Within-Patient Evolution of *Pseudomonas aeruginosa* Populations During Antimicrobial Treatment

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Abstract: Multidrug-resistant (MDR) *Pseudomonas aeruginosa* infections pose a major challenge to effective treatment. Understanding genomic adaptations during antimicrobial therapy in patients infected with this pathogen is crucial for preventing therapeutic failure. Here we investigated the population diversity and evolution of *P. aeruginosa* collected longitudinally from six patients who evolved MDR infections. Serial *P. aeruginosa* clinical isolates (n=63) and culture-enriched metagenomic populations (n=39) were collected and subjected to whole-genome sequencing. The resulting data were used to characterize and compare the species composition, multi-locus sequence types (STs), and resistance-associated mutations present within each sample type. Single-isolate sequencing showed that each patient was infected with a single *P. aeruginosa* strain that accumulated mutations and became increasingly antibiotic-resistant over time. Mutations in genes associated with β-lactam resistance, including *ampC, ftsI*, and *oprD,* arose over time and corresponded with changes in antimicrobial susceptibility in single isolates. Species profiling of culture-enriched metagenomic populations revealed that all samples contained *P. aeruginosa*, but several also contained additional Gram-negative pathogens. Metagenomic analysis of culture-enriched populations identified resistance-

associated mutations at variable frequencies, many of which were not identified in single isolates from the same sample. In some cases, resistance-associated mutations initially detected at low frequency rose to fixation after antimicrobial treatment. Overall, this study shows that population-based metagenomic sequencing effectively captures within-patient genomic diversity of *P. aeruginosa* during antimicrobial therapy, and could aid in the detection and interpretation of resistance-associated mutations in this pathogen.

Importance: *Pseudomonas aeruginosa* infections are notoriously difficult to treat and are associated with high rates of morbidity and mortality. While the genetic basis of resistance in *P. aeruginosa* is well documented *in vitro*, less is known about how resistance evolves within patients during antibiotic therapy. Standard approaches based on analysis of clonal isolates may miss within-patient diversity, potentially overlooking low-frequency mutations that contribute to treatment failure. In this study, we integrated single-colony whole-genome sequencing with culture-enriched metagenomic sequencing to monitor the evolution of *P. aeruginosa* populations in patients receiving antibiotic therapy. This approach enabled the detection of emerging resistance mutations, such as low-frequency variants in *ampC* and *ftsI*, before these variants rose to fixation. It also revealed genetically resistant subpopulations missed by isolate sequencing alone. Together, our findings highlight the value of population-based metagenomic sequencing in capturing bacterial adaptation during infection, and underscore its potential to improve resistance surveillance and guide personalized antimicrobial therapy.

Keywords: culture-enriched metagenomics; *Pseudomonas aeruginosa*; within-host evolution; antibiotic resistance

Observation: *Pseudomonas aeruginosa* is recognized as a serious public health threat by the Centers for Disease Control and Prevention and has been classified by the World Health

Organization as a critical priority pathogen, emphasizing the urgent need for new antimicrobial therapies[1]. A variety of intrinsic resistance mechanisms, combined with a remarkable capacity to acquire additional resistance determinants, make *P. aeruginosa* infections increasingly difficult to treat[2]. Infections caused by multidrug-resistant (MDR) strains are particularly concerning and often necessitate the use of newer β-lactam/β-lactamase inhibitor combinations or siderophore cephalosporins to manage[3,4]. However, the efficacy of these agents is frequently undermined by the emergence of diverse and often convergent resistance mechanisms. These include upregulation of multidrug efflux systems (e.g., MexAB-OprM), inactivation or loss of porins (OprD), mutations affecting chromosomal β-lactamase expression (AmpC), and point mutations in essential antibiotic target genes like *ftsI* (encoding penicillin-binding protein 3), *gyrA*, and *gyrB*[4,5]. Alterations in regulatory genes such as *lasR* further contribute to phenotypic heterogeneity by modulating quorum sensing pathways, which can affect antibiotic tolerance and persistence[2]. These adaptive mutations frequently arise under selective therapeutic pressures, facilitating rapid within-host evolution and complicating effective treatment[5].

