1 **Glutathione Reductase Encoding Gene (*gor*) Is Associated with Oxidative Stress and**

2 **Antibiotic Susceptibility in *Pseudomonas aeruginosa***

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24 **ABSTRACT**

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

43

1. Introduction

Pseudomonas aeruginosa is a Gram-negative human pathogen causing a wide variety of nosocomial and community-acquired infections. The range of *P. aeruginosa* infections varies from localized infections on the human body to life-threatening systemic disease, including burn wounds, cystic fibrosis, acute leukemia, renal system, bacteremia, urinary tract infection, organ transplants, and intravenous-drug addiction. (Richards *et al.*, 1999, Matta *et al.*, 2018). Treatment of *P. aeruginosa* infections however is difficult due to the presence of antibiotic resistance to a variety of antibiotics, such as aminoglycosides, quinolones, and β-lactams. The major resistance mechanisms to those antibiotics are intrinsic, acquired, and adaptive resistance. The intrinsic resistance includes decreased-membrane permeability, expression of efflux pumps that expel antibiotics out of the cell and the production of antibiotic-inactivating enzymes. The acquired resistance can be achieved by either horizontal transfer of resistance genes or alterations on the antibiotic targets. The adaptive resistance involves formation of biofilm in the lungs of infected patients where the biofilm serves as a diffusion barrier to limit antibiotic access to the bacterial cells (Zavascki *et al.*, 2010, Cerceo *et al.*, 2016, Pang *et al.*, 2019).

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

67 resistant mechanisms to antibiotics.

68

69 Cellular metabolism normally produces oxidative stresses in all aerobic organisms. In *E. coli*, the
70 oxidative stresses activate OxyR and/or SoxRS, which induces the expression of a number of genes
71 including glutathione (GSH) reductase and GSH peroxidase that neutralizes the oxidative stress
72 (Green & Paget, 2004). For example, hydrogen peroxide is reduced to water and oxygen molecules
73 by GSH peroxidase using electrons from two molecules of GSH, and the two molecules of GSH
74 are oxidized to form GSH disulfide (GSSG). The GSSG is toxic at high levels and reduced back
75 to GSH by the GSH reductase using electrons from NADPH (Green & Paget, 2004, Smirnova &
76 Oktyabrsky, 2005). In *E. coli*, the ratio of GSH/GSSG is estimated to be approximately 200 (>99%
77 of GSH) in growing cells, which corresponds to a redox potential of -240 mV, assuming a total
78 intracellular GSH concentration of 5 mM, pH 7.0, and 25ºC. This GSH-redox system plays a
79 variety of cellular functions not only in detoxifying oxidative stresses but also in deactivation of
80 toxic substances via GSH-conjugate formation (Couto *et al.*, 2016). Alterations of the GSH redox
81 potential can impair the functions of GSH-redox system (Smirnova & Oktyabrsky, 2005).
82 Therefore, the GSH-redox system is one of the oxidative-stress-scavenging systems and GSH
83 reductase is a key component for this redox system.

84

85 These observations suggest that the GSH reductase is associated with oxidative stresses and
86 antibiotic susceptibility. GSH reductase is a dimer composed by two identical subunits with a
87 molecular mass of 55 kD, a member of the Flavin-containing enzyme, encoded by a gene (*gor*) in
88 *E. coli* (Jiang *et al.*, 1995). The *gor* gene from *P. aeruginosa* PAO1 was cloned in *E. coli* (Perr *et*
89 *al.*, 1991), but the roles of the *gor* gene in oxidative stress and antibiotic susceptibility are currently

90 unclear. In this study, we aimed to understand the role of a glutathione-reductase-encoding gene

91 (*gor*) in oxidative stress and antibiotic susceptibility. The genes (*gor*) of *P. aeruginosa* (POA1 and

92 MPAO1) were knocked-out and roles of the *gor* in oxidative stress and antibiotic susceptibility

93 were determined. The results revealed that the *gor*-mutant strains were more susceptible to

94 hydrogen peroxide and antibiotics than their parental strains of *P. aeruginosa*, suggesting the *gor*

95 gene may be associated with susceptibility of antibiotics and the *gor* gene may be a possible drug-

96 target for the antibiotic resistant *P. aeruginosa*.

