute ethanol was added and the solution was left to -20°C for 1 hour. After this period of time, it was centrifuged for 15 minutes at

14000 rpm while making sure that the rotor is very cold. Subsequently, the supernatant was removed and the pellet was submitted to rapid washing with 70% ethanol. The sample thus treated was dried under a hood, and to this point, the pellet was suspended with water and buffer of the subsequent enzymatic digestion.

3.2.6. Electrophoresis of Nucleic Acids

Electrophoretic analysis was conducted by taking advantage of agarose gel 1% (p/v in TAE 1X = 40 mM EDTA, pH = 8.0). The agarose/buffer mixture was melted by heating in the microwave. Run the gel until the dye has migrated to an appropriate distance. After electrophoresis, the gel was stained in the solution containing Ethidium Bromide (1μg/ml in 1X TAE Buffer) for 10-15 minutes.

Subsequently, the decolourization was carried out soaking the gel in distilled water bath for about 5 minutes and then the gel was transferred to UV transilluminator and acquired the image of the gel. It was displayed in fluorescence having as reference to the molecular weight marker 1kb DNA Ladder (MBI Fermentas).

3.2.7. Preparation of Electrocompetent Cells

Preparation of electrocompetent cells was carried out according Krantz B. et al. (*Making Electrocompetent Cell* protocol). The bacteria were streaked out frozen glycerol stock of bacterial cells onto LB agar plate (without antibiotic) and grew plate overnight at 37°C.

A single colony of *E. coli* DH5α was selected from fresh LB agar plate and inoculated a 50 mL starter culture of LB. The culture grows overnight at 37°C in shaker.

The bacteria were incubated in a shaker at 37°C in 50 mL of LB medium to obtain an OD_{600} = 0.8. The culture was then centrifuged for 12 minutes at 6000 rpm while maintaining a temperature of 4°C. The pellet was suspended in sterile bi-distilled water and centrifuged (always at 4°C) for 10 minutes at 7000 rpm. The culture was then centrifuged with the last centrifugation of 12 minutes 6000 rpm

(again at 4°C). The supernatant was carefully aspirated with a sterile pipette and then each pellet was resuspended in 400 µl of ice cold 10% glycerol by gently swirling.

Finally, the cells were divided into sterile 1.5 mL microfuge tubes with 50µl final volume and stored frozen cells in the -80°C freezer.

3.2.8. Electroporation

Electroporation protocol was carried out according to Krantz B. et. al. For the transformation by electroporation, to a rate of 50µl of electrocompetent cells was added 1µl of ligation mixture. The whole was transferred to a cuvette for electroporation which was sterile and thickness of 0.2cm (BIO-RAD, Hercules, California, U.S.A.).

To this point it applies to 4-5 milli-seconds an electric field of 1.80 kV advantage of electroporator Micro Pulser® Apparatus (BIO-RADS, Hercules, California, U.S.A.).

The cuvettes were removed from the chamber and immediately added 1 ml SOC medium. SOC-cell mixture was transferred to an eppendorf tube then the bacterial suspension was incubated 1 hour at 37°C and subjected to constant stirring to permit expression of antibiotic resistance gene.

The whole was plating onto LB agar supplemented with appropriate antibiotic and finally the plate was incubated overnight at 37°C.

3.2.9. PCR (Polymerase Chain Reaction)

- Standard PCR Protocol

Polymerase chain reaction was used both to amplify the DNA fragments and to control their presence in the plasmid vector after cloning.

PCR reactions were performed in 50 µl volume. The reaction mixture was: Forward and reverse primers (10 pM) specific for each reaction, EmeraldAmp GT PCR Master Mix buffer 25 µl, 50 ng of template DNA and sterile MilliQ water up to the final volume.

- The PCR amplification reaction conditions

The following is the standard program of PCR, which was used for the reactions concerned by suitably varying the annealing temperature.

Table 3.3: Standard PCR Programme.

Cycle	Temperature	Time	Phase
1	94°C	1:30 minutes	Denaturation
5	94°C	0:50 minutes	Denaturation
	55°C	0:50 minutes	Annealing
	72°C	0:50 minutes	Extension
25	94°C	0:50 minutes	Denaturation
	64°C	0:50 minutes	Annealing
	72°C	0:50 minutes	Extension
1	72°C	5:00 minutes	Extension

- Colony PCR

Colony PCR is a convenient high efficiency method for determining the presence or absence of insert DNA in plasmid constructs directly from bacterial colonies.

Following transformation of the ligation reaction into competent *E. coli* cells, some of bacterial colonies were selected from the selective agar plate, boiled for 5', and centrifuged to discard cellular detritus. The supernatant was then used as the template for the Colony PCR by using conditions given in Table 3.3.

PCR reactions were performed in a total volume of 10 µl as describe above.

3.2.10. *Dpn*I Mediated Site Directed Mutagenesis Protocol

Oligonucleotides used in site directed mutagenesis (SDM) experiments were given in Table 3.4.

Table 3.4: Oligonucleotides (Sigma Aldrich) used.

Primers	**Abbreviations**	**Nucleotidic Sequences 5′→3′**
NG SOL65_EcoRI_BamHI_Rev	S_NG_Rev	GGAACCCGGCATTCACACCCGGGCTGCCCACGATC
NG SOL65_NcoI_Fw	S_NG_Fw	GATCGTGGGCAGCCCGGGTGTGAATGCCGGGTTCC
YM SOL65_ EcoRI_BamHI_Rev	S_YM_Rev	AGCGGCTGAACTTGCGCTCGAGCCCGTACATATCGCCGTGGGCGCCC
YM SOL65_ NcoI_Fw	S_YM_Fw	GGGCGCCCACGGCGATATGTACGGGCTCGAGCGCAAGTTCAGCCGCT
GN FEZ_ EcoRI_BamHI_Rev	F_GN_Rev	ATCAACCAATTTATACCCGGGATTTACGTTAATACTTCCTATAATTAC
GN FEZ_ NcoI_Fw	F_GN_Fw	GTAATTATAGGAAGTATTAACGTAAATCCCGGGTATAAATTGGTTGAT
MY FEZ_ EcoRI_BamHI_Rev	F_MY_Rev	TTATTCTTCAGATCAAAGTATCCGGCGTGCGATCCTAGAAAAA
MY FEZ_ NcoI_Fw	F_MY_Fw	TTTTTCTAGGATCGCACGCCGGATACTTTGATCTGAAGAATAA
LacZ_SphI_Fw	L_Fw	GCATGCAGCTGGCACGACAG
BRAVALLE_Rev	B_Rev	GATTTTGCCGATTTCGGCCTATTGG

Site-directed-mutagenesis was carried out according to Loening et.al. (2005). To amplify mutant DNA, PCR reactions were performed in a total volume of 50 µl as shown in Table 3.5. 50 µl reaction volume contained 10 pM primers, EmeraldAmp GT PCR Master Mix buffer, sterile MilliQ water and template DNA.

Table 3.5: PCR Program of *Dpn*I-Mediated Site-Directed Mutagenesis.

Cycle	**Temperature**	**Time**	**Phase**
1	95°C	5:00 minutes	Denaturation
18	95°C	0:50 minutes	Denaturation
	60°C	0:50 minutes	Annealing
	68°C	5:30 minutes	Extension
1	68°C	7:00 minutes	Extension

PCR products were purified with Microelute Cycle-Pure Kit (OMEGA bio-tec). Then PCR products were digested with *Dpn*I (*New England Biolabs*) in order to eliminate methylated DNA. *Dpn*I digestions were performed in a final volume of 30 µl as manufacturer's recommendations. After *Dpn*I treatment, electrocompetent *E. coli* cells were transformed with PCR products.

Following the transformation, the colonies were picked from agar plate into LB broth containing ampicillin to a final concentration of 100µg/mL. Subsequently it was incubated at 37°C for 24 hours.

After the 24 hours incubation period, plasmid DNA was isolated from the bacterial culture with EuroGold Plasmid MiniPrep Kit.

Finally, the plasmid DNA prep was digested with *Sma*I (*New England Biolabs*) restriction endonuclease looking for mutants.

3.2.11. Site-Directed-Mutagenesis Protocol-2

To create the desired mutations, it was also used an alternative approach by combining the mutagenesis primers with external ones and performing serial PCR reactions according to Ling and Robinson (1997). Firstly, two independent PCR reactions (PCR A and PCR B) were performed as shown in Table 3.6.

- PCR A; Forward primer L_Fw anneals to the *lac*Z gene on the plasmid vector. Reverse primer S_NG_Rev, which introduce the mutation, anneals to the ORF.
- PCR B; Forward primer S_NG_Fw, which introduce the mutation, anneals to the ORF. Reverse primer B_Rev, anneals on the plasmid vector.

For this purpose, primers (10 pM), EmeraldAMP® GT PCR Master Mix buffer, sterile double distilled water and DNA of interest were mixed in a final volume of 50 µl.

Table 3.6: Standard program of PCR A and PCR B.

Cycle	Temperature (◦C)	Time(minutes)	Step
1	95◦C	5:00 minutes	Initial Denaturation
18	95◦C	0:50 minutes	Denaturation
	56◦C	1:30 minutes	Annealing
	68◦C	6:00 minutes	Extension
1	68◦C	7:00 minutes	Final Extension

The «A» and «B» amplicons have been purified, mixed, denatured, allowed to anneal and extended in this step. For this purpose, a PCR reaction was performed as shown in Table 3.7. EmeraldAMP® GT PCR Master Mix buffer (TAKARA BIO INC.), sterile double deionized water and DNA of interest (equal amounts of «A» and «B» amplicons) were mixed in a final volume of 30 µl.