Although resistance mechanisms in *P. aeruginosa* have been well characterized *in vitro* and among serial clinical isolates collected from infected patients[6,7], much less is known about how infecting *P. aeruginosa* populations evolve within patients during antibiotic therapy[8]. Treatment decisions are typically guided by antimicrobial susceptibility testing and whole-genome sequencing analysis of individual bacterial clones, assuming that these reflect the entire infecting population. However, this approach offers limited insight into within-host diversity and often misses low-frequency variants that may influence treatment outcomes[9,10]. Culture-enriched metagenomic sequencing addresses these limitations by pooling all colonies from a selective culture plate and performing shotgun metagenomic sequencing, enabling a more comprehensive analysis of population-level diversity and evolution during infection[9,11].

Here we investigated the within-host evolution of *P. aeruginosa* populations in six patients who developed MDR infections during treatment and compared between results obtained from clonal and culture-enriched metagenomic sequencing from the same clinical specimens. We hypothesized that reliance on single-colony testing would underestimate the genotypic and phenotypic diversity of *P. aeruginosa* within the infected host, particularly under antimicrobial selection. A total of 63 clinical isolates and 39 culture-enriched metagenomic populations were collected from six patients, sequenced, and analyzed (**Figure 1**) (**Supplemental File 1**) (**Supplemental Methods**). In all patients, susceptibility profiles of *P. aeruginosa* isolates progressively shifted toward increasing resistance over the course of antibiotic treatment. This aligns with prior studies documenting the rapid evolution of resistance in *P. aeruginosa* under antibiotic stress, especially during prolonged therapy in chronic infections[12,13]. In two patients (Patients 1 and 6), isolates collected at the final time point exhibited a more antibiotic-susceptible profile compared to earlier isolates, suggesting that resistant bacteria may have been outcompeted or replaced, potentially due to changes in treatment, host immunity, or fitness costs associated with resistance[14].

Species profiling of culture-enriched metagenomic samples confirmed the presence of *P. aeruginosa* in all samples (**Figure 1**). Metagenomic samples from three patients (Patients 1–3) contained only *P. aeruginosa*, while the other three patients (Patients 4–6) exhibited polymicrobial infections that included *P. aeruginosa* and other organisms (**Supplemental Figure 1**). In each case, the same species were identified as part of standard-of-care testing in the clinical microbiology laboratory, suggesting that this approach accurately captured polymicrobial infections.

Single-colony sequencing revealed a single *P. aeruginosa* sequence type (ST) that was unique to each patient, and all colonies sequenced from the same patient belonged to the same ST (**Supplemental Figure 2**). Analysis of within-patient single nucleotide polymorphisms

(SNPs) among *P. aeruginosa* isolates collected from the same patient revealed low genetic diversity in five of the six patients, with pairwise SNP differences ranging from 0 to 23. This narrow range suggested that each patient was infected with a single strain that underwent clonal expansion during infection. In contrast, one patient (Patient 1) displayed markedly higher within-host diversity, with SNP distances reaching up to 411 (**Supplemental Table 2**). This elevated level of genetic variation could be attributed to the presence of hypermutators, which have increased mutation rates and rapidly diversify, particularly under selective pressures such as antibiotic treatment or the host immune response[15]. Supporting this interpretation, multiple mutations were identified in DNA mismatch repair genes from this patient's isolates, including W181R and S324P in *mutS* and L35P in *mutL*, indicating a defective mismatch repair system.

