

Compensatory Mutations, Antibiotic Resistance and the Population Genetics of Adaptive Evolution in Bacteria

Bruce R. Levin, Véronique Perrot and Nina Walker

Department of Biology, Emory University, Atlanta, Georgia 30322

Manuscript received June 21, 1999

Accepted for publication November 29, 1999

ABSTRACT

In the absence of the selecting drugs, chromosomal mutations for resistance to antibiotics and other chemotherapeutic agents commonly engender a cost in the fitness of microorganisms. Recent *in vivo* and *in vitro* experimental studies of the adaptation to these "costs of resistance" in *Escherichia coli*, HIV, and *Salmonella typhimurium* found that evolution in the absence of these drugs commonly results in the ascent of mutations that ameliorate these costs, rather than higher-fitness, drug-sensitive revertants. To ascertain the conditions under which this compensatory evolution, rather than reversion, will occur, we did computer simulations, *in vitro* experiments, and DNA sequencing studies with low-fitness *rpsL* (streptomycin-resistant) mutants of *E. coli* with and without mutations that compensate for the fitness costs of these ribosomal protein mutations. The results of our investigation support the hypothesis that in these experiments, the ascent of intermediate-fitness compensatory mutants, rather than high-fitness revertants, can be attributed to higher rates of compensatory mutations relative to that of reversion and to the numerical bottlenecks associated with serial passage. We argue that these bottlenecks are intrinsic to the population dynamics of parasitic and commensal microbes and discuss the implications of these results to the problem of drug resistance and adaptive evolution in parasitic and commensal microorganisms in general.

IN elementary classes in population genetics and evolutionary biology we teach many things that are logical, or at least pedagogically convenient, but have little or no empirical foundation. One of these things is that adaptive evolution operates by gene substitution at the specific loci coding for the phenotype(s) under selection. As a consequence of a change in the environment, a previously favored allele, A_1 , will be replaced by the now favored alternative allele at that locus, A_2 . Another of these "empirically challenged" (politically correct for virtually untested) truths is that as long as environmental conditions remain constant in any given population, the mutant genotype of highest fitness in that environment will prevail. To be sure, in finite asexual populations for any given locus, nonrevertible mutants of lower fitness can be fixed; Muller's ratchet does indeed turn. But as long as more fit mutants continue to be generated, they will eventually replace these less adapted strains from their temporary appearance at center stage. Finally, at least for this consideration, there is the view that evolution is a reversible process. When a population adapted to one environment returns to a previous environment, the genome of that population will return to its ancestral state.

The results of recent *in vitro* experimental studies with

a bacterium, *Escherichia coli*, and a retrovirus, human immunodeficiency virus (HIV), and an *in vivo* study with *Salmonella typhimurium* on the adaptation to the fitness burdens associated with drug resistance question these perceptions about the genetic basis of adaptive evolution for microorganisms. The ribosomal mutations (*rpsL*) responsible for streptomycin resistance in *E. coli* and many other bacteria reduce the efficacy of translation and, as such, engender a substantial fitness cost (Zengel *et al.* 1977; Bilgin *et al.* 1992). When *rpsL* mutants are maintained by serial passage (serial transfer) in streptomycin-free culture, the populations remain resistant to streptomycin but adapt to the fitness burden of the *rpsL* locus by compensatory mutations at other loci, which restore the efficacy of translation to nearly wild-type levels (Schrag and Perrot 1996). These streptomycin-resistant *rpsL* strains with compensatory mutations are more fit than uncompensated *rpsL* mutants but less fit than wild-type streptomycin-sensitive *rpsL*⁺ cells. In a study of the fitness cost of HIV drug resistance, Borman *et al.* (1996) found that in tissue culture the mutations responsible for resistance to a protease inhibitor severely reduce the replication rate of this retrovirus. However, in the course of passage in the absence of the protease inhibitor, the fitness burden of these resistance mutations declined and the level of resistance increased. The compensatory mutations responsible for the adaptation to these fitness costs were through additional base substitutions at the protease locus. More recently, Björkman *et al.* (1998) found that

Corresponding author: Bruce R. Levin, Department of Biology, Emory University, 1510 Clifton Rd., Atlanta, GA 30322.
E-mail: blevin@emory.edu

the majority of chromosomal mutations to streptomycin, nalidixic acid, or rifampin resistance reduce the virulence (growth rate and competitive performance) of *S. typhimurium* in laboratory mice. After sequential passages in mice in the absence of antibiotics, virulence was restored. In the majority of these cases, however, the bacteria remained resistant to the antibiotic and their fitness (virulence) was increased by mutations at other loci.

For both the *E. coli rpsL* and the HIV protease mutants, the compensatory evolution responsible for the adaptation to the cost of resistance virtually precludes those populations from returning to their ancestral, drug-sensitive state. When the streptomycin-resistant *rpsL* alleles are replaced by wild-type, streptomycin-sensitive *rpsL*⁺ in evolved, fitness-compensated *rpsL* strains, the resulting streptomycin-sensitive bacteria are less fit than either wild-type, uncompensated *rpsL* mutants or fitness-compensated *rpsL* mutants (Schrag *et al.* 1997). Stated another way, compensatory evolution establishes an adaptive valley that is difficult to traverse and thus return to the ancestral genotype, uncompensated *rpsL*⁺. This is also the case for the HIV protease inhibitor mutants. In this case, the return to the wild-type, drug-sensitive protease gene from fitness-compensated resistance alleles would require the substitution of multiple mutations, each of which would be at a selective disadvantage.

In this article, we present the results of *in vitro* experiments with *rpsL* mutants of *E. coli* and computer simulations used to explore the genetic and population dynamic processes responsible for compensatory evolution and amelioration (Cohan *et al.* 1994) in bacterial populations. The primary question we address is why, in low-fitness *rpsL* cultures, selection results in the ascent of intermediate-fitness compensatory mutants rather than high-fitness *rpsL*⁺ revertants. We argue and present evidence that this compensatory evolution is a consequence of compensatory mutations occurring at higher rates than reversion and the bottlenecks associated with serial passage. We briefly discuss the implications of these results to the problems of drug resistance and to the general theory of adaptive evolution in parasitic microorganisms, elephants, and more diminutive eukaryotes.

MATERIALS AND METHODS

Bacteria: In these experiments, we use the same *E. coli* strain employed by Schrag and Perrot (1996) and the streptomycin-resistant Str1 (*rpsL* AAC at codon 42), Str2 (*rpsL* ACA at codon 42), and compensatory mutants Str11, Str12, Str21, and Str22 described in that study. This basic strain CAB281, an O18:K:H7/K-12 chimera, was constructed by Craig A. Bloch (χ M1002 in Bloch and Rode 1996). It is prototrophic save for a nicotinic acid requirement. In addition to the wild-type Str^r CAB281, we also used a spontaneous nalidixic acid-resistant mutant of this strain (CAB281Nal).

Medium and sampling: Experimental populations were

maintained in Davis minimal medium (Carlton and Brown 1981) supplemented with 100 μ g/ml MgSO₄, 10 μ g/ml nicotinic acid, and 2 μ g/ml thiamine, with 500 μ g/ml glucose as the sole and limiting carbon source. Fluctuation tests to estimate mutation rates were performed in Luria Bertani broth (LB). The total cell densities of bacteria in these cultures were estimated by serial dilution and plating on LB agar. The frequencies of Str^r and Nal^r cell lines were estimated by plating on agar containing 50 μ g/ml of streptomycin sulfate or nalidixic acid, respectively.

Parameter estimates: Growth rates were estimated from either the changes in optical density or from colony count data. The fitness of the different strains was estimated by pairwise competition experiments. In these experiments, 0.02 ml of mixtures of stationary phase overnight cultures of the competing populations were introduced into 50-ml flasks containing 10 ml of medium. These flasks were incubated with shaking (200 rpm) at 37° for 24 hr (to achieve stationary phase), at which time the densities of the competing populations were estimated by selective plating, and 0.02 ml was transferred to fresh medium. The selection coefficient of the less fit strain, A, relative to the more fit, B, was estimated as

$$s = b/\ln(1/d),$$

where *b* is the regression coefficient of the ln(density of A/density of B) as a function of transfer and *d* is the dilution factor. With this estimator of the selection coefficient, the changes in the densities of the competing populations predicted by the simulations (described below) were virtually identical to those observed in the serial transfer competition experiments.

Mutation rates: The rates of mutation to streptomycin resistance were estimated by fluctuation tests (Luria and Delbruck 1943). Independent 5-ml LB cultures were inoculated with $\sim 10^4$ cells of CAB281, grown to stationary phase, and spun down. To each pellet we added 0.1 ml of a 0.4% streptomycin sulfate solution and 3 ml of soft agar. The soft agar cell suspensions were plated on LB Strep (50 μ g/ml) agar. For the calculation of the mutation rates, the number of mutants per culture was estimated from the number of colonies on LB-Strep agar at 48 hr.

