Glutathione Reductase Encoding Gene (*gor*) Is Associated with Oxidative Stress and Antibiotic Susceptibility in *Pseudomonas aeruginosa*

Running title: Roles of *gor* in Oxidative Stress and Antibiotic Susceptibility

Keywords: Glutathione reductase, oxidative stress, antibiotic susceptibility, *Pseudomonas aeruginosa*

ABSTRACT

Pseudomonas aeruginosa is a major causative agent of the hospital- and community-acquired infections. These infections are often antibiotic resistant and difficult to treat. Several intrinsic and acquired resistance mechanisms to antibiotics have reported in *P. aeruginosa*. Recently, oxidative-stress-scavenging-systems have suggested as a possible intrinsic resistance mechanism to antibiotics because oxidative stresses induced by bactericidal antibiotics contribute to bacterial killing effects. However, this remains controversial such that further clarification is required. Glutathione reductase is a key enzyme in the maintenance of the optimum level of intracellular glutathione-redox potential to ensure normal functioning of cellular processes including the detoxification of oxidative stress. In this study, the role of a glutathione-reductase-encoding gene (*gor*) in oxidative stress and antibiotic susceptibility was determined in *P. aeruginosa*. Results showed that a *gor*-mutant strain was more susceptible to hydrogen peroxide (but not superoxide) than the parental strain and 100% of cells were killed with 0.01% hydrogen peroxide while the parental strain survived at the same concentration of hydrogen peroxide. The *gor*-mutant strain was also more susceptible to carbenicillin, chloramphenicol, ciprofloxacin, and tetracycline than the parental strain, which was confirmed by bacterial killing-kinetics. These results suggest that the *gor* gene is associated with oxidative stress and susceptibility to bactericidal as well as bacteriostatic antibiotics and that the oxidative-stress-scavenging-systems may be a possible drug-target for multidrug resistant *P. aeruginosa*.

1. Introduction

Pseudomonas aeruginosa is a human pathogen causing nosocomial and community-acquired infections such as urinary tract, blood stream, burn, and pulmonary infections (Richards *et al.*, 1999, Matta *et al.*, 2018). Treatment of *P. aeruginosa* infections however is difficult due to the presence of intrinsic and acquired resistance to a variety of antibiotics (Falagas *et al.*, 2008, Karakonstantis *et al.*, 2020). Intrinsic and acquired resistance in *P. aeruginosa* are produced by multiple mechanisms such as enzymatic inactivation of drugs, alterations of drug targets, impermeability to the drug, and efflux of drugs (Zavascki *et al.*, 2010, Cerceo *et al.*, 2016). A single clinical isolate of multidrug resistant *P. aeruginosa* often harbors multiple drug resistant mechanisms (Zavascki *et al.*, 2010).

Antibiotics induce oxidative stresses (e.g., hydrogen peroxide, superoxide, and hydroxyl radical) in bacteria. Bactericidal antibiotics (e.g., β-lactams, aminoglycosides, and quinolones) induce hydroxyl radicals from hydrogen peroxide through the Fenton reaction (Kohanski *et al.*, 2007) whereas, in *E. coli,* bacteriostatic antibiotics (e.g., chloramphenicol) induce superoxide (Albesa *et al.*, 2004). Antibiotic-induced oxidative stresses damage cellular macromolecules and enhance antibiotic lethality (susceptibility) in addition to antibiotic-specific killing mechanisms (Van Acker & Coenye, 2017). Therefore, oxidative-stress-scavenging systems consider one of the intrinsic resistant mechanisms to antibiotics.

