Integrated Experimental and Computational Analyses Reveal Differential Metabolic Functionality in Antibiotic-Resistant *Pseudomonas aeruginosa*

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**In Brief**
Bacterial metabolism is known to play a role in antibiotic efficacy; however, less is known about how the evolution of antibiotic resistance can affect bacterial metabolism. Dunphy et al. discover systems-level changes in the metabolic functionality and growth dynamics of lab-evolved antibiotic-resistant *P. aeruginosa*. Through a combined computational and experimental approach, they predict and test the impact of observed genetic mutations on specific metabolic phenotypes.

**Highlights**
- Growth profiling of antibiotic-resistant *P. aeruginosa* on 190 carbon sources
- Quantification of growth dynamics reveals altered metabolic functionality
- Prediction of metabolic impacts of individual mutations with a computational model
- Experimental validation of predicted genotype-phenotype relationships

Dunphy et al., 2019, Cell Systems 8, 3–14
January 23, 2019 © 2018 The Author(s). Published by Elsevier Inc.
https://doi.org/10.1016/j.cels.2018.12.002
Integrated Experimental and Computational Analyses Reveal Differential Metabolic Functionality in Antibiotic-Resistant *Pseudomonas aeruginosa*

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https://doi.org/10.1016/j.cels.2018.12.002

SUMMARY

Metabolic adaptations accompanying the development of antibiotic resistance in bacteria remain poorly understood. To study this relationship, we profiled the growth of lab-evolved antibiotic-resistant lineages of the opportunistic pathogen *Pseudomonas aeruginosa* across 190 unique carbon sources. Our data revealed that the evolution of antibiotic resistance resulted in systems-level changes to growth dynamics and metabolic phenotype. A genome-scale metabolic network reconstruction of *P. aeruginosa* was paired with whole-genome sequencing data to predict genes contributing to observed changes in metabolism. We experimentally validated computational predictions to identify mutations in resistant *P. aeruginosa* affecting loss of catabolic function. Finally, we found a shared metabolic phenotype between lab-evolved *P. aeruginosa* and clinical isolates with similar mutational landscapes. Our results build upon previous knowledge of antibiotic-induced metabolic adaptation and provide a framework for the identification of metabolic limitations in antibiotic-resistant pathogens.

INTRODUCTION

With the threat of a “post-antibiotic era” looming, there is a critical need to develop new strategies to treat bacterial infections (Alanis, 2005; Falagas and Bliziotis, 2007). The design of such approaches could be guided by a better understanding of the relationship between antibiotic resistance and bacterial metabolism. Bacterial metabolism has been shown to be an important factor in the efficacy of certain classes of antibiotics (Lee and Collins, 2012; Lobritz et al., 2015; Martínez and Rojo, 2011; Meylan et al., 2017). For example, mutations to the electron transport chain have been shown to reduce proton motive force (PMF) and limit PMF-dependent influx of aminoglycosides (Martínez and Rojo, 2011; Taber et al., 1987). Such mutations are commonly observed in aminoglycoside-resistant clinical isolates (Schurek et al., 2008; Yen and Papin, 2017). Conversely, quinolone efflux is often PMF dependent, and decreased PMF can result in increased drug susceptibility (Giraudo et al., 2000). Links between metabolism and antibiotic resistance have resulted in a variety of proposed treatments to clear infections including drug cycling approaches, which aim to continuously re-sensitize resistant populations, and metabolite supplementation strategies, which jumpstart metabolism to restore drug susceptibility in antibiotic-tolerant cells (Imamovic and Sommer, 2013; Meylan et al., 2018; Peng et al., 2015). These tactics do not require new antibiotics; rather, they help to prolong the efficacy of existing drugs through the manipulation of metabolism in resistant or tolerant populations.

While it is generally understood that bacterial metabolism affects antibiotic efficacy, it is less understood how the metabolic functionality and growth dynamics of bacterial pathogens change with the development of antibiotic resistance. The direct impact of sustained antibiotic pressure on metabolic adaptation in clinical isolates is confounded by in vivo pressures, including nutrient stress, oxidative stress, host inflammation, and competition with co-infecting pathogens (Hassett and Cohen, 1989; Hoffman et al., 2010; Singh et al., 2007; Wong et al., 2013). Consequently, reports of metabolic phenotypes of clinical isolates vary across studies and even within the same patient (Hocquet et al., 2016; Jorth et al., 2015; Rau et al., 2010). There has recently been success using adaptive laboratory evolution (ALE) experiments to study the evolution of antibiotic resistance in a controlled *in vitro* environment (Banerjee et al., 2017; Dragosits and Mattanovich, 2013; Furusawa et al., 2018; Jahn et al., 2017; Toprak et al., 2012; Yen and Papin, 2017; Zampieri et al., 2017). Sequencing and expression profiling of lab-evolved resistant bacteria have revealed genetic mutations responsible for resistance phenotypes (Lázár et al., 2013; Suzuki et al., 2014); however, connecting specific mutations to metabolic phenotypes remains a significant challenge (Palmer and Kishony, 2013; de Visser and Krug, 2014).

Genome-scale metabolic network reconstructions (GENREs) can provide the framework to contextualize genetic and metabolic changes accompanying the development of antibiotic resistance (Bordbar et al., 2014; Oberhardt et al., 2009). A GENRE is a quantitative formalism that captures all known metabolic changes accompanying the development of antibiotic resistance (Bordbar et al., 2014; Oberhardt et al., 2009). A GENRE is a quantitative formalism that captures all known metabolic changes accompanying the development of antibiotic resistance (Bordbar et al., 2014; Oberhardt et al., 2009).
metabolic reactions in an organism (Orth et al., 2010). Within a GENRE, gene-protein-reaction (GPR) rules link annotated metabolic genes to the reactions that their gene products catalyze (Durot et al., 2009; Reed et al., 2003). Among other functions, GENREs can be used to mechanistically evaluate the impact of mutations in single genes on bacterial growth across many environmental conditions (Joyce et al., 2006). This analysis allows for the systematic prediction of the metabolic consequences of individual mutations identified in sequenced drug-evolved lineages. If they can be identified, robust resistant-specific changes in metabolism could be leveraged in the rational design of drug cycling and metabolite supplementation strategies.

To better understand the relationship between antibiotic resistance and bacterial metabolism, we profiled the metabolic phenotypes of previously published lab-evolved, antibiotic-resistant lineages of the opportunistic pathogen Pseudomonas aeruginosa. We evaluated the growth of piperacillin-, tobramycin-, and ciprofloxacin-resistant P. aeruginosa on 190 unique carbon sources. We also evaluated the growth of the starting ancestral lineage and a media-evolved lineage. This effort resulted in the generation of over 2,800 individual growth curves from which we captured the growth dynamics of each evolved lineage. Phenotypic data was integrated with previously collected genomic information on each lineage to probe the importance of reported resistance mutations on the observed metabolic phenotypes (Yen and Papin, 2017). Phenotypic and sequencing data were used in tandem with a recently published GENRE of P. aeruginosa strain UCBPP-PA14 to predict the individual impact of 343 genes deleted in the piperacillin-resistant lineage on loss of catabolic function (Bartell et al., 2017). Finally, a loss of catabolic function observed in the piperacillin-evolved lineage on 42 carbon sources that could be both experimentally and computationally evaluated.

