Compensatory Evolution in Rifampin-Resistant *Escherichia coli*

Mary G. Reynolds

Department of Biology, Emory University, Atlanta, Georgia 30322

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ABSTRACT

This study examines the intrinsic fitness burden associated with RNA polymerase (*rpoB*) mutations conferring rifampin resistance in *Escherichia coli* K12 (MG1655) and explores the nature of adaptation to the costs of resistance. Among 28 independent Rif^r mutants, the per-generation fitness burden (in the absence of rifampin) ranged from 0 to 28%, with a median of 6.4%. We detected no relationship between the magnitude of the cost and the level of resistance. Adaptation to the costs of rif resistance was studied by following serial transfer cultures for several Rif^r mutants both in the presence of rifampin and in the absence. For cultures evolved in the absence of rifampin, single clones isolated after 200 generations were more fit than their ancestor; we saw no association between increased fitness and changes in the level of rifampin resistance; and in all cases, increased fitness was due to compensatory mutations, rather than to reversion to drug sensitivity. However, in the parallel evolution experiments in the presence of rifampin, overall levels of resistance increased as did relative fitness—for all strains save one that had an initially high level of resistance. Among the evolved clones tested, five (of seven) demonstrated increased transcription efficiency (assessed using a semiquantitative RT-PCR protocol). The implications of these results for our understanding of adaptive molecular evolution and the increasing clinical problem of antibiotic resistance are discussed.

IN bacteria, chromosomal mutations that confer resistance to antibiotics in many cases engender structural or functional modifications in the cellular target of the drug. Prominent among these are resistance mutations in genes that control DNA coiling (gyrases, Filutowicz 1980; Herrera *et al.* 1993), transcription (Yanofsky and Horn 1981), and protein synthesis (Weisblum and Davies 1968). Because mutations that confer resistance can generate functional lesions in vital processes such as these, they are commonly associated with decreased rates of bacterial replication. In the absence of the selecting antibiotic, this reduced replication often constitutes a selective disadvantage for the resistant mutant. As a result, one would anticipate that without continuing drug selection, subsequent evolution could lead to one of two outcomes: the ascent of revertants (bacteria that have returned to the sensitive state due to a back mutation at the specific nucleotide site of the gene responsible for resistance) or the evolution of compensatory mutants (bacteria with secondary mutations that ameliorate the fitness costs of the original resistance mutation). In the absence (as well as in the presence) of selective antibiotics, compensatory mutations can be obtained without a loss of resistance (Schrag and Perrot 1996; Schrag *et al.* 1997; Bjorkman *et al.* 1998, 1999, 2000).

Address for correspondence: c/o Bruce Levin, Emory University, Department of Biology, 1510 Clifton Rd., NE, Atlanta, GA 30322.
E-mail: mreynold@biology.emory.edu

The process of adaptation to the fitness costs of chromosomally encoded resistance has been studied in some detail in *Escherichia coli* (Schrag and Perrot 1996; Schrag *et al.* 1997) and *Salmonella typhimurium* (Bjorkman *et al.* 1998, 1999, 2000) for mutations that affect translation. It is evident from these studies that the outcome of adaptation to resistance is largely dependant on the environmental context within which the adaptation occurs. For example, particular streptomycin resistance mutations in *rpsL* (ribosomal protein *S12*) and fusidic acid resistance mutations in *fusR* (elongation factor *EF-G*) have been shown to result in substantial reductions in bacterial fitness (>10% per generation) in the absence of drug, both *in vitro* and *in vivo* in laboratory mice. But for both categories of resistant mutants (*rpsL* and *fusR*) the end products of evolution in the absence of drug differ, depending on whether they were generated *in vitro* or *in vivo*.

For streptomycin-resistant (*rpsL*) *E. coli* and *S. typhimurium* mutants grown *in vitro*, amelioration of the cost of resistance is principally achieved via compensatory mutations in ribosomal proteins encoded by *rpsD* or *rpsE*. Such mutations are compensatory and foster restoration of protein elongation rates (Schrag *et al.* 1997). In contrast, however, amelioration of the cost of *rpsL* mutations *in vivo* transpires mainly via the evolution *rpsL* "replacement" alleles that retain the capacity to confer resistance, but do so with diminished cost (Cohan *et al.* 1994; Bjorkman *et al.* 1998). The story is similar for fusidic acid-resistant *S. typhymurium*. Serial

passage of these bacteria without antibiotic results in outgrowth of compensated mutants rather than drug-sensitive revertants, while evolution *in vivo* results almost exclusively in reversion at the *fusR* locus (Bjorkman *et al.* 2000).

In this report, we explore the fitness costs associated with resistance mutations affecting transcription and examine the nature of the adaptation to the fitness costs associated with these mutations. In gram-negative and gram-positive bacteria—including *E. coli* and *Mycobacterium tuberculosis*—resistance to the drug rifampin can readily be acquired by single base changes in the gene encoding the β-subunit of the DNA-dependant RNA polymerase, *rpoB*. Through *in vitro* experiments using *E. coli* K12, we consider (i) the effect of independent *rpoB* mutations on the fitness of *E. coli*; (ii) the consequences of subsequent evolution on different *rpoB* mutants maintained in medium with and without rifampin; (iii) the relationship between cost of *rpoB* mutations and rates of transcription; and (iv) the relationship between the cost of *rpoB* mutations and the magnitude of resistance to rifampin.

MATERIALS AND METHODS

Strain utilized: All experiments described in this work were conducted with *E. coli* K12 strain MG1655 (*LAM–, rph–1*; *E. coli* Genetic Stock Center no. 4837; Guyer *et al.* 1981; Jensen 1993). This strain was maintained in either glucose-limited Davis minimal medium (supplemented with 500 μg/ml glucose, 120 μg/ml $MgSO_4$, 1 μg/ml thiamine, and 40 μg/ml each of uracil, cytosine, and thymidine) or in Luria-Bertani broth (or LB agar).

