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Physiological and molecular characterization of copper and antibiotic resistance mechanisms in *Pseudomonas aeruginosa* strain PaD2

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Abstract. *Jo J, Datta DATU, Irawati W, Purnomo JS. 2025. Physiological and molecular characterization of copper and antibiotic resistance mechanisms in* Pseudomonas aeruginosa *strain PaD2. Biodiversitas 26: 1526-1536.* Environmental pollution by heavy metals and antibiotics promotes the growth of co-resistant bacteria that can spread multiple resistance genes. Bioremediation using indigenous bacteria from such polluted sites is a potential solution to this problem. We recently isolated *Pseudomonas aeruginosa* strain PaD2 from the polluted Sukolilo River in Surabaya, Indonesia, and found it to be resistant to high copper concentrations. Therefore, we investigated the resistance profiles of *P. aeruginosa* PaD2 to Cu and various antibiotics using physiological and molecular approaches. Antibiotic resistance was evaluated in the presence of 4 mM $CuSO_4$, as compared to that without Cu addition, using disk diffusion and broth macrodilution assays. The whole genome of *P. aeruginosa* PaD2 was sequenced to identify relevant resistance genes. *Pseudomonas aeruginosa* PaD2 exhibits resistance to both copper and various antibiotics (e.g., cefoxitin, erythromycin or tetracycline). Interestingly, Cu exposure did not significantly alter the antibiotic resistance profiles, indicating that Cu and antibiotic resistance mechanisms were independent. Genome analysis identified genes related to efflux pumps, antibiotic inactivation, and target modification, which may explain the multidrug resistance phenotype. In conclusion, *P. aeruginosa* PaD2 should be further tested as a bioremediation agent for copper and antibiotic pollution.

Keywords: Antibiotic resistance, bioremediation, copper resistance, indigenous bacteria, *Pseudomonas aeruginosa*

Abbreviations: MIC: Minimum Inhibitory Concentration; MBC: Minimum Bacteriocidal Concentration

INTRODUCTION

Environmental pollution caused by heavy metals and antibiotics is a major global public health concern. The rapid growth of agricultural and aquaculture industries has significantly increased heavy metal pollution. Excessive concentrations of heavy metals can disrupt metabolic reactions, reduce biodiversity, accumulate in the food chain and negatively affect human health (Fu et al. 2023). Antibiotic usages also contribute to environmental pollution. Discarded antibiotics can harm various trophic levels of biota and pose health risks to humans (Polianciuc et al. 2020). Evidently, ciprofloxacin contamination has been observed in several environmental wastes, ranging from 5.3 to 119.8 µg/kg in agricultural soil, 45.49 mg/kg in manure, 426 mg/kg in sewage sludge, 6.5 mg/L in freshwater ecosystems, and 6.5 to 31 mg/L in pharmaceutical wastewater (Ali et al. 2024). Similarly, tetracycline remains unchanged as it passes through the digestive tracts of animals and humans. Consequently, it primarily contaminates the environment through water sources (Amangelsin et al. 2023). In European aquatic environments, tetracycline has been detected at concentrations ranging from 0 to 20 ng/L (Antos et al. 2024). It has been noted that heavy metals (especially copper) coexist with antibiotic pollutants in soil and water, mainly because of their common use in hospital settings. Copper (Cu), known for its antimicrobial properties, is a popular choice for sterilization (e.g., in intensive care units) (O'Brien et al. 2023). In contrast, antibiotics are widely used to treat various infections (Deguenon et al. 2022). High exposure to Cu in the environment results in the enrichment of Cu-resistant bacterial populations (Uddin et al. 2021). Similarly, the misuse and improper disposal of antibiotics lead to their accumulation in soil and water, promoting the emergence of antibiotic-resistant strains. Furthermore, a linear correlation has been observed between bacterial resistance to heavy metals and antibiotics (Uddin et al. 2021), which is linked to the shared mechanism of heavy metal and antibiotic resistance gene regulation by relevant mobile genetic elements within the bacterial genomes. This facilitates the transfer of resistance traits, known as co-resistance (Fu et al. 2023; Zhu et al. 2023; Gillieatt and Coleman 2024; Murray et al. 2024). In summary, the coexistence of antibiotics and copper contamination in the environment is driving a selection pressure that favors the growth of co-resistant bacteria, resulting in the accumulation of resistant bacterial populations and the spreading of relevant resistance genes (Murray et al. 2024).

The potential consequences of inaction in this scenario are dire, underscoring the urgent need for intervention.

Bioremediation has emerged as an interesting alternative for controlling copper and antibiotic concentrations in the environment. It is an environment-friendly and effective decontamination method that utilizes the ability of certain microorganisms to simultaneously modify, adsorb, and/or accumulate various pollutants (Zhu et al. 2023). The use of indigenous bacteria from contaminated sites is particularly effective, because their resistance and bioremediation efficiency are enhanced by their ability to adapt to local conditions (Irawati et al. 2021). Additionally, indigenous bacteria are part of the ecosystem, which reduces the likelihood of ecological disruption caused by the introduction of foreign microbial species (Singh and Christina 2022). We previously reported the potential use of an indigenous bacterium from a contaminated site in Surabaya, Indonesia, to remove both copper- and dye-containing wastes (Irawati et al. 2023). Therefore, we speculate that a similar mechanism could be applied to decontaminate waste containing copper and certain antibiotics.

We recently isolated a Gram-negative bacterium, *Pseudomonas aeruginosa* strain PaD2, from the polluted Sukolilo River in Surabaya, Indonesia, and observed high copper resistance. This isolate survived in medium supplemented with up to 12 mM Cu, with complete tolerance to a Cu concentration of 4 mM (unpublished data). As *P. aeruginosa* is widely known for its resistance to various antibiotics (Gauba and Rahman 2023), we investigated the resistance profiles of *P. aeruginosa* PaD2 to both copper and various antibiotics using physiological and molecular approaches. Antibiotic resistance profiles were assessed using disk diffusion and broth dilution methods in the presence of 4 mM copper, and the results were compared with those obtained without copper. Finally, a molecular approach was performed by sequencing the whole genome of *P. aeruginosa* PaD2 and analyzing its copper and antibiotic resistance genes in silico.

MATERIALS AND METHODS

Bacterial maintenance

This study was conducted at the Biology Laboratory of the Department of Biology Education, Universitas Pelita Harapan, Indonesia. Bacterial isolates of *Pseudomonas aeruginosa* strain PaD2 were previously purified. The initial subculture of *P. aeruginosa* PaD2 was transferred from the isolated stock to an agar slant of Luria Bertani Agar (LBA) medium with the addition of copper (4 mM $CuSO_4$). The bacterial isolates were incubated at 37°C for 24 h. Periodic transfer to new agar slants was performed to obtain an active culture of *P. aeruginosa* PaD2.