Table 3.7: Standard PCR program to mix and denaturate of PCR A and B.

Cycle	Temperature (◦C)	Time(minutes)	Step
1	95◦C	5:00 minutes	Initial Denaturation
1	95◦C	0:30 minutes	Denaturation
1	55◦C	10:00 minutes	Annealing
1	68◦C	10:00 minutes	Extension

The external primers L_Fw and B_Rev are introduced to get the whole mutated ORF. For this purpose, to the reaction mixture of this step, 20 µl of fresh reaction buffer, containing the external primers and no template, were added.

Table 3.8: PCR program of PCR C.

Cycle	Temperature (◦C)	Time(minutes)	Step
1	94◦C	1:30 minutes	Initial Denaturation
1	94◦C	0:50 minutes	Denaturation
1	52◦C	0:50 minutes	Annealing
1	72◦C	0:50 minutes	Extension
1	72◦C	5:00 minutes	Final Extension

Following the reactions, the mutated samples were digested with *Nco*I (*Promega)* restriction enzyme and *Eco*RI (*New England Biolabs*) restriction enzyme in order to perform ligation with the plasmid pRistu and then mutated samples were

ligated to the plasmid vector pRistu, restricted with the same enzymes and purified. In this way, we obtained pR pelBSing65 plasmid DNA.

E. coli DH5α competent cells were transformed with plasmid containing the insert Sing65. Immediately after that the transformant cells were spreaded onto selective plates containing ampicillin (30μg/mL). Selective colonies were picked into a microcentrifuge tube to perform Colony PCR and after obtained by agarose gel electrophoresis.

Following the agarose gel electrophoresis, DNA fragments were cut based on size out of the agarose gel and the DNA samples were purified; it has benefited from the following kits: Promega and Genaid Kits.

The construct DNA was checked for the appearance of a *Sma*I site, introduced by the mutation.

After checking with *Sma*I, construct DNA was digested with *Sac*I restriction endonuclase and *EcoR*I restriction endonuclease enzymes and undirectionally cloning the fragment in pBCSK →pBC SING 65 vector in order to verify the presence of the metallo-β-lactamase activity.

3.2.12. Metallo-β-lactamase Activity

In order to test the enzymatic activity of the metallo-β-lactamase object of study, the recombinant clones have been subjected to Kirby Bauer Method:

- Kirby Bauer method

For this technique, the control strain (*E. coli* containing the plasmid pBCSK) and the recombinant strain (pBC SING 65) were grown overnight at 37°C on plates of LB Agar added with chloramphenicol 68 μg/ml. Subsequently, the colonies were removed to create a suspension with turbidity equal to that of point 0.5 of the standard of Mc Farland. The standard McFarland is a reference preparation useful for modulating the turbidity of the bacterial suspensions in order to obtain a specific quantity of cells: about $1,5*10^8$ CFU/ml. This corresponds to a specific absorbance value measured by a spectrophotometer: OD_{600}: 0.14.

At this point, the strains in question has been swabbed uniformly across on the MHA plate. Following that, filter-paper diskettes containing the following β-lactam

antibiotics (*Aztreonam, Piperacillin, Cefotoxime, Ceftazidime, Imipenem, Ampicillin, Cefoxatin*) have been placed on the surface of the MHA plate.

After incubation for 18 hours at 37°C, the zones of inhibition of bacterial growth around the discs were measured.

4. RESULTS

In a previous work carried out in "University of Tor Vergata, Department of Biology (Italia)", metallo-β-lactamase-like proteins from seven environmental bacteria (*Solibacter usitatus, Mucilaginibacter paludis, Opitutus terrae, Sphingopyxis alaskensis, Asticcacaulis biprosthecium, Acinetobacter radioresistens* and *A. nosocomialis*) were identified on the basis of sequence homology with 13 different functional enzymes (Table 4.1) by BLAST analysis. Putative metallo-β-lactamase (MβL) genes from these seven bacteria were cloned in *E. coli* and β-lactamase activity of each recombinant bacterium were measured. The preliminary results showed that most of the analysed enzymes, although they have high sequence similarity with the functional enzymes, had poor or no β-lactamase activity in *E. coli* [2].

Table 4.1: Functional metallo-β-lactamases (MβLs).

Enzyme	**Swiss-Prot**	**Species**	**Phylum**
L1	P52700	*Stenotrophomonas maltophilia*	γ-Proteobacteria
FEZ	Q9K578	*Fluoribacter gormanii*	γ-Proteobacteria
GOB-1	Q9RB00	*Elizabethkingia meningoseptica*	Fibrobacteres/Bacteroidetes
THIN-B	Q9AEF9	*Janthinobacterium lividum*	β-proteobacteria
CAU-1	Q8KKG1	*Caulobacter crescentus*	α-proteobacteria
BRAJA	Q89GW5	*Bradirhyzobium japonicum*	α-proteobacteria
NOVAD	Q2G7J0	*Novosphingobium aromaticivorans*	α-proteobacteria
AIM	B5DCA0	*Pseudomonas aeruginosa* (LGT)	γ-Proteobacteria
LRA-2	B5L5V0	unknown (from shotgun metagenomics)	----------
LRA-19	B5L5X0	unknown (from shotgun metagenomics)	----------
LRA-3	B5L5V2	unknown (from shotgun metagenomics)	----------
SMB-1	G5ELM3	*Serratia marcescens* (LGT)	γ-Proteobacteria
POM-1	E6Y3G9	*Pseudomonas otitidis*	γ-Proteobacteria

In order to find an answer for the lack of β-lactamase activity in the recombinant strains, one of the clones have been chosen for further analysis. ORF *Acid_6874* of *Solibacter usitatus* has been chosen since its amino acid sequence was highly homologous (54% identity, 73% similarity) to the functional MβL of *Fluoribacter gormanii* (FEZ) (Figure 4.1). *S. usitatus* MβL-like ORF *Acid_6874* protein was named Sol_65 in this study.

Figure 4.1: Alignment of amino acid sequences of MβL of *F. gormanii* (FEZ) and MβL-like protein of *S. usitatus* (Sol_65). "*" identical sequences, "**:**" conserved substitutions (amino acid is replaced by one having similar characteristics), "**.**" semi-conserved.

Predicted structure of Sol_65 (Figure 4.2a) was compared with the structure of FEZ (Figure 4.2b). Overlapping of the two protein structures had highlighted the structural differences between Sol_65 and FEZ proteins (Figure 4.3, Figure 4.4). It was predicted that amino acids at 223rd (Asn_{223}: N_{223}) and 266th (Tyr_{266} : Y_{266}) positions in the active center of Sol_65 could be responsible for the possible steric hindrance that prevent the entry of the β-lactam ring in the active site (Figure 4.4). It was decided to change those amino acids with the once present in FEZ at the same sites and vice versa by site directed mutagenesis (Figure 4.5) [2]. Present study

covers experimental results of site directed mutagenesis of 223rd amino acid in both Sol_65 (N_{223}→G) and FEZ (G_{223}→N) proteins and the effect of these mutations on MβL activity.

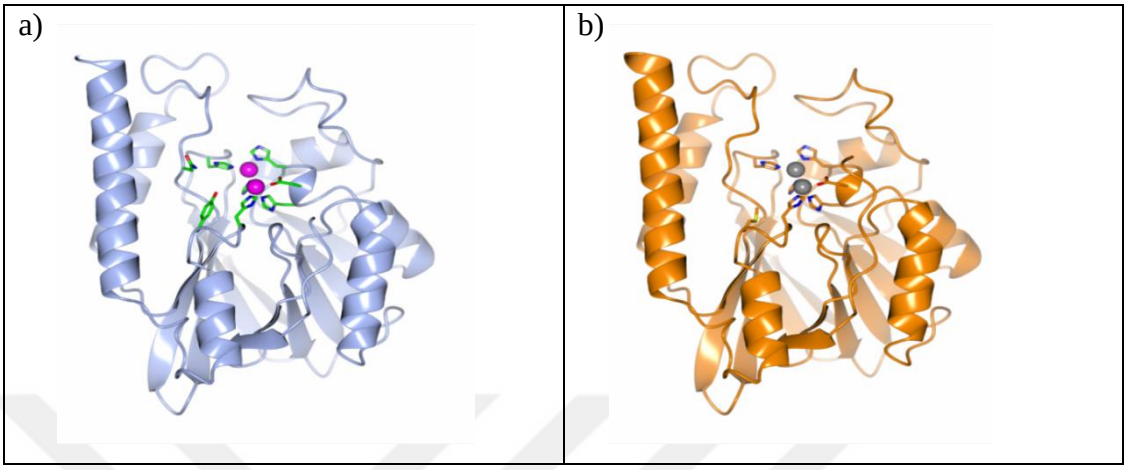


Figure 4.2: Comparison of predicted structure of Sol_65-MβL with the structure of FEZ-MβL. a) Virtual structure of Sol_65 protein: the helices, strands and loops are represented in blue. The active site of Sol_65 is consisting of two Zn (II) ions (pink spheres) bound by the indicated ligand residues (shown as sticks) and a bridging water/hydroxide molecule (red parts); b) structure of FEZ protein: the helices, strands and loops are represented in orange. The active site of FEZ is consisting of two Zn (II) ions (grey spheres) bound by the indicated ligand residues (shown as sticks) and a bridging water/hydroxide molecule (red parts).

