EXPRESSION OF THE GENES ENCODING IMPORTANT TRANSPORT SYSTEM PROTEINS IN SILVER RESISTANT ESCHERICHIA COLI MUTANTS

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ABSTRACT

EXPRESSION OF THE GENES ENCODING IMPORTANT TRANSPORT SYSTEM PROTEINS IN SILVER RESISTANT ESCHERICHIA COLI MUTANTS

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Heavy metals, such as silver (Ag), copper (Cu) or mercury (Hg), have been used as antimicrobial agents for quite some time in human history. Silver has inhibitory or lethal effects on a broad range of bacteria and fungi. Furthermore, heavy metals are useful against viruses. The applications of silver dealing with bacteria comprise industrial, agricultural, medical and healthcare areas. They were used as disinfectants in hospitals as well as in the treatment of several infectious diseases such as tuberculosis, leprosy, gonorrhea, and syphilis. Silver is not an essential metal for bacterial metabolism and low concentrations of its ions are toxic.

Increasing usage of silver has brought about a resistance problem, especially in Gram-negative bacteria. There are several reported cases in the literature indicating the acquisition of silver resistance in *Escherichia coli*, *Enterobacter cloacae*, *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa*. Silver ions must enter into the cells to show their antimicrobial effect. In order to cope with the silver stress, the Gram-negative bacteria take advantage of active efflux pumps and outer membrane-mediated decreased permeability.

In this study, the relative gene expression levels of important transport system proteins, namely as CusB, CusF, and CusS, were determined in *E.coli* ATCC 8739 strain using RT-qPCR method. At the beginning of the study, UV-induced and spontaneous silver resistant *E.coli* mutants were generated. Both the UV-induced and spontaneous mutants were examined for three of the indicated proteins. Gene expression levels of each mutant were compared with the original *E.coli* ATCC 8739 strain and among themselves. The significance of the relative expression levels of the transport proteins was determined statistically by using one-way ANOVA with Tukey's multiple comparison post-test. The results indicated that *cusB*, *cusF*, and *cusS* genes' expressions were significantly higher than the parent *E.coli* strain. It appeared that the cusCFBA efflux pump has crucial importance for the export of silver ions from the bacterial cells. The increase in gene expression levels in mutants was independent of the way that the mutants had been generated. In this regard, *E.coli* evidently relies heavily on efflux pumps to adapt themselves to the environment containing high concentrations of silver.

Keywords: RT-qPCR, Spontaneous Mutant, UV-induced Mutant, cusCFBA, Escherichia coli ATCC 8739

ÖZ

GÜMÜŞE DİRENÇLİ ESCHERICHIA COLI MUTANTLARINDA ÖNEMLİ TRANSPORT PROTEİNLERİNİ KODLAYAN GENLERİN EKSPRESYONU

Uyanık, Gökçe
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Gümüş, bakır veya cıva gibi ağır metaller, bakteriler üzerindeki toksik etkilerinden dolayı yıllardan beri antimikrobiyal ajan olarak kullanılmaktadır. Ağır metallerin bakteri üzerindeki etkilerinden endüstriyel, tarımsal, medikal ve sağlık hizmeti verilen alanlarda yararlanılmaktadır. Bunlardan biri olan gümüş, hastanelerde medikal ekipmanların dezenfekte edilmesinde kullanılmasının yanı sıra tüberküloz, cüzam, bel soğukluğu ve frengi gibi birçok bulaşıcı hastalığın tedavisinde de kullanılmıştır. Ek olarak, gümüşün bakteri, mantar ve virüsler üzerinde geniş ölçüde engelleyici ve yok edici etkileri bulunmaktadır. Bakteri metabolizması için gümüş temel bir metal olmadığından düşük derişimlerinin dahi bakteri üzerinde öldürücü etkileri vardır.

Gümüşün hem medikal hem de medikal dışındaki alanlarda kullanımının artışı, özellikle Gram-negatif bakterilerde gümüş direnci gelişmesi gibi bir problemi doğurmaya başlamıştır. Bunun sonucunda, çeşitli suşlarda gümüş direnci geliştiğini gösteren birçok vakanın olduğu saptanmıştır. Bu suşlara *Escherichia coli, Enterobacter cloacae, Klebsiellapneumoniae* and *Pseudomonas aeruginosa* örnek gösterilebilir. Literatüre göre, gümüş iyonlarının antimikrobiyal etkisini göstermesi için hücre içine ulaşması gerektiğinden, aktif dışarı atma mekanizmaları ve dış zarın

geçirgenliğini azaltmak Gram-negatif bakterilerin gümüş iyonlarının etkisini engellemek için kullandığı iki temel mekanizmadır.

Bu çalışmada, önemli dışarı atma sistem proteinlerinin, göreli gen ekspresyon seviyeleri *E.coli* ATCC 8739 suşunda RT-qPCR yöntemi kullanılarak tespit edilmiştir. Çalışma için belirlenen proteinler, CusB, CusF ve CusS'tir ve her protein için ilgili genlerin seviyeleri hem spontane hem de UV'ye maruz bırakılarak elde edilmiş *E.coli* mutantları ile saptanmıştır. Sonrasında her mutantın gen ekspresyon seviyeleri, hem orijinal *E.coli* ATCC 8739 suşu ile hem de diğer mutantlarla karşılaştırılmıştır. Göreli gen ekspresyon seviyelerinin istatistiksel önemi ise tek faktörlü varyans analizi (one-way ANOVA) ve Tukey çoklu karşılaştırma testi kullanılarak yapılmıştır. Çalışma sonucunda gümüşe dirençli *E.coli* mutantları orijinal suş ile karşılaştırıldığında, yüksek gümüş konsantrasyonunda incelenen tüm transport proteinlerinin ilgili gen seviyelerinde kayda değer bir artışın olduğu saptanmıştır. Buna dayanarak, cusCFBA dışarı atım pompasının da bir parçası olan bu proteinlerin, gümüş iyonlarının hücre dışına atımı ve yüksek konsantrasyonda gümüş içeren ortama adapte olabilmesi açısından hayati bir önem taşıdığı sonucuna varılmıştır. Fakat istatistiksel analizler, gümüşe dirençli *E.coli* mutantlarının elde ediliş yolunun (mutagenez) gen ekspresyon seviyeleri açısından bir fark yaratmadığını göstermiştir.

Anahtar Kelimeler: RT-qPCR, Spontane Mutant, UV ile Uyarılmış Mutant, cusCFBA, Escherichia coli ATCC 8739

To my family…

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ABBREVIATIONS

Ag	Silver
ANOVA	Analysis of Variance
AT	Adenine-Thymine
ATCC	American Type Culture Collection
Bp	Base pairs
Cd	Cadmium
Cdna	Complementary deoxyribonucleic acid
CFU	Colony Forming Unit
Cq	Cycle quantification
DNA	Deoxyribonucleic acid
DNase I	Deoxyribonuclease I
EDTA	Ethylenediamine tetraacetic acid
EtBr	Ethidium Bromide
EUCAST	European Committee on Antimicrobial Susceptibility Testing
GC	Guanine-Cytosine Content
GTE	Glucose Tris EDTA
Hg	Mercury
MFP	Membrane Fusion Protein
$MgCl_2$	Magnesium Chloride
MIC	Minimal Inhibitory Concentration
OMF	Outer Membrane Factor
ORF	Open Reading Frame
PCR	Polymerase Chain Reaction
RNA	Ribonucleic acid
RNase	Ribonuclease

RND	Resistance-Nodulation-Division
Rpm	Revolution per minute
RT-qPCR	Quantitative Real Time Polymerase Chain Reaction
SEM	Standard Error of Mean
TAE	Tris Base-Acetic Acid-EDTA
Tm	Melting Temperature
TRIS	Tris (hydroxymethyl) aminomethane
UV	Ultraviolet

CHAPTER 1

INTRODUCTION

1.1. Heavy Metal Resistance

Some metals are essential for bacterial cells. Essential metals take part in biochemical reactions, stabilizing the proteins and bacterial cell walls, and retain the osmotic balance (Hughes & Poole, 1989; Ji & Silver, 1995; Poole & Gadd, 1989). For instance, cobalt, copper, iron, potassium, nickel, and zinc are required; while others do not have a biological role and are non-essential. Furthermore, some of the non-essential metals such as silver, aluminum, cadmium, and mercury are even toxic to bacteria.

Metals possessing a density above $5g/cm^3$ are described as heavy metals. Due to incomplete d-orbitals, they form heavy metal cations and have the ability to form complex compounds. While some of these cations have importance in biological reactions in a bacterial cell as trace elements, they also have toxic effects at higher concentrations (Nies, 1999), and create oxygen radicals by Fenton reaction (López-Maury et al., 2002). Moreover, a conformational change in nucleic acid and protein structure, intrusion to oxidative phosphorylation, and distortion of the osmotic balance are also effects of high concentrations of heavy metals in a cell (Poole & Gadd, 1989). Especially Hg^{2+}, $Cd^{2+,}$ and Ag^{+} form toxic complexes which negatively affect the physiological state of the cell; therefore, intracellular concentrations of heavy metal cations have to be controlled by regulatory mechanisms (Nies, 1999). As a result, microorganisms tend to develop heavy-metal resistance in order to preserve their cellular components. Constitutively, preventing entry of the metals or changing cellular components are solutions applied by bacteria to reduce sensitivity against heavy metals. In fact, six mechanisms were found to operate in heavy metal resistance in microorganisms. These mechanisms are exclusion of metals with the help of

permeability barrier, efflux of metals by active transport away from the cell or organism, intracellular metal sequestration by binding of protein, extracellular sequestration, enzymatic detoxification for converting metals to a less toxic form and diminishing sensitivity of cellular targets against metals (Rouch et al., 1995; Silver, 1992).

In the early 1970s, heavy metal resistance was discovered in several microorganisms against a number of metals. Mostly, aerobic microorganisms were documented as resistant such as *Escherichia coli*, *Staphylococcus sp*., *Pseudomonas aeruginosa* and *Bacillus sp*. (Belliveau et al., 1991; Harnett & Gyles, 1984; Marques et al., 1979; Nakahara et al., 1977; Schwarz & Blobel, 1989; Wang & Shen, 1995). In addition, mercury resistance was documented in obligate anaerobic species such as *Clostridium* and *Bacteroides* (Bruins et al., 2000). In recent years, several Gram-negative and Gram-positive bacterial strains have been found to be able to survive and grow in high heavy metal concentrations (de Lima e Silva et al., 2012).

1.2. Silver Resistance in Bacteria

Due to increasing usage of silver, many bacteria especially the Gram-negative ones have acquired silver resistance (Gayle et al., 1978; Li et al., 1997; Starodub & Trevors, 1989). In Gram-negative bacteria, silver resistance was first discovered in *Salmonella typhimurium* (Larkin Mchugh et al., 1975). Several silver-resistant bacterial strains have been discovered in environmental and clinical sources such as *Escherichia coli*, *Enterobacter cloacae*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* (Hendry & Stewart, 1979; Modak & Fox, 1981).

Silver ions have to enter the cells to affect their targets. In order to reduce the intracellular silver ion concentration, active efflux mechanisms are applied by bacteria. Meanwhile, to prevent the massive entry of the ions, the permeability barrier qualities of the outer membrane is reinforced (Nikaido, 1994; Silver & Phung, 1996). In a study carried out with clinical strains of *E.coli*, decreased membrane permeability

and higher rates of silver efflux were observed in silver resistant mutants than the parental strains (Li et al., 1997).

There are two ways for bacteria to gain silver resistance; either via mutations or horizontal gene transfer (Randall et al., 2014). In a pioneering study, Gupta et al., (1999) cloned the silver related transporter and sequestration proteins of *Salmonella typhimurium* in a pMG101 plasmid. In their study, silver ions were accumulated in the periplasm with the aid of sequestration proteins and active efflux with RND-type efflux transporter expelled the ions outside the cell (Gupta et al., 2006). In the literature, bacterial silver resistance gained via plasmids has several examples (Deshpande & Chopade, 1994; Haefeli et al., 1984; Larkin Mchugh et al., 1975; Starodub & Trevors, 1989). However, occurrence of silver resistance spontaneous mutants is a rare case considering the relevant experimental studies (Drake, 1991).

1.2.1. Silver Resistance Mechanism: Cus System

Regulation of metal ion concentration in bacterial cells has vital importance in order to prevent their toxic effects. For reducing the toxic effects of silver, bacteria possess sophisticated regulatory mechanisms which enable them to gain resistance (Grass & Rensing, 2001; Grass et al., 2011; Rensing & Grass, 2003). *Escherichia coli* has Cus system in order to expel excess silver out of the cell. Cus system is reported to function in the efflux of copper and silver ions and takes its name from 'Cu-sensing' phenomena (Franke et al., 2003; Munson et al., 2000). The system is composed of two adjacent operons; *cusCFBA* and *cusRS* (Figure 1.1). While *cusCFBA* operon encodes the silver efflux pump proteins CusC, CusF, CusB and CusA; *cusRS* operon encodes CusR/CusS two-component sensor-regulator system proteins (Franke S et al., 2001a; Munson et al., 2000).

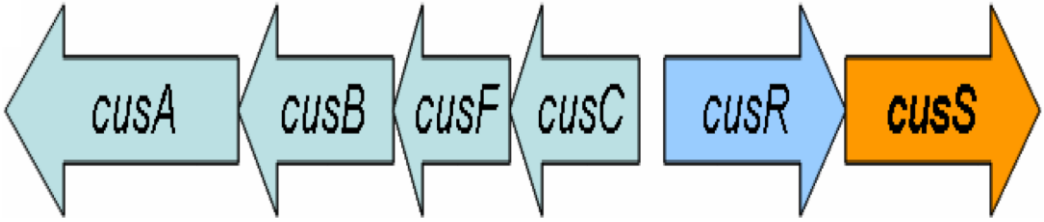

Figure 1.1. Open Reading Frames (ORFs) in *cusCFBA* and *cusRS* operons. The two operons are divergently transcribed (Gudipaty et al., 2012).

In *E.coli*, CusS is a membrane-bound sensor kinase (Franke et al., 2003; Munson et al., 2000). In the case of elevated Ag^+ concentrations, it phosphorylates the response regulator protein CusR then; phosphorylated CusR provides derepression of *CusCFBA* expression.

CusC, CusB and CusA proteins form CusCBA complex which is an RND-type efflux transporter (Figure 1.2) and ensures the export of Ag^+ from the periplasmic space. CusCBA is an antiporter exchanging proton with silver. These three proteins have been characterized to a greater extent. CusA belongs to RND (Resistance-nodulation-division) protein superfamily which consists of membrane-bound and proton-driven transporters (Saier, 2000; Saier et al., 1994; Tseng et al., 1999). CusB is classified as a membrane-fusion protein (MFP), while CusC is defined as 'Outer Membrane Factor' (OMF) (Dinh et al., 1994). In addition, CusF, which is encoded by the same operon, is a periplasmic silver-binding protein (metallochaperone). CusF brings silver ions to CusCBA complex and when it binds to CusB protein, the efflux of Ag^+ is realized (Mealman et al., 2012; Randall et al., 2014).

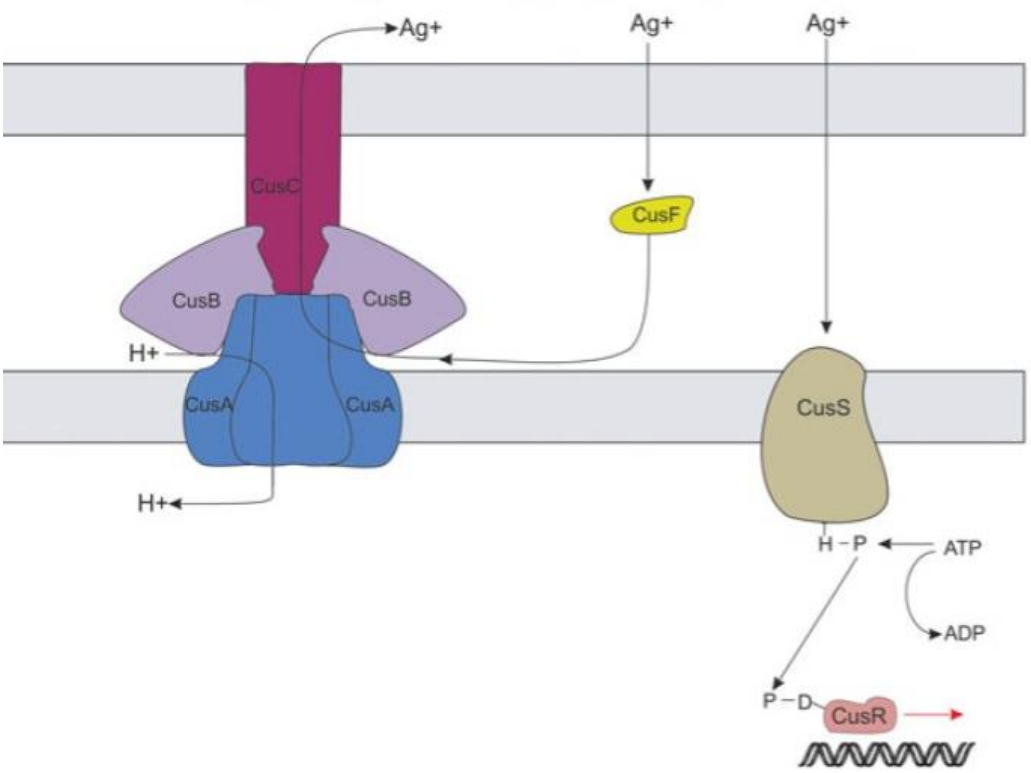

Figure 1.2. Schematic representation of the Cus system (Randall et al., 2014).

CusS binds silver and initiates downstream signaling events via a signal transduction pathway (Gudipaty et al., 2012). In addition, according to in vivo functional analysis results, in order *E.coli* to gain silver resistance, full-length CusB was reported to be crucial (Mealman et al., 2012). CusF is a periplasmic metallochaperone which is only present in copper/silver resistance systems (Bagai et al., 2008). Moreover, CusF is found requisite for the silver resistance because deletion of *cusF* causes strains to become silver-sensitive (Franke et al., 2003).

1.3. Heavy Metals as Antimicrobial Agents

For hundreds of years, heavy metals have been used as antimicrobial agents due to their toxic effects on bacterial cells (Lemire et al., 2013). In fact, as an example of historical applications, silver nitrate ($AgNO_3$) have been used to treat gonorrheal eye infections in newborns and silver foils have application in the healing of surgical wounds (Crede, 1881; Silver et al., 2006). Nowadays, metallic surfaces and metal coatings of medical devices, as well as chelates and nanomaterials which have been used in industry, healthcare and agriculture contain silver (Afessa et al., 2010; Kollef et al., 2008; Lemire et al., 2013; Saint et al., 2000). Especially silver and copper are currently used in household products, in hospital settings and industrial areas for hygiene-related purposes (Mcdonnell & Russell, 1999; Russell, 2003). In addition to silver, copper (Cu), magnesium (Mg), mercury (Hg), tellurium (Te), arsenic (As) and gold (Au) have also been used in the treatment of some infectious diseases such as tuberculosis, leprosy, gonorrhea and syphilis (Frazer & Edin, 1930; Hodges, 1889; Kayne, 1935; Keyes, 1920; Pereira, 1836).