Methods for obtaining random and representative Rif^r mutants: Rif^r mutants were obtained by selective plating of $\sim 10^9$ bacterial cells, from each of 30 independent stationary-phase cultures, onto LB rif [25 μg/ml rifampin, two times the minimum inhibitory concentration (MIC) of wild-type K12]. Resistant colonies were selected after 48 hr of incubation at 37° (incubation restricted to 48 hr may lessen the chance of obtaining plate mutants, but may bias toward more fit stains). The selection procedure was undertaken twice, once for selection of "randomly chosen" mutants and once for selection of "representative" mutants. Differences between the two selection regimens are as follows:

1. Random selection of the 28 randomly chosen Rif^r mutants was accomplished by picking the resistant colony (one per plate) within closest physical proximity to a fixed point—in this case the point of intersection of two perpendicular lines rendered on the exterior of the petri dish. This choice was made regardless of colony size or apparent resistance capacity. The parental strain for randomly chosen mutants was a K12 MG1655 derivative carrying an additional neutral marker: inability to ferment maltose [*mal*]. This marker allowed distinction between wild-type K12 MG1655 [*mal*$^+$] and Rif^r MG1655 [*mal*$^-$] on Tetrazolium (2,3,5-triphenyltetrazolium) maltose plates (Miller 1992).
2. Nine representative (primary) Rif^r mutants were specifically chosen as examples of the scope of phenotypes associated with resistance (*e.g.*, small colony size on LB rif-25, but normal colony size on LB; large colony size on both media types, etc.). These mutants were derived from a wild-type [*mal*$^+$] K12 parent. Nonmaltose-fermenting derivatives [*mal*$^-$] were generated from strains chosen for evolution experiments (rif-1, rif-2, rif-8, and rif-9).

Method for assessing fitness: competition assays: The relative fitness of the naïve and the evolved Rif^r mutants was estimated from pairwise competition experiments using a method similar to that employed in Levin *et al.* (2000). Approximately equal densities of the Rif^r mutant (or the evolved mutant) and the respective ancestral strain were mixed in 10 ml Davis minimal medium at a dilution of 1:200. These cultures were grown to stationary phase, and the relative densities of each type of bacterium were then estimated by plating on nonselective media. A total of 50 μl of the stationary phase culture was then transferred to a fresh flask and the process was repeated for four or five transfers.

The relative fitnesses (W) of the Rif^r cell lines were estimated using $W = 1 - b$, where b is the slope of the regression, $\ln(\text{Rif}^r/\text{Rif}^s)$ as a function of transfer and, T [the number of cell generations, $T = \ln(200)/\ln(2)$]. For example, if $W = 0.9$, then the fitness of the Rif^r cell line is 90% that of the Rif^s.

Assessment of rifampin resistance level: The MICs of rifampin for the randomly selected mutants were obtained by patching mutants onto LB agar containing concentrations of rifampin ranging from 0–8000 μg/ml (0, 12.5, 25, 50, 100, 200, 400, 800, 1000, 2000, 3000, 4000, 5000, 6000, 7000, and 8000). MICs for representative mutants and evolved mutants were measured in LB liquid in 96-well microtiter plates at the same range of concentrations as shown above. In both cases the MIC is denoted by the concentration interval that encompasses the upper limit of growth and the first concentration that cannot support visible bacterial growth after 24 hr.

Strain evolution: Four Rif^r mutants (rif-1, rif-2, rif-8, and rif-9) were chosen as subjects for experimental evolution, which proceeded over ~200 bacterial generations, and consisted of daily serial passage of the given strain in glucose-limited Davis minimal medium (1:200 dilution each passage; 50-μl stationary phase culture into 10 ml fresh medium). Multiple clones of each of the mutants rif-1, rif-2, and rif-8 were serially passaged with rifampin (two clones) and without further rifampin (four clones) selection. Four clones of rif-9 were serially passaged only in the presence of rifampin (25 μg/ml). Four clones of Rif^s MG1655 [*mal*] were subjected to the same regimen of serial passage (with no drug selection pressure) to serve as an experimental control.

Upon termination of serial passage (day 28) single evolved clones were picked at random from each passaged culture and assessed for phenotype.

PCR and sequence analysis: The *rpoB* genotype of representative and evolved Rif^r mutants was determined by DNA sequence analysis of the first 2226 of 4026 nucleotides of the *rpoB* locus (plus an additional 38 nucleotides 5′ to the coding sequence).

For sequence analysis, genomic DNA was harvested from each strain of interest, and two *rpoB* fragments (the first spanning nucleotides 1–1342, including resistance cluster I, and the second spanning nucleotides 1240–2226, including resistance cluster II) were amplified by PCR using QIAGEN (Valencia, CA) Taq DNA polymerase in conjunction with the following paired primers: *rpoB* I, 5′-gacagatgggtcgacttgtcagcg-3′ (sense) with 5′-aggtggtcgatatcatcgactt-3′ (antisense); *rpoB* II, 5′-tcgaaggttccggtatcctgagc-3′ with 5′-ggatacatctcgtcttcgttaac-3′. Amplified fragments were purified using QIAGEN QIAquick PCR purification prior to sequencing.

Reverse transcriptase-PCR assay: The transcriptional efficiency of a subset of Rif^r mutants and evolved mutants was established using a semiquantitative reverse transcriptase

(RT)-PCR assay (similar to that used in Houze *et al.* 1996; Gong *et al.* 1999; Mulligan-Kehoe and Russo 1999) that allowed determination of the relative abundance of a full-length induced mRNA transcript (lactose transacetylase *lacA*), as compared to that of a steady-state transcript (*recA*) at progressive time points postinduction. *RecA* was used as a low abundance, internal standard to control for sample-to-sample variation in RT and PCR reactions, and as a control for total RNA quality (Foley *et al.* 1993).

For RNA, cultures were grown to an OD_{600} of 0.5, after which transcription was induced with isopropyl β-d-thiogalactoside. Next, total cell RNA was harvested from 0.5 ml of bacterial culture recovered 3, 7, 13, and 23 min postinduction. Approximately 1 μg RNA from each time point was subjected to DNAase treatment (to destroy illegitimate amplification template), followed by reverse transcription using Sigma Chemicals' (St. Louis) enhanced avian reverse transcriptase employing gene-specific primers (same as below) for both *recA* and *lacA* transcripts. Finally, reverse transcription products were subject to 30 cycles of PCR amplification (at 55° annealing temperature) using the following paired primers: for *lacA*, 5′-atgccaatgaccgaaagaataaga-3′ (sense), with 5′-tactacccgcgc caataacagaat-3′ (antisense); and for *recA*, 5′-atctctaccggttcgcttt cactg-3′, with 5′-ctgctacgccttcgctatca-3′. We confirmed the detection of exponential-phase amplification products by measuring total product after 20, 25, 30, 35, and 40 amplification cycles. (We assume that the lag in achieving plateau phase amplification is a function of very low initial abundance of target mRNA or of inefficient RT. Either of these we anticipate being consistent across samples.)

