How nutrients shape antibiotic sensitivity of Pseudomonas aeruginosa: food for thought

Kok, M.

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Food for thought

Chapter 6

Nutrients drive the antibiotic-specific evolution of resistance in *Pseudomonas aeruginosa*

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Abstract

The pathogen *Pseudomonas aeruginosa* can exploit its metabolic flexibility during cystic fibrosis lung infections to reduce antibiotic sensitivity and offset antibiotic resistance costs, two key traits influencing its evolutionary trajectory. Although each trait has been associated with nutrient conditions, the link between nutrient conditions and antibiotic evolution remains poorly characterized. We examined how single-nutrient conditions influence antibiotic resistance evolution in *P. aeruginosa* through phenotypic and genotypic adaptations. We used adaptive laboratory evolution with different antibiotic classes in single nutrient media, we then compared these results to those obtained in nutrient-rich synthetic cystic fibrosis sputum medium (SCFM). Antibiotic susceptibility testing after evolution showed limited differences in minimal inhibitory concentrations (MIC) between single nutrient conditions for ceftazidime and imipenem, but more pronounced impact for ciprofloxacin, colistin, and tobramycin. Ciprofloxacin evolution resulted in the highest MIC increase, with at least a 4-fold increase observed in glutamate-evolved lineages, whereas glucose-evolved lineages showed up to 4-fold reduction in MICs for tobramycin, compared to lineages evolved under all other nutrient conditions for the same antibiotic. Growth kinetics of the evolved strains showed reduced growth rates specific to the antibiotic but not the nutrient condition in itself. Whole-genome sequencing showed nutrient-specific mutational profiles for tobramycin and ciprofloxacin. Tobramycin evolution resulted in glucose specific mutation in *wbpL* and a SCFM-specific mutation in *rplA,* alongside *fusA* and *pmrB* mutations in multiple conditions. Ciprofloxacin resistance was not caused by a *nfxB* mutation in glucose and arginine evolved lineages, which was present in all other lineages, with a specific mutation in *yicC* in the glutamate evolved lineages. No distinct differences between nutrient conditions for colistin were observed. Overall, these findings underscore the significant role nutrient conditions play in shaping resistance and highlight the importance of considering physiologically relevant media when studying antibiotic resistance evolution.

6.1. Introduction

Pseudomonas aeruginosa is the most prevalent pathogen causing chronic infections in the distinctive lung environment of adult cystic fibrosis (CF) patients[1]. This dominance is attributed to the exceptional metabolic versatility of *P. aeruginosa* and its rapid capacity to develop antimicrobial resistance[2–5]. Growing evidence highlights a strong interconnection between these traits, as metabolism influences antibiotic sensitivity and antibiotic resistance mechanisms impose a metabolic burden[6–9]. To advance our understanding of antibiotic resistance evolution, it is crucial to study how nutrients in the environment shape this relationship, a process that remains poorly explored.

In the CF lung, *P. aeruginosa* can utilize a diverse array of nutrients, including amino acids and short-chain fatty acids, whose composition varies substantially among different microenvironments[10,11]. *P. aeruginosa* adapts its metabolism to these varying conditions, which results in class-specific effects on antibiotic sensitivity[12–15]. Altering a single nutrient in the culture condition has been shown to significantly impact antibiotic sensitivity[16]. Because antibiotic sensitivity is a key determinant of selection strength during antibiotic resistance evolution[17], nutrient-induced changes in antibiotic sensitivity can drive the emergence of diverse antibiotic-resistant lineages.

Nutrients also play a role in reducing the fitness cost of antibiotic resistance mutations that alter vital cellular functions, by supporting efficient metabolic rearrangement[18–20]. Consequently, resistance mechanisms with high metabolic burden are less likely to prevail in nutrient-poor environments as this imposes a metabolic constraint[21].

Overall, these studies demonstrate that nutrient environments profoundly influence metabolic adaptations, antibiotic sensitivity, and fitness compensation, all of which shape the evolution of antibiotic resistance. However, the high variability within the CF lung environment, combined with pronounced phenotypic variability, complicates direct comparisons between laboratory conditions and clinical scenarios. Prior research has mainly addressed comparisons between nutrient-rich and nutrient-poor environments concerning fitness landscapes[21,22]. The subsequent essential step is understanding how specific nutrients individually impact resistance evolution. Given the well-documented metabolic flexibility of *P. aeruginosa*[23], accurately evaluating the

influence of individual nutrients within complex media remains challenging. Instead, examining central carbon metabolism responses to single-nutrient conditions can reveal precisely how nutrient-specific adaptations cascade into broader metabolic and biochemical changes[24]. Elucidating how these specific nutrient induced changes influence antibiotic resistance evolution could be essential for optimization or selection of more clinically relevant laboratory conditions.

In this study, we challenged the *P. aeruginosa* metabolic flexibility in single nutrient conditions during antibiotic resistance evolution and explored the phenotypic and genotypic adaptation capabilities in these environments. We conducted adaptive laboratory evolution (ALE) for a range of common antibiotics used to treat *P. aeruginosa* (ceftazidime, ciprofloxacin, colistin, imipenem, and tobramycin), utilizing single nutrient media. The selection of the nutrients arginine, glutamate, glucose, and lactate was based on their physiological relevance in CF mucus and their distinct roles in *P. aeruginosa* metabolism[10,24]. These nutrients have also been shown to affect antibiotic sensitivity differently[16]. Nutrient concentrations were set at 30 mM to prevent nutrient starvation, thereby maintaining stable growth conditions throughout evolution experiments. We assessed phenotypic changes of the nutrient-antibiotic combinations through antibiotic susceptibility testing and growth rate analysis, and genomic changes through whole genome sequencing. To contextualize these findings, we compared the results from single-nutrient conditions to those from a nutrient-rich synthetic CF medium (SCFM), providing insights into how *P. aeruginosa* adapts and maintains metabolic flexibility in minimal environments.