Disk-diffusion assay

The Kirby-Bauer disk diffusion method was performed based on the European Committee on Antimicrobial Susceptibility Testing (EUCAST) methodology. *Pseudomonas aeruginosa* strain PaD2 was grown on Luria Broth (LB) for four h at 37°C under shaking conditions at 150 rpm for aeration. A cell turbidity between 0.3 to 0.8 (A600) was chosen for the disk diffusion assay. The assay was performed on Mueller-Hinton (MH) agar (Himedia, India). The growth media were divided into two groups: (i) with the addition of 4 mM $CuSO_4$ and (ii) without the addition of $CuSO_4$. A total of 100 µL of bacterial culture was inoculated on the agar using the swab method (Gajic et al. 2022). Fifteen antibiotic disks (Liofilchem, Italy) were tested, including gentamicin (10 µg), cefoxitin (30 µg), sulfonamide (300 µg), vancomycin (30 µg), lincomycin (2 µg), erythromycin (15 µg), mupirocin (5 µg), methicilin (5 µg), oxacilin (1 µg), chloramphenicol (30 µg), lefamulin (20 µg), ciprofloxacin (5 µg), ofloxacin (5 µg), rifampin (5 µg), and tetracycline (30 µg). Each antibiotic disk was placed on the surface of the inoculated agar. The medium was then incubated under aerobic conditions at 37°C for 18 h. The clear zones formed on the media were measured and interpreted as bacterial resistance to the antibiotics, based on the EUCAST clinical breakpoint tables v.14.0. The measurement of the clear zones included the diameter of the antibiotic discs (6 mm). All antibiotic discs were tested three times.

Broth macrodilution assay

Pseudomonas aeruginosa strain PaD2 was grown on MH broth (Himedia, India) for four h at 37°C under shaking conditions at 150 rpm to ensure aeration. The cell turbidity, measured as optical density at 600 nm (A600), was adjusted between 0.3 and 0.8 for usage in the broth macrodilution assay using vials. Two treatment groups were compared: (i) with the addition of 4 mM $CuSO_4$ and (ii) without $CuSO_4$. Two antibiotics used in the assay were selected based on the results of the disk diffusion assay: ciprofloxacin (500 mg) and tetracycline (500 mg). Stock antibiotic solutions were prepared by dissolving antibiotics in sterile distilled water to a concentration of 5,120 mg/L. Serial dilutions of the stock solutions in MH broth were prepared to obtain working concentrations ranging from 128 mg/L to 0.25 mg/L. For the treatment with $CuSO_4$, 8 mM $CuSO_4$ was incorporated into each antibiotic dilution. 1 mL of each antibiotic was further diluted with 1 mL of the prepared bacterial cultures in vials. This resulted in final antibiotic concentrations ranging from 64 mg/L to 0.125 mg/L with twofold dilution in between (total of ten concentrations), with the concentration of $CuSO_4$ at 4 mM. Each condition was tested in triplicate. Additional controls were prepared: 2 mL of bacterial culture alone (positive control) and 2 mL of MH broth (negative control). All vials were incubated at 37°C for 18 h. Bacterial growth was assessed by comparing colony formation with that of the positive control. The Minimum Inhibitory Concentration (MIC) represented the lowest concentration that inhibited bacterial growth, which was subsequently interpreted using EUCAST MIC clinical breakpoint tables v.14.0 (Rodríguez-Melcón et al. 2021; Giske et al. 2022).

The solutions from the broth macrodilution assay were subsequently used to determine Minimum Bactericidal Concentration (MBC). A total of 100 µL from each vial that had been incubated in the broth macrodilution assay was subsequently inoculated onto the surface of the MH agar

without copper using the swab method. As each antibiotic (in the presence or absence of copper) was tested three times in the broth macrodilution assay, each MH agar plate was streaked three times per condition. Petri dishes were incubated for 24 h at 37°C. Bacterial growth was observed to determine the lowest concentration of the antibiotic that was bactericidal (Rodríguez-Melcón et al. 2021).

Whole-genome sequencing and bioinformatics analyses

Genomic DNA of *P. aeruginosa* strain PaD2 was extracted and sequenced using Oxford Nanopore Technology. The sequence was assembled de novo using FlyE v2.9.5 (https://github.com/mikolmogorov/Flye). Quality control of the final assembly was performed using QUAST v5.2.0 (https://github.com/ablab/quast). The output was annotated using dFAST v1.6.0 (https://dfast.ddbj.nig.ac.jp/). A circular map of the assembled genome was visualized using Proksee (https://proksee.ca/). Identification of the isolate was subsequently conducted using the TYGS web server (https://tygs.dsmz.de/) and visualized using iTOL (https://itol.embl.de/). Copper resistance genes were screened using the annotated results from dFAST because the initial screening with the Antibacterial Biocide and Metal Resistance Genes Database (BacMet) did not yield any results. Genes associated with Cu resistance were confirmed using NCBI-BLAST. Antibiotic resistance genes were screened using ABRicate (https://github.com/tseemann/abricate), based on the Comprehensive Antibiotic Resistance Database (CARD).

RESULTS AND DISCUSSION

Genome characteristics of *Pseudomonas aeruginosa* PaD2

The genome of PaD2 had a total length of 6,753,898 bp, with a Guanine-Cytosine (GC) content of 66.2%. A total of 6,362 genes were predicted using dFAST genome annotation tools, comprising 6,275 Coding Sequences (CDS), 75 tRNAs, 12 rRNAs, and an overall coding ratio of 89.7% (Table 1). Of the predicted CDS, 915 proteins were classified as hypothetical or unknown, whereas 5,360 proteins were presumed to be functional. A comprehensive overview of the genomic structure of isolate PaD2 is illustrated in Figure 1. Average Nucleotide Identity (ANI) values greater than 95% are commonly used to confirm species classification among prokaryotic organisms (Ciufo et al. 2018). According to the dFAST calculation of ANI, the strain showed 99% identity with *P. aeruginosa*. Phylogenetic analysis using TYGS revealed that the bacterial isolate PaD2 was closely related to *P. aeruginosa* DSM 50071 T1879, DSM 50071 T121527, and DSM 50071 T24528 (Figure 2). Taken together, the bacterial isolate PaD2 was confirmed to be a strain of *P. aeruginosa*.