RESULTS

Profiling Growth Phenotypes of Antibiotic-Resistant Pseudomonas aeruginosa

P. aeruginosa was previously evolved to lysogeny broth (LB) media through serial passaging for 20 days (Yen and Papin, 2017). In parallel, the same starting ancestral lineage of P. aeruginosa was also evolved to each of three antibiotics: ciprofloxacin, piperacillin, and tobramycin (Figure 1A). Whole-genome sequencing was also previously performed on all lineages (Figure 1B) (Yen and Papin, 2017). As formerly reported, the minimal inhibitory concentration (MIC) of each drug
measured in its respective drug-evolved lineage increased at least 32-fold, while the LB-evolved control lineage had no increase in MIC to any of the drugs relative to the ancestral lineage (Yen and Papin, 2017).

To characterize phenotypic changes in metabolism that arise with resistance, we evaluated the growth of each lineage on 190 unique carbon sources in the absence of antibiotic pressure in triplicate (Figure 1A). These growth phenotyping experiments resulted in the generation of 2,850 individual growth curves (Data S1). The median curves were taken for each lineage on each carbon source across three replicate colonies, and these 950 median growth curves are referred to for the remainder of the analysis (Figure S1). A lineage was considered to have grown on a given carbon source if the background-subtracted area under the curve (AUC) of the 48-hr growth curve was above the 3rd quartile. The growth cutoff is indicated by a dotted line.

Figure 2. Summary of Phenotypic Growth Data of Antibiotic-Resistant P. aeruginosa
(A) Binary growth calls for each lineage (Ancestor: LBE, LB-evolved; CIP, ciprofloxacin-resistant; PIP, piperacillin-resistant; TOB, tobramycin-resistant) across 190 unique carbon sources (white, no growth; gray, growth). Lineages are clustered using Jaccard distances and complete linkage. Carbon sources are sorted by growth calls for the ancestral lineage.
(B) Histogram of the area under the curve (AUC) for all measured growth curves. A lineage was considered to have grown on a given carbon source if the AUC of the 48-hr growth curve was above the 3rd quartile. The growth cutoff is indicated by a dotted line.
(C) Boxplot summarizes the maximum growth density by lineage across all carbon sources for which each lineage grew. Growth density was measured as the OD600 after subtraction of a negative control. Boxes encompass the 1st to 3rd quartile of each population. Whiskers extend up to 1.5 times the interquartile range from the closest box boundary. Data points that are out of the whisker range are outliers and are denoted by individual points. Significance relative to the ancestor denoted by Wilcoxon rank sum test with Benjamini-Hochberg correction (**p value < 0.01, ***p value < 0.001).
(D) Summary of the percent of total carbon sources that supported growth of each lineage.

measured in its respective drug-evolved lineage increased at least 32-fold, while the LB-evolved control lineage had no increase in MIC to any of the drugs relative to the ancestral lineage (Yen and Papin, 2017).

To characterize phenotypic changes in metabolism that arise with resistance, we evaluated the growth of each lineage on 190 unique carbon sources in the absence of antibiotic pressure in triplicate (Figure 1A). These growth phenotyping experiments resulted in the generation of 2,850 individual growth curves (Data S1). The median curves were taken for each lineage on each carbon source across three replicate colonies, and these 950 median growth curves are referred to for the remainder of the analysis (Figure S1). A lineage was considered to have grown on a given carbon source if the background-subtracted area under the curve (AUC) of the 48-hr growth curve was in the upper quartile of all of the data. This corresponded to a threshold of about 15.8; however, downstream analyses were not qualitatively impacted by changes to this cutoff (Figures 2A and S2; Data S2; STAR Methods). The AUC exceeded this threshold on 25.1% (238/950) of all growth curves, with only 7.4% of all carbon sources (14/190) supporting growth of all five measured lineages (Figure 2B). There were 17 instances in which one or more of the LB- or drug-evolved lineages grew where the ancestral lineage could not, indicating a gain of catabolic function. Losses of catabolic function were more common than gains. A total of 46 carbon sources supported the growth of the ancestral lineage, while one or more evolved lineages were unable to grow. Adaptive evolution to LB media and antibiotics resulted in wide-spread loss and occasional gain of catabolic functions.

To better understand growth differences across lineages, we measured and summarized the maximum growth densities across growth-supporting carbon sources for each lineage (Figures 2C and S3; Data S2). The maximum growth density was defined as the maximum background-subtracted optical density measured at 600 nm (OD600) of the median of three replicate 48-hr growth curves. Interestingly, all evolved lineages, including the LB-evolved control, exhibited a significantly decreased maximum growth density relative to the ancestral lineage (p value < 0.05). Each lineage was found to have a different number of growth-supporting carbon sources (Figure 2D). The ancestral lineage grew on 31.6% (60/190) of carbon sources.
Comparatively, ciprofloxacin- and piperacillin-evolved lineages were the most metabolically limited, only growing on 13.2% (25/190) and 23.2% (44/190) of the tested carbon sources, respectively. The LB-evolved control was able to grow only on 27.9% (53/190) of carbon sources, while the tobramycin-evolved lineage grew on 29.5% (56/190) of sources. Overall, adapted lineages were more metabolically limited and had decreased growth relative to the unevolved ancestral lineage.

**Altered Growth Dynamics of Antibiotic-Resistant* P. aeruginosa**

To more rigorously examine metabolic differences of our antibiotic-resistant lineages, we quantified the growth dynamics of each growth curve across carbon sources that supported the growth of all five lineages (Figure 1A; Data S3). For each of the 14 growth-supporting carbon sources, three parameters of bacterial growth were calculated: maximum growth density, doubling time, and time to reach mid-exponential phase (Figures 3A–3F). The maximum growth density varied by carbon source and by lineage (Figure 3A). All evolved *P. aeruginosa* lineages except for the tobramycin-evolved lineage exhibited significant decreases in maximum growth density relative to the ancestral lineage (p value < 0.05) (Figure 3D). The LB-evolved control had one of the largest decreases in growth density, indicating that media condition impacted the maximum level of growth perhaps more so than antibiotic pressure.

For each growth curve, we also calculated the doubling time and the time taken to reach mid-exponential phase (Figure 1A). A basic logistic equation was fit to each median growth curve to determine growth dynamics (Sprouffske and Wagner, 2016). Across the 14 universal growth-supporting carbon sources, the ciprofloxacin-evolved lineages had a significantly longer doubling time than the ancestral lineage (p value < 0.05) (Figures 3B and 3E). The median doubling time of the piperacillin-evolved and tobramycin-evolved lineages also increased, and the LB-evolved control slightly decreased; however, these changes were not significant (p value > 0.05). The ciprofloxacin-evolved and piperacillin-evolved lineages took significantly longer than the ancestral lineage to reach mid-exponential growth (p value < 0.05) (Figures 3C and 3F). Overall, the ciprofloxacin-evolved lineage exhibited longer lag phases and longer doubling times, while the tobramycin-evolved lineage growth curves were relatively similar to the ancestor and LB-evolved control (Figure S1). These findings demonstrate that adaptation to different antibiotics on rich media can heterogeneously impact the growth dynamics of *P. aeruginosa* across many growth-supporting conditions.

