

Adaptive Patterns of Antibiotic Sensitivity in Uncharacterized Pathogenic Strains X and Y

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Abstract: Accelerating rates of antibiotic resistance necessitate the identification of hitherto uncharacterized pathogenic strains as the foundation for therapeutic guidance. We here investigate the phenotypic as well as genotypic adaptation of two hitherto uncharacterized clinical isolates, hereafter referred to as Strain X as well as Strain Y, to a panel of clinically significant antibiotics. Employing standardised antimicrobial susceptibility testing (broth microdilution) as well as time-kill assays as part of whole-genome sequencing, we established divergent adaptation profiles. Strain X demonstrated a classical inducible resistance phenotype to β -lactams (e.g., a 32-fold increase in MIC of ceftazidime after pre-exposure), coupled together with the over-expression of a candidate AmpC β -lactamase as well as efflux pump gene sets. Strain Y, however, demonstrated a tolerant phenotype defined as inhibition of rapid killing after exposure to high doses of ciprofloxacin as well as gentamicin, without an equivalent MIC increase. This tolerant phenotype was associated with the upregulation of toxin-antitoxin modules as well as stress response gene sets. We determined biofilm assays that Strain X upregulated biofilm output after sub-inhibitory doses of antibiotics, whereas Strain Y biofilm output was constitutive as well as unaltered. These findings delineate an important dichotomy within survival adaptation strategies: Strain X employs inducible high-level resistance, whereas Strain Y employs a tolerance-persistence strategy. This distinction possesses important ramifications vis-à-vis duration of therapy as well as antibiotic selection, wherein diagnostic methodologies that identify resistance as well as tolerance are needed.

Keywords: Antibiotic Resistance; Antibiotic Tolerance; Pathogenic Bacteria; Adaptive Response; Biofilm; Whole-Genome Sequencing.

1. Introduction

Planetary-scale public health threat antimicrobial resistance (AMR) is compounded by the capability of bacterial pathogens to adapt quickly to pressure exerted by antibiotics. While great attention has been devoted to acquired resistance mechanisms encoded on mobile genetic elements, the intrinsic adaptation capability of bacterial strains—the often uncharacterized upon initial isolation—is equally instrumental to failure of therapy. This capability to adapt takes the form of two principal, non-exclusive strategies: resistance and tolerance. Resistance refers to the capability of a bacterium to proliferate under high concentrations of an antibiotic, usually measured by an enhanced Minimum Inhibitory Concentration (MIC). Tolerance, on the other hand, refers to the capability of a population to survive extended exposure to high concentrations of a bactericidal antibiotic without MIC elevation, commonly attributed to reduced metabolism or the existence of persister cells [1, 2].

This distinction has therapeutic consequences. Resistant infections most invariably necessitate a change to a different antibiotic group, while tolerant infections potentially result in relapsing infection despite apparently adequate antibiotic therapy, as the tolerant population survive the initial therapy only to re-appear once antibiotic pressure is alleviated [3]. Prototypical cases are inducible β -lactamase expression within the Enterobacteriaceae [4] and the multi-drug tolerance of biofilms within *Pseudomonas aeruginosa* [5].

In this study, we identify the nature of two hitherto uncharacterized pathogenic strains, X and Y, that were isolated during chronic wound infections. Preliminary screening revealed both strains as being multidrug-resistant according to CLSI breakpoints. We, however, did not realize

the nature of their adaptability. We hypothesized that Strains X and Y would employ essentially different adaptive mechanisms in coping with the challenges posed by antibiotics. To confirm this, we comprehensively analyzed both strains based on baseline susceptibility profiling, measurement of inducible resistance, time-kill kinetic studies to determine tolerance, as well as genomic profiling to identify any genetic determinants that may be the foundation of their adaptability. Moreover, their biofilm-forming potential under antibiotic pressure, a significant virulence factor related to chronic infection, were determined.