We next explored whether culture-enriched metagenomic sequencing could enhance the detection of resistance-associated mutations that might be missed by single-colony sequencing. In our analysis of 11 resistance-associated genes across 6 patients, culture-enriched metagenomic sequencing provided additional insight in 19/66 (29%) cases. In 84% of these cases (n=16/19), this was due to the detection of resistance-associated mutations that were not identified by single-colony sequencing (**Supplemental Table 3**). Comparison of single-colony and culture-enriched metagenomic sequencing revealed distinct interpretations depending on the method used. In Patient 3, single-colony sequencing identified a mutation in *ampC* (P243S) but failed to detect a co-occurring mutation (E247K) that was present at over 80% frequency in the population (**Figure 2A**). This highlights a key limitation of the single-colony approach, which might randomly capture minority variants while missing the dominant genotype, ultimately leading to an incomplete or misleading resistance profile. In both Patients 3 and 4, mutations in *ftsI* were identified at low frequency in population samples collected at earlier time points, and the same variants were later detected in single colonies as well as in populations at high frequency (**Figure 2A and 2B**). This illustrates how population sequencing can detect emerging

mutations before they become dominant. Finally, in Patient 6 we identified mutations at allele frequencies less than 50% that were exclusively detected in population samples and were never observed in single-colony genomes (**Figure 2C**). Together these findings emphasize the higher sensitivity of culture-enriched metagenomic sequencing for capturing within-host diversity.

Here we focused on a targeted set of genes known to be associated with antibiotic resistance in *P. aeruginosa*. While this approach allowed for in-depth analysis of clinically relevant resistance mechanisms, it likely underrepresents the broader genomic heterogeneity within infecting *P. aeruginosa* populations. Other adaptive changes (such as mutations related to antibiotic tolerance, biofilm formation, metabolic remodeling, etc.) may also contribute to treatment failure, but were not systematically assessed here. Future studies incorporating unbiased, genome-wide analyses could provide a more comprehensive view of within-host evolution during bacterial infection and antimicrobial treatment.

In summary, this study demonstrates that culture-enriched metagenomic sequencing provides enhanced resolution of within-host evolution of *P. aeruginosa* during antimicrobial treatment in human patients. We found that population-level sequencing consistently identified low-frequency variants that were missed by single-colony sequencing, revealing a more comprehensive picture of the adaptive landscape in these patients. As WGS becomes more commonly used in clinical practice, culture-enriched metagenomic sequencing could provide greater sensitivity for studies of antibiotic resistance and evolutionary dynamics, which could contribute to improved management of patients with difficult-to-treat infections.

Supplemental Materials

Supplemental Figure 1

Supplemental Figure 2

Supplemental Methods

Supplemental File 1

Supplemental Table 1

Supplemental Table 2

Supplemental Table 3

Data Availability

Genome sequencing data generated and analyzed in this study has been publicly deposited at NCBI under BioProject PRJNA1330241.