**Tobramycin-Resistant* P. aeruginosa* Shows Enhanced Growth on N-Acetyl-D-Glucosamine**

On multiple carbon sources, we observed that an antibiotic-evolved lineage exhibited enhanced growth relative to the ancestral lineage. This phenotype is potentially of greater concern in a clinical setting since a drug treatment that selects for both resistant and metabolically advantaged pathogens could have negative clinical outcomes.
One example of enhanced growth occurred in the tobramycin-evolved lineage, which grew to a higher growth density than the ancestral lineage on the carbon source N-acetyl-D-glucosamine (Figure 4A). N-acetyl-D-glucosamine is a component of human mucin as well as the cell wall of Gram-positive organisms and is thought to be present in sputum of cystic fibrosis (CF) patients (Korgaonkar and Whiteley, 2011; Palmer et al., 2007). Consistent with phenotypic profiling data, three additional tobramycin-evolved lineages of P. aeruginosa from the previous study (Yen and Papin, 2017) showed enhanced growth on N-acetyl-D-glucosamine; maximum growth densities were significantly increased in two of these three lineages relative to the ancestral lineage (p < 0.05). This phenotype was not shared with any LB-evolved control lineages, all four of which exhibited significantly decreased maximum growth densities relative to the ancestral lineage (p < 0.05) (Figures 4A and S4). These results indicate that this phenotype of enhanced growth is robust and may be affected by adaptation to tobramycin as opposed to LB media. Growth profiling revealed that the piperacillin-evolved lineage also displayed enhanced growth on N-acetyl-D-glucosamine, suggesting that multiple classes of antibiotics may impact growth on this carbon source (Figure S1A).

Although all four independent tobramycin-evolved lineages exhibited an increase in maximum growth density on N-acetyl-D-glucosamine and some genetic mutations occurred in similar pathways, there were no mutations that were universally shared, indicating that multiple evolutionary paths to tobramycin resistance could be associated with this metabolic phenotype. To explore each path, the effects of two genetic mutations for each lineage on N-acetyl-D-glucosamine utilization were evaluated. In total, eight transposon mutants were selected from the non-redundant PA14 library and grown in N-acetyl-D-glucosamine (Table S1; Figure S4B) (Liberati et al., 2006). Six out of eight of the mutants had significantly increased maximum growth densities relative to the ancestral lineage: PA14_23470, nuoB, PA14_41710, PA14_44360, nuoL, and PA14_57850 (p < 0.05) (Figure 4B). One mutant, PA14_57570, had significantly decreased growth, and another, PA14_57880, was not significantly different from the ancestor. At least one mutated gene for each of the four tobramycin-evolved lineages had enhanced growth on N-acetyl-D-glucosamine (Figure S4B).

Transposon mutants with insertions in the nuoL, nuoB, and PA14_57570 genes exhibited slight increases in resistance to tobramycin relative to the ancestral lineage; however, based on defined clinical breakpoints, the MICs were not elevated enough for the mutants to be classified as resistant (Table S1) (EUCAST, 2018). All other mutations had less than a 2-fold increase in the MIC of tobramycin. The exact mechanism by which these genes impact growth on N-acetyl-D-glucosamine needs to be resolved.
with further experimentation. However, these results demonstrate one example of how resistance-associated genetic mutations can unexpectedly and heterogeneously impact metabolism in *P. aeruginosa*.

**Network-Guided Predictions Identify Mutations that Impact Loss of Catabolic Function in Piperacillin-Resistant *P. aeruginosa***

Contrary to other evolved lineages that contained only a handful of sequenced mutations, the piperacillin-evolved lineage contained a large deletion of 343 genes (Data S4) (Yen and Papin, 2017). This deletion complicated our ability to identify clear genotype-phenotype relationships in the piperacillin-evolved lineage. To contextualize the potential impact of each gene in the large deletion on the multiple losses of catabolic function observed in our metabolic phenotyping data, we simulated single-gene knockout experiments across 42 environments with a GENRE of *P. aeruginosa* UCBPP-PA14 (Figure 1B) (Bartell et al., 2017). Each computationally tested environment contained the components of M9 minimal media and a single carbon source. For a carbon source to be considered for the *in silico* analysis, it had to meet several criteria. First, it had to have been included in our experimental screen of metabolic phenotypes. Second, the *P. aeruginosa* metabolic network reconstruction had to contain a transport reaction for the carbon source, indicating annotation evidence of a functional transporter of the metabolite across the cell membrane. A transport reaction can be thought of as a way for *P. aeruginosa* to uptake or secrete a particular metabolite. Finally, for a carbon source to be included in the analysis, it had to support the production of a non-zero biomass in the complete model, where biomass can be considered a proxy for bacterial growth. For each of the 42 carbon sources that met these requirements, we simulated single-gene knockouts to evaluate the contribution of each gene on model growth and carbon source catabolism. Genes that when knocked out in the model resulted in a total loss of biomass production were predicted to be essential for growth on the tested carbon source.

To predict which genes in the large deletion of the piperacillin-evolved lineage could have impacted observed changes in metabolic phenotype, we looked for overlap between deleted genes identified by whole-genome sequencing and model-predicted essential genes (Figure S5). In total, there were 17 genes shown to be deleted in the piperacillin-evolved lineage that were predicted to be essential for growth on at least one of the 42 experimentally measured carbon sources (Figure 5). For it to be possible to validate an essentiality prediction, the ancestral lineage needed to be able to grow on the carbon source of interest. Otherwise, we could not determine the effect of knocking out a single gene on catabolic function. We were unable to evaluate the effect of 8 of the 17 genes because they were predicted to be essential for growth on 4 carbon sources that were unable to support the growth of the ancestral lineage: D-ribose, D-serine, L-serine, and L-phenylalanine. Another 2 of the 17 genes, *bacA* and *glaA*,

![Figure 5. Model-Guided Prediction of Loss of Metabolic Function in Piperacillin-Evolved *P. aeruginosa*](image-url)
were predicted to be essential across all environmental conditions, including LB media. While neither of these genes was essential for growth in vitro, they were associated with the ability of the model to produce biomass. Predictions of the knockout of these genes, while perhaps not directly informative for our study of antibiotic resistance, provide useful new information for model curation.

The role of each of the remaining seven predicted essential genes on catabolic function was examined in more detail (Figures 6A–6D). Five of the seven remaining genes deleted in the piperacillin-evolved lineage were predicted to be essential for L-leucine utilization: gnyA, gnyB, gnyD, gnyH, gnyL. A cluster of PAO1 genes orthologous to the gnyABDHL cluster has been previously reported to be involved in L-leucine catabolism, specifically in the downstream catabolism of isovaleryl-CoA into the citric acid cycle substrate acetyl-CoA (Figure 6A) (Aguilar et al., 2006). The piperacillin-evolved lineage was found to lack the ability to grow on L-leucine, while the ancestral strain was able to grow on this carbon source (Figures 6C and S6A). Based on the predictions of the GENRE, we hypothesized that this loss of catabolic function in the piperacillin-evolved lineage was due to the deletion of the five genes in the gny operon. Our hypothesis was supported by the finding that a gnyA transposon mutant was unable to grow in minimal media with L-leucine, demonstrating that gnyA is necessary for L-leucine utilization (Figures 6C and S6A). Our model predicts that deletion of any one of the remaining gny genes would have a similar effect on L-leucine catabolism.