6.2. Material & Methods

Strains and culture conditions

Synthetic CF sputum medium (SCFM) was prepared consisting of physiologically relevant concentrations of nutrients in synthetic CF sputum as described previously[10], 0.11M phosphate buffer, ammonium chloride, potassium nitrate, ferrous sulfate, Basal Medium Eagle 1x vitamins, and trace metals (**Table S1**). Single nutrient media were prepared with the salts, vitamins and trace metals as basal medium, spiked with 4 unique nutrients including arginine, glucose,

glutamate, or lactate, at a concentration of 30 mM. The *P. aeruginosa* PAO1 laboratory strain (DSM 1117; DSMZ, Leibniz Institute, Germany) was used as the parental strain for all evolution experiments.

Antibiotics

Antibiotic stock solutions were freshly prepared on the day of the experiment and diluted to desired concentrations using an Opentrons OT-2 (Opentrons Inc., New York, NY, USA) liquid handling system. Ceftazidime pentahydrate was purchased from Thermo Fisher Scientific (Breda, The Netherlands). Ciprofloxacin, imipenem monohydrate, and tobramycin were purchased from Chem-Impex International (Wood Dale, IL, USA). Colistin sulfate was purchased from Cayman Chemical Company (Ann Arbor, MI, USA).

Laboratory evolution experiment

Three biological replicates of the *P. aeruginosa* starting cell line per media condition were propagated for 10 days under antibiotic pressure to examine the antibiotic resistance development between different culture conditions.

P. aeruginosa PAO1 was streaked out on LB agar plates, and 10 randomly selected colonies were transferred to SCFM (2 mL) and cultured overnight. The liquid cultures were diluted to an optical density at 600 nm (OD_{600}) of 0.05 to reach the starting inoculation solution, corresponding to an approximate bacterial density of $5*10^6$ CFU/mL. The bacterial inoculum (100 µL) was added to 7 wells with fresh medium with increasing antibiotic concentrations (900 µL) in a 48-well microtiter plate. After 48 hours of incubation, plates were transferred to a BMG microplate reader (Ortenberg, Germany) for OD_{600} acquisition. Cultures at the highest antibiotic concentration reaching the culture density threshold of an OD_{600} of 0.5 were transferred (100 µL) to a new range of antibiotic concentrations (900 µL) in a new 48-well microtiter plate. If the carrying capacity of any of the cultures did not exceed the OD_{600} threshold, the culture under the highest antibiotic pressure reaching at least 80% of the OD_{600} of the positive control was transferred. Cultures under ceftazidime pressure were extensively mixed and the threshold was increased to an OD_{600} of 0.65 due to the build-up of debris in the microtiter plate. At the end of the evolution experiment, the cultures reaching the OD_{600} threshold were transferred (100 µL) to an antibiotic free LB agar plate.

Colonies were collected with a pre-wet swab and stored in 20% glycerol in LB at -80 °C.

Antimicrobial susceptibility testing

Ceftazidime, ciprofloxacin, imipenem, and tobramycin minimal inhibitory concentrations were determined for the parenteral *P. aeruginosa* PAO1 strain, the antibiotic-free lineages, and the lineages evolved under the pressure of the antibiotic in SCFM by a broth microdilution method. Prior to each susceptibility test, fresh subcultures were prepared from the -80 °C stored colonies in 2 mL fresh SCFM. Susceptibility testing was conducted after 24 hours incubation at 37 °C with orbital shaking at 150 rpm or 72 hours including two 20 µL passages into 2 mL fresh SCFM medium. The starting cell density in each condition was approximately 10^6 CFU/mL in a serial twofold dilution of the antibiotics in 96-well microtiter plates (Greiner Bio-one, transparent, flat bottom) with a total volume of 200 µL. The minimal inhibitory concentration (MIC) was defined as the first concentration of antibiotic with no visible growth after 24 hours of incubation at 37 °C.

Growth rate analysis

Growth rate analysis was conducted by culturing the parental *P. aeruginosa* PAO1 strain and all lineages in the single nutrient media and SCFM. The cells were subcultured in 2 mL SCFM and incubated for 24 hours at 37 °C shaking at 150 rpm before dilution to an optical density at 600 nm (OD600) of 0.05 before inoculation. The starting cell density was approximately 10^6 CFU/mL in a transparent 96-well microtiter plate. After inoculation, microtiter plates were transferred to a Liconic StoreX STX44 119 incubator (Mauren, Principality of Liechtenstein) for incubation (95% relative humidity). A Peak Analysis and Automation KX-2 Laboratory Robot (Hampshire, United Kingdom) transferred the microtiter plate every hour between the incubator and the BMG Labtech Fluostar Omega microplate reader (Ortenberg, Germany) for time-course OD_{600} acquisition.

Genome sequencing and bioinformatics

Cells were subcultured in 2 mL SCFM and incubated for 24 hours at 37 °C shaking at 150 rpm. 500 µL of the subculture was pelleted by centrifugation for 10 minutes at 5000 x g. Genomic DNA was extracted using the QIAcube Connect automated

sample preparation system and DNeasy Blood & Tissue Kit extraction kit (Qiagen, Hilden, Germany) following the manufacturer's protocols. Extracted DNA was sent to SeqCoast Genomics for short read whole genome sequencing and bioinformatic analysis. Bioinformatic analysis was executed using Breseq (version 0.37.0) for mutation prediction and analysis, Trimmomatic (version 0.39) for read trimming and quality control, and CNOGpro (version deprecated/restored) for copy number variation analysis towards the *P. aeruginosa* PAO1 reference genome. All sequences are stored in the Sequence Read Archive (SRA) database under the PRJNA1217434 identifier[25].