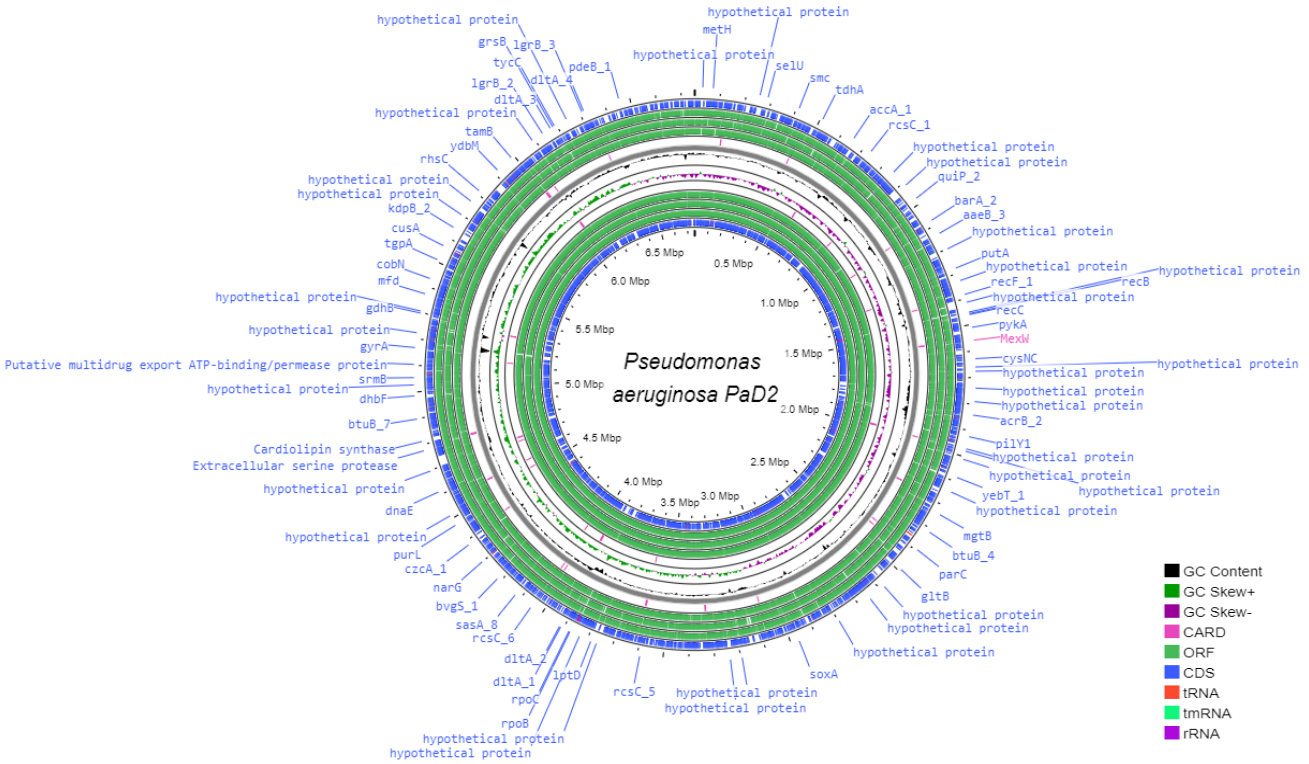

Figure 1. Visualization of the whole genome of *Pseudomonas aeruginosa* PaD2. The visualization was performed with Proksee (https://proksee.ca/). The outermost blue circle displays both forward and reverse Coding Sequences (CDS), with hypothetical genes labeled. The CDS were supplemented with tRNAs (red), rRNAs (purple), and tmRNAs (light green). The third middle circle represented the GC content (black), and the fourth inner circle represented the GC skew (dark green and dark purple). The innermost circle at the center represents the 6,753,898 base pairs that constituted the whole genome

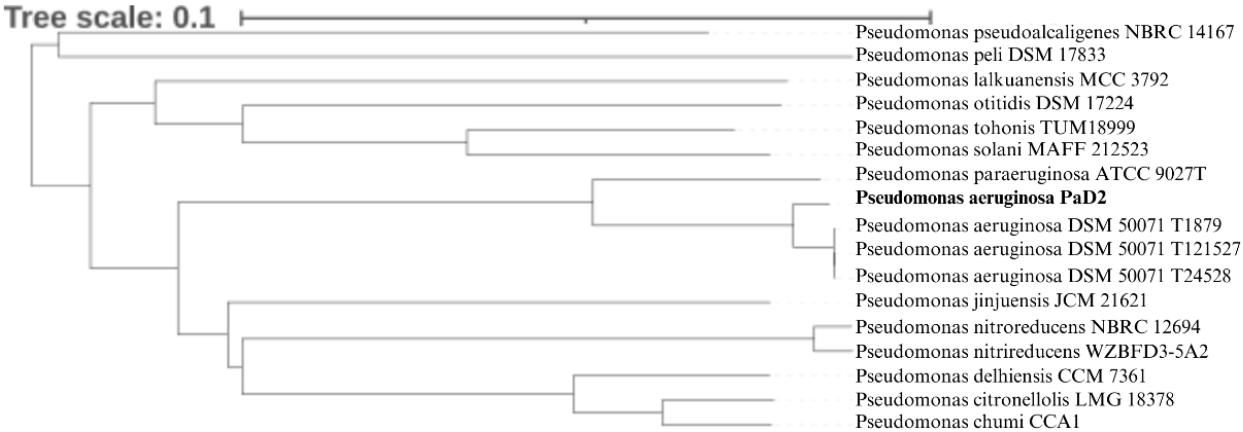

Figure 2. Phylogenetic tree of *Pseudomonas aeruginosa* PaD2. The TYGS webserver (https://tygs.dsmz.de/) was used to compare *P. aeruginosa* PaD2 with other *Pseudomonas* strains. The iTOL (https://itol.embl.de/) was used to visualize the result. The tree was generated in TGYS using the MASH algorithm to compare the bacteria with various closely related type strains. GBDP and precise intergenomic distances determined using the distance formula d5 and the "trimming" procedure were used for all pairwise comparisons among the set of genomes. The resulting intergenomic distances were used to infer a balanced minimum evolution tree with branch support via FASTME 2.1.6.1 including SPR postprocessing. From each of the 100 pseudo-bootstrap replicates, branch support was deduced. The tree was then visualized with iTOL

Table 1. Genome annotation statistics of *Pseudomonas aeruginosa* PaD2 using the dFAST annotation tool

Attribute	Value
Genome size (bp)	6,753,898
Contig	1
GC content (%)	66.2
Contig N50 (bp)	6,753,898
Contig L50	1
Plasmids	0
CDS	6,275
Total RNAs	87 (75 tRNA+12 rRNA)
Number of CRISPRs	1
Coding ratio (%)	89.7

Notes: N50 is related to the median and mean lengths of a set of sequences. It represents the length of the shortest read in the group of the longest sequences, which together account for at least 50% of the nucleotides in the set of sequences. L50 is related to N50, indicating the number of sequences that, when arranged from longest to shortest, must reach or exceed 50% of the total assembly size. bp: Base pair; GC: Guanine-Cytosine; CDS: Coding Sequence; RNA: Ribonucleic Acid; CRISPR: Clustered Regularly Interspaced Short Palindromic Repeats