The remaining three essential gene predictions were experimentally determined to be false positives. The gnyA gene was predicted to be essential for utilization of L-isoleucine; however, both the piperacillin-evolved lineage and gnyA transposon mutant were able to grow on this carbon source (Figures 5 and S6B). The exact reason that gnyA was erroneously predicted as essential remains to be determined and indicates that the current understanding of L-isoleucine utilization in P. aeruginosa as it is represented in our model is incomplete. Two genes, scoA and scoB, were predicted to be essential for the utilization of 4-hydroxybenzoic acid (Figure 5). These genes have been reported to encode for subunits A and B of a CoA-transferase (Winsor et al., 2016). According to the P. aeruginosa GENRE, scoA and scoB use a downstream product of 4-hydroxybenzoic acid catabolism to catalyze the conversion of succinyl-CoA to succinate in the citric acid cycle (Figure 6B). We found that both scoA and scoB mutants were able to grow on 4-hydroxybenzoic acid. Growth curves of both mutants matched the growth of the ancestral lineage, while the piperacillin-evolved lineage was unable to grow on 4-hydroxybenzoic acid (Figures 6D and S6C). From this result, we conclude that scoA and scoB are not individually necessary for growth on 4-hydroxybenzoic acid. Further evaluation is required to determine why these genes were falsely predicted to be essential as well as why the piperacillin-evolved lineage was unable to grow on 4-hydroxybenzoic acid.

Two independent piperacillin-evolved lineages from a previous study were found to have large deletions partially overlapping with the lineage profiled in this study (Yen and Papin, 2017). Applying the same computational approach to these two piperacillin-resistant lineages, we identified eight additional genes deleted in at least one of these lineages predicted to be essential for utilization of at least one carbon source (Figures S5B and S5C). We predict that the majority of metabolic phenotypes would be conserved across all three piperacillin-evolved lineages.
Clinical Isolates Share Loss of Metabolic Function Observed in Lab-Evolved Piperacillin-Resistant *P. aeruginosa*

To investigate the clinical relevance of the differential metabolic functionality observed in the drug-evolved lineages, we asked whether clinical isolates with large chromosomal mutations comparable to the piperacillin-resistant lineage would also exhibit an inability to catabolize L-leucine. A previous study characterized four pyomelanogenic (PM) isolates with reduced metabolic flexibility and found that they each had large chromosomal deletions (APM, BPM, CPM, and DPM). For each PM isolate, they also identified “parental” wild-type (WT) isolates that were genetically identical to the PM isolates but lacked large deletions, had significantly faster growth rates on rich media, and did not produce pyomelanin (AWT, BWT, CWT, and DWT) (Hocquet et al., 2016). The large deletions sequenced in the PM isolates were previously found to overlap with the 343 genes deleted in the piperacillin-resistant lineage (Yen and Papin, 2017). To test the hypothesis that the loss of catabolism of L-leucine observed in the piperacillin-resistant lineage and determined to be impacted by the *gnyA* gene may be a clinically relevant phenotype.

Altogether, we paired whole-genome sequencing with a GENRE to identify five genes associated with loss of metabolic function in piperacillin-resistant *P. aeruginosa*. Clinical isolates with similar mutational landscapes to lab-evolved piperacillin-resistant *P. aeruginosa* were found to share loss of the ability to catabolize L-leucine. Three additional genes predicted to be essential by the model were experimentally determined to be non-essential, highlighting gaps in our current knowledge of metabolism that need to be further investigated. While some of our predictions may be retrospectively obvious, they were all guided by the model, without which we would have been unable to reconcile direct genotype-phenotype relationships in such a complex mutational landscape. A similar approach could be applied to predict metabolic deficits in any bacterial strain with an annotated genome.

**DISCUSSION**

We have shown that *P. aeruginosa* experiences systems-level changes to metabolism when exposed to sustained antibiotic pressure. Through the analysis of 950 growth curves on 190 unique carbon sources, we determined that antibiotic-resistant *P. aeruginosa* exhibit differential growth dynamics from antibiotic-sensitive lineages. We applied a highly curated GENRE to predict which mutations in our antibiotic-evolved lineages were impacting loss of metabolic function. Finally, model predictions were validated with mutants with single-gene transposon insertions in genes of interest. Deletions of genes in the *gny*ABDHL cluster in the piperacillin-evolved lineage were found to result in loss of L-leucine utilization. Our findings emphasize the interconnectivity of antibiotic resistance and metabolism and support future efforts to consider this relationship in the design of new antibiotic regimens.
Consistent with literature, lab-evolved resistant strains displayed mostly decreased metabolic activity with occasional increases in activity across measured environments relative to the unevolved ancestor (Björkman et al., 2000; Fabich et al., 2011; Marvig et al., 2015; Rau et al., 2010). The ciprofloxacin-resistant lineage was found to be the most metabolically limited, growing on the fewest number of carbon sources, exhibiting significantly decreased maximum growth, and taking significantly longer for the population to double and reach mid-exponential phase. The piperacillin-resistant lineage was found to have decreased metabolic functionality, in part due to a large deletion that removed many metabolic genes and led to the inability to catabolize L-leucine and other carbon sources. We showed that clinical isolates with similar large chromosomal deletions also lacked the ability to catabolize L-leucine, and we predict that these isolates would share other metabolic phenotypes with the piperacillin-resistant lineage (Hocquet et al., 2016). Patients from which the isolates were collected had not been recently treated with piperacillin or other beta-lactams, indicating that such large deletions are not necessarily drug specific, but could be more likely to arise when the cell is under general stress.

The tobramycin-resistant lineage exhibited the fewest metabolic deficits and demonstrated enhanced growth on the carbon source N-acetyl-D-glucosamine. This phenotype was found to be shared with three other independent tobramycin-evolved lineages, and, while many of the genetic mutations in these lineages were found to be in similar pathways, there were no universally shared mutations. Mutation of six of these genes, each observed in a single tobramycin-evolved lineage, increased growth on N-acetyl-D-glucosamine. Two of these six mutated genes, nuoL and nuoB, exhibited slight increases in resistance to tobramycin, indicating that resistance-associated mutations had a direct impact on the observed growth phenotype. Mutations in the nuo complex encoding for subunits of the NADH dehydrogenase I have been previously associated with aminoglycoside tolerance (El-Garch et al., 2007; Schurek et al., 2008; Shan et al., 2015; Yen and Papin, 2017). More generally, disruptions to the electron transport chain have been shown to confer aminoglycoside resistance (Mayer et al., 2015; McCollister et al., 2011; Taber et al., 1987). Consistent with previous literature, mutation of the PA14_57570 gene, encoding for a subunit of the cytochrome c reductase, also resulted in increased resistance to tobramycin (Dötsch et al., 2009); however, this mutation resulted in significantly decreased growth on N-acetyl-D-glucosamine. Our results show that resistance-associated mutations can have heterogeneous impacts on the growth of P. aeruginosa. There remains more experimental work to resolve the exact mechanism by which these genes impact growth on N-acetyl-D-glucosamine, whether through direct catabolism or toxicity (Konopka, 2012).

