ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, May 2002, p. 1204–1211 Vol. 46, No. 5
0066-4804/02/$04.00+0 DOI: 10.1128/AAC.46.5.1204–1211.2002

Fitness Cost of Chromosomal Drug Resistance-Conferring Mutations

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Received 21 August 2001/Returned for modification 27 November 2001/Accepted 24 January 2002

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 nonexpansion 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 subsequently be ameliorated by compensatory mutations (4, 5, 36). However, for streptomycin resistance-conferring *rpsL* mutations, a high level of selection for no-cost drug resistance mutations was suggested to exist in vivo (6). In order to investigate whether this no-cost resistance mutation represents an isolated observation or points to a more general biological phenomenon, we examined the hypothesis that resistance mutations are costly.

MATERIALS AND METHODS

Bacteria. *Mycobacterium smegmatis rrnB* represents a variant of *M. smegmatis* mc^{2}155 SMR5 with a single rRNA allele; *M. smegmatis* normally contains two rRNA 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 rrnB rpsL3*$^+$ (strain 1682) is a streptomycin-sensitive derivative of mc^{2}155 with a single rRNA 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, *sacB* counterselection was used to inactivate the *rrnB* operon, resulting in strain mc^{2}155 *rrnB* (strain 1434) with a single rRNA allele; subsequently, two additional *rpsL* genes were introduced into the chromosome, resulting in mc^{2}155 *rrnB 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 rRNA-specific primers. Genomic DNA was isolated as described previously; standard methods were used for restriction endonuclease digestion of DNA and other manipulations. Single rRNA mutations were introduced into integrative plasmid pMV361-H-rRNA (34), which carries the complete *rrnB* 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* mc^{2}155 *rrnB* and *rrnB rpsL3*$^+$, 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 ^{32}P-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

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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:

$$S_t = \ln\left[\left(\frac{r_t/S_t}{r_{t-1}/S_{t-1}}\right)^{1/8}\right] \quad (1)$$

where r_t and s_t denote the absolute number of drug-resistant and drug-susceptible cells at a given time t, respectively, and r_{t-1} and s_{t-1} denote the number of drug-resistant and drug-susceptible cells at the preceding time point, respectively. S_t 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 were determined every eight generations.

The terms r_t/r_{t-1} and s_t/s_{t-1} give the growth rates for drug-resistant and 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_t) was calculated as

$$\mathrm{fit}_t = 1 + S_t \quad (2)$$

The cost per generation (cpg) was calculated as

$$\mathrm{cpg} = 1 - e^{S_t} \quad (3)$$

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

$$S_{tj} = \alpha_0 + \pi_j + \varepsilon_{tj} \quad (4)$$

where α_0 is a nonrandom intercept, S_{tj} denotes the selection coefficient at time t and experiment j, and ε_{tj} is the normal distributed error term at time t and experiment j. The overall S_t was estimated and tested against 0 on the basis of the null hypothesis (H_0) that α_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:

	t_0	t_6
r	r_0	r_6
s	s_0	s_6

(5)

where r_t and s_t denote the absolute number of drug-resistant and drug-susceptible cells at time t, respectively. S_t is a monotonic transformation of the odds ratio (OR) of (5)

$$S_t = \ln\left[\left(\frac{r_6/s_6}{r_0/s_0}\right)^{1/48}\right] = \ln\left[\mathrm{OR}^{1/48}\right] \quad (6)$$

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

$$\ln\frac{p}{1-p} = \beta_0 + \beta_1 \cdot t + \gamma_i \quad (7)$$

where p is the probability that a drug-susceptible colony will be picked, t is the time, γ_i is a random parameter to allow variability between the experiments, and β_0 and β_1 are the regression parameters. β_1 is an estimate for log OR, and a test for H_0 that β_1 is equal to 0 can be used to test if S_t 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):

$$\ln\frac{p}{1-p} = \beta_0 + \beta_1 \cdot t + \beta_2 \cdot t \cdot \mathrm{type} + \gamma_i \quad (8)$$

If the test for H_0 that β_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_t 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_t. 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 mc^{2}155 *rrnB* (strains 1644, 1646, and 1674) versus drug-susceptible strain mc^{2}155 *rrnB* (strain 1434); spontaneous streptomycin-resistant *rrnB rpsL3*$^+$ mutants (strains 1592, 1630, 1632, 1634, 1636, and 1647) versus a drug-susceptible *rrnB rpsL3*$^+$ strain (strain 1682); spontaneous amikacin-resistant rrnB mutants (strains 1181, 1183, 1184, 1185, 1187, and 1194) versus drug-susceptible *rrnB* strains (strains 1179, 1186, and 1193); and spontaneous clarithromycin-resistant *rrnB* mutants (strains 1082, 1086, and 1089) versus a drug-susceptible *rrnB* strain (strain 1179).

mc^{2}155 *rrnB rpsL3*$^+$ 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 (two mutant 16S rRNA alleles, one chromosomal and one plasmid derived; strains 1683, 1684, 1687, 1688, 1689, 1699, and 1700).