DNA sequencing: Genomic DNA was extracted using the QIAGEN (Valencia, CA) QIAamp Tissue kit. To amplify DNA by PCR, we designed pairs of primers external to the coding sequence of each gene based on the *E. coli* K-12 reference sequence and used a standard PCR. To amplify DNA in the *rpsL* region, we used the primer pair 5'-CGTGTTCACGAAGC AAAAGC-3' and 5'-ATTTAAGTTAAAACGTTTGGCC-3'; for *rpsE*, 5'-GCGGGTTTGAGTAATTTAATAGT-3' and 5'-AAGC TGGCCTTCAGTCTAAG-3'; for *rpsD*, 5'-AACCCTGCATTG TGTCTCT-3' and 5'-CCTCGTTTTCCAGGTTTGT-3'. The PCR fragments were then purified using the QIAGEN QIAquick PCR purification kit and sequenced from both ends using the same primers as for amplification.

EXPERIMENTAL AND SIMULATION RESULTS

Selection favors Str^s, *rpsL*⁺ mutants at all frequencies:

One possible explanation for the ascent of higher-fitness Str^r, "compensatory" mutants, rather than Str^s "revertants" in these experiments is that for some reason, when they are rare, *rpsL*⁺ bacteria cannot invade populations dominated by Str^r *rpsL* mutants. To test this frequency-dependent selection hypothesis, we performed pairwise competition experiments between the original

rpsL⁺ CAB281 and the two uncompensated *rpsL* mutants, Str1 and Str2, and the four fitness-compensated *rpsL* mutants, Str11, Str12, Str21, and Str22, studied by Schrag and Perrot (1996). To estimate the density of the Str^s bacteria when rare, we used a spontaneous nalidixic acid-resistant mutant (presumably *gyrA* or *gyrB*; Larossa 1996) of the Str^s CAB281. To control for the effect of the Nal^r marker, we performed pairwise competition experiments between otherwise isogenic Nal^s and Nal^r CAB281. At each initial frequency, the Nal marker appears to have no effect on the fitness of the *rpsL*⁺ cell line (Figure 1a).

In all experimental cultures, the Nal^r Str^s cell line had a selective advantage over the resistant line (Figure 1, b–g). The estimated selection coefficients for the Str^r cells are presented in the legend to Figure 1. The magnitude of the fitness advantage of the *rpsL*⁺ is greatest when it is competing with the uncompensated resistant mutants, Str1 and Str2. In all but one case, the intensity of selection appears to be independent of the frequency of the competing populations. The exception is the experiment with Str^s invading populations dominated by the lower-fitness *rpsL* mutant, Str2 (Figure 1c), where, for reasons we have not determined, the *rpsL*⁺ population has a greater advantage when it is rare.

The results of these experiments are inconsistent with the hypothesis that, when rare, *rpsL*⁺ mutants will not increase in frequency in populations dominated by uncompensated or fitness-compensated *rpsL* mutants. Therefore, if they were generated at all, it is reasonable to anticipate that streptomycin-sensitive *rpsL*⁺ mutants would have a selective advantage over the entire range of gene frequencies. Why then did these revertants not ascend to dominance in the Schrag and Perrot (1996) study?

The bottleneck-mutation rate hypothesis: We postulate that the failure of high-fitness revertants to evolve in the study by Schrag and Perrot (1996) is a consequence of two processes, the rate of compensatory mutation exceeding that of reversion and the bottlenecks associated with serial passage. A diagram of this hypothesis is presented in Figure 2, where we illustrate the progression of the changes in composition of a bacterial population in the course of time in a serial transfer experiment. Because of their higher mutation rate, compensatory mutants occur earlier in the growth cycle (within a transfer) than revertants and increase in relative frequency. While the revertants also occur and increase in frequency, the population reaches stationary phase and stops growing before the revertant population becomes very large. While the number of *rpsL* compensatory mutants is relatively large at that time, the number of revertants at stationary phase is going to be small. As a result, revertants that are generated in the course of a transfer are unlikely to be present in the small fraction transferred to the next flask. At each transfer, the frequency of *rpsL* compensatory mutants

increases and eventually these bacteria dominate the community. As long as uncompensated *rpsL* cells are present, higher-fitness revertants will continue to be generated, albeit in decreasing numbers. Moreover, as the frequency of fitness-compensated *rpsL* mutants increases, the rate of ascent of the *rpsL*⁺ mutants that occur will decline, due to a lower fitness differential. Although streptomycin-sensitive *rpsL*⁺ mutants may arise in the population dominated by the fitness-compensated *rpsL* mutants, they will not ascend because they are less fit than uncompensated or fitness-compensated *rpsL* mutants or *rpsL*⁺.

A computer simulation of selection in serial transfer culture: To examine this bottleneck-mutation rate hypothesis quantitatively, we used a computer simulation of mutation and selection in competing populations maintained in serial transfer culture. We consider a single resource of concentration, r , and four bacterial populations with designations and densities (bacteria per milliliter): n00, streptomycin-sensitive *rpsL*⁺; n10, uncompensated streptomycin-resistant *rpsL*; n01, streptomycin-sensitive *rpsL*⁺ with the compensatory mutation; and n11, streptomycin-resistant *rpsL* with the compensatory mutation. Within a transfer, the rates of change in the densities of these populations are determined by their growth rate, $\psi(r)$, and their relative fitness $(1 - s_{ij})$. $\psi(r)$ is a monotonically increasing function of the resource concentration, r , and s_{ij} ($0 \leq s_{ij} \leq 1$) is the selection coefficient for that population. At any time a population n_{ij} will grow at a rate $\psi(r)(1 - s_{ij})$. At the start of a transfer, the limiting resource, glucose in the experiments, is present at a concentration R ($\mu\text{g}/\text{ml}$). As the populations grow, the concentration of the limiting resource declines at a rate proportional to the growth rates of the competing populations of bacteria and a conversion efficiency parameter, e ($\mu\text{g}/\text{cell}$), which is the amount of resource required to produce a single bacterium. With these definitions and assumptions, the rates of change in the densities of the competing population and change in resource concentration in the course of a transfer are given by

$$dr/dt = -\sum_i \sum_j n_{ij} \psi(r) (1 - s_{ij}) e$$

and

$$dn_{ij}/dt = n_{ij} \psi(r) (1 - s_{ij}).$$

The population growth and resource depletion equations of this model are identical to those employed by Stewart and Levin (1973). For the resource concentration-dependent growth function, we assume a Monod model $\psi(r) = Vr/(K + r)$, where V (per hour) is the maximum growth rate and K (μg) is the concentration of the limiting resource when the growth rate is half its maximum value, $V/2$ (Monod 1949).

During the course of each transfer, mutations can occur in each population. For example, an individual of the n00 population can be produced by mutation