Antibiotic resistance profile of *Pseudomonas aeruginosa* PaD2

The antibiotic resistance of *P. aeruginosa* PaD2 was first assessed by testing it against 15 antibiotic disks from various classes using the Kirby-Bauer disk diffusion method. The assay was performed on the MH agar with 4 mM $CuSO_4$ (to observe any resistance phenotype to both antibiotics and copper) as well as the ones without $CuSO_4$. The inhibition zone results after 18 h of incubation are summarized in Table 2. The results showed that *P. aeruginosa* PaD2 displayed resistance to most of the tested antibiotics. This observation aligns with the widely recognized status of *P. aeruginosa* as a highly resistant bacterial species with multidrug resistance capabilities. Resistance arises from various mechanisms, including efflux pumps, antibiotic inactivation, antibiotic target protection, and target alteration (de Almeida et al. 2024). Compared with other *Pseudomonas* species, *P. aeruginosa* exhibited a significantly higher resistance rate. For example, *P. flurescens* and *P. putida* are notably more susceptible to several antibiotics, particularly cephalosporins (Gauba and Rahman 2023).

Intriguingly, the presence of $CuSO_4$ in MH agar did not modulate the antibiotic resistance profile of *P. aeruginosa*. The similar diameters of inhibition zone between testing condition with and without 4 mM of $CuSO_4$ could be attributed to several plausibilities. First, bacterial isolates may exhibit susceptibility or resistance to antibiotics, regardless of the presence of copper, as the action mechanisms of certain antibiotics may remain unaffected by copper, or their action mechanisms may be completely independent of those of copper. For example, certain antibiotics that inhibit protein synthesis (e.g., tetracycline) are unlikely to be affected by copper, which damages bacterial cell membranes (Uddin et al. 2021). Secondly, a copper concentration of 4 mM was fully tolerated by the isolate tested in this study (unpublished data). This suggests that a higher concentration of $CuSO_4$ may be needed to further stress the bacterial isolate and to observe the distinct presence of co-resistance mechanisms to both heavy metals and antibiotics. Third, MH agar prepared in-house from Himedia may not be suitable for detecting any impact of copper addition in further modulating resistance against certain antibiotics. It had been reported that the Himedia brand of MH dehydrated media demonstrated a relatively moderate performance among 21 brands in testing antimicrobial susceptibility via disk-diffusion method (Ahman et al 2020). Taken together, these findings suggest that the antibiotic resistance mechanisms of *P.*

aeruginosa PaD2 are not dependent on copper resistance. Instead, the results suggested the presence of multiple resistance mechanisms in *P. aeruginosa* PaD2 to copper and several antibiotics that are independent of each other. Although *P. aeruginosa* PaD2 exhibited resistance to most of the tested antibiotics, it was susceptible to gentamicin, sulfonamide, ciprofloxacin, and ofloxacin (Table 2, Figure 3). Notably, ofloxacin and ciprofloxacin belong to the same class of antibiotics (fluoroquinolones). In line with a published finding (Sihotang et al. 2022), ciprofloxacin demonstrated greater bactericidal activity against *P. aeruginosa* PaD2 than ofloxacin. Interestingly, only two antibiotics exhibited wide margins of clear-zone diameter when compared between 4 mM $CuSO_4$-supplemented MH agar and non-supplemented MH agar: ciprofloxacin (median of 28 mm versus 34 mm) and tetracycline (median of 15 mm versus 20 mm).

Table 2. Antibiotic resistance profile of *Pseudomonas aeruginosa* PaD2 based on the disk-diffusion method

Antibiotic classes	Antibiotics	4 mM $CuSO_4$		0 mM $CuSO_4$	
		Clear zone diameter (mm) (min-max)	R/I/S	Clear zone diameter (mm) (min-max)	R/I/S
Aminoglycoside	Gentamicin (10 µg) [b]	20 (19-20)	S	21 (21-21)	S
Cephalosporin	Cefoxitin (30 µg) [b]	6 (6-6)	R	6 (6-6)	R
Folate antagonist	Sulfonamide (300 µg)[f]	21 (20-21)	S	21 (21-21)	S
Glycopeptide	Vancomycin (30 µg) [d]	6 (6-6)	R	6 (6-6)	R
Lincosamide	Lincomycin (2 µg) [c]	6 (6-6)	R	6 (6-6)	R
Macrolides	Erythromycin (15 µg)[b]	6 (6-6)	R	6 (6-6)	R
Monocarboxylic Acid	Mupirocin (5 µg) [b]	6 (6-6)	R	6 (6-6)	R
Penicillins	Methicillin (5 µg) [c]	6 (6-6)	R	6 (6-6)	R
	Oxacillin (1 µg) [b]	6 (6-6)	R	6 (6-6)	R
Phenicols	Chloramphenicol (30 µg) [e]	13 (13-13)	R	15 (15-16)	R
Pleuromutilin	Lefamulin (20 µg) [b]	6 (6-6)	R	6 (6-6)	R
Fluoroquinolone	Ciprofloxacin (5 µg) [a]	28 (23-30)	I	34 (34-35)	I
	Ofloxacin (5 µg) [c]	22 (21-22)	I	24 (24-25)	I
Rifampin	Rifampin (5 µg) [b]	6 (6-6)	R	6 (6-6)	R
Tetracycline	Tetracycline (30 µg) [a]	15 (15-15)	R	20 (19-20)	R

Note: The diameter of the clear/inhibition zone is presented as the median of three experiments. [a]Based on EUCAST 2024 for *Pseudomonas* species. [b]Based on EUCAST 2024 for *Staphylococcus* species. [c]Based on EUCAST 2024 on *Staphylococcus* species in the same class of antibiotics [d]Based on EUCAST 2024 on *Enterococcus* species in the same class of antibiotics. [e]Based on EUCAST 2024 for *Enterobacterales* species. [f]Based on CLSI 2020 for *Staphylococcus* species. R: Resistant; S: Susceptible; I: Susceptible; increased exposure. Both S and I results were considered susceptible