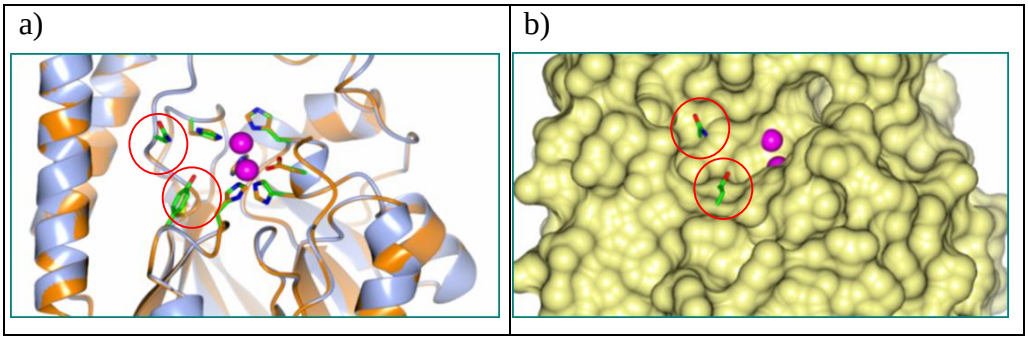


Figure 4.3: Overlapping of Sol_65 and FEZ proteins structures. Active site residues are rendered as sticks and zinc ions (purple) are shown as spheres.

Figure 4.4: Alignment of amino acid sequences of FEZ-MβL and Sol_65-MβL proteins' active sites.

Sol_65 protein	FEZ protein
N_{223}→G	G_{223}→N
Y_{266}→M	M_{266} →Y

Figure 4.5: Mutagenesis sites in Sol65 and FEZ proteins.

4.1. Preparation of template plasmid for site directed mutagenesis

Template plasmid to introduce selected mutations into FEZ (pET24Fez) was already prepared in a previous study in University of Sienna, Department of Biotechnology (Italia). The plasmid to be used in site directed mutagenesis of Sol_65 was prepared in this study.

First Sol_65 mature peptide-encoding DNA fragment was amplified by PCR (Figure 4.6). In order to clone Sol_65 into pR_Nco vector under the pelB secretion signal sequence (*Pectobacterium carotovorum*), forward primer was designed to have *Nco*I restriction enzyme site (Table 4.2). Forward primer had also some bases needed to reconstitute the correct frame. Reverse primer, which was prepared in previous studies, had *BamH*I and *EcoR*I sites which are not present in pR_Nco vector. Instead of designing new reverse primer, a stuffer plasmid was prepared to introduce desired restriction sites (*BamH*I and *EcoR*I sites) into pR_Nco vector. One of the other previously cloned ORFs (Sol_51) had also *BamH*I and *EcoR*I sites at the 3' end. Sol_51 gene was excised from pGEMT vector by *Nco*I and *Pst*I digestion (Figure 4.7) and 924 bp Sol_51 fragment was ligated to pR_Nco, digested with the same enzymes.

In this way, the desired restriction sites (*BamH*I and *EcoR*I sites) were introduced into pR_Nco vector in order to clone Sol_65. The double *Nco*I-*EcoR*I

digestions, eliminate Sol_51 fragment from this new plasmid, which was named pRistu (Figure 4.8). Eventually, the mature peptide-encoding fragment of Sol_65 ORF was amplified, cut with *Nco*I and *Eco*RI and fused with the pelB signal sequence on the plasmid pRistu. The plasmid was also digested with *Kpn*I and self-ligated to reduce the plasmid size about 600 bp. The new plasmid is called pR_pelBSol65 (Figure 4.9) in this study.

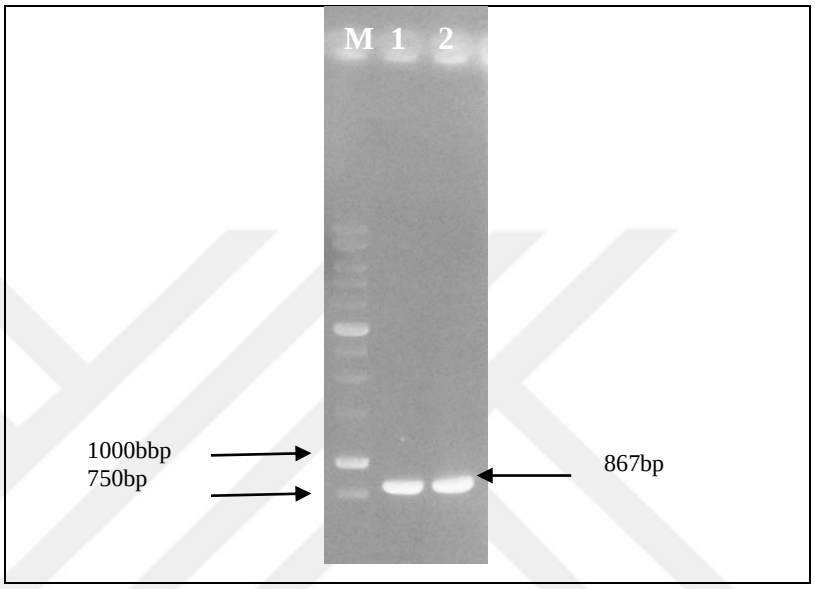


Figure 4.6: Sol_65 mature peptide-encoding DNA fragment amplified by PCR. M: DNA Ladder 1kb marker; Lane 1, 2: Sol_65.

Table 4.2: Primers used to amplify Sol_65.

Primers	**Nucleotide Sequences 5´→3´**
Sol65 NcoI Fw	TatAGCCATGg**CTGAGCCGTTCCCGCC**
Sol65 EcoRI BamHI Rev	ATATGAATTCGGATCCTGCCGCAGGGCG

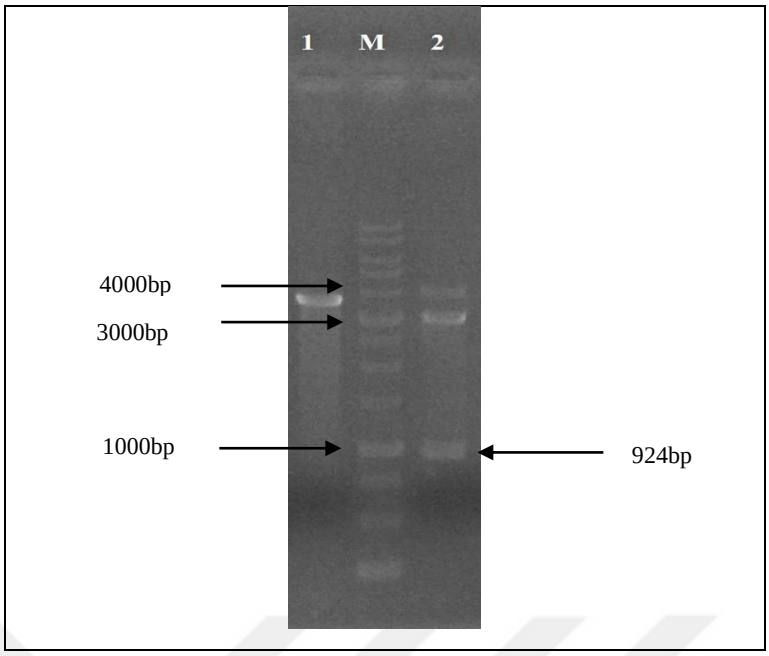


Figure 4.7: Digestion of pR_Nco and pGEMSol51 with *Nco*I and *Pst*I restriction enzymes. Lane 1: pR_Nco::NcoI::PstI; M: DNA Ladder 1kb marker; Lane 2: pGEMSol51::NcoI::PstI.

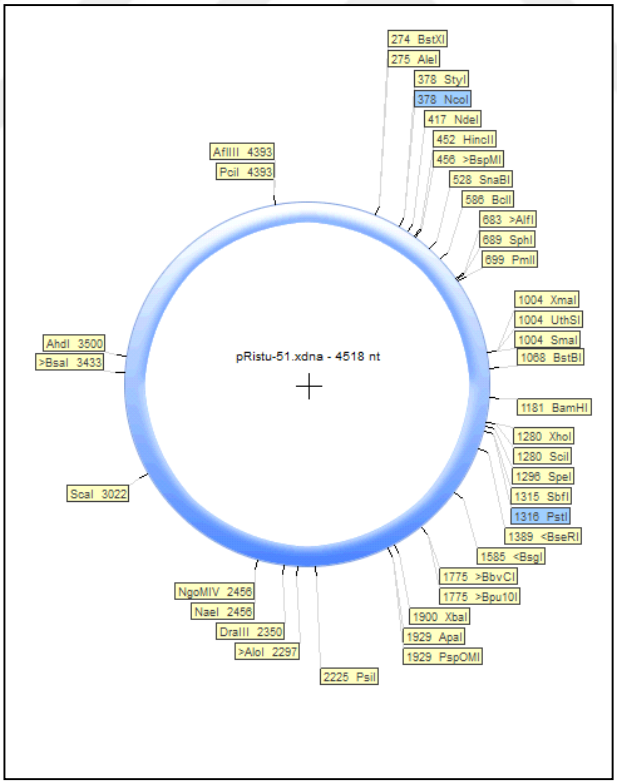


Figure 4.8: Circular map of plasmid pRistu.