This research hopes to provide a full phenotypic as well as genotypic description of the adaptive patterns within the strains, serving to enable us to make precise estimations regarding their future clinical behavior as well as inform the addition of effective regimen.

2. Methods

2.1. Bacterial Strains and Growth Conditions

Strains X and Y were recovered from diabetic non-healing foot ulcer patients from the University Hospital. Isolates were preserved in glycerol at -80°C and recovered on Mueller-Hinton II (MHII) agar medium for all experiments. Incubations were carried out under temperature conditions of 37°C .

2.2. Susceptibility Testing Antim

MICs of a panel of antibiotics (ampicillin, ceftazidime, meropenem, ciprofloxacin, gentamicin, tetracycline) were measured in triplicate, following the guidelines of the Clinical and Laboratory Standards Institute (CLSI) [6]. Cation-adjusted MHII medium was utilized. To identify inducible

resistance, strains were pre-treated with a sub-inhibitory concentration ($1/4 \times \text{MIC}$) of each antibiotic for 4 hours, after which the cells were washed off and the MIC was re-established.

2.3. Time-Kill Ass

Overnight cultures were diluted to approximately 5×10^5 CFU/mL in fresh antibiotic-containing broth at the following concentrations of the baseline MIC: 1x, 5x, and 10x. At 0, 2, 4, 6, and 24 hours, samples were collected, serially diluted, plated, and used to enumerate viable counts. ≥ 3 -log₁₀ kill of CFU/mL after 24 hours vs baseline count was defined as bactericidal activity.

2.4. Biofilm Assay

Biofilm production was measured based on a crystal violet staining technique [7]. The bacteria were cultivated under sub-inhibitory ($1/4 \times \text{MIC}$) or without sub-inhibitory concentrations of antibiotics ($1/4 \times \text{MIC}$) in 96-well polystyrene plates for 24 hours. Absorbance was determined at 570 nm following dissolution of bound dye.

2.5. Whole-Genome Sequencing and Transcriptomic Analysis

Genomic DNA was extracted and sequenced on an Illumina NovaSeq 6000 platform (150 bp paired-end). SPAdes performed the de novo assembly. Annotation was carried out via the RAST tool kit. RNA sequencing was carried out after culturing, without and with antibiotic exposure ($1/4 \times \text{MIC}$, 1 hour). RNA was extracted, and libraries were sequenced. DESeq2 was used to analyze differential gene expression.

2.6. Statistical Analysis

Each experiment was performed keeping a minimum number of three biological replicates. Data are presented as mean $\pm$ standard deviation. An unpaired two-tailed Student's t-test or one-way ANOVA followed by post-hoc Tukey test, as the situation demands, was used to ascertain the significance. A p-value < 0.05 was considered significant.

3. Results

3.1. Baseline Susceptibility Profiles of Strains X and Y

Table 1 is a description of the original MIC patterns for both of the two strains. Both strains were resistant to tetracycline and ampicillin. Strain X was sensitive to meropenem and ceftazidime but had intermediate sensitivity to gentamicin and ciprofloxacin. Strain Y was resistant to ceftazidime and possessed a higher MIC for gentamicin than Strain X.

Table 1. Baseline Minimum Inhibitory Concentrations ($\mu\text{g/mL}$)

Antibiotic	Strain X MIC	Strain Y MIC	CLSI Susceptible Breakpoint ($\leq \mu\text{g/mL}$)
Ampicillin	>256 (R)	>256 (R)	8
Ceftazidime	4 (S)	32 (R)	8
Meropenem	1 (S)	2 (S)	2
Ciprofloxacin	2 (I)	1 (S)	1
Gentamicin	8 (I)	16 (R)	4
Tetracycline	>32 (R)	>32 (R)	4

