RESEARCH ARTICLE

Linking System-Wide Impacts of RNA Polymerase Mutations to the Fitness Cost of Rifampin Resistance in *Pseudomonas aeruginosa*

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ABSTRACT **Fitness costs play a key role in the evolutionary dynamics of antibiotic resistance in bacteria by generating selection against resistance in the absence of antibiotics. Although the genetic basis of antibiotic resistance is well understood, the precise molecular mechanisms linking the genetic basis of resistance to its fitness cost remain poorly characterized. Here, we examine how the system-wide impacts of mutations in the RNA polymerase (RNAP) gene *rpoB* shape the fitness cost of rifampin resistance in *Pseudomonas aeruginosa*. Rifampin resistance mutations reduce transcriptional efficiency, and this explains 76% of the variation in fitness among *rpoB* mutants. The pleiotropic consequence of *rpoB* mutations is that mutants show altered relative transcript levels of essential genes. We find no evidence that global transcriptional responses have an impact on the fitness cost of rifampin resistance as revealed by transcriptome sequencing (RNA-Seq). Global changes in the transcriptional profiles of *rpoB* mutants compared to the transcriptional profile of the rifampin-sensitive ancestral strain are subtle, demonstrating that the transcriptional regulatory network of *P. aeruginosa* is robust to the decreased transcriptional efficiency associated with *rpoB* mutations. On a smaller scale, we find that rifampin resistance mutations increase the expression of RNAP due to decreased termination at an attenuator upstream from *rpoB*, and we argue that this helps to minimize the cost of rifampin resistance by buffering against reduced RNAP activity. In summary, our study shows that it is possible to dissect the molecular mechanisms underpinning variation in the cost of rifampin resistance and highlights the importance of genome-wide buffering of relative transcript levels in providing robustness against resistance mutations.**

IMPORTANCE **Antibiotic resistance mutations carry fitness costs. Relative to the characteristics of their antibiotic-sensitive ancestors, resistant mutants show reduced growth rates and competitive abilities. Fitness cost plays an important role in the evolution of antibiotic resistance in the absence of antibiotics; however, the molecular mechanisms underlying these fitness costs is not well understood. We applied a systems-level approach to dissect the molecular underpinnings of the fitness costs associated with rifampin resistance in *P. aeruginosa* and showed that most of the variation in fitness cost can be explained by the direct effect of resistance mutations on the enzymatic activity of the mutated gene. Pleiotropic changes in transcriptional profiles are subtle at a genome-wide scale, suggesting that the gene regulatory network of *P. aeruginosa* is robust in the face of the direct effects of resistance mutations.**

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Antibiotic resistance conferred by chromosomal mutations is typically accompanied by fitness costs that are expressed in terms of reduced growth rates, competitive abilities, and virulence of resistant mutants compared to the phenotypes of their antibiotic-sensitive ancestors in the absence of antibiotics (1–3). Fitness costs play a key role in the evolutionary dynamics of antibiotic resistance by reducing the rate at which resistance mutations spread in bacterial populations that are exposed to antibiotics and accelerating the rate at which resistance mutations are lost once antibiotic use is stopped (3). At least 80 studies have quantified the fitness costs associated with chromosomal antibiotic resistance mutations (4). Intriguingly, some of these studies show that different mutations that confer resistance to the same antibiotic can be associated with very different fitness costs (5–9). How can we explain this variation in the fitness effects of resistance mutations? Elegant experiments have shown that the variations in the fitness effects of mutations that confer resistance to fusidic acid and peptide deformylase inhibitors can be attributed to variations in the rates of protein synthesis (10) and translation initiation (11), respectively. Beyond these examples, the molecular basis of fitness in antibiotic-resistant mutants remains unresolved. In this paper, we use a systems-level approach to dissect the mechanistic basis of fitness in rifampin-resistant mutants of the pathogenic bacterium *Pseudomonas aeruginosa*.

Rifampin is a broad-spectrum antibiotic (12) that inhibits bacterial RNA synthesis by specifically targeting a small but highly conserved pocket in the β-subunit of RNA polymerase (RNAP), which is encoded by *rpoB* (13). When rifampin binds to the rifampin-binding pocket within the DNA/RNA channel of wild-type RNAP, the path of nascent RNA transcripts is directly

blocked and elongation cannot proceed beyond the first three nucleotides (14, 15). Mutations in *rpoB* can result in alterations to the structure of the rifampin-binding pocket and confer rifampin resistance by decreasing the binding affinity between rifampin and RNAP (16). Previous studies have demonstrated that rifampin resistance mutations carry various fitness costs in *Pseudomonas aeruginosa* (9), *Escherichia coli* (17), *Mycobacterium tuberculosis* (18), and *Staphylococcus aureus* (19). First, by altering the structure of a highly conserved domain of RNAP, *rpoB* mutations are thought to generate a direct fitness cost by compromising transcriptional efficiency. In support of this argument, Brandis et al. established that a rifampin-resistant mutant of *Salmonella enterica* with a lower growth rate in a rich medium showed a reduction in transcriptional efficiency relative to that of the wild type (20). Similarly, Reynolds demonstrated by using a semiquantitative β-galactosidase reporter gene assay that three *E. coli* mutants with costly rifampin resistance mutations showed reduced levels of transcriptional efficiency (21).

In addition to the direct effect of rifampin resistance mutations on RNAP activity, it is likely that *rpoB* mutations generate an indirect, pleiotropic cost by altering the transcriptional profiles of mutants at a genome-wide scale. Since RNAP is required for the transcription of every gene, *rpoB* mutations are thought to be global regulatory mutations that alter the transcriptional profiles of mutants (22). Previous work has shown that *rpoB* mutations have pleiotropic effects on carbon catabolism in *E. coli* (23), *Bacillus subtilis* (24), and *P. aeruginosa* (9), as well as lipid metabolism in *Mycobacterium tuberculosis* (25). Pleiotropy has been demonstrated to generate a fitness cost in both *Saccharomyces cerevisiae* (26) and *P. fluorescens* (27), suggesting that pleiotropic effects of *rpoB* mutations on gene expression may also contribute to the cost of rifampin resistance. However, this hypothesis has not been tested in previous work.

Although the genetic and biochemical bases of rifampin resistance have been well characterized, an important question concerning the fitness costs still remains—why do mutations in the same gene that confer resistance to the same antibiotic carry different fitness costs? If a reduction in RNAP activity is the principal cause of decreased fitness, what are the contributions of the direct effect (i.e., compromised transcriptional efficiency) and pleiotropic effects of resistance to the fitness cost of resistance? In this study, we address this problem by attempting to link the direct and pleiotropic effects of rifampin resistance mutations to the fitness cost of resistance. First, we used a luciferase reporter gene to quantify the direct effects of *rpoB* mutations on transcriptional efficiency, using luciferase reporter activity as a proxy measurement. Second, to determine the pleiotropic effects of *rpoB* mutations on gene expression, we quantified the changes in the transcript levels of a random sample of essential genes in resistant mutants relative to their levels in the rifampin-sensitive ancestral strain. By definition, essential genes are critical for the survival of an organism, and the deletion of an essential gene results in a lethal phenotype in a given environment (28). Given the functional significance and the potential fitness effects of altered expression levels of essential genes, an overall reduction in the transcript levels of essential genes relative to their levels in the ancestral strain is expected to generate a fitness cost in resistant mutants. In addition, the whole-genome transcriptional profiles of two common *rpoB* mutants and the ancestral strain were acquired through transcriptome sequencing (RNA-Seq) to uncover the genome-wide effects of rifampin resistance mutations. Using this approach, we showed that the direct effects of *rpoB* mutations on transcriptional efficiency are responsible for most of the variations in the fitness cost of *rpoB* mutations. The pleiotropic effects of *rpoB* mutations on the relative transcript levels of essential genes do not generate a fitness cost in their own right. We also demonstrated that the upregulation of the *rpoBC* operon is one of the most profound effects of *rpoB* mutations and elucidated a key molecular mechanism which is responsible for the increased expression of this operon despite the reductions in RNAP activity and transcriptional efficiency.