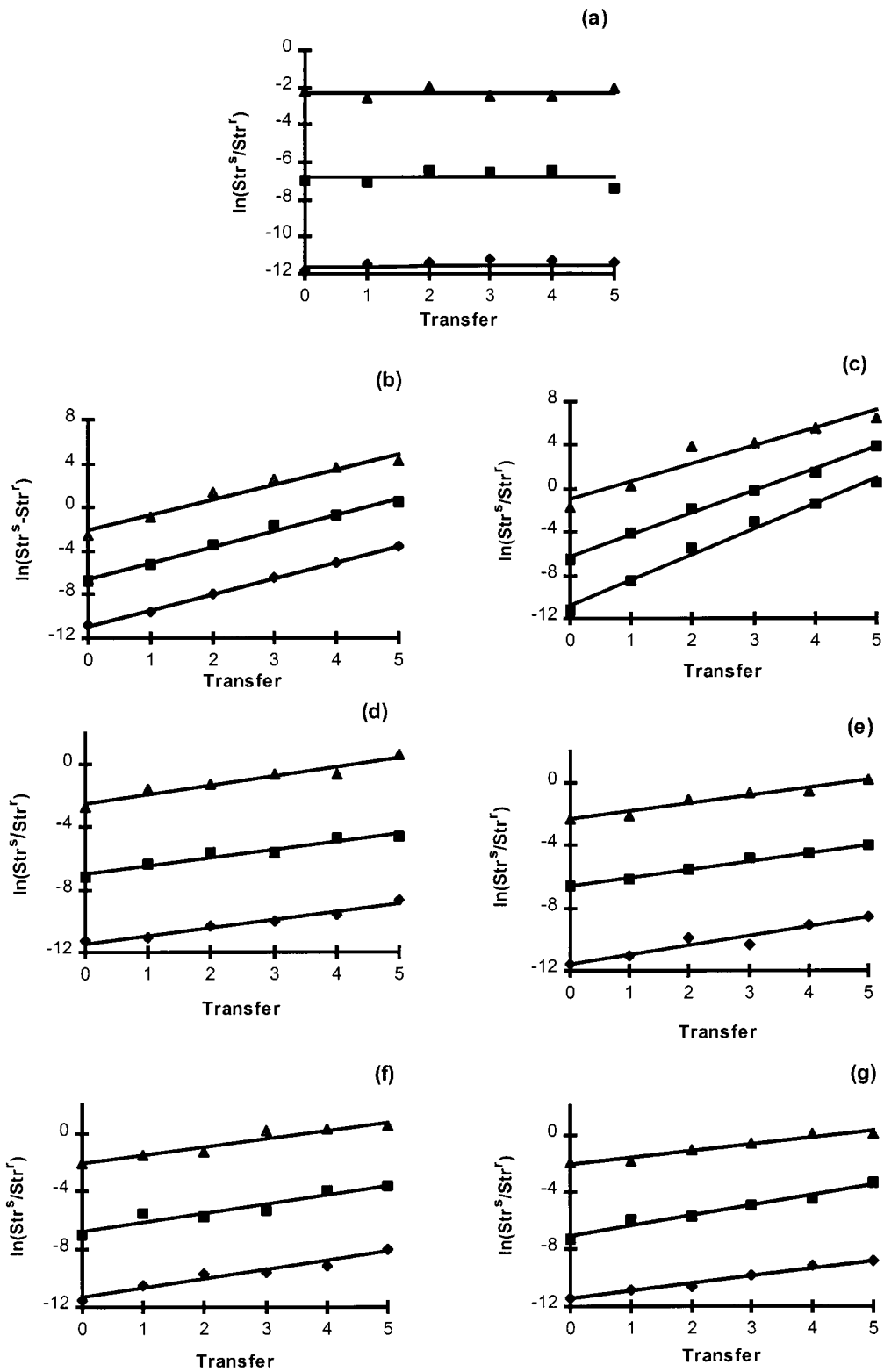


Figure 1.—Pairwise competition between Str^s and Str^r cell lines at different initial densities. The estimated selection coefficients of the $Nal^r Str^s$ strain in competition with $Nal^r Str^r$, as a control for the effect of the Nal marker, and in competition with the $Nal^s Str^r$ strains in the experimental cultures. The estimated mean selection coefficients are noted in *italic* with their corresponding 95% confidence intervals.

Invaded population	Low density			Medium density			High density		
(a) Str^s (control)	<i>0.012</i>	-0.005	0.028	<i>-0.002</i>	-0.055	0.045	<i>0.004</i>	-0.028	0.033
(b) $Str1$	<i>0.190</i>	0.185	0.196	<i>0.191</i>	0.168	0.213	<i>0.182</i>	0.138	0.221
(c) $Str2$	<i>0.276</i>	0.245	0.305	<i>0.247</i>	0.229	0.264	<i>0.208</i>	0.138	0.267
(d) $Str11$	<i>0.076</i>	0.060	0.092	<i>0.078</i>	0.051	0.103	<i>0.084</i>	0.052	0.115
(e) $Str12$	<i>0.087</i>	0.052	0.119	<i>0.079</i>	0.068	0.090	<i>0.075</i>	0.054	0.095
(f) $Str21$	<i>0.090</i>	0.061	0.117	<i>0.091</i>	0.053	0.125	<i>0.083</i>	0.053	0.112
(g) $Str22$	<i>0.080</i>	0.068	0.092	<i>0.104</i>	0.077	0.128	<i>0.070</i>	0.048	0.092

from the n01 or n10 populations. The model allows for four different mutation rates: μ_{01} and μ_{10} for mutations at the resistance, *i* (*rpsL*) locus, where μ_{01} is the mutation rate from sensitive to resistant and μ_{10} is the mutation rate from resistant to sensitive; the parameters ν_{01} and ν_{10} are, respectively, the rates of compensatory mutation and reversion to wild type from compensatory for the *rpsL* fitness compensation, *j* locus (or loci). During each step *t* to *t* + Δt in this constant step size simulation, we calculate the expected number of mutants produced in each population as the product of the mutation rate, μ_{ij} or ν_{ij} , the increment in the density (bacteria per ml) due to population growth Δn_{ij} , the total volume of the culture, vol, and the step size Δt . We then generate a rectangularly distributed random number, *y*, between 0 and 1. If that number is less than this product, $y < \mu_{ij}\Delta n_{ij} \text{ vol}\Delta t$, the density of the population of the mutant type is incremented by 1/vol and that of the donor reduced by that amount. The model does not allow for double mutations to occur at a single time. In the absence of selection, this algorithm for mutation provides a reasonably good approximation of a Luria Delbruck distribution for the number of mutants (Stewart *et al.* 1990).

The population continues to grow and mutants continue to be produced until the resource is exhausted, $r < 0.001$, at which time a fraction *d* of the population is transferred to a fresh habitat containing *R* units of the limiting resource. If the expected number of cells of a particular state being transferred is >10 , transfer is a deterministic process and that number of cells will be present at the start of the next growth cycle. If, for a particular population, the number of bacteria to be transferred is <10 , a Monte Carlo procedure is used to determine the number actually transferred. For this a Poisson distribution is assumed to calculate the probability that *x* individuals of that type are transferred, $p(x) = \lambda^x e^{-\lambda} / x!$, where $x = 0, 1, 2, \dots$, and $\lambda = d N_{ij} \text{ vol}$, where N_{ij} is the density of the n_{ij} population at the end of the transfer. A rectangularly distributed random number *y* ($0 < y < 1$) is generated. If $p(x) < y < p(x + 1)$, $x = 0, 1, 2, \dots$, then *x* cells of that type are transferred.

To solve the differential equations we use the Euler

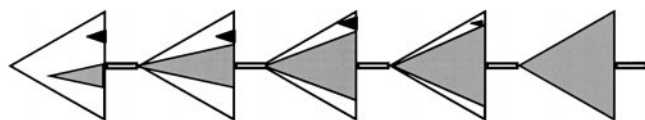


Figure 2.—The bottleneck hypothesis. There are three bacterial populations: (i) the original, Str^r (open); (ii) Str^r bacteria with the compensatory mutation (shaded); and (iii) the Str^s , *rpsL*⁺ revertants (solid). Population growth within a transfer is indicated by the horizontal triangles, with growth terminating at stationary phase. A fraction of the stationary phase population is transferred and the population growth cycle is repeated. The bottlenecks are depicted as the narrow tubes connecting the triangles.

method with a constant step size of $\Delta t = 0.01$ hr. This simulation was programmed in FORTRAN 77. The authors will make copies of this program available to interested readers.

Parameter values and simulation results: The growth rate parameters, *V* and *K*, used in the simulation runs are those estimated for CAB281 *rpsL*⁺ in glucose minimal medium, 0.85/hr and 0.25 $\mu\text{g}/\text{ml}$, respectively [Monod 1949; Levin *et al.* 1977; see also Vasi *et al.* (1994) for an interesting way to estimate *K*]. For the selection coefficients of the streptomycin-sensitive, uncompensated streptomycin-resistant, and fitness-compensated resistant cell lines n00, n10, and n11, respectively, we use values in a range similar to those estimated from the experiment depicted in Figure 1 for Str1 and its compensatory mutants, Str11 and Str12; $s_{00} = 0$, $s_{10} = 0.20$, $s_{11} = 0.08$. For the fitness of the sensitive fitness-compensated cell line n01, we use a value in a range similar to that reported in Schrag *et al.* (1997), $s_{01} = 0.35$. In these simulation runs we consider two volumes and dilution factor combinations, vol = 10 ml and $d = 0.002$, and vol = 100 ml and $d = 0.01$.