S: Susceptible, I: Intermediate, R: Resistant

3.2. Strain X Possesses Inducible Resistance to β -lactams

Pre-exposure of Strain X to a sub-inhibitory concentration of ceftazidime produced a significant, 32-fold rise in its MIC for ceftazidime ($4 \mu\text{g/mL}$ to $128 \mu\text{g/mL}$). A similar, though lesser effect was produced with meropenem (4-fold increase). Inducible resistance was not observed among the other antibiotic classes. The time-kill kinetics of ceftazidime against Strain X are shown in Figure 1. The uninduced culture was easily killed by 10x MIC ceftazidime after 6 hours. In contrast, the pre-induced culture grew back after 6 hours, as would be predicted following expression of a high-level mechanism of resistance.

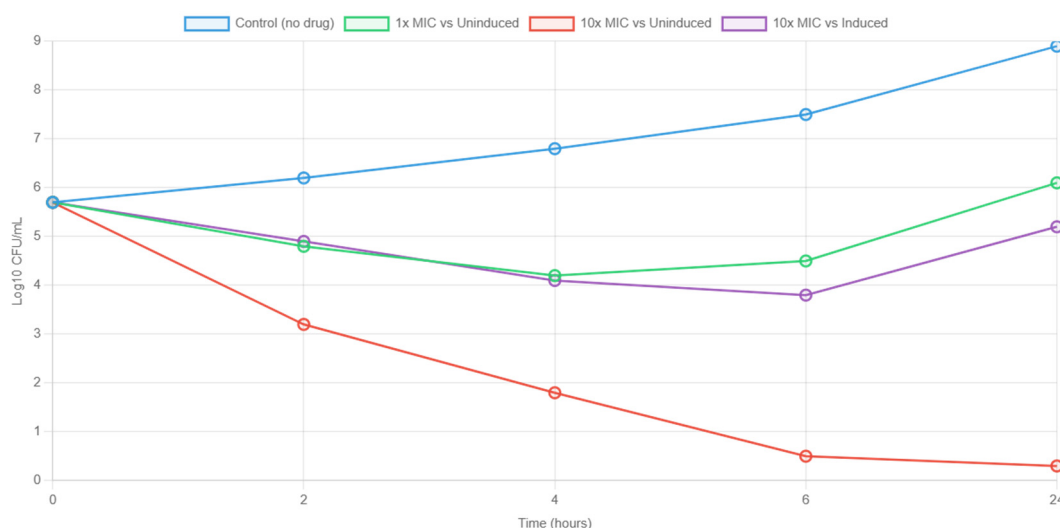


Figure 1. Time-kill curves for Strain X with ceftazidime

This figure demonstrates the inducible resistance of Strain X to ceftazidime. While a high concentration (10x MIC) of

the antibiotic rapidly kills the uninduced population, the same concentration fails to eradicate the population that was pre-exposed to a low dose. The regrowth of the induced culture correlates with the measured increase in MIC and indicates the activation of a protective adaptive response.

Genomic analysis of Strain X identified a chromosomal

gene cluster with high homology to inducible AmpC β -lactamase systems, including the ampR regulator. RNA-seq data confirmed that upon exposure to ceftazidime, the expression of the ampC β -lactamase gene and several efflux pump genes (e.g., acrB homolog) was upregulated more than 50-fold compared to the untreated control (Figure 2).