There were two potential sources of confounding error in this study that were infeasible to resolve. First, all of the growth calls and growth dynamics presented were derived from measurements of optical density over time. Biological processes such as cell widening and pigment production can confound optical measures of population growth (Stevenson et al., 2016); however, we could not have achieved the same level of temporal resolution for as many conditions with other metrics of growth. Second, we observed a significant decrease in the maximum growth density of the LB-evolved control lineage relative to the ancestral lineage, indicating that evolving to media and antibiotic at the same time may have had confounding effects on metabolism. Media condition has been shown to impact evolution of resistance, and evolving to rich media in the absence of a drug has been found to result in altered growth phenotypes and deleterious mutations (Fong et al., 2005; Kram and Finkel, 2015; Westphal et al., 2018; Zampieri et al., 2017). For future adaptive laboratory evolution studies, we recommend that the ancestral lineage be adapted to the base media and then subsequently adapted to the antibiotic of interest to control for the effect of media (Baym et al., 2016). Despite the impact of adaptation to LB media on maximum growth density, we still observed metabolic phenotypes and growth dynamics unique to antibiotic-evolved lineages.

GENREs are becoming increasingly prevalent in the study of antibiotic resistance (Banerjee et al., 2017; Carey et al., 2017; Dunphy and Papin, 2018; Zampieri et al., 2017). Although the P. aeruginosa metabolic network reconstruction does not account for many genes associated with resistance (e.g., regulation of efflux, membrane permeability), the model allowed for the rapid identification of potential mutations impacting growth phenotypes.Erroneous predictions provided useful starting points for future model curation and increased understanding of metabolic functionalities. The majority of the overlap between model genes and mutations uncovered from genome sequencing occurred in the large deletions of the piperacillin-resistant lineages. Due to the limited number of mutations in metabolic genes across the other evolved lineages, we were unable to find specific genetic mechanisms for many of the experimentally observed changes in metabolic phenotype. We speculate that metabolic adaptations that could not be explained by our model simulations may have been affected by resistance mutations that impact transcriptional regulation. We would expect the integration of a transcriptional regulatory network or transcriptomic profiling data with the metabolic network reconstruction to uncover more genotype-phenotype relationships in the resistant lineages (Blazier and Papin, 2012; Carey et al., 2017; Lister et al., 2009; Livermore, 2002; Monk et al., 2017).

While this study shows that there is systems-level metabolic changes following sustained antibiotic pressure, the evolution of resistance is a stochastic process that is sensitive to a wide variety of environmental and host factors not accounted for in our experimental design. As such, observed mutations may have varied with a different selection of antibiotics or media conditions. Nevertheless, many of the mutations we observed were consistent with those in sequenced clinical P. aeruginosa isolates, indicating that the general trends we observed may be robust to nutrient differences between LB media and CF sputum (Hocquet et al., 2016; Mayer et al., 2015). Additionally, through the interrogation of the growth of tobramycin-evolved P. aeruginosa on N-acetyl-D-glucosamine, we have shown that metabolic phenotypes can be shared across resistant lineages with independent evolutionary paths and heterogeneous mutational landscapes. While we have chosen to focus on three antibiotics and one media condition in a single bacterial species, our combined experimental and computational approach can be
applied to a large variety of organisms and environmental pressures. Moving forward, we have laid the groundwork for the interrogation of broad metabolic consequences of antibiotic resistance.

**STAR METHODS**

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- **DATA AND SOFTWARE AVAILABILITY**

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes seven figures, three tables, and four data files and can be found with this article online at https://doi.org/10.1016/j.cels.2018.12.002.

**ACKNOWLEDGMENTS**

The authors would like to thank lab-mates Anna S. Blazier and Dr. Glynis Kolling for their feedback on the experimental design and manuscript. We would like to give special thanks to Didier Hocquet for providing the clinical isolates used in this study. We would also like to thank Dr. Joanna Goldberg from Emory and Dr. Jason Yang from MIT for their advice on the project. This work was funded by the National Science Foundation Graduate Research Fellowship Program (grant number DDGE-1315231) and the National Institutes of Health (grant number R01-GM088244).

**AUTHOR CONTRIBUTIONS**

The project design was conceived by J.A.P., L.J.D., and P.Y. Experiments were performed by L.J.D. and P.Y.; L.J.D. performed all *in silico* analyses. L.J.D., P.Y. and J.A.P. all contributed to the writing and editing of the manuscript.

**DECLARATION OF INTERESTS**

The authors declare no competing interests.
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STAR METHODS

KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Bacterial and Virus Strains** | | |
| *Pseudomonas aeruginosa* UCBPP-PA14 | Yen and Papin (2017) | NCBI SRA: SRP100674, BioProject: PRJNA376615 |
| *Pseudomonas aeruginosa* transposon mutants | Liberati et al. (2006) | Mutant IDs: 32173, 42761, 53876, 45885, 53819, 34027, 28941, 54834, 35720, 40719, 43223 |
| *Pseudomonas aeruginosa* clinical isolates | Hocquet et al. (2016) | NA |
| **Chemicals, Peptides, and Recombinant Proteins** | | |
| Tobramycin | Sigma | Cat# T4014 |
| Primer synthesis | Eurofins | NA |
| **Critical Commercial Assays** | | |
| Phenotype Microarray Plates PM1 and PM2a; IF-0a GN/GP Inoculating Fluid | Biolog | Cat#12111 and Cat#12112; Cat#72268 |
| **Deposited Data** | | |
| All raw and processed data | This paper, GitHub | https://github.com/lauradunphy/dunphy_yen_papin_supplement |
| Results of statistical tests | This paper, see Table S2 | NA |
| Oligonucleotides | This paper, see Table S1 | NA |
| **Software and Algorithms** | | |
| All code used for data analysis and figure generation | This paper, GitHub | https://github.com/lauradunphy/dunphy_yen_papin_supplement |
| COBRA Toolbox | Schellenberger et al. (2011) | https://github.com/opencobra/cobra_toolbox/ |

CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Jason Papin (papin@virginia.edu).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

**Bacterial Strains and Culture Conditions**

*Pseudomonas aeruginosa* strain UCBPP-PA14 was previously evolved to one of three antibiotics (piperacillin, tobramycin, or ciprofloxacin) in lysogeny broth (LB) media for 20 days (Yen and Papin, 2017). As a control, *P. aeruginosa* was also adapted to LB media in the absence of drug for 20 days. One representative lineage from each 20-day evolution described previously (Yen and Papin, 2017) was selected for phenotypic growth profiling in this study. The lineages used were as follows: Day 0 Ancestor, Day 20 Piperacillin Replicate 1, Day 20 Tobramycin Replicate 3, Day 20 Ciprofloxacin Replicate 4, and Day 20 Control Replicate 2. All four Day 20 piperacillin-evolved replicate lineages and control replicate lineages were used in experiments on N-acetyl-D-glucosamine and their numbering was consistent with previous analyses (Figures 4A and S4A) (Yen and Papin, 2017). Day 20 Piperacillin Replicates 2 and 3 were included in expanded computational predictions (Figures S5B and S5C). Prior to phenotypic screening, frozen stocks of resistant or unevolved sensitive *P. aeruginosa* were streaked on LB agar plates (1% tryptone, 0.5% yeast extract, 1% NaCl) and incubated for 18-22 hours at 37C. The reported minimal inhibitory concentrations of tobramycin (Sigma) in Table S1 were determined in LB media using the broth microdilution method as previously described (Yen and Papin, 2017).