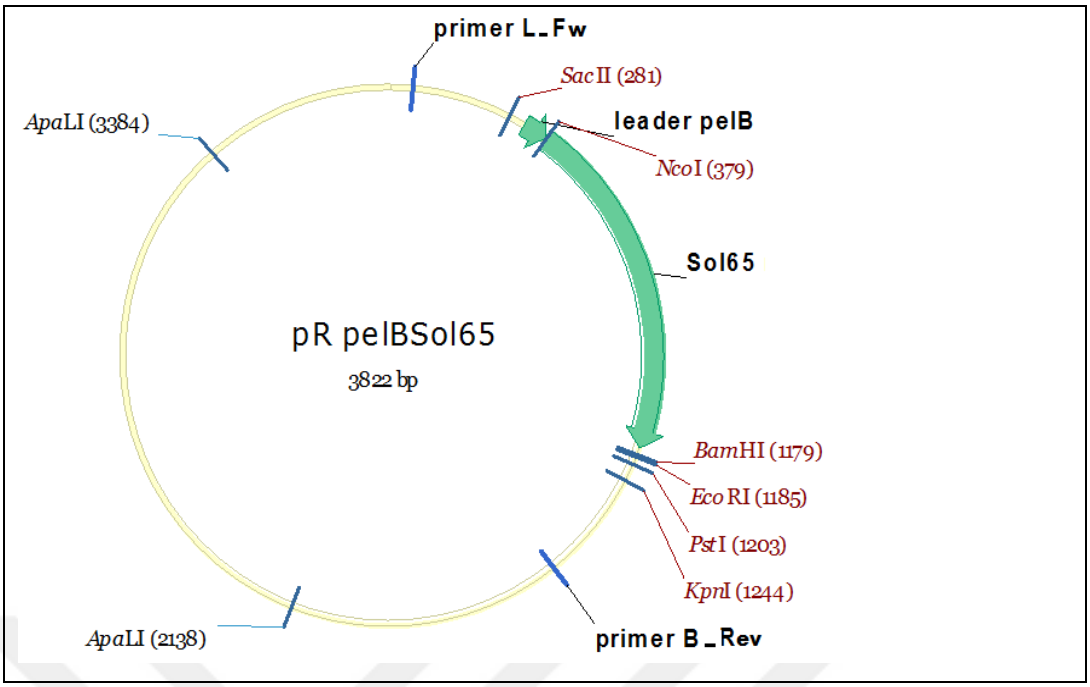


Figure 4.9: Circular structure of plasmid pR pelBSol65.

4.2. Site-Directed-Mutagenesis

pR_pelBSol65 (Figure 4.9) and pET24Fez were used as templates to introduce the desired mutations into Sol_65 and FEZ by *Dpn*I mediated site-directed mutagenesis (Loening et.al. 2005). Following the PCR reaction with the mutagenesis primers, *Dpn*I digestion was carried out in order to eliminate original template. After transforming *E. coli* DH5α competent cells with the mutagenized plasmid, plasmid extraction from the transformants was carried out and new plasmids were checked for the appearance of *Sma*I site which is a restriction site expected to occur after mutation, however none of them were cut with this enzyme (data not shown).

An alternative protocol (Ling and Robinson, 1997) used to introduce site directed mutations in FEZ and Sol_65 with the same primers. The protocol was summarized in Figure 4.10.

First two independent PCR reactions (PCR A and PCR B) were performed (Figure 4.11). PCR A: For Sol65, the forward primer “L_Fw” anneals to the plasmid vector and the reverse primer “S_NG_Rev” anneals to the ORF, introducing the mutation. For FEZ, the forward primer “L_Fw” anneals to the plasmid vector and the reverse primer “F_GN_Rev” anneals to the ORF, introducing the mutation. PCR B: For Sol65, the forward primer “S_NG_Fw” anneals to the ORF introducing the

mutation and the reverse primer “B_Rev” anneals to the plasmid vector. For FEZ, the forward primer “F_GN_Fw” anneals to the ORF introducing the mutation and the reverse primer “B_Rev” anneals to the plasmid vector.

The third PCR reaction (PCR C) was carried out with external primers “L_Fw” and “B_Rev” to get the whole mutated ORFs (2514 bp) (Figure 4.12). After the mutation, amplicons were called Sol65/NG and FEZ/GN and they were digested with *Nco*I and *Eco*RI restriction enzymes whose sites were present in the primers (Figure 4.13).

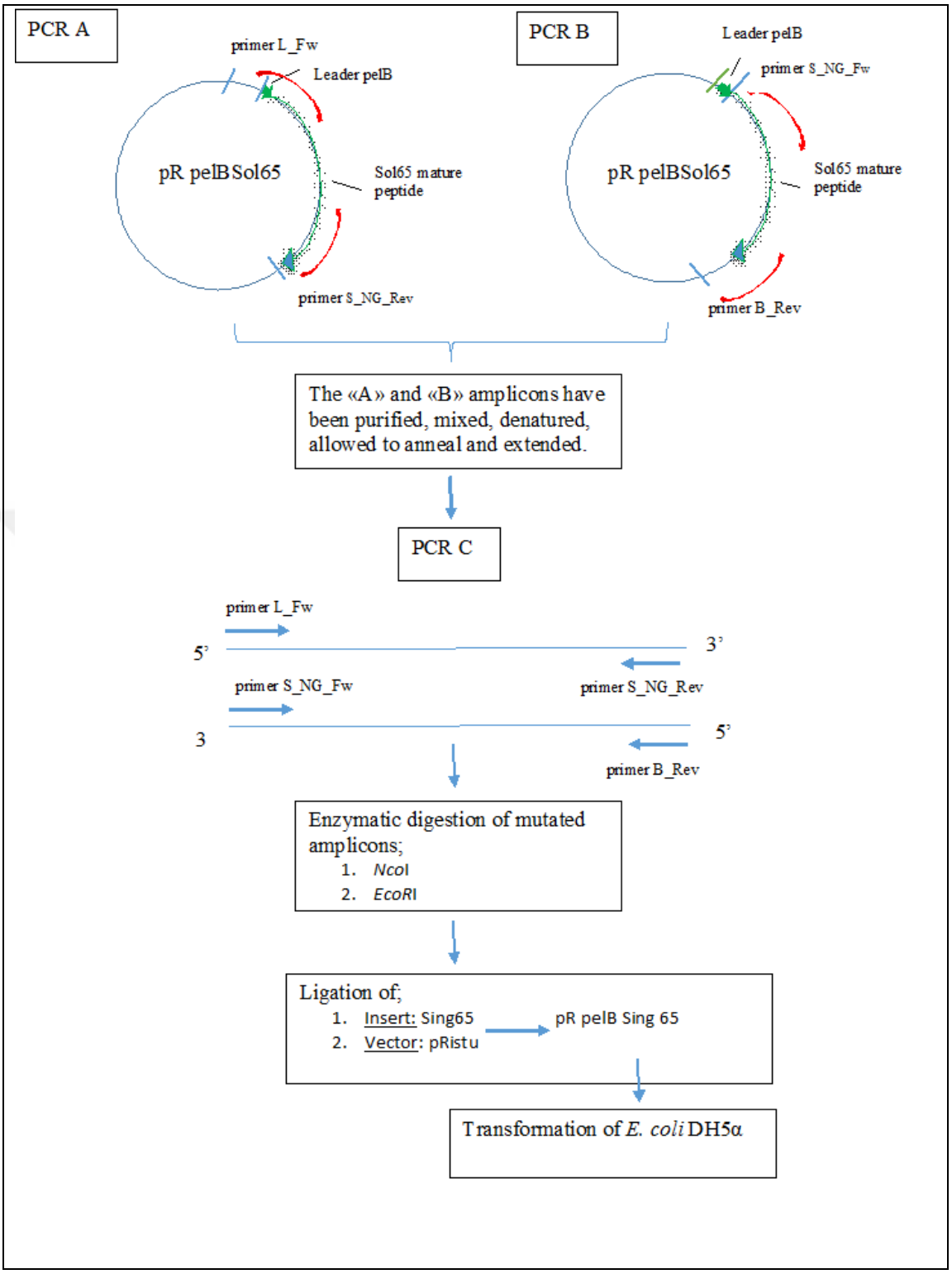


Figure 4.10: Site-directed-mutagenesis by series of PCR reactions.

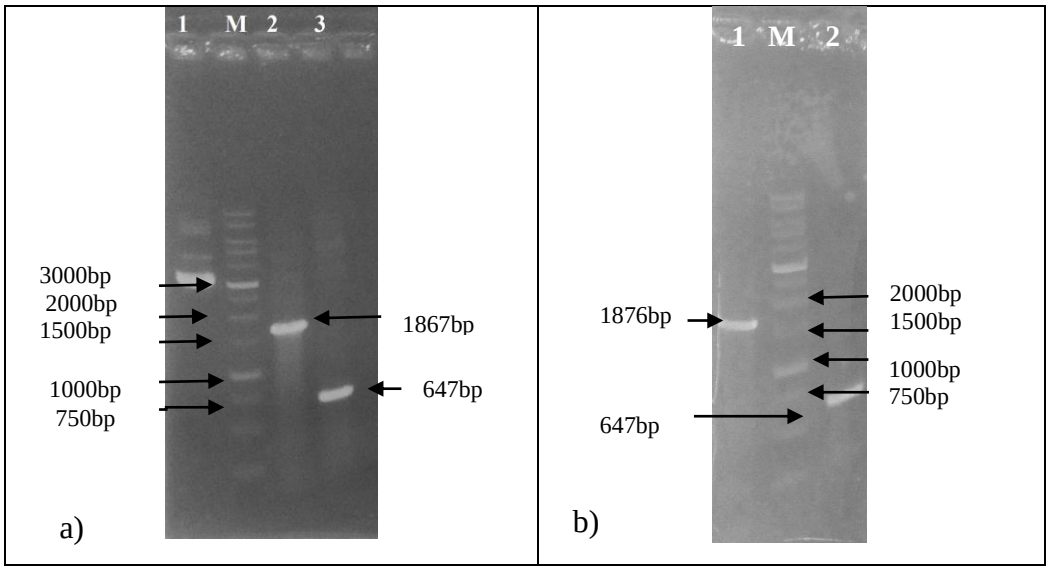


Figure 4.11: Introduction of site directed mutations by PCR A and PCR B. a) PCR A and PCR B for Sol65; Lane 1: pR pelBSol65; M: DNA Ladder 1kb marker; Lane 2: PCR A (1867bp); Lane 3: PCR B (647bp); Part b) PCR A and PCR B for Fez; Lane 1: PCR A (1867bp); M: DNA Ladder 1kb marker; Lane 2: PCR B (647bp).