RESULTS AND DISCUSSION

Determining the fitness costs carried by rifampin resistance mutations. The *rpoB* mutants were evolved from a rifampin-sensitive ancestral strain, PAO1::mini-Tn*7*-p*LAC-lux*, carrying a chromosomally integrated reporter construct designed to measure the luciferase reporter activity as a proxy for transcriptional efficiency measurement. Given the high concentration of rifampin that was used for the selection of resistant mutants, it is conceivable that some *rpoB* mutations, which could have been isolated with lower rifampin concentrations, were not isolated in our study.

To determine the fitness cost associated with rifampin resistance mutations, we subjected each resistant mutant (see Table 2) and the ancestral strain to direct competition against a green fluorescent protein (GFP)-tagged derivative of the rifampin-sensitive PAO1 control strain in rifampin-free culture medium. The average competitive fitness of the resistant mutants was lower than that of the ancestor, as shown by the results in Fig. 1 (mean fitness = 0.842, standard error [SE] = 0.059), and the competitive fitness varied significantly between mutants with different *rpoB* mutations (one-way analysis of variance [ANOVA]: $P < 0.0005$, $F_{9,\ 20} = 5.82$). With the exceptions of the mutation causing a change of serine to phenylalanine at position 536 (S536F) and the S579F mutation, all *rpoB* mutations carried significant fitness costs compared to the fitness of the ancestral strain (Dunnett's test: $P < 0.05$).

Rifampin resistance generates fitness costs by reducing transcriptional efficiency. Rifampin resistance mutations alter a highly conserved domain on RNAP that is involved in transcription, suggesting that resistance mutations are likely to generate a fitness cost by compromising the enzymatic activity of RNAP. To test this idea, we estimated the luciferase reporter activity in *rpoB* mutants relative to its activity in their rifampin-sensitive ancestor by using a chromosomally integrated luciferase reporter gene, which offers high-sensitivity measurements (29) over a wide dynamic range (30)—see Fig. 2A for an example. On average, the luciferase reporter activity levels were lower in resistant mutants than in the ancestral strain, as shown by the results in Fig. 2B (mean *lux* reporter activity = 0.672, SE = 0.040), and varied significantly between mutants with different *rpoB* mutations (one-way ANOVA: $P < 0.0001$, $F_{9,\ 20} = 80.3$). Specifically, we found that all *rpoB* mutations except S536F resulted in significantly lower levels of luciferase reporter activity in resistant mutants than in the ancestral strain (Dunnett's test: $P < 0.05$).

Previous work has shown that bacteria can compensate for the cost of rifampin resistance by fixing secondary mutations in RNAP genes *rpoA*, *rpoB*, or *rpoC* (20, 31, 32). If the decreased transcriptional efficiency observed in rifampin-resistant mutants is a cause of decreased fitness, compensatory mutations

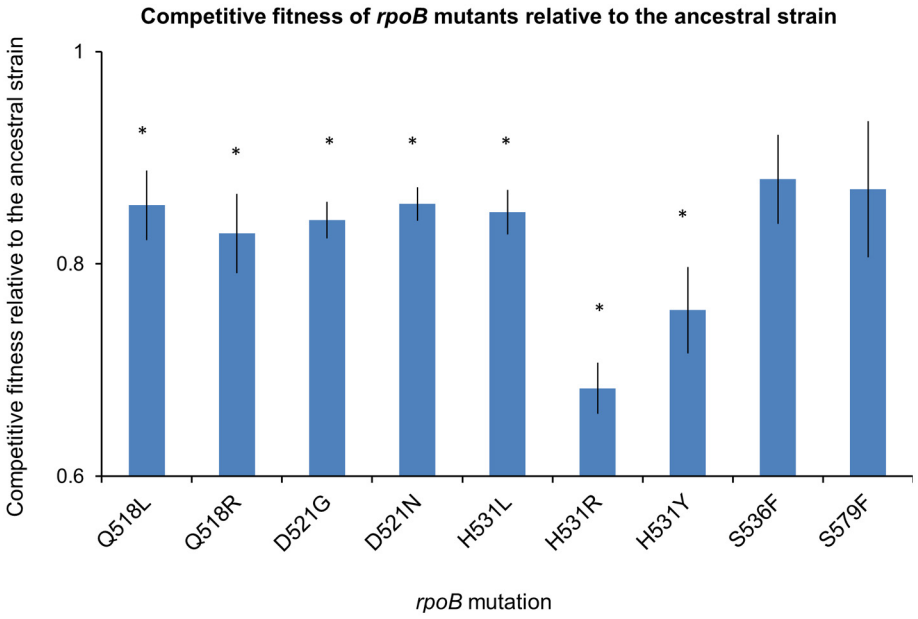

FIG 1 Rifampin resistance carries a fitness cost. Bars show the competitive fitness of rifampin-resistant mutants (mean ± SE; $n = 3$) relative to that of the rifampin-sensitive ancestral strain, as determined by competition assays in rifampin-free M9KB culture medium. On average, rifampin resistance mutations reduce fitness by 15.8%. Individual mutations that are associated with significant fitness costs, as determined by Dunnett's test, are shown with an asterisk (*, $P < 0.05$).

should recover transcriptional efficiency. To test this idea, we adopted an experimental evolution approach and allowed *rpoB* mutants to evolve in rifampin-free culture medium for a period of 30 days, or approximately 300 generations. Through whole-genome sequencing of evolved isolates, we identified strains that carried only a rifampin resistance mutation and a second-site RNAP mutation (Table 1). To establish the causal link between compromised RNAP function and fitness cost, we measured both the luciferase reporter activity and the competitive fitness in these compensated mutant isolates (Table 2). The results in Fig. 3A show that all compensated mutants showed statistically significant increases in average relative *lux* reporter activity compared to the levels of reporter activity in the original *rpoB* mutants they were evolved from (two-tailed, two-sample *t* test: $P < 0.05$). Likewise, all compensated mutants also showed increases in average relative competitive fitness (Fig. 3B). With the exception of mutants with the original resistance mutation Q518R and the compensatory mutation E528D (Q518R + E528D), the difference between the mean relative competitive fitness of each pair of a compensated mu-

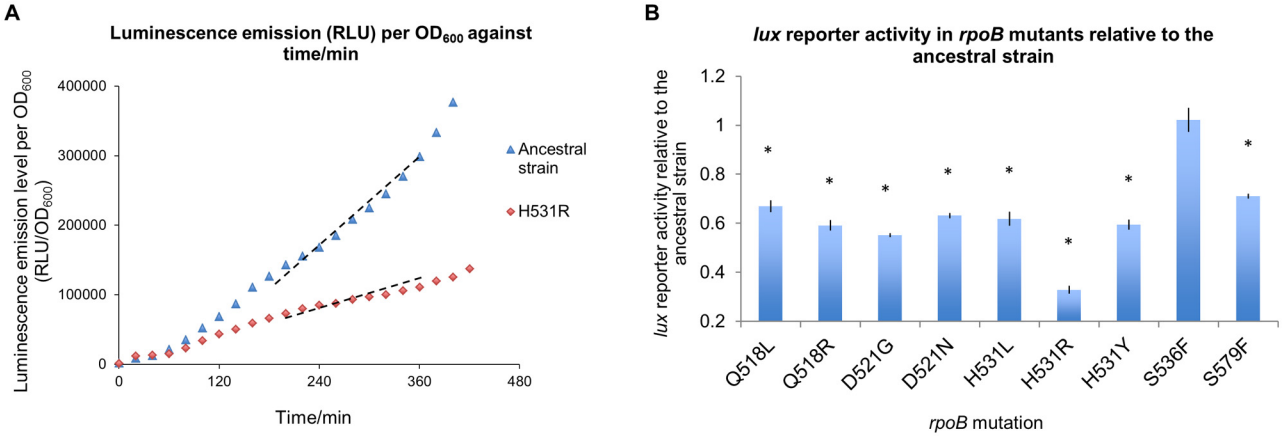

FIG 2 Rifampin resistance mutations in *rpoB* reduce transcriptional efficiency. (A) The graph shows an example of how luciferase (lux) reporter activity was determined in an *rpoB* mutant strain (H531R) relative to the reporter activity in the ancestral strain. The IPTG-induced luminescence emissions (RLU) per optical density (OD_{600}) were monitored continuously, and the maximal gradients on the RLU/OD_{600} curves during early exponential phase were calculated (dotted lines). The absolute *lux* reporter activity in each of the *rpoB* mutants was normalized to that of the ancestral strain control within each assay to obtain relative *lux* reporter activity, which is used as a proxy for relative transcriptional efficiency measurements. (B) *lux* reporter activities in *rpoB* mutants relative to that in the rifampin-sensitive ancestral strain (mean ± SE; $n = 3$), which are used as estimates for relative transcriptional efficiency. All mutations except S536F resulted in significant reductions in the relative transcriptional efficiencies of *rpoB* mutants, as determined by Dunnett's test (*, $P < 0.05$).