Amplification products were visualized on ethidium-bromide-stained 1% agarose gels. Digitized gel images were analyzed for optical density of DNA bands corresponding to *lacA* and *recA* fragments. Analysis of digital images was conducted using National Institutes of Health Image 1.61 software. Transcriptional efficiency is reported here as the slope of the line plotting the proportional change in optical density of *lacA* to *recA* per minute (*i.e.*, Δ[OD *lacA*/OD *recA*] *vs.* minutes postinduction). RNA preparations and RT-PCR reactions were performed according to specifications outlined by the manufacturers of QIAGEN Rneasy and Sigma enhanced avian RT-PCR products, respectively.

RESULTS

Characteristics of randomly chosen Rif^r mutants: To facilitate determination of the frequency with which Rif^r mutations in *E. coli* engender a fitness cost, and to learn whether or not cost and resistance level are correlated, we examined the MIC and relative fitness of 28 random mutants of MG1655 selected on LB agar supplemented with 25 μg/ml rifampin. Our goal was to define the phenotypic range of Rif^r mutants with respect to resistance level and fitness. Therefore we did not establish the *rpoB* genotypes of clones in this set. The results of this analysis are summarized in Figure 1.

The most notable observations from this data set are (i) that the frequency with which no-cost Rif^r mutations emerge in *E. coli* K12 is relatively low (64% of the Rif^r clones evaluated here exhibited a fitness burden of 2–10% per generation, and 29% had a resistance cost in excess of 10%/generation) and (ii) that resistance capacity does not correlate with resistance cost (regression coefficient = −0.02, $P = 0.29$, d.f. = 1).

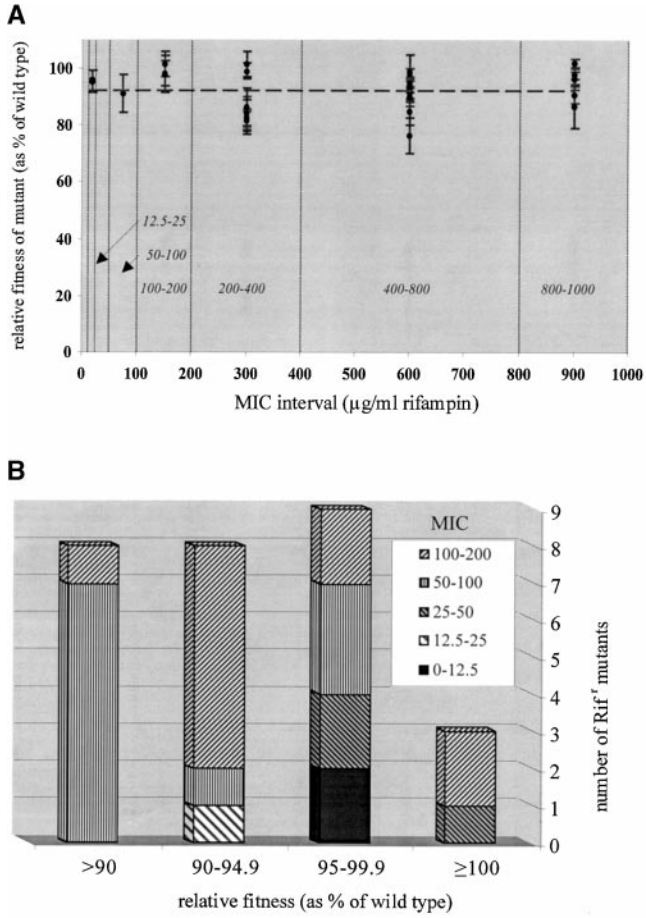

Figure 1.—(A) The cost of resistance for randomly selected mutants exhibiting different levels of resistance to rifampin. Cost is reported as percentage of growth/generation relative to the Rif^r K12 parental strain, measured in the absence of rifampin (*i.e.*, if resistance carries a 5% cost, then relative fitness is 95%). *Y*-axis error bars denote the 95% confidence interval of each fitness estimate on the basis of four independent replicates. The regression coefficient for the trend line shown is −0.02, $P = 0.29$, d.f. = 1. For purposes of simplicity, MIC values are implied with a point at the center of a given measured interval, but, in fact, the true MIC may be anywhere within the indicated interval. (B) The number of randomly selected mutants in each of four relative fitness categories. Each fitness category is subdivided to indicate the number of clones within that category exhibiting a given MIC.

Characteristics of representative Rif^r *E. coli* K12 clones: Given the broad range of phenotypes noted among the randomly selected Rif^r mutants, we sought to obtain a representative set of clones that could be analyzed in some detail to facilitate appreciation of specific relationships between *rpoB* genotype, MIC, and relative fitness. Nine clones were specifically selected as type designates (see materials and methods for detail of selection process). These mutants were derived from a wild-type [*mal*$^+$] K12 parent and were denoted rif-1 to rif-9.

The *rpoB* genotypes of these clones were determined by PCR amplification and sequence analysis of the first

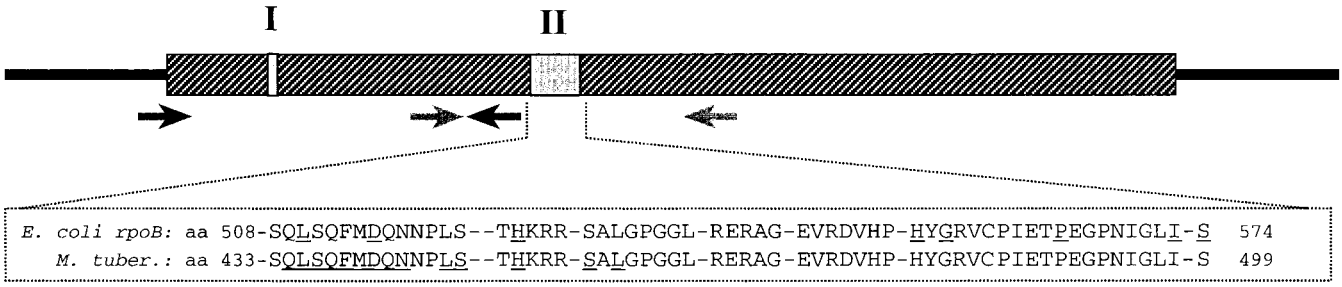

FIGURE 2.—Schematic of the *E. coli rpoB* gene, drawn to scale. Coding sequence is rendered as cross-hatched area, flanking sequence as solid bars. The two regions of the *E. coli* protein known to accumulate Rif^r mutations—cluster I (amino acids 140–148) and cluster II (amino acids 508–574)—are rendered as shaded boxes. The locations of PCR primers used to amplify clusters I and II are denoted with solid and shaded arrows, respectively. A protein sequence alignment for *E. coli* resistance cluster II with the analogous portion of the *M. tuberculosis* protein is shown; residues associated with rifampin resistance in *M. tuberculosis* (MUSSER 1995), as well as those involved with resistance (or secondary adaptation to resistance) in this study, are underscored. Nonequivalent amino acids are indicated with a dash.