1.3.1. Silver as an Antimicrobial Agent

Silver is one of the most effective and fast-acting toxic metals against bacterial cells (Silver, 2003; Silver et al., 2006). Silver is effective on a broad range of bacteria, as well as fungi and viruses (Cho et al., 2005; C N Lok et al., 2006; Silver, 2003). Moreover, the lethal effect of silver has been ascertained at very low concentrations for both Gram-negative and Gram-positive bacteria. This makes silver a favorable metal to be used in antimicrobial films, textiles, coatings and medical equipment (Fan & Bard, 2002; Ghandour et al., 1988; Ignatova et al., 2003; Schreurs & Rosenberg, 1982; Yuranova et al., 2003). In fact, in the early years, aqueous silver nitrate was also used against eye infections as a common practice for preventing the transfer of *Neisseria gonorrhoeae* during childbirth from an infected mother (Silvestry-Rodriguez et al., 2007). Then, years later, it was established that silver is the most

effective metal possessing the antimicrobial activity and the least toxic one against to animal cells (Guggenbichler et al., 1999).

Mode of action of silver on bacterial cells is not exactly determined. Nevertheless, silver-exposed bacterial cells display failure of the respiratory system preceding cell death (Bragg & Rainnie, 1974; Yudkin, 1937). It has been documented that Ag^+ detaches the respiratory chain from oxidative phosphorylation and cause dissipation of proton-motive force across the cytoplasmic membrane (Dibrov et al., 2002; Schreurs & Rosenberg, 1982). It is clear that silver ions attack the cytoplasmic membrane-associated proteins and respiratory chain enzymes. (Bragg & Rainnie, 1974; Schreurs & Rosenberg, 1982; Zeiri et al., 2004). According to the literature, in *Escherichia coli*, Ag^+ inhibits phosphate uptake (Schreurs & Rosenberg, 1982). In addition, it prevents glucose, glycerol, fumarate, and succinate oxidations by inactivating the related enzymes (Ahearn et al., 1995).

1.4. *Escherichia coli* ATCC 8739

Escherichia coli ATCC 8739, which is known as Crooks strain, is a facultative anaerobe laboratory strain. It was isolated from fecal samples and primarily used in antimicrobial assays, bioresistance tests, quality control assays, and efficacy tests or as quality control strain, according to ATCC manufacturer's product sheet (Pinto et al., 2011; Sheet, n.d.). It is a biosafety level 1 strain with no pathogenicity cases reported (Archer et al., 2011). *E.coli* ATCC 8739 differs from *E.coli* K12, by lacking one of the major porins. While *E.coli* K12 strain has OmpC and OmpF porins; *E.coli* ATCC 8739 (Crooks) expresses only OmpF porin. This is because an insertion element (IS1-13) was placed between the first 114 base-pair of *ompC* gene and its promoter (Pinto et al., 2011). According to a relevant study, lack of OmpF or both of the OmpF and OmpC porins did not show a remarkable effect on the silver resistance of different *E.coli* strains (Li et al., 1997).

1.5. Spontaneous and UV-Induced Mutants in *Escherichia coli*

Spontaneous mutations occur due to modifications in chromosomal DNA by unidentified mechanisms during DNA replication, recombination, and repair (J. W. Drake, 1970; von Borstel, 1969). Types of mutations which occur spontaneously are base substitutions, insertions, and deletions (Smith, 1992). Many spontaneous mutants in *E.coli* arise as a result of error-prone DNA repair system activities (Sargentini & Smith, 1981).

Induced mutations are obtained via the treatment of cells with an exterior chemical or physical mutagen. The mutagenic agents increase mutation frequency (Rangel & Carvalho, 2017). UV-light is one of the physical mutagens. UV irradiation generally creates mutations at certain base pairs which coincide more frequently than other base pairs (Coulondre & Miller, 1977). Especially UVC light causes harmful effects on DNA by inducing damages and gives rise to mutations such as GC-AT transitions, back mutations at specific sites and thymine dimers (Wójcik & Janion, 1997).

E.coli as being one of the representative *Enterobacteriaceae* order is widely used in spontaneous and induced mutant studies (Rangel & Carvalho, 2017). In this study, considering the wealth of accumulated information, the experiments were designed based on *E.coli*.

1.6. Aim and Scope of the Study

The aim of this study is to find out the relative gene expression levels of silver efflux system proteins in spontaneous and UV-induced silver-resistant *E.coli* ATCC 8739 mutants. The expression levels were measured by using Real-Time Quantitative Polymerase Chain Reaction (RT-qPCR). The spontaneous mutants in the study were obtained upon acute exposure to silver as opposed to the previous studies reported in the related literature in which the mutants were obtained via acclimation. Target genes for the quantitative gene expression analysis were *cusB*, *cusF,* and *cusS*. In the

literature, there has not been reported a spontaneous silver-resistant *E.coli* mutant obtained by direct exposure to high silver concentrations. So far in RT-qPCR measurements dealing with *E.coli* genes, a well-known stable housekeeping gene, 16S ribosomal RNA, have been used. However, this gene's expression was not stable in our strain under our experimental conditions. Therefore, in this study, a bacterial housekeeping gene, *tus*, was used for the first time in silver resistant *E.coli* studies as an alternative to 16S ribosomal RNA.

CHAPTER 2

MATERIALS AND METHODS

2.1. Culture Media

In the study, two different media were prepared for original strain *E.coli* ATCC 8739 and silver-resistant *E.coli* ATCC 8739 mutants. According to the instructions of the manufacturer, nutrient broth and nutrient agar media were used to grow *E.coli* ATCC 8739 cells. Because this strain was also used as a reference strain in the study, silver-nitrate was not added to its growth medium. In the selection and maintenance of silver-resistant *E.coli* mutants, 111 µg/ml silver nitrate (0,65 mM) containing nutrient agar media were used.

Composition and preparation of the culture media are given in Appendix C.

2.2. Growth Conditions and Maintenance of Bacterial Strains

Control strain, *E.coli* ATCC 8739, was grown in Nutrient Agar (Appendix C). Silver-resistant *E.coli* mutants were grown in 111 µg/ml silver nitrate-containing nutrient agar plates (Appendix C). The silver nitrate stock solution was prepared as 50µg/ml and filter-sterilized. The composition of the silver nitrate stock solution is given in Appendix B. For short term storage, each strain was sub-cultured onto relevant nutrient agar plates, monthly. Newly streaked agar plates were incubated at 37°C for 16 hours and then kept at 4°C. For long term storage, *E.coli* cells were grown in nutrient broth medium at 37°C at 180 rpm until they reach their mid-log phase. After incubation, small aliquots of broth culture were centrifuged at 3000 rpm for 5 minutes and supernatants were discarded for each aliquot. Then, 50% sterile glycerol solution was added onto the pellets. After vortexing, they were stored at -80°C.

2.3. Determination of Minimal Inhibitory Concentration (MIC)

Minimal inhibitory concentration for silver nitrate was determined according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) methodology (EUCAST, 2000). Because the following experiments will be conducted with agar media, MIC was determined with 'Agar Dilution' method. Silver nitrate stock solution was filter-sterilized before the experiments, as recommended. Agar plates with different silver nitrate concentrations ranging between 5 μg/ml to 150 μg/ml were used for the MIC tests. The plates were incubated at 37°C for 18 hours. The lowest silver concentration at which no visible growth is observed was determined as MIC of silver nitrate for *E.coli* ATCC 8739 strain.

2.4. Spontaneous Mutant Selection

In this study, the silver resistant mutants were obtained without acclimation. All mutant selection experiments were performed arranging the silver nitrate concentrations over the MIC value. *E.coli* ATCC 8739 strain was grown in nutrient broth medium (Appendix C) overnight (16-hour) at 37°C at 180 rpm in a shaker incubator. After incubation, cells were inoculated by spreading onto silver nitrate-containing nutrient agar plates. The silver nitrate concentrations ranged between 61μg/ml and 141 μg/ml with 10 μg/ml increments. Inoculated silver plates were, then, incubated at 37°C for 16 hours. 150 silver nitrate-containing agar plates were used to find spontaneous mutants. Putative silver-resistant mutant colonies were spotted on the plates after incubation. The colonies were streaked onto fresh agar media containing silver nitrate at the appropriate concentration with the media where the mutants appeared. The ones which continue to survive after passaging were designated as spontaneous mutants.

All equipment used in the study was given in Appendix A.

2.5. UV-Induced Mutant Selection

In the selection of UV-induced mutants, agar plates containing 111 µg/ml silver nitrate were used, which was higher than the MIC value. This silver nitrate concentration was determined based on the results of the spontaneous mutant selection experiments. *E.coli* ATCC 8739 cells were grown in nutrient broth media at 37°C at 180 rpm in shaker incubator for 16 hours. In order to obtain UV-induced mutants, an overnight broth culture was exposed to UVC light for 10 seconds. After that, UV-exposed cells were inoculated onto 111 µg/ml silver nitrate-containing agar plates by spreading and left for incubation at 37°C for 16 hours. After incubation, colonies seen on the silver agar plates were streaked onto fresh 111 µg/ml silver nitrate-containing plates and incubated at 37°C for 16 hours. Colonies survived after passages were designated as UV-induced silver-resistant mutants.

All equipment used in the study was given in Appendix A.

2.6. Design of Primers

Primers were designed for *cusB*, *cusF* and *cusS* genes which are the target genes of the study and for housekeeping gene, *tus*. Primers were designed according to *E.coli* ATCC 8739 genome sequence (GenBank accession number CP000946). Product sizes were chosen between 80 and 250 base pairs for reliable quantitative RT-PCR results. Usually, 80-250 base-pair length reduces the occurrence of self-dimerization or cross primer dimerization probability. GC content of all primers was adjusted between 50-60%, and their T_ms were chosen between 50-65°C. Several repeats of Gs and Cs longer than 3 bases were avoided. Primer sequences designed for each gene and their product sizes are given in Table 2.1.

Table 2.1. *List of the primers used in the study.*

Name of the Gene	Primer	Primer Sequence	Amplicon Size
tus	*tus* forward	GGGCAAGCGTGTTGTACTTG	103 bp
	tus reverse	TGCCACAGAACGCGAAGTTA	
cusB	*cusB* forward	GAGGGTAAACTGCGAGGCAT	111 bp
	cusB reverse	GCCAAAGATAACGTGGTCGC	
cusF	*cusF* forward	AAAGAAAGGTTGCCCTGCTG	89 bp
	cusF reverse	TTTACCATCACCCCGCAGAC	
cusS	*cusS* forward	ATGTCTTTACCCGCCAGTCC	249 bp
	cusS reverse	CCGCCAGGTTGAGCATTTTC	

2.7. Optimization of Primers

Primers were optimized by Polymerase Chain Reaction (PCR) using genomic DNA of mutant TG-S01 as a template. PCR products were, then, visualized under UV-spectrophotometer to determine the optimum conditions for amplification.

2.7.1. Genomic DNA Isolation

Genomic DNA of one of the silver-resistant mutants, TG-S01, was isolated in all primer optimization studies. All the primers were optimized according to this mutant's DNA. The boiling method was used for the DNA isolation procedure (Appendix I). Before starting the isolation, mutant cells (TG-S01) were streaked onto the solid media containing 111 μg/ml silver nitrate and incubated overnight at 37 °C. After cells were collected, the isolation procedure were performed as described in Appendix I. In this

procedure, TG-S01 cells were lysed in GTE (Glucose/Tris/EDTA) mix to generate a stable environment while cells were burst at a high temperature. Every component in the GTE mix has an essential role to provide this stable environment. Glucose, as a non-electrolyte, helps maintain the optimum osmolarity by preventing early lysis of the cells. Tris is used to keep the ideal pH (8.0), and preventing DNA degradation and undesired side reactions. EDTA helps prevent DNA degradation by chelating metal ions in the solution which were used in the undesired side reactions (Boyle, 2005; Casali, 2010; Dashti et al., 2009; Steehler, 2009) In addition, RNase was added to the isolation mixture to digest RNAs by preventing contamination of DNA. Preparation and composition of GTE mix are given in Appendix B.

2.7.2. Annealing Temperature Determination with Gradient PCR

In order to determine the optimum annealing temperature for each primer in PCR experiments, temperature gradient PCR was performed. After optimum $MgCl_2$ concentration was selected as 2.5mM, temperature range was assigned between 54°C and 64°C according to T_ms of the primers. Genomic DNA of TG-S01 was used as template. DMSO was also added to Taq Polymerase Mix in order to increase the specificity of primers. Because T_ms of primers were nearly identical, the same temperature range and PCR conditions were applied for all primer sets. Specifications of the PCR kit used in the procedure is given in Appendix D. Equipment used in the study is given in Appendix A. Gradient PCR conditions are given in Table 2.2 and composition of PCR mixture is given in Table 2.3.

Table 2.2. *Gradient PCR conditions applied for all primer sets.*

PCR Steps	**Temperature**	**Duration**
Initial Denaturation	94°C	05:00
Denaturation	94°C	00:45
Annealing	54-64°C	00:25
Extension	72°C	00:15
Final Extension	72°C	07:00

Table 2.3. *The composition of PCR mixtures of gradient PCR.*

Component	**Volume/Final Concentration**
Taq 2X Master Mix	12,5µl
25mM $MgCl_2$	2,5mM
Forward Primer	0,1µM
Reverse Primer	0,1µM
DMSO	4% (v/v)
Template DNA	10-50 ng
TOTAL VOLUME completed with molecular-grade H_2O	**25µl**

2.7.3. Visualization of PCR Products with Agarose Gel Electrophoresis

In order to analyze PCR products, horizontal gel electrophoresis system was used (Appendix A). Because PCR products are small in size, 1.5% (w/v) agarose gel was prepared. 1X TAE buffer (Appendix B) was used in the preparation of agarose gel and as a running buffer. After PCR products were loaded to the gel, the system was run at 100 Volts for approximately 40 minutes. A DNA ladder was used in the procedure to determine the size of the PCR product bands (Appendix E). Followed by the electrophoresis, the gel was stained with ethidium bromide solution (0.5 µg/ml EtBr in 1X TAE buffer) for 15 minutes. The DNA bands were visualized by using UV transilluminator (Appendix A).

2.8. Total RNA Isolation

Total RNA from the control strain, *E.coli* ATCC 8739, and *E.coli* silver resistant mutants was isolated using GeneJET RNA Purification Kit according to the manufacturer's instructions. Before the isolation procedure, the cell collection procedure was modified and optimized because isolation kit was designed based on the liquid culture; however, we grew our cells as solid cultures. *E.coli* ATCC 8739 cells were collected from the agar media after 4-hour incubation whereas mutant strains were collected after 5 hours.

All kits and chemicals used in the experiment are given in Appendix D. The procedure of RNA isolation experiment is given in Appendix F.

2.8.1. Qualification of RNA Isolation

In order to determine whether RNA isolation is successful or not, agarose gel electrophoresis was carried out. At this step, isolates were directly loaded on 2% (w/v) agarose gel prepared with 1X TAE buffer. System was run at 100 Volts for 50 minutes. Staining of agarose gel was done in 0.5 µg/ml EtBr containing 1X TAE buffer

(Appendix B). The gel was visualized via UV transilluminator (Appendix A). For determination of the RNA purity, Biodrop was used (Appendix A). Absorbance ratios OD_{260}/OD_{280} and OD_{260}/OD_{230} were checked for RNA samples. For pure and high-quality RNA, OD_{260}/OD_{280} ratio is expected around 2. However, lower ratios indicate lower quality of RNA due to the protein or phenol contaminations. OD_{260}/OD_{230} ratio is expected to fall between 2.0-2.2, if RNA was not contaminated with organic compounds. In the case of contamination, lower OD_{260}/OD_{230} ratios are obtained (Wilfinger et al., 1997).

2.9. DNaseI Treatment

Followed by the total RNA isolation, in order to eliminate genomic DNA fragments, DNaseI treatment was carried out. DNaseI degrades single and double-stranded DNA, and DNA-RNA hybrids formed in a nonspecific manner. DNaseI treatment procedure was performed with Thermo Fisher DNaseI (Appendix D) according to the manufacturer's instructions. The whole procedure was carried out in a PCR cabinet (Appendix A) to avoid contamination. The experimental protocol of DNaseI treatment is given in Appendix G.

2.10. cDNA Synthesis

After DNaseI-treated RNAs were obtained for all strains, cDNA synthesis was carried out with RevertAid First Strand cDNA Synthesis kit (Appendix D). The procedure was applied according to the manufacturer's instructions. In cDNA synthesis protocol, random hexamers were used as primers. For both control and mutant samples, 1 μg of DNaseI-treated RNA was used for each sample. In order to ensure the success of the cDNA synthesis, PCR was done with the housekeeping gene (*tus*) primer sets. The annealing temperature of the control PCR was selected as the same temperature as was used in the primer optimization experiment. The conditions of the PCR to check

cDNA are given in Table 2.4 and the composition of the PCR mixture is given in Table 2.5.

Table 2.4. *PCR conditions to verify cDNA synthesis.*

PCR Steps	**Temperature**	**Duration**	**Number of Cycles**
Initial Denaturation	94°C	05:00	1
Denaturation	94°C	00:45	35
Annealing	54°C	00:25	
Extension	72°C	00:15	
Final Extension	72°C	07:00	1

Table 2.5. *The composition of the PCR mixture to control the cDNA synthesis.*

Component	**Volume/Final Concentration**
Taq 2X Master Mix	12,5µl
25mM $MgCl_2$	2,5mM
Forward Primer	0,1µM
Reverse Primer	0,1µM
DMSO	4% (v/v)
Template DNA	10-50 ng
TOTAL VOLUME completed with molecular-grade H_2O	**25µl**

For visualization of the PCR products, agarose gel (1.5% w/v) electrophoresis was carried out. Then, the gel was stained with 0.5 µg/ml EtBr containing 1X TAE buffer (Appendix B). Gel electrophoresis system was run at 100 Volts for 40 minutes. The bands were visualized on the gel by the aid of a UV transilluminator (Appendix A).

2.11. Real-Time Quantitative PCR (RT-qPCR) Standard Curve Analysis

To test each primer set for the determination of efficiency in RT-qPCR, a standard curve analysis was carried out. The analyses were done using BioRad CFX Real-Time PCR Detection System. The RT-qPCR reaction mixtures were prepared with SsoAdvanced Universal SYBR Green Supermix (Appendix D) according to the manufacturer's instructions. In order to obtain the RT-qPCR standard curve, cDNA standards were prepared. The cDNA preparation of one of the mutants (TG-S01) was diluted with molecular biology-grade nuclease-free water (Appendix D) at 1:10, 1:20, 1:40, 1:80 and 1:160 ratios. All the standard curves were, then, obtained at those cDNA dilutions. In an efficient reaction, these 5 cDNA dilutions are expected to give Cq values between 15 and 25, because Cq values after 25 indicate contamination in the reaction mixture or very low expression of the target gene.

RT-qPCR reaction mixture contained SYBR Green Supermix (Appendix D), 0.5µM of each primer at the final concentration and 3µl of diluted TG-S01 cDNA at the previously mentioned dilutions. RT-qPCR reaction conditions were determined as 3 minutes at 95°C then, 35 cycles for 30 seconds at 94°C, 30 seconds at 54°C and 30 seconds at 72°C. At the end of the run, melting curve step was added which was carried out between 50°C and 99°C with 1°C increment for 00:05. Melting curve analysis was done in order to validate the presence of a single amplification product at the end of the reaction. Therefore, only one peak was expected in the melting curve if there was not any primer-dimer formation or contamination in the reaction. RT-qPCR reaction mixture’s total volume was 10µl.

2.12. Quantitative Real-Time PCR (RT-qPCR)

RT-qPCR was performed by using SsoAdvanced Universal SYBR Green Supermix with BioRad CFX Real-Time PCR Detection System. Preparation of reaction mixture and application of RT-qPCR were carried out according to the manufacturer's instructions.