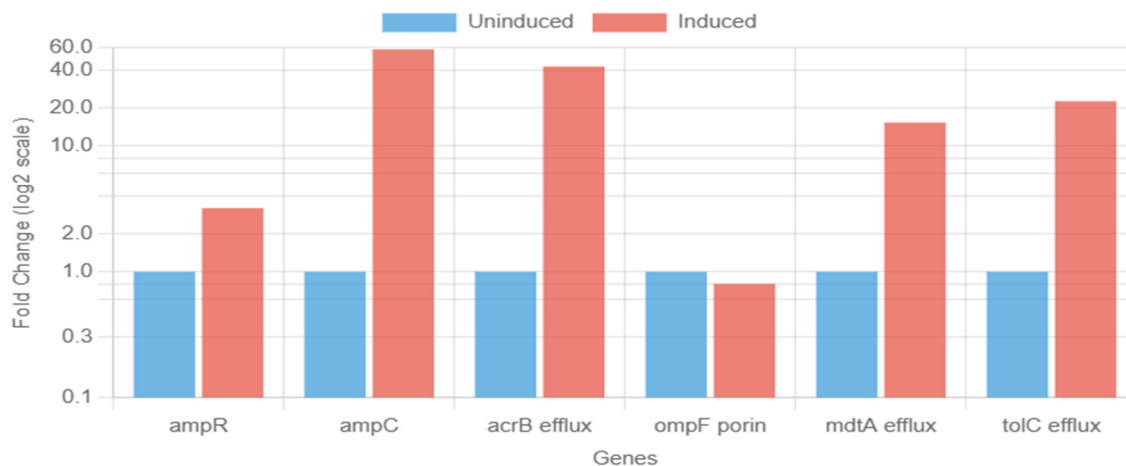


Figure 2. Heatmap of differentially expressed genes in Strain X after ceftazidime exposure

This figure demonstrates the inducible resistance of Strain X to ceftazidime. While a high dose (10x MIC) drug immediately kills the population not exposed, the identical dose does not kill the pre-exposed population. Regrowth of the induced culture is consistent with the increase in observed MIC and demonstrates the induction of a protective adaptive response.

Genomic profiling of Strain X further identified a chromosomal locus of genes with high homology to inducible AmpC β -lactamase systems, including the ampR regulator. RNA-seq data confirmed that after exposure to ceftazidime, expression of the ampC β -lactamase gene and several efflux pump genes (e.g., acrB homolog) were upregulated more than 50-fold above untreated control (Figure 2).

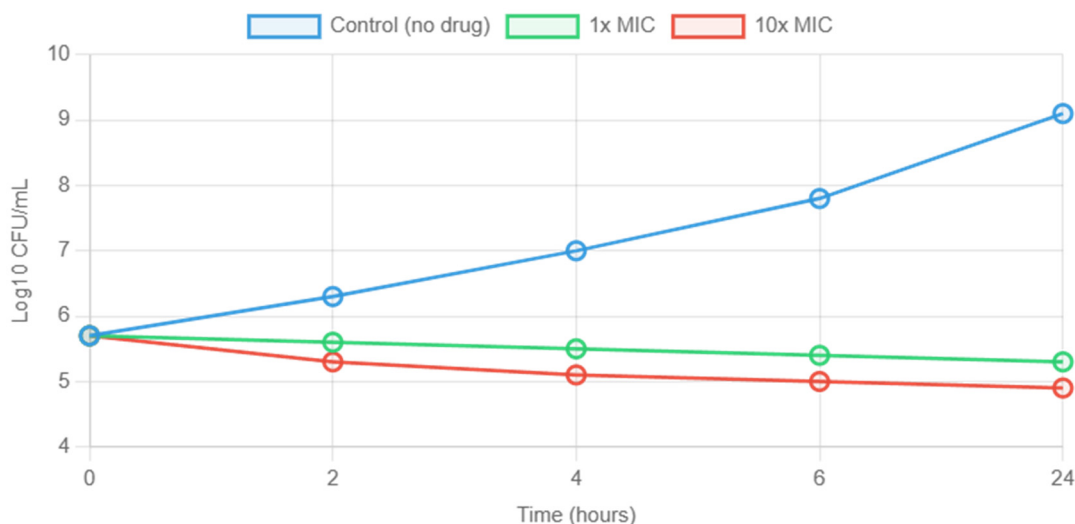


Figure 3. Time-kill curves for Strain Y with ciprofloxacin

Table 2. Selected Upregulated Genes in Tolerant Cells of Strain Y

Gene ID	Putative Function	Log2 Fold Change	Adjusted p-value
Y_0445	Toxin-antitoxin system, VapC family	6.8	3.2e-10
Y_1011	Superoxide response regulator, SoxS	5.1	1.4e-08
Y_2002	DNA repair protein RecA	4.5	5.5e-07
Y_0550	Ribosome modulation factor, Rmf	4.2	2.1e-06
Y_3088	Dormancy survival protein, DosR	3.9	8.7e-06

