CHAPTER 5:

Antibiotic resistance in biofilm

5.1 Introduction

Persister cells (PC) or persisters are a dormant subpopulation of bacterial cells that are transient antibiotic-resistant phenotype. These phenotypes are non-growing which lack transcription, translation, and proton motive force (PMF) (Hobby et al. 1942; Bigger 1944; Kwan et al. 2013). PC cell formation has been observed in *P. aeruginosa.* It is an opportunistic pathogen often associated with UTIs, CF patients, and many more. Another feature of *P. aeruginosa* is the ability to form biofilm on medical devices which makes it difficult to treat. As biofilm protects the bacterial cells from the host defense and antibiotic therapy makes it more challenging to treat biofilm infection without mechanical dispersion (Mulcahy et al. 2010; Høiby et al. 2015). Persister subpopulation that would have existed before antibiotic administration may be revealed by first eliminating the susceptible bacteria and then scaling up the metabolic pathways of persistence (Balaban et al. 2004). Major *in vitro* studies have highlighted the PC formation in *E. coli.*

Although elements of PC formation are being uncovered, the mechanism underlying remains unknown in *P. aeruginosa.* It has been demonstrated that on disruption of numerous global regulators [(p)ppGpp synthesis SpoT and RelA, RNA polymerase-binding transcription factor DksA, and alternative sigma factors RpoS and RpoN], the putative D - Nacetylase DnpA and the carbamoyl phosphate synthetase CarB, affects PC formation (Murakami et al. 2005; Viducic et al. 2006; Liebens et al. 2014; Viducic et al. 2017; Cameron et al. 2018). Recently, Baek et al., 2022 showed upregulation of *relA* in the treatment of antibiotics: ciprofloxacin, cefepime, colistin, and amikacin in the planktonic stage (Baek et al. 2020). Further, the toxin-antitoxin (TA) systems are emerging mechanisms involved in PC formation. In *E.coli,* it is known Lon protease degrades anti-toxin, resulting in the activation of toxin which inhibits cellular processes leading to PC formation (Harms et al. 2016). However, in *P. aeruginosa* aminoglycosides enhance Lon protease activity, which is required for virulence, motility, and biofilm formation (Marr et al. 2007). Persistence is increased in *P. aeruginosa* biofilm (Viducic et al. 2006; Soares et al. 2020). TA systems identified in *P. aeruginosa* include RelBE*,* HicBA, HigBA, ParDE (G. Li et al. 2016; Coskun et al. 2018; Muthuramalingam et al. 2019). The well-known TA system HigBA affects virulence factors such as pyochelin, pyocyanin, swarming motility, and biofilm formation. (Wood and Wood 2016). Further, HigB inhibits the biofilm formation by decreasing the c-di-GMP level by activating the c-di-GMP hydrolysis genes which in turn increases gene expression of T3SS (Zhang et al. 2018). Apart from this, ciprofloxacin treatment leads to PC formation in the biofilm stage through activation of

stringent response (RelA, SpoT) and TA system (HigBA) (Soares et al. 2019). Similarly to this, the TA system ParD/ParE becomes active after exposure to antibiotics that block DNA gyrase (Muthuramalingam et al. 2019). Major studies on PC formation are done for typed-strain PAO1 and PA14. And the reports on clinical strains infecting CF patients are more. Also, there is a lack of information regarding PC formation in biofilm at supra-MIC concentrations of antibiotics. This study includes PC formation in planktonic as well as biofilm stages against three antibiotics: ceftazidime, gentamicin, and ceftazidime.

Another aspect of this study was biofilm-mediated resistance through the expression of transcriptional regulator *brlR* specific to biofilm mode (Liao and Sauer 2012). A recent review has highlighted about new orphan senor SagS and its importance in biofilm development (Park and Sauer 2021). Interesting part of these two components (TCs) SagS is involved in unusual/branched pathways involving several pathways through multistep phosphorelays, also known as "one-to-many" and "many-to-one" phosphotransfer interactions, which is an intriguing feature of orphan TCSs (Laub and Goulian 2007). Resulting in their involvement in cross-communication to alter gene expression and physiological change (Rodrigue et al. 2000; Raghavan and Groisman 2010). Further, there are multiple lines of evidence pointing to c-di-GMP and c-di-GMP-dependent transcriptional regulator BrlR are potential mediators of SagS-dependent biofilm tolerance (Chambers et al. 2014; Park and Sauer 2021). BrlR is a receptor for both pyocyanin and c-di-GMP (Wang et al. 2018). Activated BrlR in turn enhances the expression of MexAB-OprM, and MexEF-OprN efflux pump resulting in biofilm tolerance (Liao et al. 2013). To date, there is no report on the effect of negative regulators mutation in efflux pumps in the biofilm stage. This study includes the genomic analysis of efflux pump (MexAB-OprM, and MexEF-OprN) along with their negative (NalC, NalD, and MexR) and positive regulators (MexT) and expression in biofilm-tolerance for three antibiotics (ceftazidime, gentamicin, and ciprofloxacin).

5.2 Materials and Methods

a) To study the effect of antibiotics in persister cell formation

5.2.1 Bacterial strains, growth conditions, and antibiotics used for the study

The present study was done using UTIs causing *P. aeruginosa* isolates (TP-10 and ST-13) and PAO1. On Pseudomonas Isolation Agar (PIA) bacterial isolates were routinely subcultured. This study includes respective antibiotics shown in below Table 5.1

Table 5.1: Antibiotics used in the study

Sr. No	Antibiotics	Mode of action
1	Ceftazidime	cell wall inhibitor
2	Gentamicin	protein inhibitor
3	Ciprofloxacin	DNA gyrase inhibitor

5.2.2 Minimum Inhibitory Concentration (MIC) determination

Preparation of antibiotic stock solution

The stock solution of antibiotics was prepared using the formula

$$W=C*V/P$$

W= weight of the antibiotics in mg to be dissolved

V= desired volume in ml

C= final concentration of the stock solution (µg/ml)

P= potency given by the manufacturer (µg/ml)

MIC determination

MIC was determined in the 96-well plate according to the CLSI guideline (Wiegand et al. 2008). In brief, 100 µl of Mueller Hinton Broth (MHB). In brief, 100 µl Mueller Hinton Broth (MHB) and 50 µl of antibiotic solution of 4X concentration were dispensed in the 96-well plate. Following that, in each well 50 µl of 1:100 diluted culture (0.8 OD at 600 nm) was added and incubated at 37 °C for 24 h under static conditions. For growth control, no antibiotics were added in those wells. The lowest concentration of antibiotics that resulted in no visible growth was concluded as the MIC for respective antibiotics. The assay was performed in triplicates for each strain with each antibiotic treatment.

5.2.3 Planktonic persister assay

The PC formation was determined by assessing the cell viability count after antibiotic treatments. According to Mulcahy *et al*. 2010, PC formation in the planktonic stage was determined for PAO1, TP-10, and ST-13 (Mulcahy et al. 2010). Briefly, overnight grown culture was diluted to 0.1 OD at 600 nm in 3 ml and treated with 5X MIC of ceftazidime, gentamicin, and ciprofloxacin and kept on shaking condition at 250 rpm for 24 hours in LB medium. By sampling one strain per tube, colony-forming units (c.f.u.) were calculated at time intervals of t = 0 h, t = 2 h, t = 4 h, t = 6 h, t = 8 h, and t = 24 h. To determine the number of c.f.u., 100 µl of culture was sampled, washed with Phosphate Buffer Saline (PBS), serially diluted, spread on Luria Agar (LA) plate, and incubated at 37 °C for 24 hours. The experiment was performed in biological triplicates for each strain.

5.2.4 Cellular Redox activity in the planktonic stage

After 4 hours of antibiotic treatment at a 5X MIC, the cellular redox activity of PC formation was studied using RSG (Redox Sensor Green) and PI (Propidium iodide) staining. As per above mentioned protocol of the planktonic persister assay, the cells are grown and treated with antibiotics. Following 4 hours of antibiotic treatment, 3 ml of the sample was aliquoted, it was washed twice with PBS wash was given, and resuspended in 300 µl of PBS. Further stained with RSG, a green fluorescent dye that is a metabolic indicator dye, and PI, a red fluorescent dye for the dead cells. For flow cytometry, the cells were stained using RSG (1 µl) and PI (1 µl of 1:100 diluted) dyes (BacLightTM RedoxSensorTM Green Vitality Kit Thermo Fisher Scientific Inc., Waltham, MA, USA) for 10 min in dark condition. No antibiotic treatment was used as a control. The FLI and FL3 channels were used in the flow cytometry (Becton Dickinson FACSCalibur, New Jersey, United States) to detect fluorescence signals. The flow cytometry data analysis was done using FlowJo software. A histogram of the RSG-positive cells using the FL1 channel was created. Three distinct biological triplicates were used to examine the findings. (Kim et al. 2018).

5.2.5 Fluorescence microscopy

PC formation in the planktonic stage was examined through BX 51 fluorescent microscope after respective antibiotic treatment. As per above mentioned protocol, antibiotic treatment was given to cells. Following 4 hours of incubation, 1 ml of sample was aliquoted; PBS wash was given to remove any residual antibiotics; 100 µl of cells were resuspended in PBS and stained using RSG (1 µl) and PI (1 µl of 1:100 diluted) for 10 min in dark condition. Further, 10 µl of the sample was spotted on a 1% agarose pad and covered with a 22 mm coverslip. A 100X oil

immersion fluorescent microscope (BX 51 Olympus microscope, Japan) was used to examine cells. No antibiotic treatment was used as a control. The experiment was performed in triplicates (Kim et al. 2018).

5.2.6 Gene expression in the planktonic stage

5.2.6.1 RNA isolation

The RNA isolation was carried out after 4 hours of antibiotic treatment. Briefly, 3 ml of cells were centrifuged at 8000 rpm for 5 minutes to remove any residue of antibiotics, then cells were washed with PBS and resuspended in 300 µl PBS. Total RNA was extracted using the Nucleospin RNA kit (Macherey Nagel, Hoerdt, France). Briefly, Cell pellet was obtained by cengtrifugation and dissolved into 100 µl TE buffer containing 1 mg/ml of lysozyme. The dissolved pellet was further incubated at room temperature (RT) for 10 minutes. After incubation, RA1 (350 µl) and β-mercaptoethanol (3.5 µl) were added to lysate and vertexed for 30 seconds. To clear lysate as well as to reduce the viscosity of lysate it was filtered through NucleoSpin® Filter (violet ring) which was placed in a collection tube of 2 ml and centrifuged at 8000 rpm for 1 minute. After discarding the NucleoSpin® filter, 350 µl of 70% ethanol was added and gently mixed with a pipette to the homogenized lysate. Thereafter, put one NucleoSpin® RNA Column (one with a blue ring) in a collecting tube (of 2 ml). The lysate was gently mixed by pipetting 2-3 times and loaded into the column (blue color filter ring) and centrifuged at 11,000 x g for 30 seconds. DNase mixture was prepared in a sterile nuclease-free microcentrifuge tube of 1.5 ml by adding 10 µl of reconstituted rDNase to 90 µl of Reaction Buffer and gently mixing. The prepared rDNase mixture (95 µl) was added to the center of the silica membrane and incubated for 15 minutes at RT. After incubation, RAW2 buffer (200 µl) was added and centrifuged at 11,000 x g for 30 seconds. Lysate was discarded from the collection, tube. Then to the NucleoSpin® RNA Column, 600 µl of RA3 buffer was added, and it was centrifuged at 11,000x g for 30 seconds (repeat the step twice). After discarding the flowthrough, the column was placed back onto collecting tube. Then 250 µl RA3 buffer was added to the NucleoSpin® RNA Column and it was centrifuged at 11,000 x g for 2 minutes to completely dry the membrane. Insert the column into the fresh nuclease-free collection tube to collect the RNA. The RNA was then eluted in the 20 µl of nuclease-free water and centrifuged at 11,000 x g for 1 minute. RNA integrity was assessed on a 2% gel, and quantification was done with Nanodrop (Thermo Fisher Scientific, Waltham, Massachusetts, USA).

5.2.6.2 cDNA synthesis

Using prime 1st Strand cDNA synthesis kit (Takara, Bio in, Japan) protocol first-strand cDNA synthesis was carried out. Reaction system-1 and Reaction system-2 were used for cDNA synthesis as shown in Table no. 5.2 and 5.3. In Brief, reaction system-1 was incubated at 65 °C for 5 minutes and placed on ice for 5 minutes. Then reaction system-2 was set up and incubated at the following conditions: 30 °C for 10 minutes; 50 °C for 1 hour and 70 °C for 5 minutes. The reaction mixture was then stored at -20 °C.

Table 5.2: Reaction system-1 for cDNA for synthesis

Reaction Component	Volume
Rand-6-mers	2 µl
dNTP mix	1 µl
Template RNA	500 ng
RNase free water	X µl
Total system	10 µl

Table 5.3: Reaction system-2 for cDNA synthesis

Reaction Component	Volume
Reaction system I	10 µl
5X Prime Script Buffer	4 µl
RNase inhibitor	0.5 µl
Reverse Transcriptase	1 µl
RNase free water	4.5 µl
Total system	20 µl

5.2.6.3 Primer designing for stringent response and TA system genes

The primers for stringent response (*relA*, *spoT*, and *lon*) and TA system (*higA* and *higB*) genes were designed using the SnapGene software. Table 5.4 depicts the list of primer sequences used in qRT-PCR.

Table 5.4: Primer used for Stringent response and toxin-antitoxin genes.

Function and Primer	The nucleotide sequence (5'-3')
RpoD_F	GGGCGAAGAAGAAATGGTC
RpoD_R	CAGGTGGCGTAGGTGGAGAA
RelA_F	GGCTACATCACGCGTGGG
RelA _R	CCGCTCATTGAGCAGCAC
SpoT_F	CGTTCAACGAGATCATGGACG
SpoT_R	GGTATGCAGCGACTGGTAGC
HigA_F	CGCGATGAGTTTCTGATGGAG
HigA_R	CCAGAACTGAGCGGACG
HigB_F	TTTGAGACGGGTCTTTCG
HigB_R	TGATGCTATGTTGGCCC
Lon_F	TATCTCGCCGTGCAAAAGC
Lon_R	AGCCGATATAGGTACGACGG

5.2.6.4 The gene expression studies using qRT-PCR

The gene expression of stringent response (*relA*, *spoT*, and *lon*) and TA system (*higA* and *higB*) genes were studied using SYBR Green (Takara Bio Inc., Shiga, Japan). The *rpoD* gene was used as a housekeeping gene. The gene expression was carried out as per below-mentioned PCR system component and carried out using the qRT-PCR cycle condition in Tables 5.5 and 5.6. The three independent biological replicates were used for relative quantification. Data normalization was done with untreated control and fold changes were calculated according to the $2^{-\Delta\Delta Ct}$ method (Guyard-Nicodème et al. 2008).

