Intrinsic Antimicrobial Resistance Determinants in the Superbug *Pseudomonas aeruginosa*

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ABSTRACT **Antimicrobial-resistant bacteria pose a serious threat in the clinic. This is particularly true for opportunistic pathogens that possess high intrinsic resistance. Though many studies have focused on understanding the acquisition of bacterial resistance upon exposure to antimicrobials, the mechanisms controlling intrinsic resistance are not well understood. In this study, we subjected the model opportunistic superbug *Pseudomonas aeruginosa* to 14 antimicrobials under highly controlled conditions and assessed its response using expression- and fitness-based genomic approaches. Our results reveal that gene expression changes and mutant fitness in response to sub-MIC antimicrobials do not correlate on a genomewide scale, indicating that gene expression is not a good predictor of fitness determinants. In general, fewer fitness determinants were identified for antiseptics and disinfectants than for antibiotics. Analysis of gene expression and fitness data together allowed the prediction of antagonistic interactions between antimicrobials and insight into the molecular mechanisms controlling these interactions.**

IMPORTANCE **Infections involving multidrug-resistant pathogens are difficult to treat because the therapeutic options are limited. These infections impose a significant financial burden on infected patients and on health care systems. Despite years of antimicrobial resistance research, we lack a comprehensive understanding of the intrinsic mechanisms controlling antimicrobial resistance. This work uses two fine-scale genomic approaches to identify genetic loci important for antimicrobial resistance of the opportunistic pathogen *Pseudomonas aeruginosa*. Our results reveal that antibiotics have more resistance determinants than antiseptics/disinfectants and that gene expression upon exposure to antimicrobials is not a good predictor of these resistance determinants. In addition, we show that when used together, genomewide gene expression and fitness profiling can provide mechanistic insights into multidrug resistance mechanisms.**

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Microbes in virtually every environment on earth are exposed to and display resistance to antimicrobials. While antimicrobial resistance has primarily been studied in the context of infection, both pathogenic and nonpathogenic bacteria in natural environments display high levels of intrinsic resistance to clinically relevant antimicrobials (1). This is true for many opportunistic bacterial pathogens, including *Pseudomonas aeruginosa*, which causes a range of chronic infections due in part to its recalcitrance to modern antimicrobial therapies (2, 3). Whether in the clinic or in the natural environment, *P. aeruginosa* encounters multiple classes of antimicrobials, including traditional antibiotics (e.g., penicillin), antiseptics (e.g., povidone-iodine), and disinfectants (e.g., bleach).

P. aeruginosa has recently been classified as an ESKAPE (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species) pathogen, one of a group of six highly antibiotic-resistant bacteria that are the primary causative agents of nosocomial (hospital-acquired) infections (4). Despite its importance, there are substantial gaps in our understanding of the intrinsic mechanisms responsible for this bacterium's ability to resist killing by antimicrobials. A primary approach to identify these intrinsic attributes has been to examine gene expression in the presence of nonlethal levels of an antimicrobial (5–8), with the idea that genes differentially regulated by low levels of an antimicrobial will provide important insights into factors important for intrinsic resistance. While these studies have provided tremendous insight into how microbes respond to antimicrobials, most of the genes identified in these studies have proven not to be important for intrinsic resistance. There could be several reasons for this; for instance, the use of antimicrobial levels that slow bacterial growth causes a conflation of antimicrobial-specific gene expression shifts with general growth-mediated ones (9), the use of undefined growth medium (5–8) results in inconsistent physiology (10, 11), and the use of different growth conditions hampers comparison between studies.

Another useful approach to identify intrinsic mechanisms of resistance has been to screen transposon mutant libraries for in-

TABLE 1 Relevant antimicrobial information, including RNA-seq and Tn-seq summaries

Antimicrobial	Class	MIC (mg/ml or as indicated)	Sub-MIC level (fraction of MIC)	No. of genes with differential results (≥2-fold, FDR <0.05) for:	
				Expression	Fitness
Gentamicin	Antibiotic, aminoglycoside	0.003	1/2	214	758
Neomycin	Antibiotic, aminoglycoside	0.0078	1/4	45	89
Tobramycin	Antibiotic, aminoglycoside	0.0008	1/2	3	77
Polymyxin B	Antibiotic, antimicrobial peptide	0.0016	1/2	59	2
Ampicillin	Antibiotic, beta-lactam	0.3125	1/2	4	75
Aztreonam	Antibiotic, beta-lactam	0.0039	1/2	0	19
Carbenicillin	Antibiotic, beta-lactam	0.0391	1/2	9	4
Cefoperazone	Antibiotic, beta-lactam	0.0031	1/2	24	389
Ciprofloxacin	Antibiotic, quinolone	0.0002	1/2	8	66
Bleach	Disinfectant, halogen releasing	1.25%[a]	1/2	88	10
Povidone-iodine	Disinfectant, halogen releasing	5%[a]	1/2	43	0
Hydrogen peroxide	Antiseptic/disinfectant, oxidizing	0.0047%[a]	1/2	99	2
Benzalkonium chloride	Antiseptic/disinfectant, quaternary ammonium	0.0153	1/4	15	0
Silver nitrate	Antiseptic, heavy metal releasing	0.3125	1/2	73	8

[a] Bleach, H_2O_2, and PVPi stock solutions were 5.25%, 30%, and 10% (1% available iodine), respectively.

creased or decreased susceptibility to sub-MIC antimicrobials (12–17). These studies have revealed novel resistance determinants; however, these experiments have been performed with limited numbers of antimicrobials and are difficult to compare due to differences in experimental design. In this study, we combined gene expression analysis under highly controlled conditions with high-throughput fitness profiling to elucidate intrinsic resistance mechanisms to 14 antimicrobials in *P. aeruginosa*.

RESULTS

Gene expression in response to sub-MIC antimicrobial levels. To examine the impact of antimicrobials on gene expression, high-throughput RNA sequencing (RNA-seq) was performed on planktonic *P. aeruginosa* cultures after 30 min of exposure to each of 14 antimicrobials (Table 1). These antimicrobials represent different classes of antibiotics and clinically relevant antiseptics and disinfectants. To eliminate gene expression changes due to variations in growth rate, cultures were exposed to the highest level of antimicrobial that did not perceptibly alter growth rate. As observed in other studies (5, 8, 16), this was generally 25 to 50% of the MIC of the antimicrobial (Table 1). To further minimize gene expression changes not directly resulting from antimicrobial exposure, all experiments were performed in a chemically defined medium with constant aeration and temperature. Genes exhibiting ≥2-fold changes in mRNA levels (false discovery rate [FDR] of <0.05) are reported (see Dataset S1 in the supplemental material).