2226 (of 4026) nucleotides of the RNA polymerase-β coding sequence (see Figure 2). The search for both primary mutations (and secondary intragenic substitutions; see below) was restricted to this portion of the *E. coli rpoB* coding sequence because this span encompasses all sites on the protein previously associated with rif resistance or suppression of resistance—the catalytic center of the protein, both principal Rif^r clusters (identified by saturation mutagenesis; see DISCUSSION), and multiple intragenic suppressor sites for temperature-sensitive rif resistance (reviewed by JIN and ZHOU 1996). The results of the *rpoB* sequence analysis, as well as the analysis of relative fitness and MIC, are summarized in Table 1.

As seen, in eight of nine Rif^r mutants (rif-4 being the exception) amino acid substitutions in either *rpoB* mutation cluster I or II were identified. The additional presence of non-*rpoB* mutations in a small number of mutants is implied by two observations: (i) we failed to identify an *rpoB* mutation rif-4 and (ii) mutants rif-5 and rif-7 possess the same *rpoB* mutation (Q148L) but exhibit measurably different relative fitnesses and MICs.

The cost of resistance among the nine mutants ranged from 16.5% (rif-8) to 4.6% (rif-4). Somewhat unexpect-

TABLE 1

Representative Rif^r *E. coli* K12 (MG1655) relative fitness

Mutant	% growth/generation (*vs.* K12 parent)[a] (±SEM)	MIC (μg/ml rif)[b]	*rpoB* substitution[c]	Mutation cluster[d]
K12	100 (±0.1)	0–12.5		
rif-1	86.4 (±1.4)	100–200	I572L (ATC to CTC)	II
rif-2	86.5 (±1.8)	25–50	L511Q (CTG to CAG)	II
rif-3	94.4 (±1.5)	100–200	H526L (CAC to CTC)	II
rif-4	104.6 (±1.4)	100–200	None identified in I or in II	
rif-5	96.3 (±0.9)	200–400	Q148L (CAG to CTG)	I
rif-6	91.3 (±1.0)	200–400	H526Y (CAC to TAC)	II
rif-7	89.6 (±2.1)	50–100	Q148L (CAG to CTG)	I
rif-8	83.5 (±1.9)	3000–4000	P564L (CCT to CTT)	II
rif-9	103.0 (±0.2)	100–200	D516G (GAC to GGC)	II

[a] Relative fitness (mutation cost) was determined via direct competition between Rif^r mutants (rif-1–9, here *mal*$^+$) and a Rif^s K12 MG1655 carrying a neutral marker [*mal*]. The standard error for the cost estimate (derived from 10 independent completion assays) is shown in parentheses. A value below 100% indicates that the strain tested was at a reproductive disadvantage relative to the wild-type reference strain. A fitness value in excess of 100% indicates that the strain tested exhibited a reproductive advantage relative to the wild type.

[b] The concentration interval indicated for MIC denotes the range within which the true MIC for rifampin exists.

[c] Substitutions are abbreviated as follows: original amino acid (single letter code) followed by amino acid number, followed by substituted amino acid (*e.g.*, I572L signifies an isoleucine has been replaced by a leucine at position 572). The altered codon is shown in parentheses.

[d] See Figure 2 for locations of resistance clusters I and II.

edly mutants rif-4 and rif-9 both demonstrated slight fitness advantages relative to the wild-type parent (P values for one-tailed t-test are 0.02 and 0.05, respectively; d.f. = 9). We cannot at this time rule out the possibility that these mutants harbor multiple substitutions and represent compensated, resistant mutants (see DISCUSSION).

Experimental evolution of *rpoB* mutants: From among these nine mutants, we selected four clones with extreme phenotypes as subjects for experimental evolution studies. These clones were rif-1 (low relative fitness, intermediate-level resistance), rif-2 (low relative fitness, low-level resistance), rif-8 (low relative fitness, high-level resistance), and rif-9 (fitness advantage, intermediate resistance). Evolution of the three low-fitness (rif-1, -2, and -8)mutants is considered first.

Multiple clones of each of the three low-fitness primary mutants were evolved over ~200 generations via serial passage both in the presence of rifampin (two clones for each mutant in 25 μg/ml rifampin) and in the absence of rifampin (three to four clones of each mutant). Both regimens were pursued so that we might learn (i) whether reversion or compensation occurs in the absence of drug selection, (ii) if continued drug selection fosters a set of potentially adaptive (compensatory) mutations different from no-drug selection (*e.g.*, discontinuation of drug use), and (iii) which, if any, conditions lead to selection for increased resistance levels.

After the period of evolution, single-clone isolates were subcultured from each evolved culture and evaluated for relative fitness (as compared to the progenitor strain), MIC, *rpoB* genotype, and transcription efficiency. The results of this analysis are shown in Table 2 and are described below.

Compensatory evolution *vs.* genetic reversion: From the final samples of each of the 200 generation evolved cultures, 500 colonies from LB agar were tested for sensitivity to rifampin by replica plating onto rifampin-agar. No Rif^s clones were detected.

Evolution of higher fitness in initially low fitness clones rif-1, -2, and -8: Of the evolved clones initiated from low-fitness drug-resistant mutants (rif-1, -2, and -8), all exhibited fitness gains (6–25%) relative to the parent mutant. This held true for strains evolved in the presence and absence of rifampin, but only the clones evolved under drug selection (25 μg/ml rifampin) exhibited heightened resistance at final accounting.

Approximately 50% of the clones analyzed demonstrated restoration of transcription efficiency (described further below) to near, or slightly better than, wild-type levels. But there was no evident correlation between amelioration of transcription and augmentation of resistance.

Five of 5 clones evolved in the presence of rifampin carry second-site mutations in the sequenced region of *rpoB*, while we detected a mutation in *rpoB* in only 3 of 12 clones evolved without rifampin. One mutation in the latter category is silent: GGT to GGG: Gly 556 Gly. The evolved mutant harboring this substitution (E-rif-1A) exhibits fully restored transcription activity. While various mechanisms might account for this result, we have not yet formally ruled out the possibility that this improvement is due to an additional, unidentified mutation.

