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Abstract

To study the cost of chromosomal drug resistance mutations to bacteria, we investigated the fitness cost of mutations that confer resistance to different classes of antibiotics affecting bacterial protein synthesis (aminocyclitols, 2-deoxystreptamines, macrolides). We used a model system based on an in vitro competition assay with defined Mycobacterium smegmatis laboratory mutants; selected mutations were introduced by genetic techniques to address the possibility that compensatory mutations ameliorate the resistance cost. We found that the chromosomal drug resistance mutations studied often had only a small fitness cost; compensatory mutations were not involved in low-cost or no-cost resistance mutations. When drug resistance mutations found in clinical isolates were considered, selection of those mutations that have little or no fitness cost in the in vitro competition assay seems to occur. These results argue against expectations that link decreased levels of antibiotic consumption with the decline in the level of resistance.
Fitness Cost of Chromosomal Drug Resistance-Conferring Mutations

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To study the cost of chromosomal drug resistance mutations to bacteria, we investigated the fitness cost of mutations that confer resistance to different classes of antibiotics affecting bacterial protein synthesis (aminocyclitols, 2-deoxystreptamines, macrolides). We used a model system based on an in vitro competition assay with defined Mycobacterium smegmatis laboratory mutants; selected mutations were introduced by genetic techniques to address the possibility that compensatory mutations ameliorate the resistance cost. We found that the chromosomal drug resistance mutations studied often had only a small fitness cost; compensatory mutations were not involved in low-cost or no-cost resistance mutations. When drug resistance mutations found in clinical isolates were considered, selection of those mutations that have little or no fitness cost in the in vitro competition assay seems to occur. These results argue against expectations that link decreased levels of antibiotic consumption with the decline in the level of resistance.

The increasing rates of recovery of antimicrobial-resistant microorganisms in hospital and community settings are of growing concern (1, 42). Resistance may emerge from a mutation in an intrinsic chromosomal gene or by acquisition of exogenous genetic material bearing resistance determinants. Resistance to antibiotics frequently reduces the fitness of bacteria in the absence of antibiotics; this is referred to as the “cost” of resistance (38). In mathematical models, the fitness cost of resistance is the primary parameter that determines both the frequency of resistance at any given level of antibiotic use and the rate at which that frequency will change with changes in antibiotic use patterns (3, 20, 21).

Restricted use of antibiotics is advocated not only to contain the dissemination of resistance but also to favor the nonexpan-
sion and, finally, the disappearance of the resistant bacteria already present in human and environmental reservoirs (3, 38). As a consequence of decreased use of antibiotics, rates of drug resistance usually fall but do not vanish, and stable rates of resistance in the apparent absence of direct selection pressure has been observed (9, 12, 32). It is not clear whether this persistence of resistant bacteria is due to (i) low-level antibiotic contamination that maintains the selective pressure, (ii) selection by means other than antibiotics, or (iii) the stability of resistance genes.

Analogous to the resistance mediated by exogenous genetic elements (13, 14, 19), chromosomal drug resistance-conferring mutations are commonly assumed to carry a fitness cost (38). This is supported by the observation that some drug resistance mutations selected in vitro involve a significant decrease in bacterial fitness (4, 20, 36); this fitness burden can subse-

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MATERIALS AND METHODS

Bacteria. Mycobacterium smegmatis rmb represents a variant of M. smegmatis mc2155 SMR5 with a single RNA allele; M. smegmatis normally contains two RNA operons (33). Spontaneous drug-resistant mutants were selected on Luria-Bertani agar plates containing clarithromycin (50 µg/ml) or amikacin (20 µg/ml). M. smegmatis mc2155 rmb L3 (strain 1682) is a streptomycin-sensitive derivative of mc155 with a single RNA allele and was used to obtain streptomycin-resistant mutants by selection on brain heart infusion (BHI) agar containing 20 µg of streptomycin per ml. In brief, suc2C counterselection was used to inactivate the rrb operon, resulting in strain mc2155 rmb (strain 1434) with a single RNA allele; subsequently, two additional rpsL genes were introduced into the chromosome, resulting in mc2155 rmb rpsL3 (39).

Drug-resistant mutants were purified by streaking them on selective agar and were immediately frozen in 15% glycerol to prevent further genetic changes; further passages were done in the absence of antibiotics.

Recombinant DNA techniques. Nucleic acids were analyzed by PCR-mediated sequencing with RNA-specific primers. Genomic DNA was isolated as described previously; standard methods were used for restriction endonuclease digestion of DNA and other manipulations. Single RNA mutations were introduced into integrative plasmid pMV361-H-rRNA (34), which carries the complete rmb operon of M. smegmatis, by PCR-mediated site-directed mutagenesis; plasmid pMV361 integrates once into the bacterial genome, thus providing a single copy of the mutated gene.