TABLE 1 *rpoB* mutations in rifampin-resistant mutants obtained through a fluctuation test and intragenic compensatory mutations in compensated mutants evolved through a selection experiment

Point mutation(s)	Amino acid substitution(s)
Original resistance mutation	
A1553T	Q518L
A1553G	Q518R
A1562G	D521G
G1561A	D521N
A1592T	H531L
A1592G	H531R
C1591T	H531Y
C1607T	S536F
C1736T	S579F
Original + compensatory mutation	
C1591T + A1592G	H531C
A1553G + G1584T	Q518R + E528D
C1591T + A1718G	H531Y + N573S

tant and the original *rpoB* mutant is statistically significant (two-tailed, two-sample *t* test: $P < 0.05$).

The key assumption of our reporter-based assay is that the luminescence emission levels reflect variations in the relative transcript levels of the *lux* operon, so that measuring the relative rate of luminescence emission through time is equivalent to measuring the rate of *lux* mRNA transcript synthesis. To test this assumption, we quantified the mRNA transcript levels of the *luxC* gene in mutants relative to the levels in the ancestral strain using quantitative real-time PCR (qPCR). The relative *luxC* transcript levels were significantly correlated with the luminescence emission levels (relative light units [RLU]) normalized to the optical density at 600 nm (OD_{600}) measured immediately prior to RNA extraction (one-way ANOVA: $P = 0.0006$, $F_{1, 8} = 30.1$, $r^2 = 0.790$) (see Fig. S1 in the supplemental material). In this analysis, we excluded a statistically significant outlier (Q518R) identified using the least trimmed squares method.

TABLE 2 Numbers of independent *rpoB* mutant isolates used in luciferase reporter gene assays, competitive fitness assays, and qPCR assays[a]

rpoB mutation(s)	No. of independent *rpoB* mutant isolates used in[b]:		
	Luciferase reporter gene assays	Competitive fitness assays	qPCR
Original resistance mutation			
Q518L	2	2	1
Q518R	1	1	1
D521G	7	2	1
D521N	2	2	1
H531L	2	2	1
H531R	2	2	2
H531Y	3	2	1
S536F	1	1	1
S579F	2	2	2
Original + compensatory mutation			
H531C[c]	4	4	1
Q518R + E528D	2	2	1
H531Y + N573S	2	2	1

[a] Isolates with the original resistance mutations in *rpoB* were obtained from the fluctuation test, and mutants with compensatory mutations were isolated from the selection experiment.

[b] The numbers of *rpoB* mutant isolates used in competitive fitness assays form subsets of those tested in luciferase reporter gene assays. Likewise, the numbers of isolates assayed using qPCR are subsets of those tested in competitive fitness assays. Small variations were observed among individual *rpoB* mutant isolates with the same *rpoB* mutations in all experiments. Three biological replicates of each isolate were assayed ($n = 3$).

[c] Two H531C isolates were evolved from the H531R *rpoB* mutant. The other two H531C isolates were evolved from the H531Y *rpoB* mutant.

To measure the contribution of reduced transcriptional efficiency to the cost of resistance, we tested for a correlation between luciferase reporter activity and competitive fitness (Fig. 4). To ensure that each *rpoB* mutant strain represents an independent data point, compensated mutants with the Q518R + E528D and H531Y + N573S mutations were excluded from all correlation analyses in this study. Consistent with our hypothesis, we found that decreased transcriptional efficiency increased the fitness cost of resistance (one-way ANOVA: $P = 0.0005$, $F_{1, 9} = 28.8$, $r^2 = 0.762$). This direct effect of *rpoB* mutations on the transcriptional efficiency can account for 76.2% of the variance in fitness among mutants.

Rifampin resistance mutations alter the transcriptional profiles of essential genes. Since RNAP is a master regulator of gene expression, changing the structure of RNAP has the potential to alter gene expression on a genome-wide scale. To test the hypothesis that *rpoB* mutations generate a pleiotropic fitness cost by altering the transcriptional profiles of resistant mutants, we quantified the mRNA transcript levels of a random sample of 15 essential genes in *rpoB* mutants relative to their transcript levels in the ancestral strain using qPCR. The distinction between measuring transcriptional activity and relative transcript levels is important. Measuring luciferase reporter activity (Fig. 2) provides an estimate of the enzymatic efficiency of RNAP. Relative transcript levels, on the other hand, provide a measure of how resistance mutations alter the transcriptional profiles of the *rpoB* mutants. In other words, our luciferase reporter activity measurements provide a proxy for how resistance mutations alter the rate at which RNAP can synthesize transcripts, whereas relative transcript level measurements reveal which genes are differentially transcribed by RNAP mutants, which can shed insight into the transcript-specific effects of *rpoB* mutations. Crucially, we expected the impact of RNAP mutations on relative transcript levels to vary between genes due to the diversity of transcriptional regulatory mechanisms in bacterial genomes (33).

In general, we found that rifampin resistance mutations had pervasive but relatively subtle effects on the relative transcript levels of essential genes (Fig. 5). To ensure that every *rpoB* mutant strain was an independent sample group, compensated mutants with the Q518R + E528D and H531Y + N573S mutations were excluded from our two-way ANOVA analysis. First, we found significant variation in the average changes in relative transcript levels between essential genes (two-way ANOVA: $P < 0.0001$, $F_{14, 420} = 5.29$). Second, we found significant variation in the average changes in relative transcript levels between *rpoB* mutations (two-way ANOVA: $P < 0.0001$, $F_{10, 420} = 6.73$). Finally, we found a significant interaction between transcript levels of essential genes and *rpoB* mutations (two-way ANOVA: $P < 0.0001$, $F_{140, 420} = 2.26$), implying that different *rpoB* mutations have different impacts on the relative transcript levels of different genes. This anal-

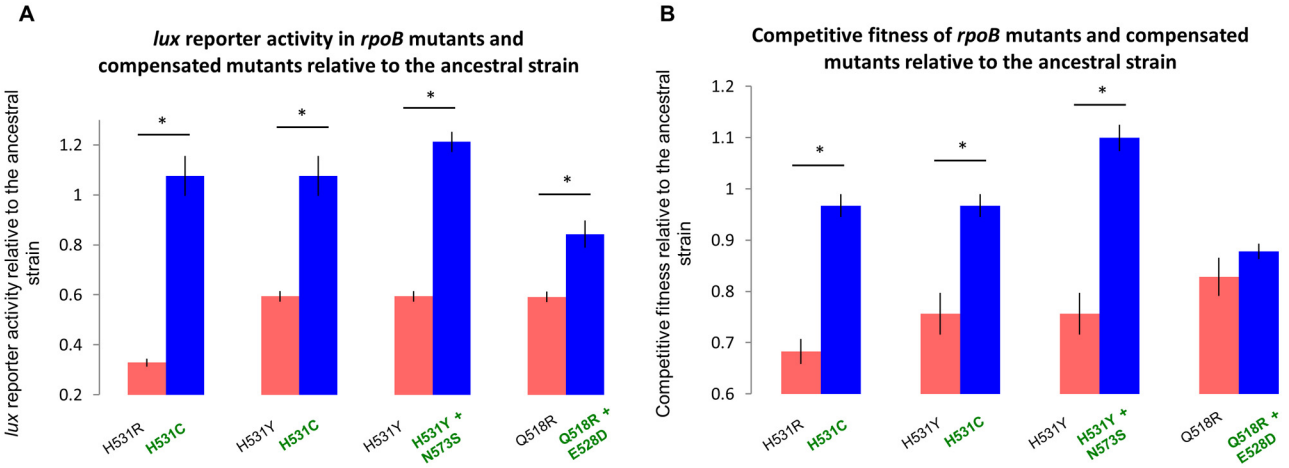

FIG 3 Compensatory mutations restore both the transcriptional efficiency and competitive fitness of resistant mutants. The graphs show the impact of compensatory mutations on transcriptional efficiency (mean ± SE; $n = 3$) (A) and competitive fitness (mean ± SE; $n = 3$) (B) relative to those of the rifampin-sensitive ancestral strain. Data are for pairs comprised of the original rifampin-resistant mutants from the fluctuation test (red bars) and their compensated strains (blue bars) carrying both an *rpoB* mutation and a second-site compensatory mutation within *rpoB*. Two-tailed, two-sample *t* test: *, $P < 0.05$.

ysis suggests that rifampin resistance mutations have various pleiotropic effects on the relative transcript levels of essential genes, which is perhaps not surprising given the central role of RNAP in gene expression and the diversity of transcriptional regulatory mechanisms for regulating gene expression.