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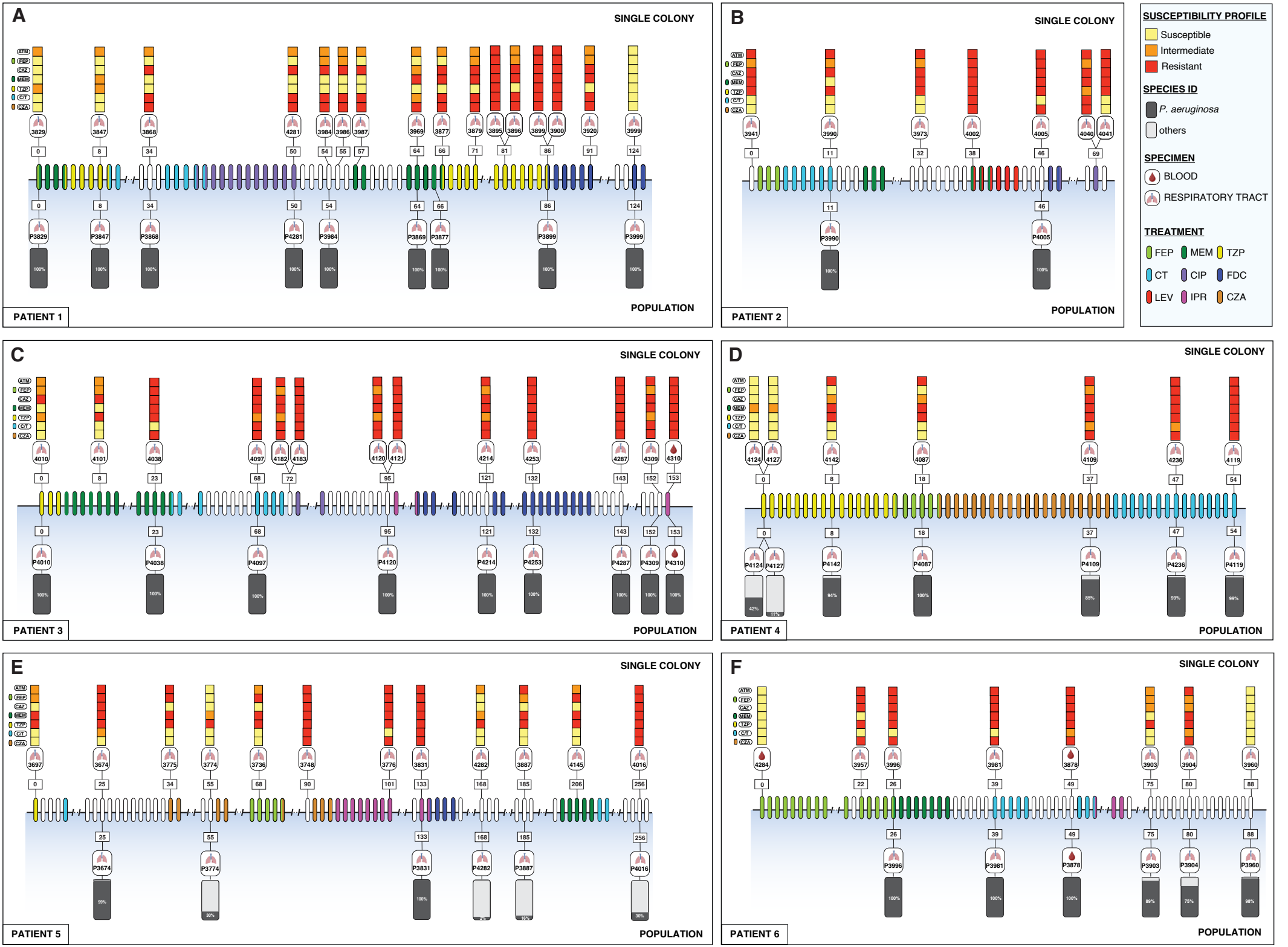
Figure Legends