The Reaction mixture of RT-qPCR contained SYBR Green Supermix (Appendix D), 0.5µM of each primer at the final concentration and 3µl of 1:40 diluted cDNA of each sample. The relative quantification analysis was carried out by comparing the mutants with the control strain (*E.coli* ATCC 8739). As a housekeeping gene (reference gene), *tus* was used. It is defined as the internal control of the reaction. This gene was selected because it is constitutively expressed and maintain constant expression levels in *E.coli* cells both in normal and under stress conditions. *cusB, cusF,* and *cusS* were selected as target genes and their relative expression levels were determined in the analysis.

RT-qPCR reaction conditions were 3 minutes at 95℃ then 35 cycles for 30 seconds at 94℃, 30 seconds at 54℃ and 30 seconds at 72℃. Stated reaction conditions were applied for all the genes; *tus*, *cusB, cusF*, and *cusS*. After every amplification reaction, melting curve step was added to the PCR to ensure the presence of only one PCR amplification product at the end of the reaction. The melting curve was obtained with 1℃ increment in 5 seconds with a temperature range from 50℃ to 99℃. For each sample, three technical replicates were used and the experiment was repeated with three independent biological replicates.

2.13. Livak ($2^{-\Delta\Delta Cq}$) Method for Expression Analysis

In the quantitative PCR study, relative quantification method was used in the analysis of the RT-qPCR data. In relative quantification, alteration in the expression of a target gene is determined relative to a reference (control) sample (Livak & Schmittgen,

2001). The control sample used throughout the study was *E.coli* ATCC 8739 strain and this strain was not being exposed to silver nitrate.

For an accurate expression analysis in order to apply the Livak method, target and reference gene efficiencies are expected to be equal or nearly equal to 100%. Only 5% efficiency difference is acceptable. Therefore, during the study, efficiency verification was carried out and all data used in the analysis were obtained from high-efficiency RT-qPCR runs (approximately 100%). Relative expression levels of the target genes; *cusB*, *cusF*, and *cusS*, were determined according to the reference gene, *tus*, by using the equations given in Table 2.6 (Livak & Schmittgen, 2001). The control sample was used as the calibrator and test samples refer to the samples of each mutant.

Table 2.6. *The $2^{-\Delta\Delta Cq}$ (Livak) Method Expression Calculation. Calibrator refers to the control sample E.coli ATCC 8739 strain; whereas, test sample refers to the samples of silver-resistant mutants.*

Steps	**Calculation**
Step 1. Normalization of the target gene according to the reference gene for the test samples and the control samples;	$\Delta Cq_{(test)} = Cq_{(target, test)} - Cq_{(reference, test)}$ $\Delta Cq_{(calibrator)} = Cq_{(target, calibrator)} - Cq_{(reference, calibrator)}$
Step 2. Normalization of the test sample ΔCq according to the control sample ΔCq	$\Delta\Delta Cq = \Delta Cq_{(test)} - \Delta Cq_{(control)}$
Step 3. Calculation of the relative expression ratio;	$2^{-\Delta\Delta Cq}$ = Normalized expression ratio according to control

The $2^{-\Delta\Delta Cq}$ method gave the expression ratio of the target gene in the samples tested compared to the calibrator (control) sample, which was normalized with respect to the reference gene (housekeeping gene, *tus*) expression. After expression levels were determined for each test sample (each mutant) for each target gene as three biological replicates, the data were subjected to statistical analyses (Livak & Schmittgen, 2001).

2.14. Statistical Analysis

In the study, 3 independent biological replicates were processed and 3 technical replicates were prepared for each sample. Expression data results were expressed as mean ± standard error of the mean (SEM). Statistical analysis of RT-qPCR data was done with one-way ANOVA (Analysis of variance) with Tukey's multiple comparison post-test using GraphPad Prism 7.04 (California, USA). The significance level of gene expression fold change of the mutants with respect to each other was determined with the help of the post statistical test. 95% confidence interval was selected and statistical significance was indicated by stars; $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***).

CHAPTER 3

RESULTS AND DISCUSSION

3.1. Determination of Minimal Inhibitory Concentration for Ag^+

Viable count was done in order to determine the MIC for Ag^+ which was carried out according to the EUCAST Agar Dilution Method. The number of colonies was on the decline while silver concentration was increased incrementally. The number of colonies taken into account on plates was between 30 and 300. During colony counting, more than 300 colonies were recorded as 'uncountable' due to overcrowding; while less than 30 colonies were not accepted for calculations. By norm, the range between 30 and 300 is regarded statistically significant (Madigan et al., 2014). CFU (Colony Forming Unit) counts are given in Table 3.1, Table 3.2 and Table 3.3.

Table 3.1. *CFU counts from MIC determination experiment with broad concentration intervals.*

The concentration of Silver nitrate	**1st Replicate**	**2nd Replicate**	**3rd Replicate**
5 µg/ml	Uncountable	Uncountable	Uncountable
25 µg/ml	Uncountable	Uncountable	Uncountable
50 µg/ml	0	0	0
75 µg/ml	0	0	0
100 µg/ml	0	0	0
125 µg/ml	0	0	0
150 µg/ml	0	0	0

Table 3.2. *CFU counts from MIC determination experiment with narrowed down concentration intervals.*

The concentration of Silver nitrate	**1st Replicate**	**2nd Replicate**	**3rd Replicate**
25 µg/ml	Uncountable	Uncountable	Uncountable
30 µg/ml	Uncountable	283	Uncountable
35 µg/ml	256	274	216
40 µg/ml	55	53	69
45 µg/ml	0	0	0
50 µg/ml	0	0	0

Table 3.3. *CFU counts from MIC determination experiment with 1µg/ml silver nitrate concentration interval.*

The concentration of Silver nitrate	**1st Replicate**	**2nd Replicate**	**3rd Replicate**
38 µg/ml	110	101	124
39 µg/ml	109	98	112
40 µg/ml	72	55	61
41 µg/ml	0	0	0
42 µg/ml	0	0	0
43 µg/ml	0	0	0
44 µg/ml	0	0	0
45 µg/ml	0	0	0

All viable counts were done in triplicates to obtain reliable results for MIC determination.

The viable counts obtained through broad silver nitrate concentration range (5-150 μg/ml silver nitrate) are given in Table 3.1. The broad range was used in order to determine silver nitrate concentration interval closest to the MIC value for Ag^+. For 5 and 25 μg/ml of silver nitrate, the number of colonies was uncountable for all 3 replicates (CFU bigger than 300). However, starting from 50 μg/ml, no growth was observed (Table 3.1). Since the number of colonies changed from 'uncountable' to 'no growth' between 25 and 50 μg/ml of silver nitrate, the intervals between these concentrations were narrowed down in the next experiment.

In the subsequent MIC determination experiment, 5 μg/ml silver nitrate concentration increments were used and inoculations were done onto agar plates containing silver nitrate from 25 μg/ml to 50 μg/ml (Table 3.2). The colonies in 25 and 30 μg/ml of silver nitrate-containing plates were uncountable except 2nd replica of 30 μg/ml silver nitrate-containing plate. However, in that plate, the counting result was very close to 300; therefore, it can be evaluated as compatible with the other replicates. After 35 μg/ml of silver nitrate, the number of colonies begins to decrease in all three replicates. In 45 and 50 μg/ml silver nitrate plates, no growth was observed. Therefore, the new silver nitrate concentration interval was arranged between 38 and 45 μg/ml for the fine tuning of the MIC value.

The last MIC determination experiment was carried out with 1 μg/ml concentration increments of silver nitrate. CFU counts are given in Table 3.3. The first concentration was selected as 38 μg/ml, not 40 μg/ml, in order to ensure the CFU count reliability in small concentration differences. While, between 38 and 40 μg/ml of silver nitrate, colony formation was observed; after 41 μg/ml of silver nitrate, number of colonies dropped to zero. Because MIC is defined as the lowest concentration which no growth was observed, 41 μg/ml (0,24 mM) of silver nitrate was determined as MIC of Ag^+ for *E.coli* ATCC 8739 strain.

In a study carried out with *E.coli* BW25113 strain, MIC for Ag^+ was determined to be 4 μg/ml in agar media (Randall et al., 2014). However, in the studies that the broth dilution method was preferred, MIC determined for Ag^+ was found between 4,8 and 38,4 μg/ml for *E.coli* cells (Kawahara et al., 2000). The reason why the MIC determined in these studies are different from one another is most likely due to the bacterial strain difference. Generally, in antimicrobial activity tests, microorganisms used in the experiments and the solubility of the chemicals are two critical factors which affect the MIC results. Moreover, the method used in the MIC determination, such as agar dilution or broth dilution, also affects the results (Valgas et al., 2007). Variability of MIC results based on the method used was also reported in other studies. In one of them conducted in Louisiana University, MICs determined by agar dilution and broth dilution methods for an antimicrobial agent, chitosan, differ from each other for *E.coli*, *Salmonella* species, and for Gram-positive bacteria (Jiang, 2011). In this study, since subsequent experiments were carried out with solid media, the MIC determination was done with agar dilution method to obtain reliable results.

3.2. Spontaneous and UV-induced Mutants

Spontaneous and UV-induced mutants were obtained by the methods described in sections 2.4 and 2.5, respectively. Spontaneous *E.coli* mutants were named as TG-S01 and TG-S03; while UV-induced ones were named as TG-S02 and TG-S04.

In this study, spontaneous mutants were obtained by acute exposure of *E.coli* cells to high silver nitrate concentration as opposed to gradual concentration increase or long term incubation. The method of obtaining mutants is one of the significances of this study because, according to the related literature, probability of the occurrence of spontaneous mutations is very rare (Drake, 1991). Moreover, in other studies, spontaneous mutations were achieved by long-term exposure of the *E.coli* cells to the different stress conditions (Sniegowski et al., 1997). In a study carried out with silver resistant *E.coli* mutants, spontaneous mutants were obtained by 6-day exposure of the

cells to silver nitrate which was carried out in sub-inhibitory concentrations (Randall et al., 2014). However, spontaneous mutants in this study, TG-S01 and TG-S03, were obtained in the silver nitrate concentration (111 μg/ml) which was much higher than the MIC value (41 μg/ml) upon overnight incubation.

3.3. Gene Expression Measurement Studies

3.3.1. Determination of Optimum Annealing Temperature for PCR

For each primer set of the determined genes, annealing temperatures were determined by performing temperature gradient PCR. The PCR temperature range was adjusted according to the T_ms of the primers (57-59 °C). Followed by the agarose gel electrophoresis, the reaction products were visualized under UV transilluminator. The best annealing temperature was expected to give the brightest band in the gel without secondary products or primer dimers. The determined temperature was used in the RT-qPCR experiments as annealing temperature.

Firstly, the primers for the amplification of a region in *cusB* gene were studied. The *cusB* gene is expressed from *cusCFBA* operon and CusB is a membrane-fusion protein which is a member of cusCBA silver-copper efflux protein complex (Dinh et al., 1994). Primers were designed to obtain small amplicon size and the expected amplicon size for *cusB* was 111 bp. The gel electrophoresis result of the temperature gradient PCR is given in Figure 3.1.

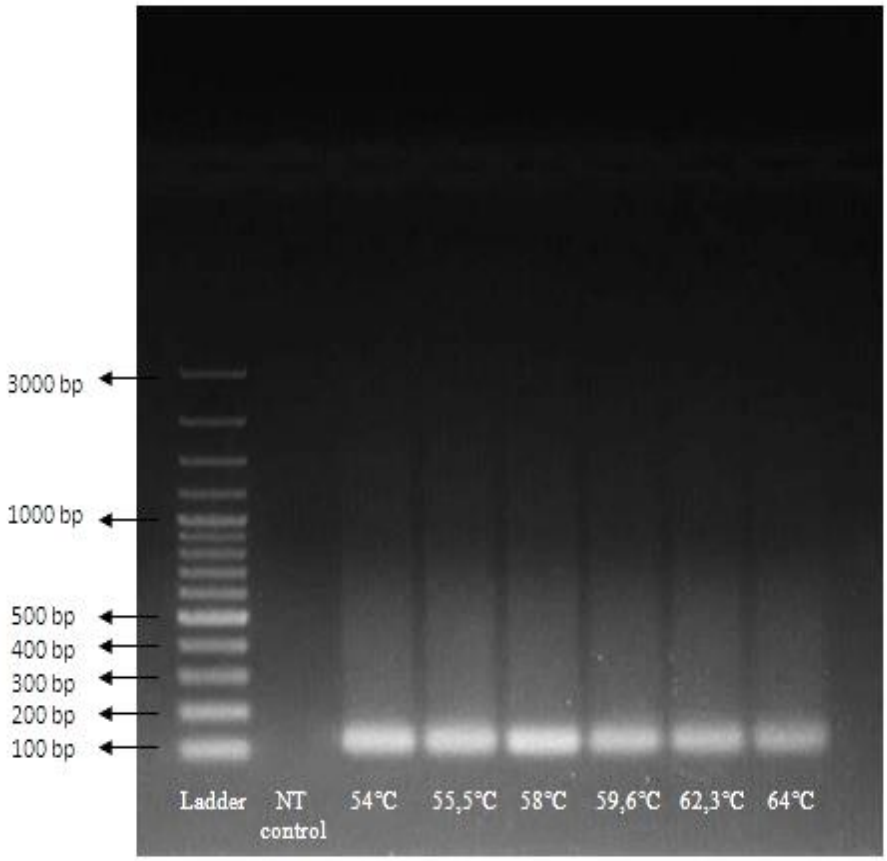

Figure 3.1. Temperature Gradient PCR for the *cusB* gene. The sizes of the ladder bands are indicated in the figure. 'L' represents GeneRuler 100 bp Plus DNA Ladder. NT: No template control (54°C).

As it is seen in the gel's photo, the brightness of the bands decreased while temperature increased from 54°C to 64°C. The first three bands were brighter than the last three bands. The amplicon bands coincided between 100 bp and 200 bp of the DNA ladder. Because designed amplicon size of *cusB* was 111 bp, all amplicons were in the expected size. In addition, primer dimerization did not occur during amplification reaction and there was no non-specific bands.

The second primer set which annealing temperature was determined was *cusF*. CusF is a silver-binding protein found in the periplasmic space of the cell and it is expressed from *cusCFBA* operon. This metallochaperone carries Ag^{+} ions to the efflux protein complex, cusCBA (Mealman et al., 2012; Randall et al., 2014). Designed amplicon size for *cusF* was 89 bp and the gel photo of temperature gradient PCR experiment is given in Figure 3.2.

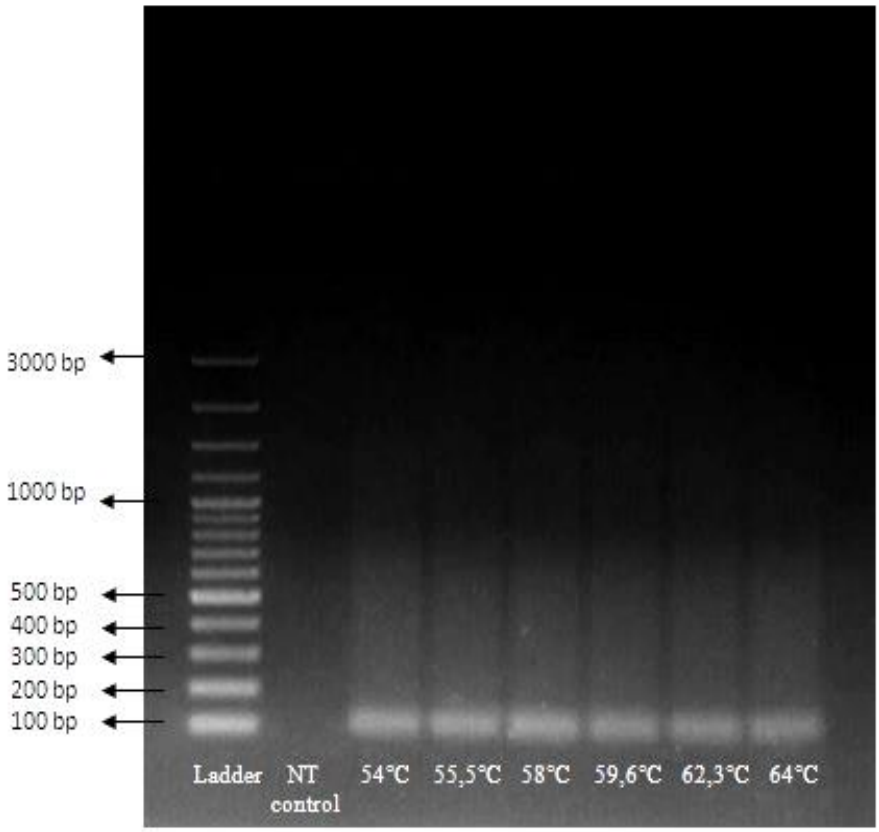

Figure 3.2. Temperature Gradient PCR for the *cusF* gene. The sizes of the ladder bands are indicated in the figure. 'L' represents GeneRuler 100 bp Plus DNA Ladder. NT: No template control (54°C).

As it is seen in the gel photo, amplicons of *cusF* were in the expected size because the last band in DNA ladder corresponded to 100 bp and *cusF* amplicon size was 89 bp. Moreover, there were no non-specific bands or primer dimers at all temperatures. However, as temperature increased, the brightness of the bands decreased; the first three bands were brighter than the others.

The third primer set for annealing temperature determination was *cusS*. CusS protein is a membrane-bound sensor kinase and constitutes a sensor-regulator system with *cusR* (Munson et al., 2000). The designed amplicon size of *cusS* was 249 bp. The gel photo representing the temperature gradient PCR experiment for *cusS* is given in Figure 3.3.

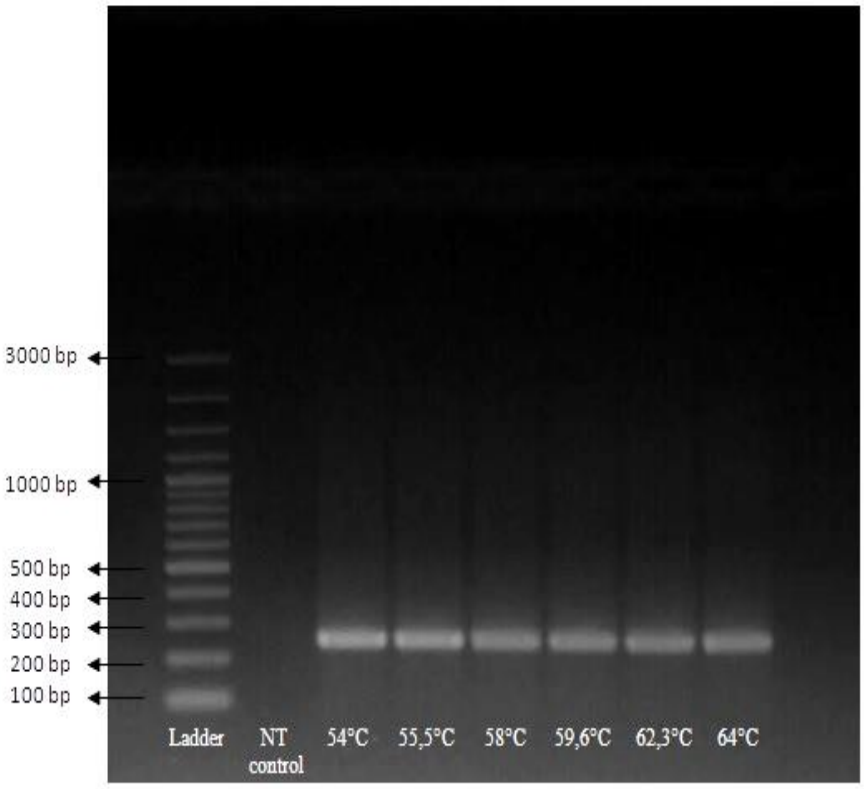

Figure 3.3. Temperature Gradient PCR for the *cusS* gene. The sizes of the ladder bands are indicated in the figure. 'L' represents GeneRuler 100 bp Plus DNA Ladder. NT: No template control (54°C).