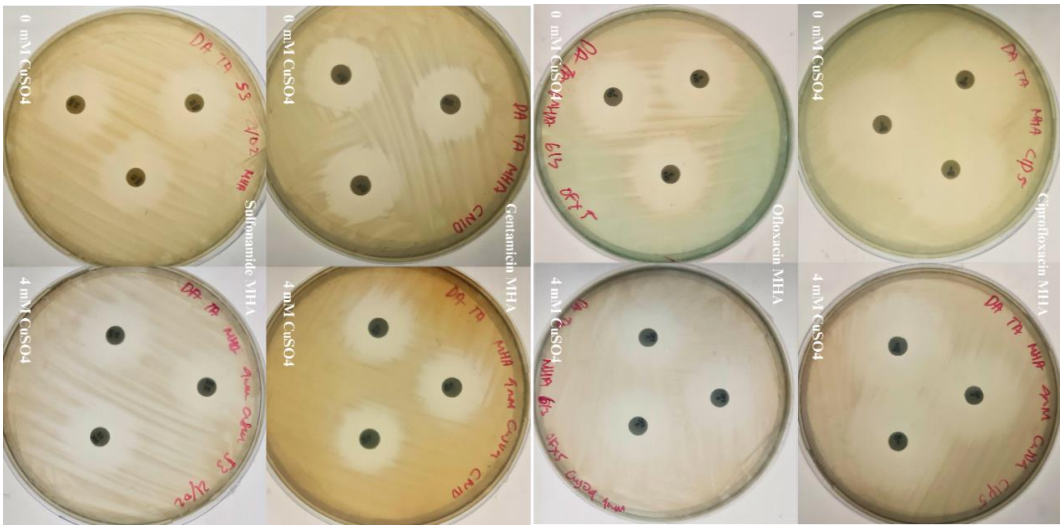

Figure 3. Representative results of disk-diffusion assay on *Pseudomonas aeruginosa* strain PaD2. Four antibiotics were displayed, i.e., gentamicin (upper left), sulfonamide (lower left), ciprofloxacin (upper right), and ofloxacin (lower right). Each plate contained three discs of the same antibiotic. The incubation was performed in an aerobic condition at 37°C for 18 h. The results of incubation in Mueller-Hinton agar without and with 4 mM of $CuSO_4$ are displayed side by side

Next, as the disk-diffusion assay could not determine the lowest concentrations of relevant antibiotics that inhibited bacterial growth (i.e., the Minimum Inhibitory Concentration (MIC)), a broth macrodilution assay was conducted. Ciprofloxacin and tetracycline were chosen as representatives of susceptible and resistant strains, respectively. The chosen antibiotic concentrations ranged from 64 to 0.125 mg/L, with two-fold dilutions in between (total of ten concentrations). Three experiments were conducted for each antibiotic. Table 3 summarizes the results of *P. aeruginosa* PaD2 after 18 h of incubation. While the MIC of *P. aeruginosa* PaD2 to ciprofloxacin was approximately 0.5 mg/L, the MIC of *P. aeruginosa* PaD2 to tetracycline was more than 64 mg/L. These findings are in line with those of the disk-diffusion assay, that is, *P. aeruginosa* PaD2 was susceptible to high doses of ciprofloxacin (supported by a relatively low MIC value) and resistant to tetracycline (supported by a high MIC value). In addition, no difference was observed in MIC values between broth without and with 4 mM $CuSO_4$. This suggests that the presence of copper did not modulate the antibiotic resistance profiles, at least for ciprofloxacin and tetracycline.

After obtaining the MIC values for ciprofloxacin or tetracycline in the presence or absence of copper, the Minimum Bactericidal Concentration (MBC) was determined. The MBC was assessed by inoculating samples from the broth macrodilution assay onto the surface of non-copper-supplemented MH agar, followed by an incubation at 37°C for 24 h. As each antibiotic was tested three times in the broth macrodilution assay, each MH agar plate was streaked onto three regions. The MBC profiles of *P. aeruginosa* PaD2 for ciprofloxacin and tetracycline are shown in Figures 4 and 5, respectively. As expected, the MBC were higher than the MIC for both antibiotics. In the presence of 4 mM $CuSO_4$, *P. aeruginosa* PaD2 began to grow only at ciprofloxacin concentrations as high as 4 mg/L, suggesting that the MBC for ciprofloxacin in the presence of 4 mM $CuSO_4$ was 8 mg/mL. The MBC of *P. aeruginosa* PaD2 for ciprofloxacin without copper was 4 mg/L, which was just one level of dilution. A plausible explanation is the lipophilic characteristics of ciprofloxacin alone, which might allow it to diffuse through the cellular membrane passively (Czyrski 2022), and its initial interaction with copper might attenuate this characteristic. This is supported by another study indicating that copper interactions with ciprofloxacin can increase the MIC for *Escherichia coli*, which may lower the effective concentration of the antibiotic and encourage subinhibitory levels of ciprofloxacin (Sutradhar et al. 2023). Nonetheless, the generated ciprofloxacin-copper compound (i.e., metalloantibiotic) could subsequently penetrate the cellular membrane and exert a stronger antibacterial effect (Ferreira and Gameiro 2021; Sousa et al. 2021). Taken together, this may explain the relative lack of difference between the sensitivity of *P. aeruginosa* PaD2 to ciprofloxacin with 4 mM CuSO4 and ciprofloxacin with ciprofloxacin alone in this experimental setting.

In contrast, consistent with the results of the disk-diffusion and broth macrodilution assays, *P. aeruginosa* PaD2 was resistant to tetracycline. It was still able to grow in the presence of tetracycline at concentrations as high as 64 mg/L (the highest concentration in this assay). This also indicates that the MBC of tetracycline for *P. aeruginosa* PaD2 exceeded 64 mg/L, which was the highest concentration tested. Similarly, no difference was observed in the MBC values of tetracycline between broth without and with 4 mM $CuSO_4$. This suggests that the presence of copper did not modulate the antibiotic resistance profiles, at least for ciprofloxacin and tetracycline.

In silico assessment of copper and antibiotic resistance genes of *Pseudomonas aeruginosa* PaD2

Bioinformatic analyses were performed to identify copper and antibiotic resistance genes in the genome of *P. aeruginosa* PaD2. Copper resistance genes in *P. aeruginosa* PaD2 were identified using dFAST annotation (Table 4), and antibiotic resistance genes in *P. aeruginosa* PaD2 were detected using ABRicate based on the Comprehensive Antibiotic Resistance Database (CARD) with standardized parameters (Table 5). Because high concentrations of copper are toxic to bacterial cells, bacteria require homeostatic mechanisms to prevent copper toxicity while maintaining sufficient copper for cellular processes. While most Gram-negative bacteria possess Cue and Cus systems to manage their Cu concentration, *Pseudomonas* species also use the Cop system (Andrei et al. 2020). The Cop system is a plasmid-mediated copper resistance mechanism identified on the chromosome (Pal et al. 2013). Several related protein systems, including CopA, CopB, CopC, and CopD, have been identified in *Pseudomonas lactis*, *Pseudomonas panacis*, and *Pseudomonas veronii* (Havryliuk et al. 2020).