97

98 **2. Materials and Methods**

99 **2.1. Bacterial strains, growth conditions, and chemicals**

100 *P. aeruginosa* PAO1 was obtained from the previous studies (Kwon & Lu, 2006). *P. aeruginosa*
101 MPAO1 and a *gor*-mutant strain (PW4508; *gor*::TnTc) were obtained from the sequence-verified
102 transposon mutant library (University of Washington, Seattle, WA). The bacterial strains routinely
103 grew on Luria-Bertani (LB; Becton, Dickinson and Company, Sparks, MD) agar plates or broth at
104 37°C. The mutant strain grew in a minimal medium for *P. aeruginosa* containing glutamate as a
105 sole carbon and nitrogen source as described (Kwon & Lu, 2006). All antibiotics, L-glutathione
106 (reduced-glutathione), and other chemicals were purchased from Sigma-Aldrich (St. Louis, MO).
107

108 **2.2. Cloning and gene-knockout**

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

121 LB agar plates containing gentamicin (80 μg/ml) for *P. aeruginosa* and chloramphenicol (15

122 μg/ml) for counter-selection against *E. coli* SM10. Authenticity of the gene-knockout was

123 confirmed by PCR amplication of the upstream and downstream of the *gor* gene.

124

125 **2.3. Glutathione reductase enzyme assay**

126 Overnight-cultured cells were diluted (1/100) into fresh LB broth (30 ml) and grown on a rotary

127 shaker (250 rpm) at 37°C. The cells (5 ml) at optical density (OD_{600}) of 1.0 were washed three

128 times with phosphate buffer (pH 7.0) and disrupted by sonication as described (Kwon *et al.*, 2013).

129 The crude extract of the cells spun down at 15,000 rpm for 5 min and the supernatant used to

130 measure levels of GSH reductase. The protein concentration of the crude extract was measured

131 using the Coomassie Plus^{TM} Protein Assay Reagent (Thermo Scientific, Rockford, IL). The amount

132 of GSH reductase (units/mg protein) was determined using a commercial kit (Sigma-Aldrich, St.

133 Louis, MO) as suggested by the vendor.

134

135 **2.4. Determination of minimum inhibitory concentration (MIC) of hydrogen peroxide,**

136 **paraquat, and antibiotics**

137 MIC levels of hydrogen peroxide, paraquat, and antibiotics were determined as guided by the

138 Clinical and Laboratory Standards Institute (CLSI) as described previously (Kwon & Lu, 2006)

139 with a minor modification. Briefly, two-fold serial dilutions of hydrogen peroxide, paraquat, and

140 antibiotics were performed using Mueller-Hinton broth (MHB; Oxoid, Ogdensburg, NY) and fresh

141 overnight cultures of *P. aeruginosa* strains (~10^{8} viable cells per ml) inoculated at each of the

142 dilutions. The cells were incubated overnight without shaking at 37°C. MIC levels defined as the

143 lowest concentration of hydrogen peroxide, paraquat, and antibiotics that completely inhibited

144 cellular growth of the inoculum. Determination of MIC levels was repeated three times to confirm
145 the results.
146
147 **2.5. Bacterial killing assay**
148 Bacterial killing of *P. aeruginosa* strains were determined as previously described (Kanagaratnam
149 *et al.*, 2017) with a minor modification. Briefly, hydrogen peroxide or antibiotics at different
150 concentrations were added in MH broth (1 mL in the Falcon culture tubes) and fresh cultures of
151 cells (~10^8 viable cells per ml) were inoculated into each of the concentrations. The cells were
152 incubated at 37°C without shaking for 18 hours and spread on plain LB agar plates with appropriate
153 dilutions. The LB agar plates were incubated for 24 hours at 37°C and colony-forming units (CFU)
154 were counted per ml. The bacterial killing assay repeated three times to confirm the results.
155
156