Cellular metabolism normally produces oxidative stresses in all aerobic organisms. In *E. coli*, the oxidative stresses activate OxyR and/or SoxRS, which induces the expression of a number of genes including glutathione (GSH) reductase and GSH peroxidase that neutralizes the oxidative stress

(Green & Paget, 2004). For example, hydrogen peroxide is reduced to water and oxygen molecules by GSH peroxidase using electrons from two molecules of GSH, and the two molecules of GSH are oxidized to form GSH disulfide (GSSG). The GSSG is toxic at high levels and reduced back to GSH by the GSH reductase using electrons from NADPH (Green & Paget, 2004, Smirnova & Oktyabrsky, 2005). In *E. coli*, the ratio of GSH/GSSG is estimated to be approximately 200 (>99% of GSH) in growing cells, which corresponds to a redox potential of -240 mV, assuming a total intracellular GSH concentration of 5 mM, pH 7.0, and 25ºC. This GSH-redox system plays a variety of cellular functions not only in detoxifying oxidative stresses but also in deactivation of toxic substances via GSH-conjugate formation (Couto *et al.*, 2016). Alterations of the GSH redox potential can impair the functions of GSH-redox system (Smirnova & Oktyabrsky, 2005). Therefore, the GSH-redox system is one of the oxidative-stress-scavenging systems and GSH reductase is a key component for this redox system.

These observations suggest that the GSH reductase is associated with oxidative stresses and antibiotic susceptibility. GSH reductase is a dimer composed by two identical subunits with a molecular mass of 55 kD, a member of the Flavin-containing enzyme, encoded by a gene (*gor*) in *E. coli* (Jiang *et al.*, 1995). The *gor* gene from *P. aeruginosa* PAO1 was cloned in *E. coli* (Perr *et al.*, 1991), but the roles of the *gor* gene in oxidative stress and antibiotic susceptibility are currently unclear. In this study, *gor* genes of *P. aeruginosa* (POA1 and MPAO1) were knocked-out and the roles in oxidative stress and antibiotic susceptibility were determined. The results revealed that the *gor*-mutant strains were more susceptible to hydrogen peroxide and antibiotics than their parental strains of *P. aeruginosa*.

2. Materials and Methods

2.1. Bacterial strains, growth conditions, and chemicals

P. aeruginosa PAO1 was obtained from the previous studies (Kwon & Lu, 2006). *P. aeruginosa* MPAO1 and a *gor*-mutant strain (PW4508; *gor*::TnTc) were obtained from the sequence-verified transposon mutant library (University of Washington, Seattle, WA). The bacterial strains routinely grew on Luria-Bertani (LB; Becton, Dickinson and Company, Sparks, MD) agar plates or broth at 37°C. The mutant strain grew in a minimal medium for *P. aeruginosa* containing glutamate as a sole carbon and nitrogen source as described (Kwon & Lu, 2006). All antibiotics, L-glutathione (reduced-glutathione), and other chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

2.2. Cloning and gene-knockout

Genomic DNA extracted from *P. aeruginosa* PAO1 was used to amplify a full length of a glutathione reductase encoding gene (*gor*) encompassing upstream (570-bp) and downstream (60-bp) of the *gor* gene (1980-bp: forward PCR-primer: 5'-cagggaatcggcagcgcct-3'; reverse PCR-primer: 5'-tgcgcgcgaaaatgaaagaa-3'). The PCR fragment was inserted into an *E. coli*/*P. aeruginosa* shuttle vector (named pAU250) and used to determine DNA sequence from the commercial service (GENEWIZ, South Plainfield, NJ). The gene-knockout experiment was performed as described previously (Kwon & Lu, 2006). Briefly, the cloned *gor* gene was inactivated by inserting a gentamicin-resistance gene cassette (Gm) from pGMΩ1 into a middle of the gene (*Eco*47III) and the knocked-out gene cassette (*gor*::Gm) was transferred into a suicidal conjugative vector (pRTP1). *E. coli* SM10 carrying the resulting plasmid was used as a donor strain and *P. aeruginosa* PAO1 was used for the recipient strain. Bi-parental conjugation was performed as described (Kwon & Lu, 2006) and the gene-replaced *P. aeruginosa* (*gor*::Gm) was selected on

LB agar plates containing gentamicin (80 μg/ml) for *P. aeruginosa* and chloramphenicol (15 μg/ml) for counter-selection against *E. coli* SM10. Authenticity of the gene-knockout was confirmed by PCR amplication of the upstream and downstream of the *gor* gene.