Transformation of mycobacteria. Strains M. smegmatis mc155 rmb and M. smegmatis rmb L3, which have single rRNA alleles, were used for the transformation experiments. Transformants were selected primarily on BHI plates containing hygromycin (50 µg/ml); subsequently, RecA-mediated gene conversion was used to obtain strains carrying homozygous mutant rRNA alleles (30); mutants were passaged once on selective agar. The homogeneity of the mutations introduced was verified by manual sequence analysis with 32P-labeled CTP and Sequenase (U.S. Biochemicals); MICs were determined to verify the resistance phenotype.

Determination of bacterial fitness. The cost of a resistance mutation was determined by direct competition against the drug-susceptible parental strain. Equal densities of drug-susceptible and drug-resistant strains were mixed and
incubated in antibiotic-free BHI medium; every 24 h, 0.05 ml of the grown culture was transferred into 5 ml of fresh BHI medium for growth. Aliquots were plated every 24 h onto drug-free BHI agar to count the number of colonies. The number of drug-resistant bacterial cells was determined by plating the colonies on BHI agar containing the respective drug (streptomycin, 10 μg/ml; amikacin, 50 μg/ml; clarithromycin, 50 μg/ml); the number of parental drug-susceptible cells was calculated as the total number of bacterial cells minus the number of drug-resistant bacterial cells. The experiments were performed in triplicate with three independent cultures. Serial dilutions of each aliquot were plated three times, and a weighted mean according to the level of dilution was used for analysis.

The difference in fitness between two competing strains at time \( t \) was computed by use of the following function:

\[
S_i = \ln \left( \frac{r_iS_i}{r_jS_j} \right)^{1/\gamma}
\]

where \( r_i \) and \( s_i \) denote the absolute number of drug-resistant and drug-susceptible cells at a given time \( t \), respectively, and \( r_{i-1} \) and \( s_{i-1} \) denote the number of drug-resistant and drug-susceptible cells at the preceding time point, respectively. \( S \) is called the selection coefficient at time \( t \). The quotient of the ratios of the cell numbers was standardized with the exponent 1/8 because cell numbers is equal to 0 if there is no difference in the natural logarithm of the quotient of the growth rates of the competing strains.

Drug-susceptible strains, respectively (8). Hence, \( S \) can be interpreted as the natural logarithm of the quotient of the growth rates of the competing strains. \( S \) is equal to 0 if there is no difference in fitness between the competing strains; \( S \) is negative if antibiotic resistance reduces bacterial fitness, and \( S \) is positive if resistance increases bacterial fitness relative to that of the drug-susceptible competitor strain.

Relative bacterial fitness at time \( t \) (fit) was calculated as

\[
\text{fit} = 1 + S_i
\]

The cost per generation (cpg) was calculated as

\[
cpg = 1 - e^{\gamma}
\]

The cost per generation can be interpreted as 1 minus the quotient of the growth rates. An analysis of variance was performed with \( S_i \) as the dependent variable and the experiment as the random explanatory factor \( \pi \) (35):

\[
S_i = \alpha_0 + \pi_i + e_i
\]

where \( \alpha_0 \) is a nonrandom intercept, \( S_i \) denotes the selection coefficient at time \( t \) and experiment \( j \), and \( e_i \) is the normal distributed error term at time \( t \) and experiment \( j \). The overall \( S_i \) was estimated and tested against 0 on the basis of the null hypothesis (H0) that \( \alpha_0 \) is equal to 0. The data for different time points from one experiment were assumed to be independent. Given that the random factor “experiment” had no significant influence, subsequent analyses were performed without this factor and the data were assumed to be fully independent of each other.

To eliminate dilution errors and to determine the cost of resistance more precisely for no-cost and low-cost resistance mutations, additional experiments were performed. Aliquots from the competition assay were taken at time zero (\( t_0 \) 0 h) and time 6 (\( t_6 \); 144 h, corresponding to 48 generations) and plated onto nonselective agar; subsequently, \( >100 \) individual colonies were picked at random and the resistance phenotype was investigated for each colony individually to determine the ratio of susceptible and resistant cells (at least three independent experiments were performed for each mutant selected). This situation can be reflected by the following cross tabulation:

\[
\begin{array}{c|c|c|c}
  & S & \text{R} \\
  \hline
  S & 1 & 0 & 1 \\
  \text{R} & 0 & 1 & 0 \\
  \hline
  \text{OR} & 1 & 1 & 1 \\
\end{array}
\]

Estimations of OR can be obtained by a logistic model (17):

\[
\ln \frac{p}{1-p} = \beta_0 + \beta_1 \cdot t + \gamma
\]

where \( p \) is the probability that a drug-susceptible colony will be picked, \( t \) is the time, \( \gamma \) is a random parameter to allow variability between the experiments, and \( \beta_0 \) and \( \beta_1 \) are the regression parameters. \( \beta_1 \) is an estimate for log OR, and a test for \( H_0 \) that \( \beta_1 \) is equal to 0 can be used to test if \( S_i \) is 0. To test whether the bacterial fitness differed between bacteria with mutations introduced by site-directed mutagenesis and spontaneous drug-resistant mutants, an interaction term between time and the type of the mutation is added to the model (7):

\[
\ln \frac{p}{1-p} = \beta_0 + \beta_1 \cdot t + \beta_2 \cdot \text{type} + \gamma
\]