For the standard rates of reversion and compensatory mutation we use $\mu_{01} = \mu_{10} = 2 \times 10^{-10}$ and $\Delta_{01} = \Delta_{10} = 4.0 \times 10^{-9}$, respectively. The former is based on the results of two fluctuation test experiments estimating the rate of spontaneous mutation to *rpsL*, 3.0×10^{-10} and 4.8×10^{-10} for 22 and 19 cultures, respectively. Streptomycin-resistant mutants that form colonies within 48 hr on LB agar containing streptomycin all involve single-base substitutions at the 42nd codon (AAA) to AAC, ACA, and AGA. Reversion to AAA from each of these *rpsL* requires specific single-base substitutions. Assuming all else is equal, that rate should be approximately one-third as great as the rate of mutation from *rpsL*⁺ to *rpsL* or $\sim 1.3 \times 10^{-10}$. In point of fact it could be somewhat higher, as the AAC and ACA mutations have a marked selective disadvantage. Consequently, they would be less well represented at stationary phase than they would be if they were neutral, as assumed by the fluctuation test estimator of mutation rates (Stewart *et al.* 1990). For this reason, we used $\mu_{10} = 2 \times 10^{-10}$ as our standard estimate of the reversion rate. The standard rate of compensatory mutation we use in these simulations, $\nu_{01} = 4 \times 10^{-9}$, is somewhat, but as we shall argue, not entirely, arbitrary. As long as they are small, the rate of mutation to Str^r , μ_{01} , and the rate of reversion at the compensatory locus, ν_{10} , are effectively irrelevant for this consideration, and for these simulations we assume them to be equal to the rates of mutation to their respective alternative allelic states.

With these standard parameter values, an initial population composed solely of uncompensated *rpsL* (n10) mutants and the 10-ml, $d = 0.002$ serial transfer protocol similar to that used by Schrag and Perrot (1996), by the 50th transfer, 284 out of 500 simulations (56.8%) were dominated by and often fixed for the fitness-com-

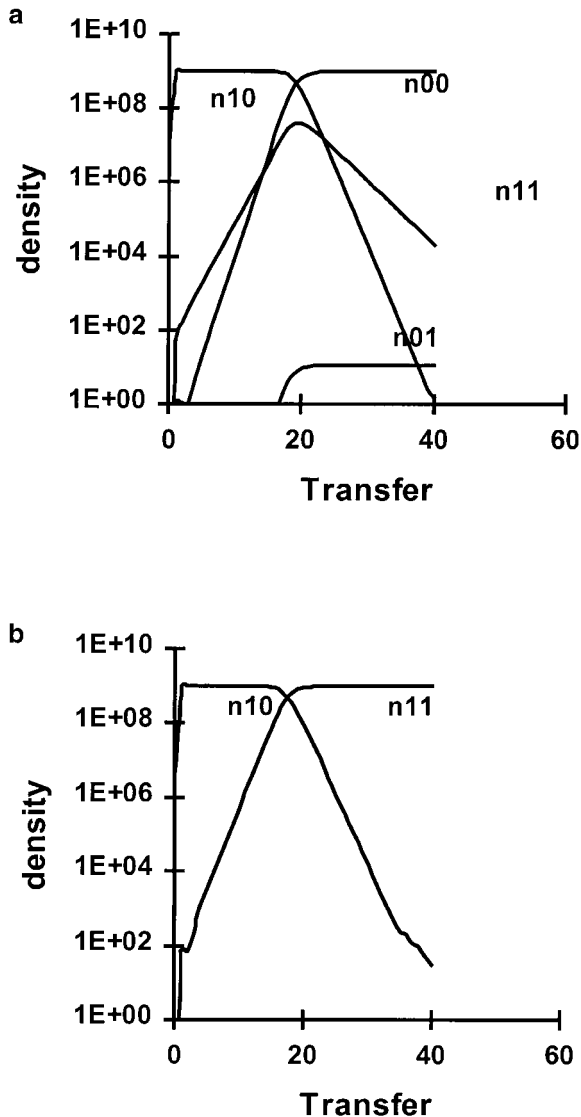


Figure 3.—Simulation of mutation and selection in serial transfer culture. Standard parameters, $V = 0.85$, $K = 0.25$, $s_{00} = 0.00$, $s_{10} = 0.35$, $s_{01} = 0.20$, $s_{11} = 0.08$, $R = 500$, $e = 5 \times 10^{-7}$, $\mu_{01} = \mu_{10} = 2 \times 10^{-10}$, $\nu_{01} = \nu_{10} = 4.0 \times 10^{-9}$, $d = 0.002$, and $vol = 10$ ml. (a) Sample run ending with the fixation of n_{11} . (b) Sample run ending with the fixation of n_{00} .

compensated *rpsL* mutants, n_{11} . The remaining 43.2% of the simulations were dominated by streptomycin-sensitive revertants, n_{00} . Among the latter, in 38.9% n_{00} achieved dominance before the frequency of the first-occurring n_{11} mutant cell line exceeded 0.50. Among the runs in which the fitness-compensated cell line dominated by the 50th transfer, the mean and standard deviation in the time for n_{11} to reach a relative frequency of 0.50 were 8.50 and 0.80 transfers, respectively. The corresponding values for the runs where n_{00} exceeded 0.5 were 17.57 and 10.47, respectively. The population dynamics of this evolutionary process are illustrated in Figure 3 for single simulation runs. In Figure 3a, the ascending n_{11} cell line is replaced by the more

fit revertants, n_{00} , while in the run depicted in Figure 3b, the revertants do not pass through the bottlenecks and the run terminates with the fixation of the resistant compensatory mutant n_{11} .

To examine the sensitivity of this process to the mutation rates, we did a series of runs with different mutation rates. If we assumed standard values for the other parameters but let the rate of mutation at the resistance locus be half its standard value, $\mu_{01} = \mu_{10} = 1 \times 10^{-10}$, the frequency of runs terminating with a dominant population of revertants was lower than noted in the $\mu_{01} = \mu_{10} = 2 \times 10^{-10}$ runs, 28.4 vs. 43.2%. If the rate of compensatory mutation was reduced to 10 times rather than 20 times the reversion rate, $\nu_{01} = \nu_{10} = 2 \times 10^{-9}$, by the 50th transfer, 57% of 500 simulations were dominated by compensatory mutants rather than revertants. If the rate of reversion and rate of compensatory mutation were equal but low, $\mu_{01} = \mu_{10} = \nu_{01} = \nu_{10} = 2 \times 10^{-10}$, by the 50th transfer, revertants dominated 76.4% of the 500 simulations. Finally, if the mutation rates were higher but equal, $\mu_{01} = \mu_{10} = \nu_{01} = \nu_{10} = 4 \times 10^{-9}$, by the 50th transfer all 500 simulations were dominated by revertants.

With a larger habitat volume (total population size) and greater fraction of the population transferred, $vol = 100$ ml and $d = 0.01$ with the other parameters the same as those in the legend of Figure 3, by the 50th transfer all 500 simulations were dominated by the revertants, n_{00} . The average number of transfer before the frequency of the n_{00} population exceeded 0.50 was 9.97 with a standard deviation of 1.41.

Predictions and their tests: If the bottleneck-mutation rate mechanism accounts for the compensatory evolution results reported by Schrag and Perrot (1996), the following three computer-assisted (simulation-based) predictions should hold:

1. The rate of mutation from *rpsL* to *rpsL*⁺ should be sufficiently low that only few revertants are produced at each transfer and the rate of compensatory mutation should be on the order of 10 times greater than of reversion. If this were not the case, many of the *rpsL* experimental evolution cultures studied by Schrag and Perrot (1996) would have been dominated by high frequencies of revertants by the 20th transfer. They were not.
2. Revertants should be more likely to evolve in *rpsL* populations maintained in serial transfer cultures with greater numbers of bacteria (greater volumes) and higher transfer fractions than in cultures with lower numbers of bacteria and lesser fractions transferred.
3. When *rpsL*⁺ bacteria are introduced at very low densities, they should be more likely to successfully invade populations of lower-fitness, uncompensated *rpsL* mutants than populations of higher-fitness, compensated *rpsL* mutants. Moreover, in the latter popula-

tions, stochastic processes should play a more prominent role in the invasion process.

1. *Rates of reversion and compensatory mutation:* At this juncture, there is no way to directly estimate the rates of reversion at the *rpsL* locus or the rate at which second-site mutations will occur that compensate for the cost of *rpsL* mutants. Nevertheless, from what we know about mutation, it seems highly unlikely that the rate of reversion at the *rpsL* locus would be greater than that from wild type to resistant, on the order of 2×10^{-10} . As noted earlier, reversion requires specific base substitutions, AAC or ACA to AAA (or possible AAG, which also codes for lysine) at the 42nd codon. If all else were equal, we would expect the rate of compensatory mutation to exceed that of reversion if there were more ways to generate compensatory mutants than revertants. To ascertain if this is the case, for 24 independently evolved *rpsL* compensatory mutants, 12 for each of Str1 and Str2, and the ancestral CAB281, we sequenced two candidate genes for the compensatory mutants, *rpsD* (621 bp) and *rpsE* (504bp), which code for the S4 and S5 ribosomal proteins, respectively. We chose these candidates in part because of sage advice from Julian Davies. They also seemed to be reasonable candidates for the compensatory mutations, because they are the known sites of ribosome ambiguity (*ram*) mutants (Rosset and Gorini 1969; Hasenbank *et al.* 1973; Andersson *et al.* 1982) that revert streptomycin-dependence phenotypes to streptomycin independence.