As it is seen in the gel photo, all *cusS* bands were in the expected size range. The amplicon size was 249 bp and amplicon bands were detected between 200 and 300 bp markers of the ladder. As in the other primer sets, while temperature increased from 54°C to 64°C, the brightness of the bands diminished. The bands in the first two lanes seemed brighter than the others. In addition, there were no indication of non-specific bands or primer dimerization, which was the desired result for an optimized PCR.

As a housekeeping gene, *tus* was used as a reference for the transcriptional activity. Tus is a DNA-binding protein and needed to end the DNA replication by binding at the terminator sites (Roecklein et al., 1991). Thus far, rrsA-encoding 16S ribosomal RNA is the mostly used reference gene in RT-qPCR studies in *E.coli*; however, its stability has not been verified yet (Zhou et al., 2011). In this study, before *tus* was used as a reference gene, 16S rRNA gene had been tested but it was not stable under high silver nitrate-driven stress conditions. Although *tus* was used as reference gene in the previous RT-qPCR studies performed with UPEC (*E.coli* CFT073) strain (Cai et al., 2013; Ma et al., 2018), it was firstly used with *E.coli* ATCC 8739 strain in our study to determine the relative expression of transport system proteins. In addition, up to now, there has not been any studies carried out with spontaneous mutants using *tus* gene as a reference in RT-qPCR experiments.

For the temperature gradient PCR, designed amplicon size for *tus* gene was 103 bp. The gel photo of temperature gradient PCR experiment is given in Figure 3.4.

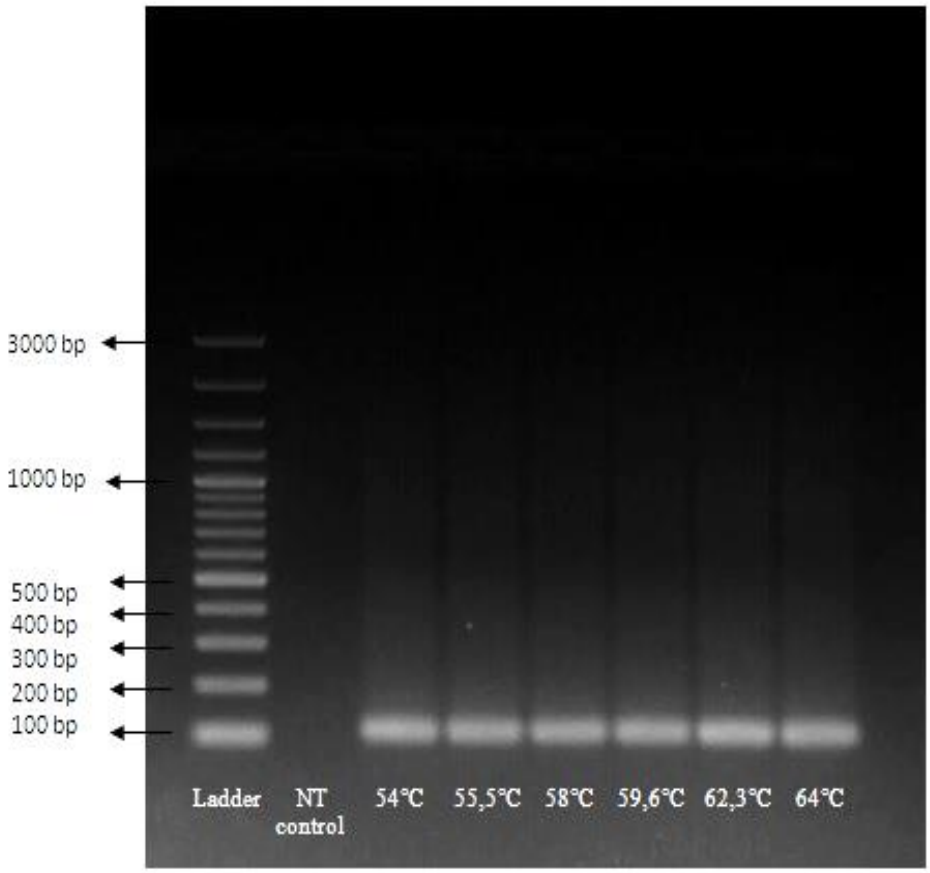

Figure 3.4. Temperature Gradient PCR for the *tus* gene. The sizes of the ladder bands are indicated in the figure. 'L' represents GeneRuler 100 bp Plus DNA Ladder. NT: No template control (54°C).

Designed amplicon size for *tus* gene was 103 bp and all PCR products were seen as coinciding with 100 bp of DNA ladder. Therefore, according to the gel photo, all amplicons of *tus* gene were in the expected size. All bands had similar brightness. Moreover, no primer dimerization or non-specific products formed during the amplification.

Considering all the gel photos, the bands were brighter at lower temperatures than the ones at higher temperatures. Especially, for *cusB*, *cusF*, and *cusS*, brighter bands were obtained at 54℃, 55,5℃ and at 58℃. For *tus*, there was no detectable difference between temperatures. As a result, annealing temperature for RT-qPCR was determined as 54℃ for all genes because at this temperature, bright and thick bands were observed for all genes, and non-specific products did not form. Moreover, using the same annealing temperature for all genes in all RT-qPCR procedures eased the plate arrays in the runs.

3.3.2. Total RNA Isolation from Spontaneous and UV-induced Silver Mutants

Total RNA was isolated from control strain *E.coli* ATCC 8739, spontaneous and UV-induced *E.coli* mutants. The purity and quality of RNA samples were validated with BioDrop by determining OD_{260}/OD_{280} and OD_{260}/OD_{230} absorbance ratios. OD_{260}/OD_{280} ratio was found very close to 2 in all samples; which is expected for pure and high-quality RNA samples because lower ratios indicate protein, phenol or other contaminations (Wilfinger et al., 1997). OD_{260}/OD_{230} ratios were found between 2.0-2.2 in all RNA samples, which is the acceptable range for high-quality RNA. These values also indicated that RNA samples were not contaminated with organic compounds (Wilfinger et al., 1997). In addition, RNA samples were checked with agarose gel electrophoresis to detect 16S and 23S ribosomal RNA bands on the gel for all RNA samples. In a successful RNA isolation procedure, 23S and 16S ribosomal RNA bands should be seen in the gel for prokaryotic cells (Oelmiiller et al., 1990). Gel photo of RNA isolation is given in Figure 3.5.

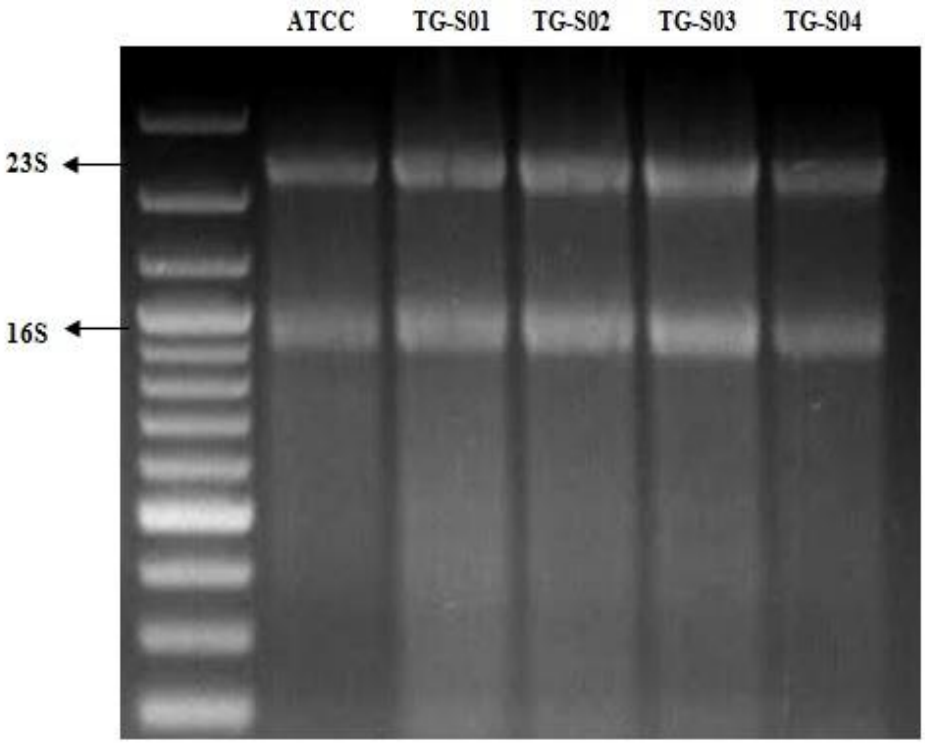

Figure 3.5. Gel photo of RNA isolation for all samples. ATCC represents the control sample *E.coli* ATCC 8739; TG-S01, TG-S02, TG-S03, and TG-S04 represent silver-resistant *E.coli* mutants.

3.3.3. cDNA Synthesis

Followed by the RNA isolation, all RNA samples were treated with DNaseI enzyme to eliminate genomic DNA contamination. 1 μg of DNaseI-treated RNA was used for all mutant samples and for the control sample *E.coli* ATCC 8739. In order to check the success of the cDNA synthesis, PCR was performed with the housekeeping gene primer sets, *tus*. The PCR products were visualized followed by the agarose gel electrophoresis. The gel photo of the control PCR is given in Figure 3.6.

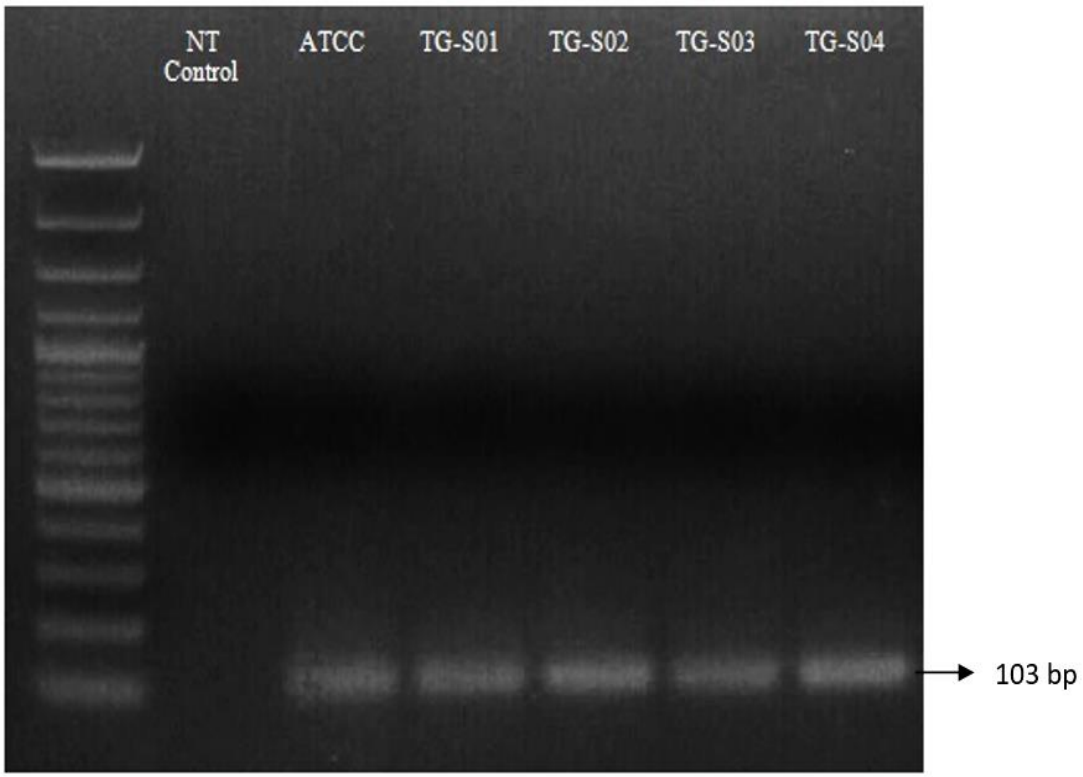

Figure 3.6. Gel photo of control PCR for cDNA synthesis. GeneRuler 100 bp Plus DNA Ladder was used as size markers. NT: No template control, Control strain: *E.coli* ATCC 8739, Silver resistant *E.coli* mutants: TG-S01, TG-S02, TG-S03, and TG-S04.

The gel in Figure 3.6 indicated that cDNA synthesis was successful for all samples. There were clear bands in the expected locations in all lanes. In addition, there were not any non-specific products. Otherwise, repetition of cDNA synthesis have been required.

3.3.4. RT-qPCR Standard Curve Analysis

Standard curve analysis was carried out in order to determine the amplification efficiency of the RT-qPCR reaction (Rogers-Broadway & Karteris, 2015). The curve represents a linear regression plot of C_q values versus the logarithm of input nucleic acid. In this study, for each primer set, standard curve analysis was done along with no-template control samples. No-template controls verify the absence of any contamination in RT-qPCR process (Derveaux et al., 2010). Standard curves were obtained by using 5 dilutions of cDNA of TG-S01 silver resistant mutant and outliers were excluded from the analysis. The aim was to obtain optimum PCR efficiency, which is 100% efficiency (Livak & Schmittgen, 2001).

Standard curve analysis result for the *cusB* gene is given in Figure 3.7 and the melt peak of the PCR run is given in Figure 3.8.

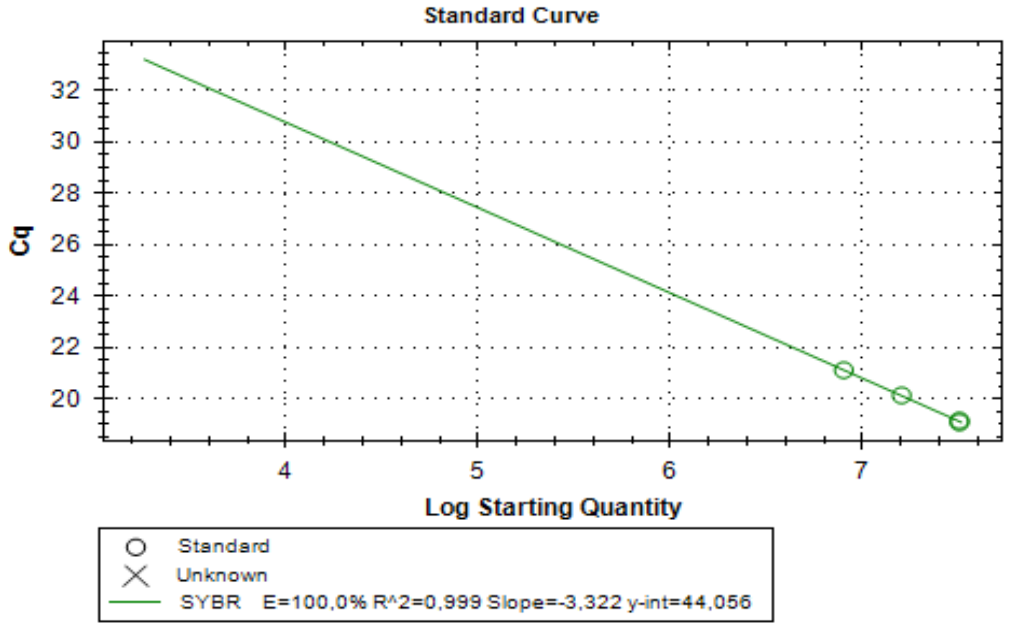

Figure 3.7. The standard curve for the *cusB* gene. E indicates the PCR efficiency of the run.

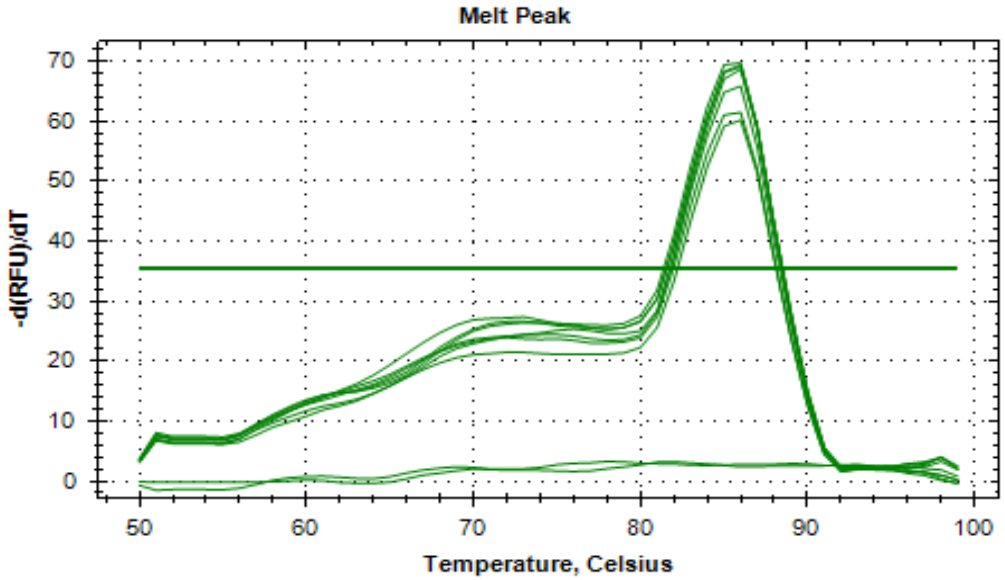

Figure 3.8. Melt peak of PCR reaction for *cusB* primer set.

The standard curve of *cusB* indicated an efficient run for the primer set (100% PCR efficiency). This is accepted as an optimum result. In addition, the slope of the curve was -3.317 which is very close to the expected optimum result (-3.32) in an efficient run. In the melt peak of *cusB*, there was only one peak for each sample.

Standard curve analysis result for the *cusF* gene is given in Figure 3.9 and the melt peak of the PCR run is given in Figure 3.10.

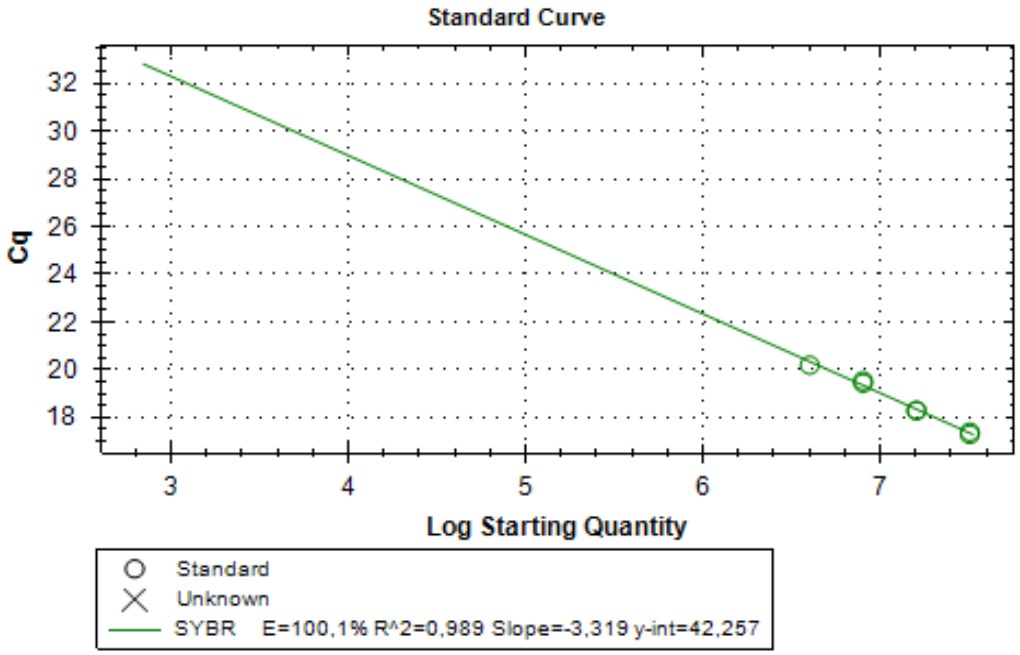

Figure 3.9. The standard curve for the *cusF* gene. E indicates the efficiency of the run.