If the test for \( H_0 \) that \( \beta_2 \) (another regression parameter) is equal to 0 is not significant, the preceding model would be sufficient and no influence of the type of mutation on \( S_i \) would be present. If the test is significant, an interaction term would be necessary for the model; i.e., the type of mutation would have a significant influence on \( S_i \). All statistical calculations were performed with SAS release 8.01.

**Strains used for competition experiments.** The following strain combinations were used in the competition experiments: spontaneous streptomycin-resistant mutants of mc155 rmb (strains 1644, 1646, and 1674) versus drug-susceptible strain mc155 rmb (strain 1434); spontaneous streptomycin-resistant rmb plnL33 mutants (strains 1592, 1630, 1632, 1634, 1636, and 1647) versus a drug-resistant rmb plnL33 strain (strain 1682); spontaneous amikacin-resistant rmb mutants (strains 1181, 1183, 1184, 1185, 1187, and 1194) versus drug-susceptible rmb strains (strains 1179, 1186, and 1193); and spontaneous clarithromycin-resistant rmb mutants (strains 1082, 1086, and 1089) versus a drug-susceptible rmb strain (strain 1179).

mc155 rmb plnL33 transformed with pMV361-H-rRNA2058 (strain 1691) was used as a drug-susceptible competitor strain (containing two wild-type 16S rRNA alleles, one chromosomal and one plasmid derived) for the strains into which the respective streptomycin resistance mutation was introduced by transformation with mutagenized plasmid pMV361-H-rRNA2058 and subsequent RecA-mediated gene conversion (strains 1701, 1702, and 1703).

An rmb mutant transformed with pMV361-H-rRNA2058 (strain 1516) was used as a susceptible competitor for the strains into which the 1408A-G resistance mutation was introduced by transformation with mutagenized plasmid pMV361-H-rRNA2058 and subsequent RecA-mediated gene conversion (strains 1512, 1513, 1514-A, and 1515-B).

An rmb mutant transformed with pMV361-H-rRNA (strains 2004, 2005) was used as a susceptible competitor for the strains into which the 2058A-G (strains 1998 and 1999) and 2059A-G (strains 2006 and 2007) resistance mutations were introduced by transformation with mutagenized plasmid pMV361-H-rRNA.

For a summary of the strains used in this investigation, see Table 1.

**Frequency of resistance mutations in clinical isolates.** The literature was searched for published molecular biology-based analyses of mycobacterial drug resistance by use of the key words mycobacteria and drug resistance. Elimination of reviews and descriptions of clonal outbreaks and multiple isolates resulted in identification of 93 unique streptomycin-resistant strains of Mycobacterium tuberculosis for which the mechanism of resistance was characterized at the molecular level: 79 of these strains had one of the resistance mutations investigated here and were included in the analysis; 14 strains had a resistance mutation not investigated in this study (in Rpsl at amino acid 88 and in rrs at positions 501, 912, and 913).

**RESULTS**

Ribosomal resistance due to point mutations in ribosomal nucleic acids (rrn) has been recognized as a cause of clinically acquired resistance to drugs that affect the translation apparatus in such important pathogens as *M. tuberculosis*, *Helicobacter pylori*, and *Mycoplasma pneumoniae* (10, 11, 16, 22, 23, 24, 29, 31, 33, 40, 41).

A genetically well defined system was chosen to investigate the cost of resistance of *rrn* mutations to avoid the influence of an unknown or ill-defined genetic background, like those that occur in clinical isolates. Derivatives of *M. smegmatis* with a
single rRNA allele (33) were used to saturate the ribosomal nucleic acids with drug resistance-conferring mutations by plating them on selective agar. A total of 40 independent in vitro mutants were obtained. The resistance mutations were mapped and found to locate in the **rrn** operon. With the exception of the 16S rRNA 524G→C mutation (which was detected in 12 of 22 streptomycin-resistant mutants investigated), all other mutations obtained are representative of those mutations found in pathogens with clinically acquired drug resistance (Table 2).