157 **3. Results**

158 **3.1. Mutant strains of *P. aeruginosa***

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

168

169 **3.2. Effect of a GSH reductase-encoding gene (*gor*) on oxidative stresses**

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

180 strains (Fig. 1). These results suggest that the GSH reductase-encoding gene (*gor*) is associated
181 with oxidative stress in *P. aeruginosa*.
182
183 **3.3. Role of a GSH reductase-encoding gene (*gor*) in antibiotic susceptibility**
184 Antibiotics induce oxidative stresses that contribute bacterial killing effect (Van Acker &
185 Coenye, 2017). Since the *gor* gene is associated with oxidative stress as shown by the above
186 results, the *gor* gene may be also associated with antibiotic susceptibility. To test this possibility,
187 the *gor*-mutant strains were used to determine antibiotic susceptibility in comparison to their
188 parental strains. Results revealed that the mutant strains were both more susceptible to
189 carbenicillin, chloramphenicol, ciprofloxacin, and tetracycline than their parental strains (MICs
190 fall 2- to 4-fold) (Table 2). To corroborate these results bacterial killing assay was determined at
191 different concentrations of antibiotics. As shown in Fig. 2, the *gor*-mutant strains were
192 completely killed at much lower concentrations of each antibiotic (carbenicillin,
193 chloramphenicol, and ciprofloxacin) than their parental strains. These results are consistent with
194 the MIC results (Table 2) and suggest that the *gor* gene is also associated with antibiotic
195 susceptibility in *P. aeruginosa*.
196
197

198 **4. Discussion**

199 *P. aeruginosa* is a common Gram-negative rod-shaped bacterium associated with a variety of
200 infections in hospitalized and immunocompromised people. Infections with *P. aeruginosa* can lead
201 to severe illness and death. The CDC (Centers for Disease Control and Prevention) reported 51,000
202 health-care-associated *P. aeruginosa* infections per year and 440 of them were lethal. These 440
203 deaths were among 6,700 infections with multidrug resistant *P. aeruginosa*
204 (https://www.cdc.gov/hai/organisms/pseudomonas.html). *P. aeruginosa*, unlike other Gram-
205 negative bacterial pathogens, constitutively expresses chromosomal-encoded genes for AmpC β-
206 lactamase and multidrug efflux pumps and has a low permeability outer membrane, which
207 produces high-level intrinsic resistance to diverse antibiotics. Additionally, *P. aeruginosa* can
208 acquire resistance to most commercially available antibiotics (Falagas *et al.*, 2008, Zavascki *et al.*,
209 2010). Recently, oxidative-stress-scavenging-system (OSSS) has suggested as one of the intrinsic
210 resistant mechanisms to antibiotics based on the evidence that OSSS detoxifies oxidative stresses
211 induced by antibiotics. This hypothesis however requires further clarification (Van Acker &
212 Coenye, 2017).

213

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

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

225

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

236

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

244 Kohanski et al. However, our results showed that the *gor*-mutant strains were also more susceptible
245 to bacteriostatic antibiotics (chloramphenicol and tetracycline) than their parental strain. These
246 results may be related to the fact that the *gor* gene controls the GSH-redox system that detoxifies
247 a broad range of toxic substances.
248 Overall, this study demonstrates that the *gor* gene is associated with oxidative stress as well as
249 antibiotic susceptibility in *P. aeruginosa*. The *gor* gene is a key component of the GSH-redox
250 system and thus the GSH-redox system may be a possible drug target to treat the multidrug
251 resistant *P. aeruginosa*.
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253