2.3. Glutathione reductase enzyme assay

Overnight-cultured cells were diluted (1/100) into fresh LB broth (30 ml) and grown on a rotary shaker (250 rpm) at 37ºC. The cells (5 ml) at optical density (OD_{600}) of 1.0 were washed three times with phosphate buffer (pH 7.0) and disrupted by sonication as described (Kwon *et al.*, 2013). The crude extract of the cells spun down at 15,000 rpm for 5 min and the supernatant used to measure levels of GSH reductase. The protein concentration of the crude extract was measured using the Coomassie Plus™ Protein Assay Reagent (Thermo Scientific, Rockford, IL). The amount of GSH reductase (units/mg protein) was determined using a commercial kit (Sigma-Aldrich, St. Louis, MO) as suggested by the vendor.

2.4. Determination of minimum inhibitory concentration (MIC) of hydrogen peroxide, paraquat, and antibiotics

MIC levels of hydrogen peroxide, paraquat, and antibiotics were determined as guided by the Clinical and Laboratory Standards Institute (CLSI) as described previously (Kwon & Lu, 2006) with a minor modification. Briefly, two-fold serial dilutions of hydrogen peroxide, paraquat, and antibiotics were performed using Mueller-Hinton broth (MHB; Oxoid, Ogdensburg, NY) and fresh overnight cultures of *P. aeruginosa* strains (~10^8 viable cells per ml) inoculated at each of the dilutions. The cells were incubated overnight without shaking at 37°C. MIC levels defined as the lowest concentration of hydrogen peroxide, paraquat, and antibiotics that completely inhibited

cellular growth of the inoculum. Determination of MIC levels was repeated three times to confirm the results.

2.5. Bacterial killing assay

Bacterial killing of *P. aeruginosa* strains were determined as previously described (Kanagaratnam *et al.*, 2017) with a minor modification. Briefly, hydrogen peroxide or antibiotics at different concentrations were added in MH broth (1 mL in the Falcon culture tubes) and fresh cultures of cells (~10^8 viable cells per ml) were inoculated into each of the concentrations. The cells were incubated at 37ºC without shaking for 18 hours and spread on plain LB agar plates with appropriate dilutions. The LB agar plates were incubated for 24 hours at 37ºC and colony-forming units (CFU) were counted per ml. The bacterial killing assay repeated three times to confirm the results.

3. Results

3.1. Mutant strains of *P. aeruginosa*

A mutant strain of *P. aeruginosa* PAO1 (*gor*::Gm) was constructed as described in the Materials and Methods. The mutant strain of *P. aeruginosa* MPAO1 (*gor*::TnTc) obtained from the mutant library (University of Washington, Seattle, WA) was confirmed by PCR-sequencing as suggested by the mutant library. The two mutant strains were used to determine GSH reductase activity with comparison to their parental strains. Results showed that the GSH reductase activity (units/mg protein) of the mutant strains was significantly lower than that of their parental strains [2.25±0.14 for PAO1 and 1.69±0.25 for MPAO1; 0.18±0.03 for PAO1 (*gor*::Gm) and 0.12±0.02 for MPAO1 (*gor*::TnTc)]. The growth rate of the *gor*-mutant strains was similar to their parental strains in the minimum medium (data not shown).