Table 5.5: Reaction system used for qRT-PCR

Reaction Component	Volume
SYBR Green master mix	5 µl
AMQ water	2 µl
Forward Primer	1 µl (10 pmol)
Reverse Primer	1 µl (10 pmol)
cDNA Template	1 µl
Total System	10 µl

Table 5.6: Condition used for qRT-PCR

Step	Temperature ºC	Time
Initial denaturation	95	3 minutes
Denaturation	94	30 seconds
Annealing	58	30 seconds
Primer Extension	72	30 seconds
Repeat cycle 35 X		
Final extension	72	5 minutes
Melt curve	65 to 95 increments 0.5	0.05 seconds

5.2.7 Biofilm Model:

For the biofilm model, *P. aeruginosa* isolates TP-10, ST-13, and PA01 (used as a reference strain) were used in the study. Biofilm model: 3 ml of bacterial culture (0.01 OD at 600 nm) was added to the 6 well polystyrene plates and incubated at 37 °C for 24 hours under static conditions. The following day, planktonic cells were removed and biofilms were gently rinsed to remove planktonic cells with 1 ml of 0.85% NaCl. After that, 3 ml of fresh MHB was added and challenged with a 5X MIC concentration of antibiotics for 24 hours (Soares et al. 2019).

5.2.8 Biofilm quantification

In 96-well plate, biofilm was grown according to Stepanovic *et al* 2004. In a 96-well plate, 230 µl of LB was dispensed into the well and 20 µl of overnight culture (0.2 OD at 600 nm) was incubated at 37 °C for 24 hours in static conditions. Only LB was used as a blank control,. The following day, biofilm was rinsed with 0.85% NaCl to remove planktonic cells; fixed with

methanol for 15 minutes; stained with 0.1% crystal violet (CV) for 15 minutes; CV was removed and excess CV was rinsed with distilled water (DW) twice; air-dried and then attach CV was dissolved in 33% glacial acetic acid. Biofilm quantification was done at 570 nm and further categorized into weak, moderate, and strong biofilm producers as per Stepanović *et al.* 2004. The experiment was performed in triplicates (Stepanović et al. 2004).

5.2.9 Biofilm Time kill-curve assay

The biofilm was formed as per above mentioned protocol and treated with respective antibiotics (ceftazidime, gentamicin, and ciprofloxacin) with 5X MIC concentration for 24 hours. Untreated well served as a control. According to Soares *et al.* 2019, the cell viability was assessed for each antibiotic treatment at t=0 h, t=2 h, t=4 h, t=6 h, t=8 h, and t=24 h (Soares et al. 2019). All experiments were done in three biological replicates for each strain and antibiotic treatment.

5.2.10 Cellular Redox activity in the biofilm stage

As per the above-described protocol, biofilm was formed for 24 hours and then treated with respective 5X MIC of antibiotics for 4 hours. To remove planktonic cells biofilm was rinsed with 1 ml of 0.85% NaCl. In 300 µl of 0.85% NaCl biofilm was resuspended and stained with 1 µl RSG, and 1 µl of 1: 100 diluted PI dyes were incubated for 10 minutes in dark conditions. The biofilm that was untreated with antibiotics served as a control. For flow cytometry (Becton Dickinson FACSCalibur, New Jersey, United States), FL1 and FL 3 channels were used to detect fluorescence signals. FlowJo software was used for data analysis. A histogram of the RSG-positive cells and the FL1 channel was created. Three independent biological replicates were done for each strain.

5.2.11 Confocal Laser Scanning Microscopy of Biofilm

Biofilm was grown in the 6-well plate for 24 hours as per the above-aforementioned protocol and treated with 5X MIC of antibiotics for 4 hours at 37 °C. Thereafter, the biofilm was rinsed with 0.85% NaCl to remove planktonic cells as well as antibiotics. Then biofilm was stained with 1 µl RSG, and 1 µl of 1: 100 diluted PI dyes in a dark condition for 10 minutes. After incubation, biofilm was rinsed with 0.85% to remove excess stain and then covered coverslips and sealed with nail polish. As a control biofilm that was untreated with antibiotics was used. Thereafter, PC formation in the biofilm stage was observed under Carl Zeiss CLSM 780 microscopes. Fiji software was used for analysis.

5.2.12 The gene expression in the biofilm stage

The biofilm was formed as per above mentioned protocol and treated with respective antibiotics at 5X MIC concentration for 4 hours. Biofilm was rinsed with 1 ml of 0.85% NaCl; biofilm was then pooled in 1 ml of 0.85% NaCl; centrifuged at 8000 rpm for 5 min, and resuspended in 300 µl of 0.85% NaCl. RNA extraction was carried out using Nucleospin RNA kit (Macherey Nagel, Hoerdt, France). The detailed protocol was followed as aforementioned in **section 5.2.6.1**. The cDNA synthesis was done as per the protocol mentioned in **section 5.2.6.2**. The primer used to study stringent response and TA system were as per the above for planktonic stage gene expression as shown in Table 5.4 in **section 5.2.6.3**. qRT-PCR was done using the primer listed in Table 5.4 using the reaction mixture and reaction condition listed in Tables 5.5 and 5.6 in **section 5.2.6.4.**

b) To study the biofilm-mediated antibiotic resistance through *brlR*

5.2.13 Whole genome sequence analysis

Whole genome sequences of clinical isolates were previously available in the lab. TP-10 and TP-11 isolates were selected and further analysis was done. Genomic DNA extraction was done using XpressDNA Bacterial Isolation kit (MagGenome, USA) and using Ion Xpress™ Plus gDNA Fragment Library preparation kit (ThermoFisher Scientific) library preparation was done according to the manufacturer's instructions. Genomic libraries were loaded onto the Ion 530Tm chip, and sequencing was carried out in accordance with established methods using the Ion S5TM system (Ion torrent, Thermo Fisher Scientific). Bioinformatic analysis of WGS: after sequencing the quality of each raw file was examined using the FastQC (v.0.11.5) tool (S. Andrews. 2010). Using the FASTX toolkit (http://hannonlab.cshl.edu/fastx_toolkit/) the low-quality reads and ambiguous bases were diverted. Using the SPAdes (v. 3.11.1) tool the De novo assembly of high-quality reads was prepared (Bankevich et al. 2012). Each isolate draft genome sequence was constructed using a technique that used read error correction algorithms, and the assembled sequence's quality was evaluated using the QUAST (v.5.0.2) program (Gurevich et al. 2013). For gene annotation 11 Prokka (v. 1.13.30 available at: https://github.com/tseemann/prokka) tool was used (Seemann 2014). Sequences of target genes were found for PAO1 using Pseudomonas Genome DB (Winsor et al., 2016). Whole genome sequences of clinical isolates were imported to Geneious Prime version 2021.2.2 using gff file. Sequences of target genes in clinical isolates were found using Geneious Prime and the

Comprehensive Antibiotic Resistance Database (CARD). DNA and Protein sequences of target genes were compared through multiple sequence alignments done using MEGAX software.

5.2.14 Minimum Inhibitory Concentration (MIC) determination

MIC was determined according to CLSI guidelines in the 96-well format (Wiegand et al. 2008). The detailed protocol for MIC determination is mentioned in **section 5.2.2.** The MIC was determined for gentamicin and ciprofloxacin antibiotics for PAO1, TP-10, and TP-11 isolates.

5.2.15 Biofilm formation and quantification assay:

Biofilm formation and quantification were done according to Stepanovic *et al* 2004. The detailed protocol is mentioned in **section 1.2.8.**

5.2.16 Biofilm antibiotic susceptibility testing

Overnight grown culture was diluted to 0.08 OD600. Further, a 1:100 dilution of 0.08 OD at 600 nm was inoculated in 6 well culture plates and incubated at 37 ˚C for 24 hours. After 24 hours of incubation, formed biofilm was rinsed with 0.85% saline to remove planktonic cells. Then biofilm was treated with 5X MIC of gentamicin and ciprofloxacin antibiotics and further incubated for 2 hours. After treatment with antibiotics, biofilm was again rinsed with 1 ml of 0.85% NaCl, homogenized, and resuspended in 1 ml of 0.85% NaCl. Further, the serially diluted culture of 20 µl was spread on LA plates and incubated at 37 °C for 24 hours. The next day, colonies were counted and cfu/ml was determined (Soares et al. 2019). The experiment was performed in three biological replicates.

5.2.17 Pyocyanin quantification

Biofilm was formed in 6 well plates as per above mentioned protocol. Briefly, in the 6-well plate, 1:100 dilution of 0.08 at OD600 overnight grown culture was inoculated and incubated at 37 °C for 24 hours. After 24 hours of incubation, the biofilm was rinsed with 0.85% saline to remove planktonic cells; the biofilm was treated with respective antibiotics at 5X MIC and incubated at 37 °C for 2 hours. After incubation, the biofilm was rinsed with 1 ml of 0.85% NaCl to remove planktonic cells, homogenized, and resuspended in 1 ml of 0.85% NaCl. Cells were centrifuging at 8,000 rpm for 10 minutes to obtain pellet and the supernatant was extracted in another tube. To supernatant 500 µl of chloroform was added and vortexed for 10 seconds. It was again centrifuged at 10,000 rpm for 10 minutes; the bottom phase was transferred to a new tube and 250 µl 0.2N HCl was added to it. The tube was vortexed 2 times for 10 seconds and centrifuged for 2 minutes at 10,000 rpm. The OD of the top phase (pink to light pink) was measured at 520 nm and multiplied by 17.072 to calculate the micrograms of pyocyanin per ml

(Essar et al. 1990). The 0.2N HCL was used as blank. The experiment was performed in triplicates for each isolate.

5.2.18 Quantitative RT-PCR (qRT-PCR)

Biofilm was formed for PAO1, TP-10, and TP-11 in a 6-well plate for 24 hours at 37 °C, and the next day biofilm was treated with 5X MIC gentamicin and ciprofloxacin antibiotics as per above mentioned protocol. After treatment with antibiotics for 2 hours at 37 °C, planktonic cells, as well as antibiotics, were removed by rinsing the biofilm with 1 ml of 0.85% NaCl; the biofilm was homogenized and resuspended in 1 ml of 0.85% NaCl. Cells were centrifuged at 8500 rpm for 5 minutes to obtain pellet; which was resuspended in 1 ml of 0.85% NaCl and again centrifuged to remove any residues of antibiotics. The cell pellet was resuspended in 100 µl TE buffer containing 1 mg/ml lysozyme and RNA was extracted as per the protocol mentioned in the Nucleospin RNA kit (Macherey Nagel, Hoerdt, France) as per the detailed protocol mentioned in **section 5.2.6.1** and DNase treatment was given to remove any DNA in the sample according to the manufacturer's instructions. Further, using nanodrop (Thermo Fisher Scientific, Waltham, MA, USA) RNA was quantified and RNA integrity was checked on 2% agarose gel. The cDNA synthesis was carried out using the 1st Strand cDNA synthesis kit (Takara, Bio in, Japan) as per the protocol mentioned in **section 5.2.6.2**. The primers were designed using SnapGene software. The below-listed primer sequence in Table 5.7 of *sagS, brlR, nalD, nalC* gene. The qRT-PCR reaction system mentioned in Table 5.8 and the qRT-PCR cycling condition mentioned in below Table 5.9 were used in gene expression studies. *rpoD* gene was used as housekeeping control. The untreated control was used for data normalization and fold changes were calculated according to the $2^{-\Delta\Delta Ct}$ method (Guyard-Nicodème et al. 2008). All runs were done in the three biological experiments for each strain and antibiotic treatment.

Table 5.7: Primers used for qRT-PCR

Genes	Primer sequence (5'→3')
***sagS* (F)**	GGGCAACTGGTCCTGGAAC
***sagS* (R)**	GTTGCTGACGATCTGGCG
***brlR* (F)**	GCAACGACACCAGCACAC
***brlR* (R)**	GAAGCGTTCCCAGAGCTG
***mexA*(F)**	GATCTACGTCGACGTCACCC

***mexA* (R)**	CTCGGAGAATTCGAGGCGAC
***mexF* (F)**	TTCAACTCGCTGACCCTGTC
***mexF* (R)**	AAGCCGAAGTAGGTCAGCAC
***nalC* (F)**	GAAATCTTCGACGACAGCGC
***nalC* (R)**	TGCTCGTAGAAGGACTTGCC
***nalD* (F)**	AGGCCGTGCATCGTTG
***nalD* (R)**	CGAACAACTGCTCGCAGAG
***rpoD*(F)**	GGGCGAAGAAGGAAATGGTC
***rpoD*(R)**	CAGGTGGCGTAGGTGGAGAA

Table 5.8: Reaction system for qRT-PCR

Reaction Component	Volume
SYBR Green master mix	5 µl
AMQ water	2 µl
Forward Primer	1 µl (10 pmol)
Reverse Primer	1 µl (10 pmol)
cDNA Template	1 µl
Total System	10 µl

Table 5.9: Reaction conditions used for qRT-PCR

Step	Temperature ºC	Time
Initial denaturation	95	3 minutes
Denaturation	94	30 seconds
Annealing	57	30 seconds
Primer Extension	72	30 seconds
Repeat cycle 35 X		
Final extension	72	5 minutes
Melt curve	65 to 95 increments 0.5	0.05 seconds

5.2.19 Statistical analysis

Each experiment was performed in biological triplicates for each strain. Corresponding data represents mean ± standard deviation (SD). The student's t-test one way ANOVA and two-way ANOVA were performed using GraphPad Prism 9 software (GraphPad, San Diego, USA). Differences were statistically significant at $P<0.05$.

5.3 Results

a) To study the effect of antibiotics in persister cell formation

5.3.1 MIC determination for *P. aeruginosa* isolates

The MIC values of the three different antibiotics (ceftazidime, gentamicin, and ciprofloxacin) against the clinical isolates of *P. aeruginosa*: TP-10, ST-13, and PAO1 (used as a reference strain) are displayed in Table 5.10. According to CLSI guidelines, PAO1 and ST-13 isolates were ciprofloxacin-resistant, whereas susceptible to ceftazidime and gentamicin. The TP-10 isolate was found to be susceptible to all antibiotics.