Our results revealed that exposure to antimicrobials resulted in relatively minor changes in transcription, with the majority of antimicrobials affecting the expression of less than 100 (1.7%) of the 5,978 *P. aeruginosa* PA14 genes. *P. aeruginosa* showed differential expression of the greatest number of genes in response to gentamicin, altering the expression of 214 (3.6%) genes. In contrast, the beta-lactam antibiotic aztreonam altered the expression of no genes by ≥2-fold (see Dataset S1 in the supplemental material). Furthermore, H_2O_2 and ciprofloxacin exposure resulted in differential regulation of 109 and 8 genes, respectively, in our study, while previous studies showed differential regulation of 223 and 941 *P. aeruginosa* genes (5, 7). Despite the fact that we observed fewer changes in gene expression than were found in previous studies, there was significant overlap between the gene expression profiles generated in this study and those of previous studies examining the *P. aeruginosa* response to sub-MIC ciprofloxacin and hydrogen peroxide (using the Fisher exact test, $P = 3.6 \times 10^{-6}$ for ciprofloxacin and $P = 0.002$ for hydrogen peroxide) (5, 7). This overlap occurred despite the fact that different antimicrobial exposure times and growth conditions were used in these studies.

To view the global features of our transcriptomic data, we first conducted hierarchical clustering of RNA-seq data, focusing on genes that showed differential regulation under at least two antimicrobial conditions. Our results revealed that the data for antimicrobials do not cluster by class or by mechanism of action (Fig. 1). For example, the aminoglycosides gentamicin and neomycin cluster strongly with each other but not with the aminoglycoside tobramycin, while the beta-lactams cefoperazone and carbenicillin cluster strongly with each other but not with aztreonam or ampicillin. While most of the genes exhibiting transcriptional changes upon exposure to multiple antimicrobial conditions were of unknown function, genes previously shown to be important for modulating the susceptibility of *P. aeruginosa* to oxidative stress were differentially regulated upon exposure to the oxidizing agents H_2O_2, bleach, and povidone-iodine, as seen previously (18) (Fig. 1, yellow box). Additionally, multiple classes of antimicrobials induced general stress response genes, such as those encoding universal stress proteins (Fig. 1, blue box). These results reveal that *P. aeruginosa* possesses specific and general responses to different antimicrobials and that these responses are not predictive of antimicrobial class or mechanism of action.

Identification of fitness determinants in response to sub-MIC antimicrobial levels. While our transcriptomic results revealed gene expression changes in response to antimicrobials, it is not known whether these genes are important for intrinsic resistance. To address this question, we performed transposon sequencing (Tn-seq) of *P. aeruginosa* in the presence of sub-MIC