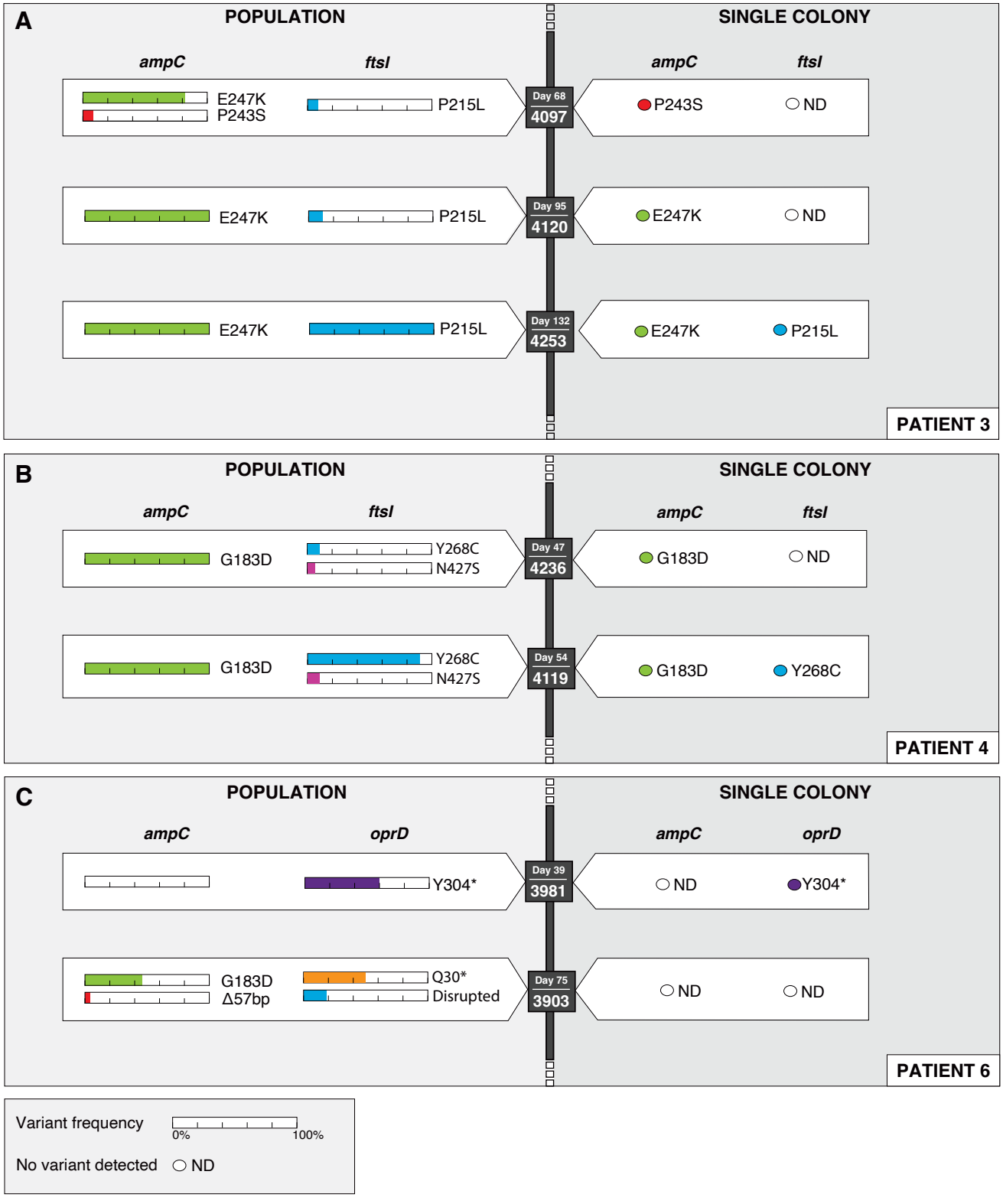
Figure 1. Timelines of *P. aeruginosa* infection and treatment in six patients with MDR infections. Each panel shows longitudinal sampling of *P. aeruginosa* single colony isolates (above timeline) and culture-enriched metagenomic populations (below timeline) during recurrent infection for each patient. Colored squares indicate the antimicrobial susceptibility profile of each isolate, and grey bars show the proportion of each population composed of *P. aeruginosa* or other bacterial species. β-lactam antibiotics tested included aztreonam (ATM), cefepime (FEP), ceftazidime (CAZ), meropenem (MEM), piperacillin/tazobactam (TZP), ceftolozane/tazobactam (C/T), and ceftazidime/avibactam (CZA). Ovals in each timeline are colored based on antimicrobial treatment administered to each patient, including FEP, MEM, TZP, C/T, ciprofloxacin (CIP), cefiderocol (FDC), levofloxacin (LEV), imipenem/relebactam (IPR), and CZA. Sample identifiers, specimen source (respiratory tract or blood), and collection timepoints (in days) are indicated above and below each timeline.