Data Analysis

All data analyses were performed using R. Minimal inhibitory concentrations (MICs) for each evolutionary lineage were determined by calculating the median value from antibiotic susceptibility tests performed in quadruplicate (n = 4 biological replicates). Fold changes in MIC were calculated relative to the median MIC of the parental strain. Antibiotic-free evolution did not result in any significant change in MIC compared to the parental strain (data not shown). To assess differences in MIC between conditions, a Welch two-sample t-test was conducted between the SCFM group and each single nutrient medium.

Maximal growth rates (μmax) were determined using the splines function from the grofit package [26], fitted to growth curves (n = 3 biological replicates) for each evolutionary lineage. Growth measurements were recorded at regular intervals to ensure accurate curve fitting. Fold changes in μmax for antibiotic-evolved lineages were calculated relative to the corresponding antibiotic-free lineages in the same evolution medium. A Welch two-sample t-test, using the R base function, was used to compare the evolved lineages with the antibiotic-free controls evolved under the same medium condition.

To determine how mutation profiles differed between nutrient conditions, a partial least squares discriminant analysis (PLS-DA) was performed separately for each antibiotic using the mixOmics package [27]. The first four components were extracted to generate Variable Importance in Projection (VIP) scores, which were used to evaluate the distinguishability of mutated genes across nutrient conditions. VIP scores provided a measure of each gene's contribution to differentiation between nutrient environments, aiding in the identification of key mutations.

6.3. Results

Single nutrients conditions differentially shape antibiotic resistance evolution

We assessed the impact of specific single nutrient conditions on antibiotic resistance acquisition in *P. aeruginosa* PAO1. To this end we performed serial passaging under stepwise increasing antibiotic concentrations for five different antibiotics for a period of 10 days. *P. aeruginosa* PAO1 evolved in nutrient-rich SCFM medium was used as control. We evaluated changes in antibiotic susceptibility for the lineages under differential medium and antibiotic conditions through determination of the relative change in minimum inhibitory concentration (MIC) of SCFM-lineages in comparison to the parental strain (**Fig. 1**).

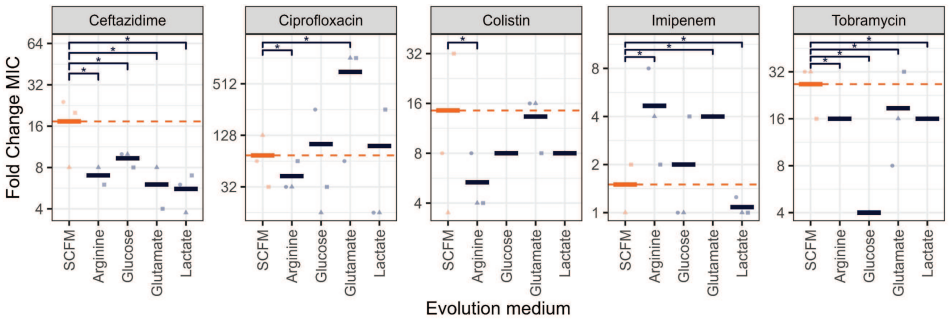

Figure 1. Change in minimum inhibitory concentrations (MICs) for five antibiotics in lineages evolved under different medium conditions. *P. aeruginosa* PAO1 strain was evolved in single nutrient media (arginine, glucose, glutamate, lactate) and synthetic cystic fibrosis sputum media (SCFM) as control, under incrementally increasing antibiotic concentrations. All MICs were determined in SCFM. Bold horizontal lines represent the mean fold change in MIC of the evolution condition relative to the parental strain. Different evolutionary lineages are indicated by shapes: (•) for replicate 1, (▲) for replicate 2, and (▪) for replicate 3. A Welch two-sample t-test was performed between the SCFM group and each single nutrient medium, with * indicating $p < 0.05$.

When considering the differential effects of media on resistance acquisition, for ceftazidime, the magnitude of MIC increase was consistently lower across all single nutrient media compared to SCFM, with limited differences between specific minimal media. For all four remaining antibiotics, substantial differences in MIC were found across single nutrient media. For ciprofloxacin, evolution in glutamate medium led to a substantially increased MIC, while the arginine condition results in a comparatively smaller MIC. Imipenem MICs only increased in arginine and glutamate medium, with no significant MIC change after evolution in SCFM, glucose, or lactate. For tobramycin, evolution in glucose medium resulted in substantially lower MICs compared to all other conditions. For colistin, the largest MIC increases were observed after evolution in SCFM and glutamate medium, while the smallest MIC decrease was noted in arginine. Across all antibiotics, it can be concluded that evolution in single nutrient media with arginine, glucose, and lactate often results in attenuated resistance acquisition, while resistance acquisition in glutamate media showed higher outcomes (**Fig. S1**).

Resistance evolution under single-nutrient conditions results in limited fitness changes

To evaluate if differences after evolution across single nutrient media could be explained by differences in fitness, we compared the maximal growth rate (μ_{max}) of all evolution lineages under antibiotic-free conditions in the original evolution media and SCFM. Overall, largest effects on μ_{max} were found when the original growth medium was used. Significant reductions in μ_{max} were observed for ceftazidime and tobramycin lineages evolved in glucose, and for ciprofloxacin lineages evolved in arginine and lactate (**Fig. 2**). These reductions were however absent for μ_{max} estimated derived in SCFM. The magnitude of changes in μ_{max} did not correlate with the changes in MIC in the evolution lineages (**Fig. S2**).