Table 5.10: MIC of *P. aeruginosa* isolates

Sample	MIC (mg/L)		
	CAZ	GEN	CIP
PAO1	1 (S)	0.5 (S)	8 (R)
TP-10	0.5 (S)	0.5 (S)	0.0625 (S)
ST-13	2 (S)	1 (S)	8 (R)

CAZ: ceftazidime; GEN: Gentamicin; CIP: Ciprofloxacin. According to CSLI guidelines, clinical breakpoints for antibiotics were ≤8 µg/mL (S), 16 µg/mL (I), and ≥32 µg/mL (R) for ceftazidime (CAZ); ≤4 µg/mL (S), 8 µg/mL, (I) ≥16 µg/mL, and (R) for gentamicin (GEN); ≤1 µg/mL (S), 2 µg/mL (I) ≥4 µg/mL, and (R) for ciprofloxacin (CIP); R: resistant strains; S: Sensitive strains; I: Intermediate

5.3.2 Formation of PCs during the planktonic stage

Three bactericidal antibiotics: ceftazidime (Cephalosporin), gentamicin (aminoglycoside), and ciprofloxacin (fluoroquinolone) were used to study the development of PCs in *P. aeruginosa* isolates (PAO1, TP10, and ST13). The time-kill curve assay was done for the isolates PAO1, TP-10, and ST-13 as shown in Figure 5.1 against three antibiotics at 5X MIC at the specified time points for 24 hours. The hallmark of persistence “Biphasic kill curve” differed among the isolates. In the PAO1 strain, ceftazidime treatment resulted in the least log reduction and formed a greater number of 9 log10 cfu/ml survival fractions (Figure 5.1a). Treatment with gentamicin produced 8 log10 cfu/ml, whereas treatment with ciprofloxacin produced 6 log10 cfu/ml survival fractions (Figure 5.1a). Additionally, in the TP-10 isolate gentamicin and ceftazidime treatment resulted in a 9 log10 cfu/ml survival fraction (Figure 5.1b) while ciprofloxacin treatment produced a cell survival fraction of 5-6 log10 cfu/ml (Figure 5.1b).

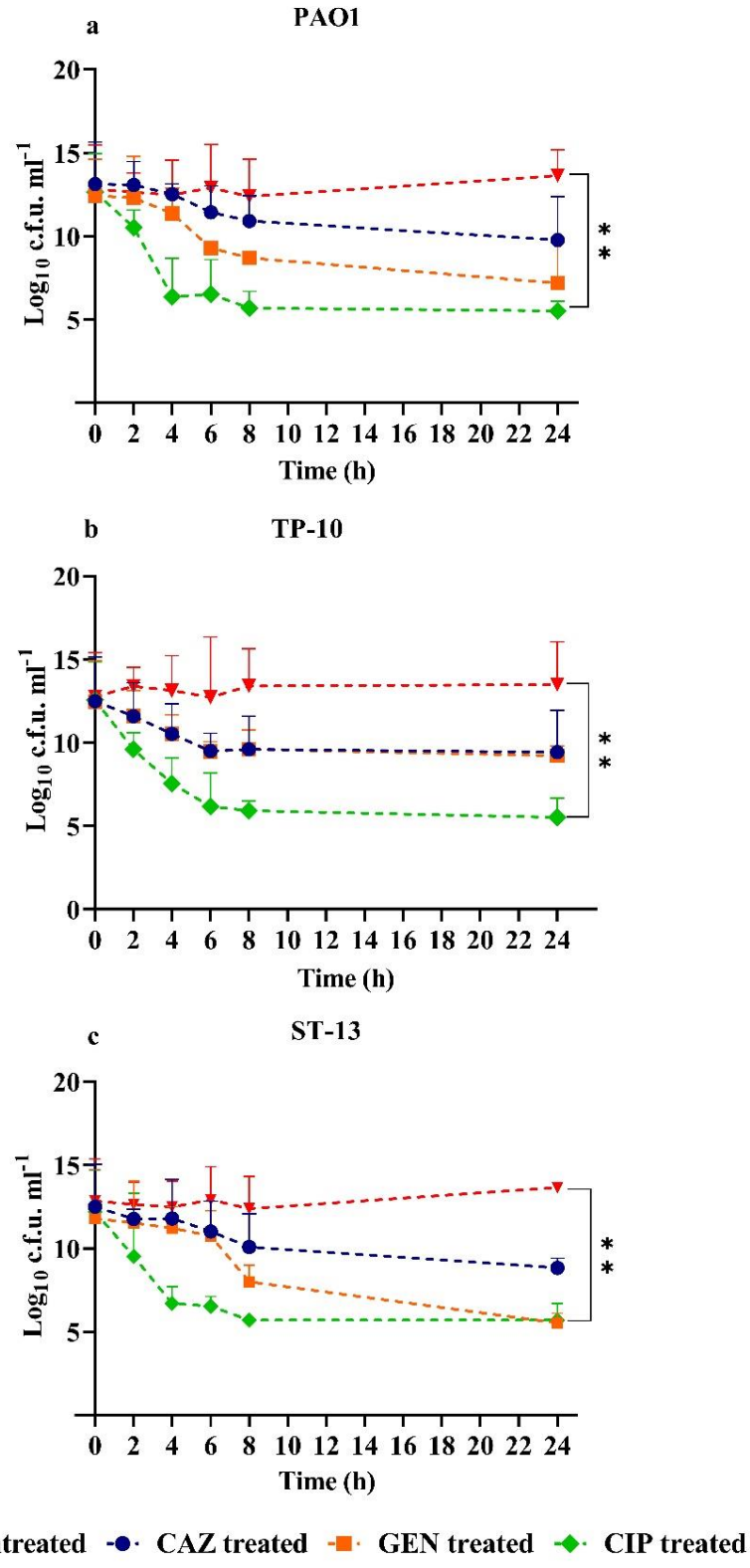

Figure 5.1: Planktonic kill curve assay. The time-kill curve assay was performed in the planktonic stage of (a) PAO1 (b) TP-10 and (c) ST-13 which were grown in LB and then treated with 5X MIC of antibiotic (CAZ, GEN, and CIP). At the indicated time point of t=2, 4, 6 8-, and 24-hours cells were plated for the viable count. As a control untreated was used. CAZ: ceftazidime; GEN: gentamicin, and CIP: ciprofloxacin. One-way ANOVA was used for statistical analysis, which was $*P < 0.05$, $**P < 0.01$.

In the ST-13 isolate, ceftazidime treatment resulted in 9-10 log10 cfu/ml (Figure 5.1c). While on gentamicin and ciprofloxacin treatment resulted in a range of survival fraction of 5-6 log10 cfu/ml (Figure 5.1c). In all isolate the maximum log reduction and least PC formation was observed on ciprofloxacin treatment.

5.3.3 Redox activity in planktonic stage

The cellular redox activity of isolates PAO1, TP-10, and ST-13 was examined in the planktonic stage using RSG and PI staining. When reduced by bacterial reductases, the RSG dye readily penetrates bacteria and produces green fluorescence that can be associated with cellular metabolic activity(Orman and Brynildsen 2013; Orman et al. 2016). The flow cytometry results of the PAO1, TP-10, and ST-13 isolates after 4 hours of antibiotics treatment are shown in Figures 5.2a–c. In the PAO1 isolate, an increase in redox activity was observed on ceftazidime treatment compared to untreated (Figure 5.2a). While there was a decrease in redox activity on gentamicin and ciprofloxacin treatment (Figure 5.2a). In the TP-10 isolate, redox activity was increased on ceftazidime treatment compared to untreated and was followed by gentamicin and ciprofloxacin treatment (Figure 5.2b). In the ST-13 isolate, there was an increase in redox activity on ceftazidime treatment compared to untreated, with gentamicin and ciprofloxacin treatment following (Figure 5.2c). As shown in Figures 5.2d–f the number of RSG-positive cells after antibiotic treatment and without treatment is analyzed using flow cytometry. In PAO1, on ceftazidime treatment, there is a significant increase in redox activity compared to untreated. While on gentamicin and ciprofloxacin treatment redox activity was decreased (Figure 5.2d). In the TP-10 isolate, on ceftazidime and ciprofloxacin treatment there was a significant difference observed in RSG fluorescence compared to untreated (Figure 5.2e). Whereas in the ST-13 isolate, there was no significant difference observed (Figure 5.2f).

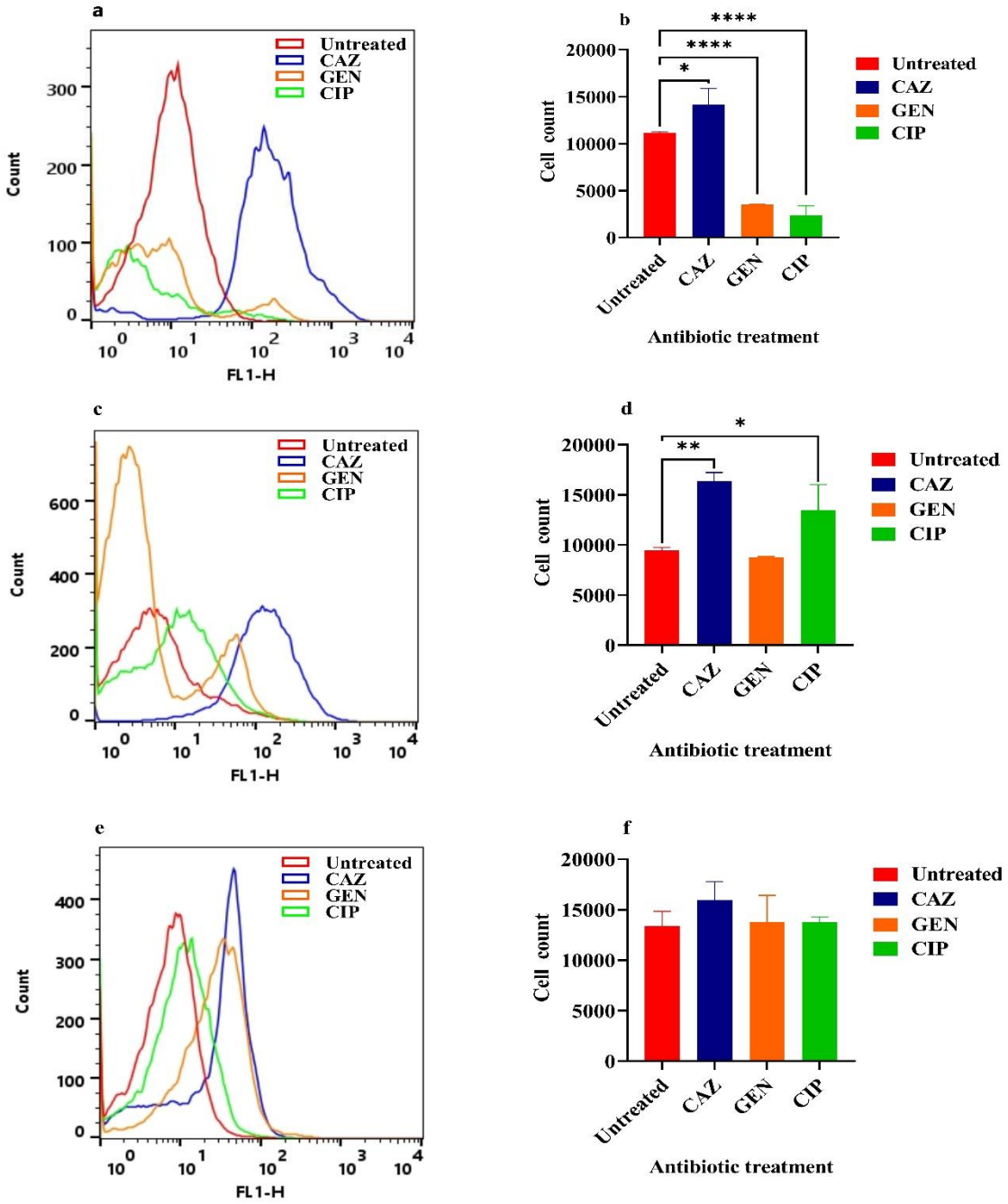

Figure 5.2: Cellular Redox activity within the planktonic stage. After 4 hours of antibiotics (CAZ, GEN and CIP) treatment the flow cytometry was done for (a) PAO1, (b) TP-10, and (c) ST-13 using RSG and PI dyes. Untreated served as control. The quantitative data of RSG-positive cells in (d) PAO1 (e) TP-10 and (f) ST-13 using flow cytometry analysis after treated with antibiotics and compared with untreated control. CAZ: ceftazidime; GEN: gentamicin, and CIP: ciprofloxacin. The above data is represented as mean ± standard deviation. The experiment was performed in triplicates for each strain. One-way ANOVA was used for statistical significance, were *P < 0.05, and **P < 0.01, ***P < 0.001, ****P < 0.0001.

5.3.4 Fluorescent Microscopy of persisters in planktonic stage

Figure 5.3 depicts the fluorescence microscopy images of PCs formed following 4 hours of each antibiotic treatment at 5X MIC and untreated used as control. In the PAO1 strain, treatment with ceftazidime resulted in the filamentous form of cells, whereas those with gentamicin and ciprofloxacin treatment lead to rod-shaped cells (Figures 5.3b-d). In the TP-10 isolate, ceftazidime treatment leads to the filamentous forms of cells similar to PAO1 (ceftazidime treatment), whereas rod-shaped cells were observed on gentamicin and ciprofloxacin treatment (Figures 5.3f-h). Besides these, on ceftazidime and gentamicin treatment a high number of redox-active cells were observed in the PAO1 (Figures 5.3b, and c), and TP-10 (Figures 5.3f, and g). Additionally, the number of redox-active cells was less observed and killing was more on ciprofloxacin treatment (Figures 5.3d, and h). In the ST-13 isolate, elongated cells with high-redox activity were observed on ceftazidime treatment. While the number of high redox-active cells was fewer in number on gentamicin and ciprofloxacin treatment (Figures 5.3k and l).