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324

325

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

327

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

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

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

330

331

332

333 **Table 2.** Antibiotic susceptibility in *P. aeruginosa*

334

	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

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

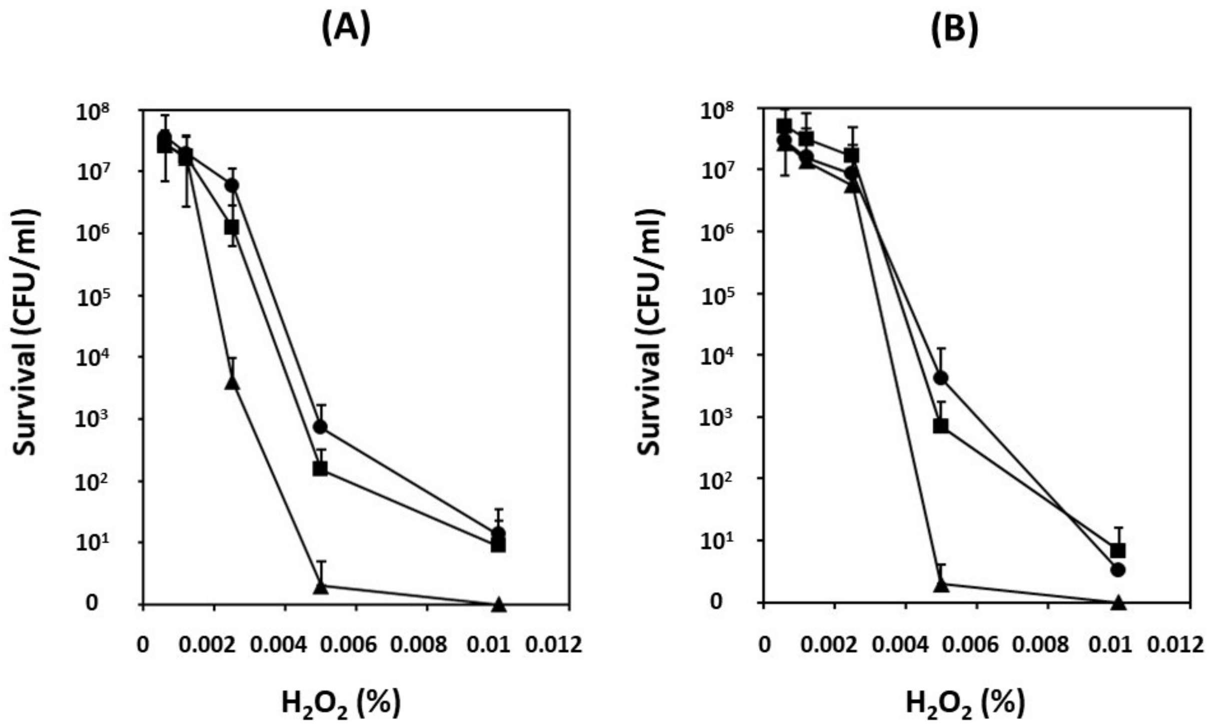
336 Note: ATM: aztreonam; CAR: carbenicillin; CAZ: ceftazidime; MEM: meropenem; CHL: chloramphenicol; CIP:

337 ciprofloxacin; GEN: gentamicin; TET: tetracycline

338 ND: not determined

339

340 **Figures**
341



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343

344 **Fig. 1. Bacterial killing of hydrogen peroxide (H_2O_2) on *P. aeruginosa*.** The bacterial killing
345 assay performed as described in Materials and Methods. (A) is *P. aeruginosa* PAO1 and (B) is *P.*
346 *aeruginosa* MPAO1. For (A) and (B), circle is wild type strains, triangle is mutant strains
347 (*gor*::Gm for PAO1 and *gor*::TnTc for MPAO1), and square is *gor*-mutant stains carrying an
348 intact *gor* gene (pAU250). Three independent measurements used for the standard deviation.
349