**Fitness costs of spontaneous mutations.** The changes in the relative fitness of *M. smegmatis* strains carrying mutations that confer ribosomal drug resistance were examined in a competition model in the absence of antibiotics. The model was validated by using well-characterized mutations at amino acid 42 of the **rpsL** gene, which confers resistance to streptomycin;

---

**TABLE 1. Strains used in this study**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Transformed plasmid</th>
<th>Relevant phenotype</th>
<th>Parental strain</th>
</tr>
</thead>
<tbody>
<tr>
<td>0058 mc²155</td>
<td>Wild type</td>
<td>STR'</td>
<td>0058</td>
<td></td>
</tr>
<tr>
<td>003 mc²155 SMR5</td>
<td>rpsL 42 Lys→Arg</td>
<td>KAN'</td>
<td>0058</td>
<td></td>
</tr>
<tr>
<td>1434 mc²155 rmB</td>
<td>rmB::aph</td>
<td>KAN' GEN'</td>
<td>1434</td>
<td></td>
</tr>
<tr>
<td>1682 mc²155 rmB rpsL3⁺</td>
<td>rmB::aph rpsL3⁺</td>
<td>KAN'</td>
<td>0003</td>
<td></td>
</tr>
<tr>
<td>1179 rmB</td>
<td>rmB::aph</td>
<td>KAN'</td>
<td>0003</td>
<td></td>
</tr>
<tr>
<td>1193 rmB</td>
<td>rmM::aph</td>
<td>KAN'</td>
<td>0003</td>
<td></td>
</tr>
<tr>
<td>1186 rmA</td>
<td>rmB::aph</td>
<td>KAN'</td>
<td>0003</td>
<td></td>
</tr>
</tbody>
</table>

**Spontaneous resistant mutants**

- 1644 RpsL 42 Lys→Asn STR' 1434
- 1646 RpsL 42 Lys→Thr STR' 1434
- 1674 RpsL 42 Lys→Arg STR' 1434
- 1592 rrs 526C→T STR' 1682
- 1630 rrs 526C→C STR' 1682
- 1632 rrs 523A→C STR' 1682
- 1634 rrs 522C→T STR' 1682
- 1636 rrs 522C→T STR' 1682
- 1647 rrs 524G→T STR' 1682
- 1682 Rrs 524G→T STR' 1682
- 1181 rrs 1408A→G AMK' 1179
- 1183 rrs 1408A→G AMK' 1179
- 1184 rrs 1408A→G AMK' 1179
- 1185 rrs 1408A→G AMK' 1179
- 1187 rrs 1408A→G AMK' 1179
- 1194 rrs 1408A→G AMK' 1179
- 1082 rrl 2058A→G CLR' 1179
- 1086 rrl 2058A→G CLR' 1179
- 1089 rrl 2059A→G CLR' 1179

**Genetically engineered strains**

**Streptomycin resistant**

- 1691 pMV361-H-rRNA2058G HYG' CLR' 1682
- 1683 pMV361-H-rRNA 524C 2058G HYG' CLR', STR' 1682
- 1684 pMV361-H-rRNA 524C 2058G HYG' CLR', STR' 1682
- 1687 pMV361-H-rRNA 526T 2058G HYG' CLR', STR' 1682
- 1688 pMV361-H-rRNA 526T 2058G HYG' CLR', STR' 1682
- 1689 pMV361-H-rRNA 526T 2058G HYG' CLR', STR' 1682
- 1699 pMV361-H-rRNA 523C 2058G HYG' CLR', STR' 1682
- 1700 pMV361-H-rRNA 523C 2058G HYG' CLR', STR' 1682

**Aminoglycoside resistant**

- 1516 pMV361-H-rRNA2058G HYG' CLR' 1193
- 1512 pMV361-H-rRNA 1408G 2058G HYG' CLR', AMK' 1193
- 1513 pMV361-H-rRNA 1408G 2058G HYG' CLR', AMK' 1193
- 1514A pMV361-H-rRNA 1408G 2058G HYG' CLR', AMK' 1193
- 1515B pMV361-H-rRNA 1408G 2058G HYG' CLR', AMK' 1193

**Clarithromycin resistant**

- 2014 pMV361-H-rRNA HYG' CLR' 1193
- 2015 pMV361-H-rRNA HYG' CLR' 1193
- 1998 pMV361-H-rRNA2058G HYG' CLR' 1193
- 1999 pMV361-H-rRNA2058G HYG' CLR' 1193
- 2006 pMV361-H-rRNA 2059G HYG' CLR' 1193
- 2007 pMV361-H-rRNA 2059G HYG' CLR' 1193
the mutations chosen have been characterized previously and are known to affect bacterial fitness and virulence to different degrees: the nonrestrictive Lys→Arg mutation is a no-cost resistance mutation which does not affect fitness, and the restrictive Lys→Asn and Lys→Thr mutations have significant effects on fitness (4, 5, 36). The growth of the mutant with the nonrestrictive mutation was similar to that of the wild type, while the wild type outgrew the restrictive mutants (Fig. 1; the costs of resistance were calculated as 14.1 and 14.9% per generation, respectively). The costs of these resistance determinants are in close agreement with those observed in previous experiments for resistant Escherichia coli and Salmonella enterica serovar Typhimurium strains (5, 36).