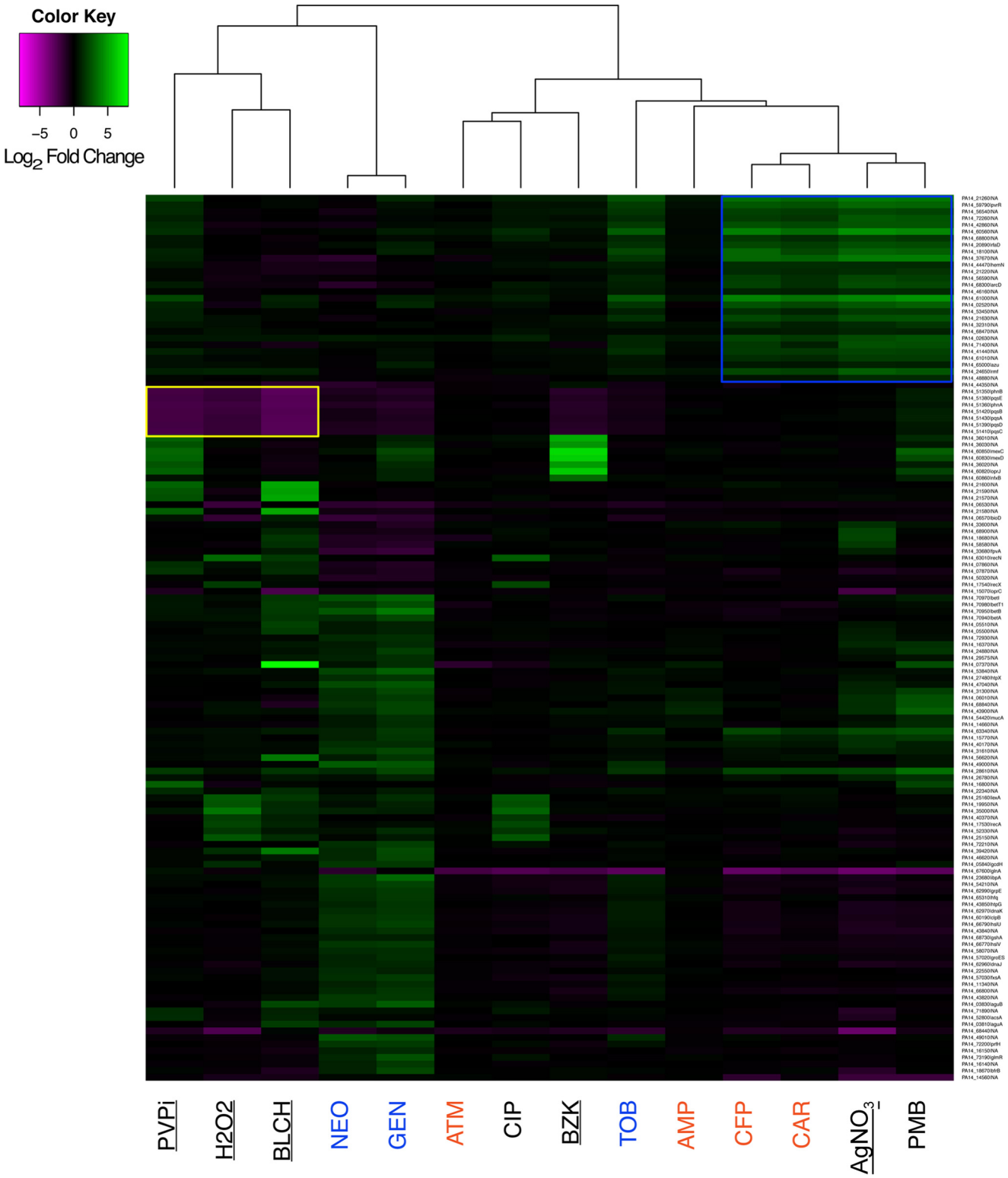


FIG 1 Hierarchical clustering and heat map of RNA-seq data. Clustering of RNA-seq data generated in response to sub-MIC levels of 14 antimicrobials using the Spearman rank correlation coefficient. The heat map was generated using genes that were differentially expressed by at least 2-fold under two or more conditions. Downregulation of a gene is indicated in red, and upregulation is indicated in green. The antimicrobials used were gentamicin (GEN), neomycin (NEO), tobramycin (TOB), carbenicillin (CAR), ampicillin (AMP), aztreonam (ATM), cefoperazone (CFP), ciprofloxacin (CIP), hydrogen peroxide (H_2O_2), povidone-iodine (PVPi), polymyxin B (PMB), benzalkonium chloride (BZK), sodium hypochlorite (BLCH), and silver nitrate ($AgNO_3$); aminoglycoside antibiotics are in blue, beta-lactams in orange, and antiseptics and disinfectants are underlined. The yellow box denotes genes important for susceptibility of *P. aeruginosa* to oxidative stress, and the blue box denotes general stress response genes.

levels of the 14 antimicrobials. For these experiments, a transposon mutant library containing ~300,000 *P. aeruginosa* PA14 mutants, each with a single transposon insertion, was constructed. This number of mutants represents an insertion approximately every 20 bp along the ~6.5-Mb *P. aeruginosa* genome. The transposon mutant library was subjected to sub-MIC levels of each of the 14 antimicrobials and allowed to grow for approximately 12 generations, and quantitative sequencing of genomic DNA adjacent to the transposon allowed the abundance of each insertion mutant to be measured (19–21). By comparing mutant abundance in the presence and absence of an antimicrobial, transposon insertion sites that affect fitness in the presence of an antimicrobial can be identified.

Most treatments (12 out of 14) resulted in the identification of less than 100 fitness determinants (transposon mutant abundance changes of ≥2-fold, with an FDR of <0.05) (Table 1; see also Dataset S2 in the supplemental material). Gentamicin resulted in the largest number of genes being identified, with ~13% of annotated genes being important for fitness, while for benzalkonium chloride and povidone-iodine, no fitness determinants with fold changes of ≥2 were identified (Table 1; see also Dataset S2). Hierarchical clustering analysis of the Tn-seq data revealed that, unlike the results from RNA-seq, both the aminoglycosides and the beta-lactams clustered by class (Fig. 2). As would be expected by their mechanisms of action, aminoglycoside fitness determinants consisted of heat shock genes that are important for coping with misfolded proteins (8), and beta-lactam antibiotic fitness determinants included cell wall biosynthetic genes (22). Unlike the antibiotics, few mutations affected the fitness of *P. aeruginosa* in the presence of the antiseptics and disinfectants, indicating that the inactivation of single genetic elements has little effect on the intrinsic resistance of *P. aeruginosa* to these antimicrobials. Finally, the inactivation of two genetic loci not previously linked to antimicrobial resistance showed enhanced fitness upon exposure to most antimicrobials (Fig. 2), indicating that *P. aeruginosa* can acquire mutations in individual loci that render it more resistant to multiple classes of antimicrobials. These two genetic elements are presumed to be involved in amino acid biosynthesis, one being a putative cysteinyl-tRNA synthetase and the other a putative arginine tRNA (23).

Gene expression and fitness are not well correlated. The generation of RNA-seq and Tn-seq datasets for the same antimicrobial under identical growth conditions provides the opportunity to determine whether expression data can be used to identify fitness determinants. The null hypothesis is that gene expression and mutant fitness are anticorrelated, because intuitively, we would expect that genes that are upregulated in the presence of an antimicrobial would be important for fitness when mutated. Thus, we determined the level of correlation between gene expression and mutant fitness across the *P. aeruginosa* genome for all antimicrobials tested. For this analysis, only nonessential genes were tested, since essential genes cannot be studied with Tn-seq. Our results revealed that the correlation between expression and fitness is weak (Fig. 3A and B show results for a specific example and for all antimicrobials, respectively), with the average correlation for all 14 antimicrobials being insignificant (−0.01). Confining our correlation analysis to the genes that were induced most highly by antimicrobial addition in the RNA-seq experiments (≥8-fold) did not result in a more significant correlation with mutant fitness (0.02), indicating that even genes that are the most responsive to antimicrobial addition are not enriched for fitness determinants. These data are consistent with previous work from our group and others showing weak correlation between expression and fitness (24, 25); however our previous study revealed that for some functionally related groups of genes, expression can be more predictive of fitness determinants (25). To examine this possibility for antimicrobials, we first tested whether fitness determinants for each antimicrobial were enriched in functionally related subsets of genes defined by the *Pseudomonas aeruginosa* Community Annotation Project (PseudoCAP) (23) and Kyoto Encyclopedia of Genes and Genomes (KEGG) (26). We then assessed whether the fitness determinants within these enriched subsets showed differential regulation in RNA-seq data. Our results reveal that only four antimicrobials showed enrichment of genes that were both fitness determinants and differentially regulated within individual PseudoCAP categories or KEGG pathways. For example, the data for ciprofloxacin showed enrichment for genes involved in DNA replication, recombination, modification, and repair, and 25% of the genes differentially regulated in this category were fitness determinants upon exposure to ciprofloxacin (Fig. 3C). Higher percentages were observed for other antimicrobial/gene category combinations, including gentamicin and the category cell wall/lipopolysaccharide (LPS)/capsule, in which 75% of differentially regulated genes were fitness determinants (Fig. 3C). These data indicate that, while the correlation between expression and fitness across the *P. aeruginosa* genome is weak, differentially regulated genes within particular PseudoCAP categories and KEGG pathways can be more predictive of fitness determinants.