For growth experiments on the carbon sources N-acetyl-D-glucosamine (>95% (HPLC), Sigma), L-leucine (Sigma), and L-isoleucine (Sigma), frozen stocks of the appropriate *P. aeruginosa* lineages were streaked on an LB agar plate and incubated for 24 hours at 37C. Single colonies were isolated from the streak plates and grown for 24 hours in 5mL of LB media at 37C and shaken at 120rpm. Overnight cultures were diluted down to a starting OD600 of 0.001 (10^6 CFU/mL) in 200uL of M9 minimal media containing the designated carbon source. Prior to inoculation, overnight cultures were washed three times in 1X PBS (6,000rpm for 5 minutes).
A concentration of 20mM was used for all carbon sources except L-leucine, which was used at a concentration of 40mM. The OD600 was measured every 10 minutes for 48 hours (Tecan Infinite M200 Pro).

**P. aeruginosa UCBPP-PA14 Transposon Mutants**

*P. aeruginosa* mutants with transposon insertions in the *nuoL* (mutant ID 32173), *nuoB* (mutant ID 42761), and PA14_41710 (mutant ID 53876) genes were selected from the non-redundant PA14 genome-scale transposon library (PA14NR Set) (Liberati et al., 2006). The mutants were grown on an LB agar plate at 37°C. Transposon mutants with insertions in the following genes were grown LB agar plates supplemented with gentamicin and incubated at 37°C for 18-22 hours: *gnyA* (mutant ID 45885), *scoA* (mutant ID 53819), *scoB* (mutant ID 34027), PA14_23470 (mutant ID 28941), PA14_57570 (mutant ID 54834), PA14_57880 (mutant ID 35720), PA14_44360 (mutant ID 40719), and PA14_57850 (mutant ID 43223). Single colonies of each mutant were then selected and grown in LB liquid culture at 37°C overnight, shaken at 125rpm and were frozen in 25% glycerol. The presence of transposon insertions in the expected genes were verified by PCR (Table S2).

**P. aeruginosa Clinical Isolates**

Four pairs of clinical isolates were collected by Hocquet et al. as described in their study (Hocquet et al., 2016). Four replicate colonies of each of the eight isolates were grown in 40mM of L-leucine for 48-hours as described above.

**METHOD DETAILS**

**Whole-Genome Sequencing**

Whole-genome sequencing was previously performed on the evolved strains of *P. aeruginosa* used in this study (NCBI SRA: SRP100674, NCBI BioProject: PRJNA376615) (Yen and Papin, 2017). In summary, samples of adaptively evolved strains and wild type ancestor were streaked on LB plates and incubated at 37°C before being submitted to Genewiz Incorporation for sequencing (Illumina HiSeq 2500). Sequence reads were aligned to the *P. aeruginosa* UCBPP-PA14 reference genome (NCBI Reference Sequence: NC_008463.1).

**Phenotypic Growth Profiling via Single-Carbon Source Utilization Screens**

Following incubation on an LB agar plate at 37°C for 18-22 hours, bacteria were scraped from a lawn on an LB agar plate and transferred to 1.5mL of IF-0a GN/GP inoculating fluid (Biolog). The sample was vortexed until the liquid culture appeared turbid and contained no visible clumps of bacteria. The culture was then diluted to a starting OD600 of 0.07 in 10-15mL of inoculating fluid. 100uL of liquid culture was transferred to each well of a Phenotype Microarray plate (PM1 or PM2a, Biolog), mixing thoroughly to dissolve lyophilized carbon sources at the bottom of each well, and OD600 was measured every 10 minutes for 48 hours (Tecan Infinite M200 Pro). Three PM1 and three PM2a Phenotype Microarray plates each were used for the ancestral, LB-evolved, piperaclillin-evolved, ciprofloxacin-evolved, and tobramycin-evolved lineages. To ensure the homogeneity of each lineage, a different lawn was used for each plate. Each starting lawn was considered a biological replicate. No antibiotics were used in these growth profiling experiments. Plots of all 950 median growth curves can be found in the supplement (Figure S1).

**QUANTIFICATION AND STATISTICAL ANALYSIS**

**Growth Cutoff for Phenotypic Profiling**

Each well of the Biolog Microarray plates contained a single carbon source, totaling 190 unique carbon sources and two negative control wells across two, 96-well plates. The median of the negative control wells (median of three colonies for each strain) were subtracted from the median growth curves (across three colonies for each strain). Bacteria were considered to have grown on a given carbon source if the AUC of the growth curve after subtracting the negative control was greater than or equal to a defined threshold. The AUC was chosen to avoid noise associated with single-time point measurements such as the maximum or endpoint optical densities. AUC values correlated well with the maximum optical density across all of the data (Pearson correlation = 0.943, Figure S7). A lineage was considered to have grown on a given carbon source if the AUC was in the upper quartile of data (3rd quartile = 15.8). This threshold was generally consistent with growth calls made by manual inspection of the curves. To rigorously examine the effect of the growth cutoff value on the results of this study, we increased and decreased the cutoff by up to 50% and recalculated the growth dynamics for each lineage on growth-supporting carbon sources. While the carbon sources included in the analyses varied slightly, trends in growth dynamics remained stable across the full range of threshold perturbations (Figure S2).

**Calculation of Growth Dynamics**

Three growth parameters were calculated on the background-subtracted median growth curves collected from the Biolog Microarray plates: maximum growth density, doubling time, and time to mid-exponential phase. The maximum growth density was reported as the maximum OD600 value along each growth curve. The doubling time and time to mid-exponential phase were calculated using the growthcurver package in R, which fits a basic logistic equation to growth data (Sprouffske and Wagner, 2016). In brief, the
doubling time was taken to be the natural log of 2 divided by the growth rate, where the growth rate was the maximum slope of the natural log of the growth curve. The time to reach mid-exponential phase was defined as the time at which the culture reached half of the maximum stationary phase OD600 or theoretical maximum OD600 if the curve did not reach stationary phase in 48 hours.

The maximum growth densities were calculated for each replicate colony in Figures 4, 6C, 6D, 7, and S4C. Prior to calculating the maximum optical density, the minimum OD600 value of each replicate colony growth curve was subtracted from every time point. The maximum OD600 was taken from each of these minimum-subtracted growth curves and the distributions of maximums were reported.

**Genome-Scale Metabolic Network Reconstruction of *P. aeruginosa***

The recently published genome-scale metabolic network reconstruction of wild type *P. aeruginosa* UCBPP-PA14 was used for this study (Bartell et al., 2017). The reconstruction, iPau1129, accounts for the functions of 1129 genes and 1495 reactions. Previous analysis revealed that the model was 81% accurate at predicting growth phenotypes of wild type *P. aeruginosa* on 91 experimentally measured carbon sources (Bartell et al., 2017). This accuracy was maintained in the subset of carbon sources examined in this study.

**Gene Essentiality Predictions**

75 carbon sources of the 190 measured carbon sources had exchange reactions in the genome-scale metabolic network reconstruction, iPau1129. An exchange reaction represents the ability to import a carbon source into the model system. This is different from a transport reaction, which can be thought of as a way to transport a metabolite between the media and the cell cytosol. We temporarily added exchange reactions for another nine carbon sources that had transport reactions but lacked exchange reactions. The base model supported growth, defined as non-zero biomass, on 42 of these 84 carbon sources. We used iPau1129 to simulate growth of *P. aeruginosa* in each of these carbon sources with minimal media. Using the singleGeneDeletion function in the COBRA Toolbox (Schellenberger et al., 2011), we simulated single-gene knockouts of each gene in the presence of each unique carbon source. From these simulations, we predicted genes for each media condition that when knocked out, resulted in the model no longer being able to support growth. We refer to genes predicted to be required for growth as essential genes for a given environment. We looked for overlap between essential genes predicted by the model and mutated genes in our evolved resistant strains. This overlap was used to predict resistance-specific essential genes as well as to identify incorrect model predictions.