To further evaluate whether observed μ_{max} changes were antibiotic or medium dependent, we performed growth rate analysis of all evolution lineages across all conditions (**Fig. S3**). The observed reduction in μ_{max} for colistin- and imipenem-resistant lineages was consistent in glucose minimal media, regardless of the evolution media. Ciprofloxacin-resistant lineages generally exhibited reduced μ_{max} in minimal arginine media, except for those evolved in lactate. Notably, ciprofloxacin lineages evolved in lactate exhibited reduced

growth in all other single nutrient media, suggesting a unique metabolic adaptation or trade-off specific to lactate-driven resistance evolution.

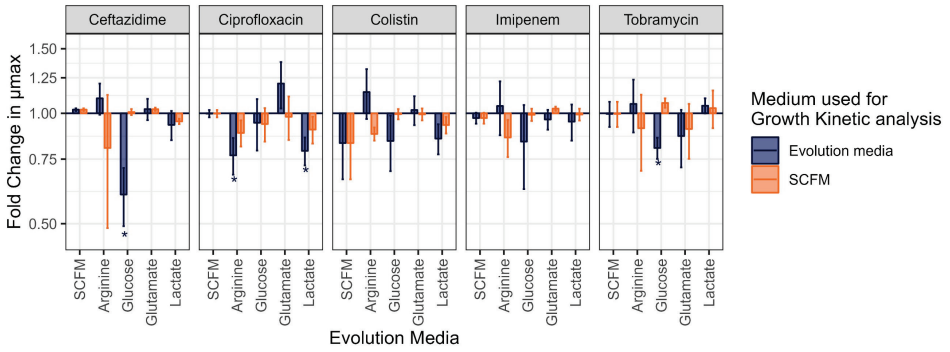

Figure 2. Changes in maximal growth rate of antibiotic evolved lineages upon re-culturing in their evolution medium and SCFM. The maximal growth rates (µmax) were determined by applying a spline function to triplicate growth curves obtained under antibiotic-free conditions. Fold changes are calculated relative to lineages without antibiotic in the corresponding medium. A Welch two-sample t-test was used to determine the difference between the evolved lineages and the antibiotic free controls evolved in the same medium (* indicating $p<0.05$).

Medium-specific genetic variations differentiate between evolution conditions.

Whole genome sequencing was performed for final evolution lineages for ciprofloxacin, colistin, and tobramycin to evaluate genetic variants across parallel evolved biological replicates (**Fig. 3**). Single nucleotide polymorphisms (SNPs), insertions, and deletions observed with a frequency higher than 20% and present within more than 1 lineage were included due to large heterogeneity within evolutionary lineages.

To determine how mutations differ between the evolution media, a partial least squares discriminant analysis (PLS-DA) was applied for each antibiotic. This analysis indicates the discriminatory importance of mutations that allow differentiation across specific medium conditions, quantified using a

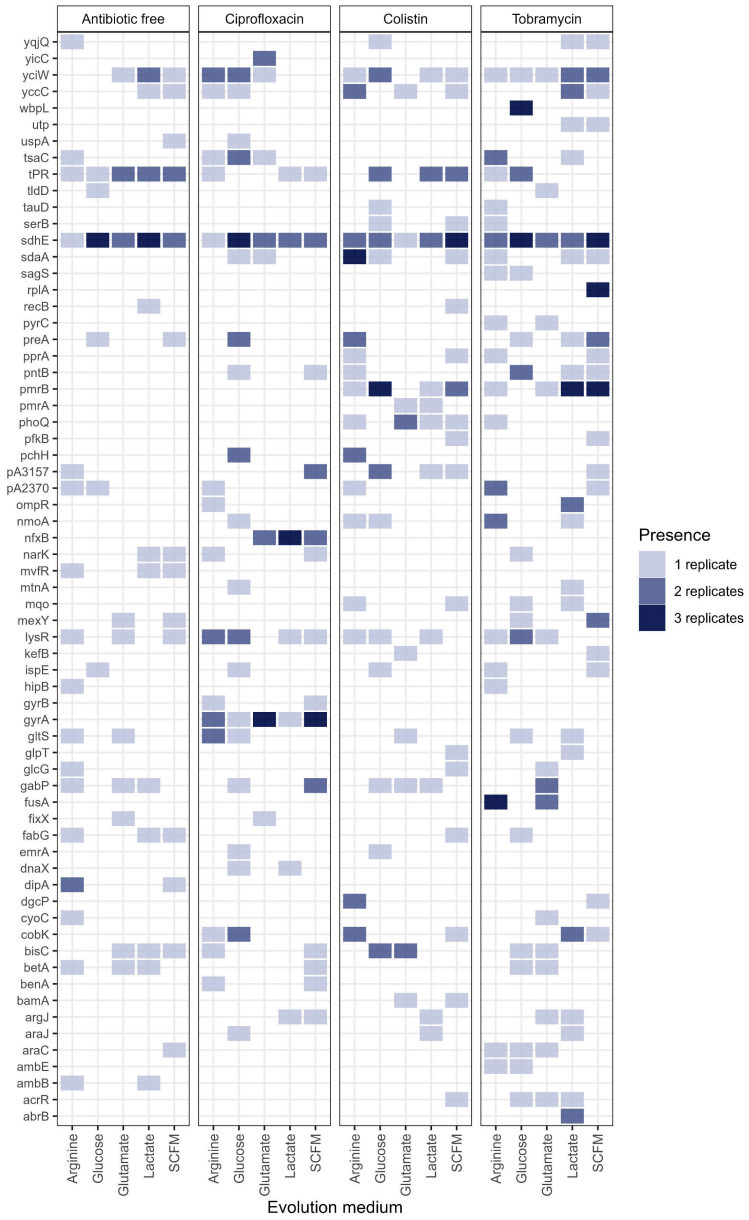

Figure 3. Whole-genome sequencing-derived mutations in evolution lineages from different antibiotic and nutrient conditions. The occurrence of single nucleotide polymorphisms (SNPs), deletions, and insertions associated with annotated genes are indicated. The color scale represents the number of evolutionary lineages under the same condition where a mutation was observed. All mutations detected only once are excluded from the figure.