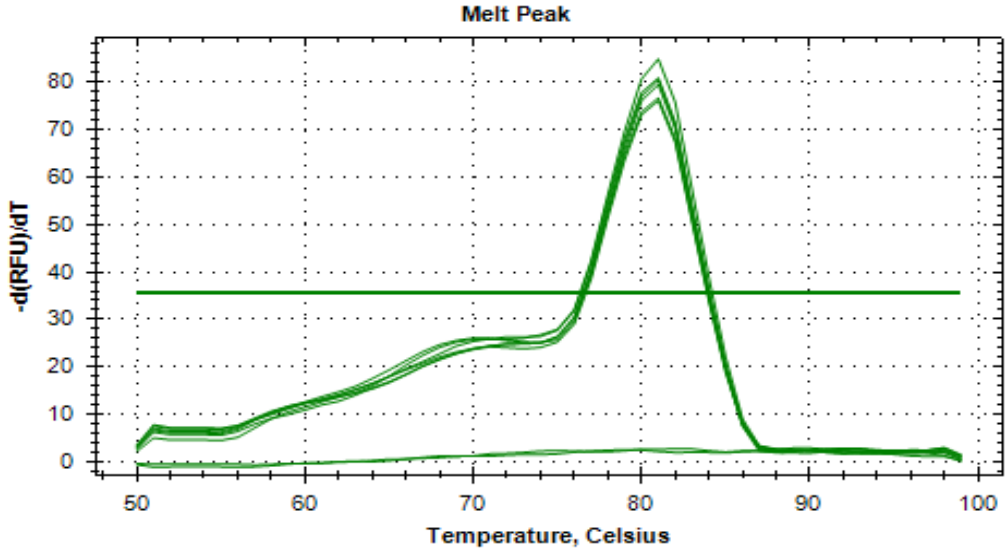

Figure 3.10. Melt peak of PCR reaction for *cusF* primer set.

The standard curve for the *cusF* gene was obtained with an optimum efficiency of 100.1% which was almost 100%. In addition, the slope of the curve was -3.307 which was very close to -3.32. In the melt peak, there was only one peak for each sample.

Standard curve analysis result for the *cusS* gene is given in Figure 3.11 and melt peak of the PCR run is given in Figure 3.12.

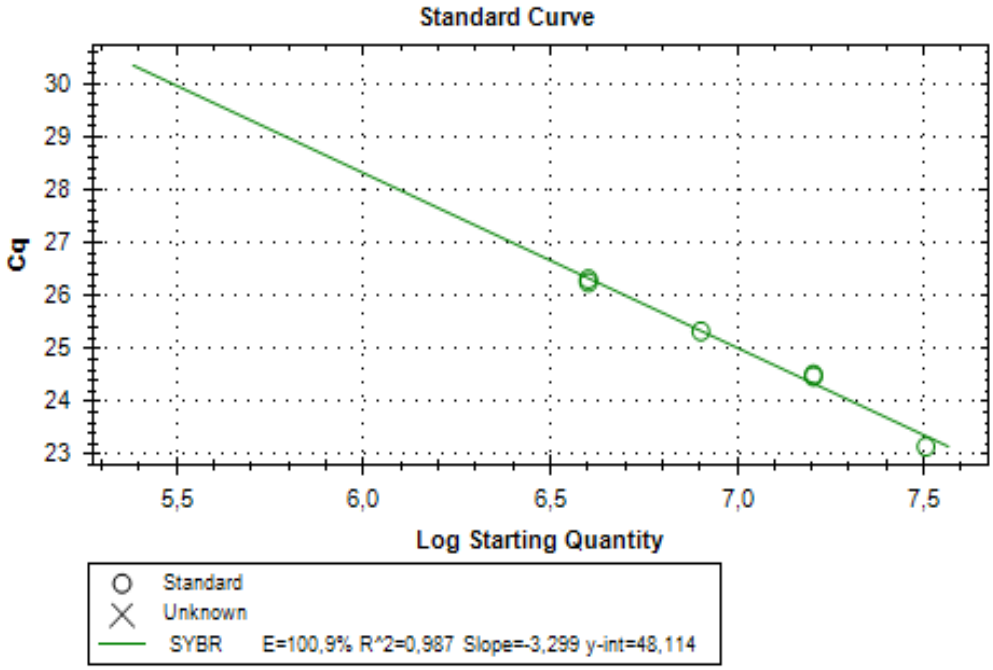

Figure 3.11. The standard curve for the *cusS* gene. E indicates the efficiency of the run.

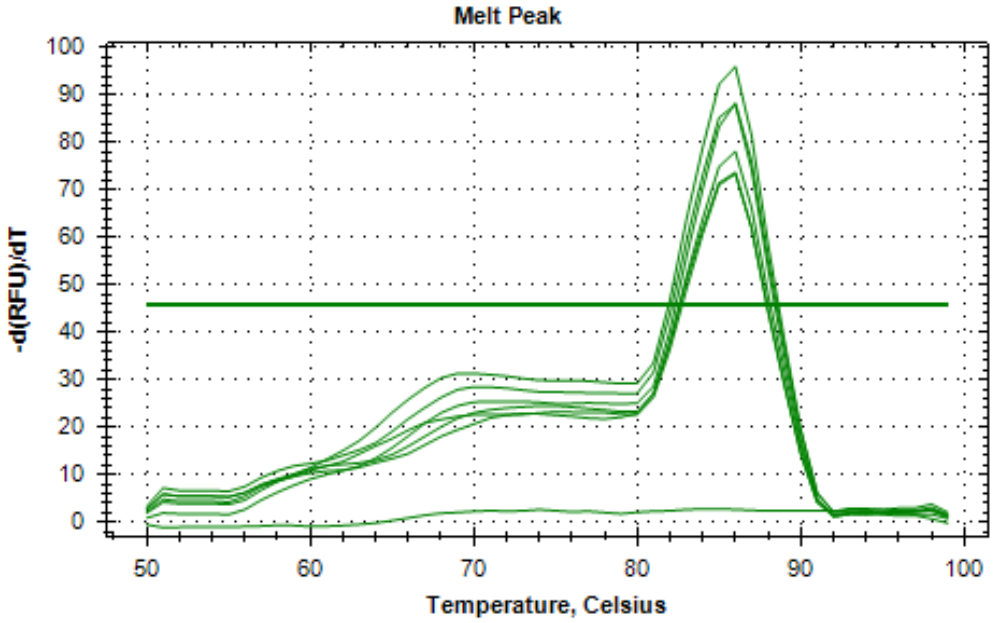

Figure 3.12. Melt peak of PCR reaction for *cusS* primer set.

The standard curve for *cusS* primer set was obtained with an efficiency of 100.9%. This was also very close to the optimum efficiency and slope of the curve was close to -3.32. In addition, in the melt peak of the run, there was only one peak for each sample indicating the region of interest was amplified.

Standard curve analysis result for *tus* gene is given in Figure 3.13 and the melt peak of the PCR run is given in Figure 3.14.

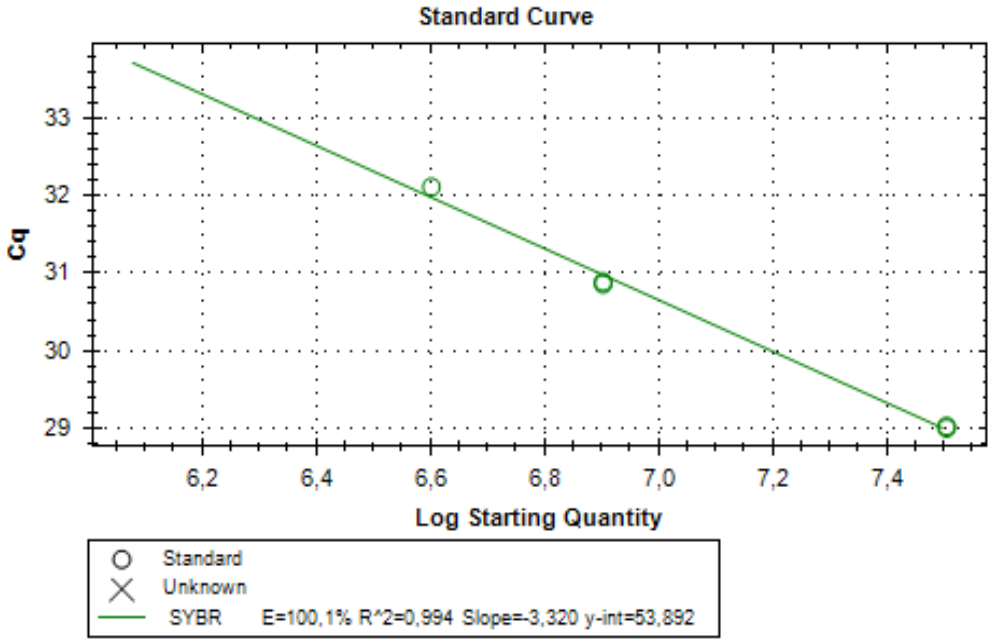

Figure 3.13. The standard curve for the *tus* gene. E indicates the efficiency of the run.

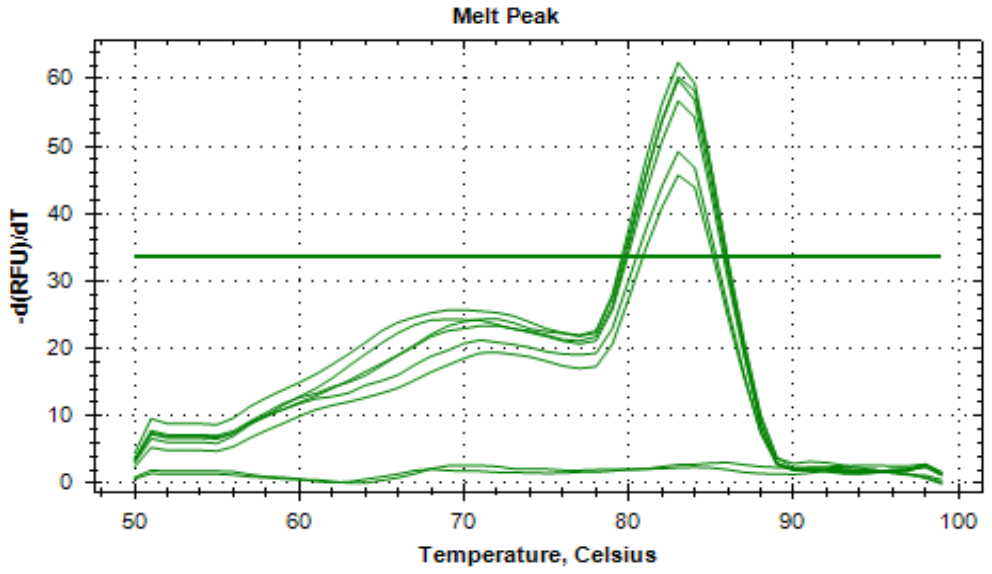

Figure 3.14. Melt peak of PCR reaction for *tus* primer set.

The standard curve for *tus* primer set was obtained with 100.1% efficiency and slope of the curve is exactly -3.32. Moreover, in the melt peak, there was only one peak for each sample.

In an RT-qPCR run, a slope of the standard curve which is close to -3.32 gives an efficient PCR reaction with 100% efficiency. The efficiency of an RT-qPCR run is calculated with the equation $E = (10^{-1/slope} - 1) \times 100$. While the slope of the standard curve becomes more negative, the efficiency of the PCR reaction decreases. Low PCR efficiency is related to assay performance which consists of primer and template sequences. Secondary structures or undesired PCR products decrease PCR efficiency (Svec et al., 2015). If efficiency is well above 100%, it points out the problems related with sample quality or pipetting errors in the preparation of the samples (Biosystems, 2013). In addition, the presence of inhibitors or interfering substances remained from upstream steps increases PCR efficiency value giving unrealistic efficiency results (Svec et al., 2015). If the efficiency of a reaction is 100% obtained through 2-fold cDNA dilutions, the PCR amplicon will be doubled in every single cycle of the PCR. The results are obtained from the equation 2^n = dilution factor, where 'n' represents the number of cycles. Ideally, minimal 3 or 4 different cDNA dilutions are required for a quality standard curve. (Bustin & Huggett, 2017).

In this study, 5 cDNA dilutions were used (1:10, 1:20, 1:40, 1:80, 1:160), which was in the ideal range, and the outliers were excluded. In all primer sets, efficiencies were 100%, which translated into efficient PCR runs. In all runs, no-template control samples fell under the threshold line as single lines as they should be, which indicated the non-amplified samples. In melt curve analyses, only one peak was observed in the graphs of all genes in all samples. This result meant that only one region of interest was amplified in the reactions, and there was not any non-specific products, primer dimers, non-specific site bindings of primers or contamination. If this was not to be

the case, primer dimer peaks would have been seen at the lower temperatures; while contamination peaks would have appeared at the higher temperatures in the graph.

Standard curves were obtained through 2-fold cDNA dilutions; therefore, expected C_q change in a 2-fold increase in the amplification was 1, which also means 1 PCR cycle. Following the exclusion of outliers, 1 Cq change was recorded between 2 cycles of the PCR and the efficiencies of the reactions were 100%. Results showed that the ideal amplification reactions were obtained for all primer sets with optimal reaction efficiencies.

3.3.5. RT-qPCR Expression Analyses for Spontaneous and UV-induced Silver Mutants

For determining the gene expression level of silver efflux mechanism proteins in silver-resistant *E.coli* mutants, the RT-qPCR method was used. Expression levels of *cusB*, *cusF* and *cusS* genes were determined in reference to *tus*, a constitutively expressed-housekeeping gene. After standard curve analyses were completed, in each run for the determination of the gene expression, a standard curve was also constructed. It was found to be necessary to verify the efficiency of runs so that the generated data can be used for the analyses. The cDNA dilutions used for the standard curve were 1:10, 1:20, 1:40, 1:80, and 1:160. In each run of the given target gene, all mutant samples (TG-S01, TG-S02, TG-S03 and TG-S04) and the control sample (*E.coli* ATCC 8739) were used, and cDNA dilution factor for all the samples was determined as 1:40. Experiments were performed with 3 independent biological replicates, and each one consisted of 3 technical replicates.

In all experiments, efficiencies were 100% which were given under the standard curve graphs. In the amplification curves of the runs, the values were stated as RFU versus PCR cycles. The point where the fluorescence line interrupts threshold line is defined as Cq of the sample. Cq values obtained from the graphs were used in the analyses.

Standard curve and amplification plot for *cusB* are given in Figure 3.15 and Figure 3.16, respectively. Melt curve is given in Figure 3.17 and the melt peak is given in Figure 3.18.

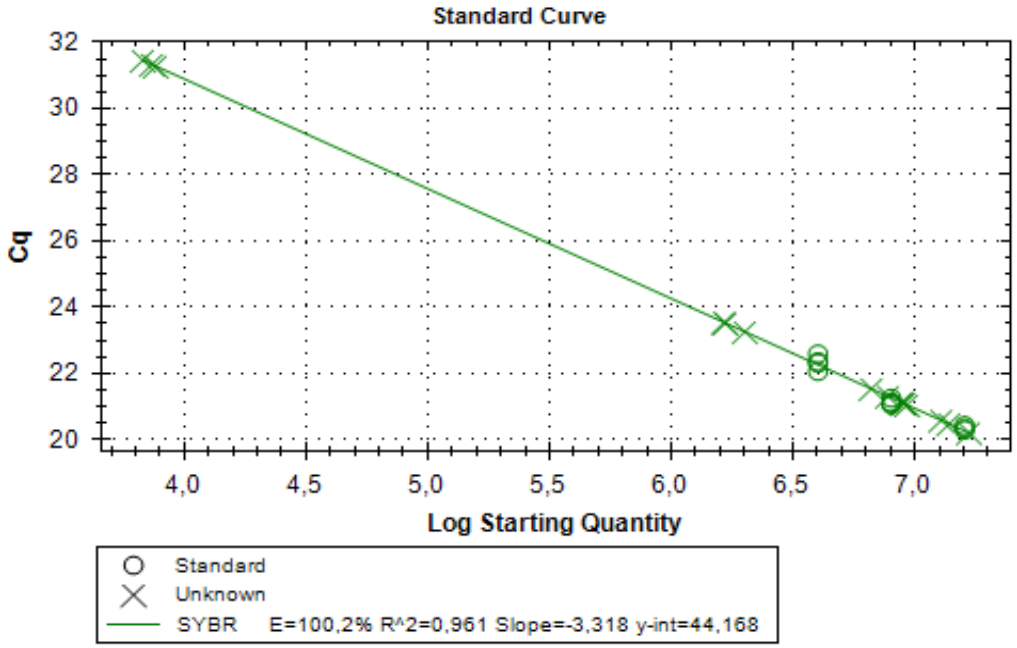

Figure 3.15. Standard curve for the *cusB* gene. Unknown samples (X) were spontaneous and UV-induced *E.coli* mutants and control sample; TG-S01, TG-S02, TG-S03, TG-S04 and *E.coli* ATCC 8739. E indicates the efficiency of the run.

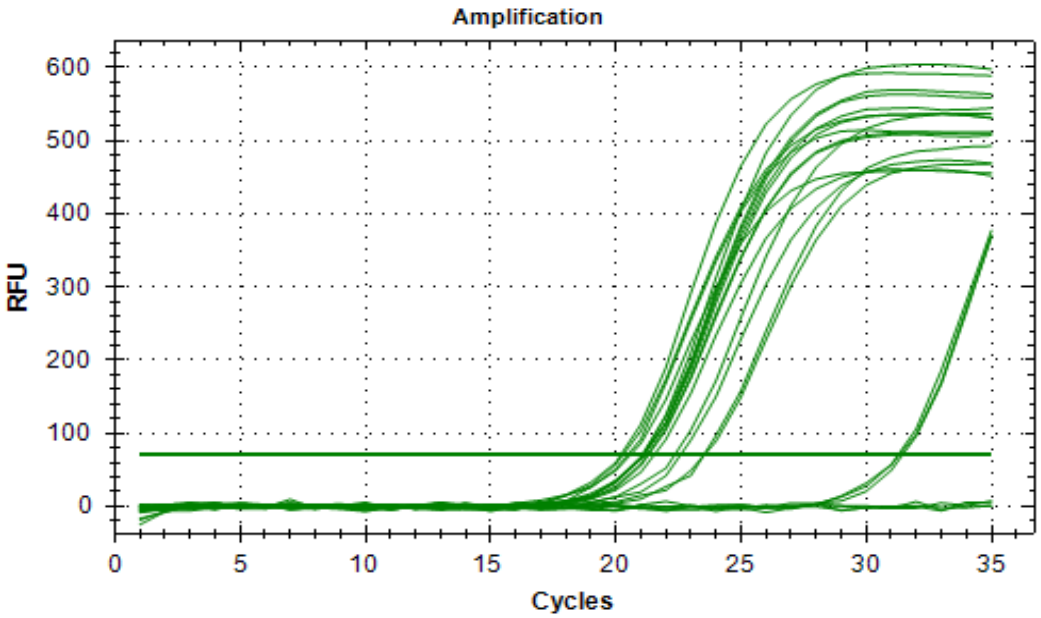

Figure 3.16. Amplification plot for the *cusB* gene.