Interestingly, the compensated mutants (with H531C, H531Y + N573S, and Q518R + E528D mutations) clustered much more closely with the ancestral strain than the *rpoB* mutants they were evolved from (with H531R, H531Y, and Q518R mutations), as indicated by our two-way clustering analysis (Fig. 5), implying that these intragenic compensatory mutations have the dual effect of recovering transcriptional efficiency and restoring the relative transcript levels of essential genes in resistance mutants to ancestral strain levels.

Previous work in *E. coli* strongly supports the idea that the expression levels of essential genes are under stabilizing selection (34). If this is the case, both increases and decreases in the expression of essential genes are expected to carry a fitness cost. Therefore, we quantified the pleiotropic effects of *rpoB* mutations by calculating the average magnitude of deviation in the transcript levels of the 15 essential genes in each *rpoB* mutant relative to that in the ancestral strain. We did not find any evidence of a correlation between pleiotropy and fitness after correcting for variations in the transcriptional efficiency of *rpoB* mutants (Pearson partial

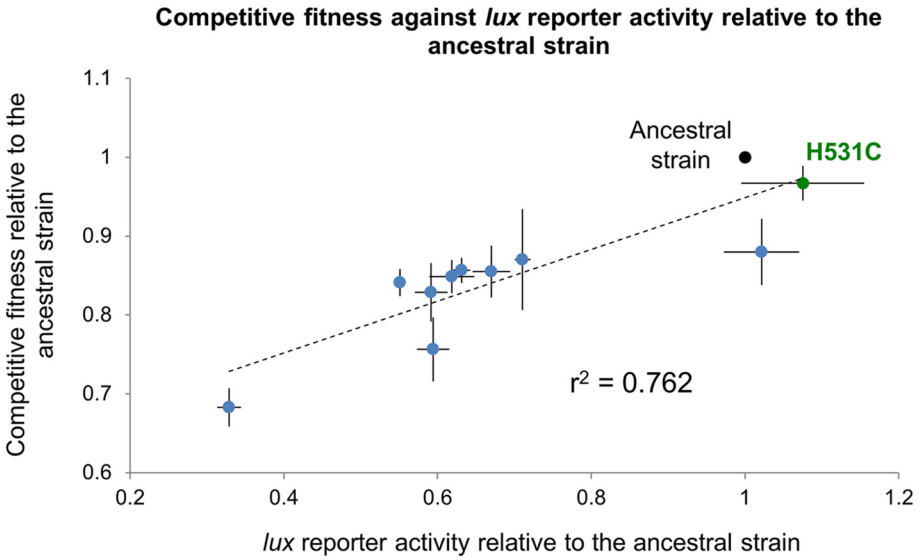

FIG 4 Variation in the fitness cost of rifampin resistance can be explained by variation in transcriptional efficiency. The correlation between levels of competitive fitness (mean ± SE; $n = 3$) and transcriptional efficiency (mean ± SE; $n = 3$) of resistant mutants relative to those of the ancestral strain is shown. Variation in relative transcriptional efficiency can explain 76.2% of the variation in relative competitive fitness, as determined by linear regression (dashed line). The ancestral strain is shown in black, and the compensated mutant H531C in green. To ensure that every *rpoB* mutant strain is an independent data point, compensated mutants Q518R + E528D and H531Y + N573S were excluded from the correlation analysis.

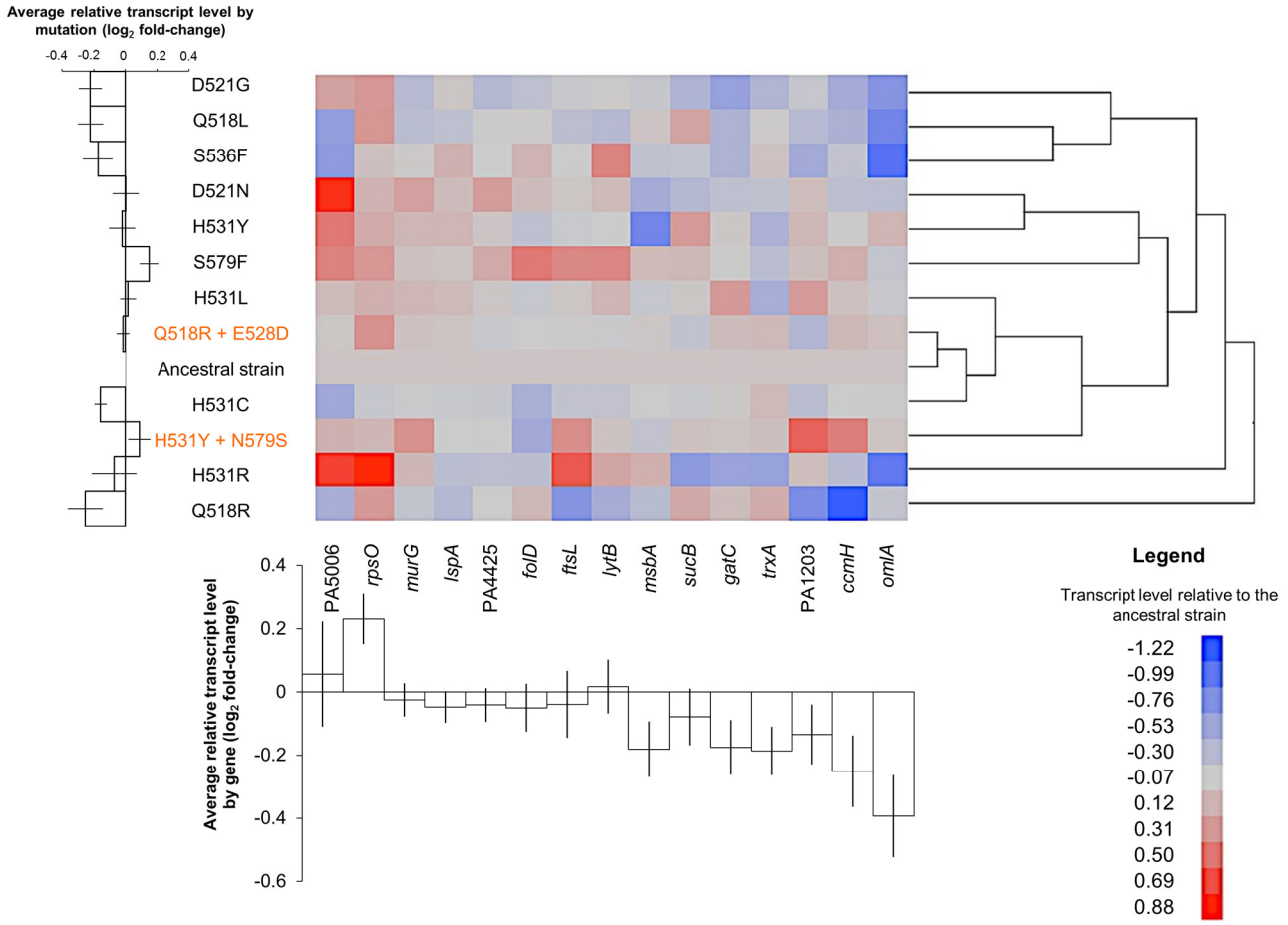

FIG 5 Impact of *rpoB* mutations on relative transcript levels of essential genes. The heat map shows the average transcript levels of 15 randomly selected essential genes in the *rpoB* mutants and three mutants with intragenic compensatory mutations relative to the transcript levels in the ancestral strain, expressed in terms of $\log_2$-fold change. Significant variation in relative transcript levels is observed between *rpoB* mutations (vertical axis: mean ± SE; $n = 11$) and between essential genes (horizontal axis: mean ± SE; $n = 15$). Interaction between mutations and genes also contributed significantly to the observed variation in the relative transcript levels of essential genes. The Q518R + E528D and H531Y + N573S mutations (highlighted in orange font) were excluded from the two-way ANOVA analysis. The dendrogram from the two-way clustering analysis shows that the compensated mutants cluster more closely with the ancestral strain than do the original *rpoB* mutants they were evolved from.