Expression and fitness data can be used to determine the genetic basis of antimicrobial antagonism. Due to the increasing occurrence of antimicrobial-resistant infections, clinicians often prescribe combinations of antibiotics (27). Most studies focus on the effects of simultaneous antibiotic treatment; however, due to differential rates of diffusion through host tissue, bacteria likely encounter antibiotics individually and in succession (27, 28). Therefore, we sought to predict the mechanistic basis of enhanced resistance or susceptibility resulting from combinatorial antimicrobial treatment using our expression and fitness data. The rationale is that if exposure to an antimicrobial induces the transcription of a fitness determinant for a second antimicrobial, then we hypothesize that exposure to the first antimicrobial would enhance resistance to the second. As a test of this hypothesis, we examined how exposure to sub-MIC polymyxin B affects resistance to aminoglycosides and ciprofloxacin. Polymyxin B induces the transcription of two genes, PA14_38410 (*mexY* orthologue) and PA14_38395 (*mexX* orthologue), encoding components of a tripartite efflux pump. The *mexY* gene is a fitness determinant for gentamicin, neomycin, tobramycin, and ciprofloxacin, while *mexX* is a fitness determinant for gentamicin and tobramycin. Based on these data, we reasoned that exposure to sub-MIC polymyxin B would promote *P. aeruginosa* resistance to these antibiotics. To test this, *P. aeruginosa* was exposed to sub-MIC polymyxin B and subsequently tested for susceptibility to gentamicin, neomycin, tobramycin, and ciprofloxacin. For these experiments, a luminescent *P. aeruginosa* strain was used in which light production serves as a proxy for antimicrobial susceptibility. Our results reveal that preexposure to sub-MIC polymyxin B significantly increases resistance to gentamicin, neomycin, tobramycin, and ciprofloxacin (Fig. 4) but, as predicted by the RNA-seq results, not polymyxin B (see Fig. S1 in the supplemental material). To test

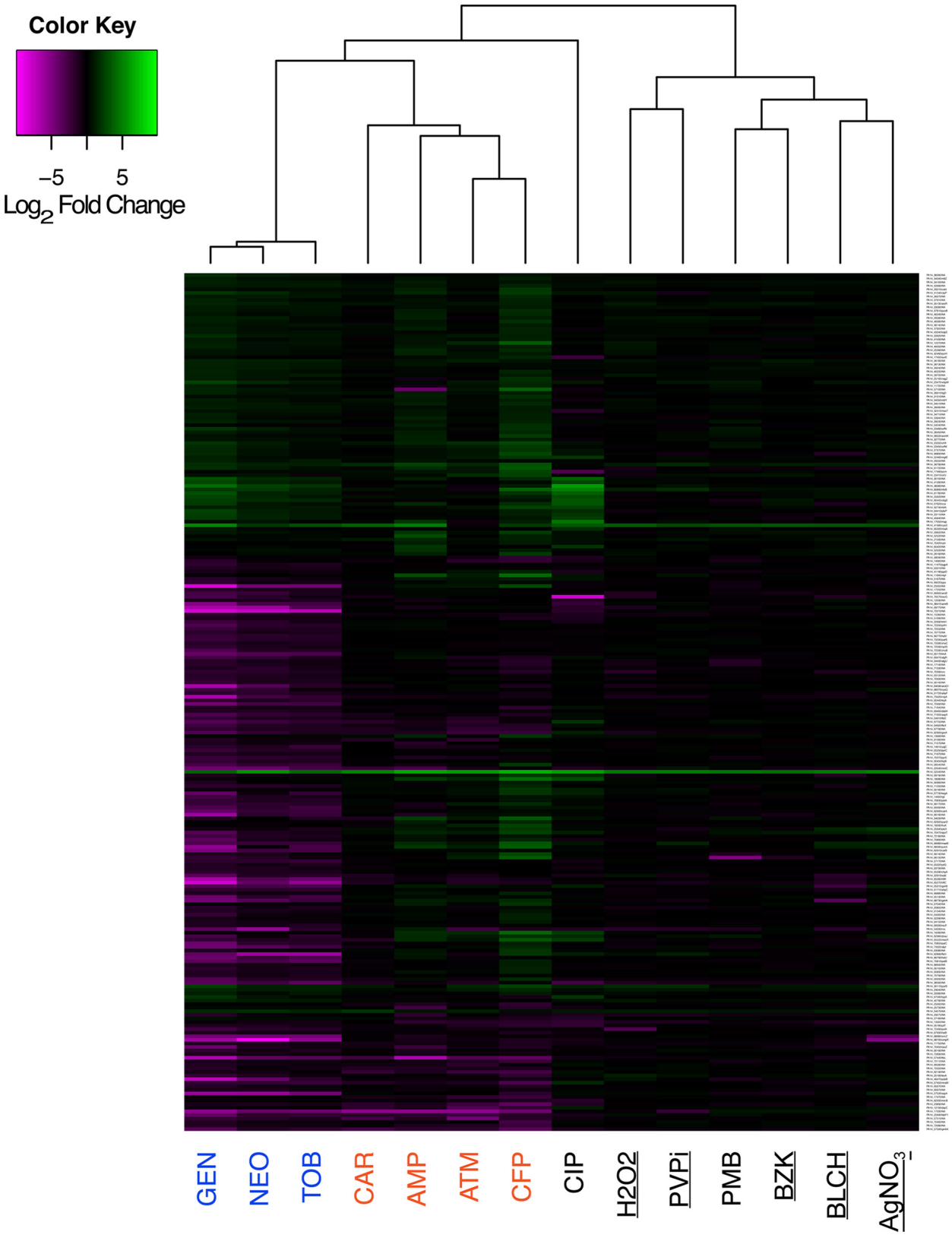
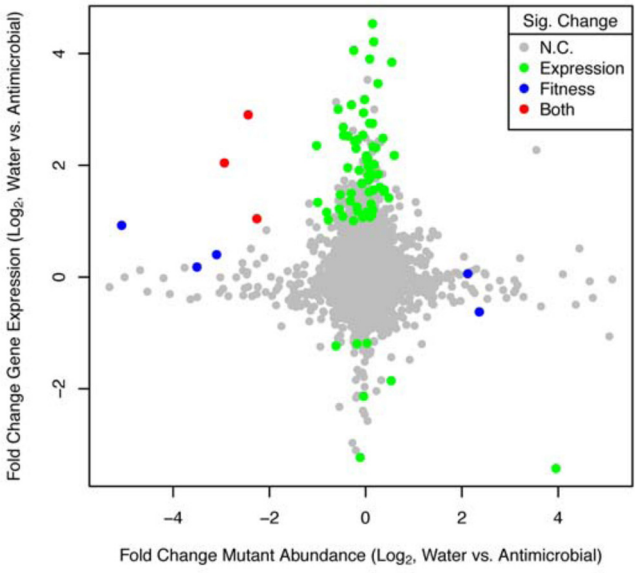