variance importance in projection (VIP) score. Mutations in the top six genes ranked by VIP score were selected and visualized as potential mutations associated with media-specific adaptation for each antibiotic (**Fig. 4**). Among the top distinguishing genes, only three loci mutations appeared in a single evolution condition. Two were in tobramycin lineages: *rplA* for SCFM and *wbpL* for glucose medium (**Fig. 4A**). Other genes specific for tobramycin were mutations in the *pmrB* gene, which was mutated in all media except glucose, and the *fusA* gene, which was specific to arginine and glutamate minimal media. For ciprofloxacin, mutations in *yicC* were unique for the ciprofloxacin-glutamate condition, while mutations in *nfxB* were present in lineages evolved glutamate, lactate, and SCFM media (**Fig. 4B**). Finally, mutations in *bisC, cobK, dgcP, PA3157, preA, pchH,* and *sdaA* had high VIP scores for distinguishing evolution conditions when stratified by antibiotic, but were also present in various other media conditions across different antibiotics, including antibiotic-free evolution. The top VIP scores after colistin evolution consist only of these genes, indicating a limited presence of condition-specific mutations in colistin resistance (**Fig. 4C**).

6.4. Discussion

We demonstrated that *P. aeruginosa* can rapidly develop antibiotic resistance in single nutrient media, with the magnitude of MIC increase varying substantially between evolution conditions as well as antibiotics. Despite the MIC variation, changes in mutant growth rates (μ_{max}) were generally consistent among lineages evolved under the same antibiotic.

Across the single nutrient conditions, we found the smallest increase in MIC in lineages evolved for the beta-lactam antibiotics imipenem and ceftazidime. This may be explained by resistance development against imipenem requiring multiple mutation steps[28] and the limited duration of the evolution experiment. Previous findings demonstrated similar limited MIC increases for ceftazidime during evolution in minimal media conditions[29–31]. Lineages evolved during exposure to ciprofloxacin, colistin, and tobramycin, larger differences between MICs for different nutrient conditions were observed, which were further genetically characterized.

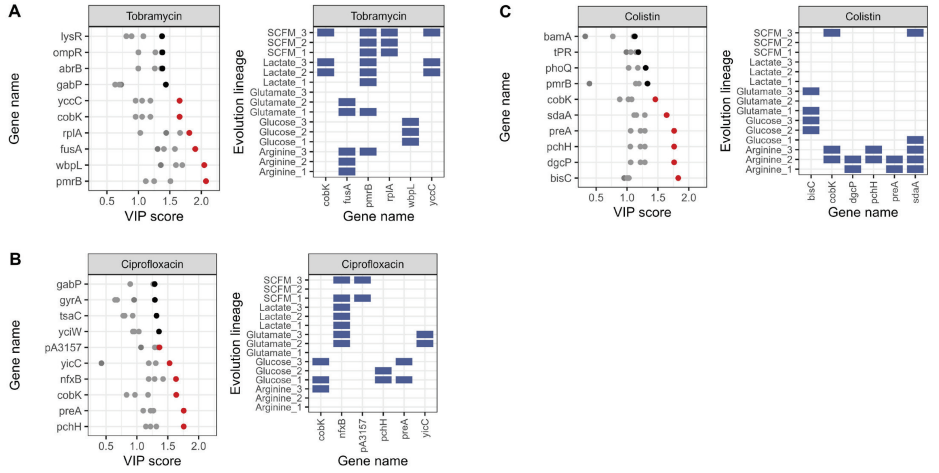

Figure 4. Key mutations distinguishing mutational profiles of culture conditions across (**A**) tobramycin, (**B**) ciprofloxacin and (**C**) colistin. (Left) VIP (Variable Importance in Projection) scores from a 4-dimensional partial least squares discriminant analysis (PLS-DA) highlight key mutations distinguishing culture conditions. The three lowest VIP scores for each mutation are colored grey, while the highest VIP score per drug is highlighted in black, and in red if identified as a key distinguishing mutation. (Right) Heatmaps display the six highest-scoring locus mutations, with colored tiles indicating mutation presence (x-axis) across evolutionary lineages (y-axis). These visualizations provide a comparative overview of mutation patterns across culture conditions and evolutionary lineages for the different antibiotics.

For ciprofloxacin lineages, the lowest MIC increases were found in arginine-lineages and the highest increases in glutamate-lineages. These differences may be explained due to differences in accumulation of specific established fluoroquinolone resistance mutations, particularly *gyrA* and *nfxB*[32]. The *gyrA* gene encodes a subunit of DNA gyrase, which is the primary target of fluoroquinolones, and mutations in this gene typically reduce the binding affinity of the antibiotic. Mutations in *nfxB* lead to overexpression of the MexCD-OprJ efflux pump, which exports various antibiotics. Whilst mutations in *gyrA* were common across all media, the glutamate-lineages with the highest MIC increases also carried *nfxB* mutations. We observed no changes in μ_{max} for these mutants, in line with previous reports for *P. aeruginosa* on compensatory effects

