

The Competitive Cost of Antibiotic Resistance in *Mycobacterium tuberculosis*

Sebastien Gagneux,^{1,4*}† Clara Davis Long,^{2*} Peter M. Small,^{4,5} Tran Van,¹ Gary K. Schoolnik,^{1,3} Brendan J. M. Bohannon²

Mathematical models predict that the future of the multidrug-resistant tuberculosis epidemic will depend on the fitness cost of drug resistance. We show that in laboratory-derived mutants of *Mycobacterium tuberculosis*, rifampin resistance is universally associated with a competitive fitness cost and that this cost is determined by the specific resistance mutation and strain genetic background. In contrast, we demonstrate that prolonged patient treatment can result in multidrug-resistant strains with no fitness defect and that strains with low- or no-cost resistance mutations are also the most frequent among clinical isolates.

Antimicrobial resistance has become a worldwide problem in many pathogens, incurring both economic costs and loss of human lives (1, 2). Antibiotic resistance is also often associated with a reduced competitive ability against antibiotic-sensitive strains, in the absence of the antibiotic (3). In a variety of model systems, it has been shown that this fitness “cost” depends on the specific drug resistance-conferring mutation and the strain genetic background and that it can be ameliorated by compensatory mutations (4–9). However, there are few studies linking the predictions of these model systems to epidemiological data from human populations (10–12). Here, we connect the in vitro cost of resistance to the prevalence of clinically important mutants of *Mycobacterium tuberculosis*, the causative agent of human tuberculosis.

The emergence of multidrug-resistant strains of *M. tuberculosis* is threatening global disease control efforts (13). Multidrug-resistant tuberculosis (MDRTB) is defined as tuberculosis caused by organisms resistant to isoniazid and rifampin, the two most potent first-line anti-tuberculosis drugs. MDRTB represents a major public health burden, requiring prolonged treatment with more costly and less effective agents (13, 14). To date, most investigations of factors determining the spread of MDRTB have focused on the quality of tuberculosis-control programs and patient adherence to treatment. However, recent studies suggest that bacterial factors, such as the relative fitness of drug-resistant *M. tuberculosis* strains, are also important (15–18). *M. tuberculosis* develops drug resistance by the sequential acquisition of chromo-

somal mutations (19), but there are only limited data on the effect of different drug resistance-conferring mutations on the relative fitness of *M. tuberculosis* (12, 20). Furthermore, there has been no attempt to investigate the effects of different genetic backgrounds on the competitive fitness of drug-resistant pathogens, including *M. tuberculosis*.

The relative competitive fitness of bacteria can be quantified experimentally with the use of competition assays originally developed for *Escherichia coli* (21). In these experiments, the drug-susceptible and the drug-resistant organisms compete for limited resources in a common environment. In *M. tuberculosis*, as in other bacteria, resistance to rifampin is mediated through missense mutations in *rpoB*, the gene encoding the β subunit of the RNA polymerase (19). To determine the impact of different *rpoB* mutations on the relative competitive fitness of rifampin-resistant *M. tuberculosis*, we selected a panel of spontaneous rifampin-resistant mutants by growing *M. tuberculosis* CDC1551 on media containing rifampin. CDC1551 is a clinical strain and part of a lineage of *M. tuberculosis* associated with Europe and the Americas

(22–24), and it is susceptible to currently used antituberculosis drugs. We sequenced known rifampin-resistance regions in the *rpoB* gene of all of the 52 colonies recovered and found that all of them had a missense substitution in *rpoB* (table S1). We chose nine mutants with different *rpoB* mutations and had them compete in vitro against their rifampin-susceptible ancestor, with the use of an assay that included lag, exponential, and early stationary phases of growth (21). All of the rifampin-resistant mutants had a statistically significant fitness cost compared with the susceptible ancestor, which is defined to have a competitive fitness of 1.0 (Fig. 1 and table S2). Among the different *rpoB* mutants, there were significant differences in competitive fitness, with values ranging from 0.58 to 0.91. We found a significant effect of mutation on fitness [analysis of variance (ANOVA), $F_{8,38} = 12.1563$, $P < 0.0001$]. The Ser⁵³¹→Leu⁵³¹ (S531L) mutant exhibited the lowest cost, whereas the Arg⁵²⁹→Gln⁵²⁹ (R529Q) mutant had the greatest fitness cost of all mutants (Fig. 1 and table S2). These results confirm that a wide range of *rpoB* mutations can have markedly different effects on the competitive fitness of *M. tuberculosis* in vitro, consistent with earlier work that examined the competitive cost of a small number of *rpoB* mutations (12, 20).