FIG 2 Hierarchical clustering and heat map of Tn-seq data. Clustering of Tn-seq data generated in response to sub-MIC levels of 14 antimicrobials using Spearman rank correlation coefficient. The heat map was generated using genes that showed at least 2-fold differential fitness under two or more conditions. Fitness determinants are indicated in red, and beneficial mutations are indicated in green. Antimicrobial abbreviations are defined in the legend to Fig. 1; aminoglycoside antibiotics are in blue, beta-lactams in orange, and antiseptics and disinfectants are underlined.

a



b

Antimicrobials	Abbreviation	Genome wide Correlation
Ampicillin	AMP	-0.03
Aztreonam	ATM	0.01
Benzalkonium Chloride	BZK	-0.03
Bleach	BLCH	-0.10
Carbenicillin	CAR	-0.01
Cefoperazone	CFP	0.00
Ciprofloxacin	CIP	0.14
Gentamicin	GEN	0.01
Hydrogen Peroxide	H2O2	-0.09
Neomycin	NEO	0.03
Polymyxin B	PMB	0.01
Povidone-Iodine	PVPi	-0.10
Silver Nitrate	AgNO3	-0.01
Tobramycin	TOB	0.01

c

Category/Pathway	Antimicrobial	% differentially regulated genes that are fitness determinants
PseudoCAP		
Cell division	GEN	25
Cell wall / LPS / capsule	GEN	75
Chaperones & heat shock proteins	GEN	40
DNA replication, recombination, modification and repair	CIP	25
Transcription, RNA processing and degradation	GEN	50
Two-component regulatory systems	AgNO3	25
KEGG		
beta-Lactam resistance	AMP	100
Two-component system	AgNO3	50

FIG 3 Genome-wide *P. aeruginosa* gene expression and knockout fitness in the presence of antimicrobials are not correlated. (a) Log_2-transformed fold change in gene expression (*y* axis) and abundance (*x* axis) of *P. aeruginosa* strains with gene knockouts in $AgNO_3$-treated cultures compared to the results for untreated cultures. Significant changes in gene expression (fold change ≥2, FDR < 0.05) and mutant abundance (fold change ≥2, FDR of <0.05) are colored as shown in the key (N.C., no change). (b) Spearman rank correlation coefficients between gene expression and mutant abundance for all antimicrobial treatments. (c) PseudoCAP categories and KEGG pathways in which expression data were enriched for fitness determinants. Enrichment

(Continued)

whether the mechanism for this increased resistance was mediated through *mexXY*, these genes were deleted and aminoglycoside/ciprofloxacin resistance assessed following pretreatment with polymyxin B. The results reveal that deletion of *mexXY* eliminates the enhanced resistance to aminoglycosides and ciprofloxacin observed upon exposure to sub-MIC polymyxin B (Fig. 4). To ensure that pretreatment was required for enhanced resistance, wild-type *P. aeruginosa* and the *mexXY* deletion mutant were treated simultaneously with polymyxin B and gentamicin (see Fig. S2). As expected, simultaneous treatment with polymyxin B did not lead to increased resistance to aminoglycosides and ciprofloxacin. These results demonstrate that whole-genome expression and fitness data can be used to identify genes important for antimicrobial cross-resistance.

DISCUSSION

The goal of this study was to identify determinants of bacterial fitness in response to antimicrobials, using the model superbug *P. aeruginosa*. Our highly controlled experimental parameters allowed us to define a detailed transcriptome and fitness landscape upon exposure to sub-MIC levels of antimicrobials. This is the first paired gene expression and fitness analysis in any bacterium in response to a large number of antimicrobials and provides a valuable resource for the antimicrobial research community. The clustering of the data for antimicrobials revealed that fitness profiles are a more accurate proxy for antimicrobial class than gene expression profiles (Fig. 1 and 2), indicating that the use of bacterial fitness data will likely better inform the classification of novel antimicrobials. Our analysis demonstrated that, on the whole-genome level, expression and fitness are not well correlated, indicating that the use of expression data to identify antimicrobial fitness determinants may not be the best strategy. However, we discovered that for four antimicrobials, RNA-seq can be highly predictive of fitness determinants (Fig. 3C), and thus, focusing on these categories may be a viable alternative when Tn-seq experiments are not possible. The lack of correlation between gene expression and fitness data may be due to a number of factors, one of which is the potential that antimicrobials also serve as cues, eliciting responses not required to protect against them (29). Another possible explanation is "adaptive prediction," a process wherein a bacterium is able to anticipate a future environment by expressing genes not required for fitness in the first environment but required in a second (30). In addition, because of the pooled nature of the experiments, Tn-seq may also be affected by interactions between individual mutants that result in community-based antimicrobial resistance (31). Finally, one could argue that the differences in length of antimicrobial exposure for RNA-seq (30 min) and Tn-seq (7 to 12 h) may account for the lack of correlation. We do not favor this hypothesis, since our RNA-seq data show significant overlap with data from previous transcriptomic studies that used different antimicrobial exposure times (albeit less than 12 h), indicating that the duration of exposure does not alter the funda-

Figure Legend Continued

analysis was performed with the one-tailed Fisher exact test (FDR was ≤0.05 except for ciprofloxacin, where FDR was 0.16). The percentages of differentially regulated genes that are fitness determinants (far right column) were determined by dividing the total number of fitness determinants that were differentially expressed within each category by the total number of differentially expressed genes within the category.