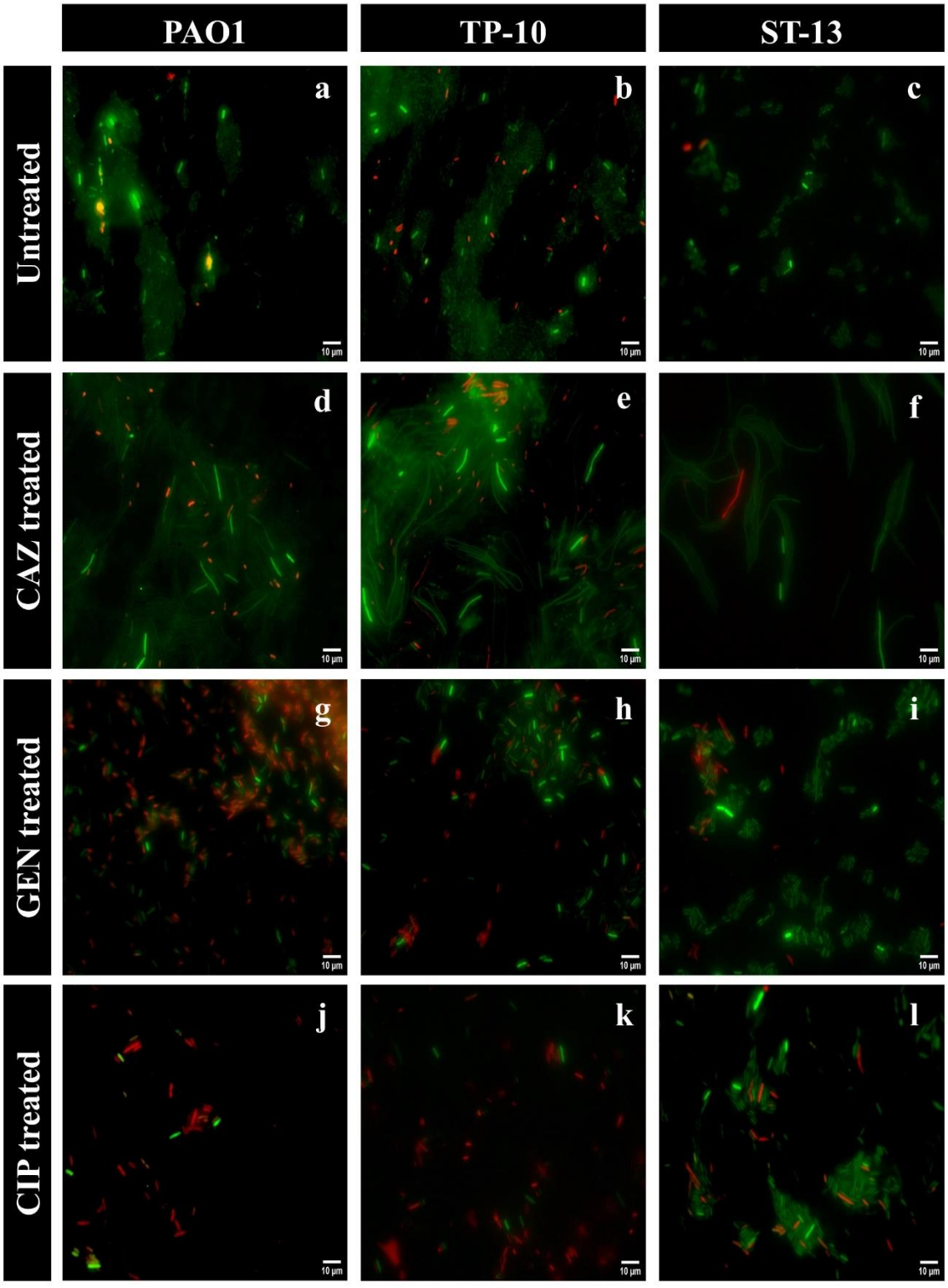

Figure 5.3: Fluorescence Microscopy of PCs within the planktonic stage. The representative images of PCs formed in PAO1, TP-10, and ST-13 after 4 hours of antibiotic treatment with (b, f, and j) CAZ, (c, g, and k) GEN, and (d, h, and l) CIP which were grown in LB. Before staining the cells with RSG and PI dyes the cells were washed to remove residues of antibiotics. (a, e, and i) As a control, untreated

cells were used. RSG staining was used to measure the cellular redox activity. CAZ: ceftazidime; GEN: gentamicin, and CIP: ciprofloxacin. Images of each isolate were analyzed using Fiji software

5.3.5 Expression of stringent response and TA system in the planktonic stage

To better understand the mechanism behind the persistence of *P. aeruginosa* isolates (PAO1, TP-10, and ST-13) in the planktonic stage on the following antibiotic treatments: ceftazidime, gentamicin, and ciprofloxacin, the gene expression responsible for antibiotic tolerance which includes (i) stringent response (RelA, SpoT, and Lon), (ii) toxin-antitoxin (HigBA), was examined.

Figure 5.4 shows the differences in the gene expression of stringent response and TA system among isolates (PAO1, TP-10, and ST-13) during the planktonic stage, where antibiotic treatments lead to upregulation of genes compared to untreated. In PAO1, on ciprofloxacin treatment, the expression of *relA* gene was 2.30 ± 0.44-fold higher, while in TP-10 on ceftazidime treatment there was 2.63 ± 0.32-fold higher (Figure 5.4a). Additionally, in PAO1 on ceftazidime treatment, there was 3.44 ± 0.43-fold upregulation of *spoT* gene, while in TP-10 on gentamicin and ciprofloxacin treatment there was 4.11 ±0.72 and 4.40 ± 0.34-fold upregulation (Figure 5.4b). In the ST-13 isolate, on gentamicin treatment *spoT* gene was 3.81 ± 0.78-fold upregulated (Figure 5.4b). As shown in Figure 5.4c, in PAO1 on ceftazidime treatment *lon* gene was 7.99 ± .96-fold upregulated, and in ST-13 there were 3.94 ± 0.30 and 3.36 ± 0.074-fold increases on ceftazidime and gentamicin treatment. Further, in PAO1 there was 15.26 ± 0.70 and 16.88 ± 0.30-fold upregulation of *higA* gene on ceftazidime and gentamicin treatment, respectively (Figure 5.4d). Also, in TP-10 on ceftazidime and gentamicin treatment there was a 2.72 ± 0.52 and 5.05 ± 0.42-fold increase in gene expression of *higA,* and ST-13 there was a 28.77 ± 0.80-fold higher on gentamicin treatment (Figure 5.4d). In addition, PAO1 on ceftazidime and gentamicin treatments leads to 7.90 ± 0.33 and 33.33 ± 1.02-fold upregulation of *higB* gene. While in TP-10 there was 2.74 ± 1.3 and 51.63 ± 0.35-fold higher *higB* gene expression on ceftazidime and gentamicin (Figure 5.4e).

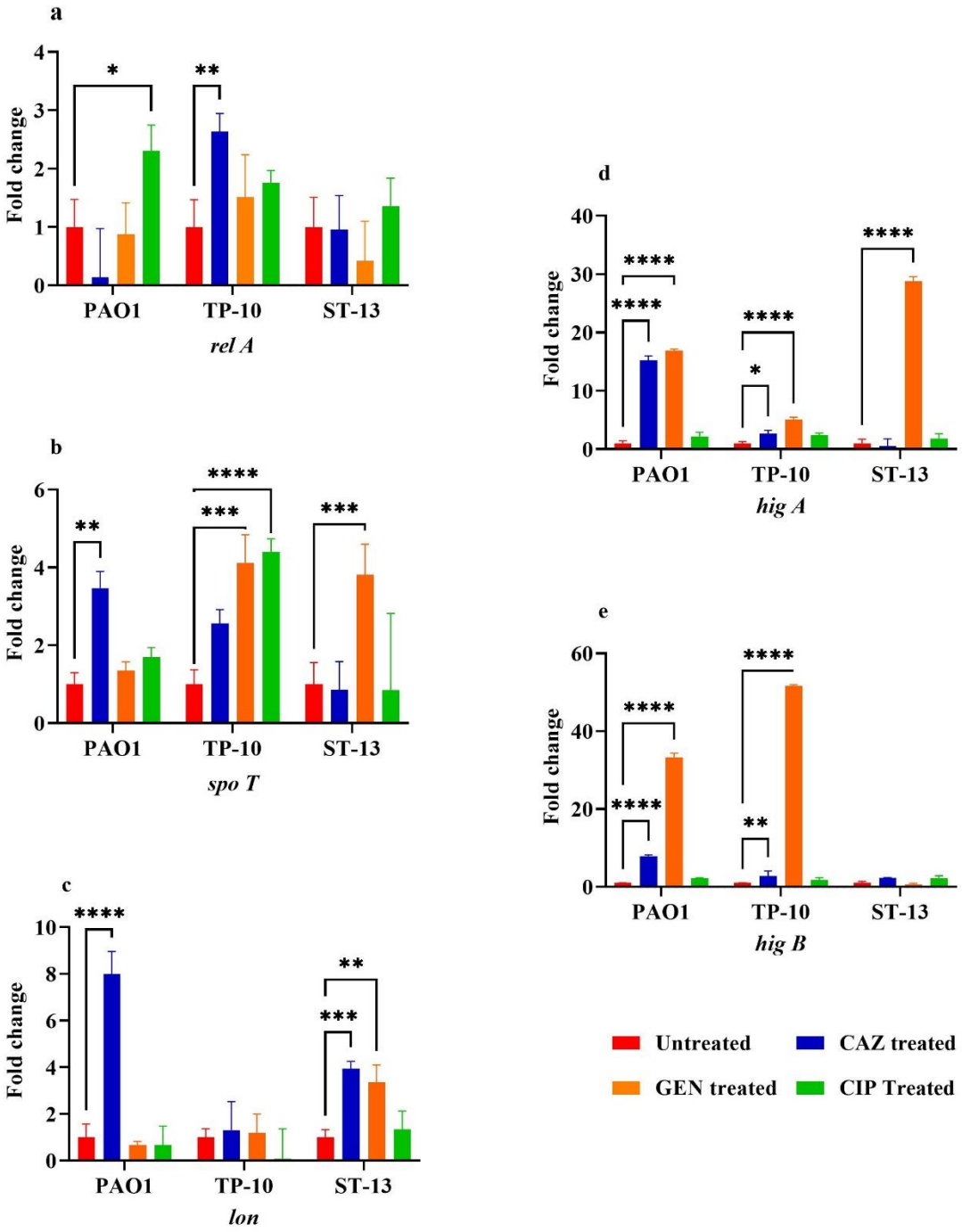

Figure 5.4: The gene expression studies in the planktonic stage. The gene expression studies were carried out after 4 hours of antibiotics (CAZ, GEN, and CIP) treatment in PAO1, TP-10, and ST-13 isolates. The following gene expression of (i) stringent response genes; (a) *relA*, (b) *spoT*, and (c) *lon* in addition to (ii) TA system genes: (d) *higA* and (e) *higB*. The data normalization was done with untreated control. The gene expression studies were carried out in three biological triplicates for each strain. The corresponding data represents as mean ± standard deviation. Two-way ANOVA was used for statistical analysis were *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001.

5.3.6 Formation of PCs during the biofilm stage

The crystal violet assay was used to study the biofilm formation of all three isolates, and it observed that PAO1 and ST-13 were strong biofilm producers while TP-10 was a weak biofilm

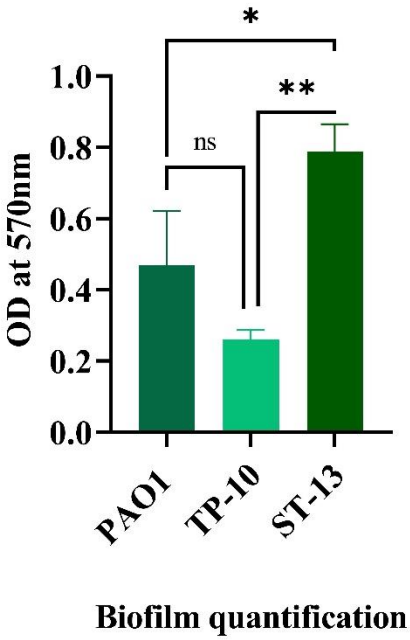

Figure 5.5: Biofilm quantification. The biofilm quantification was done in the 96-well plate using CV assay for PAO1, TP-10 and ST-13 isolate. The experiment was performed in triplicates for each strain. Student *t*-test was performed for statistical analysis, were *P < 0.05, **P < 0.01.

producer (Figure 5.5). Further to study PC formation in the biofilm state of PAO1, TP-10, and ST-13 isolates, a biofilm time-kill curve experiment was carried out against three antibiotics (Figure 5.6). Between isolates, the mean inoculum ranged from 5 to 6 log10 cfu/ml for TP-10 and ST-13 to 11 to 12 log10 cfu/ml for PAO1. Biofilm was regrown as a growth control in all isolates after ceftazidime treatment for 24 hours (Figures 5.6a-c). The biphasic kill curve was observed in all isolates after treatment with gentamicin and ciprofloxacin antibiotics (Figures

5.6a-c). In PAO1 biofilm, gentamicin and ciprofloxacin treatment resulted in a biphasic kill curve and resulted in cell survival fraction of 8-9 log10 cfu/ml and 8 log10 cfu/ml Figures 5.6a. While in TP-10 biofilm, on gentamicin and ciprofloxacin treatment approximately 2-3 log10 cfu/ml survival fractions were formed in a biphasic kill curve (Figure 5.6b). Further ST-13 biofilm on gentamicin and ciprofloxacin treatment resulted in 3-4 log10 cfu/ml survival fractions (Figure 5.6c).