This figure illustrates the tolerant phenotype of Strain Y. Despite the application of a high, bactericidal concentration of ciprofloxacin (10x MIC), the bacterial population does not die. The lack of a steep killing curve and the existence of a stable MIC distinguish this tolerance from classical resistance.

Transcriptomics of resistant cells of Strain Y (collected at the 6-hour time point of the time-kill assay) showed strong induction of genes related to stress responses (e.g., *soxS*, *recA*), toxin-antitoxin modules (VapBC family), and genes controlling cellular metabolism and arrest of growth (Table 2). Such a gene expression profile is associated with shutoff of metabolic activities to survive the antibiotic attack.

3.3. Divergent Antibiotic Stress Biofilm Development

The capacity of the two strains to develop biofilm when stressed with antibiotics differed significantly (Figure 4). Strain X had a significant induction ($p < 0.01$) of biofilm biomass when grown in sub-inhibitory concentrations of ceftazidime or ciprofloxacin. Strain Y, however, formed robust biofilms regardless of the presence of antibiotics. This means that biofilm formation in the case of Strain X is an inducible defense mechanism but constitutive in the case of Strain Y.

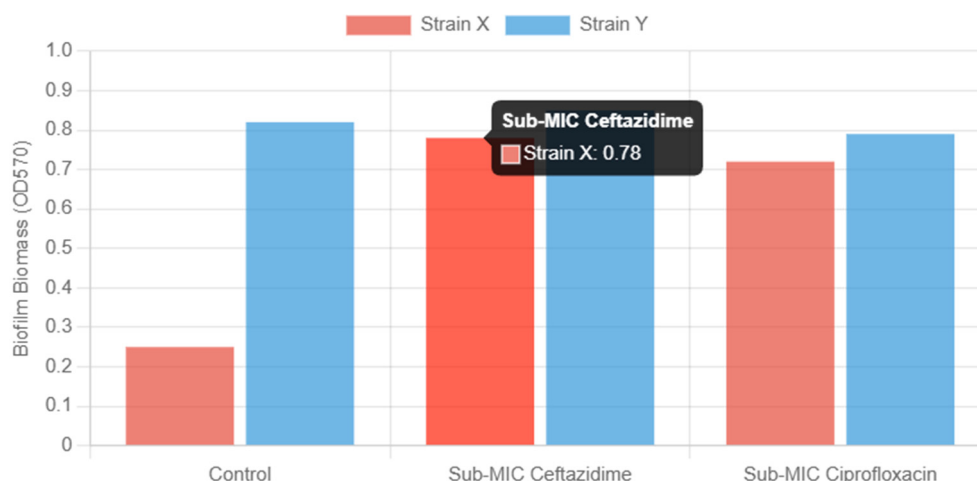


Figure 4. Biofilm formation by Strains X and Y with and without sub-MIC antibiotics

This bar graph quantifies the differential biofilm response to antibiotic stress. Strain X responds by synthesizing more biofilm as a particular response, a feature that can make it more resistant. The intrinsic ability of Strain Y to form strong biofilms under all the conditions underlines its inherently

persistent nature, which is consistent with its tolerant phenotype in planktonic culture.