As shown in Fig. 1, three rrn mutations causing resistance to streptomycin, i.e., 523A→C, 526C→T, and 522C→T, had costs of resistance of 5.9, 8.5, and 8.9% per generation, respectively. The strain with the 16S rRNA mutation 524G→C, which is the streptomycin resistance mutation most frequently generated in vitro but which is not found in clinical drug-resistant isolates, had a greater than 30% growth disadvantage per generation relative to that of the streptomycin-resistant parental strain in this assay (39).

In contrast to their effect on resistance to streptomycin, alterations of ribosomal proteins do not play a major role in acquired chromosomal resistance to 2-deoxystreptamines and macrolides, but resistance is predominantly due to mutations in rrn (24, 29, 33). None of the resistance-conferring mutations (16S rRNA 1408A→G, 23S rRNA2058A→G, 23S rRNA2059A→G) was found to affect bacterial fitness to a major degree (Table 3).

The fitness cost was determined more precisely for resistance mutations with low or no costs (1408G, 2058G, and 2059G; see Materials and Methods for details). Mutations 1408G and 2059G demonstrated small but significant fitness costs (2 to 3% per generation). Mutation 2058G had a non-significant cost of 0.5 to 1.4% per generation, well within the range of the no-cost RpsL 42 Arg mutation (Table 4).

**Low fitness costs are not due to compensatory mutations.** The frequency with which mutants appeared on the selection plate corresponded to the frequency of appearance of single point mutations in M. smegmatis rRNA (10⁻⁸ to 10⁻⁹) (33). The frequency of a double mutation is so low that it would be unlikely that any of the mutants studied would have another, compensatory mutation. This tentative conclusion was verified experimentally. Selected mutations were introduced by recombinant DNA techniques to ensure that the strains used for competition were isogenic; these mutations were 16S rRNA 523A→C, 524G→C, 526C→T, 1408A→G, 23S rRNA2058A→G, and 2059A→G. The strains into which all of the mutations were introduced by site-directed mutagenesis showed fitness characteristics similar to those of the spontaneous drug-resistant mutants (Fig. 1; Tables 3 and 4); a significant difference between genetic and spontaneous mutants could not be found for any of the mutations (523C, P = 0.73; 524C, P = 0.66; 526T, P = 0.65; 1408G, P = 0.73; 2058G, P = 0.06; 2059G, P = 0.84). These experiments appear to disprove

<table>
<thead>
<tr>
<th>Type of rRNA mutation</th>
<th>No. of spontaneous mutants obtained by selective plating/total no. of isolates tested</th>
<th>Resistance phenotype</th>
<th>MIC (µg/ml)</th>
<th>Resistance mutation observed in clinical pathogens⁴⁶</th>
</tr>
</thead>
<tbody>
<tr>
<td>16S rRNA 522 C→T</td>
<td>2/22</td>
<td>Streptomycin</td>
<td>250</td>
<td>+</td>
</tr>
<tr>
<td>16S rRNA 523 A→C⁵</td>
<td>1/22</td>
<td>Streptomycin</td>
<td>125</td>
<td>+</td>
</tr>
<tr>
<td>16S rRNA 524 G→C⁵</td>
<td>12/22</td>
<td>Streptomycin</td>
<td>&gt;500</td>
<td>–</td>
</tr>
<tr>
<td>16S rRNA 526 C→T</td>
<td>7/22</td>
<td>Streptomycin</td>
<td>125</td>
<td>+</td>
</tr>
<tr>
<td>16S rRNA 1408 A→G⁶</td>
<td>10/10</td>
<td>2-Deoxystreptamines</td>
<td>&gt;500⁴</td>
<td>+</td>
</tr>
<tr>
<td>23S rRNA2058 A→G⁷</td>
<td>7/8</td>
<td>Macrolides, lincosamides, streptogramin B</td>
<td>128⁵</td>
<td>+</td>
</tr>
<tr>
<td>23S rRNA2059 A→G⁷</td>
<td>1/8</td>
<td>Macrolides, streptogramin B</td>
<td>128⁵</td>
<td>+</td>
</tr>
</tbody>
</table>

⁴ The indicated mutations were also introduced into M. smegmatis by recombinant DNA techniques.
⁵ A total of 40 independent laboratory mutants were obtained in vitro, 22 were resistant to streptomycin, 10 were resistant to 2-deoxystreptamines, and 8 were resistant to macrolides.
⁶ The MICs of drug-susceptible M. smegmatis strains are as follows: streptomycin, 1.0 µg/ml; amikacin, 0.6 µg/ml; clarithromycin, 0.06 µg/ml.
⁷ Tabulated from previous reports (10, 11, 16, 22, 23, 24, 29, 31, 33, 40, 41).
⁸ Amikacin was chosen as a representative of the 2-deoxystreptamines.
⁹ Clarithromycin was chosen as a representative of the macrolides.