Next, we investigated the influence of the bacterial genetic background on the cost of rifampin resistance-conferring mutations. In the laboratory, we selected a second panel of spontaneous rifampin-resistant mutants in T85, another pan-susceptible isolate that is part of a distinct lineage of *M. tuberculosis* strongly associated with East Asia, commonly referred to as the Beijing strain (24, 25). All 63 rifampin-resistant colonies selected in T85 had a non-synonymous substitution in *rpoB* (table S3). We measured the relative competitive fitness of four different mutants with *rpoB* mutations whose fitness costs had been measured in CDC1551. Similar to the results in the CDC1551 back-

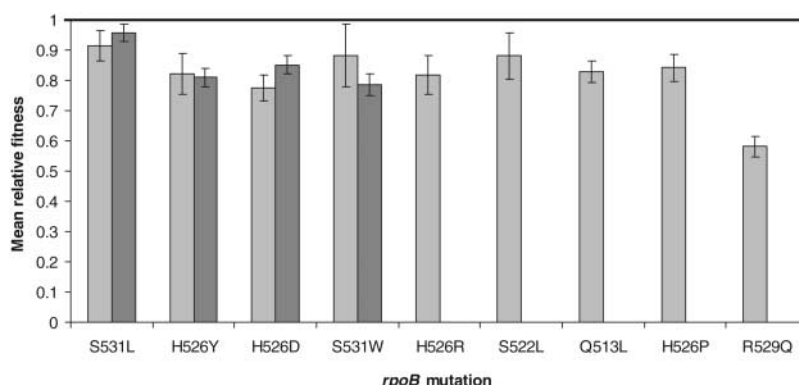


Fig. 1. Relative competitive fitness of laboratory-derived rifampin-resistant mutants of *M. tuberculosis*. All mutants had a statistically significant fitness cost (error bars indicate 95% confidence intervals). This cost was less in *rpoB* S531L mutants than in other *rpoB* mutants, irrespective of the strain background. Light gray bars, CDC1551 mutants; dark gray bars, T85 mutants. Y, Tyr; W, Trp; P, Pro.

¹Division of Infectious Diseases and Geographic Medicine, ²Department of Biological Sciences, ³Department of Microbiology and Immunology, Stanford University, Stanford, CA 94305, USA. ⁴Institute for Systems Biology, Seattle, WA 98103, USA. ⁵Bill and Melinda Gates Foundation, Seattle, WA 98102, USA.

*These authors contributed equally to this work.

†To whom correspondence should be addressed. E-mail: sgagneux@systemsbiology.org

ground, all T85 rifampin-resistant mutants had a significant fitness cost (Fig. 1 and table S2). We tested for an effect of genetic background by comparing the fitness costs of the four mutations found in each of the two backgrounds. We found an overall significant effect of genetic background when nested in mutation (nested ANOVA, $F_{7,34} = 6.8962$, $P < 0.0001$), and, as observed in the CDC1551 background, the *rpoB* S531L mutant had higher competitive ability than other mutations in the T85 background (Fig. 1 and table S2). The fitness of the S531L mutation did not significantly differ in the two genetic backgrounds (i.e., the confidence intervals overlap; Fig. 1 and table S2). However, genetic background did significantly alter the fitness of the His⁵²⁶→Asp⁵²⁶ (H526D) mutation (i.e., the confidence intervals do not overlap; Fig. 1 and table S2). Taken together, our data confirm that the in vitro relative fitness of rifampin-resistant *M. tuberculosis* depends on both the resistance-conferring mutation and the strain genetic background.

The relevance of empirically measured costs of laboratory-derived drug resistance to resistance that emerges in tuberculosis patients has not been established. Therefore, we measured the relative competitive fitness of rifampin-resistance in paired isolates (one that was drug resistant and one that was drug susceptible) from 10 patients who acquired rifampin resistance during antibiotic treatment (Fig. 2 and table S4). We used a standard DNA fingerprinting method to confirm that the paired isolates belonged to the same bacterial clone (26). In all 10 patients, both isolates had identical DNA fingerprinting patterns (table S5), which supports the assertion that the rifampin-resistant isolates recovered later were indeed derived from the corresponding rifampin-sensitive isolates. However, DNA fingerprinting methods are limited and may not reveal small genetic differences which could have been present

between isolates. All of the clones were distinct but belonged to one of the two strain lineages represented by CDC1551 and T85 (tables S4 and S5). Five of the resistant isolates harbored the *rpoB* S531L mutation and five had other *rpoB* mutations (Fig. 2, table S4).

All of the clinical isolates with mutations other than S531L had a relative fitness of less than 1.0, although the measured relative fitness was not always the same as that observed in the mutants selected in vitro (Fig. 2 and table S4). In contrast, four of the five clinical strains with the *rpoB* S531L mutation had a relative fitness of greater than or equal to 1.0. Notably, these four strains were the only rifampin-resistant mutants that had no fitness cost compared with their rifampin-susceptible ancestor.