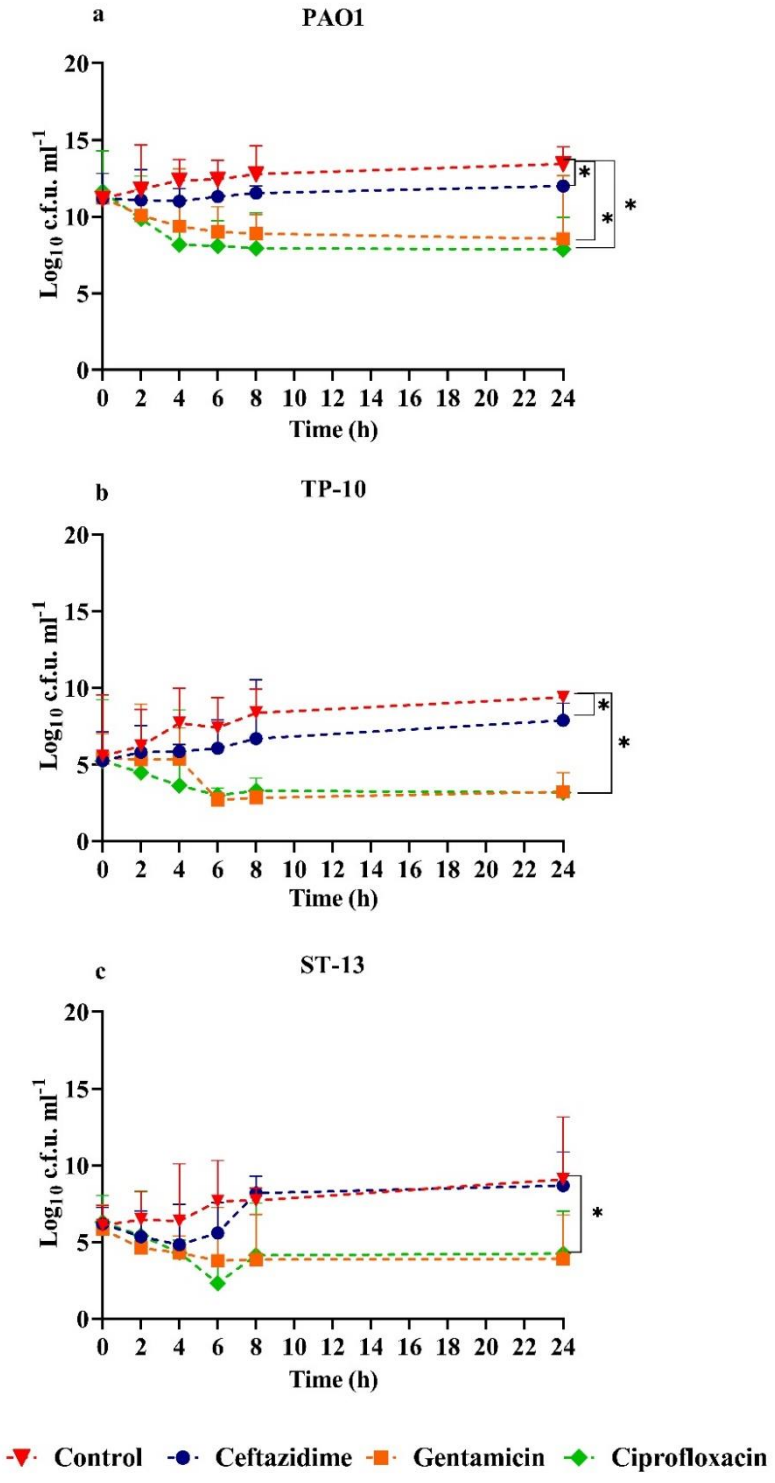

Figure 5.6: Biofilm kill curve assay. The time-kill curve assay for performed in the biofilm stage of (a) PAO1 (b) TP-10 and (c) ST-13 which were grown in MHB and then treated with 5X MIC of antibiotics (CAZ, GEN, and CIP). At the indicated time point of t=2, 4, 6 8-, and 24-hours cells were plated for the viable count. As a control untreated was used. CAZ: ceftazidime; GEN: gentamicin, and CIP: ciprofloxacin. One-way ANOVA was used for statistical analysis, which was *$P < 0.05$.

5.3.7 Redox activity in biofilm stage

Using flow cytometry, the cellular redox activity within the biofilm stage was studied after 4 hours of following antibiotic treatments: ceftazidime, gentamicin, and ciprofloxacin (Figure 5.7). In PAO1 biofilm, on ceftazidime treatment increase in redox-active cells was observed compared to the control (Figure 5.7a). However, gentamicin and ciprofloxacin treatments did not increase redox-active cells (Figure 5.7a). In TP-10 biofilm, ceftazidime treatment resulted in high redox active cells followed by gentamicin and ciprofloxacin compared to untreated (Figure 5.7b). Further in ST-13 biofilm, ceftazidime treatment leads to high redox active cells followed by ciprofloxacin and gentamicin compared to untreated (Figure 5.7c). Following 4 hours of antibiotic treatments, RSG-positive cells are analyzed by flow cytometry as shown in Fig. 5.7d–f. In PAO1 biofilm, on ceftazidime treatments, a high number of redox-active cells were observed compared to untreated Figure 5.7d. Whereas, gentamicin and ciprofloxacin treatment resulted in a decrease in redox-active cells Figure 5.7d. In TP-10 and ST-13 biofilm, a negligible difference was observed in redox activity compared to untreated control (Figures 5.7e and f).

5.3.8 CLSM of persisters in biofilm stage

After 4 hours of antibiotic treatment, the cellular redox activity within biofilm formed by PAO1, TP-10, and ST-13 was observed by CLSM using redox dyes (RSG and PI dye) (Figure 5.8). In PAO1 biofilm, compared to treated biofilm untreated biofilm had less number of redox active cells (Figure 5.8 a). Further, on ceftazidime treatment elongated cells were observed having less number of high redox-active cells (Figure 5.8d). While on gentamicin and ciprofloxacin treatment had rod-shaped high redox-active cells (Figure 5.8 g and j) and number of dead cells were increased after ciprofloxacin treatment in PAO1 biofilm (Figure 5.8j). In TP-10 biofilm, on ceftazidime treatment elongated cells were observed with few redox-active cells compared to the untreated (Figure 5.8 b and e). Further, on gentamicin treatment rod-shaped cells were observed with high redox-active cells (Figure 5.8h). Whereas on ciprofloxacin treatment TP-10 biofilm was disrupted and fewer number of rod-shaped cells were observed (Figure 5.8k). In ST-13 biofilm, ceftazidime treatment had very less number of elongated cells were observed with a small number of redox-active cells compared untreated having thick biofilm (Figure 5.8c and f). While, on gentamicin treatment rod-shaped with high redox-active cells were observed (Figure 5.8i). Additionally, on ciprofloxacin treatment less number of cells with few redox-active cells were observed compared to untreated (Figure 5.8l).

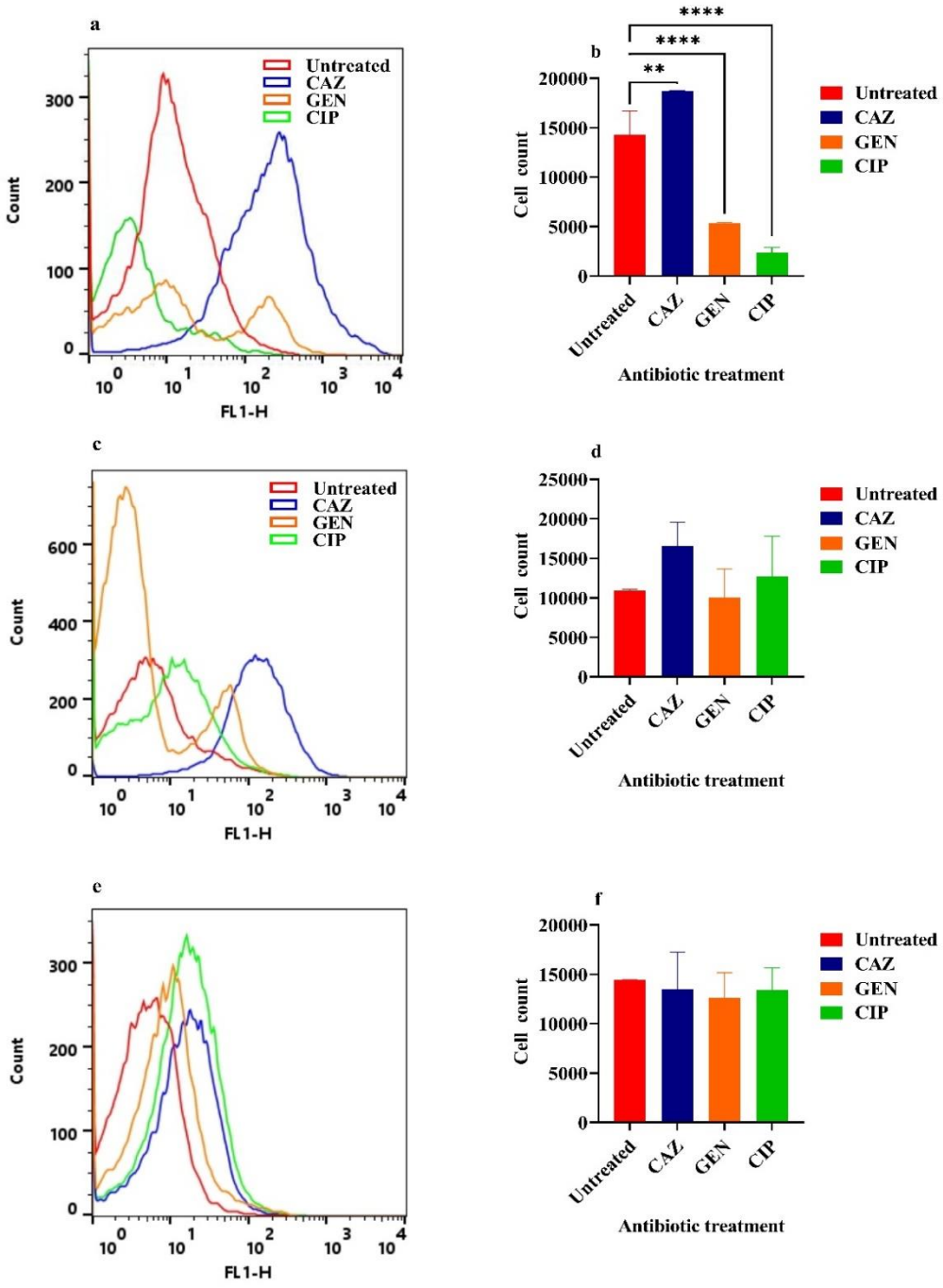

Figure 5.7: Cellular Redox activity within the biofilm stage. After 4 hours of antibiotic (CAZ, GEN, and CIP) treatment the flow cytometry was done for (a) PAO1, (b) TP-10, and (c) ST-13 using RSG and PI dyes. Untreated served as control. The quantitative data of RSG-positive cells in (d) PAO1 (e) TP-10 and (f) ST-13 using flow cytometry analysis after being treated with antibiotics and compared with untreated control. CAZ: ceftazidime; GEN: gentamicin, and CIP: ciprofloxacin. The above data is represented as mean ± standard deviation. The experiment was performed in triplicates for each strain.

One-way ANOVA was used for statistical significance, where *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

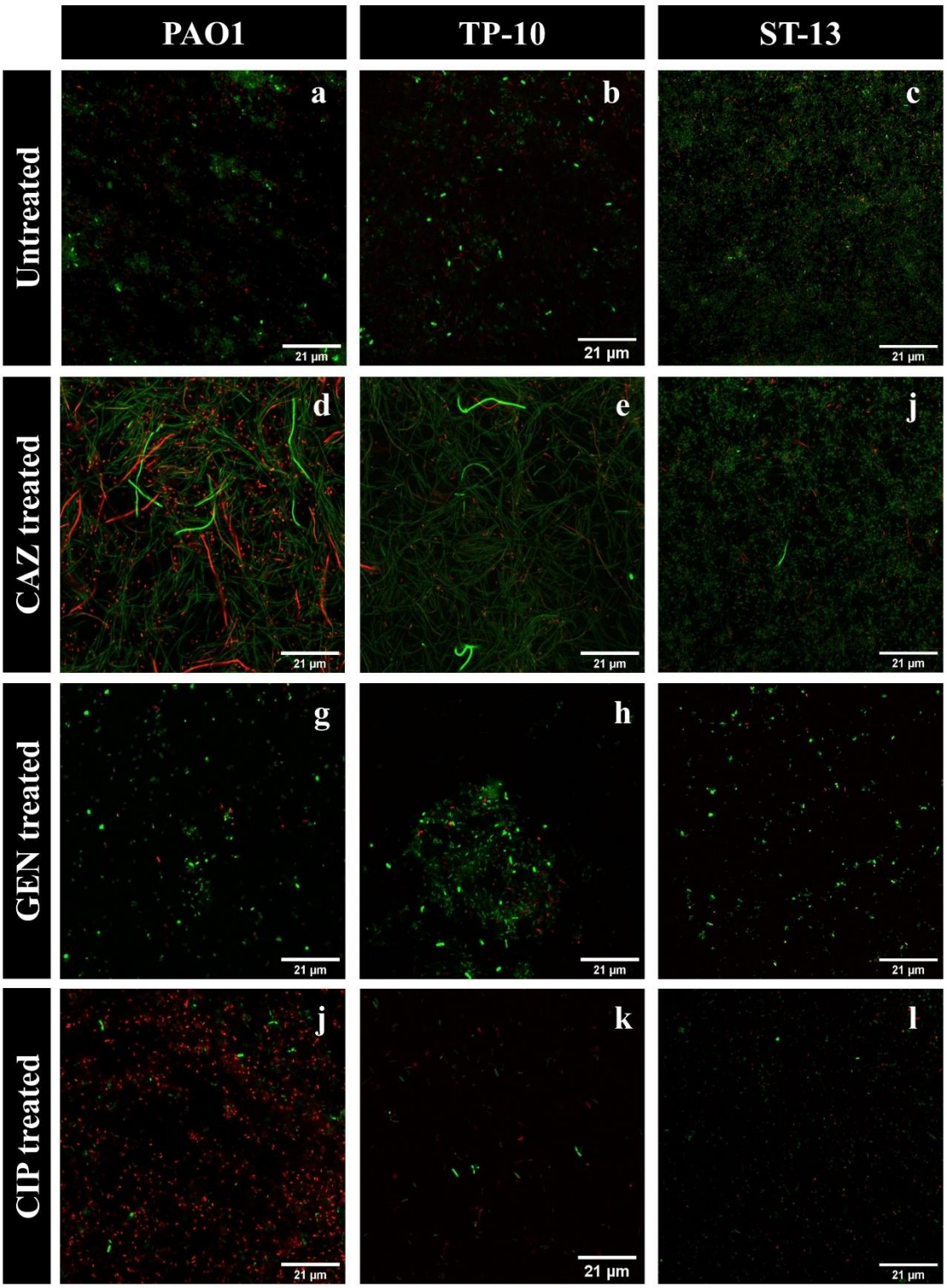

Figure 5.8: CLSM of PCs within the biofilm stage. The representative images of Pcs formed in PAO1, TP-10, and ST-13 biofilms after 4 hours of antibiotic treatment with (b, f, and j) CAZ, (c, g, and k) GEN, and (d, h, and l) CIP which were grown in MHB. Before staining the cells with RSG and PI dyes

the biofilm was gently rinsed to remove planktonic cells and any residues of antibiotics. (a, e, and i) As a control, untreated cells were used. RSG staining was used to measure the cellular redox activity. CAZ: ceftazidime; GEN: gentamicin, and CIP: ciprofloxacin. Images of each isolate were analyzed using Fiji software

5.3.9 Expression of stringent response and TA system in the biofilm stage

The gene expression responsible for PC formation is stringent response genes (*relA, spoT,* and *lon*), as well as toxin-antitoxin genes (*higA* and *higB*), which were studied after 4 hours of antibiotic treatments and gene, was normalized to untreated control (Figure 5.9). In PAO1 and TP-10, on ceftazidime treatment, there was upregulation of *relA* gene by 3.78 ± 0.50-fold and 4.40 ± 3.50-fold, respectively (Figure 5.9a). Whereas in ST-13, on gentamicin and ciprofloxacin treatment there was upregulation of *relA* gene by 2.94 ± 0.25 and 3.50 ± 0.52-fold (Figure 5.9a). In TP-10, on ceftazidime treatment, the *spoT* gene was elevated by 3.61 ± 0.47-fold, whereas in ST-13 on gentamicin and ciprofloxacin resulted in upregulation by 2.31 ± 0.311 and 2.60 ± 0.68-fold (Figure 5.9b). In PAO1 and TP-10, ceftazidime treatment resulted in *lon* gene expression by 9.45 ± 0.42 and 5.01 ± 0.02-fold increase (Figure 5.9c). Further, in TP-10 on ceftazidime treatment *higA* gene was elevated by 2.24 ± 0.12-fold while in ST-13 on all three-antibiotic treatments there was upregulation by 1.91 ± 0.68, 3.50 ± 0.34, and 2.24 ± 0.48-fold (Figure 5.9d). In addition, in ST-13 biofilm on ceftazidime and ciprofloxacin treatment there was an expression of *higB* gene by 2.50 ± 1.05, and 2.09 ± 0.68-fold high (Figure 5.9e).