associated with *gyrA* mutations and *nfxB*-induced MexCD-OprJ efflux pump upregulation[19,33]. The burden of these compensatory effects could however still influence the evolutionary selection of these mutations. Ciprofloxacin-lineages demonstrated reduced μ_{max} in arginine medium, the condition in which this specific mutation combination was absent. The absence of *gyrA* and *nfxB* mutations in arginine-lineages, along with the lower μ_{max} of ciprofloxacin-lineages in arginine, suggests that metabolic constraints may hinder the establishment of these mutations under such conditions. In the case of *nfxB*-mediated upregulation of the MexCD-OprJ efflux pump, an active proton motive force (PMF) is required for export[34]. Arginine metabolism may lead to a reduced PMF, as energy can also be derived via the arginine deiminase pathway rather than PMF-generating oxidative phosphorylation[35]. Glutamate, by contrast, serves as a central metabolite in nitrogen metabolism and has been shown to reduce ciprofloxacin sensitivity in *P. aeruginosa*[36–38]. Glutamate has also been reported to enhance antibiotic penetration and counters efflux, thereby increasing intracellular ciprofloxacin concentration[39–41]. This combination raises the evolutionary pressure of ciprofloxacin in glutamate medium, which can result in faster and different acquisition of resistance[17]. We speculate that this pressure contributes to the unique *yicC* mutation observed in glutamate medium. Although less studied in *P. aeruginosa*, *yicC* in *E. coli* has been linked to DNA stress responses and RNA degradation[42,43], both processes linked to ciprofloxacin exposure.

Colistin resistance development showed considerable variation in MICs between lineages and across nutrient conditions. Similarly, no distinct patterns in mutations emerged across different nutrient conditions. Colistin resistance in *P. aeruginosa* primarily arises from lipid A modifications in the lipopolysaccharide (LPS) layer[44,45], with no observed fitness cost[45,46]. This aligns with our findings of increased MIC and unaffected μ_{max} in lineages carrying mutations in the lipid A regulatory genes *phoQ* and *pmrAB*. Interestingly, we observed a reduced μ_{max} when re-growing colistin-evolved lineages in glucose medium, except for those lineages originally evolved in glucose. Glucose is central to glycolysis and the pentose phosphate pathway, both of which supply precursors essential for the lipid A modifications regulated by *phoQ* and *pmrAB*[47]. *P. aeruginosa* possesses an inherently less efficient glycolytic pathway[24], and our findings suggest this

efficiency is further compromised if glucose utilization is not actively selected during colistin exposure. Under colistin exposure, *P. aeruginosa* has been suggested to utilize glucose as an osmotic regulator[48–50]. The fact that glucose-lineages exhibited a distinct phenotype, which could not be readily explained from high diversity in mutational profiles across all lineages, underscores the plasticity of colistin resistance in *P. aeruginosa*.

The evolution of tobramycin resistance exhibited significant variation in MIC across nutrient conditions. Substantial differences in mutational patterns among lineages were found, consistent with the extensive aminoglycoside resistome[51]. One key mutation observed was in *fusA*, a gene encoding elongation factor G, which is involved in ribosomal translocation. Mutations in the *fusA* gene have been identified in clinical isolates and are known to confer tobramycin resistance by altering ribosome function, the primary target of aminoglycosides[52]. Although these mutations are often associated with a fitness cost, they had not previously been linked to specific nutrient conditions. In our study, *fusA* mutations were observed exclusively in lineages evolved in arginine and glutamate media. One possible explanation is the known enrichment of *fusA* mutations in biofilm-grown populations[53]. This may be relevant given the reduced motility of *P. aeruginosa* in arginine, a phenotype commonly associated with biofilm formation[54]. Additionally, *fusA* mutations are known to influence quorum sensing, particularly through interaction with *lasR*, a quorum sensing regulator that increases aminoglycoside resistance[55]. Since glutamate nitrogen metabolism plays a critical role in quorum sensing pathways in other species[56,57], this may suggest a metabolic link between glutamate availability and selection for *fusA* mutations during tobramycin exposure. The *fusA* mutations co-occurred twice with *pmrB* mutations but did not lead to significant MIC changes to single mutations. The *pmrB* gene encodes a sensor kinase involved in LPS modification, a mechanism linked to both increased tobramycin resistance and increased susceptibility in some contexts[58,59]. The highest increase in tobramycin MIC was observed when *pmrB* mutation was accompanied with an SCFM-specific *rplA* mutation. The rplA gene is involved in ribosome assembly, and is known to reduce aminoglycoside binding and induce efflux pump overexpression in other species[60,61]. The combination of *pmrB-rplA* mutation seemed to be specific to nutrient rich conditions, yet there is no direct link between these mutations and

nutrient conditions in the literature. The *pmrB* mutations were found in lineages evolved in all nutrient conditions except glucose. Similar to the observed fitness cost of LPS-modifying mutations under colistin pressure in glucose medium, the absence of *pmrB* mutations in glucose-evolved lineages may be related to the altered and less efficient glycolytic pathway of *P. aeruginosa*[24]. Instead, glucose-evolved lineages harbored mutations in *wbpL*, another gene essential for LPS biosynthesis. Alteration of *wbpL* likely reduces aminoglycoside uptake[51,62]. Consistent with this, we observed no changes in μ_{max} across all tobramycin lineages. This demonstrates that, even in the absence of significant fitness costs, nutrient-induced adaptations favor distinct mutational patterns, as the importance of cellular processes related to tobramycin activity varies between conditions. These findings align with previous observations showing that LPS-modifying mutations are common in sessile biofilm populations, whereas transcriptional regulator mutations dominate in planktonic populations[53]. Together, our data support the conclusion that nutrient environments strongly influence both the metabolic state and lifestyle of *P. aeruginosa*, thereby shaping the adaptive pathways used to acquire tobramycin resistance.