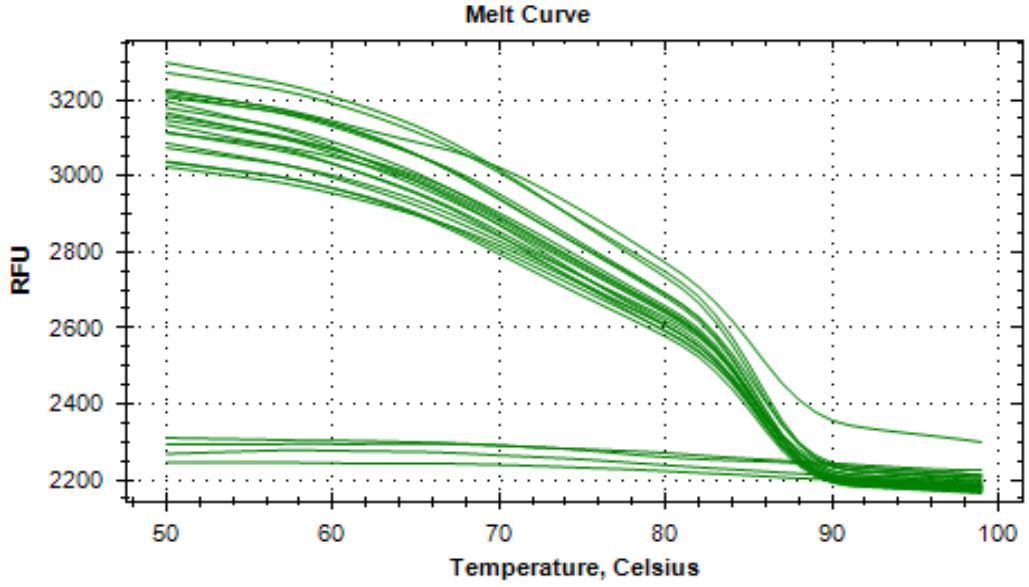

Figure 3.17. Melt curve for the *cusB* gene.

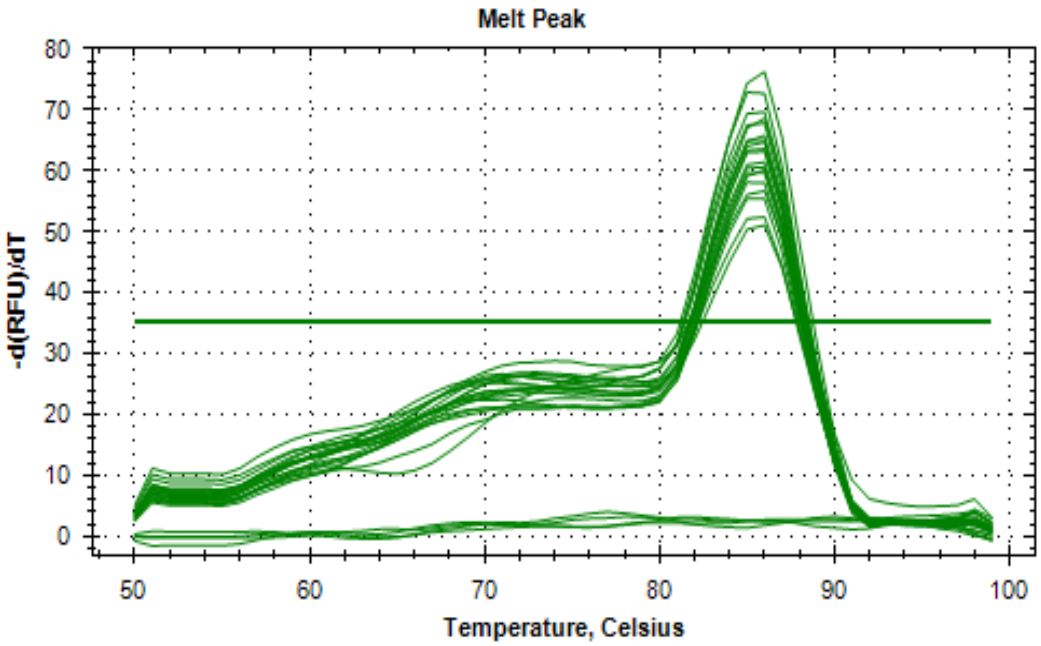

Figure 3.18. Melt peak for the *cusB* gene.

According to the standard curve of *cusB*, the efficiency of the run was 100.2 % and the slope of the curve was -3.318. In the melt peak, there was only one peak for each sample and the no-template controls were seen as a single line under the threshold line.

Standard curve and amplification plot for *cusF* are given in Figure 3.19 and Figure 3.20, while results of melt curve and melt peak are given in Figure 3.21 and Figure 3.22, respectively.

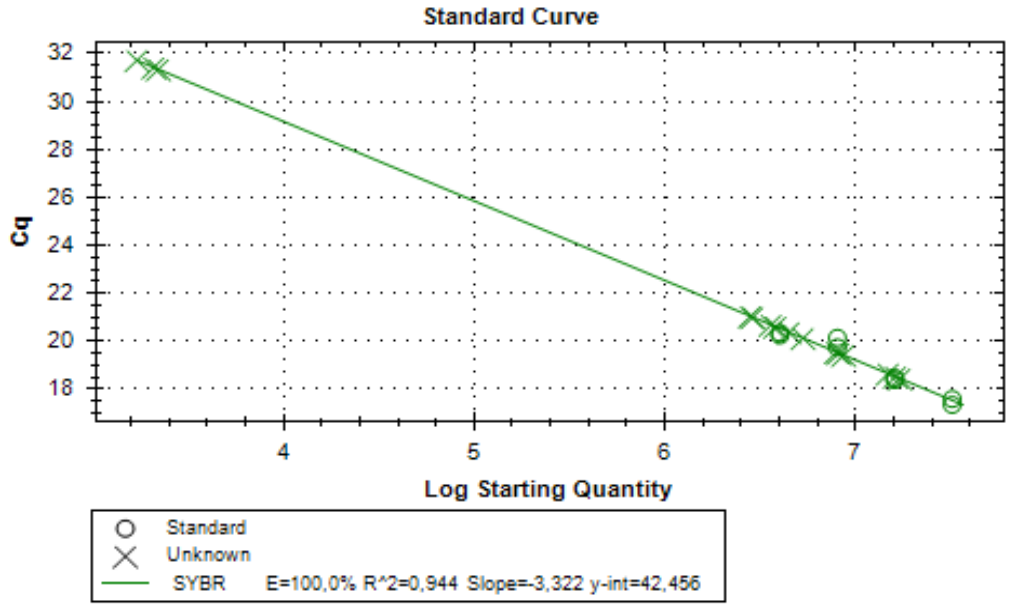

Figure 3.19. Standard curve for the *cusF* gene. Unknown samples (X) were spontaneous and UV-induced *E.coli* mutants and the control sample; TG-S01, TG-S02, TG-S03, TG-S04 and *E.coli* ATCC 8739. E indicates the efficiency of the run.

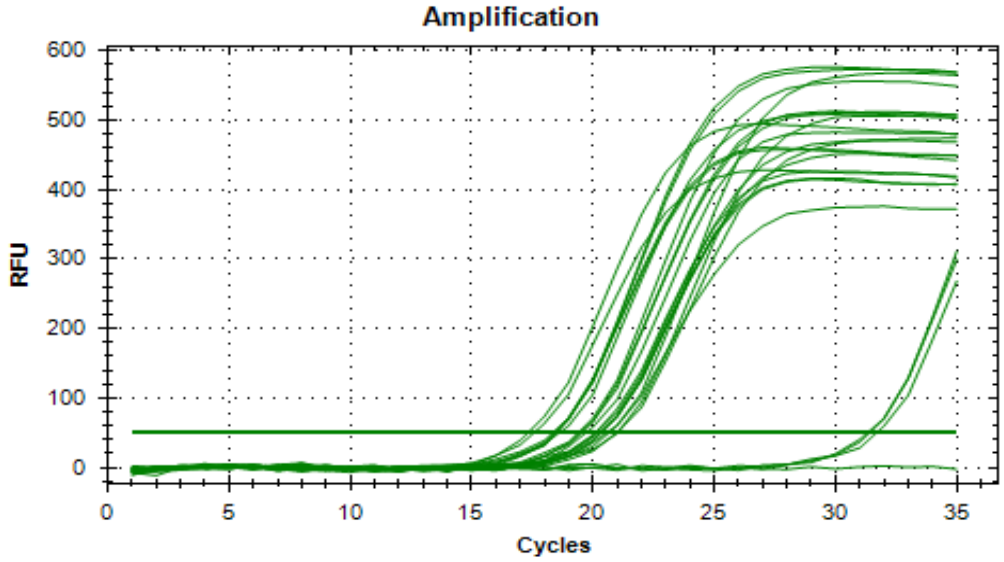

Figure 3.20. Amplification plot for the *cusF* gene.

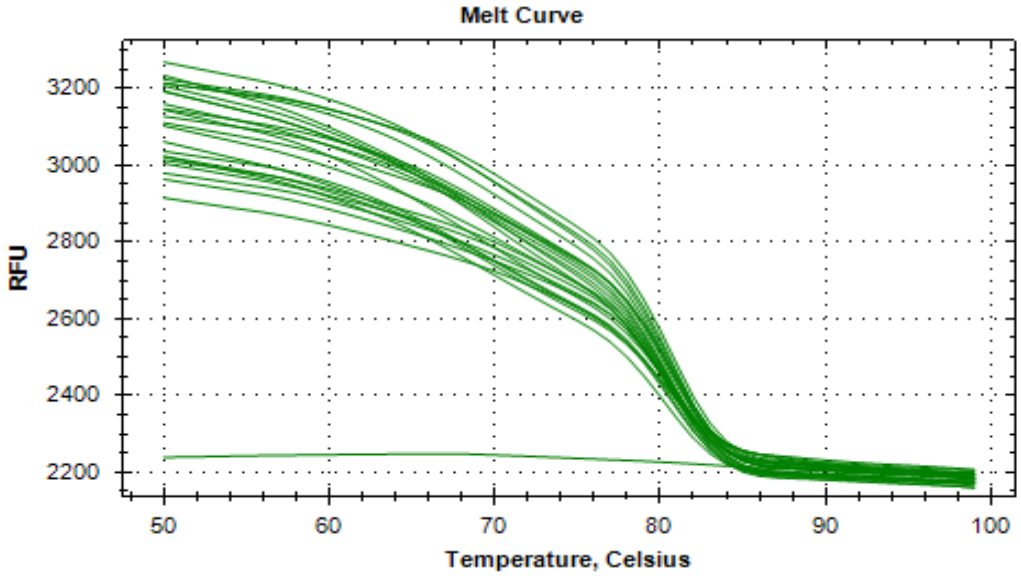

Figure 3.21. Melt curve for the *cusF* gene.

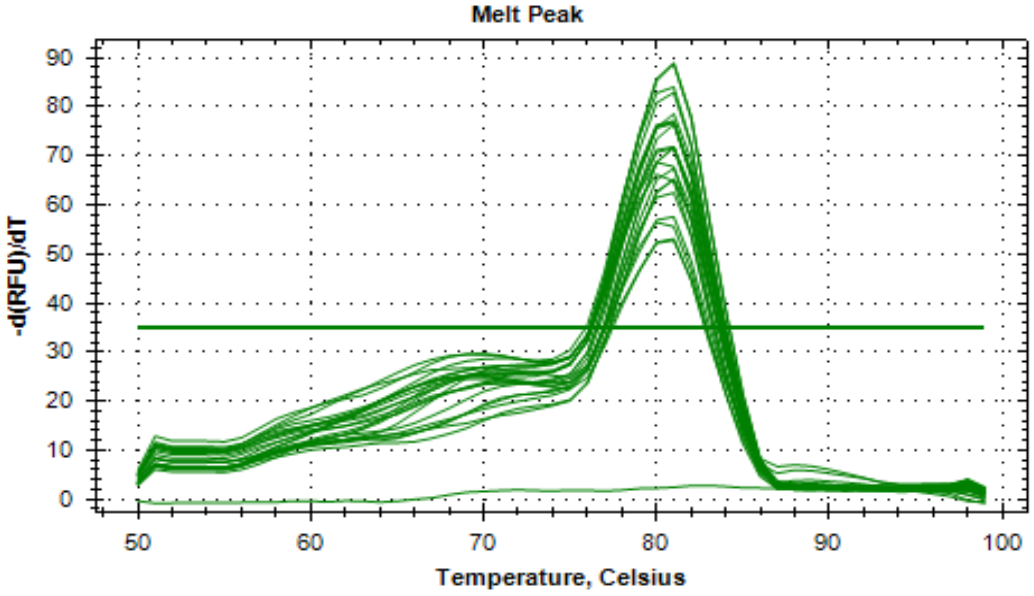

Figure 3.22. Melt peak for the *cusF* gene.

The efficiency of the reaction was exactly 100.0 % and the slope of the curve was -3.322. Only a single peak was observed for each sample in the melt peak. No-template controls were found under the threshold line as a single line.

Standard curve and amplification plot for *cusS* are given in Figure 3.23 and Figure 3.24. The graphs of the melt curve and melt peak are given in Figure 3.25 and Figure 3.26, respectively.

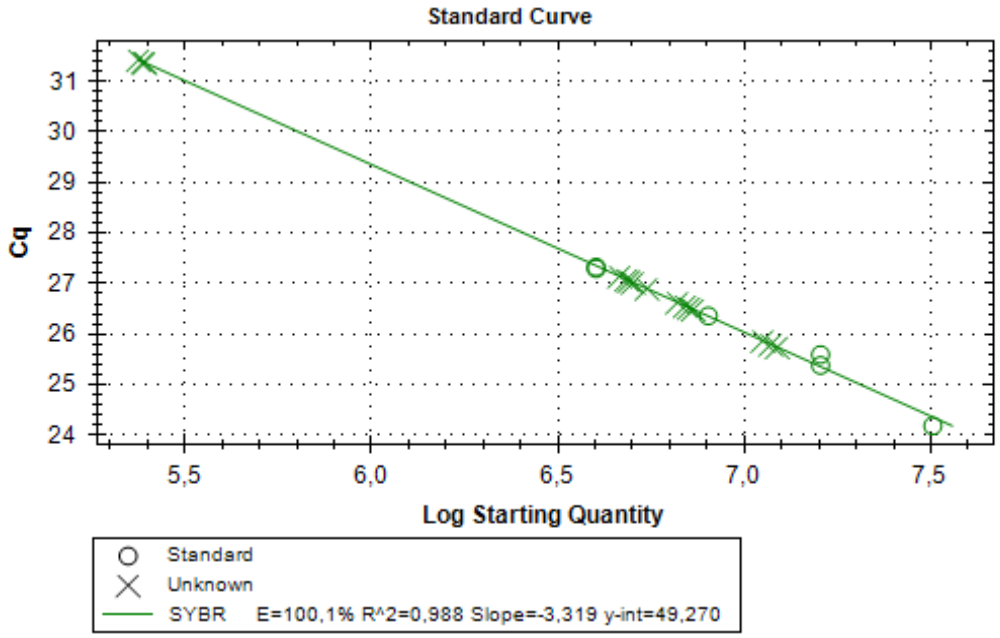

Figure 3.23. Standard curve for the *cusS* gene. Unknown samples (X) were spontaneous and UV-induced *E.coli* mutants and control sample; TG-S01, TG-S02, TG-S03, TG-S04 and *E.coli* ATCC 8739. E indicates the efficiency of the run.

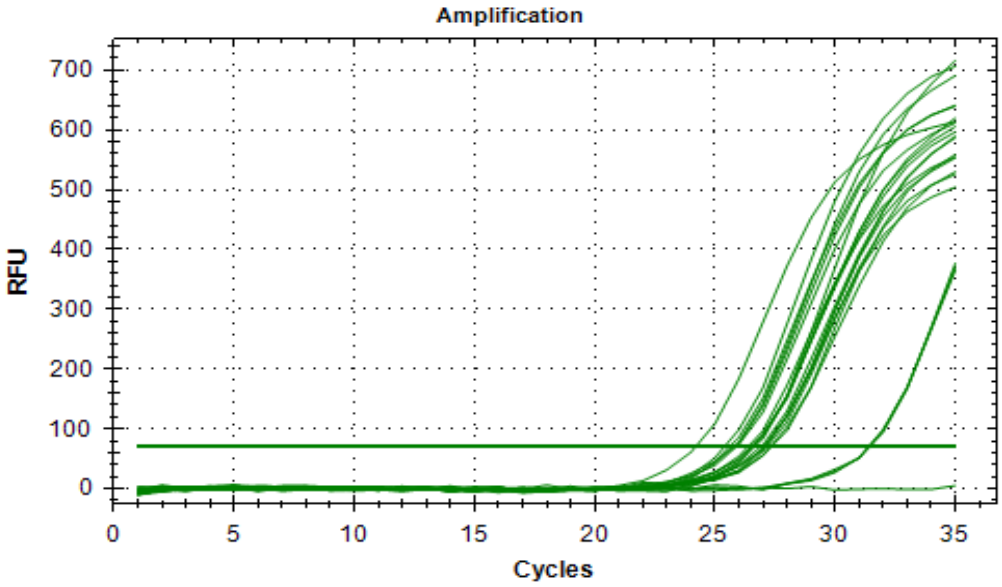

Figure 3.24. Amplification plot for the *cusS* gene.

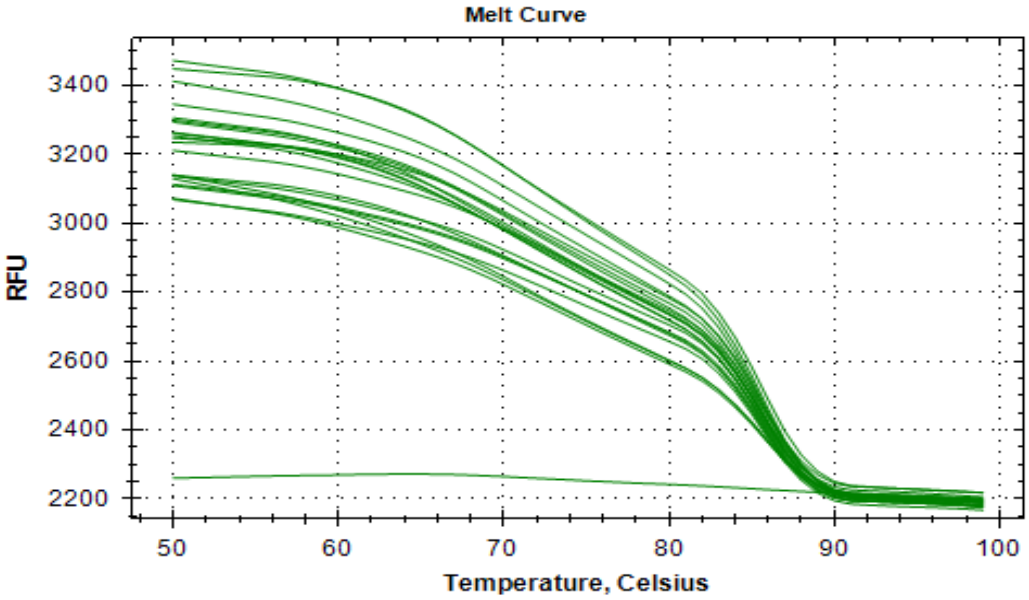

Figure 3.25. Melt curve for the *cusS* gene.