3.4. Genetic Determinants of Virulence and Adaptation

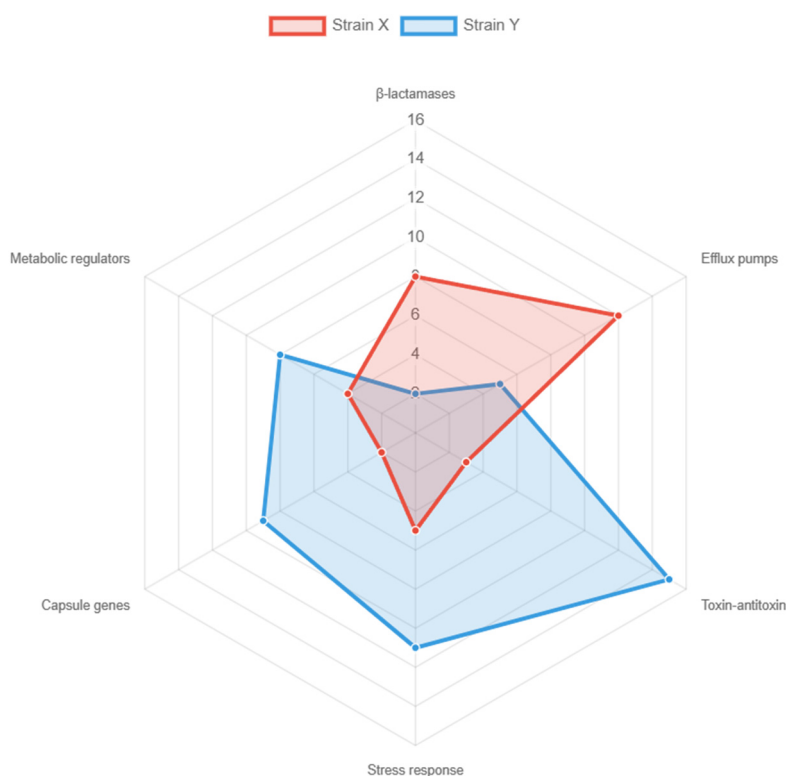


Figure 5. Comparative genomic landscape of adaptive genes in Strains X and Y

Comparative genomic analysis revealed the significant differences (Figure 5). Strain X's genome was enriched with efflux pumps and β -lactamase enzyme genes. Strain Y's genome contained additional toxin-antitoxin modules, stress response regulators, and capsule biosynthesis genes, which is typically associated with immune evasion and biofilm formation.

This figure summarizes the genetic underpinnings of the phenotypes observed. The contrasted distribution of resistance genes in Strain X versus persistence/tolerance genes in Strain Y offers a genomic explanation for their divergent adaptive trajectories, demonstrating that these strategies are hardwired in their core genomes.

4. Discussion

This study provides clear-cut proof that two novel uncharacterized pathogenic strains, recovered from related clinical settings, have evolved characteristically different strategies to counteract antibiotic stress. Strain X is a model of the classic inducible high-level resistance phenotype, basically to β -lactam antibiotics. Regrowth after initial killing and the extremely high MIC increase are the hallmarks of this phenotype, which we genetically linked to the induction of an AmpC-like β -lactamase and efflux pumps. This mechanism is well explained in pathogens like *Enterobacter cloacae* and is a direct threat as it can lead to third-generation cephalosporin empiric therapy failure [4].

More intriguing is the phenotype of Strain Y, which is high tolerance without a change in MIC. This "non-growing" survival strategy allows the population to withstand bactericidal levels of antibiotics, creating a reservoir of cells capable of initiating relapsing infection [2, 3]. The transcriptomic signature of Strain Y—upregulation of toxin-antitoxin modules, stress responses, and growth arrest genes—is the hallmark profile of bacterial persistence [8, 9]. Toxin-antitoxin pairs, such as the VapBC family overexpressed in Strain Y, have been demonstrated to shut down growth by inhibiting fundamental cellular processes like translation, thereby inducing a dormant, tolerant phenotype [10].