To our knowledge, nutrient-specific differences in resistance evolution have not been explored beyond this study, although several studies have examined the impact of nutrient-rich versus nutrient-poor environments on antibiotic resistance development[18–21,29,63]. In this light, for ceftazidime and tobramycin, we observed a significantly higher MIC increase in nutrient-rich SCFM compared to the single nutrient conditions. For colistin, ciprofloxacin, and imipenem, such differences were less evident, with only ciprofloxacin evolution in glutamate minimal medium resulting in a significantly higher MIC compared to SCFM. Overall, our findings highlight the importance of carefully considering medium nutrient composition during in vitro experimental evolution studies.

In this study, all MIC testing of evolved lineages was conducted in SCFM. This approach enabled direct comparisons of permanent phenotypic and genotypic changes among evolved lineages, as confirmed through sequencing data. Conducting MIC assays in SCFM rather than in the specific evolution media resulted in the loss of a phenotypic dimension related to nutrient-specific antibiotic sensitivity adaptations. Although addressing this additional complexity was beyond the scope of the current study, changing nutrient conditions are

known to significantly influence antibiotic sensitivity[13,16]. Consequently, our results may underestimate the full spectrum of phenotype variability, further underscoring the importance of identifying the biochemical mechanisms driving antibiotic sensitivity changes. The use of -omics approaches offers valuable insights into how cells adapt to different environments during antibiotic exposure. For instance, metabolomics studies would be relevant to further evaluate changes in cellular energy metabolism or metabolic rewiring that support membrane modifications[64], whereas proteomics has been employed to unravel a broad range of cellular adaptations[65]. By integrating these system-level analysis with extensive genotypic data[66], we can better understand the nutrient-specific differences in phenotype-genotype relationships observed between laboratory findings and clinical isolates. Multiple studies in other pathogens have demonstrated that adjusting media composition to better mimic in vivo conditions leads to in vitro antibiotic responses more closely mirroring clinical observations[67–71], underscoring the importance of environmental specific conditions to enhance clinical relevance of ALE.

We employed a stepwise increase in antibiotic concentration to rapidly drive resistance evolution over short timescale[72], enabling us to investigate a broad set of nutrient-antibiotic combinations. While this strategy provides valuable insights, additional confirmatory experiments with increased replicates and extended evolution periods would offer a more comprehensive view of how nutrients influence evolutionary trajectories in the long run. In clinical settings, antibiotic concentrations are highly dynamic, whereas nutrient profiles, though variable across infection sites, tend to remain relatively stable within the larger volumes of human tissues and fluids[11,73]. Building on our findings from stepwise adaptation studies, future work could leverage continuous culture systems to better control nutrient levels and mimic realistic antibiotic concentration profiles[74,75].

Overall, our findings demonstrate that evolution in single nutrient conditions results in significant variation in antibiotic resistance acquisition in *P. aeruginosa*. By focusing on multiple antibiotics and nutrient environments, we showed that the effects are specific to combinations of antibiotics and nutrients. These findings lay the groundwork for broader incorporation of nutrient composition as a key factor in antibiotic resistance evolution studies. Understanding the mechanisms behind these nutrient-induced differences is an important step toward unraveling the complex evolutionary trajectories seen in the CF lung.

6.6. References

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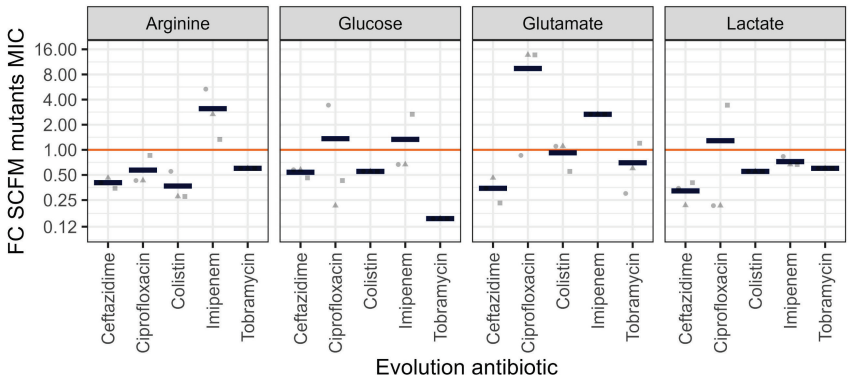
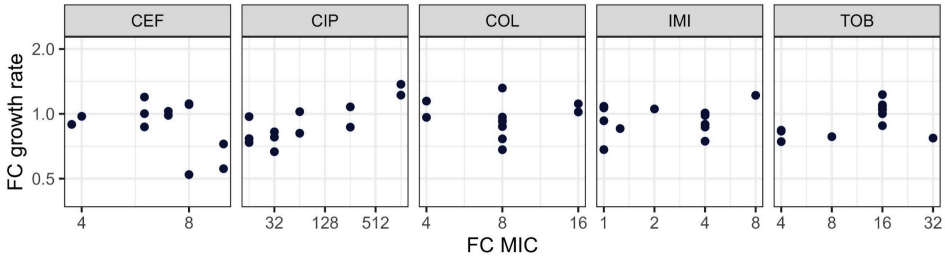
6.7. Supplementary Materials

Supplemental table 1. Detailed content list of synthetic cystic fibrosis sputum media