Two alternative explanations could account for the high fitness we observed in clinical strains harboring the *rpoB* S531L mutation. First, this mutation may indeed be a no-cost (versus just a low-cost) mutation in these particular strains. Second, given that sufficient time had passed between the isolation of the susceptible and resistant members of each pair (table S4), compensatory mutations could have arisen and ameliorated the low initial fitness cost associated with this mutation, similar to what has been observed in other species of bacteria (4, 5, 8). In support of this possibility, we found that the clinically derived *rpoB* S531L mutants had a mean competitive fitness of 1.04 (95% confidence interval: 1.00 to 1.08). This was statistically significantly higher than the laboratory-derived *rpoB* S531L mutants, which had a mean competitive fitness of 0.93 (95% confidence interval: 0.90 to 0.96). Given that all clinical ancestors of the *rpoB* S531L mutants were already resistant to isoniazid or isoniazid and streptomycin (table S4), this increase in fitness could be due to amelioration of fitness costs associated with the other resistance mutations rather than specifically the rifampin-resistance mutation. In addition,

general increases in fitness could have occurred during long-term infection, rather than specific amelioration of the cost associated with a resistance mutation. We cannot differentiate between these alternative hypotheses with our current data. Nevertheless, our results clearly demonstrate that clinical strains of multidrug-resistant *M. tuberculosis* can have a significantly higher fitness than their progenitors, either because of no-cost resistance mutations, compensatory evolution, or both.

We hypothesized that those drug-resistant strains with the least costly and potentially most easily compensated resistance mutation will be more likely to spread and become prevalent in human populations. This scenario is particularly likely given the recent observation that many patients harbor subpopulations of the same mycobacterial clone with different drug resistance-conferring mutations (27). Many studies have reported the frequency of different drug-resistance alleles in a variety of clinical settings (28), providing an opportunity to test our hypothesis. When we compared the relative fitness of the different *rpoB* mutants to their clinical frequency, an association became evident (Figs. 1 and 2 and tables S2 and S4). The *rpoB* S531L mutation, which exhibited the lowest fitness cost in laboratory-derived mutants and no fitness cost in clinical strains, is the most prevalent rifampin resistance-conferring mutation, overall accounting for 54% of rifampin-resistant isolates, even in parts of the world associated with phylogenetically distinct strain lineages (24, 28). In contrast, the *rpoB* R529Q mutant, which carried the highest fitness cost of all mutants, has never been observed in clinical settings (28). Taken together, these findings are consistent with the idea that drug-resistant strains harboring low- or no-cost mutations such as *rpoB* S531L are selected in patients during treatment and that such strains are more likely to spread in human populations.

Several mathematical models have been developed to predict the future of MDRTB epidemics. Some have assumed a universal fitness cost to drug resistance and concluded that MDRTB will remain a localized problem (15). More recent mathematical models have allowed for variable fitness among strains and have come to very different conclusions depending on the specific fitness assumed (17). Our work supports the more complex models and suggests that the heterogeneity in fitness is a function of the drug-resistance mutation, the strain genetic background, and potentially compensatory evolution as well. These more complex models suggest that resistance will not spread below a relative fitness of 0.7. Interestingly, all the *rpoB* mutants tested had a relative fitness >0.7, except for the one that has not been observed in clinical settings (Figs. 1 and 2). None of these models has allowed for changing fitness through time, either as a result of compensatory evolution or other increases in fitness

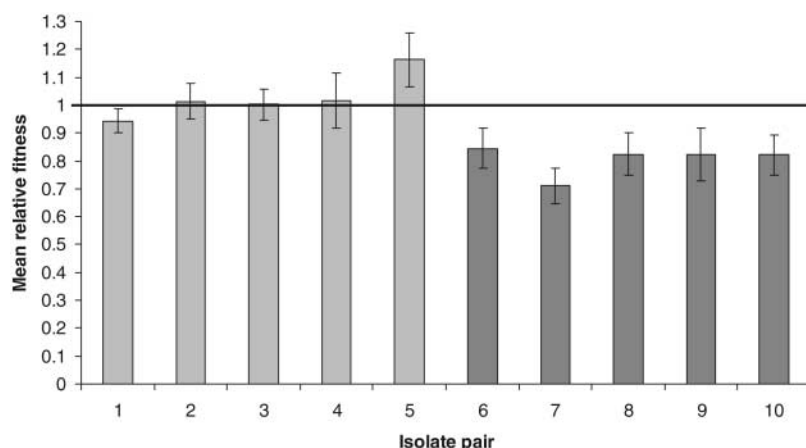


Fig. 2. Relative competitive fitness of clinically derived rifampin-resistant mutants of *M. tuberculosis*. Four of the five mutants with the *rpoB* S531L mutation (light gray bars) had no fitness cost compared with their rifampin-susceptible ancestors. All mutants with other *rpoB* mutations (dark gray bars) had significant fitness defects (error bars indicate 95% confidence intervals).

due to within-host evolution. Future mathematical treatments of the MDRTB epidemic should include such changes.