Figure 2. Comparison of resistance-associated mutations detected in culture-enriched metagenomic populations versus single colonies. Each panel represents a subset of the isolate and population data collected from each patient, and displays mutations identified in populations (left) and in single colony isolates (right) collected from the same specimens at different sampling timepoints. Nonsynonymous mutations, nonsense mutations, and structural variants are shown in β-lactam resistance-associated genes *ampC*, *oprD*, and *ftsI*. Colored bars represent the allele frequency (%) of each mutation in culture-enriched metagenomic populations. Sample ID and collection day are indicated for each comparison.

Supplemental Figure 1. Relative abundance of different bacterial species in culture-enriched metagenomic population samples collected from each patient.

Supplemental Figure 2. (A) Core genome phylogenetic tree of 63 single-colony *P. aeruginosa* isolates, colored by patient. Tree branches are marked with the multilocus sequence type (ST) that all isolates from the same patient belong to. Isolate source is noted for each isolate. (B-G) Time-dated phylogenetic trees of single-colony *P. aeruginosa* isolates collected from each patient.


A
SINGLE COLONY
ATM
FEP
CAZ
MEM
TZP
C/T
CZA
3829 3847 3868 4281 3984 3986 3987 3969 3877 3879 3895 3896 3899 3900 3920 3999
0 8 34 50 54 55 57 64 66 71 81 86 91 124
P3829 P3847 P3868 P4281 P3984 P3869 P3877 P3899 P3999
100%
PATIENT 1
POPULATION
B
SINGLE COLONY
3941 3990 3973 4002 4005 4040 4041
0 11 32 38 46 69
P3990 P4005
PATIENT 2
POPULATION
SUSCEPTIBILITY PROFILE
Susceptible
Intermediate
Resistant
SPECIES ID
P. aeruginosa
others
SPECIMEN
BLOOD
RESPIRATORY TRACT
TREATMENT
FEP MEM TZP
CT CIP FDC
LEV IPR CZA
C
SINGLE COLONY
4010 4101 4038 4097 4182 4183 4120 4121 4214 4253 4287 4309 4310
0 8 23 68 72 95 121 132 143 152 153
P4010 P4038 P4097 P4120 P4214 P4253 P4287 P4309 P4310
PATIENT 3
POPULATION
D
SINGLE COLONY
4124 4127 4142 4087 4109 4236 4119
0 8 18 37 47 54
P4124 P4127 P4142 P4087 P4109 P4236 P4119
42% 51% 94% 100% 85% 99% 99%
PATIENT 4
POPULATION
E
SINGLE COLONY
3697 3674 3775 3774 3736 3748 3776 3831 4282 3887 4145 4016
0 25 34 55 68 90 101 133 168 185 206 256
P3674 P3774 P3831 P4282 P3887 P4016
99% 30% 100% 2% 15% 30%
PATIENT 5
POPULATION
F
SINGLE COLONY
4284 3957 3996 3981 3878 3903 3904 3960
0 22 26 39 49 75 80 88
P3996 P3981 P3878 P3903 P3904 P3960
100% 100% 100% 89% 75% 98%
PATIENT 6
POPULATION


A
POPULATION
SINGLE COLONY
ampC
ftsI
ampC
ftsI
E247K
P243S
P215L
Day 68
4097
P243S
ND
E247K
P215L
Day 95
4120
E247K
ND
E247K
P215L
Day 132
4253
E247K
P215L
PATIENT 3
B
POPULATION
SINGLE COLONY
ampC
ftsI
ampC
ftsI
G183D
Y268C
N427S
Day 47
4236
G183D
ND
G183D
Y268C
N427S
Day 54
4119
G183D
Y268C
PATIENT 4
C
POPULATION
SINGLE COLONY
ampC
oprD
ampC
oprD
Y304*
Day 39
3981
ND
Y304*
G183D
Δ57bp
Q30*
Disrupted
Day 75
3903
ND
ND
PATIENT 6
Variant frequency
0%
100%
No variant detected
ND