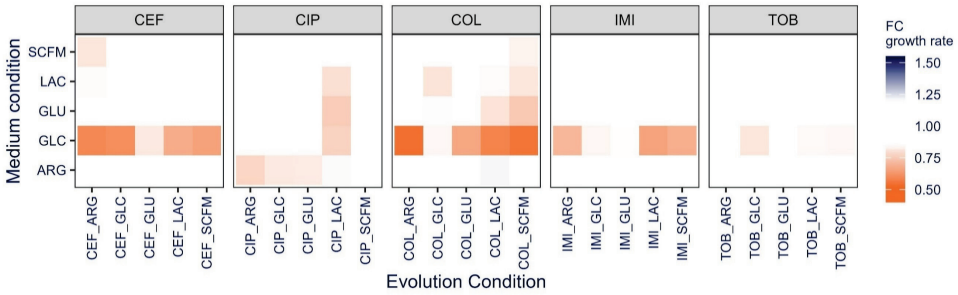
	Name	Concentration (mM)	Company information
M9 buffer	di-sodium hydrogen phosphate (Na_2HPO_4)	90.2	Thermo Fisher Scientific
	Potassium di-hydrogen phosphate (KH_2PO_4)	22.0	VWR International
	Sodium chloride (NaCl)	8.5	Merck KGaA (Avantor™)
	Ammonium chloride (NH_4Cl)	18.6	Alfa Aesar
	Magnesium sulphate hepta-hydrate ($MgSO_4$)	1.0	VWR International
	Calcium chloride ($CaCl_2$)	0.1	Acros Organics
Salts	Potassium nitrate (KNO_3)	0.35	Acros Organics
	Iron sulphate ($FeSO_4$)	0.0036	Alfa Aesar
Suppl.	BME Vitamin solution	*1x*	Thermo Fisher Scientific
Trace metals	Di-sodium Ethylene di-amine tetra-acetic acid (EDTA)	0.002 (mg/mL)	J.T. Baker (Avantor™)
	Zinc Sulphate hepta-hydrate ($ZnSO_4$)	0.23 (mg/mL)	Alfa Aesar
	Boric acid (H_3BO_3)	0.111 (mg/mL)	Acros Organics
	Manganese chloride tetra-hydrate ($MnCl_2$)	0.051 (mg/mL)	Sigma Aldrich (Avantor™)
	Cobalt chloride ($CoCl_2$)	0.017 (mg/mL)	Alfa Aesar
	Copper Sulphate penta-hydrate ($CuSO_4$)	0.015 (mg/mL)	Sigma Aldrich (Avantor™)
	Ammonium hepta-molybdate tetra hydrate ($(NH_4)_6\ Mo_7O_2$)	0.01 (mg/mL)	Alfa Aesar
Nutrients	Alanine (Ala)	1.8	Chem-Impex International
	Arginine (Arg)	0.3	Chem-Impex International
	Aspartate (Asp)	0.8	Chem-Impex International
	Cysteine (Cys)	0.2	Chem-Impex International
	Glucose (GLC)	3.2	Alfa Aeser
	Glutamate (Glu)	1.5	Chem-Impex International
	Glycine (Gly)	1.2	Acros Organics
	Histidine hydrochloride (His)	0.5	Chem-Impex International
	Isoleucine (Ile)	1.1	Chem-Impex International
	Lactate (LAC)	9.0	Biosynth International
	Leucine (Leu)	1.6	Chem-Impex International
	Lysine hydrochloride (Lys)	2.1	Thermo Fisher Scientific
	Methionine (Met)	0.6	Chem-Impex International
	Phenylalanine (Phe)	0.5	Chem-Impex International
	Proline (Pro)	1.7	Thermo Fisher Scientific
	Serine (Ser)	1.4	Chem-Impex International
	Threonine (Thr)	1.0	Chem-Impex International
	Tryptophan (Trp)	0.01	Chem-Impex International
	Tyrosine (Tyr)	0.8	Chem-Impex Internationa
	Valine (Val)	1.1	Chem-Impex International



Supplemental figure 1. Comparison of minimum inhibitory concentrations (MIC) between lineages evolved in single-nutrient media and nutrient-rich synthetic cystic fibrosis sputum medium (SCFM). *P. aeruginosa* PAO1 was evolved in single-nutrient media (arginine, glucose, glutamate, lactate) with incrementally increasing antibiotic concentrations. The difference in evolution conditions is shown as the fold change in MIC of lineages evolved in single-nutrient media relative to lineages evolved in SCFM. All MICs were determined in SCFM. Bold horizontal lines indicate the mean fold change in MIC of the SCFM lineages. Symbols represent distinct evolutionary replicates: (•) replicate 1, (▲) replicate 2, and (▪) replicate 3.



Supplemental Figure 2. Correlation between the maximal growth rate (μmax) and minimal inhibitory concentration (MIC) of evolved *P. aeruginosa* PAO1 lineages. Maximal growth rates were determined by spline fitting of triplicate growth curves under antibiotic-free conditions and expressed as fold changes relative to lineages evolved without antibiotic in the same medium. MICs were measured for lineages evolved in single-nutrient media under incrementally increased concentrations of ceftazidime (CEF), ciprofloxacin (CIP), colistin (COL), imipenem (IMI), or tobramycin (TOB). MIC fold changes are shown relative to the parental strain.



Supplemental figure 3. Changes in the maximal growth rates of antibiotic-evolved lineages after re-culturing in antibiotic-free media. Lineages were obtained by incrementally increasing concentrations of five antibiotics (ceftazidime (CEF), ciprofloxacin (CIP), colistin (COL), imipenem (IMI), and tobramycin (TOB)) under various nutrient conditions. Maximal growth rates were determined by spline fitting of triplicate growth curves in antibiotic-free synthetic cystic fibrosis sputum medium (SCFM) or in single-nutrient media (arginine (ARG), glucose (GLC), glutamate (GLU), lactate (LAC)). Fold changes are shown relative to lineages evolved under antibiotic-free conditions in each respective medium.