Table 3. Minimum inhibitory concentration of *Pseudomonas aeruginosa* via the broth macrodilution assay

Antibiotic classes	Antibiotic	$CuSO_4$ concentration	MIC (mg/L)
Fluoroquinolone	Ciprofloxacin	4 mM	0.5
		0 mM	0.5
Tetracycline	Tetracycline	4 mM	>64
		0 mM	>64

Note: The assay was repeated three times for each antibiotic

Table 4. Copper resistance genes in the genome of *Pseudomonas aeruginosa* PaD2

Locus	Gene	Role
LOCUS_30630	*senC*	Cu-binding protein
LOCUS_39670	*copA1*	Cu-transporting ATPase
LOCUS_52250	*copD*	Importing Cu into The Cytoplasm
LOCUS_52260	*copC*	Cu ions binder
LOCUS_52300	*copB*	Reducing Cu(I) to Cu (II)
LOCUS_52320	*cusR*	Regulates the expression of the Cus system

Pseudomonas aeruginosa PaD2 has several copper resistance genes. Firstly, the *cusR* gene, which encodes a key component responsible for exporting Cu(I) ions from cells in the Cus system (Havryliuk et al. 2020), was discovered in *P. aeruginosa* PaD2. Secondly, the genes *copA1*, *copB*, *copC*, and *copD*, which are linked to the copper-resistant Cop system, were observed. Finally, *senC*, which encodes an SCO1/SenC-type protein that plays a crucial role in copper binding and delivery to cytochrome c oxidases, was identified. SenC functions as a terminal enzyme in the aerobic respiration of *P. aeruginosa*, particularly under conditions of copper limitation, and aids *P. aeruginosa* in adapting to copper deprivation (Quintana et al. 2017).

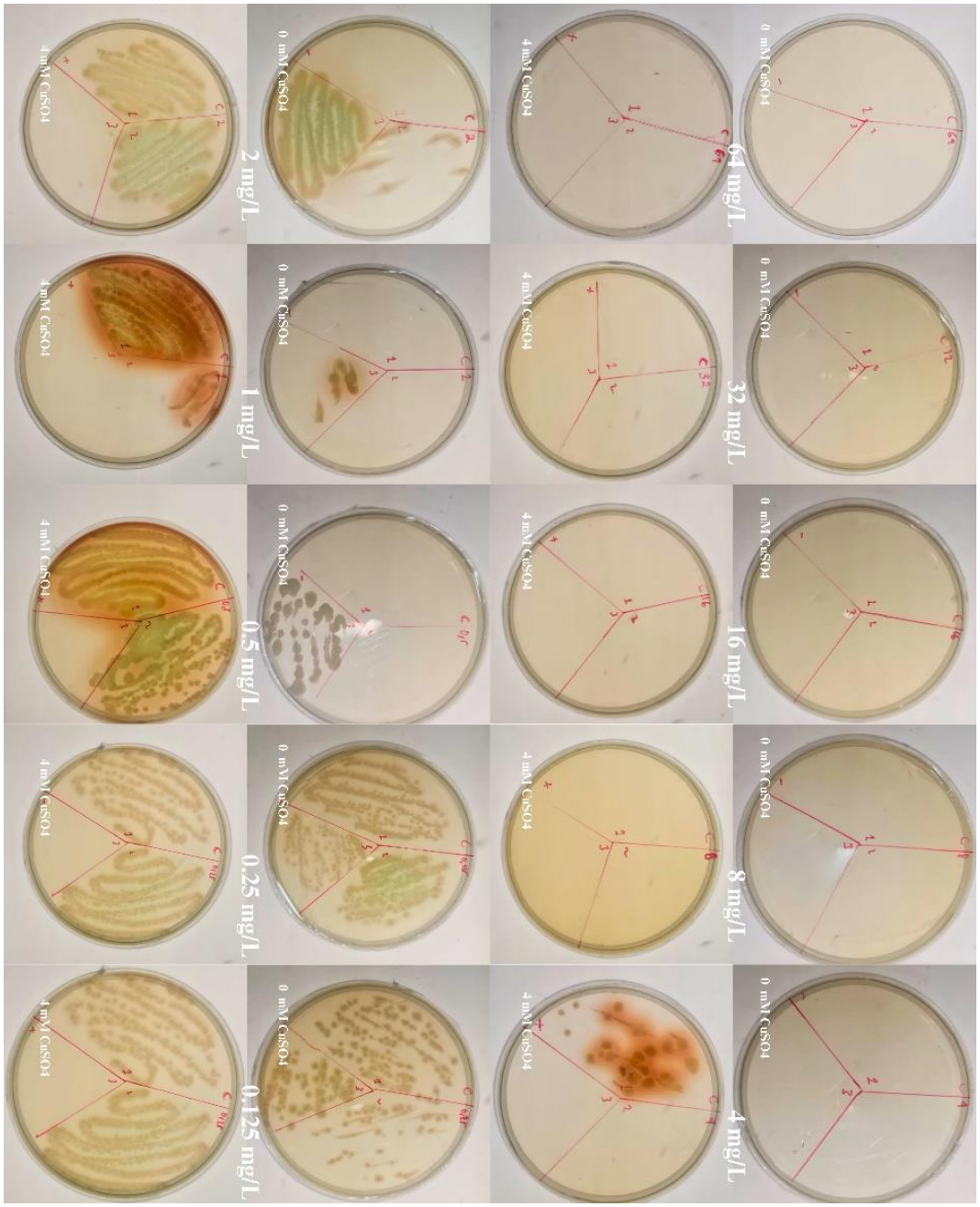

Figure 4. Minimum bactericidal concentration of *Pseudomonas aeruginosa* PaD2 in the presence of ciprofloxacin. The assay was performed on non-copper-supplemented Mueller-Hinton agar by subsequently testing the solutions from the previous broth macrodilution assay in determining the minimum inhibitory concentration. Each agar plate was streaked three times by using independent solutions (i.e., each quadrant was streaked using solution from each experiment). Concentrations of ciprofloxacin were stated as well, i.e., 64 - 32 - 16 - 8 - 4 - 2 - 1 - 0.5 - 0.25 - 0.125 mg/L. The first and third rows represent bacterial solutions tested with ciprofloxacin without copper. The second and fourth rows represent bacterial solutions tested with ciprofloxacin with 4 mM CuSO4. The media was incubated in an aerobic condition at 37°C for 24 h