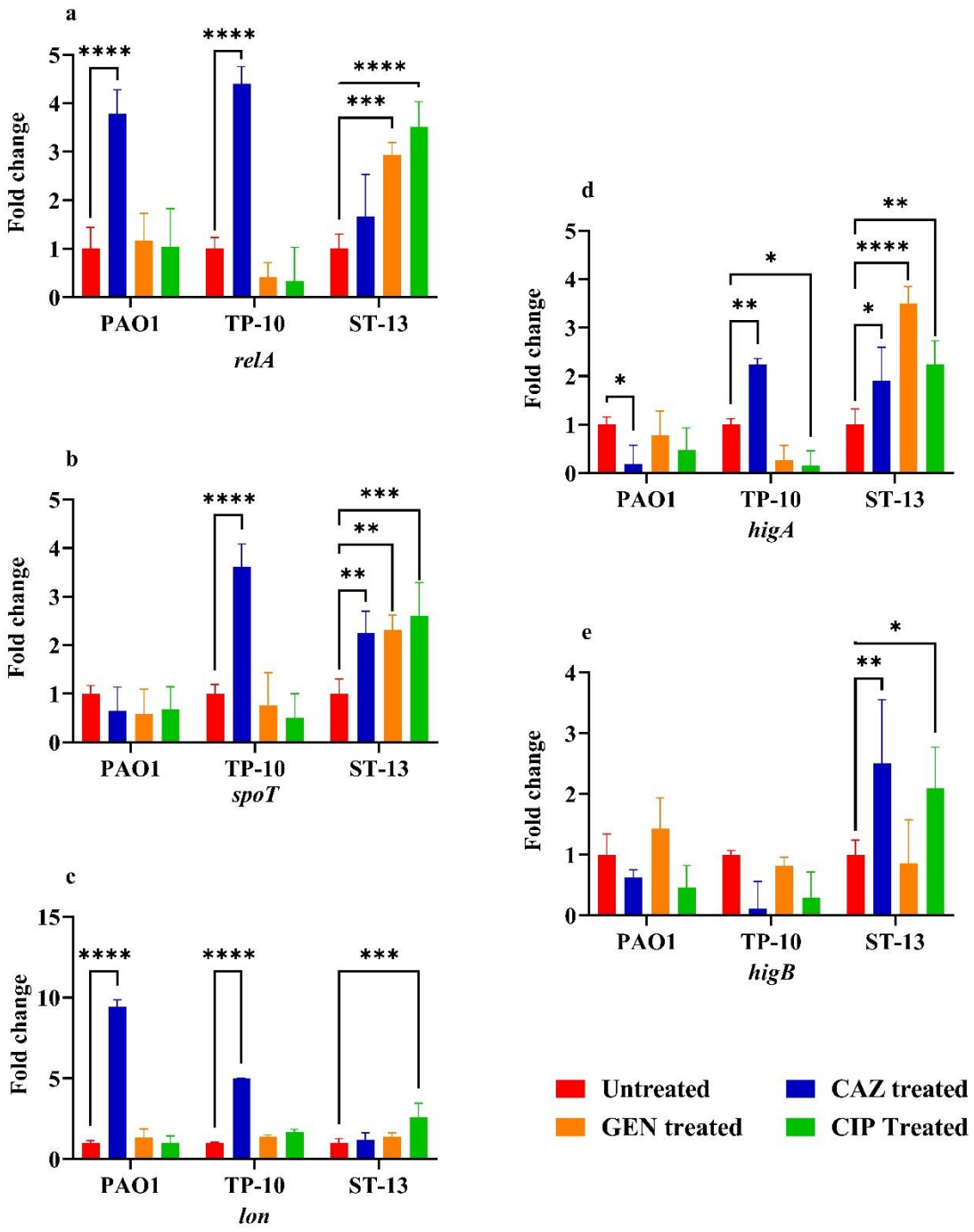

Figure 5.9: The gene expression studies in the biofilm stage. Biofilm was formed in the 6-well plates for expression studies. The gene expression studies were carried out after 4 hours of antibiotic (CAZ, GEN, and CIP) treatment in PAO1, TP-10, and ST-13 biofilm. The following gene expression of (i) stringent response genes; (a) *relA*, (b) *spoT*, and (c) *lon* in addition to (ii) TA system genes: (d) *higA* and (e) *higB* was carried out. The data normalization was done with untreated control. The gene expression studies were carried out in three biological triplicates for each strain. The corresponding data

represents as mean ± standard deviation. Two-way ANOVA was used for statistical analysis were *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001.

b) To study the biofilm-mediated antibiotic resistance through *brlR*

5.3.10 Whole genome sequence analysis

The gene sequence of *sagS*, *brlR*, efflux pump (*mexAB-oprM* and *mexEF-oprN*) genes, and their negative and positive regulators were analyzed for mutations and all mutations found in the individual genes are listed in Table 5.11. Through analysis, it was observed that TP-10 and TP-11 isolates have few silent and few synonymous mutations in the efflux pump and their regulator genes. A combination of both transitional and transversional point mutations, either silent or leading to changes in amino acid substitutions were observed in *brlR, mexA, mexB, nalC, mexE, nalD* in TP-10 and having *sagS*, *brlR, mexA, mexB, nalC, mexE* in TP-11 isolate. However, in it was also observed that *sagS* gene was found to be truncated in TP-10 isolate with deletion in DNA sequences from 1110-1560 position (Figure 5.10). Additionally, TP-11 had *nalD* gene mutated having 2 base pair deletion mutation at 398-399 position resulting in truncated amino acid sequence as shown in Figure 5.11.

Table 5.11: Summary of genetic analysis of mutations in genes in clinical isolates

Gene	Sample	Nucleotide	Amino acid
sagS	TP-10	^{279}G→C, ^{282}C→T, ^{465}C→G, ^{582}T→C, ^{903}T→C, ^{1026}G→A, ^{1029}A→G, $^{1110\text{-}1560}$bp deletion, ^{1770}T→C, ^{1867}C→T, ^{2151}A→G, ^{2298}T→C ^{2334}T→C	Truncated protein
	TP-11	^{282}C→T, ^{294}G→A, ^{465}C→G, ^{582}T→C, ^{654}C→T, ^{861}A→G, ^{903}T→C, ^{1578}G→A, ^{1770}T→C, ^{1867}C→T, ^{2151}A→G, ^{2298}T→C, ^{2334}T→C	^{155}H→Q
brlR	TP-10	^{16}C→A, ^{510}T→C, ^{549}C→T, ^{615}A→G	^{6}Q→K
	TP-11	^{75}C→A, ^{483}C→T, ^{537}C→T, ^{573}G→A, ^{615}A→G	-

mexA	TP-10	^{117}G→A, ^{333}T→C, ^{345}A→G, ^{447}G→A, ^{678}G→T, ^{697}C→T, ^{729}T→C, ^{1002}T→C	-
	TP-11	^{117}G→A, ^{333}T→C, ^{345}A→G, ^{447}G→A, ^{655}T→C, ^{678}G→T, ^{729}T→C, ^{997}C→T	-
mexB	TP-10	^{495}A→G, ^{567}C→T, ^{804}A→C, ^{1749}T→C, ^{1938}A→G, ^{2067}T→C, ^{2079}A→G, ^{2226}T→C, ^{2280}T→C, ^{2730}T→C, ^{2892}A→G, ^{2949}C→T, ^{2955}C→T, ^{3117}G→A	-
	TP-11	^{198}G→A, ^{315}C→T, ^{351}A→G, ^{495}A→G, ^{804}A→C, ^{1749}T→C, ^{1938}A→G, ^{2067}T→C, ^{2079}A→G, ^{2226}T→C, ^{2280}T→C, ^{2730}T→C, ^{2892}A→G, ^{2949}C→T, ^{2955}C→T, ^{3120}G→T, ^{3121}T→G, ^{3122}C→A, ^{3123}C→G, ^{3125}T→C	^{1041}S→E, ^{1042}V→A
nalC	TP-10	^{12}T→G, ^{69}T→C, ^{434}C→T, ^{444}T→C, ^{447}T→C, ^{625}A→C	^{145}A→V, ^{209}S→R
	TP-11	^{12}T→G, ^{69}T→C ^{444}T→C, ^{447}T→C, ^{625}A→C	^{209}S→R
nalD	TP-10	^{135}C→A	-
	TP-11	^{135}C→A, ^{398,399}2bp mutation	Truncated protein
mexE	TP-10	^{180}G→A, ^{189}G→A, ^{213}G→A, ^{252}A→C, ^{498}G→A, ^{540}C→T	
	TP-11	^{23}C→T, ^{180}G→A, ^{189}G→A, ^{213}G→A, ^{236}C→G, ^{252}A→C, ^{498}G→A, ^{567}C→T, ^{691}G→A, ^{723}G→C	^{8}S→F, ^{79}A→G, ^{231}A→T

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TP11_sagS       GTGCTCGACCTCTCCAAGTTCGAGGCCGGGCAACTGGTCCTGGAACAGATCCCGTTCGAC
TP10_sagS       GTGCTCGACCTCTCCAAGTTCGAGGCCGG------------------------------
PAO1_sagS       GTGCTCGACCTCTCCAAGTTCGAGGCCGGGCAACTGGTCCTGGAACAGATCCCGTTCGAC
                ****************************

TP11_sagS       CTGGGCGTCCTGGTGGAAGACACCGCCAGCCTCCTCTCGCAGAACGCCGCGGCCGGCGTG
TP10_sagS       ------------------------------------------------------------
PAO1_sagS       CTGGGCGTCCTGGTGGAAGACACCGCCAGCCTCCTCTCGCAGAACGCCGCGGCCGGCGTG

TP11_sagS       GAGTTGACCTGCCTGGTCGACCCCGCCCTGCCCGCCCAGGTTTCCGGCGACCCGACGCGG
TP10_sagS       ------------------------------------------------------------
PAO1_sagS       GAGCTGACCTGCCTGGTCGACCCCGCCCTGCCCGCCCAGGTTTCCGGCGACCCGACGCGG

TP11_sagS       ATTCGCCAGGTCGTCAGCAACCTGCTGTCCAACGCGCTGAAGTTCACCCGCCTGGGGCGG
TP10_sagS       ------------------------------------------------------------
PAO1_sagS       ATTCGCCAGATCGTCAGCAACCTGCTGTCCAACGCGCTGAAGTTCACCCGCCTGGGGCGG

TP11_sagS       GTCGACGTTCGCGTGGAAGCCACCGCCGAGGGTGTGCGCATCAGTGTGCGCGACACCGGC
TP10_sagS       ----------------TGCCGCCGTATTGG------------------------------
PAO1_sagS       GTCGACGTTCGCGTGGAAGCCACCGCCGAGGGTGTGCGCATCAGTGTGCGCGACACTGGC
                               :***.*** . :**

TP11_sagS       ATCGGCATCGCCCAGGAGGCGCTGGATCGCATCTTCCAACCGTTCACCCAGGCCGACGCG
TP10_sagS       ------------------------------------------------------------CG
PAO1_sagS       ATCGGTATCGCCCAGGAGGCGCTGGATCGCATCTTCCAACCGTTCACCCAGGCCGACGCG
                                                                          **

TP11_sagS       GGTATCACCCGCCAATACGGCGGCACCGGCCTCGGCCTGGCCCTCACGCGCAAGCTGTGC
TP10_sagS       GGTGATACCCG------------------------------------------------
PAO1_sagS       GGTATCACCCGCCAATACGGCGGCACTGGCCTCGGCCTGGCCCTCACGCGCAAGCTGTGC
                ***.: *****

TP11_sagS       GAGGCCATGCAGGGCGAACTGACGGTGGAGTCGACGGTCGGCCTGGGCAGCCTGTTCAGC
TP10_sagS       ------------------------------------------------------------C
PAO1_sagS       GAGGCCATGCAGGGCGAGCTGACGGTGGAGTCGACGGTCGGCCTGGGCAGCCTGTTCAGC
                                                                          *
```

Figure 5.10: Gene alignment of *sagS*. Schematic representation of *sagS* gene alignment in PAO1, TP-10 (having *sagS* gene truncated), and TP-11. The alignment was through MegaX software.

```
PAO1_nalD       GAACTGCGCGAGGCGCAGGAACGCAACAACGCCTTCGTGCAGATGTTCATCGAACTCTGC
TP-11           GAACTGCGCGAGGCGCAGGAACGCAACAACGCCTTCG--CAGATGTTCATCGAACTCTGC
TP10_nalD       GAACTGCGCGAGGCGCAGGAACGCAACAACGCCTTCGTGCAGATGTTCATCGAACTCTGC
                ************************************  ********************
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Figure 5.11: Gene alignment of *nalD* gene. Schematic representation of *nalD* gene alignment in PAO1, TP-11 (having 2bp mutation), TP-10. Gene alignment was done using MegaX software.

5.3.11 MIC determination

MIC was determined for each antibiotic as shown in below Table 5.12, as per CLSI guidelines. The PAO1 was found to be susceptible to gentamicin while it was resistant to ciprofloxacin antibiotics. Whereas TP-10 isolate was susceptible to both (gentamicin and ciprofloxacin) antibiotics while TP-11 isolate was highly resistant to both antibiotics.