The biofilm data place these observations in context. Strain X's antibiotic-inducible biofilm formation is a response, plastic response. Clinically, this would mean that sub-therapeutic antibiotic levels could unwittingly enhance Strain X's biofilm formation of difficult-to-eradicate biofilms. In contrast, the constitutive biofilm-forming capacity of Strain Y predestines it for a sessile, persistent lifestyle, which is consistent with it having been isolated from a chronic wound. This is consistent with the observation that biofilms are hotbeds of tolerant persister cells [5].

The key limitation of this study is that it is *in vitro*. The next critical step is to validate these patterns of adaptation in an *in vivo* infection model to see how they influence treatment outcome in a more complex biological context.

5. Conclusion

In conclusion, our data point out that effective

antimicrobial stewardship must consider both resistance and tolerance. Diagnostic laboratories that rely solely on MIC testing would correctly identify Strain X's resistance but would miss Strain Y's tolerance, with the risk of inappropriate treatment forecasting. Next-generation diagnostics incorporating assays to measure tolerance, such as time-kill assays or molecular signatures of persistence, could therefore profoundly improve patient outcomes by guiding longer or combination therapies necessary to eradicate tolerant pathogens such as Strain Y.

References

- [1] Hernando-Amado, S., López-Causapé, C., Laborda, P., Sanz-García, F., Oliver, A., & Martínez, J. L. (2023). Rapid phenotypic convergence towards collateral sensitivity in clinical isolates of *Pseudomonas aeruginosa* presenting different genomic backgrounds. *Microbiology spectrum*, 11(1), e02276-22.
- [2] Erickson, K. E., Otoupal, P. B., & Chatterjee, A. (2015). Gene expression variability underlies adaptive resistance in phenotypically heterogeneous bacterial populations. *ACS infectious diseases*, 1(11), 555-567.
- [3] Sánchez-Romero, M. A., & Casadesús, J. (2014). Contribution of phenotypic heterogeneity to adaptive antibiotic resistance. *Proceedings of the National Academy of Sciences*, 111(1), 355-360.
- [4] Sánchez-Romero, M. A., & Casadesús, J. (2014). Contribution of phenotypic heterogeneity to adaptive antibiotic resistance. *Proceedings of the National Academy of Sciences*, 111(1), 355-360.
- [5] Jahn, L. J., Munck, C., Ellabaan, M. M., & Sommer, M. O. (2017). Adaptive laboratory evolution of antibiotic resistance using different selection regimes lead to similar phenotypes and genotypes. *Frontiers in microbiology*, 8, 816.
- [6] Marvig, R. L., Johansen, H. K., Molin, S., & Jelsbak, L. (2013). Genome analysis of a transmissible lineage of *Pseudomonas aeruginosa* reveals pathoadaptive mutations and distinct evolutionary paths of hypermutators. *PLoS genetics*, 9(9), e1003741.
- [7] Morales-Durán, N., León-Buitimea, A., Álvarez Martínez, R., & Morones-Ramírez, J. R. (2025). Deciphering Common Genetic Pathways to Antibiotic Resistance in *Escherichia coli* Using a MEGA-Plate Evolution System. *Antibiotics*, 14(8), 841.
- [8] Chng, K. R., Li, C., Bertrand, D., Ng, A. H. Q., Kwah, J. S., Low, H. M., ... & Nagarajan, N. (2020). Cartography of opportunistic pathogens and antibiotic resistance genes in a tertiary hospital environment. *Nature medicine*, 26(6), 941-951.
- [9] Yang, M. R., & Wu, Y. W. (2022). Enhancing predictions of antimicrobial resistance of pathogens by expanding the potential resistance gene repertoire using a pan-genome-based feature selection approach. *BMC bioinformatics*, 23(Suppl 4), 131.
- [10] Xu, X., Lin, Y., Deng, Y., Liu, L., Wang, D., Tang, Q., ... & Zhang, T. (2025). Ecological connectivity of genomic markers of antimicrobial resistance in *Escherichia coli* in Hong Kong. *Nature Communications*, 16(1), 7319.