We detected two independent second-site mutations derived from rif-8 occurring outside regions defined as resistance, or suppressor, clusters (L194R in E-rif-8A; R211P in ER-rif-8A). In addition, rif-8 twice arrived at the same evolutionary outcome, a second-site mutation at S574F: once when evolved under rifampin selection (ER-rif-8B) and once when evolved without (E-rif-8D). (Four clones of wild-type K12 MG1655 were evolved in parallel to serve as an "evolution control." The average gain in relative fitness of these four lines after ~200 generations of serial passage was 1.5%, not significantly different from zero in a two-tailed t-test [$P = 0.19$; d.f. = 9].)

Experimental evolution of the initial high fitness clone rif-9: Three clones of rif-9 (D516G) were serially passaged in rifampin, but not in rif-free medium, as this mutant already demonstrated high relative fitness in the absence of rifampin. Three independent evolution experiments yielded similar results: the rif-evolved clones exhibited increased MICs (150 μg/ml starting, 600 μg/ml concluding), aggregated second-site mutations in resistance cluster II (H554Y, S574Y), and slightly *diminished* fitness (1.3–2.5%) relative to their Rif^r parent as measured by competition in the absence of drug. In all three cases (for ER-rif-9A, -9B, and -9C) transcription efficiency of the rif-evolved clones declined relative to the parental rif-9.

Evolution of transcription efficiency: The transcription efficiency of a subset of primary Rif^r mutants and evolved clones was examined using a semiquantitative RT-PCR assay (see Figure 3). This assay measured the kinetics of production of a full-length induced transcript, lactose transacetylase (*lacA*; the 3′-most mRNA encoded on the lac operon), relative to that of an internal steady-state control, *recA*, as a function of time post-induction (see MATERIALS AND METHODS). We selected this method to measure transcription efficacy for a number of reasons: First, it affords extraordinary sensitivity of detection, especially for low numbers of transcripts (FOLEY *et al.* 1993). Second, it shows a high degree of fidelity for specimens similarly prepared and of nearly equivalent genotype. And third, it has been successfully employed in other situations requiring measurements of subtle variations in the timing of transcript production (HOUZE *et al.* 1996; GONG *et al.* 1999; MULLIGAN-KEHOE and RUSSO 1999). Instead of attempting to separate and measure the individual components of RNA polymerase function (initiation, clearance, elongation, and termination) we chose a single assay that would encompass the sum of these events.

TABLE 2

Characteristics of evolved Rif^r *E. coli* K12 (rif-1, rif-2, rif-8, rif-9)

Mutant	Relative fitness: % growth/generation (*vs.* mutant parent) (±SEM)	MIC (μg/ml rif)	Original *rpoB* substitution[a]	Mutation cluster[c]	Secondary *rpoB* substitution[a]	Mutation cluster	Transcription efficiency[d] (±SEM)
K12(MG1655)		0–12					0.058 (0.008)
rif-1	100	100–200	I572L (ATC to CTC)	II			0.029 (0.004)
E-rif-1A	119.9 (±1.5)	100–200	I572L (ATC to CTC)	II	G556G (GGT to GGG)	II	0.058 (0.014)
E-rif-1B	114.1 (±1.3)	100–200	I572L (ATC to CTC)	II	None identified		0.042 (0.009)
E-rif-1C	112.1 (±0.7)	100–200	I572L (ATC to CTC)	II	None identified		ND
E-rif-1D	114.2 (±0.8)	100–200	I572L (ATC to CTC)	II	None identified		ND
ER-rif-1A[b]	117.1 (±1.1)	400–800	I572L (ATC to CTC)	II	D516G (GAC to GGC)	II	0.051 (0.018)
rif-2	100	25–50	L511Q (CTG to CAG)	II			0.021 (0.001)
E-rif-2A	110.7 (±1.5)	25–50	L511Q (CTG to CAG)	II	None identified		0.067 (0.014)
E-rif-2B	110.8 (±1.2)	25–50	L511Q (CTG to CAG)	II	None identified		ND
E-rif-2C	105.9 (±1.4)	25–50	L511Q (CTG to CAG)	II	None identified		0.029 (0.002)
E-rif-2D	107.7 (±1.9)	25–50	L511Q (CTG to CAG)	II	None identified		ND
ER-rif-2A[b]	111.0 (±0.7)	800–1000	L511Q (CTG to CAG)	II	D516G (GAC to GGC)	II	0.060 (0.014)
ER-rif-2B[b]	113.2 (±1.9)	800–1000	L511Q (CTG to CAG)	II	D516G (GAC to GGC)	II	0.059 (0.009)
rif-8	100	3000–4000	P564L (CCT to CTT)	II			0.019 (0.003)
E-rif-8A	109.6 (±0.6)	3000–4000	P564L (CCT to CTT)	II	R211P (CGC to CCG)	X	0.040 (0.001)
E-rif-8B	113.5 (±1.0)	3000–4000	P564L (CCT to CTT)	II	None identified		0.027 (0.009)
E-rif-8C	114.1 (±0.7)	5000–6000	P564L (CCT to CTT)	II	None identified		ND
E-rif-8D	115.6 (±0.6)	5000–6000	P564L (CCT to CTT)	II	S574F (TCT to TTT)	II	0.031 (0.009)
ER-rif-8A[b]	114.9 (±3.0)	3000–4000	P564L (CCT to CTT)	II	L194R (CTG to CGG)	X	0.039 (0.019)
ER-rif-8B[b]	115.9 (±8.7)	5000–6000	P564L (CCT to TTT)	II	S574F (TCT to TTT)	II	0.044 (0.005)
rif-9	100	100–200	D516G (GAC to GGC)	II			0.059 (0.009)
ER-rif-9A[b]	97.5 (±0.7)	400–800	D516G (GAC to GGC)	II	S574Y (TCT to TAT)	II	0.038 (0.003)
ER-rif-9B[b]	98.7 (±0.7)	400–800	D516G (GAC to GGC)	II	H554Y (CAC to TAC)	II	0.047 (0.007)
ER-rif-9C[b]	98.6 (±0.5)	400–800	D516G (GAC to GGC)	II	S574Y (TCT to TAT)	II	ND

[a] All evolved strains retained their original (parental) mutation. Secondary mutations are additional substitutions identified in evolved strains. All evolved strains were subjected to sequence analysis across the first 2226 nucleotides of the *rpoB* gene.

[b] E strains were passaged without drug. ER strains were evolved under drug selection pressure (25 μg/ml rifampin).

[c] See Figure 2 for description of mutation clusters I and II. X refers to positions outside defined mutation clusters.

[d] See Figure 3 legend for details regarding empirical determination of transcription efficiency. Values here represent the average of two to four independent efficiency determinations. Standard error is shown in parentheses. ND, not determined.