3.2. Effect of a GSH reductase-encoding gene (*gor*) on oxidative stresses

The two *gor*-mutant strains and their parental strains were used to determine levels of MIC against hydrogen peroxide and superoxide-producing paraquat. Results showed that MIC levels of the *gor*-mutant strains against hydrogen peroxide were 2-fold lower than that of their parental strains (MICs fall from 0.01 to 0.005%). The levels of MIC were fully restored in the mutant strains harboring a plasmid carrying an intact *gor* gene. MIC levels of the mutant and their parental strains against paraquat were all 200 μg/ml (Table 1). To clarify the effects of *gor* on hydrogen peroxide, bacterial killing assay was determined at different concentrations of hydrogen peroxide. As shown in Fig. 1, both *gor*-mutant strains were completely killed at 0.01% hydrogen peroxide while their parental strains survived significantly at the same concentration of hydrogen peroxide. Survival of the mutant strains harboring a plasmid carrying an intact *gor* gene was similar as their parental

strains (Fig. 1). These results suggest that the GSH reductase-encoding gene (*gor*) is associated with oxidative stress in *P. aeruginosa*.

3.3. Role of a GSH reductase-encoding gene (*gor*) in antibiotic susceptibility

Antibiotics induce oxidative stresses that contribute bacterial killing effect (Van Acker & Coenye, 2017). Since the *gor* gene is associated with oxidative stress as shown by the above results, the *gor* gene may be also associated with antibiotic susceptibility. To test this possibility, the *gor*-mutant strains were used to determine antibiotic susceptibility in comparison to their parental strains. Results revealed that the mutant strains were both more susceptible to carbenicillin, chloramphenicol, ciprofloxacin, and tetracycline than their parental strains (MICs fall 2- to 4-fold) (Table 2). To corroborate these results bacterial killing assay was determined at different concentrations of antibiotics. As shown in Fig. 2, the *gor*-mutant strains were completely killed at much lower concentrations of each antibiotic (carbenicillin, chloramphenicol, and ciprofloxacin) than their parental strains. These results are consistent with the MIC results (Table 2) and suggest that the *gor* gene is also associated with antibiotic susceptibility in *P. aeruginosa*.

4. Discussion

P. aeruginosa is a common Gram-negative rod-shaped bacterium associated with a variety of infections in hospitalized and immunocompromised people. Infections with *P. aeruginosa* can lead to severe illness and death. The CDC (Centers for Disease Control and Prevention) reported 51,000 health-care-associated *P. aeruginosa* infections per year and 440 of them were lethal. These 440 deaths were among 6,700 infections with multidrug resistant *P. aeruginosa* (https://www.cdc.gov/hai/organisms/pseudomonas.html). *P. aeruginosa*, unlike other Gram-negative bacterial pathogens, constitutively expresses chromosomal-encoded genes for AmpC β-lactamase and multidrug efflux pumps and has a low permeability outer membrane, which produces high-level intrinsic resistance to diverse antibiotics. Additionally, *P. aeruginosa* can acquire resistance to most commercially available antibiotics (Falagas *et al.*, 2008, Zavascki *et al.*, 2010). Recently, oxidative-stress-scavenging-system (OSSS) has suggested as one of the intrinsic resistant mechanisms to antibiotics based on the evidence that OSSS detoxifies oxidative stresses induced by antibiotics. This hypothesis however requires further clarification (Van Acker & Coenye, 2017).

Three major OSSS exist in bacteria such as an enzymatic system (e.g., catalases and superoxide dismutases), a GSH-redox system, and a thioredoxin redox system (Lu & Holmgren, 2014). In this study, we determined roles of a *gor* gene encoding GSH reductase in oxidative stress and antibiotic susceptibility. GSH reductase activity was significantly decreased in the *gor*-mutant strains but was not fully deficient, suggesting an alternative pathway (or enzyme) to salvage the role of GSH-redox system. Similar growth rates of the *gor*-mutant and the parental strains also support this possibility. GSH reductase is a key component of the GSH-redox system that plays a key role in

maintaining an optimum level of the intracellular redox potential and is required for normal cellular processes in *E. coli* (Smirnova & Oktyabrsky, 2005). In eukaryotic cells, GSH-redox and thioredoxin redox systems crosstalk to compensate roles for each other (Lu & Holmgren, 2014) and this may also be the case in *P. aeruginosa*.