An *rrnB* 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 *rrnB* mutant transformed with pMV361-H-rRNA (strains 2014, 2015) was the 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 *rrn* 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

TABLE 1. Strains used in this study

Strain	Genotype	Transformed plasmid	Relevant phenotype	Parental strain
Parental strains				
0058 mc^{2}155	Wild type		STR^r	
0003 mc^{2}155 SMR5	*rpsL 42* Lvs→Arg			0058
1434 mc^{2}155 *rrnB*	*rrnB*::*aph*		KAN^r	0058
1682 mc^{2}155 *rrnB rpsL3*$^+$	*rrnB*::*aph rpsL3*$^+$		KAN^r GEN^r	1434
1179 *rrnB*	*rrnB*::*aph*		KAN^r	0003
1193 *rrnB*	*rrnA*::*aph*		KAN^r	0003
1186 *rrnA*	*rrnB*::*aph*		KAN^r	0003
Spontaneous resistant mutants				
1644	RpsL 42 Lys→Asn		STR^r	1434
1646	RpsL 42 Lys→Thr		STR^r	1434
1674	RpsL 42 Lys→Arg		STR^r	1434
1592	*rrs* 526C→T		STR^r	1682
1630	*rrs* 526C→T		STR^r	1682
1632	*rrs* 523A→C		STR^r	1682
1634	*rrs* 522C→T		STR^r	1682
1636	*rrs* 522C→T		STR^r	1682
1647	*rrs* 524G→T		STR^r	1682
1181	*rrs* 1408A→G		AMK^r	1179
1183	*rrs* 1408A→G		AMK^r	1179
1184	*rrs* 1408A→G		AMK^r	1179
1185	*rrs* 1408A→G		AMK^r	1179
1187	*rrs* 1408A→G		AMK^r	1186
1194	*rrs* 1408A→G		AMK^r	1193
1082	*rrl* 2058A→G		CLR^r	1179
1086	*rrl* 2058A→G		CLR^r	1179
1089	*rrl* 2059A→G		CLR^r	1179
Genetically engineered strains				
Streptomycin resistant				
1691		pMV361-H-rRNA2058G	HYG^r, CLR^r	1682
1683		pMV361-H-rRNA 524C 2058G	HYG^r, CLR^r, STR^r	1682
1684		pMV361-H-rRNA 524C 2058G	HYG^r, CLR^r, STR^r	1682
1687		pMV361-H-rRNA 526T 2058G	HYG^r, CLR^r, STR^r	1682
1688		pMV361-H-rRNA 526T 2058G	HYG^r, CLR^r, STR^r	1682
1689		pMV361-H-rRNA 526T 2058G	HYG^r, CLR^r, STR^r	1682
1699		pMV361-H-rRNA 523C 2058G	HYG^r, CLR^r, STR^r	1682
1700		pMV361-H-rRNA 523C 2058G	HYG^r, CLR^r, STR^r	1682
Aminoglycoside resistant				
1516		pMV361-H-rRNA2058G HYG^r	HYG^r, CLR^r	1193
1512		pMV361-H-rRNA 1408G 2058G	HYG^r, CLR^r, AMK^r	1193
1513		pMV361-H-rRNA 1408G 2058G	HYG^r, CLR^r, AMK^r	1193
1514A		pMV361-H-rRNA 1408G 2058G	HYG^r, CLR^r, AMK^r	1193
1515B		pMV361-H-rRNA 1408G 2058G	HYG^r, CLR^r, AMK^r	1193
Clarithromycin resistant				
2014		pMV361-H-rRNA	HYG^r	1193
2015		pMV361-H-rRNA	HYG^r	1193
1998		pMV361-H-rRNA2058G	HYG^r CLR^r	1193
1999		pMV361-H-rRNA2058G	HYG^r CLR^r	1193
2006		pMV361-H-rRNA 2059G	HYG^r CLR^r	1193
2007		pMV361-H-rRNA 2059G	HYG^r CLR^r	1193