**Statistical Tests and Clustering**

Statistical comparisons between the ancestral lineage and all evolved lineages and transposon mutants were made with the non-parametric two-tailed Wilcoxon rank sum test, which does not require data to be normally distributed. The Benjamini-Hochberg procedure was used to adjust the P-value when there were multiple comparisons (Benjamini and Hochberg, 1995). Tests were performed on distributions of biological replicates or on distributions of medians across biological replicates. A P-value below 0.05 was considered significant (Table S3). Samples were assumed to be independent. The Jaccard distances between lineages were calculated on binary growth calls across all carbon sources using the vegan package in R (Oksanen et al., 2018) and the distance matrix was clustered using the complete-linkage method.

**DATA AND SOFTWARE AVAILABILITY**

All code and data used to calculate growth dynamics, make gene essentiality predictions, as well as generate figures and supplemental data (excluding Figure 1) can be found at https://github.com/lauradunphy/dunphy_yen_papin_supplement. Complete growth data, binary growth calls, growth dynamics, and computational gene essentiality predictions can be found in the supplement (Data S1, S2, S3, and S4).
Supplemental Information

Integrated Experimental and Computational Analyses
Reveal Differential Metabolic Functionality
in Antibiotic-Resistant *Pseudomonas aeruginosa*

Laura J. Dunphy, Phillip Yen, and Jason A. Papin
Figure S1. Growth curves of all lineages on all carbon sources. Related to Figures 1A and 2. Median 48-hour growth curves of the five lineages (ancestor, LB-evolved, CIP-evolved, PIP-evolved, TOB-evolved) on 190 carbon sources. (A) Growth on carbon sources from Phenotypic Microarray Plate PM1. (B) Growth on carbon sources from Phenotypic Microarray Plate PM2a. Each curve represents the median of three colonies from a single evolved lineage. Growth is measured as the OD600 - background, where the background was an inoculated well containing media with no carbon source.
Figure S2. The effect of varying the AUC growth cutoff value on growth dynamics. Related to Figure 3. The growth cutoff was increased or decreased by 10%, 25%, and 50%. Growth curves exceeding each cutoff were included in the calculation of the maximum growth density (top row), doubling time (middle row), and time to mid-exponential phase (bottom row). The calculated growth dynamics with the initial AUC cutoff from Figure 3 are shown in the middle column.
Figure S3. Area under the curve (AUC) on all carbon sources by lineage. Related to Figure 2. The total AUC for each lineage on 190 carbon sources where the AUC = sum(growth curve - background). (A) Phenotypic Microarray Plate PM1. (B) Phenotypic Microarray Plate PM2a. The median growth curves and backgrounds were taken across three colonies for each lineage. A lineage was considered to have grown on a given carbon source if the AUC was greater than the upper quartile of all data.
Figure S4. Growth curves of tobramycin-evolved *P. aeruginosa* and transposon mutants on N-acetyl-D-glucosamine and LB media. Related to Figure 4. (A) Median 48-hour growth (OD600) of four independent tobramycin-evolved lineages (blue) and four independent LB-evolved lineages (dark gray) on 20mM of N-acetyl-D-glucosamine relative to the unevolved ancestor (gray) and uninoculated media (black). Numbers refer to replicate lineages from a previous study (Yen and Papin, 2017). TOB 3 and LBE 2 correspond to TOB and LBE from the phenotypic profiling analysis. Shading shows the interquartile range of four replicate colonies for each lineage. (B) Median 48-hour growth curves of eight transposon mutants (green) on 20mM of N-acetyl-D-glucosamine relative to ancestor (gray) and uninoculated media (black). Shading shows the interquartile range of four replicate colonies for each lineage. (C) Maximum growth (OD600) of eight transposon mutants (green) relative to ancestor (gray). Significance relative to the ancestor determined by Wilcoxon rank sum test with Benjamini-Hochberg correction (*P*-value < 0.05). Crossbar denotes median and error bars show the interquartile range of four replicate colonies. (D) Median 48-hour growth curves of eight transposon mutants (green) on LB media relative to ancestor (gray). Shading shows the interquartile range of four replicate colonies for each lineage.
Figure S5. Complete essentiality predictions in the PIP large deletion and unique essentiality predictions in other piperacillin-evolved lineages. Related to Figure 5. (A) Expansion of Figure 5 that includes all genes that were deleted in the piperacillin-evolved lineage and are included in the iPau1129 GENRE. Unique gene essentiality predictions for genes in the large deletions of additional lineages (B) Day 20 Piperacillin Replicate 2 and (C) Day 20 Piperacillin Replicate 3 from Yen and Papin, 2017 (In vitro: white = no growth, gray = growth; in silico: black = essential gene, white = non-essential gene).
Figure S6. Expanded validation of model-guided gene essentiality predictions. Related to Figures 6 and 7. (A) The median 48-hour growth (OD600) of the ancestral lineage (gray), piperacillin-evolved lineage (red), and a gnyA transposon mutant on 40mM of L-leucine. (B) The median growth (OD600) of gnyA (orange) on 20mM of L-isoleucine. (C) The median 48-hour growth (OD600) of the ancestral lineage (gray), piperacillin-evolved lineage (red), and scoA (solid orange) and scoB (dashed orange) transposon mutants on 4-hydroxybenzoic acid. (D) The median 48-hour growth (OD600) of four clinical isolate pairs (AWT and APM, BWT and BPM, CWT and CPM, DWT and DPM) on 40mM L-leucine. Negative controls show the OD600 of uninoculated media (black). Shading shows the interquartile range of at least four replicate colonies for each lineage or clinical isolate.
Figure S7. Correlation between maximum optical density and the area under the curve. Related to Figures 2 and 3. The area under the curve and maximum OD600 for each lineage on each carbon source colored by lineage (pearson correlation coefficient = 0.943). The growth cutoff, defined as the upper quartile measurement, is shown with a dashed line.
Table S1. Relevant minimal inhibitory concentrations of tobramycin. Related to Figure 4.

| P. aeruginosa mutant | Lineage mutation found in | MIC Tobramycin |
|----------------------|---------------------------|----------------|
| Ancestor             | NA                        | 0.5 - 1 μg/ml  |
| nuoB transposon mutant | TOB 2                    | 2 μg/ml        |
| nuoL transposon mutant | TOB 4                    | 2 μg/ml        |
| PA14_23470 transposon mutant | TOB 1                | 1 μg/ml        |
| PA13_41710 transposon mutant | TOB 2                | 1-2 μg/ml      |
| PA14_44360 transposon mutant | TOB 3                | 1 μg/ml        |
| PA14_57570 transposon mutant | TOB 1                | 2 μg/ml        |
| PA14_57850 transposon mutant | TOB 4                | 1 μg/ml        |
| PA14_57880 transposon mutant | TOB 3                | 1 μg/ml        |
Table S2. Primer sequences for verification of insertions in transposon mutants. Related to Star Methods.