MIC levels of the *gor*-mutant strains against hydrogen peroxide were lower than their parental strains. In addition, killing-rate of the *gor*-mutant strains was faster than their parental strains at the same concentrations of hydrogen peroxide. These results suggest that the lack of GSH reductase (*gor*-mutation) is associated with susceptibility to hydrogen peroxide. This may be due to an unbalance of the ratio of GSH/GSSG, which directly depends on the GSH reductase and relates to the function of GSH peroxidase that detoxifies hydrogen peroxide to water and oxygen molecules. Superoxide susceptibility of the *gor*-mutant strains was the same (or similar) level as their parental strains, suggesting that GSH reductase may not be responsible for detoxifying superoxide. Superoxide dismutase normally detoxifies the superoxide in bacteria, and *P. aeruginosa* encodes superoxide dismutase (http://www.pseudomonas.com/).

Kohanski et al. and other investigators reported that hydroxyl radicals were induced by bactericidal antibiotics (e.g., ampicillin, kanamycin, norfloxacin) but not by bacteriostatic antibiotics (e.g., chloramphenicol, rifampicin, and tetracycline), which enhanced the bacterial killing effect (Kohanski *et al.*, 2007). We showed that the *gor* gene was associated with oxidative stress, thus, the *gor*-mutant strains should be more susceptible to the bactericidal antibiotics than their parental strains. We observed that the *gor*-mutant strains were more susceptible to bactericidal antibiotics (carbenicillin and ciprofloxacin) than their parental strain, which is consistent with the report from

Kohanski et al. However, our results showed that the *gor*-mutant strains were also more susceptible to bacteriostatic antibiotics (chloramphenicol and tetracycline) than their parental strain. These results may be related to the fact that the *gor* gene controls the GSH-redox system that detoxifies a broad range of toxic substances.

Overall, this study demonstrates that the *gor* gene is associated with oxidative stress as well as antibiotic susceptibility in *P. aeruginosa*. The *gor* gene is a key component of the GSH-redox system and thus the GSH-redox system may be a possible drug target to treat the multidrug resistant *P. aeruginosa*.

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Table 1. Hydrogen peroxide and paraquat susceptibility in *P. aeruginosa*

Strain	Minimum Inhibitory Concentration (MIC)[a]	
	Hydrogen peroxide (H_2O_2; %)	**Paraquat (μg/ml)**
PAO1	0.01	200
PAO1 (*gor*::Gm)	0.005	200
PAO1 (*gor*::Gm/pAU250)[b]	0.01	200
MPAO1	0.01	200
MPAO1 (*gor*::TnTc)	0.005	200
MPAO1 (*gor*::TnTc/pAU250)	0.02	200

[a] MIC measurement repeated three times with an identical result.

[b] pAU250 carries an intact *gor* gene.

Table 2. Antibiotic susceptibility in *P. aeruginosa*

	Minimum Inhibitory Concentration (MIC; μg/ml) [a]							
	ATM	**CAR**	**CAZ**	**MEM**	**CHL**	**CIP**	**GEN**	**TET**
PAO1	4	128	4	2	64	0.12	ND	8
PAO1 (*gor*::Gm)	4	64	4	2	32	0.062	ND	4
MPAO1	2	256	4	1	128	0.25	0.5	ND
MPAO1 (*gor*::TnTc)	2	128	4	1	32	0.062	0.25	ND

[a] MIC measurement repeated three times with an identical result.