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 2. Resistance mutations investigated and corresponding resistance phenotype

Type of rRNA mutation	No. of spontaneous mutants obtained by selective plating/total no. of isolates tested[b]	Resistance phenotype	MIC[c] (μg/ml)	Resistance mutation observed in clinical pathogens[d]
16S rRNA 522 C→T	2/22	Streptomycin	250	+
16S rRNA 523 A→C[a]	1/22	Streptomycin	125	+
16S rRNA 524 G→C[a]	12/22	Streptomycin	>500	−
16S rRNA 526 C→T[a]	7/22	Streptomycin	125	+
16S rRNA 1408 A→G[a]	10/10	2-Deoxystreptamines	>500[e]	+
23S rRNA2058 A→G[a]	7/8	Macrolides, lincosamides, streptogramin B	128[f]	+
23S rRNA 2059 A→G[a]	1/8	Macrolides, streptogramin B	128[f]	+

[a] The indicated mutations were also introduced into *M. smegmatis* by recombinant DNA techniques.
[b] 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.
[c] 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.
[d] Tabulated from previous reports (10, 11, 16, 22, 23, 24, 29, 31, 33, 40, 41).
[e] Amikacin was chosen as a representative of the 2-deoxystreptamines.
[f] Clarithromycin was chosen as a representative of the macrolides.

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-sensitive 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 rRNA 2059A→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 nonsignificant 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 3. S_t and cost of resistance of mutations investigated[a]

Mutation	S_t (±1 SE [10^{-2}])[b]	P value	Cost per generation (%)	Relative fitness (95% confidence interval)
Streptomycin				
RpsL 42 Arg	−1.0 ± 1.5	0.48	0.99	95.8–102.1
RpsL 42 Asn	−15.2 ± 1.9	<0.01	14.10	79.9–89.6
RpsL 42 Thr	−16.2 ± 3.3	<0.01	14.98	76.4–91.1
rrn 522 T[c]	−9.3 ± 1.3	<0.01	8.88	88.0–93.3
rrn 523 C[c]	−6.1 ± 1.5	<0.01	5.92	90.7–97.0
rrn 523 C[d]	−5.4 ± 1.4	<0.01	5.26	91.4–97.7
rrn 524 C[c]	−39.3 ± 5.1	<0.01	32.50	44.5–77.0
rrn 524 C[d]	−41.6 ± 2.9	<0.01	34.03	51.4–65.4
rrn 526 T[c]	−8.9 ± 1.8	<0.01	8.52	77.1–95.0
rrn 526 T[d]	−7.9 ± 0.9	<0.01	7.60	90.2–94.0
2-Deoxystreptamines				
rrn 1408G[c]	−1.7 ± 2.5	0.52	1.69	92.6–104.1
rrn 1408G[d]	−3.0 ± 2.2	0.13	2.96	92.4–101.7
Macrolides				
rrn 2058G[c]	−2.0 ± 2.1	0.36	1.98	93.4–102.6
rrn 2059G[c]	−2.0 ± 3.4	0.57	1.98	90.5–105.6

[a] Determined by analysis of variance (F test).
[b] Estimated by direct competition against an equal density of the drug-susceptible parental strain.
[c] Spontaneous mutants.
[d] Recombinant mutants.