The DNA sequences of *rpsD* of Str^s CAB281 and the original *rpsL* mutants, Str1 and Str2, were identical to that of a Str^s ancestral strain of *E. coli* K-12 (MG1655). Among the 24 fitness-compensated *rpsL* mutants sequenced, for *rpsD* we found a minimum of five different sequences. Three of these were single-nucleotide changes leading to three amino acid substitutions, one of which appeared in two independently evolved Str1 compensatory mutations. Two of these *rpsD* variants involved tandem duplications leading to the insertion of three or five amino acids in the middle of the S4 protein. The DNA sequence of the *rpsE* gene of Str^s CAB281 differs from that of *E. coli* K-12 by five silent nucleotide substitutions. The same silent mutations were found in the original *rpsL* mutants, Str1 and Str2, and all of the fitness-compensated, evolved strains. The DNA sequence of 9 of the 24 sequenced fitness-compensated *rpsL* mutants differed from that of their uncompensated *rpsL* CAB281 ancestor by five different single-base changes leading to four different amino acids. One of these nonsilent mutations appeared in 5 independently evolved compensatory mutants, 2 for Str1 and 3 for Str2. In no case did we find compensatory mutants with changes in both *rpsD* and *rpsE*. Finally, for two fitness-compensated strains we failed to find changes in either *rpsD* or *rpsE*. At this juncture, as compelling as these sequence data may be—multiple changes and the substi-

tution of neutral mutations would not be anticipated in these short-term experiments—the evidence that the observed mutations in *rpsD* and *rpsE* are responsible for compensating for the costs of *rpsL* is only circumstantial. Reconstruction experiments will have to be performed to formally demonstrate that these changes in the *rpsD* and *rpsE* loci do in fact compensate for the costs imposed by the Str1 and Str2 *rpsL* mutations. If, however, we accept the hypothesis that the observed sequence changes in *rpsD* and *rpsE* are compensatory mutations and assume the two compensatory mutations that are at neither of these loci represent at least one additional compensatory mutant, we can say that there are at least 10 different ways to generate *rpsL* compensatory mutations. Stated another way, the rate of compensatory mutation exceeds that of reversion by at least a factor of 10.

2. *The effect of culture volume and dilution factor:* Sixteen cultures were set up for this experiment, four 10-ml, $d = 0.002$ cultures each for Str1 and Str2 and four 100-ml, $d = 0.01$ cultures each for Str1 and Str2. To minimize the likelihood of unrecognized contamination with Str^s CAB281, the Str1 and Str2 bacteria we used in these evolution experiments carried spontaneous Mal⁻ and Gal⁻ mutations, respectively. The parental strains and all other CAB281 strains in our lab were able to ferment these sugars. These cultures were maintained by serial passage for 40 transfers, ~350 and 270 generations, respectively, for the 10-ml, $d = 0.002$ and the 100-ml, $d = 0.01$ cultures. By at least the 12th transfer all 16 of these cultures were dominated by *rpsL* cells carrying compensatory mutations. At the final transfer, 200 colonies were picked from each of the 16 cultures and tested for resistance to streptomycin. No Str^s mutants were detected in the eight 10-ml cultures or the four 100-ml cultures with Str1. On the other hand, two of the four 100-ml Str2 cultures were dominated by streptomycin-sensitive *rpsL*⁺ bacteria.

These results are consistent with the predictions of the bottleneck model in the sense that more of the 100-ml cultures were dominated by *rpsL*⁺ bacteria than the 10-ml cultures, 2/8 vs. 0/8. But alas, this difference, at least from the objective perspective of a contingency chi-square test with Yates correction, is not significant, $\chi^2 = 0.571$, $P > 0.05$. If we include the data from the earlier study by Schrag and Perrot (1996), who maintained their 10-ml Str1 and Str2 cultures for 180 generations, obtained compensatory mutants, but failed to detect revertants, the score looks better for this hypothesis, 2/8 for the 100-ml cultures and 0/12 for the 10-ml cultures. Nevertheless, the Yates-corrected contingency χ^2 , 1.34, is still not significant at the 5% level.

3. *Invasion of *rpsL*⁺:* Reconstruction experiments were performed with 10-ml, $d = 0.002$ serial transfer cultures initiated with an average of 0.4 *rpsL*⁺ NaI^r cells per milliliter and $\sim 10^7$ of either Str1, Str2, and two independent compensatory mutants for these *rpsL* Str1

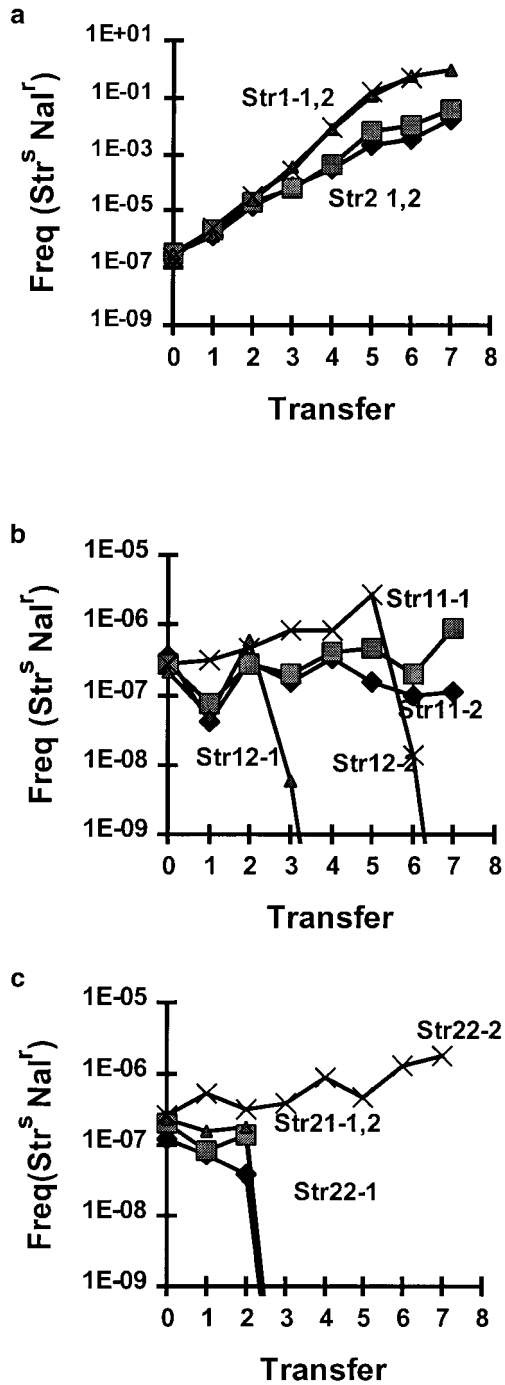


Figure 4.—Reconstruction experiments. Invasion of rare *Str^s Nal^r* cells cultures dominated by (a) uncompensated *Str^r* mutants Str1 and Str2; (b) fitness-compensated *Str^r* mutants Str11 and Str12; and (c) fitness-compensated *Str^r* mutants Str21 and Str22. On average, these 10-ml ($d = 0.002$) cultures initially contained 0.4 ml *Str^s Nal^r* bacteria. Each line shows the results of a single reconstruction experiment.

mutants, Str11, Str12, and two for the Str2, Str21 and Str22. Each experiment was replicated twice. The results of these experiments are presented in Figure 4. The rare *rpsL⁺* cell line successfully invaded and rapidly rose in frequency in each of the cultures initially dominated

by the uncompensated *rpsL* mutants, Str1 and Str2 (Figure 4a). This was not the case in the experiments with the *rpsL⁺* cell line introduced in a majority population of the higher-fitness compensatory mutants. In five of these eight experiments (Figure 4, b and c) the uncompensated *rpsL⁺* cell line was lost by the seventh transfer, when the experiment was terminated. Moreover, in only one experiment, with *rpsL⁺* invading one of the Str2 compensatory mutants, was there clear evidence for the wild-type gene increasing in frequency (Figure 4c).