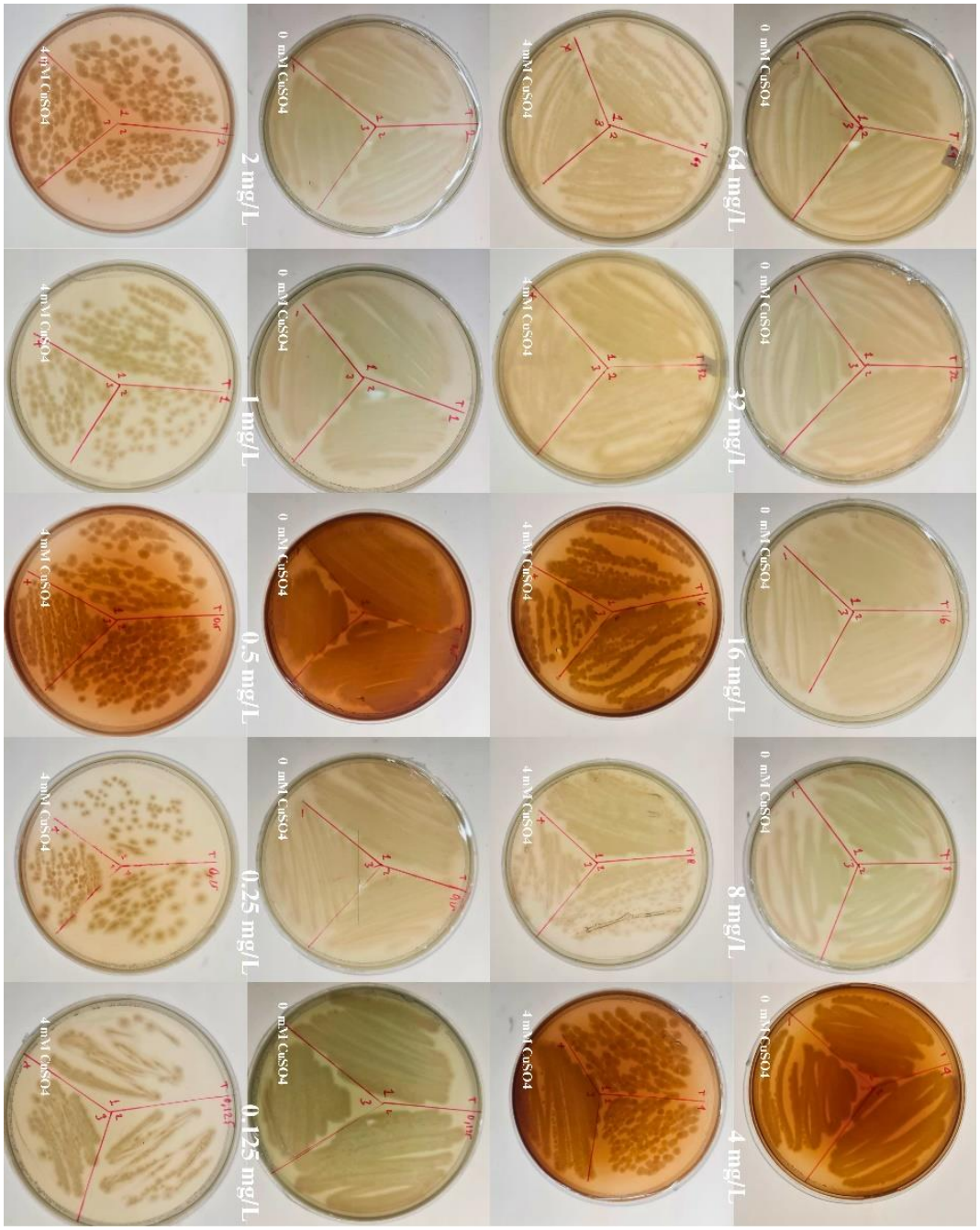

Figure 5. Minimum bactericidal concentration of *Pseudomonas aeruginosa* PaD2 in the presence of tetracycline. The assay was performed on Mueller-Hinton Agar by subsequently using solutions from the broth dilution assay in determining the minimum inhibitory concentration. Each agar plate was streaked three times by using independent solutions (i.e., each quadrant was streaked using solution from each experiment). Concentrations of tetracycline were stated as well, i.e., 64 - 32 - 16 - 8 - 4 - 2 - 1 - 0.5 - 0.25 - 0.125 mg/L. The first and third rows represent bacterial solutions tested with tetracycline without copper. The second and fourth rows represent bacterial solutions tested with ciprofloxacin with 4 mM $CuSO_4$. The media was incubated in an aerobic condition at 37°C for 24 h

Various antibiotic resistance genes were observed within the genome of *P. aeruginosa* PaD2, which can be classified into (i) efflux pumps (*armR*, *cpxR*, *emrE*, *mexA*, *mexB*, *mexC*, *mexD*, *mexE*, *mexF*, *mexG*, *mexH*, *mexI*, *mexJ*, *mexK*, *mexL*, *mexM*, *mexN*, *mexP*, *mexQ*, *mexV*, *mexW*, *muxA*, *muxB*, *muxC*, *opmB*, *opmD*, *opmE*, *oprJ*, *oprM*, *oprN*, *pmpM*, and *soxR*); (ii) antibiotic inactivation (*catB7*, *crpP*, *oxa-448*, and *aph(3')-IIB*); as well as (iii) antibiotic target alteration (*pdc-7*). Pertaining to the tetracyline-resistant phenotype of *P. aeruginosa* PaD2, the identified resistance genes included *armR*, *cpxR*, *mexA*, *mexB*, *mexC*, *mexD*, *mexG*, *mexH*, *mexI*, *mexJ*, *mexK*, *mexL*, *mexP*, *mexQ*, *mexV*, *mexW*, *muxA*, *muxB*, *muxC*, *opmB*, *opmD*, *opmE*, *oprJ*, *oprM*, and *soxR*. Notably, these genes encode proteins that are components of multidrug efflux systems, such as MexAB-OprM, MexCD-OprJ, MexEF-OprN, MexGHI-OpmD, MexPQ-OpmE, MexVW-OprM, and MuxABC-OpmB (Lorusso et al. 2022).

Table 5. Antibiotic resistance genes in the genome of *Pseudomonas aeruginosa* PaD2