Table 5.12: MIC determination

MIC	GEN (µg/ml)	CIP (µg/ml)
PAO1	0.5	8
TP-10	0.5	0.0625
TP-11	64000	32

GEN: gentamicin; and CIP: ciprofloxacin antibiotics used in this study. According to CLSI guidelines, the clinical breakpoints are as follows: (I) ≥16 µg/mL, and (R) for gentamicin (GEN); ≤1 µg/mL (S), 2 µg/mL (I) ≥4 µg/mL, and (R) for ciprofloxacin (CIP); R: resistant strains; S: Sensitive strains; I: Intermediate

5.3.12 Biofilm quantification

Biofilm quantification was done according to Stepanovic *et al* 2004 (Stepanović et al. 2004), where biofilm formation ability of isolates was categorized into strong, moderate, and weak biofilm producers. Figure 5.12 shows TP-10 is weak biofilm producer while TP-11, and PAO1 are strong biofilm producers. There was a significant difference between strong and weak biofilm producers as well as between strong biofilm producers.

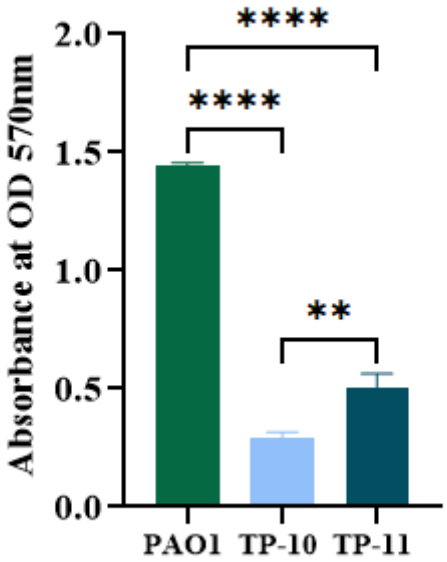

Figure 5.12: Biofilm quantification. Biofilm quantification of PAO1, TP-10 and TP-11. The experiment was performed in three biological triplicates for each strain. The data represents mean and standard deviations. The statistical significance represents **p<0.01 and ***p<0.001 (one-way ANOVA)

5.3.13 Biofilm antibiotic susceptibility testing

Biofilm susceptibility was carried out for PAO1, TP-10, and TP-11 biofilm formed for 24 hours and treated with gentamicin and ciprofloxacin antibiotic at 5X MIC level for 2 hours. Figure 5.13 represents the biofilm susceptibility in log cfu/ml. There was no significant difference found in biofilm log cfu/ml (PAO1, TP-10, and TP-11) in any of the antibiotic treatments compared to 0 hours.

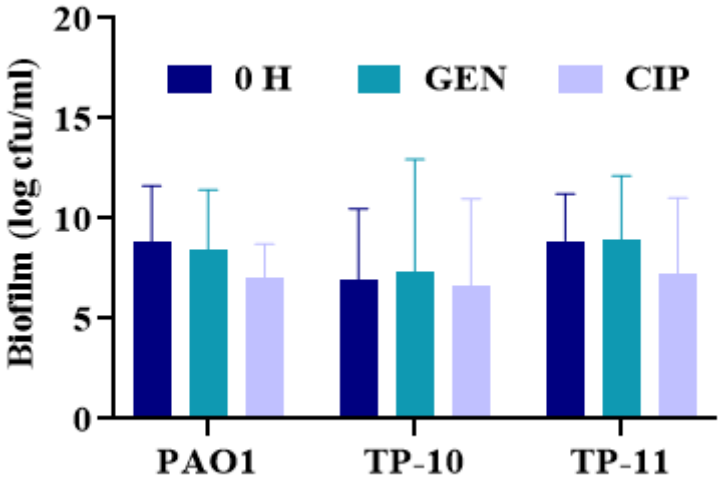

Figure 5.13: Biofilm susceptibility assay. Biofilm antibiotic susceptibility for PAO1, TP-10, and TP-11 biofilm after 2 h of antibiotic treatment prior to it biofilm grown for 24 hours. After treatment, biofilm log10 cfu/ml was plated on LA for the viable count. The experiment was performed in three biological triplicates for each strain. The statistical significance represents ns $p>0.05$.

5.3.14 Pyocyanin quantification

Pyocyanin quantification was done as described by (Essar et al., 1990), in the 6-well plate after 2 hours of antibiotic treatments. Figure 5.12 represents the pyocyanin quantification in PAO1, TP-10, and TP-11 biofilm. In PAO1 and TP-10 isolate pyocyanin production was decreased after antibiotic treatment of 2 hours compared to untreated control (Figure 5.14). It was found that TP-11 isolate produced high levels of pyocyanin in growth control as well as in antibiotic treatment (gentamicin and ciprofloxacin). There was a significant difference in pyocyanin production after 2 hours of antibiotics (Gentamicin and ciprofloxacin) treatment compared to untreated control Figure 5.14.

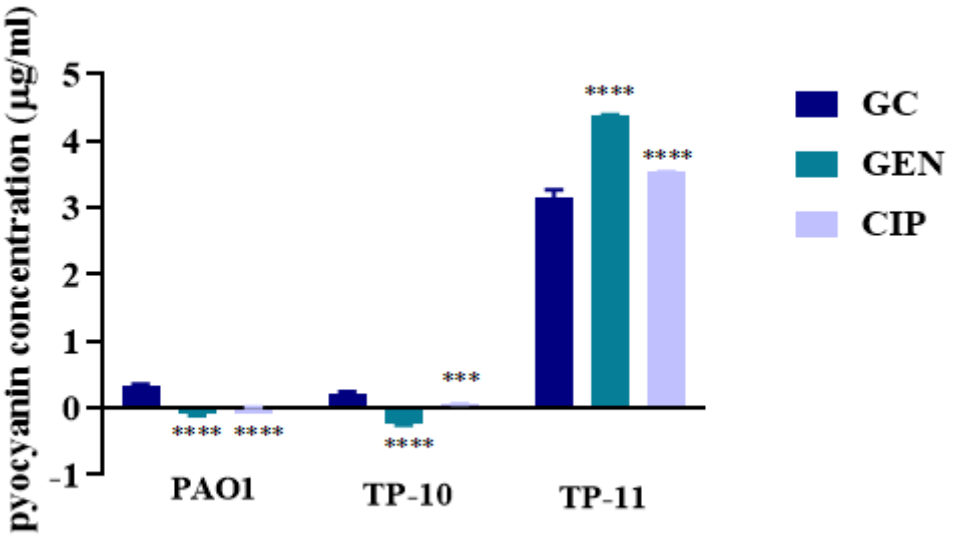

Figure 5.14: Pyocyanin quantification. Pyocyanin quantification of PAO1, TP-10 and TP-11 biofilm. The experiment was performed in three biological triplicates for each strain. One-way ANOVA was used for statistical difference representation were represents ****p<0.001 and ***p<0.01.

5.3.15 Gene expression of biofilm-mediated antibiotic resistance genes

It is well known that SagS contributes to BrlR-dependent biofilm drug tolerance, by increasing the *brlR* transcript levels and also contributes to BrlR-DNA binding to its target promoters. In this study through WGS, it was found that the TP-10 isolate has Δ*sagged* and has no mutation in *brlR*, *mexA*, *mexF*, *nalD,* and *nalC* genes while the TP-11 isolate has Δ*nalD* and has functional *sagS*, *brlR*, *mexA*, *mexF* genes. Figure 5.13 shows the gene expression of *sagS, brlR*, *mexA*, *mexF, nalC, nalD* in PAO1 and TP-11 biofilm after respective antibiotic treatments. The relative gene expression was compared with TP-10 (Δ*sagS*) in growth control (untreated) and both antibiotic treatments. The transcript levels of *sagS* in TP-11 were 6.7-fold high in untreated as well as in gentamicin and ciprofloxacin treated were 19.1-, and 7.7-fold as compared to TP-10 (ΔsagS) (Figure 5.15a). The *brlR* transcripts level in TP-11 was 3.4-, 12-, and 15-fold high in untreated, gentamicin and ciprofloxacin, where the transcript levels were statistically significantly high compared to TP-10 (Figure 5.15b). Also, there was 2.7-, and 2.2-fold upregulation of efflux pump *mexA* and *mexF* gene on gentamicin and ciprofloxacin treatment (Figure 5.15c, and d). There was no upregulation of *nalD* gene observed in TP-11 (Δ*nalD*)

(Figure 5.15e). However, another negative regulator *nal*C was 2.3-, and 6.9-fold upregulated in untreated and ciprofloxacin treatment (Figure 5.15f).

In PAO1 isolate, there was no significant upregulation of *sagS* (Figure 5.15a), however *brlR* levels increased by 11-fold only in ciprofloxacin treatment (Figure 5.15b). Further, there was no upregulation of efflux pump (*mexA* and *mexF*) as well as negative regulator *nalD* gene in any of antibiotic treatments (Figure 5.15c, and d). One of all the negative regulators, *nalC* was found to be upregulated by 3-fold in untreated (Figure 5.15f).

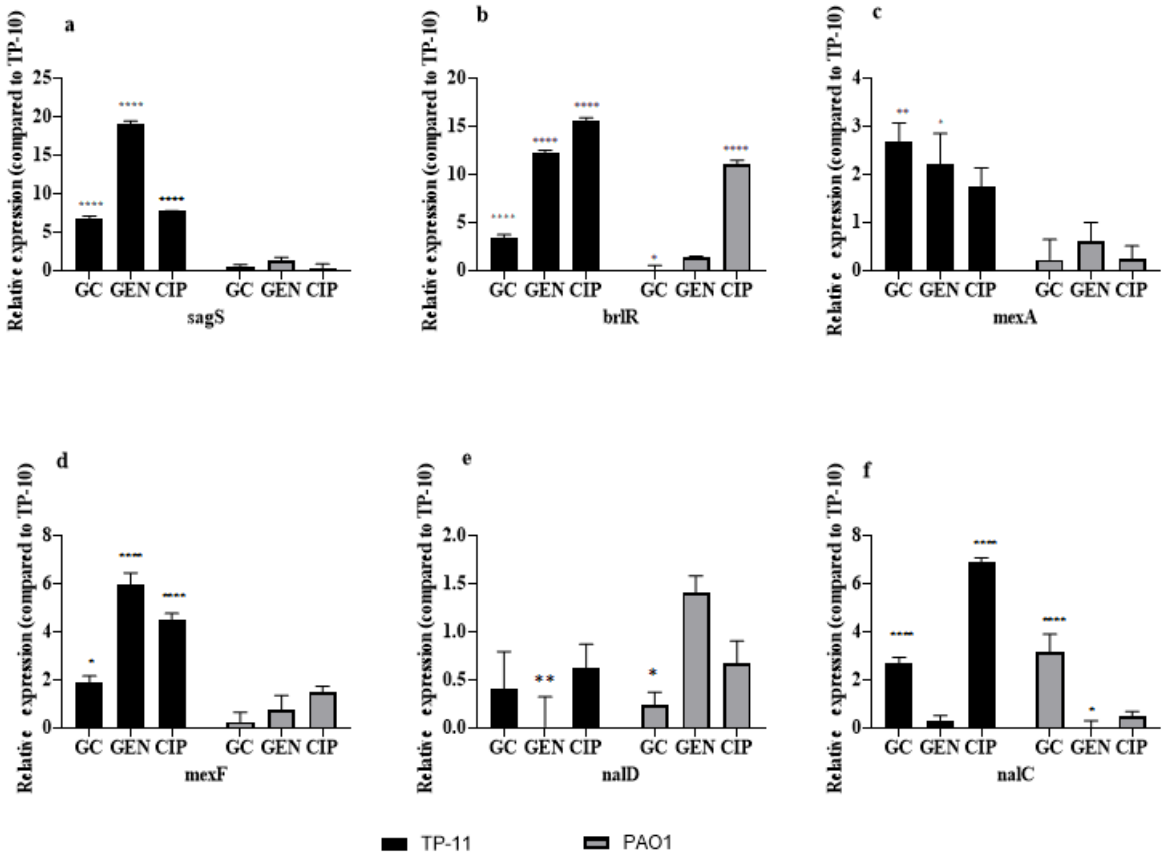

Figure 5.15: Gene Expression studies of biofilm-mediated antibiotic resistance. Relative Gene expression of (a) *sagS*, (b) *brlR*, (c) *mexA*, (d) *mexF*, (e) *nalD*, and (f) *nalC* in PAO1 and TP-11 (ΔnalD) biofilms. The data normalization was done TP-10 biofilm. The experiment was performed in triplicates for each strain. Two-way ANOVA was used for the statistical significance where p value represents as *p<0.5, **p<0.01, ***p<0.001 and ****p<0.0001.

5.4 Discussion

a) To study the effect of antibiotics in persister cell formation

P. aeruginosa poses a serious threat to human health, especially in hospital settings owing to the form of antibiotic-tolerant biofilms (Clark et al. 2018; Sweere et al. 2019; Tarafder et al. 2020). Within the deepest layers of biofilms resight a subset of metabolically inactive cells known as PC. This subpopulation is capable of a phenotypic switch, survives antibiotic exposure, and is one of the reasons for antibiotic treatment failure. Studies have shown that PC formation is a complex mechanism and depends on numerous factors such as the growth phase of bacteria, gene expression of the stringent response, TA system, QS, and SOS response (Soares et al. 2020). Many studies have highlighted PCs formation in the typed strain of *P. aeruginosa* (PAO1 and PA14) upon antibiotic exposure (Viducic et al. 2006; Coskun et al. 2018; Soares et al. 2019). In the present study, PC formation was studied in PAO1, TP-10 and ST-13 strains on exposure to three different antibiotics (ceftazidime, gentamicin, and ciprofloxacin) from distinct under two physiological stages (planktonic and biofilm). The impact of the different classes of antibiotic treatment on PC formation was assessed by measuring cellular redox activity, gene expression of the stringent response, and TA systems genes in planktonic as well as biofilm stages.

In the present study, during the planktonic stage biphasic kill curve was observed in all isolates on ceftazidime treatment, indicating the formation of PCs. Although, the biphasic kill curve varied across the isolates on gentamicin and ciprofloxacin treatments (Figure 5.1). Spoering and Lewis group observed similar findings that β-lactam (carbenicillin) treatment had a greater number of survival fractions than fluoroquinolone (ofloxacin) and aminoglycoside (tobramycin). The persister cell formation was observed on ciprofloxacin and colistin treatment which varied across the POA1 and clinical isolate (Golmoradi Zadeh et al. 2022). Also, on ciprofloxacin treatment, the cell survival rate was reduced as it kills non-growing cells and there was variability in survival fractions among the clinical isolates of *P. aeruginosa* (Brooun et al. 2000; Baek et al. 2020).