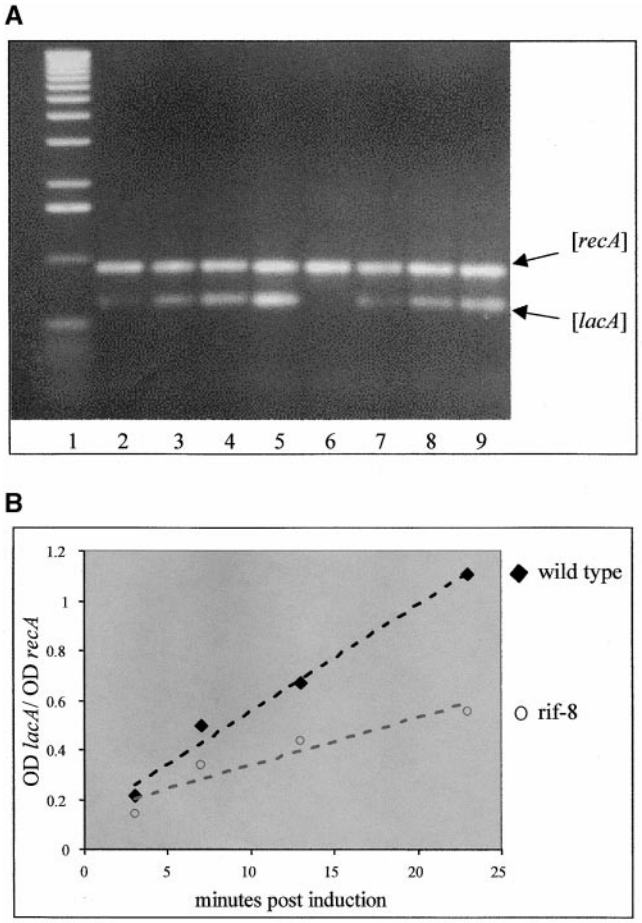

FIGURE 3.—Results of RT-PCR assay showing data representative of that used to determine the kinetic efficiency of mRNA transcript production for Rif^r *rpoB* mutants and evolved mutants. (A) Ethidium-bromide-stained agarose gel showing RT-PCR amplification products for *recA* and *lacA* transcripts (indicated by arrows). Lanes 2–5 and 6–9 contain products generated from total cell RNA harvested at 3, 7, 13, and 23 min post-*lacA* induction for wild-type K12 (lanes 2–5) and for rif-resistant mutant rif-8 (*rpoB* Pro 564 Leu; lanes 6–9). The RT-PCR reaction products shown here were processed using QIAGEN Qia-quick PCR purification reagents and were loaded on the gel such that *rec*A concentration was roughly constant. (B) Optical analysis of RT-PCR results. The slopes of the lines plotting transcript ratio *vs.* time postinduction are utilized here as a measure of overall RNA polymerase efficiency.

The results of this analysis indicate that the fitness burden of initial rif-resistance can be attributed to diminished transcription efficiency (see Figure 4) and that this functional lesion can, in many (but not all) cases, be ameliorated by second-site *rpoB* mutations that arise during experimental evolution. Mutations in other components of the RNA polymerase holoenzyme (α subunits, one β, one β′, and one σ factor), or in the final 1800 nucleotides of *rpoB*, may account for instances of transcription amelioration not accompanied by secondary mutations in the sequenced portion of *rpoB* (*e.g.*, E-rif-1B and E-rif-2A).

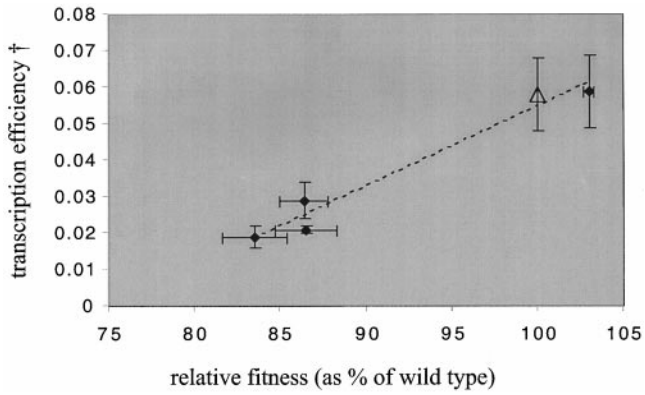

FIGURE 4.—Relationship between transcription efficiency and relative fitness for wild-type (△) K12 and the four Rif^r mutants (◆) selected for experimental evolution (rif-1, -2, -8, and -9). *x* and *y* error bars indicate the standard error for the respective measurements. The R^2 for the trend line shown is 0.97, the *P* value for the *x* variable is 0.002 (d.f. = 1). (†) See MATERIALS AND METHODS for details of transcription assay.

In two instances, increased relative fitness was not accompanied by increased transcription efficiency (E-rif-2C and E-rif-8B). In these cases something *other than* direct enhancement of transcription is presumed to account for the observed fitness gains.

Evolution for higher levels of rif-resistance: While we did not see evolution of increased rif resistance in rifampin-free cultures, the MICs of all mutants evolved in the presence of rifampin did increase. One explanation for this is that, although the level of drug used in these cultures (25 μg/ml) was below each strain's MIC, it nevertheless still imposed selection (see Figure 5). However, evolution via serial passage in rifampin can select both for higher replication rates *and* higher-level resistance; therefore we sought to determine if we could derive a similar genetic outcome as seen above, but with resistance as the sole selection. To test this, two parallel cultures of rif-2 (L511Q) bacteria (grown overnight) were plated directly on LB-rif agar at two concentrations of rifampin (200 and 800 μg/ml). Two resistant clones from each of the four plates were selected for DNA sequencing as described above. A D516G second-site mutation was detected in all eight independent clones. One clone selected from each 200-μg/ml and 800-μg/ml plate (R200-rif-2 and R800-rif-2) was phenotypically characterized and found to be indistinguishable from ER-rif-2A, a batch-culture-evolved isolate with equivalent *rpoB* genotype (see Table 3).