Note: ATM: aztreonam; CAR: carbenicillin; CAZ: ceftazidime; MEM: meropenem; CHL: chloramphenicol; CIP: ciprofloxacin; GEN: gentamicin; TET: tetracycline

ND: not determined

336 **Figures**
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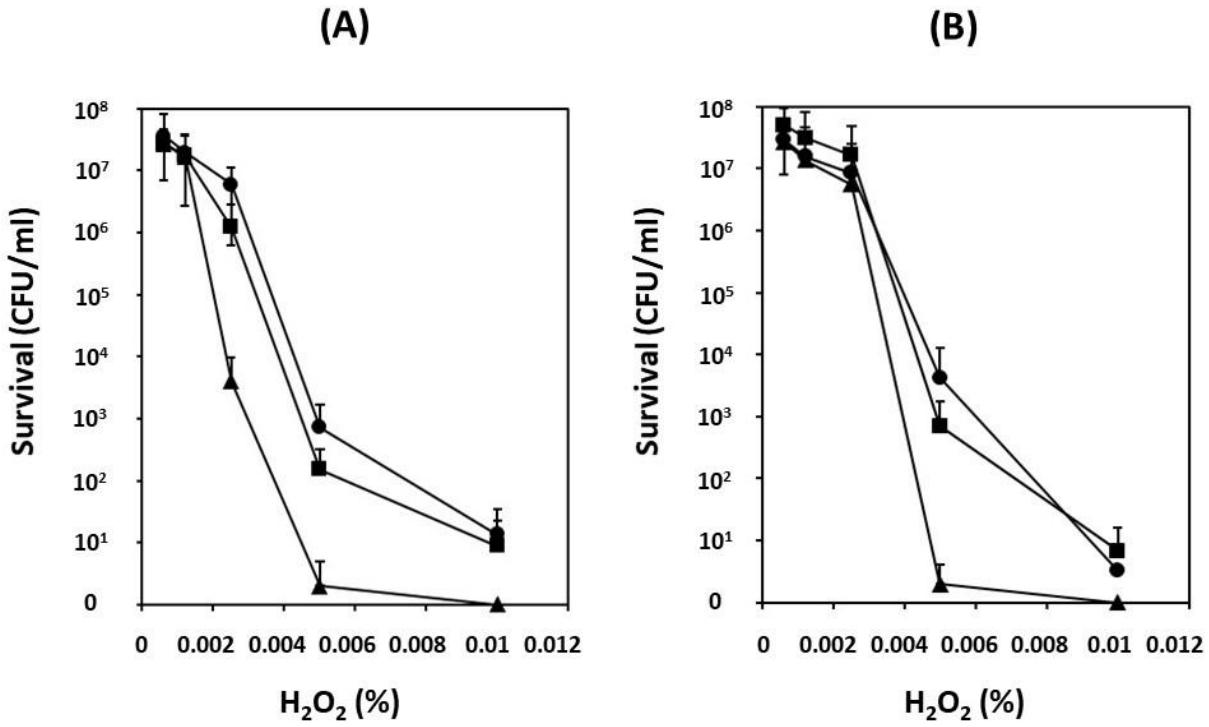

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340 **Fig. 1. Bacterial killing of hydrogen peroxide (H_2O_2) on *P. aeruginosa*.** The bacterial killing
341 assay performed as described in Materials and Methods. (A) is *P. aeruginosa* PAO1 and (B) is *P.*
342 *aeruginosa* MPAO1. For (A) and (B), circle is wild type strains, triangle is mutant strains
343 (*gor*::Gm for PAO1 and *gor*::TnTc for MPAO1), and square is *gor*-mutant stains carrying an
344 intact *gor* gene (pAU250). Three independent measurements used for the standard deviation.