To compare these results to what would be anticipated from the bottleneck hypothesis, we performed a series of independent simulations of these reconstruction experiments with *rpsL⁺* cells introduced in populations of uncompensated and compensated *rpsL* mutants. In these simulations $vol = 10$ ml and $d = 0.002$. Each run was initiated with an average of 0.4 *rpsL⁺* and 2×10^6 *rpsL* per milliliter with the actual number of *rpsL⁺* bacteria at the start of a given run being calculated by a Monte Carlo routine assuming a Poisson distribution. In our analysis we considered simulations only when, at the start of the second transfer, there was at least one *rpsL⁺* cell per milliliter, which was the case for all of the experimental cultures depicted in Figure 4. In the simulations with *Str^s* introduced in a population of Str1-uncompensated *rpsL* (n10), in all 100 simulation runs (Figure 5a) the sensitive n0 ascended at rates similar to that observed in the corresponding experiments (Figure 5a). In the simulation of n0 (the wild type) introduced in a population of n11 (the fitness-compensated *rpsL* mutants), n0 appeared to do better in the simulations than they did in the experiments; compare Figure 5b to Figure 4, b and c. Moreover, while in the experiments, five out of eight minority populations of *Str^s* cells were lost by the seventh transfer, in the simulations only six were lost out of 100 independent runs. Thus, if anything, stochastic processes played a less significant role in determining the outcome of the n0 invading n11 simulations than they did in the experimental cultures.

DISCUSSION

We interpret the results of this study as support for the hypothesis that in the study of Schrag and Perrot (1996) the ascent of intermediate-fitness *Str^r* compensatory mutations, rather than highest-fitness *Str^s* revertants, is a consequence of the rate of compensatory mutation being substantially higher than that of reversion and of the bottlenecks associated with serial passage. While the bacteria are growing within a transfer, higher-fitness *Str^s* (*rpsL⁺*) revertants may be generated in the cultures initially dominated by *Str^r* *rpsL* mutants. However, because of the lower rate at which they are produced by mutation, these higher-fitness revertants make their debut later than the intermediate-fitness *rpsL* compensatory mutants. Although they are proliferating at a higher rate than the compensatory mutants or the

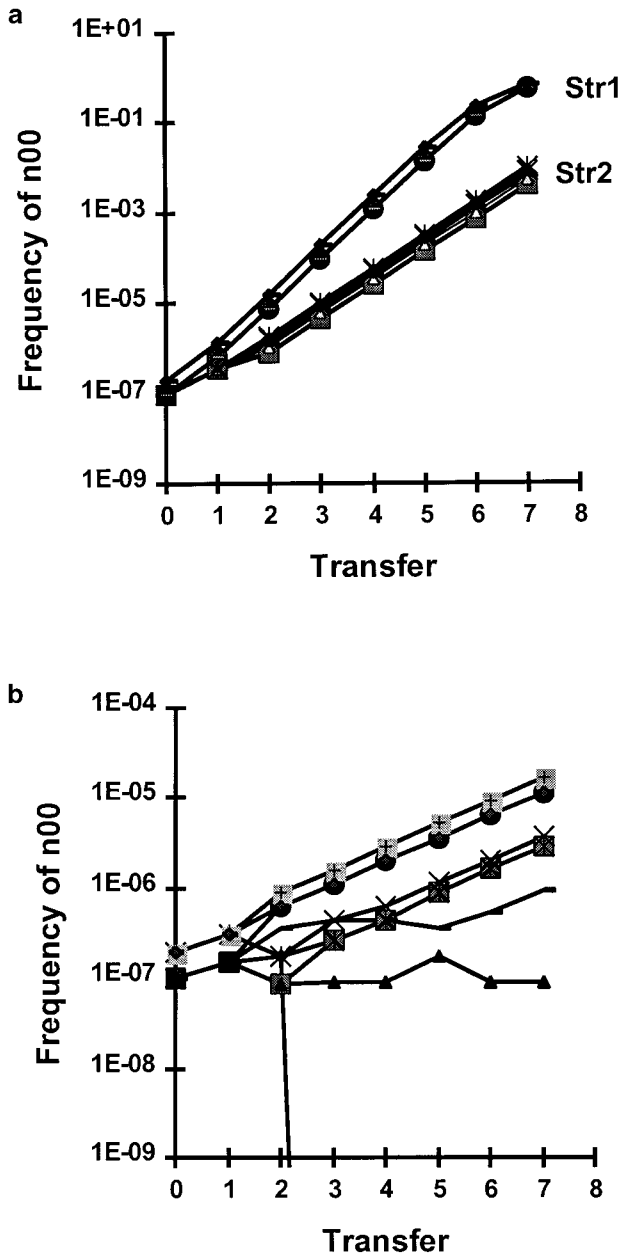


Figure 5.—Reconstruction simulations. Invasion of uncompensated Str^s ($n00$) in 10-ml $d = 0.002$ habitats. (a) Five randomly chosen simulations each of $n00$ invading $n10$ populations with fitness values similar to those estimated for $\text{Str}1$ and $\text{Str}2$ (see the legend to Figure 2). (b) Ten randomly chosen simulations of $n00$ bacteria invading a population initially dominated by fitness-compensated *rpsL* mutants ($n11$); $s_{11} = 0.08$. In these simulations, the average initial density of the invading $n00$ population was 0.4/ml.

original *rpsL* cell line, by the end of the transfer these come-lately revertants will still be in lower numbers than the compensatory mutants and are less likely to be included in the small fraction of the population that is transferred. Once they are present, at each successive transfer the numbers of *rpsL* compensatory mutants will increase because of their advantage over the uncompen-

sated *rpsL* population. Although they continue to be produced by mutation from the original *rpsL* cell line, the number of revertants at the end of each successive transfer gets progressively smaller, due to the accumulation of compensatory mutants. Eventually, because of the bottlenecks only *rpsL* compensatory mutants will be present in a succeeding transfer. While *rpsL*⁺ bacteria with the compensatory mutations will also be produced, because of their selective disadvantage relative to all other cell lines, their densities will remain low.

We conjecture that this same mutation rate/bottleneck mechanism accounts for the compensatory evolution observed by Borman *et al.* (1996) in their tissue culture serial transfer experiments with HIV resistant to a protease inhibitor. We believe this mechanism may also account for the ascent of either compensatory mutants or higher-fitness resistant mutants rather than sensitive revertants in the *S. typhimurium in vivo* (laboratory mouse) serial passage results of Björkman *et al.* (1998). However, for this bottleneck process to account for their *rpsL* results, it is necessary to assume that the rate of reversion at the 42nd codon AAC to AAA or AAG is less than the rate at which AAC yields the higher-fitness AGA streptomycin-resistant mutants, which evolved in the majority of their *rpsL* experiments.

While these examples of compensatory evolution are from laboratory experiments, we believe the phenomenon is general and can be anticipated to occur for most asexual pathogenic and commensal microorganisms in natural settings. Only a small fraction of the populations of microparasites infecting a host are transmitted to and successfully colonize new hosts. Stated another way, bottlenecks are a general feature of the structure of microparasite populations in communities of infected and uninfected hosts (Bergstrom *et al.* 1999). If, in addition, the tenure of microparasite populations in an infected host is limited, and these populations are cleared rather than continue to replicate and evolve in an individual host, then the first-occurring rather than the overall best genotype in any given host may achieve dominance and may not be replaced by a better genotype before that lineage is cleared. If the transmission bottleneck is small enough, and the duration of colonization brief enough, only rarely will the best-adapted but low frequency genotypes be transmitted to a new host.

To further examine the effect of the size of the bottleneck on evolution in these serial passage systems, we used the Monte Carlo simulation described earlier to explore the effects of different values of d and vol on the frequency of runs that are (i) fixed for the highest-fitness uncompensated Str^s genotype, $n00$, (ii) fixed for the compensatory mutant, $n11$, and (iii) polymorphic for more than one genotype by the 50th transfer. In these simulations, we use the standard parameter values noted in the legend to Figure 3 and with 10^7 uncompensated Str^s bacteria per milliliter, $n10$, as the sole initial

TABLE 1
Monte Carlo simulation of mutation, bottlenecks, and selection in serial transfer culture

Dilution factor	$d = 10^{-3}$	$d = 10^{-4}$	$d = 10^{-5}$	$d = 10^{-6}$
10 ml				
Fixed for n00	<i>98</i> (37.4 ± 3.8)	<i>53</i> (29.8 ± 4.2)	<i>20</i> (32.7 ± 7.5)	<i>0</i> (—)
Fixed for n11	<i>324</i> (44.0 ± 30.5)	<i>356</i> (33.7 ± 1.8)	<i>118</i> (34.3 ± 3.2)	<i>28</i> (34.1 ± 6.7)
Polymorphic	<i>78</i>	<i>91</i>	<i>362</i>	<i>472</i>
100 ml				
Fixed for n00	<i>371</i> (41.2 ± 2.1)	<i>193</i> (34.3 ± 2.5)	<i>53</i> (29.1 ± 4.1)	<i>14</i> (22.9 ± 6.6)
Fixed for n11	<i>3</i> (44.0 ± 30.5)	<i>273</i> (29.7 ± 1.8)	<i>423</i> (27.8 ± 1.3)	<i>195</i> (31.2 ± 2.3)
Polymorphic	<i>126</i>	<i>34</i>	<i>24</i>	<i>291</i>