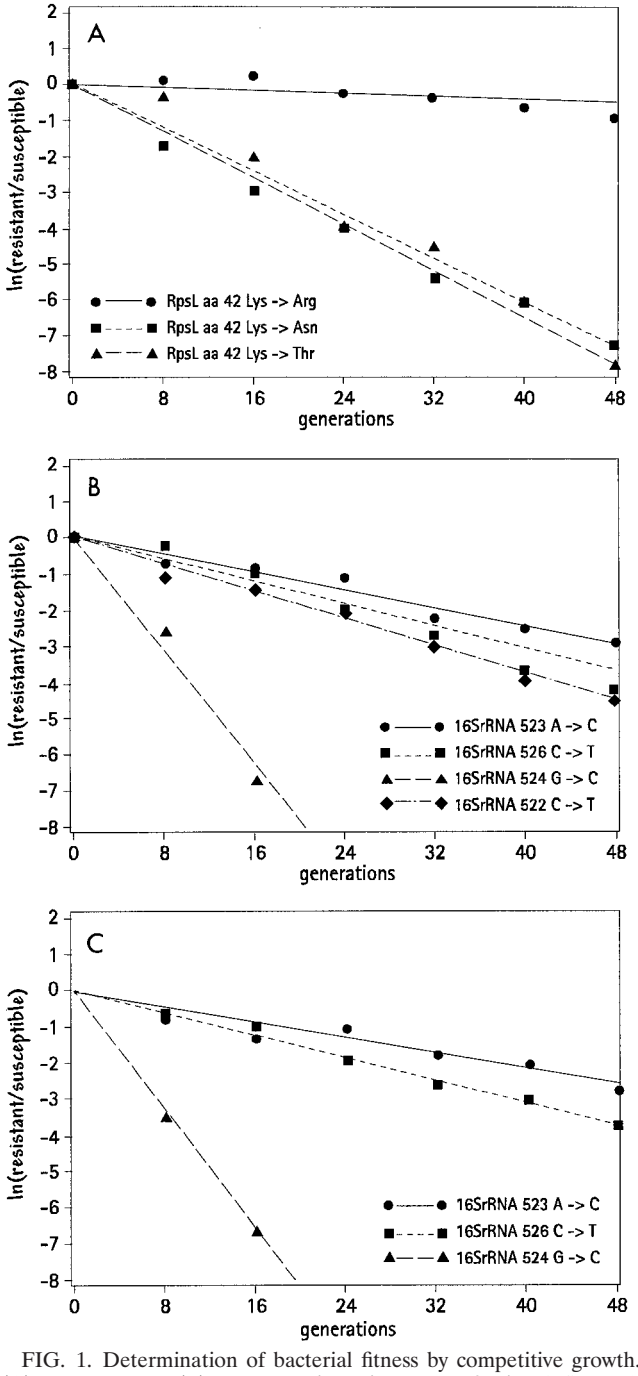

FIG. 1. Determination of bacterial fitness by competitive growth. (A) *rpsL* mutants; (B) streptomycin resistance-conferring 16S rRNA mutations (spontaneous mutants); (C) streptomycin resistance-conferring 16S rRNA mutations (mutations introduced by site-directed mutagenesis). The mean of at least two 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 523A→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_t and relative fitness of mutations investigated

Mutation	No.[d]	S_t (±1 SE [10^{-2}])[a]	*P* value	Cost per generation (%)	Relative fitness (95% confidence interval)
RpsL 42 Arg	9	−0.98 ± 0.37		0.98	98.23–99.74
rrn 1408G[b]	18	−3.10 ± 0.74	<0.01	3.05	95.45–98.35
rrn 1408G[c]	13	−2.88 ± 0.59	<0.01	2.84	95.96–98.28
rrn 2058G[b]	6	−0.46 ± 0.29	0.12	0.46	98.96–100.12
rrn 2058G[c]	3	−1.40 ± 0.40	<0.01	1.39	97.81–99.39
rrn 2059G[b]	9	−2.63 ± 0.83	<0.01	2.60	95.74–99.00
rrn 2059G[c]	6	−2.47 ± 0.23	<0.01	2.44	97.08–98.99

[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.

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, 7, 18, 36).

In vivo frequency of resistance mutations. The multiplicity of different chromosomal mutations that conferred resistance to streptomycin (*rpsL* and *rrn*) 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 equal to 100% and can be described by $y = 1 - a \cdot e^{bx-c}$. 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 cost. In contrast to clinically acquired resistance mutations, no correspondence between the frequency of a resistance mutation and the fitness cost was observed (Fig. 2).

DISCUSSION

Competition experiments of the type used here are able to detect statistically significant fitness differences larger than 2% per generation (4, 5, 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-