DISCUSSION

In this investigation we have examined the effects of RNA polymerase mutations associated with rifampin resistance on the fitness of bacteria and adaptation to the cost of this resistance. There were four principal findings:

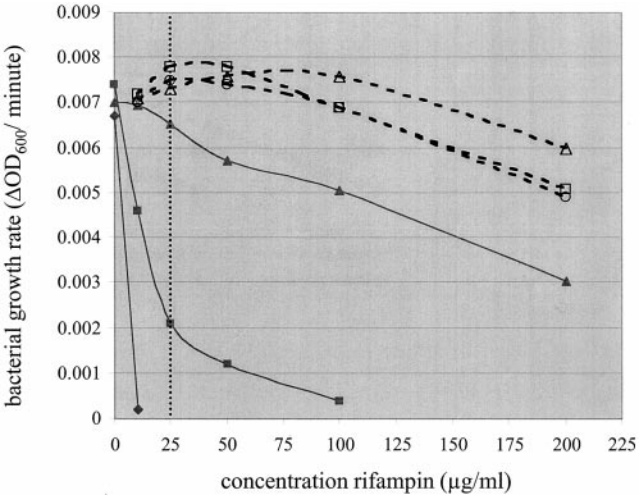

FIGURE 5.—Bacterial growth rate shown as a function of rifampin concentration for various K12 MG1655 derivatives. Plot shows representative data from an experiment in which K12 (Rif^s), two Rif^r derivatives (rif-2, rif-9), a derivative of rif-2 evolved in the presence of rifampin (ER-rif-2A), and two plate-selected derivatives of rif-2 (R200-rif-2, R800-rif-2) were assessed for growth rate in glucose-limited Davis minimal medium containing concentrations of rifampin ranging from 0 to 800 μg/ml (results shown to 200 μg/ml). The vertical dotted line indicates the level of rifampin at which rif-2 and rif-9 were initially selected and at which rif-2 was serially passaged for ~200 generations to yield ER-rif-2A. R200-rif-2 and R800-rif-2 were generated in a single step by plating rif-2 *E. coli* on agar plates at 200 or 800 μg/ml rifampin, respectively. The *rpoB* genotypes of the strains are as follows: L511Q, rif-2; D516G, rif-9; and L511Q + D516G, ER-rif-2A, R200-rif-2, R800-rif-2. (◆) K12MG1655, (■) rif-2, (▲) rif-9, (○) ER-rif-2A, (△) R200-rif-2, (□) R800-rif-2.

First, and somewhat unexpectedly, our analysis of randomly selected Rif^r mutants revealed no obvious association between the magnitude of rif resistance (MIC) and its allied cost (Figure 1A). This was unanticipated as one might have predicted that an *rpoB* mutation capable of rendering the polymerase more refractory to rifampin might also diminish the polymerase's affinity for its natural substrate(s), thus linking resistance level and polymerase function. This, however, appears to not hold true in this situation.

Second, these results indicate that there *is* a direct relationship between the fitness costs of *rpoB* mutations and their effects on transcription; the higher the cost, the greater the transcription impairment (Figure 4). Moreover, for nearly all of the evolved (fitness compensated) mutants, the observed gains in fitness were coincident with improved transcription efficiency (exceptions discussed below).

Third, the majority of the primary Rif^r *E. coli* mutants examined here exhibited some measurable fitness burden. Perhaps more significant, however, is the observation that modification of the β-subunit of RNA polymerase can occur without engendering any discernible effect on fitness, at least as it is measured by the *in vitro* procedures employed here.

And last, in step with previous *in vitro* studies of *E. coli*—for example, *in vitro* adaptation to the cost of ribosomal mutations conferring resistance to streptomycin (SCHRAG and PERROT 1996; SCHRAG *et al.* 1997) and adaptation to deleterious mini-*Tn10* insertions (MOORE *et al.* 2000)—adaptation to the fitness costs of RNA polymerase (rif-resistance) mutations occurs by mitigation of the deleterious effects of the resistance mutations (compensatory evolution) rather than through reversion to the drug-sensitive state. As noted, this compensatory evolution occurred in the absence as well as in the presence of the selecting antibiotic. The most conspicuous differences between the mutants evolved under continuing drug pressure and those evolved without were

TABLE 3

Characteristics of double mutants selected for higher-level resistance on plates *vs.* batch-culture-evolved equivalents

Test strain[a]	*rpoB* genotype	Reference strain[a]	*rpoB* genotype	Relative fitness of test strain[b]	IC_{50} test strain[c]	IC_{50} reference strain[c]
ER-rif-2A	L511Q/D516G	K12	Wild type	101.4 (0.5)	377.5 (7.5)	6.0 (1.0)
ER-rif-2A	L511Q/D516G	rif-9	D516G	99.4 (0.7)	—	144.6 (13)
R200-rif-2	L511Q/D516G	K12	Wild type	101.5 (1.2)	387.5 (7.5)	—
R200-rif-2	L511Q/D516G	rif-9	D516G	97.6 (1.2)	—	—
R800-rif-2	L511Q/D516G	K12	Wild type	101.4 (1.1)	390.0 (20)	—
R800-rif-2	L511Q/D516G	rif-9	D516G	97.8 (1.5)	—	—

[a] Relative fitness was determined via direct competition between a test strain carrying a neutral marker ([*mal*]) and an unmarked reference strain. (R200-rif-2 and R800-rif-2 are plate-evolved mutants; ER-rif-2A is a culture-evolved mutant.)

[b] See Table 1 legend for details of relative fitness determination.

[c] IC_{50} values indicate the concentration of rifampin (in micrograms/milliliters) at which the rate of bacterial growth is 50% maximal for a given strain. The value in parentheses is the standard error of the IC_{50} based on two to three independent determinations. The IC_{50} for rif-2 (L511Q) is 28.6 (±1.0).

the resultant changes in drug resistance level and the loci involved in compensation.

Our results suggest that the trend toward enhanced resistance was a function of the concentration of drug used for selection. That concentration (25 μg/ml) was well below the MIC of any of the resistant mutants yet was sufficient to reduce the growth rate of low or moderately resistant mutants (Figure 5), thus establishing the selective context for enhanced resistance. The exception to this generalization comes from a mutant (rif-8) that possessed a very high level of resistance at the outset. The evolutionary outcomes for this mutant were similar whether it was maintained in medium with or without rifampin.

There are, as always, a number of caveats associated with these results. First, it may be that Rif^r *rpoB* mutants, for which we were unable to detect a cost, may well have a profound cost in other habitats. Conversely, some of the costly Rif^r mutants may be more fit than their Rif^s ancestors in other environments.

Second, we cannot ignore the possibility that any cost attributable to resistance in, for example, the hyper-fit mutants rif-4 and rif-9 may have been masked either by a fortuitous compensatory mutation or possibly by the fact that the original MG1655 parent may have already been a "down" mutant owing to a lesion in *de novo* pyrimidine biosynthesis. If true, the latter could furnish a mechanism for augmented fitness in rif-9 or in any of the evolved mutants via increased RNA polymerase affinity for pyrimidines, or enhanced intracellular transport or retention of pyrimidine nucleotides. This possibility, however, hinges on the assumption that *E. coli* K12 MG1655 are to some degree starved, even when provided with an exogenous source of pyrimidines, which is probably not the case, since clones of the wild-type MG1655 evolved in parallel with the Rif^r mutants did not demonstrate any significant gains in fitness (something one might expect to see in bacteria undergoing adaptation to conditions of partial starvation). Nevertheless, this possibility warrants further investigation.