correlation coefficient: $r = -0.541$, $P = 0.106$), which implies that there is no overall correlation between fitness and pleiotropic changes in the average relative transcript levels of essential genes.

We then hypothesized that the average magnitudes of deviation in the transcript levels of essential genes in *rpoB* mutants relative to the levels in ancestral strains depend on the extent to which transcriptional efficiency is altered. Among the original *rpoB* mutants evolved from the fluctuation test (Table 1), the average magnitudes of deviation in relative transcript levels of essential genes showed significant variations between mutants with different *rpoB* mutations (one-way ANOVA: $P < 0.0001$, $F_{9,\ 140} = 4.47$). In addition, we observed that decreases in transcriptional efficiency increase the average magnitudes of deviation in the relative transcript levels of essential genes (one-way ANOVA: $P = 0.0303$, $F_{1,\ 9} = 6.59$, $r^2 = 0.423$) (Fig. 6). Taken together, our results support the view that alterations in the relative transcript levels of essential genes do not generate an additional fitness cost in *rpoB* mutants.

RNA-Seq profiling of two *rpoB* mutants. To extend our qPCR measurements of gene expression to a genome-wide scale, we used RNA-Seq to obtain the transcriptome profiles of the ancestral strain and two *rpoB* mutants that carry the *rpoB* mutations D521G and H531R, which are associated with significant fitness costs. At a genome-wide scale, both *rpoB* mutants displayed expression profiles similar to that of the rifampin-sensitive ancestral strain, as demonstrated by the high correlated expression scores of the mutants and the sensitive strain (see Fig. S3A and B in the supplemental material). In the D521G mutant, only 77 genes, or 1.36% of the total number of genes in the genome, showed significantly different expression levels, while in the H531R mutant, 475 genes, or 8.36% of all genes, were differentially expressed ($P < 0.05$ after correcting for false discovery rate). In line with this observation, we found that the genome-wide expression profiles of the two mutants were very similar to each other (Fig. S3C). In addition, 59 genes showed significantly different expression levels ($P < 0.05$) in both mutants relative to their expression in the ancestral strain (see Fig. S4 and Annex B in the supplemental material).

At a genome-wide level, we calculated the average magnitudes of deviation for the transcript levels of the following three categories of genes in *rpoB* mutants with the D521G and H531R muta-

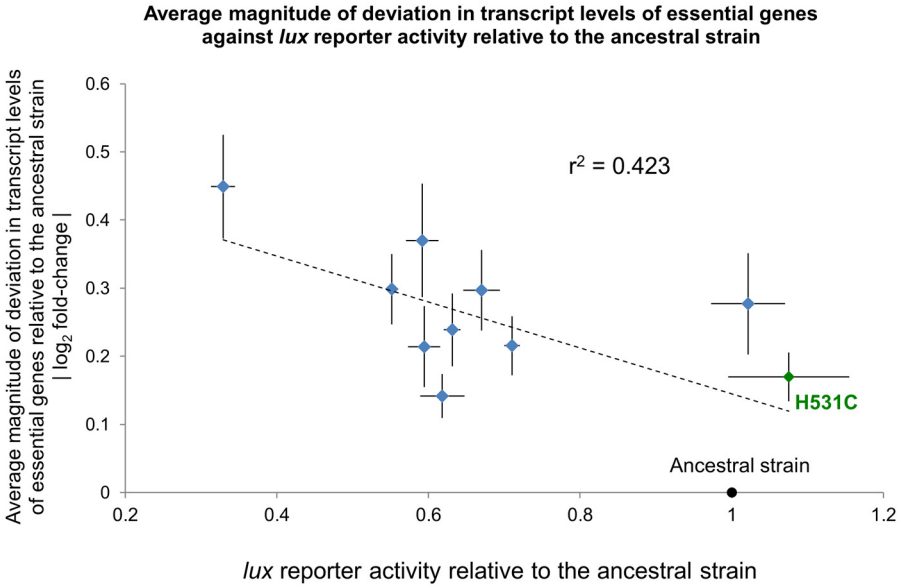

FIG 6 Decreased transcriptional efficiency increases the average magnitude of deviation in transcript levels of essential genes in *rpoB* mutants relative to the levels in the ancestral strain. The graph shows the correlation between relative transcriptional efficiencies (mean ± SE; $n = 3$) and average magnitudes of deviation in relative transcript levels of essential genes in resistant mutants (mean ± SE; $n = 15$). Decreased relative transcriptional efficiency is associated with elevated average deviation in relative transcript levels of essential genes in *rpoB* mutants, as determined by linear regression (dashed line). The ancestral strain is shown in black, and the compensated mutant H531C in green. Q518R + E528D and H531Y + N573S mutants were omitted from the correlation analysis.

tions relative to the transcript levels in the ancestral strain: essential genes, highly conserved core genes that play vital roles in bacterial physiology, and lineage-specific accessory genes that probably provide bacteria with niche-specific benefits (Fig. 7). Based on the whole-genome sequencing data for 36 *Pseudomonas* species (35), 1,435 core genes were identified (36), of which 196 are also essential genes (37). Accessory genes include all remaining genes in the genome that are neither core nor essential. In agreement with the idea that the expression levels of essential and core genes are under stabilizing selection (35), we found that the average magnitudes of genome-wide deviation in relative transcript levels are higher for accessory genes than for the combined group of essential and core genes in both the D521G and the H531R mutants (two-tailed, two-sample *t* test: D521G mutant, $t = -4.10$, df $= 3.64 \times 10^3$, $P < 0.0001$; H531R mutant, $t = -5.68$, df $= 3.86 \times 10^3$, $P < 0.0001$).

For all three categories of genes, the average magnitudes of deviation in transcript levels from ancestral strain levels are significantly greater in the H531R mutant than in the D521G mutant (two-tailed, two-sample *t* test: essential genes, $t = -7.21$, df $= 4.66 \times 10^3$, $P < 0.0001$; core genes, $t = -16.3$, df $= 1.97 \times 10^3$, $P < 0.0001$; accessory genes, $t = -24.0$, df $= 5.00 \times 10^3$, $P < 0.0001$). Since the H531R mutant has a lower relative transcriptional efficiency than the D521G mutant, these results suggest that reduced relative transcriptional efficiency is linked to altered transcriptional profiles at a genome-wide scale, which supports our qPCR-based measurements of relative transcript levels of essential genes, shown in Fig. 6.

Differential expression of the *rpoBC* operon in *rpoB* mutants. Previous work in *E. coli* demonstrated that increased expression of the *rpoBC* operon was observed in the presence of a sublethal concentration of rifampin (38). A similar upregulation of *rpoBC* was observed when a partial restriction of RNAP activity was induced in an *E. coli* mutant with conditionally defective RNAP (39). Given the adverse effects of *rpoB* mutations on RNAP functions, we hypothesized that the transcript levels of *rpoBC* would be higher in *rpoB* mutants than in the rifampin-sensitive ancestral strain and that the extent of *rpoBC* upregulation should depend on the extent to which RNAP function is impaired. Consistent with this idea, we found that the relative transcript levels of *rpoB* correlated significantly with relative transcriptional efficiency estimated by using the luciferase reporter gene (one-way ANOVA: $P = 0.0091$, $F_{1,9} = 10.9$, $r^2 = 0.549$) (Fig. 8A and B). Interestingly, *rpoB* and *rpoC* were among the 59 genes that were significantly upregulated in both the D521G and the H531R mutant according to our RNA-Seq dataset, suggesting that the upregulation of *rpoBC* is one of the most profound effects of *rpoB* mutations, at least in strains carrying mutations that lead to substantial reductions in relative transcriptional efficiency.