**TABLE 3. Sₜ and cost of resistance of mutations investigated**

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Sₜ (±1 SE [10⁻⁷])</th>
<th>P value</th>
<th>Cost per generation (%)</th>
<th>Relative fitness (95% confidence interval)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Streptomycin</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RpsL 42 Arg</td>
<td>−1.0 ± 1.5</td>
<td>0.48</td>
<td>0.99</td>
<td>95.8–102.1</td>
</tr>
<tr>
<td>RpsL 42 Asn</td>
<td>−15.2 ± 1.9</td>
<td>&lt;0.01</td>
<td>14.10</td>
<td>79.9–89.6</td>
</tr>
<tr>
<td>RpsL 42 Thr</td>
<td>−16.2 ± 3.3</td>
<td>&lt;0.01</td>
<td>14.98</td>
<td>76.4–91.1</td>
</tr>
<tr>
<td>522 T⁶</td>
<td>−9.3 ± 1.3</td>
<td>&lt;0.01</td>
<td>8.88</td>
<td>88.0–93.3</td>
</tr>
<tr>
<td>523 C⁶</td>
<td>−6.1 ± 1.5</td>
<td>&lt;0.01</td>
<td>5.92</td>
<td>90.7–97.0</td>
</tr>
<tr>
<td>523 C⁶</td>
<td>−5.4 ± 1.4</td>
<td>&lt;0.01</td>
<td>5.26</td>
<td>91.4–97.7</td>
</tr>
<tr>
<td>524 C⁶</td>
<td>−39.3 ± 5.1</td>
<td>&lt;0.01</td>
<td>32.50</td>
<td>44.5–57.0</td>
</tr>
<tr>
<td>524 C⁶</td>
<td>−41.6 ± 2.9</td>
<td>&lt;0.01</td>
<td>34.03</td>
<td>51.4–65.4</td>
</tr>
<tr>
<td>526 T⁶</td>
<td>−8.9 ± 1.8</td>
<td>&lt;0.01</td>
<td>8.52</td>
<td>77.1–95.0</td>
</tr>
<tr>
<td>526 T⁶</td>
<td>−7.9 ± 0.9</td>
<td>&lt;0.01</td>
<td>7.60</td>
<td>90.2–94.0</td>
</tr>
</tbody>
</table>

| 2-Deoxystreptamines |                   |         |                         |                                           |
| 1408G⁶            | −1.7 ± 2.5       | 0.52    | 1.69                    | 92.6–104.1                                 |
| 1408G⁶            | −3.0 ± 2.2       | 0.13    | 2.96                    | 92.4–101.7                                 |

| Macrolides |                   |         |                         |                                           |
| 2058G⁷        | −2.0 ± 2.1       | 0.36    | 1.98                    | 93.4–102.6                                 |
| 2059G⁷        | −2.0 ± 3.4       | 0.57    | 1.98                    | 90.5–105.6                                 |

⁵ Determined by analysis of variance (F test).
⁶ Estimated by direct competition against an equal density of the drug-susceptible parental strain.
⁷ Spontaneous mutants.
⁸ Recombinant mutants.
the hypothesis that compensatory mutations which could have compensated for an initial cost of resistance had taken place during the procedure used for the isolation of the spontaneous drug-resistant mutants (4, 5, 18, 36).

**In vivo frequency of resistance mutations.** The multiplicity of different chromosomal mutations that conferred resistance to streptomycin *(rpsL and *rrm*) allowed us to test if the frequency of a resistance mutation in clinical drug-resistant isolates is a function of the relative fitness of the mutation, as determined with the model system presented here. To address this issue, previously described mutations that confer resistance to streptomycin in clinical isolates of *M. tuberculosis* were determined from the literature. *M. tuberculosis*, like most other species of clinically relevant mycobacteria, carries a single rRNA operon in its genome, which allows a comparison with results obtained in our model system using single rRNA allelic strains of *M. smegmatis*. Figure 2 demonstrates that a strong correlation between the frequency of clinical isolates with a given resistance mutation and the relative fitness of the corresponding mutation exists. Assuming that the fitness of resistant strains is less than or equal to the fitness of the drug-susceptible parental strain, the correspondence function should be asymptotic to $y = 100\%$ and can be described by $y = 1 - a \cdot e^{b - c}$. The model was fit to the measured data by using an implementation of the nonlinear least-squares Marquardt-Levenberg algorithm; and the values of the parameters were estimated to be as follows: $a = 0.18$, $b = 3.13$, and $c = 0.46$.