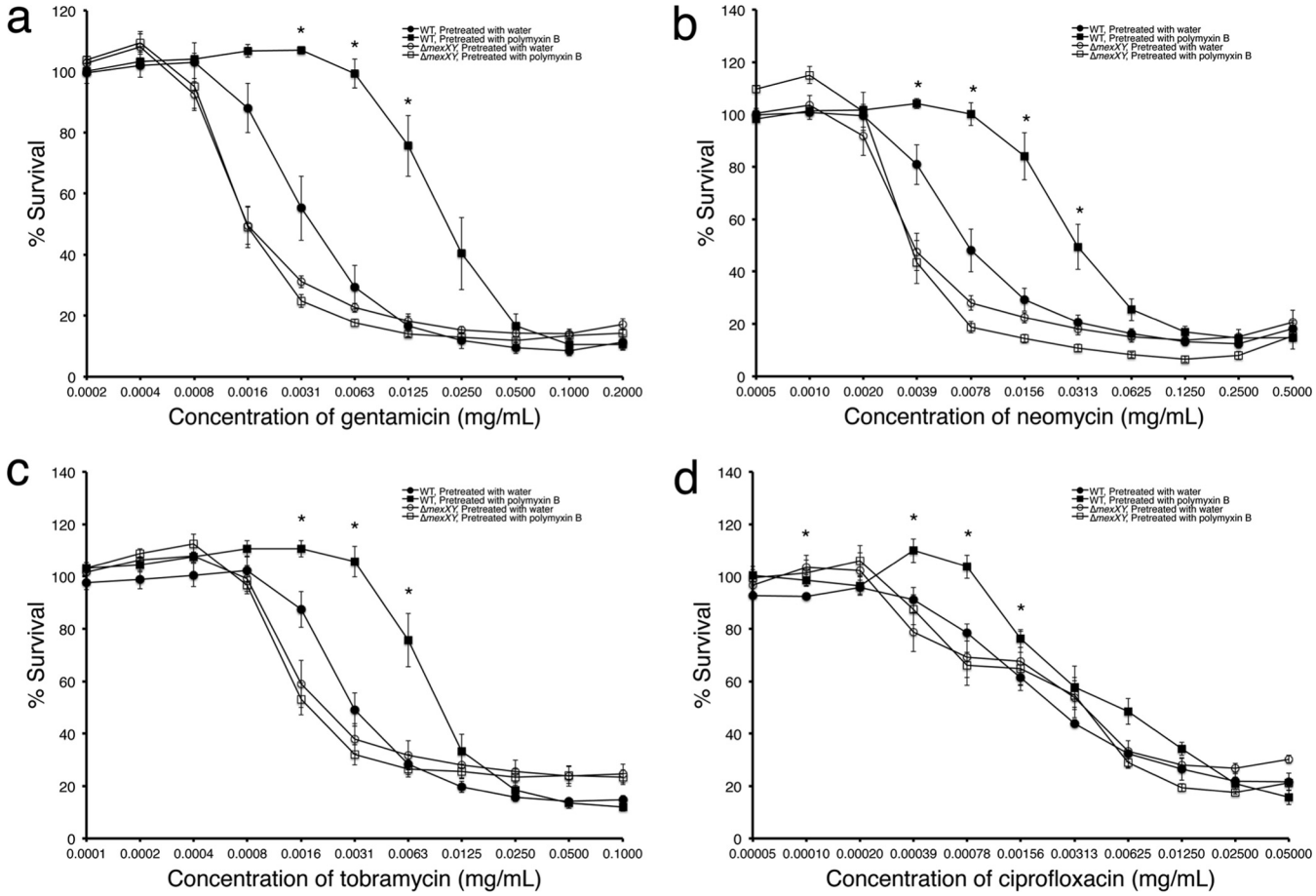


FIG 4 Pretreatment with sub-MIC polymyxin B induces resistance to aminoglycosides and ciprofloxacin. Antimicrobials were applied to luminescent *P. aeruginosa*, and luminescence was followed over time using a luminometer. Results at 60 min (a to c) and 180 min (d) post-antimicrobial treatment are shown. Decreases in luminescence correlate with antimicrobial activity. Data are shown relative to the luminescence of a control in which fresh medium or sub-MIC polymyxin (in the case of pretreatment assays) was added. The antimicrobials were tested with and without pretreatment with sub-MIC polymyxin B. *, $P < 0.05$ ($n = 3$ for gentamicin, neomycin, and tobramycin and $n = 4$ for ciprofloxacin) by the two-tailed Student *t* test. Error bars represent standard errors of the means.

mental transcriptional response. Regardless, our results indicate that the typical experimental designs used to study the transcriptional responses to sub-MIC antimicrobials do not generally capture fitness determinants.

Our data also provide new insight into how bacteria respond to and resist exposure to antibiotics and disinfectants/antiseptics. While the data for disinfectants/antiseptics clustered by fitness determinants (Fig. 2), there were remarkably few fitness determinants for antiseptics/disinfectants compared to the number for antibiotics, suggesting that *P. aeruginosa* possesses either multiple or redundant mechanisms for antiseptic/disinfectant resistance or lacks intrinsic resistance to these antimicrobials altogether. The fact that fitness profiling identified mutations that have also been observed in antimicrobial-resistant clinical isolates suggests that these datasets may be used to predict *P. aeruginosa* mutations that lead to increased antimicrobial resistance. Included among these are transcriptional regulators controlling the beta-lactamase-encoding gene *ampC*, multidrug efflux pumps, and porin proteins that result in decreased cell permeability upon inactivation (2, 32).

Finally, these studies have the potential to provide insights into resistance mechanisms that occur in individuals receiving multiple antimicrobials. For example, our results (Fig. 4) expand on previous studies showing that clinical strains of *P. aeruginosa* overexpressing the efflux pump components MexXY in *P. aeruginosa* PAO1 are more resistant to aminoglycosides/quinolones and that transcription of *mexXY* is increased in the presence of antimicrobial peptides (33). The ability to correlate transcriptional regulation and fitness profiles for multiple antibiotics offers the opportunity to develop specific hypotheses regarding the mechanisms controlling drug-induced antibiotic resistance. Indeed, while we formally tested the antagonistic interactions between polymyxin B and aminoglycosides/ciprofloxacin (Fig. 4), several additional interactions are predicted by the data (see Dataset S3 in the supplemental material). Thus, these data provide a framework to investigate the intrinsic resistance mechanisms of *P. aeruginosa* and will likely inform similar studies of other bacteria that possess orthologous genes.