Number of runs initiated solely with n10 that are fixed for either n00 or n11, or polymorphic at the 50th transfer (*italic*), and the mean and standard deviation of the transfer at which fixation occurred. The resource concentration, population growth, and mutation and selection parameters used in these simulations are identical to those in the legend to Figure 3. These runs terminated at the end of the 50th transfer or when, at the end of an earlier transfer, n00 was fixed (n10 = n01 = n11 = 0) or n11 was fixed (n00 = n10 = n01 = 0).

population. A total of 500 independent runs were made with each set of parameters. The results of these simulations are presented in Table 1. For both the 10-ml and 100-ml cultures, the ratio of the number of runs fixed at the 50th transfer for the lower-fitness, n11 population relative to those fixed for the highest-fitness cell line, n00, increases as the fraction of the population transferred, d , declines. For the 10-ml cultures, the proportion of populations that are polymorphic at the 50th transfer increases as the dilution factor declines. For the 100-ml simulations, on the other hand, the number of runs that were polymorphic at the 50th transfer initially declined with the decline in the dilution factor, but then increased. This can be attributed to the loss of the n11 as well as the n00 genotypes, when the bottleneck becomes too small. Each time these mutants are lost completely, the run effectively starts over with just n10.

This situation, where the more frequently occurring rather than the best-adapted mutants become fixed, is exacerbated by the fact that the intermediate mutant states, Str^s bacteria with compensatory mutations and Str^r bacteria without compensatory mutations, are at a selective disadvantage relative to the original, sensitive genotype and the resistant genotypes with the compensatory mutation, as observed by Borman *et al.* (1996) and Schrag *et al.* (1997). The fixation of the compensatory mutation establishes an adaptive valley that virtually precludes a return to the original state. This adaptive valley mechanism almost certainly accounts for why, in the 10,000-generation experimental evolution study of Richard Lenski and his collaborators (Lenski and Travisano 1994), the originally streptomycin-resistant *rpsL* *E. coli* B strain they studied remained resistant to this antibiotic, despite the absence of this drug (see Schrag *et al.* 1997). At this juncture, we do not know how common this kind of negative epistasis is for mutations that compensate for the fitness costs of drug resistance, or

other environment-specific adaptations, of microparasites.

To be sure, the ascent and fixation of the earlier-occurring rather than the best-adapted genotypes due to this bottleneck-mutation rate mechanism is a non-equilibrium result. On Equilibrium Day deterministic processes will prevail and the best genotypes will inherit the earth. If antibiotic use ceases and sensitive bacteria are more fit than resistant (with or without compensatory mutations), they will eventually replace the resistant. This would happen even if the intermediates, sensitive bacteria with compensatory mutations, are at a selective disadvantage. The rate at which the equilibrium with only drug-sensitive bacteria will be achieved can be quite slow, however, and particularly so if resistance engenders little cost to the fitness of bacteria in the absence of antibiotics and if the antibiotics that favor resistance continue to be used.

The rate of approach to the best-prevails equilibrium outcome could be accelerated and compensatory evolution negated if hosts are commonly infected with multiple lineages of the same microparasites. In the cases considered in Table 1, if hosts are either simultaneously or subsequently infected with n00 as well as n11, the higher fit cell line, n00, is more likely to ascend than if infections are monoclonal. For example, if instead of initiating the runs presented in Table 1 with n10 as the sole population, there were also 20 cells per milliliter each of n00, n01, and n11, a much higher fraction of the simulations would be fixed for the n00 bacteria relative to that fixed for n11. For example, under these conditions for the 100-ml cultures with $d = 10^{-4}$, 499 out of 500 runs were fixed for n00 by the 50th transfer (as opposed to 193), with the mean and standard error of the transfer of fixation being 19.6 and 0.83, respectively. In this initially polyclonal simulation, only 1 run (as opposed to 273) was fixed for n11. For a more general and more mathematical consideration of the

contribution of bottlenecks to selection in bacterial populations, see Gerrish (1998).

Recombination can also negate the effects of compensatory evolution. In the situation considered here, if the rates of mutation from n_{11} to n_{01} and the rates of recombination between n_{10} and n_{01} are substantial, the most fit, n_{00} genotypes would be produced by recombination as well as by mutation. This would increase the likelihood and rate of the fixation of this most fit genotype in any given population. A more quantitative and mathematically formal consideration of the role of recombination in an analogous epistatic compensatory evolution process has been considered by Stephan (1996). In his model, unlike the one analyzed here, the fitness of the n_{00} is equal to that of n_{11} and that of the lower-fitness n_{10} population is the same as that of n_{01} . As the rate of recombination increases, the rate at which the n_{11} population evolves from the n_{00} state declines as long as the intermediates are at a marked disadvantage, as they are here.

There is no way to interpret these compensatory evolution-bottleneck mechanisms in a positive light with respect to the drug resistance problem. It is clear that if antibiotics and other antimicrobial chemotherapeutic agents continue to be used, microparasites resistant to those compounds will eventually ascend and persist for extensive periods. For commensal microbes transmitted through common reservoirs in open communities (as opposed to hospitals and similar institutions), relatively little drug use can maintain substantial equilibrium frequencies of resistant organisms that would be at a selective disadvantage in the absence of these drugs (Levin *et al.* 1997; Stewart *et al.* 1998). For directly (and presumably sexually and vector-) transmitted pathogens responsible for acute (mortal and/or cleared) infections in open communities, whether resistance ascends at all depends on the level of drug use. If relative to drug-sensitive bacteria, resistance engenders (i) a transmission disadvantage, (ii) a competitive disadvantage in mixed infections, and/or (iii) higher rates of clearance in (or greater rates of mortality of) infected hosts, resistance may not ascend at all even when drugs are employed (Massad *et al.* 1993; Austin *et al.* 1997). On the other hand, if the level of drug use is sufficiently high, the populations will eventually be dominated by resistant microbes.

One effect of the continued use of antibiotics is to make compensatory evolution all that more likely than reversion. There is no evolutionary alternative for bacteria in hosts that are under treatment or in an environment that abounds with active antibiotics. In the presence of these chemotherapeutic agents, sensitive revertants would be at a selective disadvantage, even if they may be more fit than resistant bacteria in the absence of these drugs. Stating this another way, as a consequence of the extensive use of antibiotics during the

past half century, we have established not only an environment where not-so-natural selection favors resistance, but also an environment where selection would almost necessarily favor mutations that compensate for the fitness costs of those resistance genes and plasmids, rather than drug-sensitive revertants and segregants. Moreover, it may well be that this compensatory evolution in bacteria has established genetic backgrounds where susceptible revertants (or segregants, in the case of plasmids) are at a selective disadvantage relative to resistant, as has been observed experimentally by Borman *et al.* (1996) and Schrag *et al.* (1997) for chromosomal resistance and by Bouma and Lenski (1988) and Modi and Adams (1991) for plasmid-borne resistance.

Nevertheless, despite all of these wonderful experimental results, models, and verbal arguments, at this juncture it remains unclear just how important this kind of compensatory evolution actually is for the persistence of drug resistance in natural populations of bacteria and other microparasites. This remains a currently unanswered empirical question, but one that could be addressed by retrospective genetic (molecular) studies of drug-resistant microparasites isolated from treated, untreated, and previously treated infected hosts. It could well be that compensatory evolution has played little or no role in natural populations of drug-resistant bacteria and other microparasites. In accord with the present theory, drug-sensitive revertants (or segregants in the case of accessory element-encoded resistance), rather than compensatory mutants, could ascend in a substantial fraction of treated patients following the cessation of drug use. And, in the course of time before a microparasite population colonizes a new drug-treated host or enters an extrahost environment where drug-mediated selection favors resistance, drug-sensitive members of that population could ascend and replace those that are resistant. As long as drug-sensitive microparasites remain in the population and are more fit than resistant members of that species (clone or ecotype) with and without compensatory mutations, reductions in rates of use of antimicrobial agents can lead to reductions in the frequencies of resistance. This may have been the reason for the decline in the frequencies of resistance observed in countries that have reduced the rates at which specific classes of antibiotics have been used (Austin *et al.* 1997; Seppala *et al.* 1997).

While this investigation was motivated by concern with adaptation to the cost of antibiotic resistance, we believe the evolutionary consequences of these bottleneck mechanisms are more general. The processes considered here will affect the ascent of other adaptive characters in pathogenic and commensal microparasites in general. Within-host selection would favor characters that increase their rate of proliferation and term of persistence in a host and the expansion of their niches for proliferation and persistence in other sites and tissues (Levin and Bull 1994). If, however, because of

the bottlenecks, these locally adapted genotypes are not transmitted or for physical reasons cannot be transmitted (*e.g.*, they reside in cerebrospinal fluid), the within-host evolution that occurred may have little or no effect on global evolution of that microparasite.