| Name | Mutant ID | Locus tag   | Insertion Position | Forward Primer Sequence (5'-3')                     | Reverse Primer Sequence (5'-3')                     |
|------|-----------|-------------|--------------------|-----------------------------------------------------|-----------------------------------------------------|
| gnyA | 45885     | PA14_38480  | 3432231            | GTGCACAGCGACATCGAC                                   | CAGGGGCGTTCATTTC                                   |
| scoA | 53819     | PA14_38660  | 3448424            | CATCTGGGGAACCTCTTTG                                  | ACAATCACAAGAGCCGGAAG                                |
| scoB | 34027     | PA14_38640  | 3447400            | CTTGCATGGATGGTTCTCTCAT                              | ACTACGTCAACCTCGGCAATT                              |
| nuoL | 32173     | PA14_29880  | 2587857            | GAGCAGCAGGTAGGGTTTCA                                 | AGACCGATATCAAGCGCATC                                |
| nuoB | 42761     | PA14_30010  | 2598643            | CCTGGGACCACCGAATAGATG                                | TTCATGCTAGGGGTTCGC                                 |
|      |           | PA14_41710  | 3723046            | AAGCAGAAGCTTGTCTTCGAC                                | AGGATCAGGATCACCAGCAC                                |
|      |           | PA14_23470  | 2041628            | TTTGAGTTGCTGGATGGTTGCA                               | AGTCGGCCATGAAATACTGG                                |
|      |           | PA14_57570  | 5127878            | TAGGACATCTCGACCCCAAGG                                | GAGCAAGCTGTATTCATCGT                                 |
|      |           | PA14_57880  | 5152471            | ATGTCGCCGCTTGTATTCATCGT                              | ATCGAGGTTCTGGGCTATGT                                |
|      |           | PA14_44360  | 3949500            | GGCAAGGAGCATCGAGAAGAA                                | GAGCAGTATGTTGGCAGAAACAT                            |
|      |           | PA14_57850  | 5150645            | GAGTGCCGCTAGAGCTTTGC                                 | CAACGGCGTACAAAGTA                                  |
Table S3: P-values for every statistical test performed throughout the results. Related to Figures 2-4, 6, and S4. Wilcoxon Rank Sum test was used for all tests. We used a nonparametric test because we could not justify the assumptions of normality and homogenous variance required for a parametric test (e.g. t-test). Benjamini-Hochberg procedure was used for all tests except those in Figure 7, which did not require multiple-comparisons corrections. The null hypothesis was rejected if the P-value < 0.05.

| Figure | Comparison | Value | Corrected P-value | Null (H₀: μ₁=μ₂) |
|--------|------------|-------|-------------------|------------------|
| Figure 2C | Ancestor vs LBE | Max Growth | 1.715363e-05 | Reject |
| Figure 2C | Ancestor vs CIP | Max Growth | 9.246727e-05 | Reject |
| Figure 2C | Ancestor vs PIP | Max Growth | 1.715363e-05 | Reject |
| Figure 2C | Ancestor vs TOB | Max Growth | 1.920510e-03 | Reject |
| Figure 3D | Ancestor vs LBE | Max Growth | 0.00345813 | Reject |
| Figure 3D | Ancestor vs CIP | Max Growth | 0.010989347 | Reject |
| Figure 3D | Ancestor vs PIP | Max Growth | 0.009433061 | Reject |
| Figure 3D | Ancestor vs TOB | Max Growth | 0.175272139 | Accept |
| Figure 3E | Ancestor vs LBE | Doubling Time | 0.2602848339 | Accept |
| Figure 3E | Ancestor vs CIP | Doubling Time | 0.0004989341 | Reject |
| Figure 3E | Ancestor vs PIP | Doubling Time | 0.2602848339 | Accept |
| Figure 3E | Ancestor vs TOB | Doubling Time | 0.2474878772 | Accept |
| Figure 3F | Ancestor vs LBE | Time to Mid-Exp | 0.4213482327 | Accept |
| Figure 3F | Ancestor vs CIP | Time to Mid-Exp | 0.0006009329 | Reject |
| Figure 3F | Ancestor vs PIP | Time to Mid-Exp | 0.0081646317 | Reject |
| Figure 3F | Ancestor vs TOB | Time to Mid-Exp | 0.4213482327 | Accept |
| Figure 4A | Ancestor vs LBE 1 | Max Growth | 0.03472323 | Reject |
| Figure 4A | Ancestor vs LBE 2 | Max Growth | 0.03472323 | Reject |
| Figure 4A | Ancestor vs LBE 3 | Max Growth | 0.03472323 | Reject |
| Figure 4A | Ancestor vs LBE 4 | Max Growth | 0.03472323 | Reject |
| Figure 4A | Ancestor vs TOB 1 | Max Growth | 0.03472323 | Reject |
| Figure 4A | Ancestor vs TOB 2 | Max Growth | 0.03472323 | Reject |
| Figure 4A | Ancestor vs TOB 3 | Max Growth | 0.03472323 | Reject |
| Figure 4A | Ancestor vs TOB 4 | Max Growth | 0.06060197 | Accept |
| Figure 4B | Ancestor vs nuoL | Max Growth | 0.03472323 | Reject |
| Figure 4B | Ancestor vs nuoB | Max Growth | 0.03472323 | Reject |
| Figure 4B | Ancestor vs PA14_41710 | Max Growth | 0.03472323 | Reject |
| Figure 4B | Ancestor vs PA14_23470 | Max Growth | 0.03472323 | Reject |
| Figure 4B | Ancestor vs PA14_57570 | Max Growth | 0.03472323 | Reject |
| Figure 4B | Ancestor vs PA14_57880 | Max Growth | 0.03472323 | Reject |
| Figure 4B | Ancestor vs PA14_44360 | Max Growth | 0.03472323 | Reject |
| Figure 6C | Ancestor vs PIP | Max Growth | 0.03038282 | Reject |
| Figure 6C | Ancestor vs gnyA | Max Growth | 0.03038282 | Reject |
| Figure 6D | Ancestor vs PIP | Max Growth | 0.09114847 | Accept |
| Figure 6D | Ancestor vs scoA | Max Growth | 0.88523391 | Accept |
| Figure 6D | Ancestor vs scoB | Max Growth | 0.88523391 | Accept |
| Figure 7A | AWT vs APM | Max Growth | 0.02940105 | Reject |
| Figure 7B | BWT vs BPM | Max Growth | 0.03472323 | Reject |
| Figure 7C | CWT vs CPM | Max Growth | 0.02940105 | Reject |
| Figure 7D | DWT vs DPM | Max Growth | 0.03472323 | Reject |
| Figure S4C | Ancestor vs nuoL | Max Growth on LB | 0.04051043 | Reject |
| Figure S4C | Ancestor vs nuoB | Max Growth on LB | 0.04051043 | Accept |
| Figure S4C | Ancestor vs PA14_41710 | Max Growth on LB | 0.04051043 | Reject |
| Figure S4C | Ancestor vs PA14_23470 | Max Growth on LB | 0.06925939 | Accept |
| Figure S4C | Ancestor vs PA14_57570 | Max Growth on LB | 0.04051043 | Reject |
| Figure S4C | Ancestor vs PA14_57880 | Max Growth on LB | 0.04051043 | Reject |
| Figure S4C | Ancestor vs PA14_44360 | Max Growth on LB | 0.04051043 | Reject |
| Figure S4C | Ancestor vs PA14_57850 | Max Growth on LB | 0.04051043 | Reject |

0.05.