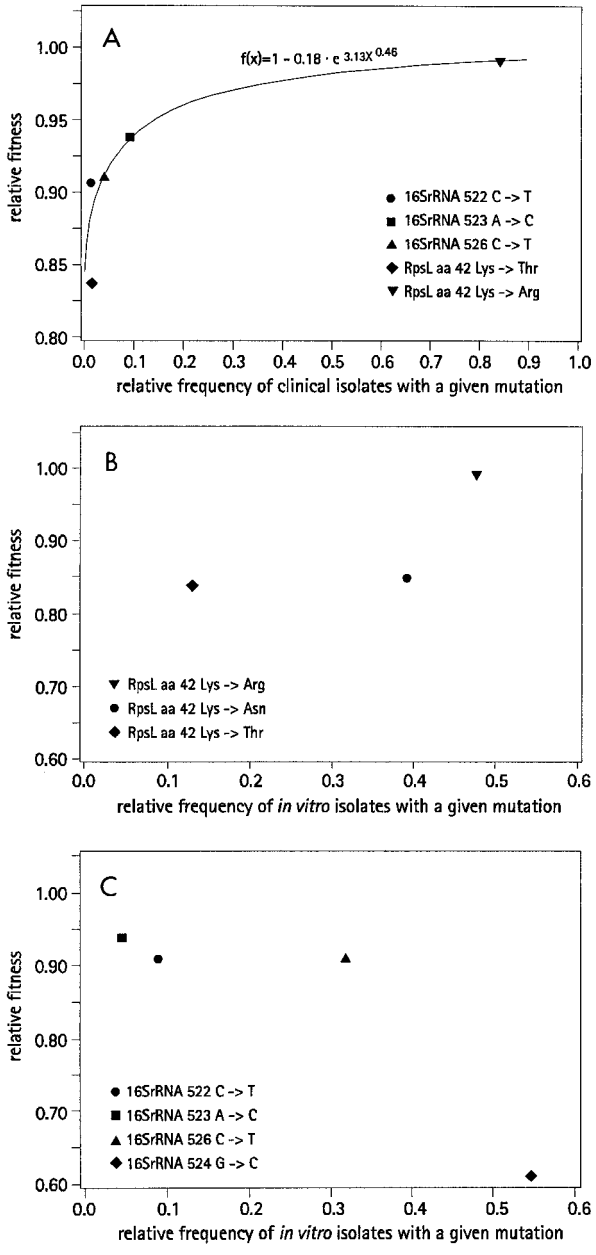

FIG. 2. Relationship between frequency of streptomycin resistance mutations and relative fitness. (A) Clinical *M. tuberculosis* isolates: ◆, RpsL 42 Lys→Thr; ▼, RpsL 42 Lys→Arg; ●, 16S rRNA 522C→T; ■, 16S rRNA 523A→C; ▲, 16S rRNA 526C→T; data were tabulated from previous reports (10, 11, 15, 23, 26, 28, 40). (B) In vitro *rpsL* mutants of *M. smegmatis* mc^2155: ▼, 42 Lys→Arg; ◆, 42 Lys→Thr; ●, 42 Lys→Asn; data were tabulated from reference 6. (C) In vitro 16S rRNA mutants of *M. smegmatis rrnB rpsL3*$^+$: ◆, 524G→C; ▲, 526C→T; ●, 522C→T; ■, 523A→C.

tral; (ii) the failure to detect the costs of resistance in these spontaneous mutants, and probably also in mutants detected in nature (clinical isolates), is not due to compensatory mutations; and (iii) under natural conditions (clinical isolates), strong selection pressure seems to exist for those drug resistance mutations which impose little if any burden on fitness, i.e., mutations which do not confer a substantial cost in the absence of antibiotics.

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 mutants provides data on the frequency and type of 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

Cost of resistance (%)	No. of generations for:	
	Fixation by compensatory mutation[a]	Elimination[b]
2	912	1,139
5	359	449
10	175	218
20	83	103

[a] Assuming a frequency of 10^{-6} 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: $t = \ln[(rc_t/r_t)/(rc_{t-1}/r_{t-1})]/\ln(1 - \text{cost per generation})$, where rc_t and rc_{t-1} are absolute numbers of resistant mutants with a compensatory mutation.

[b] 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 levels of spontaneous drug resistance frequency (10^{-8}) determined by the following formula: $t = \ln[10^{-8}\ (r_0/S_0)]/\ln(1 - \text{cost per generation})$.

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 *M. tuberculosis* 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^{-6} (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^{-8}). For *M. tuberculosis*, 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 chromosomal 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).

ACKNOWLEDGMENTS

We thank J. Buer and J. Colston for critical review of the manuscript; W. R. Jacobs, Jr., for providing *M. smegmatis* mc^{2}155; C. K. Stover for plasmid pMV361; K. Ellrott for expert technical assistance; A. Goebel for initial help with the statistical analysis; and S. Maibom, K. Härri, and F. Mitterecker for typing the manuscript.

This study was supported in part by grants from the Deutsche Forschungsgemeinschaft (Schwerpunkt Ökologie bakterieller Krankheitserreger), the Swiss National Research Foundation (grant SRP 49), and the Niedersächsischer Verein zur Bekämpfung der Tuberkulose e.V.

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