We investigated the molecular mechanism underpinning the differential expression of *rpoBC* by quantifying the transcriptional read-through of the intergenic region between the *rplJL* and *rpoBC* operons. In *P. aeruginosa*, a partial terminator (also known as an attenuator), P26, lies immediately downstream from *rplL* (Fig. 8C). In *E. coli*, nonbacteriostatic concentrations of rifampin, which partially inhibited RNAP activity and decreased transcriptional efficiency, were shown to have a small stimulatory effect on the expression of *rplJL* (40) but a large stimulatory effect on the expression of *rpoBC* (41, 42). Morgan and Hayward demonstrated that this was a direct effect of an increase in read-through of the t_{L7} partial *rho*-independent terminator between *rplL* and *rpoB*, which is the *E. coli* homologue of P26 and has a termination efficiency of 80% in the wild-type strain (42).

Since many *rpoB* mutations are known to alter the termination phenotypes of RNAP in *E. coli* (43), we hypothesized that the observed increases in the relative transcript levels of *rpoBC* in

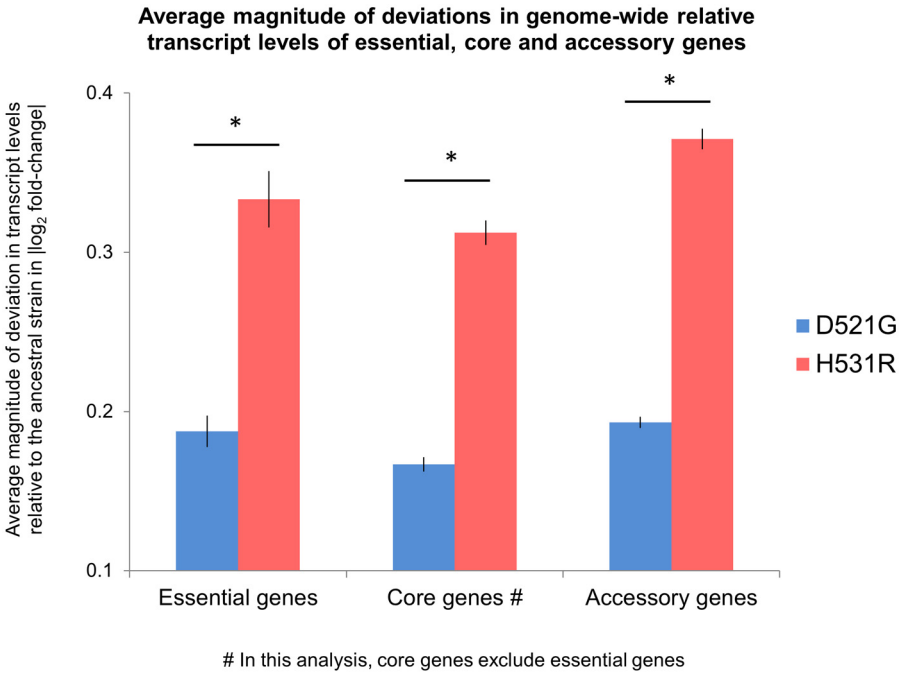

FIG 7 Average magnitudes of deviation in genome-wide transcript levels of essential, core, and accessory genes in *rpoB* mutants D521G and H531R relative to the levels in the ancestral strain. The 4,765 genes of the *P. aeruginosa* PAO1 genome that were successfully sequenced in our RNA-Seq dataset were classified as essential, core, and accessory genes. Regardless of the classification of genes, the average magnitudes of deviation in relative transcript levels are significantly greater in the H531R *rpoB* mutant than in the D521G mutant (two-tailed, two-sample *t* test: *, $P < 0.05$).

rifampin-resistant mutants with a compromised RNAP activity can be attributed to an increase in read-through of the P26 partial terminator in *P. aeruginosa*. To test this idea, we quantified the relative transcript levels of two regions spanning the start and end of P26 (Fig. 8C) using qPCR. One amplicon (*rplL*_P26) spans the junction between *rplL* and P26, while the other (P26_intergenic) spans the junction between P26 and the start of the remaining intergenic region. The extent of P26 read-through was characterized by the transcript level of the P26_intergenic amplicon relative to that of the *rplL*_P26 amplicon within each *rpoB* mutant strain.

The results in Fig. 8D show that the percentage of change in P26 read-through is significantly correlated with the relative expression of *rpoB* in rifampin-resistant mutants (one-way ANOVA: $P = 0.0013$, $F_{1,\ 9} = 21.3$, $r^2 = 0.702$), confirming that altered P26 read-through contributes to the differential expression of *rpoB* in rifampin-resistant mutants. To establish the causality of the correlation between *rpoB* expression and P26 read-through, we used qPCR to measure the transcript levels of *rpoB* and alteration in P26 read-though in the three compensated mutants listed in Table 2 relative to the transcript levels and P26 read-through in the ancestral strain. Consistent with our hypothesis, the compensated mutants with the H531C and H531Y + N573S mutations showed statistically significant reductions in relative *rpoB* transcript levels compared to the levels in the original *rpoB* mutants they were evolved from (see Fig. S5A in the supplemental material). The extent of P26 read-through also showed a statistically significant decrease in the H531C mutant compared to the P26 read-through in the H531R mutant (Fig. S5B). Taken together, these observations support the idea that alterations in P26 read-through and, consequently, *rpoBC* expression depend on the extent to which RNAP function is compromised in *rpoB* mutants. However, we emphasize that altered P26 read-through may not be the only mechanism that contributes to altered expression of *rpoBC*.

Conclusions. In summary, we found that rifampin resistance resulted in significant reductions in transcriptional efficiency (Fig. 2), and this direct effect of rifampin resistance explains 76.2% of the variation in the fitness of *rpoB* mutants isolated in this study (Fig. 4). The indirect, pleiotropic consequence of compromised RNAP activity was that rifampin resistance mutations showed altered transcriptional profiles at a genome-wide scale. The pleiotropic changes in relative transcript levels were modest in comparison to the large changes in relative transcriptional efficiency associated with rifampin resistance, and this helps to explain why we do not detect any evidence that global transcriptional responses contribute to the cost of rifampin resistance. For example, the D521G mutation was associated with a 44.9% reduction in relative transcriptional efficiency, which is typical of rifampin resistance mutations (Fig. 2), but this mutation resulted in significant changes in the expression of only 71 genes (see Fig. S5 in the supplemental material), which corresponds to 1.25% of the *P. aeruginosa* genome.