Gene	Resistance	Location in genome (bp)
Efflux pump		
pmpM	Aminoglycoside, fluoroquinolone	448143- 449576
mexN	Phenicol	603452-606562
mexM	Phenicol	606559-607716
mexV	Fluoroquinolone, macrolide, phenicol, tetracycline	1556584- 1557714
mexW	Fluoroquinolone, macrolide, phenicol, tetracycline	1557765- 1560821
oprJ	Aminoglycoside, cephalosporin, fluoroquinolone, macrolide, phenicol, tetracycline	1970068- 1971507
mexD	Aminoglycoside, cephalosporin, fluoroquinolone, macrolide, phenicol, tetracycline	1971513- 1974644
mexC	Aminoglycoside, cephalosporin, fluoroquinolone, macrolide, phenicol, tetracycline	1974672- 1975835
emrE	Aminoglycoside	2426597- 2426929
mexA	Cephalosporin, fluoroquinolone, macrolide, phenicol, sulfonamide, tetracycline	3603508- 3604659
mexB	Inner membrane Cephalosporin, fluoroquinolone, macrolide, phenicol, sulfonamide, tetracycline	3604675- 3607815
oprM	Cephalosporin, fluoroquinolone, macrolide, phenicol, sulfonamide, tetracycline	3607817- 3609274
opmD	Fluoroquinolone dan tetracycline	3938866- 3940329
mexI	Fluoroquinolone dan tetracycline	3940326- 3943415
mexH	Fluoroquinolone dan tetracycline	3943428- 3944540
mexG	Fluoroquinolone dan tetracycline	3944548- 3944994
armR	Cephalosporin, fluoroquinolone, macrolide, phenicol, sulfonamide, tetracycline	4523547- 4523708
mexL	Macrolide, tetracycline	4568323- 4568961
mexJ	Macrolide, tetracycline	4569057- 4570160
mexK	Macrolide, tetracycline	4570165- 4573242
mexP	Macrolide, phenicol, tetracycline	4789287- 4790444
mexQ	Macrolide, phenicol, tetracycline	4790441- 4793602
opmE	Macrolide, phenicol, tetracycline	4793599- 4795074
cpxR	Aminoglycoside, cephalosporin, fluoroquinolone, macrolide, phenicol, sulfonamide, tetracycline	5129285- 5129962
muxA	Macrolide, tetracycline	6003751- 6005031
muxB	Macrolide, tetracycline	6005028- 6008159
muxC	Macrolide, tetracycline	6008156- 6011266
opmB	Macrolide, tetracycline	6011263- 6012762
oprN	Fluoroquinolone, phenicol	6046048- 6047466
mexF	Fluoroquinolone, phenicol	6047463- 6050651
mexE	Fluoroquinolone, phenicol	6050673- 6051917
soxR	Cephalosporin, fluoroquinolone, phenicol, rifamycin, tetracycline, triclosan	6345068- 6345538
Antibiotic inactivation		
catB7	Chloramphenicol	1390892-1391530
crpP	Ciprofloxacin (Fluoroquinolone)	1761088- 1761285
oxa-448	Cephalosporin	3075615- 3076403
aph(3')-IIb	Aminoglycoside	4037494- 4038300
Antibiotic target alteration		
pdc-7	Cephalosporin	4523547- 4523708

Note: bp: base pair

In both Gram-negative and Gram-positive bacteria, efflux pumps play a key role in removing toxic substances (including antibiotics) from cells, thereby reducing intracellular antibiotic concentrations and preventing them from reaching their target sites (Laborda et al. 2022). Four primary efflux pump systems (MexAB-OprM, MexXY, MexCD-OprJ, and MexEF-OprN) in *P. aeruginosa* have been linked to antibiotic resistance. Taken together, the presence of various resistance genes in the genome of *P. aeruginosa* strain PaD2 corroborated its resistance phenotype to tetracycline based on disk diffusion and broth microdilution assays.

Pseudomonas aeruginosa is widely recognized for its resistance to both heavy metals and antibiotics. Studies have shown that hospital strains of *P. aeruginosa* exhibit higher levels of antibiotic multiresistance, alongside resistance to mercury (Hg) and copper (Cu), while environmental strains demonstrate greater resistance to zinc (Zn) and cadmium (Cd) (Deredjian et al. 2011). In residential areas of Egypt, similar multidrug-resistant strains have been reported, where common antibiotics (i.e., rifampicin, kanamycin, ampicillin, chloramphenicol, streptomycin and tetracycline) as well as heavy metals (i.e., Zn, Hg, Cd and lead (Pb)) were ineffective in inhibiting growth of those strains of *P. aeruginosa* (Soltan 2001).

Additionally, strains isolated from agricultural water sources were found resistant to various antibiotics (i.e., ampicillin, ceftriaxone, chloramphenicol, cephalothin, cefotaxime, nitrofurantoin, kanamycin, streptomycin and tetracycline) as well as metal ions (i.e., Cu, Zn, barium (Ba), Pb and selenium (Se)) (Gutiérrez Cárdenas et al. 2017). Interestingly, several *P. aeruginosa* strains have

shown potential as bioremediation agents for metal ions. For example, strain BC15 exhibited resistance to Cd, chromium (Cr), nickel (Ni), and Pb, along with antibiotics, including ampicillin, tetracycline, chloramphenicol, streptomycin, kanamycin and erythromycin (Raja et al. 2006). Another strain, *P. aeruginosa* RA-14 demonstrated resistance to Hg, Pb, Zn, Cu, Cd and Ni, as well as to penicillin G, nalidixic acid, ceftazidime, cefotaxime, kanamycin and ampicillin (Al-Ansari et al. 2021).

This study's findings regarding multidrug resistance to copper and several common antibiotics can be interpreted from various perspectives. First, heavy metal contamination warrants significant attention. Metal-polluted environments increase the virulence of *P. aeruginosa* by facilitating the uptake of ferric iron, which is a crucial nutrient for pathogen growth within the host (Lear et al. 2022). Metal-contaminated environments can also drive the emergence of antibiotic-resistant phenotypes in *P. aeruginosa* (Lear et al. 2023). This suggests that copper remediation (e.g., via bioremediation) should be continuously performed to prevent the emergence of dangerous strains of *P. aeruginosa*. Second, our results suggest that *P. aeruginosa* PaD2 could be tested to remediate both metal and antibiotic pollutants, as this bacterial isolate could survive in the presence of both copper and certain antibiotics (e.g., tetracycline). If this method was successful, a subsequent study was conducted to test the viability of the bioremediation method for heavy metal and antibiotic pollution. Third, as these results indicated that *P. aeruginosa* PaD2 was still sensitive to several antibiotics (e.g., sulfonamide, gentamicin, and ciprofloxacin), these antibiotics could be used to control the use of this bacterial isolate to remediate copper pollution.

In conclusion, in the first phase of this project, we observed that *P. aeruginosa* PaD2 possessed the ability to survive in environments contaminated with both copper and various antibiotics, owing to its multi-resistance properties. In subsequent experiments characterizing the resistance to copper, ciprofloxacin, or tetracycline, we observed that the presence of copper did not increase or reduce the antibiotic resistance profiles towards ciprofloxacin and tetracycline. The presence of certain genes in the genome of *P. aeruginosa* PaD2 (i.e., genes involved in the efflux pumps, antibiotic inactivation and antibiotic target alteration), which may act specifically against copper or certain antibiotics, may explain the resistance phenotypes of *P. aeruginosa* PaD2 against both copper and antibiotics; these resistance mechanisms were independent of each other. In a subsequent phase of this project, the ability of *P. aeruginosa* PaD2 to remediate Cu and other antibiotic pollutants was investigated.

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