Further, RSG staining was used to confirm the PC formation where variations in cellular redox activity were observed among isolates and high redox activity was observed for ceftazidime treatment which was followed by ciprofloxacin and gentamicin (Figure 5.2). The variation in redox activity might be due to variation observed in survival fraction (CFU/ml data) (Figure 5.1). In addition, fluorescence microscopy using RSG staining revealed that ceftazidime

treatment had the filamentous formation of PCs having high redox activity in the planktonic stage (Figure 5.3). The filamentous formation observed on ceftazidime treatment inhibits septal division by binding to Penicillin Binding Protein 3 (PBP3) after doubling cells (Buijs et al. 2008). The PBP 2 and PBP 3 are aided in the formation of the bacterial cell wall and inhibition of these proteins leads to spherical or filamentous morphology of cells. Most recent studies on PCs in *E. coli* reported that filamentous formation of PC having high reactive oxygen species (ROS) on ampicillin treatment (Sulaiman and Lam 2020), while rod-shaped metabolically inactive cells observed on rifampicin treatment (Kim et al. 2018) and elongation of cells were reported (Völzing and Brynildsen 2015). Further, colistin treatment leads to elongation, and the cell wall/membrane was damaged while in amikacin treatment DNA condensation was observed in persister, and on ciprofloxacin treatment, outer membrane vesicles were observed through TEM analysis in *P. aeruginosa* (Baek et al. 2020). These suggest that morphological changes observed above in the PC formation might depend on the antibiotic used and/or environmental stress they are subjected to.

For PCs formation, the well-characterized elements in *P. aeruginosa* of stringent response and persistence include (p)ppGpp alarmone, RelA, SpoT, DksA, and TA systems which all together arrest the cell growth by directly interacting with RNA polymerase which leads to halt in transcription process and provides fitness advantage (Nguyen et al. 2011; Moradali et al. 2017). In the present study, the variation of gene expression of stringent response (*relA*, *spoT,* and *lon*) as well as TA system (*higA* and *higB)* was observed across the isolates (PAO1, TP-10 and ST-13) on treatment with different antibiotics (Figure 5.4). A recent study reported that on treatment with amikacin, colistin, ciprofloxacin, and cefepime in the mid-exponential phase there was upregulation of *relA* gene in *P. aeruginosa* clinical isolates (Baek et al. 2020). In the same study, there was upregulation of *spoT* gene on colistin treatment and no regulation of the gene was observed on other treatments. The most well studied type II TA system HigBA in *P. aeruginosa* have reported to prevalent among clinical strains of *P. aeruginosa* (Williams et al. 2011; Andersen et al. 2017). These TA systems are also found on IncC plasmid and are associated with antimicrobial resistance genes (Qi et al. 2021). In this study, *higA* and *higB* were differentially expressed on treatment with different antibiotics across the isolates (Figure 5.4 d, and e). The study by Li *et.,* reported that *higB* gene was elevated on sub-MIC exposure to ciprofloxacin and thus increasing the PC formation by 1000 times in planktonic culture (M. Li et al. 2016). The same study also reported activation of HigB toxin upregulates the T3SS and bacterial cytotoxicity. These TA systems affect the DNA gyrase activity, regulate ribosome

maturation, and inhibit metabolism as well as growth to enhance PC formation (Page and Peti 2016). Further, on gentamicin treatment, Lon protease gets activated which degrades HigA antitoxin and activates the HigB toxin. The free HigB toxin now can bind to HigA decreasing its DNA binding ability and thus increasing the expression of *higB* toxin (Marr et al. 2007; M. Li et al. 2016). The HigB now repress the swarming motility, virulence motility and biofilm motility (Wood and Wood 2016). Recent studies have shown HigA represses the *mvfR* (one of the **m**ultiple **v**irulence **f**actor **r**egulators) gene expression virulence genes in the planktonic stage (Guo et al. 2019). Apart from this, HigA regulates various processes such as biofilm formation, ion uptake, and carbon metabolism. In this study, there was an increase in the expression of *relA, spoT, higA,* and *higB* genes in susceptible isolate (TP-10) however in resistant isolate *spoT* and *lon* genes were expressed (Figure 5.5). In susceptible isolates, the stringent response and TA system are thought to have been activated, leading to PC formation.

This study also examines the PC development in biofilm in relation to a different class of antibiotic treatment at supra-MIC levels. The opportunistic pathogen *P. aeruginosa* is frequently associated with biofilm-related infections. These biofilm formed are difficult to eradicate due to high tolerance to many antibiotics (Costerton et al. 1999; R. M. Donlan 2001). One of the biofilm tolerance mechanisms known is PC formation (Rahbari et al. 2017) and thus causing recurrent biofilm-related infection. The phenotypic switch to persister phenotype within the *P. aeruginosa* biofilm is not fully elucidated at supra-MIC levels of antibiotics. The PC formation was studied using a biofilm kill curve where on ceftazidime treatment biofilm was regrown as control. The biphasic killing pattern was observed for gentamicin and ciprofloxacin and varied across the isolates (Figure 5.6). Studies have shown quinolones, colistin, and aminoglycosides are effective in killing based on concentration or time-dependent killing at certain MBIC/MIC levels (Hengzhuang et al. 2011; Wang et al. 2012; Hengzhuang et al. 2013; Macià et al. 2014). The antibiotic exposure leads to the killing of the subset of cells and biphasic killing was observed where a subset of cells remained in the biomass of biofilm. The PC formations in *P. aeruginosa* biofilm have been studied using CLSM with Syto9 (stains live cells) and PI (stains dead cells) dye where a high concentration of azithromycin leads to filamentation of cells within the biofilm, disruption of biofilm and regrowth of biofilm was also observed (Rojo-Molinero et al. 2016). In addition, aminoglycoside treatment leads to a deep decline in biofilm biomass, and in the inner layer of the biofilm live cells were observed (Rojo-Molinero et al. 2016). These might be due to oxygen restriction, lower antibiotic penetration through biomass, and low metabolic activity within the inner layers of biofilm

(Hatch and Schiller 1998; Walters III et al. 2003; Borriello et al. 2004). On the other hand, fluoroquinolone treatment especially ciprofloxacin leads to a deep decline in the biofilm, disruption of biomass, and dead cells (Soares et al. 2019). As ciprofloxacin causes vacuole formation and cell lysis, which leads to intracellular components being extruded (Elliott et al. 1987). In this study, the biofilm thickness varied across the three isolates (PAO1, TP-10, and ST-13), and also the thickness varied across the strong biofilm producers (Figure 5.5). These might be due to the variations in cell adhesion to the surface and twitching motility across the isolates behavior (Patel and Gajjar 2022). In addition, the survival rates of the isolates demonstrated unpredictability when antibiotics with a different mode of action were employed. This finding suggests that variation could be due to difference in antibiotic penetration rate due to varying biofilm thickness across the isolates. It has previously been shown that other bacteria exhibit a similar wide difference in PC formation among isolates in response to various antibiotics (Gallo et al. 2017; Rahbari et al. 2017; Drescher et al. 2019). This shows that persistence depends not only on the type of antibiotics used, but also on the growth phase and the heterogeneity of the isolates.

Further expression of stringent response genes (*relA, spoT,* an*d lon*) and TA system (*higB*, and *higA*) varied across the isolates (Figure 5.9). The stringent response and TA system genes were significantly upregulated on ceftazidime treatment followed by gentamicin (Figure 5.8). Studies have reported an increased susceptibility to fluoroquinolones, meropenem, colistin, and gentamicin was seen in biofilms produced by a *P. aeruginosa relAspoT* double knockout mutant (Nguyen et al. 2011). In this study, despite enhanced *lon* expression on ceftazidime treatment, however, the *higA* expression was reduced and no change in the expression of *higB* gene was observed in the PAO1 biofilm (Figure 5.9 c-e). This suggests that *higB* and *higA* at the transcriptional level are controlled by the unknown transcriptional regulator during the biofilm stage and similar findings were reported by Song et al 2020 on azithromycin antibiotic (Song et al. 2020). Furthermore, the persister level was unaffected on ceftazidime treatment through lon protease activation (Chowdhury et al. 2016). When gentamicin exposure to *P. aeruginosa* biofilm activates the Lon protease, which further degrades the HigA anti-toxin and in turn activates the transcription of *higB* toxin (Marr et al. 2007; M. Li et al. 2016). Another study reported that during the late stationary phase gentamicin exposure of about 30 minutes leads to the upregulation of *higB* gene due to HigA protein being degraded by Lon protease (Guo et al. 2019). On 1 hour of ciprofloxacin exposure to *P. aeruginosa* biofilm also upregulated the stringent response genes (*relA*, *spoT,* and *lon*) and toxin-antitoxin gene *higBA.* (Soares et al.

2019). The same study also reported the constant expression of the stringent response gene after 4 hours and no change in *higB* gene. Further, HigA levels are controlled transcriptionally, and post-transcriptionally are regulated by a concentrated-dependent DNA binding manner. Further, they are modified by making complexes with HigB. A recent study has shown the decrease in the level of HigA due to antibiotic stress/ cytotoxicity can upregulate the *pslO*, *PA2440,* and *pelA* genes of biofilm formation (Song et al. 2020). Which enables PC formations in biofilm as well as the survival of these cells within host cells. We hypothesize that the persistence within the biofilm might be strain-specific, depending on biofilm development and the degree of antibiotic exposure/penetration within the biofilm. This study provides new findings in PC development in clinical isolates of *P. aeruginosa*. Thus, complete transcriptomic studies would give a better informative idea of molecular mechanisms in PC formation.

In summary, ceftazidime treatment leads to PC formation in the planktonic stage compared to other antibiotic treatments, however, in the biofilm stage PC formation was observed on gentamicin and ciprofloxacin treatments. Considering this study, it is possible the production of PCs in response to various antibiotics was influenced in distinct ways by the type II TA (HigBA) system and the stringent responses.

b) To study the biofilm-mediated antibiotic resistance through *brlR*

P. aeruginosa is capable of causing chronic bacterial-related infections and exceptionally difficult to eradicate with antibiotic treatments due to the high degree of tolerance/resistance of cells within the biofilm. The biofilm resistance is multifactorial and mediated via the expression of resistance gene preferentially in the biofilm state than the planktonic (exponential phase) state. One such mechanism known is *brlR,* which is known to express in biofilm state (Mah et al. 2003; Zhang and Mah 2008; Liao et al. 2013; Zhang et al. 2013; Hall and Mah 2017b).

It is known that SagS contribute to drug tolerance via the activation of *brlR* through c-di GMP, thus activated *brlR* upregulates the efflux pump genes in biofilm mode (Gupta et al., 2013). Further, the present study focuses on efflux pumps (*mexAB-oprM* and *mexEF-oprM*) and their regulators in biofilm drug tolerance with different antibiotics. Recent studies have found that the periplasmic sensory HmsP domain is a control point for biofilm development as well as biofilm tolerance. Further, within HmsP domain have distinct residues of amino acids that contribute to biofilm development and biofilm tolerance. The same study also showed the SagS variants having alanine substitution with D105, R116 and L126 residues were unable to restore

susceptibility to tobramycin resulting in reduced *brlR* abundance in biofilm (Dingemans et al. 2018). In this study, the TP-10 isolate was found to possess truncated *sagS* (Surface attached growth sensor) and no mutations in TP-11 whereas in the TP-11 isolate *nalD* gene had a 2 bp deletion mutation. However, in TP-10 and TP-11 the above-mentioned residues were the same. It is known that substitution of L154 residue with alanine reduced attachment as well as mature biofilm formation as compared to wild type. The biofilm formation as well as attachment (data not shown) was less in the TP-10 isolate though there was no substitution in L154 residue. This can be due to the truncated *sagS* in TP-10. The biofilm formation was more in TP-11 as compared to TP-10 as there was no mutation in the *sagS* gene.

Further, log reduction was observed in *ΔsagS* biofilm on treatment with tobramycin and norfloxacin in a 3-day-old biofilm (Gupta et al. 2013). However, we did not observe any log reduction on treatment with gentamicin and ciprofloxacin at 5X MIC concentrations in PAO1, TP-10, and TP-11 biofilm (Figure 5.13). No log reduction observed in this study might be due to differences in experimental conditions.

SagS has been shown to modulate the levels of c-di-GMP which directly or indirectly activates the BrlR which leads to drug tolerance (Park & Sauer, 2021). Further, in presence of glucose-6-phosphate the levels of *sagS* and *brlR* were increased (Park et al., 2021). However, in presence of antibiotic treatment, the gene expression of *sagS* studies has not been studied. Gupta *et al* 2013 studied that ΔsagS biofilm cells harbor reduced levels of brlR promoter activity and promoters of multidrug efflux pump operon, mexAB-open, and mexEF-oprN (Gupta et al., 2013). In this study, it was observed that in TP-11 (compared to TP-10) the relative gene expression of *sagS* and *brlR* was significantly increased in the present growth control, gentamicin, and ciprofloxacin treatment (Figure 5.15a and b). Also, the relative gene expression of *mexA* and *mex F* was upregulated in gentamicin and ciprofloxacin treatment, and no upregulation of the negative regulator *nalD* gene (which 2 bp deleted) (Figure 5.15 c and d). Wang *et al*., 2018 have reported pyocyanin and c-di-GMP enhance the BrlR and BrlR-DNA binding of the efflux pump which leads to drug tolerance. In this study, TP-11 biofilm had high pyocyanin production after antibiotic treatment of 2 h which can be one of the reasons for drug tolerance (Figure 5.14). The limitation of this study is the quantification of c-di-GMP is not done. It is also previously reported that disruption in *nalD* gene increases efflux pump genes (*mexAB-oprM*) expression in the planktonic stage (Morita et al., 2006; Sobel et al., 2005). However, there are no reports regarding effects of negative regulator mutation with respect to biofilm drug tolerance

It has been reported that SagS not only directly contributes to the motile-sessile switch to enable *P. aeruginosa* biofilm formation but also contributes indirectly to both *brlR* expression and BrlR-DNA binding and enhance the biofilm-specific mechanism of drug tolerance in *P. aeruginosa*. From the above study, it is can be concluded that TP-11 has more drug tolerance due to more gene expression of *sagS, brlR, mexA, mexF* in both antibiotic treatments and GC as well as high pyocyanin production.