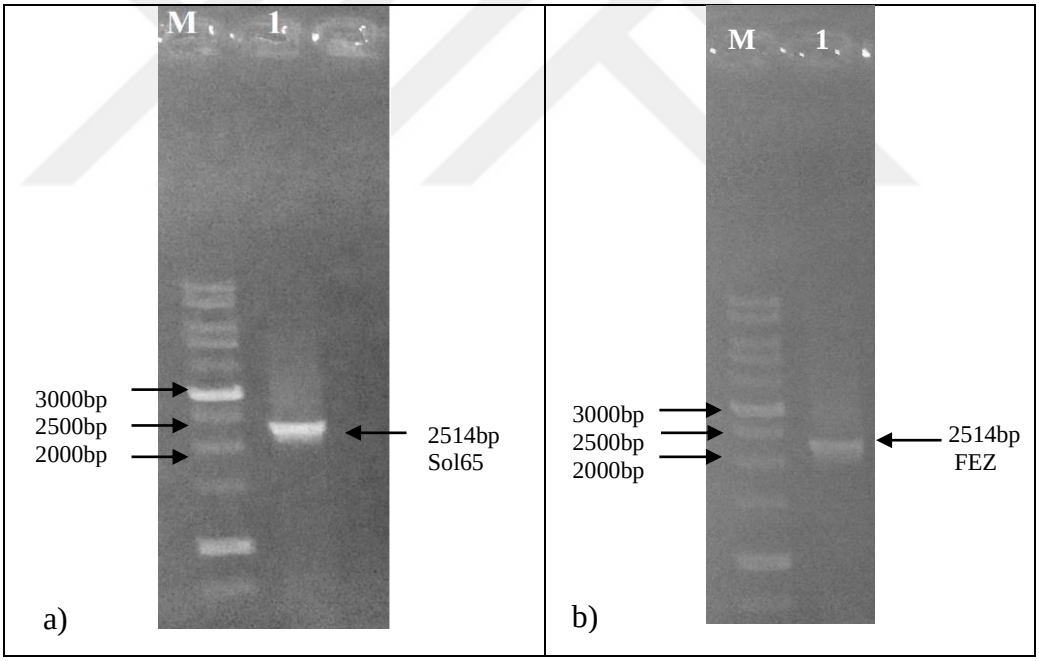


Figure 4.12: Overlap PCR result to fuse PCR A and PCR B amplicons. a) Sol65; M: DNA Ladder 1kb marker; Lane 1: PCR C (Sol_65); b) FEZ; M: DNA Ladder 1kb marker; Lane 1: PCR C (FEZ).

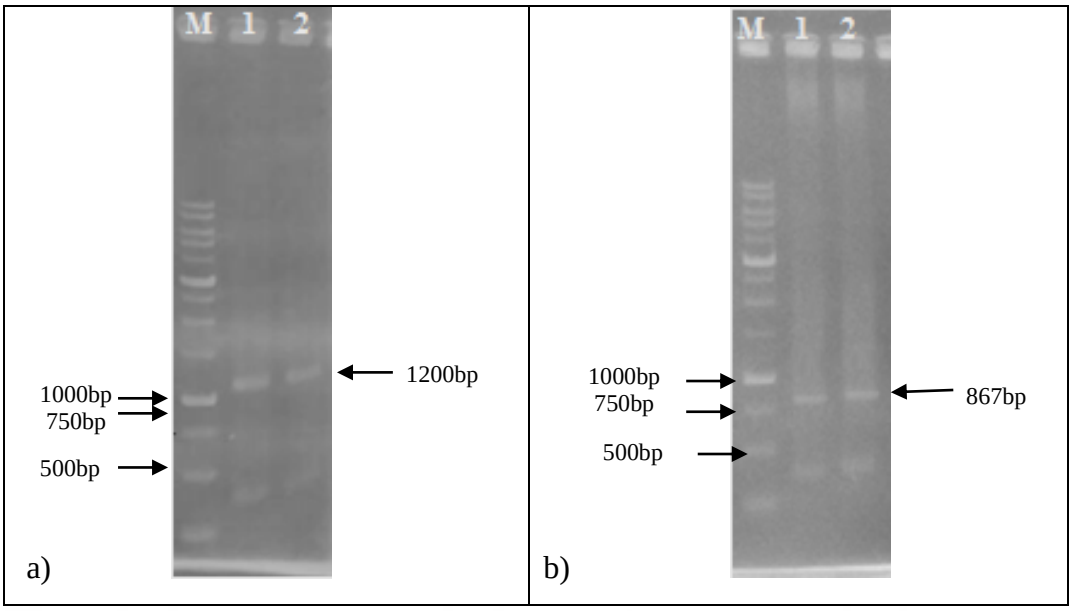


Figure 4.13: Digestions of Sol65/NG and Fez/GN PCR with *Nco*I and *Eco*RI. a) M: DNA Ladder 1kb marker; Lane 2: Sol65/NG::*Nco*I; Lane 3: Fez/GN::*Nco*I b) M: DNA Ladder 1kb marker; Lane 2: Sol65/NG::*Nco*I::*Eco*RI; Lane 3: Fez/GN::*Nco*I::*Eco*RI.

After *Nco*I and *EcoR*I digestions, 867 bp DNA fragment Sol65/NG (Sing 65) ligated into the pRistu vector cut with the same enzymes under the pelB signal sequence. The new vector was called pR pelB Sing 65 in this study (Figure 4.14). Cloning of 800 bp Fez/GN was not completed in this study, this part is going on with another researcher in Prof. Maria Cristina Thaller's laboratory.

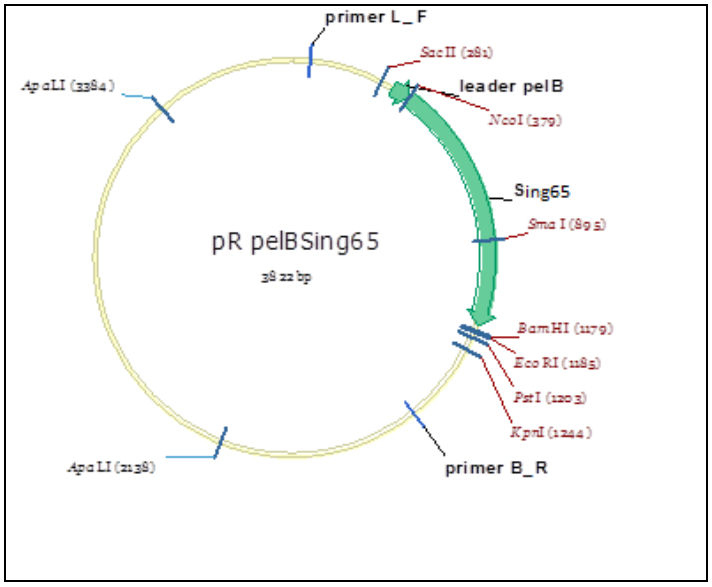


Figure 4.14: Circular structure of plasmid pR pelBSing65.

4.3. Transformation of *E. coli* DH5α cells with pR pelBSing65

E. coli DH5α competent cells were transformed with pR pelBSing65. Plasmid isolated from one of the transformants was digested with *Sma*I to check for the presence of this enzyme site which is expected to form after mutation. Although pR pelBSol65 was not digested with *Sma*I, pR pelBSing65 was cut with the same enzyme (Figure 4.15) prooving that the desired mutation (N_{223}→G in Sol_65 protein) was inserted into *S. usitatus* MβL protein.

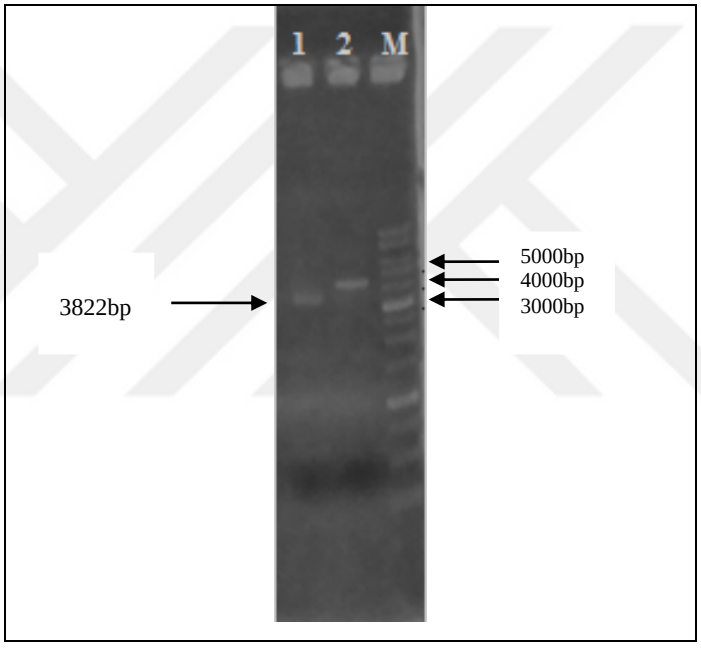


Figure 4.15: *Sma*I digestion result of pR pelBSol65 and pR pelBSing65. Lane 1: pR pelBSing65 (linearized) (3822bp); Lane 2: pR pelBSol65; M: Ladder 1kb marker.