One of the most important conclusions of this study is that the transcriptional regulatory network of *P. aeruginosa* displays robustness against *rpoB* mutations. First, we found that rifampin resistance mutations were associated with an increase in the expression of *rpoBC*, implying that the loss of RNAP activity in *rpoB* mutants was buffered by increased RNAP abundance via increased read-through at a partial terminator upstream from *rpoB*. Transcription is a complex, multistep biochemical process that can be divided into the three distinct stages of initiation, elongation, and termination. *rpoB* mutations which affect any stage of

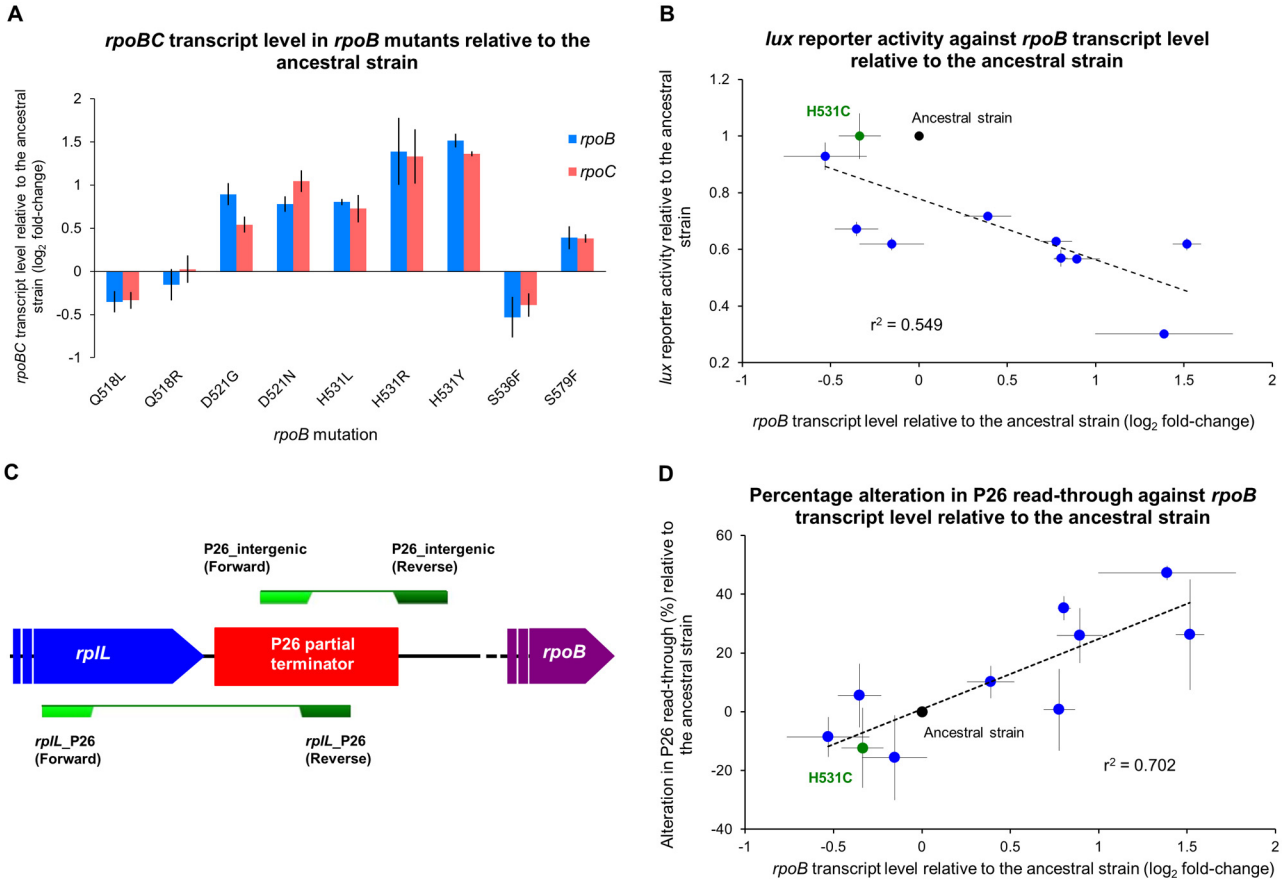

FIG 8 *rpoB* mutations increase the expression of the *rpoBC* operon via increased read-through at a partial terminator. (A) Transcript levels of *rpoB* and *rpoC* ($\log_2$-fold change) in rifampin-resistant mutants relative to the levels in the ancestral strain (mean ± SE; $n = 3$), as determined by qPCR. (B) *rpoB* transcript levels and transcriptional efficiencies in resistant mutants relative to those in the ancestral strain (mean ± SE; $n = 3$). Decreased relative transcriptional efficiency is associated with elevated relative *rpoB* transcript level, as determined by linear regression (dashed line). (C) Schematic of the qPCR strategy used to quantify read-through at the P26 partial terminator, which is located immediately upstream from *rpoB*. One amplicon (*rplL*_P26) spans the junction between *rplL* and P26, while the other (P26_intergenic) spans the junction between P26 and the start of the remaining intergenic region. The transcript levels of the P26_intergenic amplicon were normalized to those of *rplL*_P26 to quantify the extent of the partial terminator read-through within each *rpoB* mutant strain. The lengths of *rplL* and *rpoB* genes are not drawn to scale. (D) Significant correlation is observed between alterations in P26 read-through (mean ± SE; $n = 3$) and *rpoB* transcript levels (mean ± SE; $n = 3$) relative to those in the ancestral strain. Decreased termination at P26 can explain 70.2% of the variation in relative transcript levels of *rpoB*, as determined by linear regression (dashed line).

transcription, promoter selectivity of the RNAP core enzyme, or interactions between RNAP and transcriptional regulatory elements, such as transcriptional attenuators, can potentially affect the transcriptional profiles of mutants. It is unclear whether reduced attenuation is specific to the P26 partial terminator or whether this phenomenon can be observed at a genome-wide scale. We plan to further investigate these questions using the RNA-Seq datasets we have obtained for the D521G and H531R mutants.

Second, we found that pleiotropic changes in the average magnitudes of genome-wide changes in relative transcript levels are much smaller for core and essential genes than for accessory genes. The molecular mechanisms underpinning this are not clear, but it has been shown that essential genes and genes with a high degree of evolutionary conservation show much less cell-to-cell variation in expression than accessory genes in *E. coli* (34). This suggests that bacteria have evolved very general mechanisms to minimize variation in the expression of essential and core genes. If this is the case in rifampin-resistant mutants of *P. aeruginosa*, the small pleiotropic changes in the expression of essential genes in *rpoB* mutants might have very little to do with altered RNAP activity *per se*.

Chromosomal antibiotic resistance mutations often alter the structure of highly conserved enzymes with pivotal biochemical functions, such as ribosomes, RNAP, and DNA gyrase. Intuition suggests that alterations to these cellular components are likely to have extensive pleiotropic effects at a genome-wide scale. In line with our findings, previous studies have found that the fitness effects of resistance mutations correlate strongly to the direct effects of resistance mutations (10, 11). Our study raises the intriguing possibility that despite the strong correlation between the direct effects of rifampin resistance mutations and fitness, the buffering capacity of *rpoB* mutants against changes in relative transcript levels of essential genes could diminish the fitness costs that are associated with the pleiotropic effects of *rpoB* mutations. At the same time, the significant reductions in relative transcriptional efficiency and the associated fitness cost observed in the *rpoB* mutants generated in this study imply that elevated *rpoB*

expression alone is not sufficient to eliminate the cost of resistance. One motivation for studying the costs of antibiotic resistance is that understanding the costs of resistance could theoretically allow for the development of new therapeutics aimed at exploiting potential weaknesses of resistant bacteria. If bacteria are well buffered against resistance mutations, it is possible that resistant mutants may lack Achilles' heels.

MATERIALS AND METHODS

Generating the IPTG-inducible luciferase-tagged ancestral strain and isolating *rpoB* mutants. The DNA fragment between the SalI and BamHI restriction sites from the pUC18-mini-Tn*7*T-Gm-*LAC* mini-Tn*7* vector (accession no. AY599234 [44]), which contains the *lac* operon promoter and its repressor gene, was cloned upstream from the *luxCDABE* operon in the pUC18-mini-Tn*7*T-Gm-*lux* vector (accession no. AY962893 [44]) at the same sites to generate the mini-Tn*7* transposon delivery vector shown in Fig. S6 in the supplemental material. Sucrose-treated electrocompetent *P. aeruginosa* wild-type (PAO1) cells were cotransformed with this vector and a helper plasmid, pUX-BF13 (45), that encodes the Tn*7* site-specific transposition pathway to generate the PAO1::mini-Tn*7*-p*LAC-lux* ancestral strain, which bears an isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible *luxCDABE* reporter gene construct at the *att*Tn*7* site.

Using a fluctuation test (46), *rpoB* mutants were evolved from the ancestral strain (Table 1). The ancestral strain was grown overnight in LB medium at 37°C and diluted 10^6-fold. The diluted cultures were transferred to 96-well Nunc microplates (Fisher Scientific, USA) and incubated overnight at 37°C. Overnight cultures were pin replicated onto M9KB agar containing 64 μg ml^{-1} rifampin and incubated at 37°C overnight. Rifampin-resistant mutants were isolated the following day. Genomic DNA was extracted from each mutant strain using the Wizard genomic DNA extraction kit (Promega, USA). Two regions of *rpoB* were sequenced using procedures as previously described (47).