To test if the higher frequency of particular mutations in vivo is due to the more frequent generation of those mutations, i.e., a higher mutation rate, we plotted the frequency of resistance mutations generated in vitro in the laboratory versus the frequency of the corresponding mutation investigated. The mean of at least two independent experimental determinations is given. (A) RpsL 42 Lys → Arg (●, strain 1674); RpsL 42 Lys → Asn (■, strain 1644); RpsL 42 Lys → Thr (▲, strain 1646). (B) 16S rRNA 522C → T (●, strains 1634 and 1636); 16S rRNA 523A → C (■, strain 1632); 16S rRNA 524G → C (▲, 1647); 16S rRNA 526C → T (■, strains 1592 and 1630). (C) 16S rRNA 522A → C (●, strains 1699 and 1700); 16S rRNA 524G → C (▲, strains 1684 and 1683); 16S rRNA 526C → T (■, strains 1687, 1688, and 1689). Cultures were inoculated with equal numbers of the drug-resistant mutant and the drug-susceptible parental strain, and the ratio of the number of resistant organisms/number of susceptible organisms recovered was determined after the indicated number of generations; *M. smegmatis* has a doubling time of 3 h.

**TABLE 4.** $S_{i}$ and relative fitness of mutations investigated

<table>
<thead>
<tr>
<th>Mutation</th>
<th>No. $^c$</th>
<th>$S_{i}$ (±1 SE $^d$)</th>
<th>$P$ value</th>
<th>Cost per generation (%)</th>
<th>Relative fitness (95% confidence interval)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RpsL 42 Arg</td>
<td>9</td>
<td>$-0.98 \pm 0.37$</td>
<td>0.98</td>
<td>98.23–99.74</td>
<td></td>
</tr>
<tr>
<td><em>rrm</em> 1408G$^b$</td>
<td>18</td>
<td>$-3.10 \pm 0.74$</td>
<td>&lt;0.01</td>
<td>3.05</td>
<td>95.45–98.35</td>
</tr>
<tr>
<td><em>rrm</em> 1408G$^b$</td>
<td>13</td>
<td>$-2.88 \pm 0.59$</td>
<td>&lt;0.01</td>
<td>2.84</td>
<td>95.96–98.28</td>
</tr>
<tr>
<td><em>rrm</em> 2058G$^b$</td>
<td>6</td>
<td>$-0.46 \pm 0.29$</td>
<td>0.12</td>
<td>0.46</td>
<td>98.86–100.12</td>
</tr>
<tr>
<td><em>rrm</em> 2058G$^b$</td>
<td>3</td>
<td>$-1.40 \pm 0.40$</td>
<td>&lt;0.01</td>
<td>1.39</td>
<td>97.81–99.39</td>
</tr>
<tr>
<td><em>rrm</em> 2059G$^b$</td>
<td>9</td>
<td>$-2.63 \pm 0.83$</td>
<td>&lt;0.01</td>
<td>2.60</td>
<td>95.74–99.00</td>
</tr>
<tr>
<td><em>rrm</em> 2059G$^b$</td>
<td>6</td>
<td>$-2.47 \pm 0.23$</td>
<td>&lt;0.01</td>
<td>2.44</td>
<td>97.08–98.99</td>
</tr>
</tbody>
</table>

$^a$ Estimated by direct competition against an equal density of the drug-susceptible parental strain.

$^b$ Spontaneous mutants.

$^c$ Recombinant mutants.

$^d$ Number of independent experiments.

**DISCUSSION**

Competition experiments of the type used here are able to detect statistically significant fitness differences larger than 2% per generation (4, 5, 18, 36). Although smaller fitness differences might have some effects, our data suggest the following conclusions: (i) chromosomal resistance mutations found in laboratory (in vitro)-derived spontaneous mutants may be cost neu-
As a starting point, we searched the literature for different resistance mutations occurring in a variety of pathogens; the experimental investigations, however, were conducted with M. smegmatis, which was used as a model system. We suggest that the findings observed in our model system allow us to draw more general conclusions, as comparable costs of resistance were determined for mutations investigated previously in other microorganisms, i.e., rpsL mutations 42 Lys → Arg, Lys → Asn, Lys → Thr, which exhibited fitness costs of 0, 14, and 15%, respectively, in E. coli, S. enterica serovar Typhimurium, and M. smegmatis (5, 36) (Table 3).

Different constraints have been postulated to affect the translation machinery under various in vitro and in vivo conditions (2, 25). The selection of mutant genotypes observed in vivo and determined in the in vitro competition model to be no-cost mutations suggests that the measurements obtained in vitro adequately reflect the measurements obtained in the in vivo situation. While determination of a mutation as no cost (within the limitations that accompany the use of a model organism) conflicts with the assumption that resistance mutations are costly, one cannot exclude a hypothetical condition in which some sort of difference in fitness would become manifest. However, the selection of no-cost resistance mutations observed in clinical isolates exposed to complex and fluctuating conditions as well as heterogeneous habitats makes this possibility unlikely.