Mutation in the Sol_65, was also checked by DNA sequencing. For this purpose, pR pelBSing65 was digested with *SacI*I and *Eco*RI and DNA fragment isolated from the gel was sent to sequence analysis (Figure 4.16). Sequence result was prove that "N" at 223rd position has been changed with "G" in *S. usitatus* MβL protein (Figure 4.17).

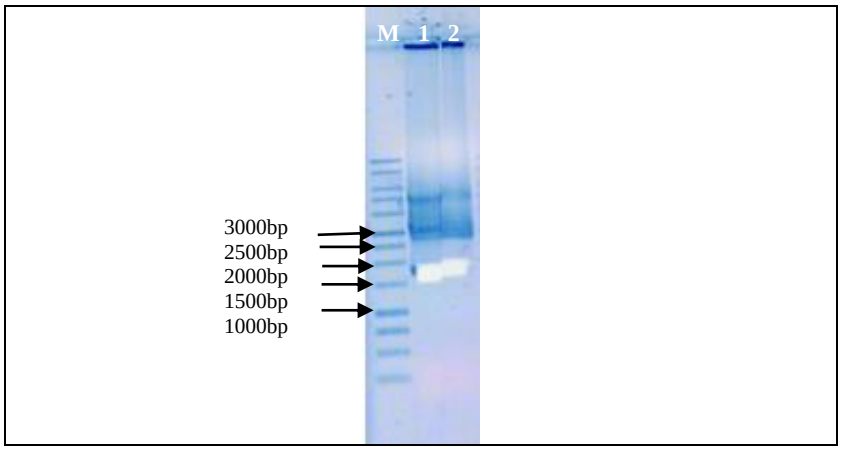


Figure 4.16: *Sac*II and *Eco*RI digestion of pR pelBSing65. M: 1kb DNA ladder; Lane 1, 2: Sol_65 NG::*Sac*II::*Eco*RI.

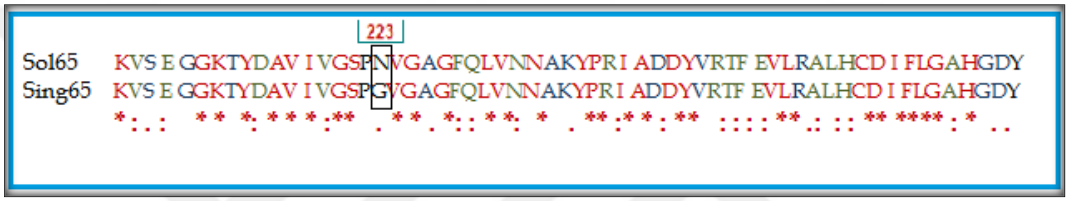


Figure 4.17: Sequence result that show, "N" at 223rd position has been changed with "G" in *S. usitatus* MβL protein.

4.4. Subcloning of sing 65 into pBCSK

pR pelBSing65 was not suitable to measure β-lactamase activity since it has ampicillin resistance gene as a selective marker. Ampicillin is a beta-lactam antibiotic and it is target for the β-lactamase enzyme. In order to assay metallo-β-lactamase activity, sing 65 was excised from pR pelBSing65 with *Sac*II and *Eco*RI and directionally cloned into pBCSK cut with the same enzymes (Figure 4.18) which has chloramphenicol resistance gene for selection. The resulting 4261 bp plasmid was called pBC SING 65 in this study (Figure 4.19).

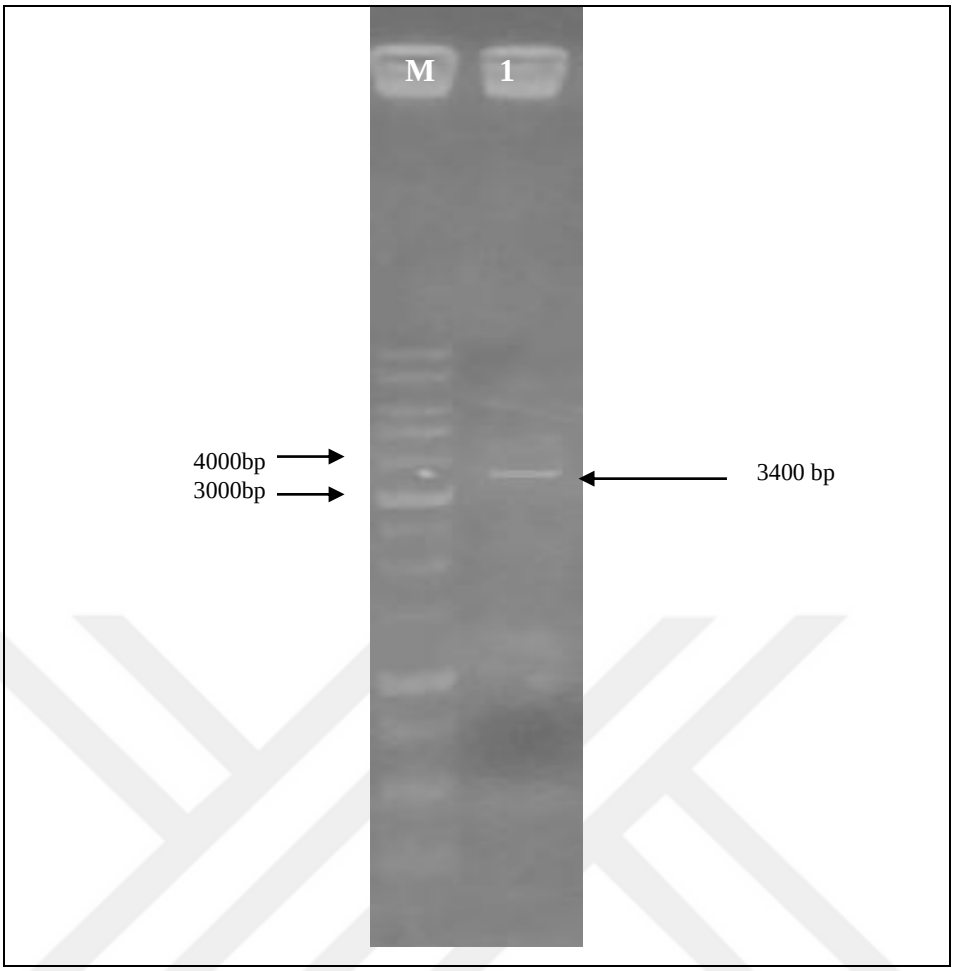


Figure 4.18: Digestion of pBCSK with *Sac*II and *EcoR*I restriction enzymes. M: Ladder 1kb marker; Lane 1: pBCSK cut with *Sac*II and *Eco*RI (3400 bp).

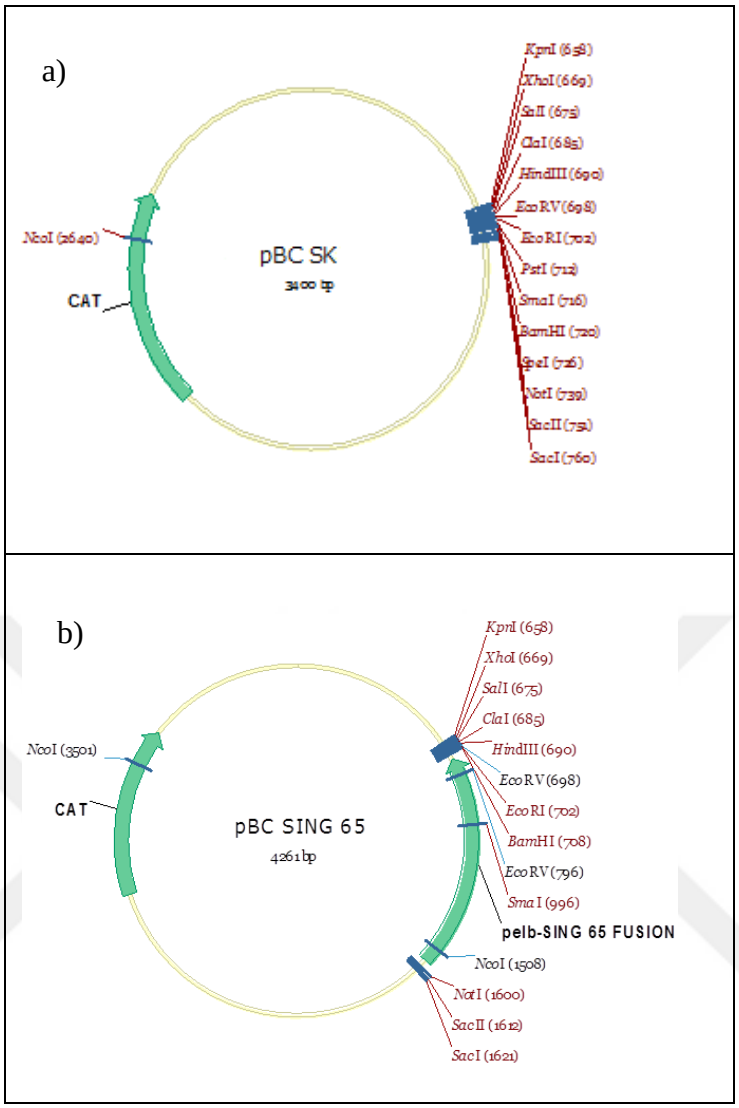
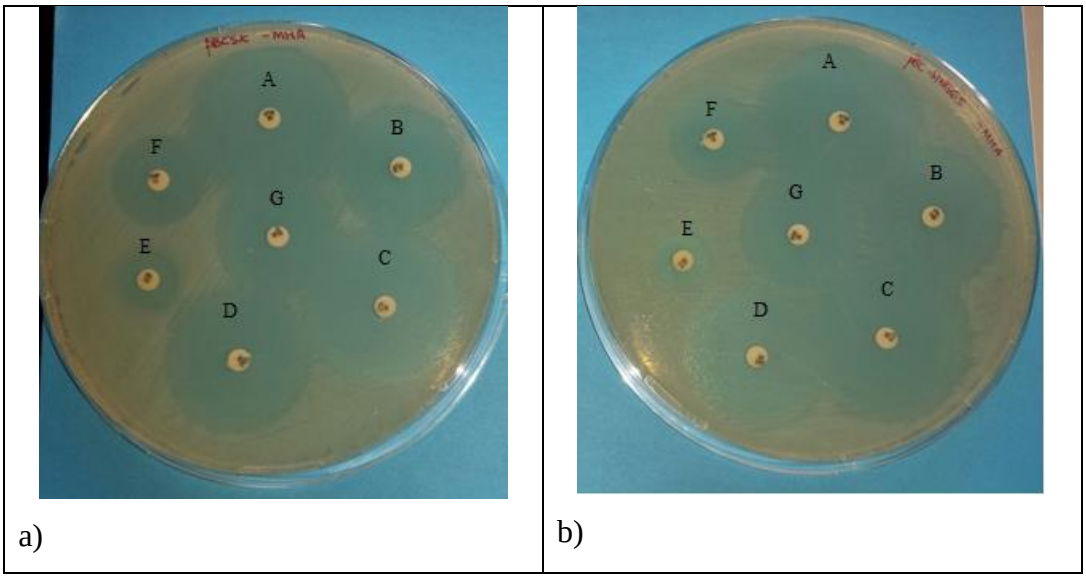