MATERIALS AND METHODS

Bacterial strains and growth media. *P. aeruginosa* strain UCBPP-PA14 (34) was obtained from the MGH-Para-biosys:NHLBI Program for Genomic Applications (http://pga.mgh.harvard.edu/cgi-bin/pa14/mutants/retrieve.cgi). The luminescent *P. aeruginosa* UCBPP-PA14 strain carrying pQF50-lux was used for pretreatment assays. pQF50-lux con-

tains a 1,489-bp fragment (genomic location, positions 6323103 to 6324591 of the UCBPP-PA14 genome) from the *P. aeruginosa* genome that provides a high level of expression of *luxCDABE*. Liquid cultures were grown in chemically defined medium (CDM) supplemented with 20 mM succinate (35) at 37°C with shaking at 250 rpm.

Construction and confirmation of deletion mutants. The PA14 Δ*mexXY* (PA14_38395-38410) deletion mutant was constructed by amplifying ~700-bp fragments flanking these two genes by PCR with Phusion hot start II DNA polymerase (Thermo Scientific, Waltham, MA) to replace the coding sequence of the genes with the sequence 5′-GCGGCCGCC-3′ flanked by the native start codon of PA14_38395 and stop codon of PA14_38410. The primers used for PCR were 5′-TTC TGCAGGTCGACTCTAGACCAGGGTGCCGCAGATGC-3′ and 5′-GC ATCAGGCGGCCGCCATGGGTGTCCCTCGATTCGTG-3′ for the upstream region and 5′-CCATGGCGGCCGCCTGATGCCCCTAGCGAAA CTCTCGC-3′ and 5′-GAATTCGAGCTCGAGCCCGGGCCCGGAAGT TCTCCCTGGGC-3′ for the downstream region. These two amplicons and the suicide vector pEXG2 (36) were assembled using Gibson assembly as described previously (37), transformed initially into *Escherichia coli* DH5α λpir, and then transformed into *E. coli* SM10 λpir for conjugation into strain PA14. This construct was introduced into PA14 by conjugation with selection for gentamicin-resistant transconjugates, followed by selection on sucrose to obtain the chromosomal deletion. This mutation was then verified by PCR.

MIC determination. For MIC determination, logarithmic *P. aeruginosa* cells (optical density at 600 nm [OD_{600}] of 0.5) were diluted to an OD of 0.001 and added to a 96-well microtiter plate. Antimicrobials were added, using serial 2-fold dilutions across the plate. The MIC was determined as the lowest concentration with no visible growth.

RNA-seq growth conditions. Cultures for RNA-seq analysis were grown overnight in CDM, diluted to an OD_{600} of 0.03 in 10 ml CDM, grown to an OD_{600} of 0.5, and then treated with 100-μl amounts of sub-MIC concentrations of antimicrobials or with 100 μl water as a control. Sub-MIC antibiotic levels were determined as the highest concentration at which *P. aeruginosa* growth was not inhibited (either 1/2 or 1/4 MIC) (Table 1). Cultures were grown for 30 min at 37°C with continuous shaking at 250 rpm. After 30 min, the OD_{600} was between 0.7 and 1.0, and the culture was immediately diluted with an equal volume of RNAlater.

Tn-seq growth conditions. Cultures for Tn-seq analysis were grown as follows: frozen aliquots of the *P. aeruginosa* PA14 transposon insertion library (38) were washed three times with 1 ml 20 mM MOPS (morpholinepropanesulfonic acid) buffered to pH 7.2, inoculated into 25 ml CDM at 2.5×10^5 CFU/ml, and grown for 30 min at 37°C with shaking at 250 rpm. Then, 250-μl amounts of sub-MIC antimicrobials or water were added and the culture was grown for approximately 12 generations (to 10^9 CFU/ml) and immediately placed on ice. Cells were pelleted, the supernatant was discarded, and the pellet was frozen.

RNA-seq Illumina library preparation. Cultures stored in RNAlater were pelleted, resuspended in RNA Bee, and transferred to 2-ml bead-beating tubes containing 0.1-mm beads (MP Biomedical). Cells were lysed by bead beating 3 times for 60 s, and the tubes placed on ice for 1 min between each homogenization. Amounts of 200 μl of chloroform were added, and the tubes were shaken vigorously for 30 s and incubated on ice for 5 min. Samples were centrifuged for 15 min at 4°C to separate the aqueous and organic phases. The top aqueous phase from each tube was transferred to a new microcentrifuge tube to which 0.5 ml isopropanol was added, and the tubes were incubated at room temperature for 10 min. Amounts of 20 μg of linear acrylamide were added to the tubes, and the samples were centrifuged at 12,000 × *g* for 5 min at 4°C. The pellets were washed with 1 ml 75% ethanol, air dried for 10 min, and resuspended in 50 μl of RNase-free water. The RNA concentration for each sample was determined with a NanoDrop spectrophotometer (Thermo Scientific). DNA contamination was assessed with PCR amplification of the *clpX* gene, and RNA integrity was verified with agarose gel electrophoresis of glyoxylated samples (Ambion). Ribosomal RNA was depleted using the RiboZero bacteria kit (Epicentre) and purified by ethanol precipitation using 12.5 μg linear acrylamide to precipitate the RNA. The depleted RNA was fragmented, and cDNA libraries were prepared as described previously (39). Libraries were sequenced at the Genome Sequencing and Analysis Facility at the University of Texas at Austin on an Illumina HiSeq 2000 using a 1 × 100-bp single-end run.