However, the former suggestion (that rif-4 and rif-9 harbor compensatory mutations) we consider more doubtful, as the probability of obtaining two mutually favorable mutations during the course of an ~20-generation culture ($N = 2 \times 10^{10}$ cells), with no mechanism to selectively enrich for either single-mutant subpopulation, is exceedingly low.

On the other hand, our results do suggest that *either* rif-5 *or* rif-7 does harbor a second, non-*rpoB* mutation. (Both have the same *rpoB* genotype but register measurably distinct phenotypes.) In this case, though, we are unable to discriminate between two hypotheses: (i) that the double mutant is a compensated strain (*e.g.*, rif-5; unlikely for the reason outlined above) or (ii) that the double mutant harbors a slightly deleterious mutation that has "piggy-packed" along with the rif-resistance substitution (*e.g.*, rif-7). Both possibilities can be addressed with allelic replacement studies.

A third caveat is that the compensatory mutants that evolved may *not* represent the final stage of evolution in the Rif^r populations maintained without drug selection. With additional generations, drug-sensitive revertants might indeed have ascended in the evolving populations, provided they had fitnesses greater than those of compensated mutants (Levin *et al.* 2000). In this study, however, compensated mutants were the first to arrive.

While compensatory adaptation to the costs of rif resistance may have been a foreseeable result, there were two aspects of compensation that we did not expect: (i) that adaptation to a functional lesion (here transcription) *can* be lesion-nonspecific; and (ii) that selection for enhanced target (polymerase) function does not always result in enhanced resistance.

Previous studies have shown that the fitness consequences of streptomycin resistance mutations in *rpsL* (ribosomal protein *S12*) map to diminished rates of protein elongation (Schrag and Perrot 1996) and that *in vitro* adaptation to the resistance cost entails compensation in other components of the translation apparatus (Bjorkman *et al.* 1998, 2000). In other words, the compensatory changes were specific for the functional lesion. Similarly, lesion-specific adaptation was inferred by Moore *et al.* (2000) in the context of adaptations to deleterious mini-*Tn10* transposon insertions in *E. coli*.

On the other hand, here we demonstrate two classes of fitness-compensated clones: those exhibiting ameliorated transcription and those not. (The former category may or may not harbor secondary mutations in *rpoB*.) From this we surmise that repair of fitness owing to transcription deficits can be achieved without a direct fix of the original functional lesion (nonspecific compensation). However, inasmuch as our RT-PCR assay was optimized for the detection of small differences in transcription efficacy between multiple genotypes (rather than for explicit measurement of a single genotype) we were still constrained in the absolute number of genotypes that could be analyzed. Consequently, though we have been able to demonstrate different potentialities for adaptation—lesion specific *vs.* nonspecific—we would be hard pressed to speculate as to the commonality of one vein relative to the other.

There also remains the possibility that some aspect of transcription function measured here only indirectly, such as level of active polymerase in the cell, may indeed be altered in those compensated strains that do not appear to exhibit ameliorated transcription. Our results argue against this explanation, however, since such changes would probably themselves engender some fitness costs due to wholesale changes in the regulation of gene expression (Jensen 1988). Nevertheless, this remains a viable possibility, which can be addressed in the future by performing direct measurements of the

individual components of transcription and by assaying intracellular concentrations of active polymerase.

The result that Rif^r *rpoB*s can evolve to states of heightened efficiency without demonstrating a collateral gain in resistance was the opposite of our expectations that improved *rpoB* efficiency would lead to enhanced apparent resistance. Other studies have shown that for drug-resistant *Plasmodium falciparum* (Reynolds and Roos 1998) and HIV (Borman *et al.* 1996), amelioration of fitness (or functional) deficits is coincident with increased resistance, even (as in the case of HIV) when evolution has taken place in the absence of drug selection. This observation might lead one to hypothesize that, if a secondary mutation makes a sickly resistant enzyme better able to perform its allotted task, then the mutation may *appear* to increase resistance even though it has not directly altered the enzyme's affinity for the drug. Thus we anticipated that evolution of low-fitness resistant mutants would result in the ascent of compensated mutants with ameliorated fitness and enhanced resistance. This was not the case. But this situation may vary for different pathogens, different drug targets, or different selections.

It is safe to say that few would suggest *E. coli* is an especially good surrogate for *M. tuberculosis*. Nevertheless our results are consistent with what has been observed for that pathogen with respect to fitness costs, MIC, and the composition of certain multiply substituted alleles, although the number of *M. tuberculosis* strains for which direct comparisons can be made is limited (Musser 1995; Billington *et al.* 1999; Pozzi *et al.* 1999). Single substitutions at the equivalent of D516 (rif-9) are found in ~2–8% of resistant isolates of *M. tuberculosis* (Musser 1995) and have been reported in resistant *Streptococcus pneumoniae* (Padayachee and Klugman 1999) and *Staphylococcus auereus* (Aubry-Damon *et al.* 1998), but valine substitutions at this site are in the minority for *M. tuberculosis* and have not been reported for the other pathogens.

It is worth noting that a similar combination of *rpoB* substitutions derived here multiple times among independent mutants (*e.g.*, L511Q + D516G) has also been observed in Rif^r *M. tuberculosis* (Pozzi *et al.* 1999). In the present study the L511Q + D516G arose under conditions of selection primarily for enhanced resistance. Yet this double mutant also exhibits a relative fitness either greater than or roughly equal to either single mutant (or the wild type; Table 3), suggesting that this allele is favored not merely as a combination of two low-level rest mutations but also because these particular mutations together boost resistance *and* preserve fitness. Whether the same will be true for complex alleles in *M. tuberculosis* in general, or even in this specific case, remains to be seen.

Finally, with respect to *M. tuberculosis*, the fact that selection for heightened resistance occurred for three of the four parent genotypes in this study strongly points to the idea that the combination of sublethal drug exposure and intermediate- or low-level resistance may have unfortunate repercussions in the context of extended clinical care, particularly if heightened resistance can be achieved without a decline in fitness.

Evolutionary studies of drug resistance have benefited substantially from practical *in vivo* systems used to estimate the costs of resistance and to provide more realistic environments for experimental evolution. This notwithstanding, using *in vitro* approaches remains an important means with which to address a number of as-yet-unanswered questions relevant to both studies of adaptive evolution and real-world drug resistance.

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