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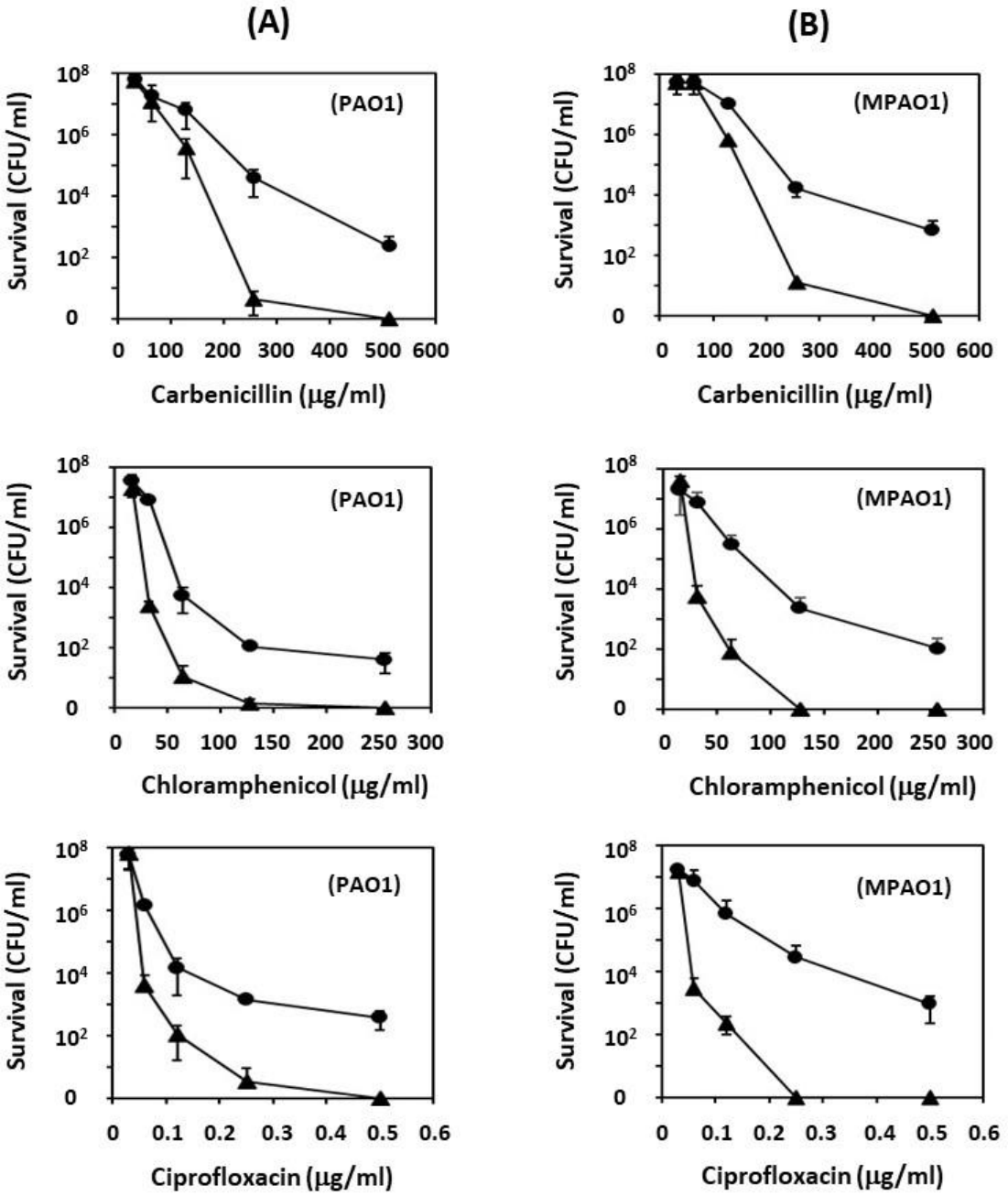

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348 **Fig. 2. Bacterial killing of antibiotics on *P. aeruginosa*.** The bacterial killing assay performed

349 as described in Materials and Methods. (A) is *P. aeruginosa* PAO1 and (B) is *P. aeruginosa*

350 MPAO1. For (A) and (B), circle is wild type strains and triangle is mutant strains (*gor*::Gm for

351 PAO1 and *gor*::TnTc for MPAO1). Three independent measurements used for the standard

352 deviation.