Figure 4.19: Circular structure of plasmid pBCSK a) and pBC SING 65 b) vector.

4.5. Measuring β-lactamase activity

The new strain carrying pBC SING65 was tested for possible β-lactamase activity by Kirby Bauer method with 7 different antibiotic. *E. coli* carrying pBCSK plasmid was used as negative control (Figure 4.20). Even if N_{223}→G mutation in Sol_65 protein was achieved, the result of the antibiotic test shows that the new strain carrying pBC SING65 has poor β-lactamase activity (Figure 4.20; Table 4.3).



a) b)

Figure 4.20: Determining β-lactamase activity. a) *E. coli* carrying pBCSK; b) *E. coli* carrying pBC SING65. A: ATM (Aztroneam); B: PRL (Piperacillin); C: CTX (Cefotoxime); D: CAZ (Ceftazidime); E: IMI (Imipenem); F: AMP (Ampicillin); G: FOX (Cefoxatin).

Table 4.3: The measurement of zone diameter (mm).

Antibiotics	*E. coli* with pBCSK	*E. coli* with pBC SING65
ATM (Aztroneam)	1.7	1.8
PRL (Piperacillin)	1.3	1.45
CTX (Cefotoxime)	1.7	1.8
CAZ (Ceftazidime)	1.6	1.7
IMI (Imipenem)	0.7	0.6
AMP (Ampicillin)	1	0.9
FOX (Cefoxatin)	1.4	1.2

5. DISCUSSION

Antibiotic resistance is recognised as a global public health problem. Nowadays, on one hand, new drugs are being developed rapidly all over the world, on the other hand pathogen microorganisms are rapidly gaining resistance to antibiotics. The dimensions of this problem is growing day by day.

β-lactam antibiotics are the most widely used antibiotic groups in medicine and they are distinguished from other antibiotics by their common chemical molecules called "β-lactam" ring. The most common mechanism of resistance to β-lactam antibiotics is the enzymatic degradation caused by β-lactamases. Among different classes of β-lactamases, metallo-β-lactamases are the most dangerous ones because of their ability to inactivate carbapenems, which are affective against bacteria which are resistant to other β-lactams in general use.

Metallo-β-lactamase-like (MβL) proteins from seven environmental bacteria were identified on the basis of sequence homology with 13 different functional enzymes by BLAST analysis in a previous study carried out in "University of Tor Vergata, Department of Biology (Italia)". No β-lactamase activity was obtained from any of the clones obtained by cloning putative metallo-β-lactamase genes from these seven bacteria. In order to find an answer for the lack of β-lactamase activity, one of the clones (containing ORF *Acid_6874* of *Solibacter usitatus*) have been chosen for further analysis. *S. usitatus* MβL-like ORF (Sol_65) has been chosen since its amino acid sequence was highly homologous to the functional MβL of *Fluoribacter gormanii* (FEZ). Comparison of the Sol_65 and FEZ protein's 3D structures predicted that amino acids at 223^{rd} (Asn_{223} : N_{223}) and 266^{th} (Tyr_{266} : Y_{266}) positions in the active center of Sol_65 could be responsible for the possible steric hindrance that prevent the entry of the β-lactam ring in the active site. It was decided to change those amino acids with the once present in FEZ at the same sites (223^{rd} and 226^{th}) and vice versa by site directed mutagenesis.

Site-directed-mutagenesis is a powerful tool used in the analysis of proteins, structure-function relationships and protein engineering. This method allows researchers to make specific changes in the structure of the protein. In the present study, the first attempt has been performed with the *Dpn*I mediated site-directed mutagenesis protocol (Loening et.al., 2005), but the result was negative, colonies

was without the desired mutation. Changing the *E. coli* host, increasing the *Dpn*I digestion time or increasing the amount of *Dpn*I used and decreasing the number of PCR cycles didn't change the result. Therefore, an alternative protocol (Ling and Robinson, 1997) used to introduce site-directed mutations in FEZ and Sol65 by performing serial PCR reactions with the same primers. Restriction enzyme digestion and DNA sequence results proove that "N" at 223rd position has been changed with "G" in *S. usitatus* MβL protein.

Ampicillin is a beta-lactam antibiotic and is target for the β-lactamase enzyme. In order to assay metallo-β-lactamase activity, mutated *S. usitatus* MβL gene was excised from the recombinant vector (pR pelBSing65), which has ampicillin resistance gene as a selectable marker, and cloned into pBCSK which has chloramphenicol resistance gene for selection. The new strain carrying pBC SING65 was tested for possible β-lactamase activity. Although, N_{223}→G mutation in Sol_65 protein was achieved, the result of the antibiotic test shows that recombinant strain has very poor β-lactamase activity. In order to eliminate possible steric hindrance that prevent the entry of the β-lactam ring in the active site, another site directed mutation (Y_{266}→M mutation in Sol_65 protein) is necessary. This thesis covers only one site directed mutagenesis, the other mutation experiment is going on with another researcher in Professor Maria Cristina Thaller's laboratory. It is possible to obtain β-lactamase activity after changing both 223rd and 226th amino acids with the once present in FEZ at the same sites.

New experimental data are needed before concluding that the original cloned genes or the mutated ones are not expressed or the products are not functional. Transcriptional studies can be carried out in order to detect RNA products. Moreover, western blot can show the presence or absence of the products. Optimization of culture conditions and specific parameters is very important for the expression and activity of the enzymes especially for the recombinant ones. For example, in a study performed by Mercuri and coworkers [17], FEZ-1 β-lactamase activity in *E. coli* was detectable when the cultures were grown at 28°C in the presence of 100 or 500 μM IPTG but β-lactamase activity was not seen when the cultures were grown at 37°C in the presence of 100 or 500 μM IPTG. In our study we didn't induce the promoter with IPTG and we didn't grow the cells in different temperatures. These optimizations has to be carried out in the concept of another study. Furthermore, different expression vectors can be used, or the genes can be

cloned under strong and inducible promoters. In order to produce metallo-β-lactamase in a soluble form and in larger amounts, it can be fused with a leader signal peptide, for example PelB.

In 2001 Mercuri et. al. [14] showed that deletion of proline-rich sequence (PMPNPFPPF) close to the FEZ-1 amino terminus resulted very high level of enzyme production. This strategy can be also used for Sol-65 and Sing-65 in future studies. If it will be possible to obtain enzyme activity in the future trials, the enzymes can be purified and the kinetic parameters can be determined. Moreover, pH and temperature dependence of the enzyme activity and zinc content of the β-lactamase can be investigated.

The rapid emergence of resistant bacteria is occurring worldwide. Studies performed in the field of bacterial genomics and determination of resistance mechanisms will help our understanding of the evolution of resistant strains and their resistance mechanisms. Development of new antibiotics and designing effective drugs with new targets are good strategies for combating resistant bacteria.

REFERENCES

[1] Davies J., Davies D., (2010), "Origins and Evolution of Antibiotic Resistance", Microbiol Molecular Biology Review, 74(3), 417–433.

[2] Di Meo I., (2012), "Post-genomic study of Pfam00753 superfamily proteins showing homologies with B3 metallo-β-lactamases", Master Thesis, Universita Degli Studi Di Roma Tor Vergata.

[3] Web 1, (2004), http://www.wiley.com/college/pratt/0471393878/student/, (Accession Date: 14/05/2016).

[4] Web 2, (2015), http://tmedweb.tulane.edu/pharmwiki/doku.php/antibiotic_targets (Accession Date: 14/05/2016).

[5] Web 3, (2015), http://www.who.int/mediacentre/factsheets/fs194/en/, (Accession Date: 14/05/2016).

[6] Rossolini G. M., Thaller M. C., (2010), "Coping with antibiotic resistance: contributions from genomics", Genome Medicine, 2,15.

[7] Web 4, (2008-2012), http://textbookofbacteriology.net/resantimicrobial.html, (Accession Date 15/04/2016).

[8] Liras P., Martín J. F., (2006), "Gene clusters for b-lactam antibiotics and control of their expression: why have clusters evolved, and from where did they originate?", International Microbiology, 9, 9-19.

[9] Ambler, R.P., (1980), "The structure of beta-lactamase", Philosophical Transactions of the Royal Society of London B.: Biological. Science 298, 321-331.