In contrast to competitive environments such as clinical in vivo conditions, the use of a compartmentalized experimental environment, i.e., solid medium, for selection of drug-resistant variants largely irrespective of a fitness cost (variants with all possible alleles capable of surviving the selection procedure will grow). The observation that clinically acquired resistance rarely involves mutations with a cost can be ascribed to two different mechanisms: (i) a priori, mutations that confer a substantial cost (e.g., mutations for resistance to aminoglycosides of the 2-deoxystreptamine type) do not arise even under in vitro conditions in the laboratory, as the functional constraints of the target molecule seem to allow only mutations that are both cost neutral and able to produce a resistance phenotype; and (ii) mutations that confer a cost occur under in vitro conditions in the laboratory (e.g., mutations for resistance to streptomycin), but there is selection in vivo for low-cost resistance mutations. This selection might be explained by fluctuating environments, i.e., expansion of mutants experiencing a low cost of resistance in the absence of antibiotics during periods in which selection for antibiotic resistance is removed.

While our findings do not deny the existence of compensatory mutations (4, 5, 36), they indicate that under natural in vivo conditions these may be of minor relevance to the epidemiology of drug resistance. The likelihood that a costly resistance mutation is ameliorated by an additional compensatory mutation is far greater than the likelihood that a no-cost resistance mutation (occurring roughly at the same frequency as a costly resistance mutation) is ameliorated, making the scenario of costly but compensated resistance mutations unlikely in nature. In principle, resistance mutations that are acquired in vivo and that carry a cost might therefore be found only when a cost-neutral resistance mutation does not exist for a given drug (5, 27). The eventual observation that a costly
TABLE 5. Fixation and elimination of drug resistance mutations carrying different costs of resistance

<table>
<thead>
<tr>
<th>Cost of resistance (%)</th>
<th>Fixation by compensatory mutation&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Elimination&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>912</td>
<td>1,139</td>
</tr>
<tr>
<td>5</td>
<td>359</td>
<td>449</td>
</tr>
<tr>
<td>10</td>
<td>175</td>
<td>218</td>
</tr>
<tr>
<td>20</td>
<td>83</td>
<td>103</td>
</tr>
</tbody>
</table>

<sup>a</sup> Assuming a frequency of 10<sup>−6</sup> of a compensatory mutation restoring fitness; the values are the number of generations (t) required for the compensated resistance genotype to become fixed, i.e., to represent 99% of the drug-resistant population, determined by the following formula: \( t = \ln\left(\frac{r_c}{r_c + r_{c-1}}\right)/\ln(1 - \text{cost per generation}) \), where \( r_c \) and \( r_{c-1} \) are absolute numbers of resistant mutants with a compensatory mutation.

<sup>b</sup> Calculated for a ratio of resistant to susceptible organisms of 99:1 at time zero; the values are the number of generations (t) to reach peaks of spontaneous drug resistance frequency (10<sup>−6</sup>) determined by the following formula: \( t = \ln(10^{−6} / r_0/S_0)/\ln(1 - \text{cost per generation}) \).

resistance mutation will emerge in a single patient (e.g., streptomycin resistance mutation RpsL 42 Lys→Thr, which was found in 1 of 79 streptomycin-resistant <i>M. tuberculosis</i> isolates) probably reflects the stochastic probability of a resistance mutation in a bacterial population of limited size. It is under these circumstances that compensatory mutations that ameliorate the cost of resistance are likely to develop (27, 37).

Two outcomes of resistance mutations are feasible: fixation or elimination (Table 5). For a costly resistance mutation, e.g., the streptomycin resistance mutation 523A→C, which carries a cost of resistance of 5% per generation, 359 generations would be required for the mutation to become fixed, i.e., for isolates of the compensated resistance genotype to become the dominant (>99%) drug-resistant population, assuming that the frequency of a compensatory mutation is 10<sup>−6</sup> (5). These calculations are in line with the finding that the compensated resistance genotype is less frequent in vivo than in vitro (27).

On the other hand, it will be difficult to eliminate resistance mutations with a low fitness cost even in the absence of compensatory mutations. By assuming a population of 99% resistant cells and a cost per generation of 2%, more than 1,000 generations are required to lower the existing frequency of resistance to the frequency of resistance resulting from spontaneous mutations (frequency of spontaneous drug-resistant mutants, 10<sup>−8</sup>). For <i>M. tuberculosis</i>, which has an estimated generation time of 24 h, this would correspond to more than 3 years of total absence of the corresponding drug.

Our investigations focused on the fitness cost of chromosomosomal drug resistance. The epidemiology of drug-resistant pathogens is more complex and involves transmission and clearance of microbes from infected hosts. Although we hesitate to generalize our findings, the observation of no-cost or low-cost mutation types in clinical situations for resistance to different classes of antibiotics that act on the ribosome suggests that these observations may reflect a general biological phenomenon. Our results indicate the limitations of strategies for the containment of resistance based solely on restrictive drug use because, due to a missing fitness burden, negative selection pressure for such mutations does not seem to exist in an antibiotic-free environment. These results are consistent with the hypothesis that even if the rate of antibiotic consumption is reduced, in places where resistance is already common, the frequency of resistance will not decline rapidly, if at all (1, 20).

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