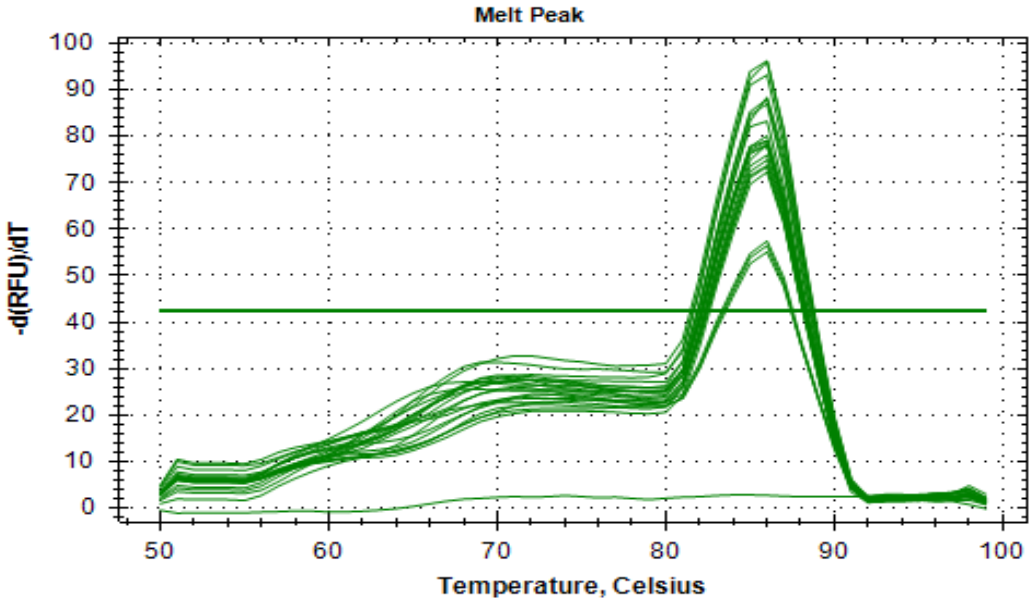

Figure 3.26. Melt peak for the *cusS* gene.

The efficiency of the reaction for *cusS* was 100.1% and the slope of the curve was -3.319. Only one peak was seen for each sample. No-template controls appeared as a single line under the threshold line.

Standard curve and amplification plot for *tus*, the reference gene, are given in Figure 3.27 and Figure 3.28. The melt curve and melt peak for *tus* are given in Figure 3.29 and Figure 3.30, respectively.

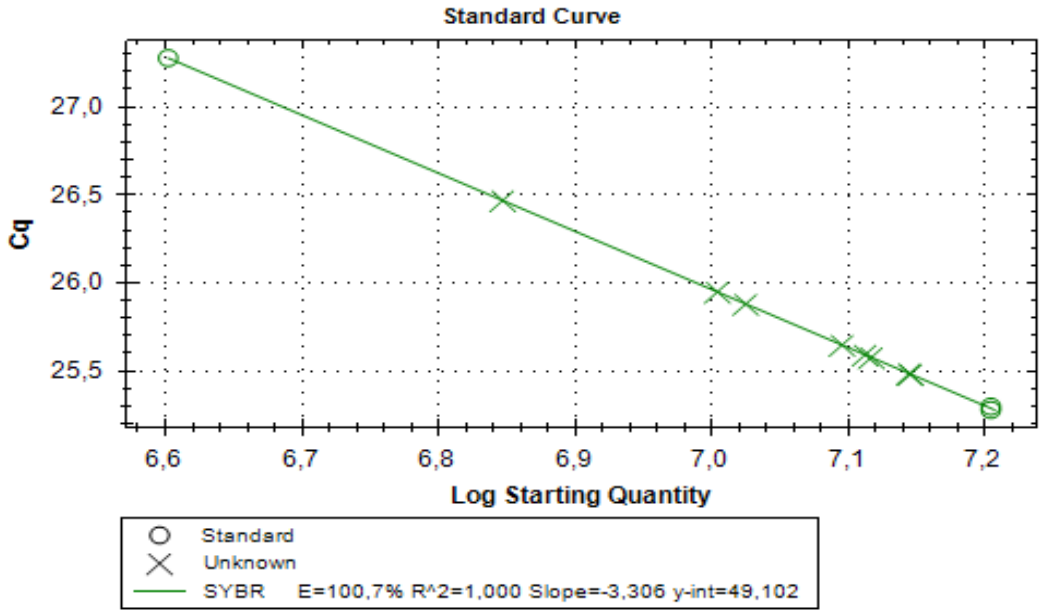

Figure 3.27. Standard curve for the *tus* gene. Unknown samples (X) were spontaneous and UV-induced *E.coli* mutants and control sample; TG-S01, TG-S02, TG-S03, TG-S04 and *E.coli* ATCC 8739. E indicates the efficiency of the run.

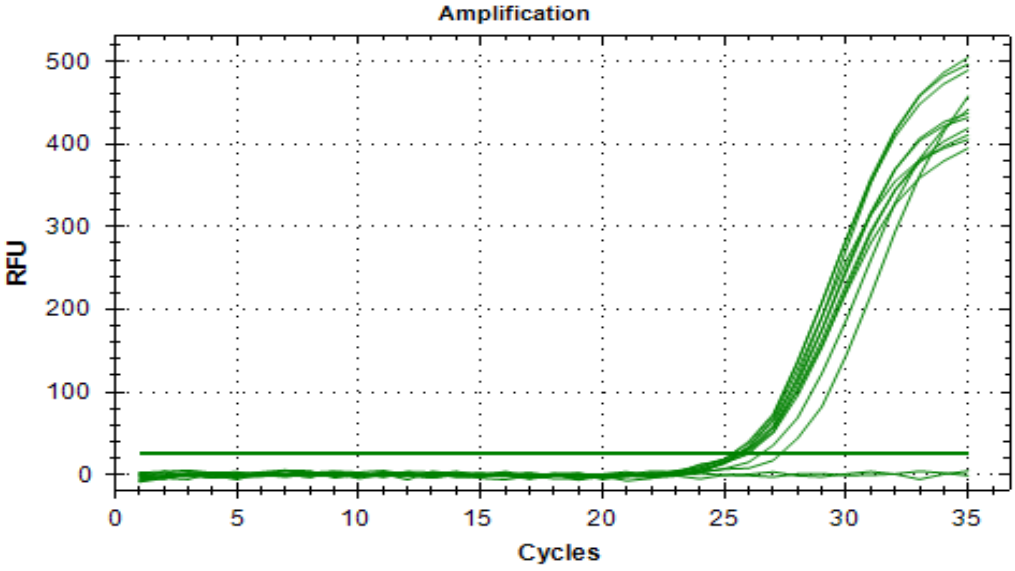

Figure 3.28. Amplification plot for the *tus* gene.

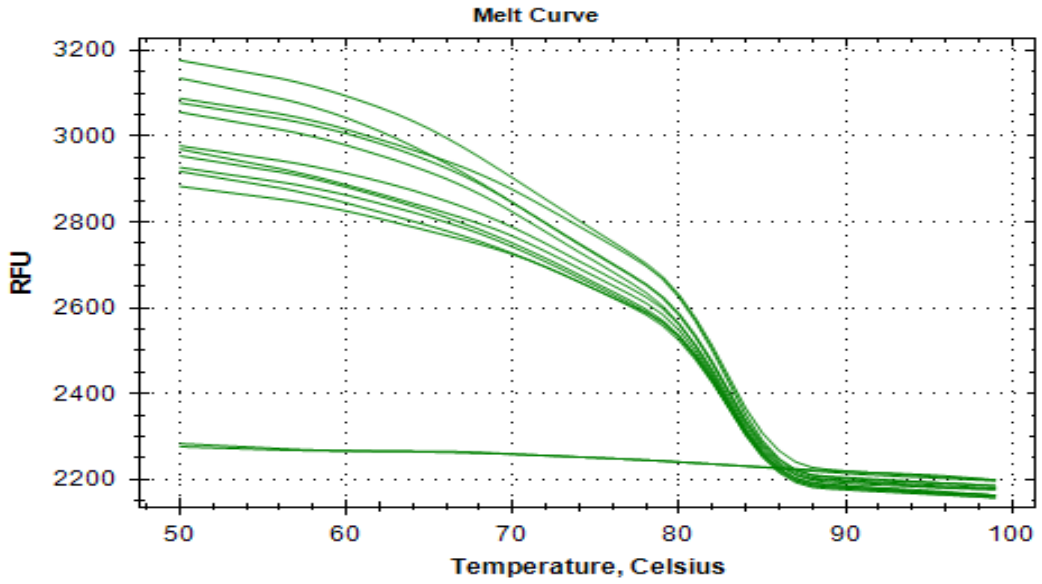

Figure 3.29. Melt curve for the *tus* gene.

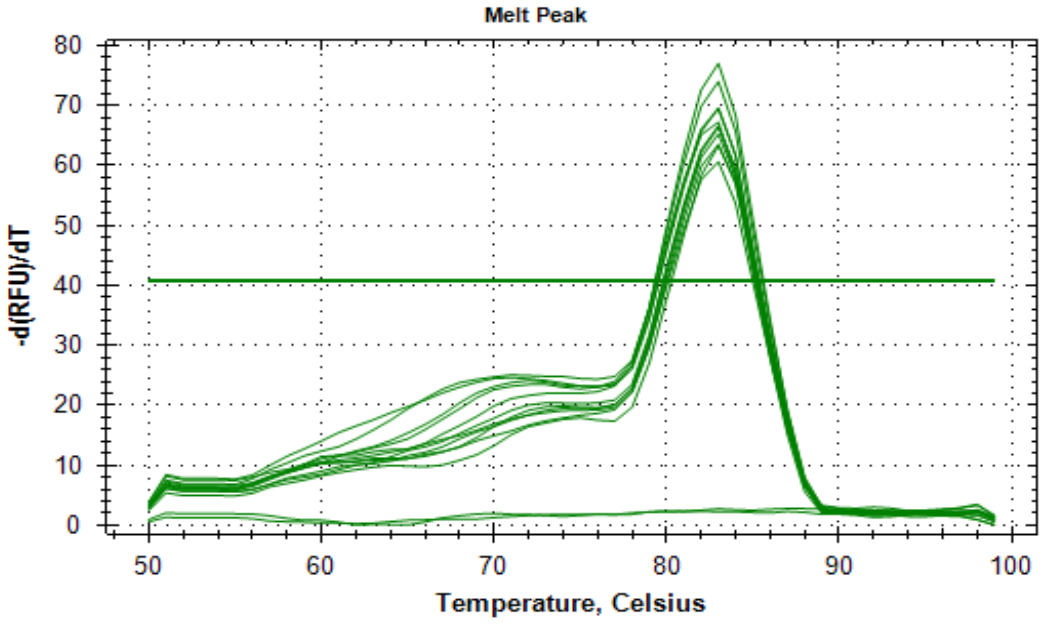

Figure 3.30. Melt peak for the *tus* gene.

The efficiency of the reaction for *tus* was 100.7% and the slope of the curve was -3.306. No-template controls appeared as a single line under the threshold line and in the melt peak, only one peak was observed for each sample.

In the expression analyses of the target genes (*cusB*, *cusF* and *cusS*) and the reference gene (*tus*), regarding the melt curves and melt peaks for all genes, for each run, only one single PCR product was amplified. There were no secondary peaks. It showed that there were no non-specific products, primer dimer formation, and contamination in the reactions. Moreover, no template control samples were seen as a single line indicating that there were no amplification of these samples. No-amplification certifies that primer dimer formation and nucleic acid contamination were not present in the runs. In addition, for all runs, reaction efficiencies were remarkably close to 100% with a slope of -3.32. Considering all the results, the runs of the each gene gave the optimum results and the amplification reactions were completed ideally at the end of the RT-qPCR procedure (Biosystems, 2013).

3.3.5.1. Relative Expressions of *cusB, cusF* and *cusS*

The relative expression levels of *cusB*, *cusF* and *cusS* genes in silver-resistant *E.coli* mutants were determined by using the Livak ($2^{-\Delta\Delta Cq}$) method. Since efficiencies of RT-qPCR experiments were found to be 100%, this method was chosen as the most appropriate method for the expression analysis. As a reference gene, *tus* was used and all strains were normalized according to the reference gene in the fold change analysis. For determining the expression fold changes, the baseline for the control strain (*E.coli* ATCC 8739) was set to 1 and the fold changes of the mutants were determined relative to the control strain. TG-S01 and TG-S03 represent spontaneous silver-resistant *E.coli* mutants, TG-S02 and TG-S04 represent UV-induced silver-resistant mutants. After relative expressions were determined, samples were compared among themselves by statistical analyses. One-way ANOVA with Tukey's multiple comparison post-test was performed.

Figure 3.31 represents the relative expression of *cusB* gene in spontaneous and UV-induced silver-resistant mutants.

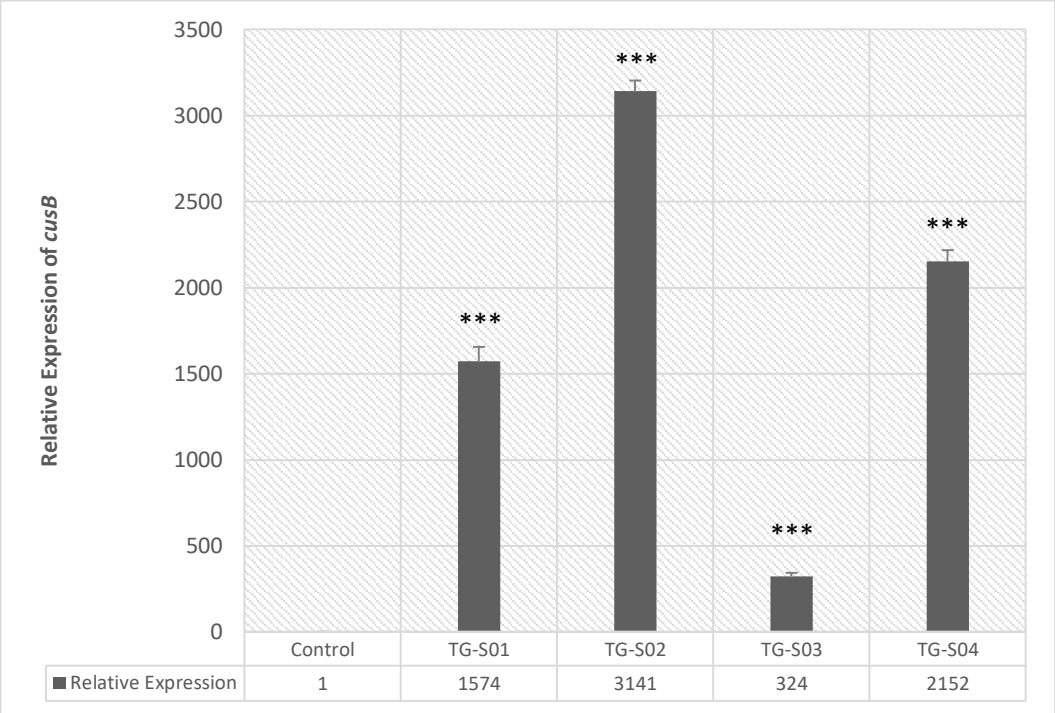

Figure 3.31. Relative expression of *cusB* gene. Fold changes in *cusB* expression are given at the bottom of the bar chart. Relative expression was calculated in reference to *tus.* Expression of the control sample (*E.coli* ATCC 8739) was set to 1. Error bars represent standard error of mean (SEM) of the samples. TG-S01 and TG-S03 are spontaneous silver-resistant *E.coli* mutants; TG-S02 and TG-S04 are UV-induced *E.coli* mutants. $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***).

Results of one-way ANOVA with Tukey's multiple comparison post-test for *cusB* gene is given in Table 3.4.

Table 3.4. *One-way ANOVA with Tukey's multiple comparison post-test for cusB. Ns: No statistical significance. p ≤ 0.05 (*), p ≤ 0.01 (**), p ≤ 0.001 (***). Control: E.coli ATCC 8739; TG-S01, TG-S02, TG-S03, TG-S04: Silver resistant mutants.*

Samples Compared	Significance	p Value
Control vs. TG-S01	***	<0.0001
Control vs. TG-S02	***	<0.0001
Control vs. TG-S03	***	<0.0001
Control vs. TG-S04	***	<0.0001
TG-S01 vs. TG-S02	ns	0.1470
TG-S01 vs. TG-S03	ns	0.9940
TG-S01 vs. TG-S04	ns	0.1704
TG-S02 vs. TG-S03	ns	0.0810
TG-S02 vs. TG-S04	ns	>0.9999
TG-S03 vs. TG-S04	ns	0.0944

The relative expression results showed that in all mutants, TG-S01, TG-S02, TG-S03, and TG-S04, the *cusB* expression increased relative to the control strain *E.coli* ATCC 8739. Although the relative expression of TG-S03 was less than the other mutants in terms of the expression fold change, it was also significant compared to the control. All mutants (spontaneous and UV-induced ones) gave a significant increase in *cusB* expression compared to the control strain. There were no statistical significance in the expression fold changes among mutant samples (Table 3.4).

In the previous studies dealing with the expression of the *cusB* gene, it was found that the gene expression increases in the silver-resistant *E.coli* strain when it was exposed to silver ions (Delmar et al., 2014; Chun Nam Lok et al., 2008; Xue et al., 2008). Therefore, findings of this study are in accordance with the literature about over-expression of *cusB* in silver-resistant strains.

Figure 3.32 represents the relative expression of *cusF* gene in silver-resistant *E.coli* mutants.

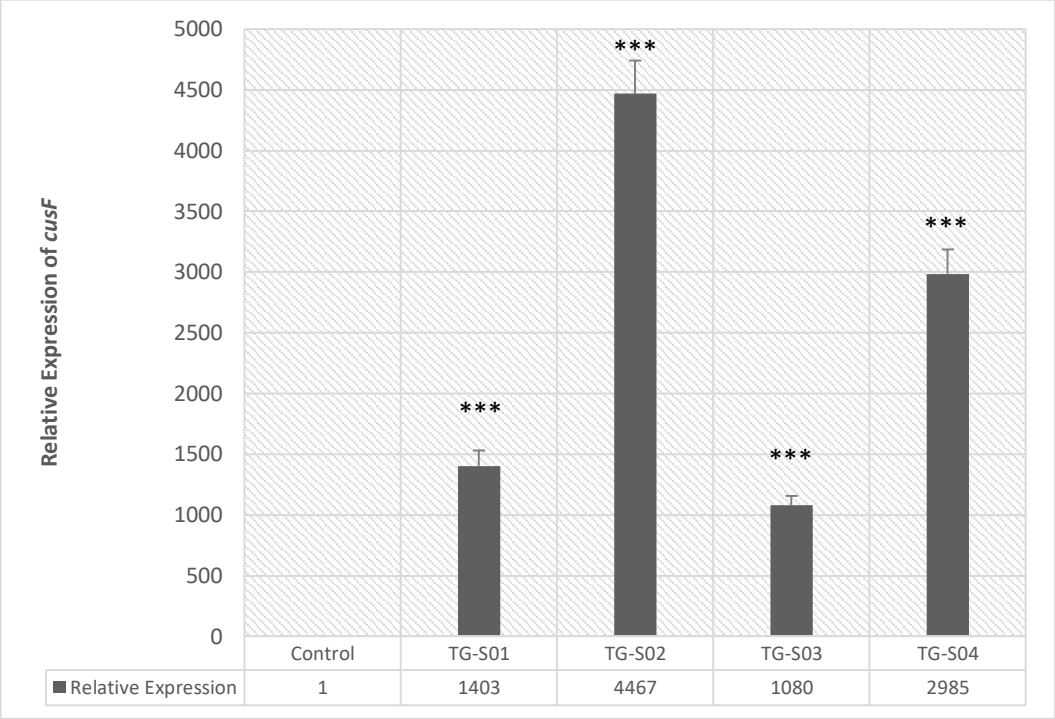

Figure 3.32. Relative expression of *cusF* gene. Fold changes in *cusF* expression are given at the bottom of the bar chart. Relative expression was calculated in reference to *tus.* Expression of the control sample (*E.coli* ATCC 8739) was set to 1. Error bars represent standard error of mean (SEM) of the samples. TG-S01 and TG-S03 are spontaneous silver-resistant *E.coli* mutants; TG-S02 and TG-S04 are UV-induced *E.coli* mutants. $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***).