Our work suggests that in vitro competition assays can be predictive of biological differences important to *M. tuberculosis* ecology and evolution. Our finding that genetic background is a factor in the measured cost of resistance suggests that the cost of a given mutation may vary from location to location worldwide owing to the phylogeography of this pathogen (24). The possibility that fitness changes can occur during infection of a single patient should be taken into account when applying evolutionary ecology to infectious disease treatments or to the prediction of epidemics. The availability of such predictive methodology permits the incorporation of fitness considerations into the design and deployment of new drug regimens for controlling tuberculosis.

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Supporting Online Material

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Lhx2 Maintains Stem Cell Character in Hair Follicles

Horace Rhee, Lisa Polak, Elaine Fuchs*

During embryogenesis, stem cells are set aside to fuel the postnatal hair cycle and repair the epidermis after injury. To define how hair follicle stem cells are specified and maintained in an undifferentiated state, we developed a strategy to isolate and transcriptionally profile embryonic hair progenitors in mice. We identified Lhx2 as a transcription factor positioned downstream of signals necessary to specify hair follicle stem cells, but upstream from signals required to drive activated stem cells to terminally differentiate. Using gain- and loss-of-function studies, we uncovered a role for Lhx2 in maintaining the growth and undifferentiated properties of hair follicle progenitors.

Hair follicle morphogenesis involves a temporal series of reciprocal interactions between the ectoderm and its underlying mesenchyme (fig. S1) (1–3). In response to an inductive Wnt and an inhibitory Bmp signal (Noggin), small hair placodes bud from the epithelium, giving rise to larger hair germs (4–7). In the presence of the mitogen Shh, these hair germs develop further and grow downward to form a mature follicle that actively produces hair (8–10). Although the molecular details of bud formation are still poorly defined, the general features of this process are repeated at the start of each postnatal hair cycle when multipotent stem cells in the hair follicle bulge become activated to initiate a new round of hair growth. In addition, the early epithelial

remodeling to form the hair germ shares many features with the development of other epithelial tissues and organs, including feathers, teeth, and mammary glands (11–13). Understanding how tissues form buds that then progress along different lineages is predicated on elucidating the molecular mechanisms that funnel these early signaling pathways into a transcriptional program that drives morphogenesis.

To examine the genetic changes that occur during epithelial bud formation, we developed a strategy to isolate embryonic hair progenitors. To this end, we generated mice doubly transgenic for a *Keratin 14–GFP* gene expressed in skin keratinocytes and the Wnt reporter gene *TOPGAL*, transcribed in hair placodes and germs where β -catenin/Lef1 complexes are active (4, 14). In these early hair progenitors, E-cadherin is down-regulated and P-cadherin is up-regulated (Fig. 1A) (7). By embryonic day 17 (E17), we could use disperse to separate the epidermis, including hair placodes and

germs, from the underlying dermis, which harbored more mature hair pegs and follicles (fig. S2). With the use of fluorescence-activated cell sorting (FACS) on the epidermal fraction, the early “PCAD+” hair progenitors (K14-GFP+, α 6-integrin+, P-cadherin+) were then separated from the “PCAD–” interfollicular epidermis (K14-GFP+, α 6-integrin+, P-cadherin–) on the basis of their differential surface P-cadherin expression (fig. S3). Characterization of these two cell populations confirmed that they had similarities in the expression of K5 and β 4-integrin but distinct activities of *TOPGAL* and the expression of known hair-placode markers (Fig. 1, B to D, and fig. S4).

The gene expression profiles of purified PCAD+ hair progenitors and PCAD– interfollicular basal keratinocytes were further analyzed using oligonucleotide microarrays. Using fold differences of known hair-placode markers as a sensitivity gauge, a twofold cutoff was assigned as a genuine difference between the two populations. A total of 1394 probes (660 in PCAD+ and 734 in PCAD–) were preferentially expressed in one population relative to the other (table S1).

A short list of differentially expressed genes that are relevant to the present study is provided in table S2. As anticipated, a number of these genes have documented roles in either hair morphogenesis (PCAD+) or epidermal differentiation (PCAD–). The interfollicular epidermal population was typified by adhesive and cytoskeletal components, Notch signaling factors, c-Myc, Kruppel-like factors, and Bmp-responsive transcription factors (Grainyhead-like and Ovo1) previously implicated in epidermal differentiation (15–18). In contrast, the hair germ signature featured Wnts, Shh, Bmps, transforming growth

Howard Hughes Medical Institute, The Rockefeller University, 1230 York Avenue, New York, NY 10021.

*To whom correspondence should be addressed. E-mail: fuchs@rockefeller.edu

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