Tn-seq Illumina library preparation. The frozen pellets were resuspended in 1 ml 1× buffer A (40) with 0.1% SDS, homogenized in a bead-beating tube for 1 min, and then placed on ice. Proteinase K (1 mg/ml) was added, and the samples were incubated for 1 h. Samples were extracted with equal volumes of 25:24:1 phenol-chloroform-isoamyl alcohol, pH 8.0. DNA was ethanol precipitated (0.1 volume 3 M sodium acetate and 3 volumes of 100% ethanol) from the aqueous phase, and the pellet was washed with 75% ethanol 2 times, air dried for 10 min, and resuspended in 100 μl water. DNA concentrations for each sample were determined with a NanoDrop spectrophotometer (Thermo Scientific). DNA was sheared to ~300 bp in a Q880R sonicator (Qsonica), and the size was confirmed on an agarose gel. The sheared DNA was treated with terminal deoxynucleotidyltransferase (TdT), followed by two PCRs as described previously (38, 41). The libraries were sequenced at the Genome Sequencing and Analysis Facility at the University of Texas at Austin on an Illumina HiSeq 2500 using a 1 × 100-bp single-end run.

RNA-seq bioinformatic analyses. After discarding reads with no call ("N") or low complexity (not containing all four nucleotides), we mapped reads to the *P. aeruginosa* UCBPP-PA14 genome (GenBank accession number NC_008463.1; downloaded from http://www.pseudomonas.com on 31 July 2013) using the Burrows-Wheeler Aligner, Smith-Waterman algorithm (BWA-SW) implemented on version 0.7.10 (42), and the best nucleotide match for each read according to its mapping quality score was selected. To remove ambiguous reads, we discarded the reads having multiple best hits with the same mapping quality score. Because our library preparation method is designed to capture essentially all transcripts, including small RNAs (sRNAs), the aligned length of the reads was highly variable. Therefore, instead of read count, we counted the number of nucleotides covering each gene (using the GFF3 annotation file obtained from PseudoCAP [43]) and used the length of the gene and total number of nucleotides mapped on genic regions of the genome as normalization factors, analogous to transcripts per millions of reads (TPM) (44). We identified genes showing significantly different RNA levels under each condition using edgeR (45), with false discovery rates (FDRs) of less than 0.05 and differences of at least 2-fold. Detailed procedures and related scripts are available at https://github.com/marcottelab/HTseq-toolbox/wiki/ProkRNAseq.

Tn-seq bioinformatic analyses. As with the RNA-seq data, after discarding reads with no call or low complexity, we mapped the Tn-seq reads against the *P. aeruginosa* UCBPP-PA14 genome using BWA-SW and selected the best nucleotide match for each read according to its mapping quality score. We further filtered out mapped reads if their aligned length was shorter than 10 bp (too short to infer the genomic DNA sequence flanking the transposon). We then identified transposon insertion sites as a junction of the transposon flanking sequence (TAAGAGTCA) and the mapped genomic DNA sequence. For fitness analysis, the occurrence of all transposon insertion sites within a gene was summed based on PseudoCAP GFF3 annotation (43) and normalized by the total number of reads, and genes showing significant fitness changes under each condition, with FDRs of less than 0.05 and differences of at least 2-fold, were identified by using edgeR. Detailed procedures and related scripts are available at https://github.com/marcottelab/HTseq-toolbox/wiki/ProkTNseq.

Overlap between RNA-seq and existing gene expression data. Brazas and Hancock reported microarray data from sub-MIC ciprofloxacin-treated *P. aeruginosa*, and genes showing at least 2-fold differences in expression and *P* values of less than 0.05 were compared to genes showing at least 2-fold differences in expression and FDRs of less than 0.05 in the RNA-seq data in this study (5). Chang et al. reported the 30 *P. aeruginosa* genes that were most highly upregulated in response to hydrogen perox-

ide, which were compared to genes showing at least 2-fold differences in expression and FDRs of less than 0.05 in the RNA-seq data in this study (7). The significance of overlaps in these data was assessed using the Fisher exact test.

Heat map and cluster analyses. Heat maps were generated in R with the function heatmap.2 of the gplots package. Clustering was performed using genes exhibiting at least 2-fold differences in expression or fitness and FDRs of less than 0.05 across at least 2 conditions using Spearman correlation coefficients.

Enrichment analyses. Enrichment of differentially regulated genes and differentially fit genes in a given PseudoCAP category or Kyoto Encyclopedia of Genes and Genomes (KEGG) (46) pathway was determined by comparing the prevalence of genes with differences of at least 2-fold and FDRs of less than 0.05 assigned to a specific PseudoCAP category or KEGG pathway to the prevalence of genes in the genome assigned to that PseudoCAP category or KEGG pathway using the one-tailed Fisher exact test. Only annotated genes were used in the analysis. *P* values for enriched categories were adjusted for multiple testing using a Benjamini-Hochberg correction, giving the resulting false discovery rate/*q* values (47).

Pretreatment assay. PA14 and PA14 Δ*mexXY*, constitutively expressing luminescence from the plasmid pQF50-lux, were grown overnight in CDM or Lysogeny Broth with carbenicillin (300 μg/ml), washed 3× with fresh CDM containing no carbenicillin, diluted to an OD_{600} of 0.05 in 10 ml CDM, and grown to mid-logarithmic phase (OD_{600} of 0.5). The cultures were then treated with 100 μl of subinhibitory (1/2 MIC) polymyxin B or with 100 μl water as a nontreatment control for 30 min. Treated cells were added to 96-well microtiter plates, and antibiotics (gentamicin, neomycin, tobramycin, or ciprofloxacin) were added, using serial dilutions. Luminescence was monitored at 0, 5, 15, 30, 60, and 180 min using a Luminoskan Ascent microplate luminometer, and luminescence at 60 min (gentamicin, neomycin, and tobramycin) and 180 min (ciprofloxacin) was reported. The internal temperature within the luminometer was maintained at 37°C, and the plates were shaken at 240 rpm; prior to luminescence measurement, the plates were shaken at 1,200 rpm for 10 s.

Microarray data accession numbers. RNA-seq and Tn-seq sequencing data are available at the National Center for Biotechnology Information Sequence Read Archive (NCBI SRA) under accession number SRP062243.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.01603-15/-/DCSupplemental.

Figure S1, JPG file, 0.6 MB.
Figure S2, JPG file, 0.6 MB.
Dataset S1, XLSX file, 2.8 MB.
Dataset S2, XLSX file, 2.9 MB.
Dataset S3, XLSX file, 0.02 MB.

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