Isolation of *rpoB* mutants with intragenic compensatory mutations. *rpoB* mutants with intragenic compensatory mutations were evolved from the rifampin-resistant mutants isolated in this study through a selection experiment and were identified using Illumina whole-genome sequencing, which we have described in an unpublished study (see Annex A in the supplemental material). Briefly, overnight cultures of the original *rpoB* mutant strains were diluted 1,000-fold in fresh M9KB culture medium (48) without rifampin and incubated overnight with shaking at 30°C. The serial transfer was repeated for 30 consecutive days under the same experimental conditions. Genomic DNA was extracted from 3 colonies of the initial and final populations from the selection experiment using the DNeasy blood and tissue kit (Qiagen, Netherlands). Paired-end high-throughput sequencing using the Illumina HiSeq system for next-generation sequencing (Illumina, USA) was performed by the Wellcome Trust Centre for Human Genetics, University of Oxford. Raw whole-genome sequencing data were analyzed using an experimentally validated in-house computational pipeline (36). The compensated mutants used in this study are listed in Table 1.

Competitive fitness assay. The fitness cost of *rpoB* mutations was determined using standard competitive fitness assays. The PAO1::mini-Tn*7*-p*LAC-lux* ancestral strain, *rpoB* mutant strains (Table 2), and a GFP-tagged strain derived from PAO1, which had been generated by inserting a mini-Tn*7* construct carrying the *gfpmut3* gene (accession no. DQ493877) into the chromosomal mini-Tn*7* site of the PAO1 wild-type strain (44), were precultured in M9KB medium overnight at 37°C with continuous shaking. On the next day, all precultures were diluted in M9KB and regrown to early exponential phase at 30°C. The nonfluorescent strains (the ancestral strain and *rpoB* mutants) were individually mixed with the GFP-tagged control strain, diluted 1:200 in fresh M9KB medium, and incubated in Nunc 96-well microplates (Thermo Scientific, USA) at 30°C overnight for approximately 24 h with continuous shaking.

The proportions of fluorescent and nonfluorescent cells in each competition experiment were determined before and after overnight incubation by using an Accuri C6 flow cytometer (BD Biosciences, USA) according to the manufacturer's instructions. The competitive fitness of each nonfluorescent strain was calculated as the ratio of the population doublings of each mutant and the GFP-tagged control strain it was competing against. The relative competitive fitness levels of the *rpoB* mutants were obtained by standardizing their competitive fitness levels to that of the ancestral strain within each round of competition. Three biological and three technical replicates of each strain were assayed.

Bacterial luciferase reporter gene assay. The PAO1::mini-Tn*7*-p*LAC-lux* ancestral strain and the *rpoB* mutant strains (Table 2) were precultured in M9KB medium overnight at 37°C with continuous shaking (225 rpm). The overnight precultures were diluted 200-fold in fresh M9KB and transferred to black 96-well Costar microplates with clear bottoms (Corning, USA). Luciferase (*lux*) expression was induced using 1 mM IPTG. Bacterial cultures were incubated in a Synergy 2 microplate reader (Bio-Tek, USA) at 30°C, during which the level of luminescence emission (RLU) per OD_{600} was measured every 20 min with shaking prior to each read. The maximal gradient of each RLU/OD_{600} curve was calculated using 10 consecutive data points during the early exponential phase. Within each assay, the relative *lux* reporter activity in each *rpoB* mutant strain was obtained by normalizing each gradient to that of the ancestral strain control in the same plate. For each strain, three biological and three technical replicates were assayed.

RNA extraction, reverse transcription, and quantitative real-time PCR. Total RNA samples were extracted from the PAO1::mini-Tn*7*-p*LAC-lux* ancestral strain, *rpoB* mutant derivative strains (Table 2), and three *rpoB* mutants with compensatory mutations (Table 2). Three biological replicates of total RNA were isolated from each strain, with the inclusion of the ancestral strain control in every round of RNA extraction. The strains were inoculated in M9KB medium and precultured overnight at 37°C with continuous shaking. The overnight cultures were diluted in M9KB with 1 mM IPTG to induce luciferase expression and incubated at 30°C with shaking. RNA was extracted after 2 h during early exponential phase using the SV (spin or vacuum) total RNA isolation system (Promega, USA) according to the manufacturer's instructions, with minor modifications. To eliminate genomic DNA, DNase treatment was performed during and after RNA extraction. cDNA was synthesized using the GoScript reverse transcription system (Promega, USA). Quantitative real-time PCR (qPCR) assays were performed using the relative quantification method. Each gene was amplified using the Fast SYBR green master mix (Applied Biosystems, USA) on the StepOnePlus real-time PCR system (Applied Biosystems, USA).

Given the deleterious effects of *rpoB* mutations on RNAP activity, a key challenge of this study was to identify a group of stably expressed internal reference genes for normalizing target gene transcript levels to obtain relative transcript levels. We used the *GeNorm* algorithm (49) to screen the expression stability of six candidate internal reference genes in all sample groups and selected 16S rRNA, *atpA*, *acpP*, and *rpoD* as our internal reference genes. These results were further verified using the *NormFinder* algorithm (50). Normalization factors were calculated based on the geometric means of the uncorrected transcript levels of the four internal reference genes. Next, the relative transcript levels of target genes in each cDNA sample were determined by normalizing the uncorrected transcript level of every target gene to the transcript levels of the normalization factors. The oligonucleotide sequences of all qPCR primers used in this study are shown in Table S2 in the supplemental material. Finally, the relative transcript levels of target genes in *rpoB* mutants were normalized to those of the ancestral strain within the same batch of biological replicates to correct for the batch effect. Further experimental details are described in Annex A in the supplemental material.

RNA-Seq. Two biological replicates of RNA samples extracted from the ancestral strain and the D521G and H531R mutants were sequenced at the Wellcome Trust Centre for Human Genetics, University of Oxford. Ribosomal RNA was depleted using the Ribo-Zero magnetic kit for Gram-

negative bacteria (Epicentre, USA). cDNA libraries were prepared using the NEBNext mRNA library prep master mix set for Illumina (New England BioLabs, USA) according to the manufacturer's instructions, with modifications. Paired-end high-throughput sequencing of the cDNA libraries was performed using the Illumina MiSeq system for next-generation sequencing (Illumina, USA).

Raw sequence reads from FASTQ files were mapped to the reference genome of the PAO1::mini-Tn*7*-p*LAC-lux* ancestral strain using TopHat version 1.4 (51) to generate BAM files, which can be downloaded from the Dryad Digital Repository (http://doi.org/10.5061/dryad.vj35n). Raw gene counts were generated from the BAM files using the *htseq-count* Python script with default parameters and analyzed using BioConductor (52). The gene count data were filtered to eliminate genes with an average count of less than 10 reads in all sequenced samples. Differential expression analysis of the remaining 4,765 genes was performed using *DESeq2* (53). The read counts of genes in the *rpoB* mutant strains were normalized to those in the ancestral strain from within the same batch of biological replicates to correct for the batch effect. Logarithmic fold changes in transcript levels were calculated relative to the transcript levels in the ancestral strain, and raw *P* values for each gene were calculated using a generalized linear model based on the negative binomial distribution (54). Raw *P* values were corrected for multiple testing using the Benjamini-Hochberg procedure with a significance threshold of $P < 0.05$ (55).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.01562-14/-/DCSupplemental.

Figure S1, TIF file, 0.2 MB.
Figure S2, TIF file, 0.2 MB.
Figure S3, TIF file, 0.5 MB.
Figure S4, TIF file, 0.2 MB.
Figure S5, TIF file, 0.1 MB.
Figure S6, TIF file, 1 MB.
Table S1, DOCX file, 0.01 MB.
Table S2, DOCX file, 0.02 MB.
Annex A, DOCX file, 0.02 MB.
Annex B, DOCX file, 0.02 MB.

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