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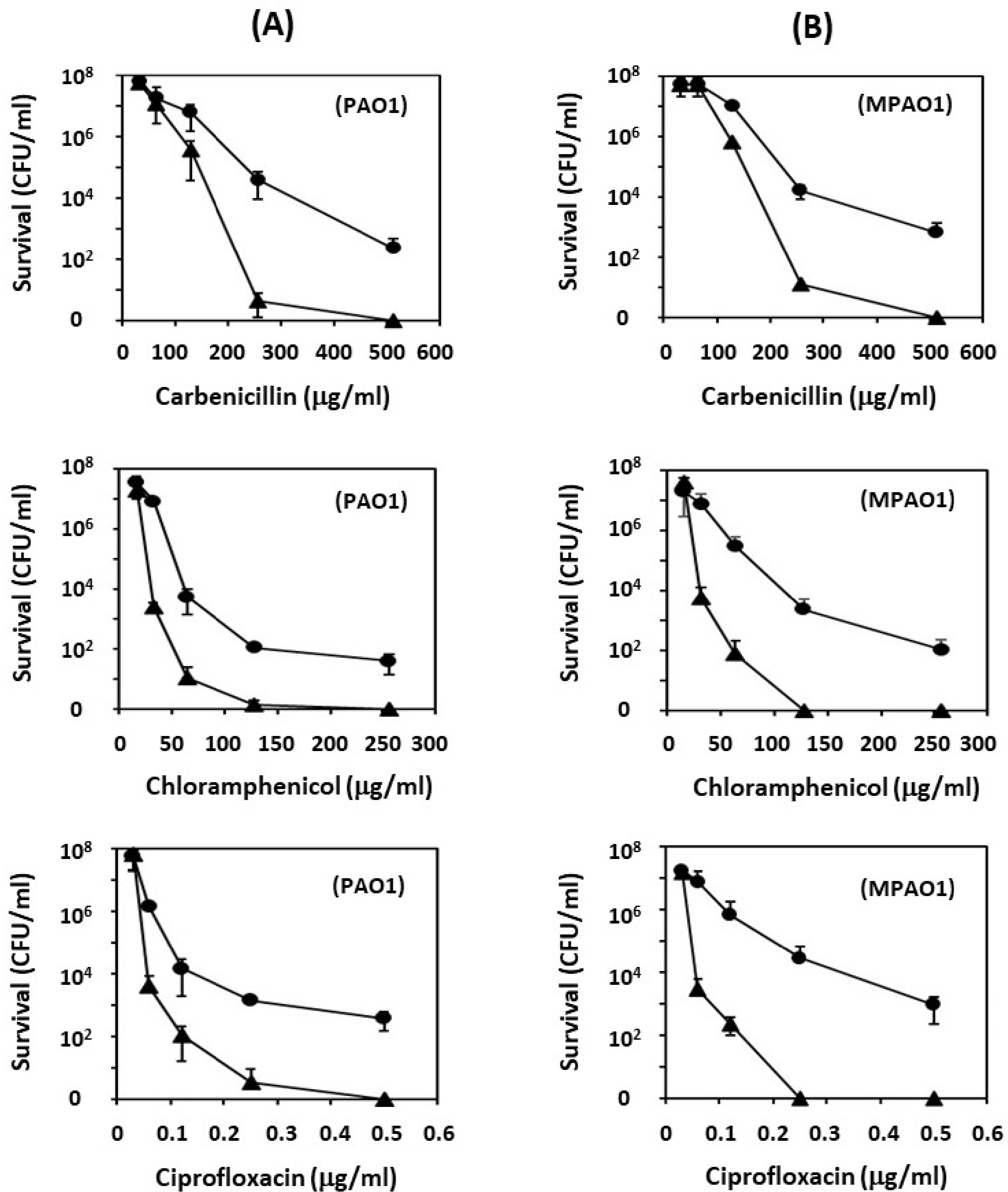

351

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

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

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

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

356 deviation.