[10] Schofield C. B., **(**2011), "The anarchy of antibiotic resistance: mechanisms of bacterial resistance", MLO: Medical Laboratory Observer, 43(5), 10-2, 14-6; quiz 18-9.

[11] Bebrone C., (2007), "Metallo-β-lactamases (classification, activity, genetic organization, structure, zinc coordination) and their superfamily", Biochemical Pharmacology 74 (1), 686-1701.

[12] Edelheit O., Hanukoglu A., Hanukoglu I., (2009), "Simple and efficient site-directed mutagenesis using two single-primer reactions in parallel to generate mutants for protein structure-function studies", BMC Biotechnology, 9-61.

[13] Challacombe JF., Eichorst SA., Hauser L., Land M., Xie G., et al., (2011), "Biological Consequences of Ancient Gene Acquisition and Duplication in the Large Genome of *Candidatus Solibacter usitatus Ellin6076*", PLoS ONE 6(9) : e24882. doi: 10.1371/journal.pone.0024882.

[14] Mercuri, S. P., Bouillenne, F., Boschi, L., Lamotte-Brasseur, J., Amicosante, G., Devreese, B., Van Beeumen, J., Frere, J. M., Rossolini, G. M., Galleni, M., (2001), "Biochemical Characterization of the FEZ-1 Metallo-β-Lactamase of *Legionella gormanii* ATCC 33297^{T} Produced in *Escherichia coli",* Antimicrobial Agents and Chemotherapy, p. 1254–1262.

[15] Web 5, (2015), https://www.promega.com/, (Accession Date: 15/04/2016).

[16] Loening, A. M., (2006), "Technologies for Imaging with Bioluminescently Labelled Probes, Site-Directed-Mutagenesis Protocol", PhD Thesis, Stanford University.

[17] Mercuri P. S., Garcia-Saez I., De Vriend K., Thamm I., Devreese B., Van Beeumen J., Dideberg O., Rossolini G. M., Fre J. M., Galleni M., (2004), **"**Probing the Specificity of the Subclass B3 FEZ-1 Metallo-β-lactamase by Site-directed Mutagenesis**"** Journal of Biological. Chemistry, 279, 33630-33638.

[18] Thaller M. C., Borgianni L., Di Lallo G., Chong Y., Lee K., Dajcs J., Stroman D., Rossolini G. M., (2011), "Metallo-β-Lactamase Production by *Pseudomonas otitidis*: A Species-Related Trait", Antimicrobial Agents and Chemotherapy, 118-123.

[19] Abraham EP, Chain E, (1940), "An enzyme from bacteria able to destroy penicillin", Nature, 146, 837-837.

[20] Falconer S. B., Brown E. D., (2009), "New screens and targets in antibacterial drug discovery", Current Opinion in Microbiology, 12, 497-504.

[21] Bush, K., (1998), "Metallo-beta-lactamases: a class apart", Clinical Infectious Disease, 27, 48-53.

[22] Boschi, L., Mercuri, S. P. Riccio, M. L., Amicosante, G., M., Galleni, J. M., Rossolini, G., (2000), "The *Legionella* (*Fluoribacter*) *gormanii* Metallo-b-Lactamase: A New Member of the Highly Divergent Lineage of Molecular-Subclass B3 β-Lactamases", Antimicrobial Agents and Chemotherapy, 1538–1543.

[23] Guillemot D., Courvalin P., and the French Working Party to Promote Research to Control Bacterial Resistancea, (2001), "Better Control of Antibiotic Resistanc", Clinical Infectious Disease, 33 (4), 542-547.

[24] Xu D., Xie D., Guo H, (2006), "Catalytic Mechanism of Class B2 Metallo-β-lactamase", Journal of Biological Chemistry, 281, (13), 8740-8747.

[25] Challacombe, J.F., Eichorst, S.A., Hauser, L., Land, M., Xie, G., Kuske, C.R., (2011), "Biological Consequences of Ancient Gene Acquisition and Duplication in the Large Genome of *Candidatus Solibacter usitatus Ellin6076*", PLoS One, 6, e24882.

[26] D'Costa V. M., King C. E., Kalan L., Morar M., Sung W. W. L., Schwarz C., Froese D., Zazula G., Calmels F., Debruyne R., Golding G. B., Poinar H. N., Wright G. D., (2011), "Antibiotic resistance is ancient", Nature, 10388.

[27] Challacombe J. F., Kuske C. R., (2012), "Mobile Genetic Elements in The Bacterial Phylum Acidobacteria", Mobile Genetic Elements, 2 (4), 179–183; Landes Bioscience.

[28] Ennis D. G., (2001), "Mutagenesis", Encyclopedia of Life Sciences / Nature Publishing Group, Published online: April 2001.

[29] Weickert M. J., and Chambliss G. H., (1990), "Site-Directed-Mutagenesis of a catabolite repression operator sequence in *Bacillus subtilis*", Proceedings of National Academy of Science of the USA, 87, 6238-6242.

[30] Deng W. P., and Nickoloff J. A., (1992), **"**Site-Directed Mutagenesis of Virtually Any Plasmid by Eliminating a Unique Site", Analytical Biochemistry, 200, 81-88.

[31] Carter P., (1986), "Site-directed mutagenesis", Biochemical Journal, 237, 1-7.

[32] Garau G., Di Guilmi A. M., Hall B. G., (2005), "Structure-Based Phylogeny of the Metallo-β-Lactamases", Antimicrobial Agents and Chemotherapy, 2778-2784.

[33] Garcia-Saez I., Mercuri P. S., Papamicael C., Kahn R., Frere J. M., Galleni M., Rossolini G. M., Dideberg O., (2003), "Three-dimensional Structure of FEZ-1, a Monomeric Subclass B3 Metallo-b-lactamase from Fluoribacter gormanii, in Native Form and in Complex with D-Captopril", Journal of Molecular Biology, 325, 651–660.

[34] Daiyasua H., Osakaa K., Ishinob Y., Toha H., (2001), "Expansion of the zinc metallo-hydrolase family of the L-lactamase fold", FEBS Letters, 503, 1-6.

[35] Ullah JH, Walsh TR, Taylor IA, Emery DC, Verma CS, Gamblin SJ, Spencer J., (1998), "The crystal structure of the L1 metallo-β-lactamase from *Stenotrophomonas maltophilia* at 1.7 A resolution", Journal of Molecular Biology, 20;284 (1), 125-36.

[36] Web 6, (2010-2016), http://www.creative-biogene.com/, (Accession Date 15/04/2016).

[37] Crouse J., Amorese D., (1987), "Ethanol Precipitation: Ammonium Acetate as an Alternative to Sodium Acetate", Focus, 9 (2), 3–5.

[38] Ling, M., Robinson, B. H., (1997), "Approaches to DNA Mutagenesis: An Overview", Analytical Biochemistry, 254, 157–178.

[39] Andrews, J. M., (2001), “Determination of Minimum Inhibitory Concentration”, Journal of Antimicrobial Chemotherapy, 48, Suppl. S-1, 5-16.

[40] Senda K., Arakawa Y., Nakashima Y., Ito H., Ichiyama S., Shimokata K., Kato N., Ohta M., (1196), “Multifocal Outbreaks of Metallo-β-Lactamase-Producing Pseudomonas aeruginosa Resistant to Broad-Spectrum β-Lactams, including Carbapenems”, Antimicrobial Agents and Chemotherapy, 349-353.

[41] Web 7, (2014), http://www.slideserve.com/morty/ant-b-yot-kler-n-etk-mekan-zmasi, (Accession Date: 14/05/2016).

[42] Zeng X., Lin J., (2013), “Beta-lactamase induction and cell wall metabolism in Gram-negative bacteria”, Frontiers in Microbiology, 4, 128, 1-9.

[43] Albayrak T. G., (2008), “Hastane İnfeksiyonu Etkeni Olan Pseudomonas aeruginosa Kökenlerinde Çift Disk Sinerji Testi ve Kombine Çift Disk Sinerji ile Metallo-Beta-Laktamaz Varlığının Araştırılması”, T.C. Sağlık Bakanlığı Haydarpaşa Numune Eğitim ve Araştırma Hastanesi, Dissertation (Uzmanlık Tezi).

[44] Oncul O., (2002), “Antibiyotikler I”, Akılcı Antibiyotik Kullanımı ve Erişkinde Toplumdan Edinilmiş Enfeksiyonlar, Sempozyum Dizisi, 31, 23-38.

[45] Web 8, (2003), http://kbb.uludag.edu.tr/antibiyotik05.htm, (Accession Date: 14/05/2016).

[46] Ghafourian S., Sadeghifard N., Soheili S., Sekawi Z., (2015), “Extended Spectrum Beta-lactamases: Definition, Classification and Epidemiology”, Current Issues in Molecular Biology, 17, 11-22.

BIOGRAPHY

Gozde GORGULU born on 16th September, 1986 in Balıkesir Turkey. She began her undergraduate study at Ege University in Department of Biology in year 2004. Before completing her bachelor's degree, she worked at crew department of two different shipping companies as a personnel assistant (2009-2011). By working and studying in parallel, she completed her bachelor's degree in 2010. In order to improve her biology knowledge and skills, she started her Master of Science degree in Molecular Biology and Genetic Depatment at Gebze Technical University in 2011. During her Ms study she went to University of Rome "Tor Vergata" as an exchange student (Erasmus) and she carried out experimental part of her thesis under the supervision of Prof. Dr. Maria Cristina Thaller in Department of Biology (Italia). While continuing her graduate studies, she is working as a molecular microbiologist at Episome Biotechnology, Inc., Department of R&D since 2015.