Results of one-way ANOVA with Tukey's multiple comparison post-test for *cusF* is given in Table 3.5.

Table 3.5. *One-way ANOVA with Tukey's multiple comparison post-test for cusF. Ns: No statistical significance. p ≤ 0.05 (*), p ≤ 0.01 (**), p ≤ 0.001 (***). Control: E.coli ATCC 8739; TG-S01, TG-S02, TG-S03, TG-S04: Silver resistant mutants.*

Samples Compared	Significance	P Value
Control vs. TG-S01	***	<0.0001
Control vs. TG-S02	***	<0.0001
Control vs. TG-S03	***	<0.0001
Control vs. TG-S04	***	<0.0001
TG-S01 vs. TG-S02	ns	0.1949
TG-S01 vs. TG-S03	ns	0.9568
TG-S01 vs. TG-S04	ns	0.6439
TG-S02 vs. TG-S03	ns	0.0705
TG-S02 vs. TG-S04	ns	0.8515
TG-S03 vs. TG-S04	ns	0.3015

According to relative expression results of *cusF* gene, mutants TG-S01, TG-S02, TG-S03, and TG-S04 showed an increase in the gene expression. Although expression fold changes of spontaneous mutants (TG-S01:1269; TG-S03:1090) were less than the UV-induced mutants (TG-S02:4270; TG-S04:3304), statistical analysis showed that *cusF* expression in all mutants increased significantly compared to the control strain. However, there was no significant difference between spontaneous (TG-S01 and TG-S03) and UV-induced (TG-S02 and TG-S04) mutants when they were compared among themselves (Table 3.5).

According to the previous studies, it was found that *cusF* (a component of cusCFBA efflux transporter) was over-expressed in the silver-resistant strains (Delmar et al., 2014; Chun Nam Lok et al., 2008; Xue et al., 2008). In addition, deletion of *cusF* in a silver-resistant *E.coli* strain caused the loss of silver resistance (Chun Nam Lok et al., 2008).

As mentioned before, *cusB* and *cusF* are found in the same operon and therefore, they are elements of a polycistronic mRNA. Interestingly, by looking at the expression fold increases of *cusB* and *cusF*, it was seen that *cusF* expression was higher than that of *cusB* eventhough they are co-transcribed. According to the studies carried out on *E.coli*, expression of a specific gene which is found in an operon increases depending on the operon length and the position of the gene in the operon. As the gene is found farther from the operon end, the expression of the gene increases. This term is called 'Transcription distance', which stems from the situation that more time remains for the gene translation at the beginning of the operon during transcription (Lim et al., 2011).

Figure 3.33 represents the relative expression of *cusS* gene for silver-resistant mutants.

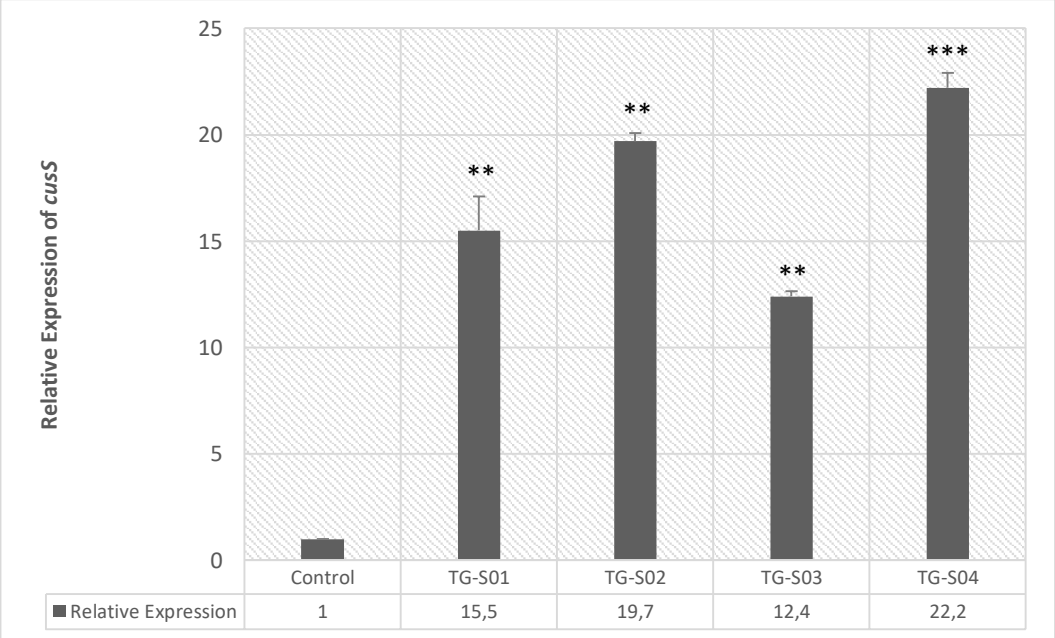

Figure 3.33. Relative expression of *cusS* gene. Fold changes in *cusS* expression are given at the bottom of the bar chart. Relative expression was calculated in reference to *tus*. Expression of the control sample (*E.coli* ATCC 8739) was set to 1. Error bars represent standard error of mean (SEM) of the samples. TG-S01 and TG-S03 are spontaneous silver-resistant *E.coli* mutants; TG-S02 and TG-S04 are UV-induced *E.coli* mutants. $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***).

Results of one-way ANOVA with Tukey's multiple comparison post-test is given in Table 3.6 for *cusS*.

Table 3.6. *One-way ANOVA with Tukey's multiple comparison post-test for cusS. Ns: No statistical significance. p ≤ 0.05 (*), p ≤ 0.01 (**), p ≤ 0.001 (***). Control: E.coli ATCC 8739; TG-S01, TG-S02, TG-S03, TG-S04: Silver resistant mutants.*

Samples Compared	Significance	P Value
Control vs. TG-S01	**	0.0068
Control vs. TG-S02	**	0.0024
Control vs. TG-S03	**	0.0021
Control vs. TG-S04	***	0.0008
TG-S01 vs. TG-S02	ns	0.9448
TG-S01 vs. TG-S03	ns	0.9156
TG-S01 vs. TG-S04	ns	0.5475
TG-S02 vs. TG-S03	ns	>0.9999
TG-S02 vs. TG-S04	ns	0.9127
TG-S03 vs. TG-S04	ns	0.9426

As it is seen in Figure 3.33, all mutants showed an increase in the *cusS* gene expression compared to the control strain. According to the statistical analysis (Table 3.6), the increases in *cusS* expression in the mutant cells were significant compared to the control. However, when mutants were compared among themselves, no statistical significance was indicated in their *cusS* expression levels (Table 3.6).

In the literature, it was reported that cusR/S two-component system is responsible for the regulation of *cusCFBA* transcription. The *cusS* becomes prominent in the case of silver stress (Franke S et al., 2001b; Munson et al., 2000). In the research carried out on *E.coli*, more than 2-fold increase in the transcription of *cusS* was observed when

cells were exposed to silver ions (Yamamoto & Ishihama, 2005). Our findings agree with the findings in their research, in which from 12 to 23-fold expression increase was observed.

Based on our results, gene expression levels of all target genes increased significantly in all mutant samples compared to the control samples. However, when mutants were compared among themselves, there was no significant change in gene expressions although 2 of the mutants were spontaneous (TG-S01 and TG-S03) and the others were UV-induced (TG-S02 and TG-S04). As a result, it can be concluded that the way that mutations occurred did not have any remarkable effect on the expressions of the important transport system proteins.

CHAPTER 4

CONCLUSION

In this study, the relative gene expression levels of transport system proteins were determined in spontaneous and UV-induced *E.coli* mutants using RT-qPCR method.

Minimal inhibitory concentration of silver nitrate for *E.coli* ATCC 8739 strain was determined as 41 μg/ml by agar dilution method. Then, silver-resistant *E.coli* mutants were obtained spontaneously or by UV-induced mutagenesis, growing above the determined MIC value. In all investigated genes of transport system proteins, namely *cusB, cusF,* and *cusS,* gene expressions showed an increase in silver-resistant *E.coli* mutants compared to the control strain *E.coli* ATCC 8739 at a high silver nitrate concentration (111 μg/ml). Therefore, it is concluded that examined transport proteins, which are the elements of the cusCFBA efflux pump, were considered vital for the efflux of silver ions from the bacterial cells. All expression level fold increases were statistically significant. When spontaneous silver resistant mutants were compared to the UV-induced ones, there was no significant difference. This means that the way in which mutagenesis was occurred giving rise to silver resistant mutants (by UV-induction or spontaneously) did not affect the gene expression levels of transport system proteins. Our study indicated that *tus* gene can be used as a reference gene in RT-qPCR studies which were conducted by using laboratory strain ATCC 8739 due to its stability and consistent expression levels under high silver concentration. It is likely that in any metal stress studies, *tus* can be used as a reference constitutively expressed gene. The importance of this study arises from the uniqueness of discovering spontaneous mutants in the silver-resistance studies for the first time, and using *tus* gene as a housekeeping gene in RT-qPCR experiments at high silver concentration-driven stress conditions.

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APPENDICES

A. EQUIPMENTS

Table A.1. *List of equipments and suppliers of the equipments used in the study*

EQUIPMENT	SUPPLIER OF THE EQUIPMENT
Incubator	Binder, Germany
Shaker Incubator	Zhicheng, China
Magnetic Stirrer	Velp Scientifica, Italy
Autoclave	Nuve, Turkey
pH Meter	Jenco, USA
Vortex	Velp Scientifica, Italy
Microcentrifuge	Sigma, Germany
Class II Biological Safety Cabinet	ESCO, USA
UV-Spectrophotometer	SOIF, China
Biodrop	Biodrop, UK
Heat-block	Bioer, China
Gel Electrophoresis System	Biometra, Germany
Shortwave UV Transilluminator	UVP, Canada
PCR Machine	Thermo Scientific, USA
RT-qPCR Machine	Bio-RAD, USA
PCR Cabinet	N-Biotek, Korea

B. SOLUTIONS AND BUFFERS

TAE Buffer (50X)

- Tris-base 242 g.
- Glacial acetic acid 57.1 ml.
- EDTA (0.5 M, pH 8.0) 100 ml.
- Distilled water up to 1000 ml.

DNA Loading Dye (6X)

- Tris-HCl (pH 7.6) 10 mM
- Bromophenol Blue (w/v) 0.03 %
- Xylene cyanol FF (w/v) 0.03 %
- Glycerol (w/v) 60 %
- EDTA 60 mM

GTE Mix

- Glucose 50 mM
- EDTA 10 mM
- Tris-HCl 25 mM
- RNase 0.1 mg/ml

pH is adjusted to 8.0.

TE Buffer

- Tris-HCl 10 mM
- EDTA 1 mM

Final pH is adjusted to 8.0.

50µg/ml Silver-nitrate Stock Solution

- Silver nitrate 2.5 g
- Distilled Water up to 50 ml

After dissolving silver nitrate in distilled water completely, solution is filter sterilized with 0.22 µm filters (Pall, USA).

C. COMPOSITION AND PREPARATION OF CULTURE MEDIA

Nutrient Agar:

- Meat Extract 3.0 g
- Peptone from meat 5.0 g
- Agar 15.0 g
- Distilled Water up to 1000 ml

Final pH is adjusted to 7.0 (+/- 0.2). Medium is sterilized at 121℃ for 20 minutes.

Nutrient Broth:

- Meat Extract 3.0 g
- Peptone from meat 5.0 g
- Distilled Water up to 1000 ml

Final pH is adjusted to 7.0 (+/- 0.2). Medium is sterilized at 121℃ for 20 minutes.

111 µg/ml Silver-nitrate containing Nutrient Agar:

- Meat Extract 3.0 g
- Peptone from meat 5.0 g
- Agar 15.0 g
- Distilled Water up to 1000 ml

Final pH is adjusted to 7.0 (+/- 0.2). Medium is sterilized at 121℃ for 20 minutes. After cooling, 111 µg/ml silver nitrate is added to the media before solidifying of agar.

D. SUPPLIERS OF CHEMICALS, KITS AND ENZYMES

Chemicals

Agar	Sigma
Agarose	Sigma
DMSO	ThermoFisher Scientific
EDTA	Sigma
Ethanol (Molcular Biology Grade)	Sigma
Ethidium Bromide	AppliChem
Glacial Acetic Acid	Merck
Glycerol	Fluka
HCl	Merck
NaCl	Sigma
NaOH	Merck
Nutrient Broth	Merck
Silver-nitrate	Merck
Trizma Base	Sigma
Tris-HCl	Fluka
Water (Molecular Biology Grade)	Fisher BioReagents
2-mercaptoethanol	Aldrich

Kits

DNase I Kit, RNase-free	Thermo Fisher
GeneJet RNA Purification Kit	Thermo Scientific
RevertAid First Strand cDNA Synthesis Kit	Thermo Fisher
SsoAdvanced Universal SYBR Green Supermix	Bio-Rad
Taq DNA Polymerase 2X Master Mix	Ampliqon

Enzymes

RNase A Solution	5Prime

E. DNA LADDER

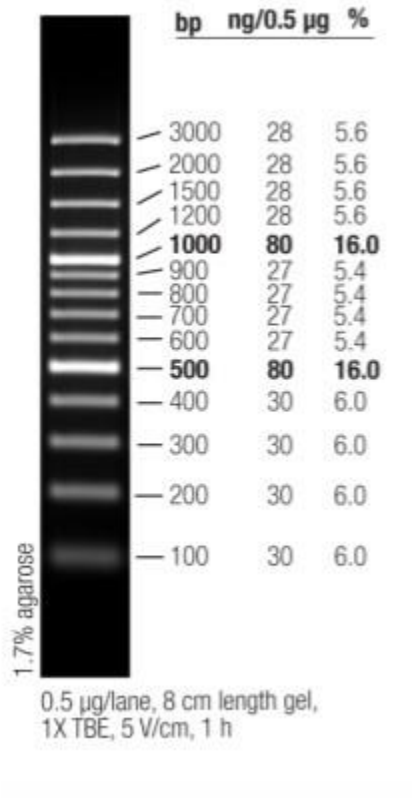

Figure E.1. DNA Ladder. GeneRuler 100 bp Plus (Thermo Scientific #SM0321)

F. RNA ISOLATION PROCEDURE

- Before starting RNA isolation, recommended amount of molecular grade ethanol was added to Wash Buffer 1 and Wash Buffer 2 (Ethanol 96-100%).

- In order to prepare wash buffers, ethanol amount added was indicated below;

Table F.1. *Preparation of wash buffers*

	Concentrated Amount	**Ethanol Added**	**Total Amount**
Wash Buffer 1	40 ml	10 ml	**50 ml**
Wash Buffer 2	23 ml	39 ml	**62 ml**

- For Lysis Buffer preparation, 20 µl of 14.3 M β-mercaptoethanol was added to 1 ml of Lysis Buffer to be used in the procedure. This step was carried out before each RNA purification and prepared freshly.

- TE Buffer (10 mM Tris HCl, pH 8.0, 1 mM EDTA) was prepared by adding lysozyme in a way to adjust the final concentration to 0.4mg/ml.

RNA Isolation Procedure

- At the exponential phase of bacterial growth, cells were collected.
- Into a 1.5 ml microcentrifuge tube, 1 x 10^9 cells (approximately 1.5 ml of bacterial culture) were placed and centrifuged for 2 min at ≥12000 x g. After centrifugation, supernatant was disposed to obtain a pellet as dry as possible.
- In a 100 µL TE Buffer which was freshly prepared with lysozyme (0.4mg/ml), the pellet was resuspended and tubes were mixed by inverting several times.
- Cells resuspended at TE Buffer were left in incubation at 15-25°C for 5 min.
- In each sample tube, 300 µl Lysis Buffer prepared with appropriate amount of β-mercaptoethanol were added and each tube was vortexed for 15 sec for homogenization.
- Into each sample tube, 180 µL of ethanol (96-100%) were added and pipetting was done for mixing.
- GeneJET RNA Purification Column was inserted into a collection tube and lysate, up to 700 µl, was transferred to it. Then, each column was centrifuged for 1 min at ≥12000 x g and flowthrough was disposed and purification column was put back in the collection tube.
- Into GeneJET RNA Purification Column, 600 µl of ethanol added Wash Buffer 2 were added and 1 min centrifugation was done at ≥12000 x g. Flowthrough was disposed and purification column was put back in the collection tube.
- Into GeneJET RNA Purification Column, 250 µl of Wash Buffer 2 was added and column was centrifuged at ≥12000 x g for 2 min. Then, collection tube was discarded with the flowthrough solution and into a sterile 1.5 mL RNase-free microcentrifuge tube, GeneJET RNA Purification Column was placed.
- To the center of GeneJET RNA Purification Column membrane, 100 µl of nuclease-free, molecular grade water was added and column was centrifuged at ≥12000 x g for 1 min. At the end of the procedure, RNA will be eluted.
- GeneJET RNA Purification Column was discarded.

G. DNaseI TREATMENT PROTOCOL

- RNA, reaction buffer and DNase enzyme were added into an RNase –free tube with the given order;

  RNA……...…….….1 µg

  10X Reaction Buffer with $MgCl_2$…….1 µl

  DNase I, RNase-free…….1 µl (1U)

  RNase-DNase-free Water……....complete to 10 µl

- Then the tube was incubated at 37 °C for 30 min.

- 1 µL of EDTA (50 mM) was added and incubated for 10 min at 65 °C.

H. cDNA SYNTHESIS PROTOCOL

- Reagents provided by the kit were added into a nuclease free tube in the order given in the table;

Table H.1. *Preparation of cDNA synthesis mixture*

Template RNA	0.1 ng - 5 µg
Primer	1 µl
Nuclease-free Water	Completed to 12 µl
5X Reaction Buffer	4 µl
RiboLock RNase Inhibitor (20U/µl)	1 µl
10 mM dNTP Mix	2 µl
RevertAid M-MuLV RT (200 U/µl)	1 µl

- Tubes were mixed gently and centrifuged.
- Then, tubes were incubated for 5 min at 25°C and for 1 hour at 42°C.
- At the final step, tubes were placed at 70°C for 5 min to terminate the reaction.

I. BOILING METHOD

- The selected mutant's cells were incubated on agar plates overnight at 37°C.
- Cells were collected and dissolved in the GTE Mix solution (1 ml) which was prepared by adding RNase.
- After dissolving the cells in the GTE mix by vortexing, eppendorf containing the mixture was placed in the heat block for 10 minutes at 100°C.
- The cells were centrifuged for 5 minutes at 1000 rpm.
- The extracted DNA was found in the supernatant.