Bottlenecks may be particularly critical in situations where microparasites infect novel host species. Even if mutants can be produced that allow for the adaptation to the new host, if the infection is cleared or the host dies before a substantial fraction of the transmitted parasites are of the adapted genotypes, the evolved host-adapted lineages may be lost. The net effect would be to delay the rate of adaptation of a parasite to the new host species (see, *e.g.*, Bergstrom *et al.* 1999). Bottlenecks are not all bad.

A molecular geneticist of some repute is said to have suggested that "what is true for *E. coli* is true for elephants, only more so." While it would take a rather large bottle for an individual elephant to pass through its neck, in the course of time populations of elephants like those of virtually all species will contract to relatively low numbers and pass through metaphorical bottlenecks of the sort considered here. Moreover, populations of elephants, as well as those of other eukaryotes, will occasionally include individuals bearing mutations that augment their fitness in the current environment. A population and evolutionary geneticist of some repute once suggested that adaptive mutants of less effect in increasing fitness are going to occur more frequently than those of greater effect (Fisher 1930; see also Kimura 1983). While there is little direct evidence for Fisher's conjecture being true for elephants or other eukaryotes, a very elegant experimental study by Burch and Chao (1999) provides compelling evidence in its support for the Pseudomonas phage $\phi 6$. Bottlenecks were the key to their demonstration. The narrower the bottleneck, the smaller the increase in fitness of this RNA virus. Stated another way, because they are generated at a lower rate, higher-fitness mutants are less likely to pass through the bottlenecks than more frequently occurring mutants of a lower fitness and are thus less likely to ascend, at least in the short run. If, in general, the rates at which adaptive mutants occur are inversely proportional to the extent to which they augment fitness and there are periodic contractions in population size, it may well be that the mutation rate-bottleneck mechanisms considered here have played (and continue to play) a role in the adaptive evolution of elephants and more diminutive higher organisms. While it is easy to raise this possibility, at this time we have neither the theory to evaluate nor data to support or refute this extrapolation of the present results.

We thank Carl Bergstrom, Mary Reynolds, and two (somewhat) anonymous reviewers for useful comments and suggestions. This research and the preparation of this report has been supported by a grant from the National Institutes of Health, grant no. AI40662, and the Swedish National Science Foundation, NFR to B.R.L.

LITERATURE CITED

- Andersson, D. I., K. Bohman, L. A. Isaksson and C. G. Kurland, 1982 Translation rates and misreading characteristics of *rpsD* mutants in *Escherichia coli*. *Mol. Gen. Genet.* **187**: 467-472.
- Austin, D. J., M. Kakehashi and R. M. Anderson, 1997 The transmission dynamics of antibiotic-resistant bacteria: the relationship between resistance in commensal organisms and antibiotic consumption. *Proc. R. Soc. Lond. Ser. B* **264**: 1629-1638.
- Bergstrom, C. T., P. McElhany and L. A. Real, 1999 Transmission bottlenecks as determinants of virulence in rapidly evolving pathogens. *Proc. Natl. Acad. Sci. USA* **96**: 5095-5100.
- Bilgin, N., F. Claesens, H. Pahverk and M. Ehrenberg, 1992 Kinetic properties of *Escherichia coli* ribosomes with altered forms of S12. *J. Mol. Biol.* **224**: 1011-1027.
- Björkman, J., D. Hughes and D. I. Andersson, 1998 Virulence of antibiotic-resistant *Salmonella typhimurium*. *Proc. Natl. Acad. Sci. USA* **95**: 3949-3953.
- Bloch, C. A., and C. K. Rode, 1996 Pathogenicity island evaluation in *Escherichia coli* K1 by crossing with laboratory strain K-12. *Infect. Immun.* **64**: 3218-3223.
- Borman, A. M., S. Paulous and F. Clavel, 1996 Resistance of human immunodeficiency virus type 1 to protease inhibitors: selection of resistance mutations in the presence and absence of the drug. *J. Gen. Virol.* **77**: 419-426.
- Bouma, J. E., and R. E. Lenski, 1988 Evolution of a bacteria/plasmid association. *Nature* **335**: 351-352.
- Burch, C. L., and L. Chao, 1999 Evolution by small steps and rugged landscapes in the RNA virus $\phi 6$. *Genetics* **151**: 921-927.
- Carlton, B. C., and B. J. Brown, 1981 Gene mutation, pp. 222-242 in *Manual of Methods for General Microbiology*, edited by P. Gerhardt. American Society for Microbiology, Washington DC.
- Cohan, F. M., E. C. King and P. Zawadzki, 1994 Amelioration of the deleterious pleiotropic effects of an adaptive mutation in *Bacillus subtilis*. *Evolution* **48**: 81-95.
- Fisher, R. A., 1930 *The Genetical Theory of Natural Selection*. Oxford University Press, Oxford. Reprinted by Dover Press.
- Gerrish, P. E., 1998 *Dynamics of Mutation and Selection in Asexual Populations*. Ph.D. Dissertation, Michigan State University, East Lansing, MI.
- Hasenbank, R., C. Guthrie, G. Stöffler, H. G. Wittmann, L. Rosen *et al.*, 1973 Electrophoretic and immunological studies on ribosomal proteins of 100 *Escherichia coli* revertants from streptomycin dependence. *Mol. Gen. Genet.* **127**: 1-18.
- Kimura, M., 1983 *The Neutral Theory of Molecular Evolution*. Cambridge University Press, Cambridge, United Kingdom.
- Larossa, R. A., 1996 Mutant selections: linking physiology, inhibitors and genotype, pp. 2527-2587 in *Escherichia and Salmonella*, edited by F. C. Neidhart. ASM Press, Washington, DC.
- Lenski, R. E., and M. Travisano, 1994 Dynamics of adaptation and diversification: a 10,000-generation experiment with bacterial populations. *Proc. Natl. Acad. Sci. USA* **91**: 6808-6814.
- Levin, B. R., and J. J. Bull, 1994 Short-sighted evolution and the virulence of pathogenic microorganisms. *Trends Microbiol.* **2**: 76-81.
- Levin, B. R., F. M. Stewart and L. Chao, 1977 Resource-limited growth, competition, and predation: a model and experimental studies with bacteria and bacteriophage. *Am. Nat.* **111**: 3-24.
- Levin, B. R., M. Lipsitch, V. Perrot, S. Schrag, R. Antia *et al.*, 1997 The population genetics of antibiotic resistance. *Clin. Infect. Dis.* **24**: S9-S16.
- Luria, S., and M. Delbruck, 1943 Mutations of bacteria from virus sensitivity to virus resistance. *Genetics* **28**: 491-511.
- Massad, E., S. Lundberg and H. M. Yang, 1993 Modeling and simulating the evolution of resistance against antibiotics. *Int. J. Biomed. Comput.* **33**: 65-81.
- Modi, R. I., and J. Adams, 1991 Coevolution in bacteria-plasmid populations. *Evolution* **45**: 656-667.
- Monod, J., 1949 The growth of bacterial cultures. *Annu. Rev. Microbiol.* **3**: 371-394.
- Rosset, R., and L. Gorini, 1969 A ribosomal ambiguity mutation. *J. Mol. Biol.* **39**: 95-112.
- Schrag, S., and V. Perrot, 1996 Reducing antibiotic resistance. *Nature* **381**: 120-121.
- Schrag, S., V. Perrot and B. R. Levin, 1997 Adaptation to the

- fitness cost of antibiotic resistance in *Escherichia coli*. Proc. R. Soc. Lond. Ser. B **264**: 1287–1291.
- Seppala, H., T. Llaukka, J. Vuopio-Varkila, A. Muotiala, H. Henttonen *et al.*, 1997 The effects of changes in the consumption of macrolide antibiotics of erythromycin resistance in Group A Streptococci in Finland. N. Engl. J. Med. **331**: 120–121.
- Stephan, W., 1996 The rate of compensatory mutation. Genetics **114**: 419–426.
- Stewart, F. M., and B. R. Levin, 1973 Resource partitioning and the outcome of interspecific competition: a model and some general considerations. Am. Nat. **107**: 171–198.
- Stewart, F. M., D. M. Gordon and B. R. Levin, 1990 Fluctuation analysis: the probability distribution of the number of mutants under different conditions. Genetics **124**: 175–185.
- Stewart, F. M., R. Antia, B. R. Levin, M. Lipsitch and J. E. Mittler, 1998 The population dynamics of antibiotic resistance II: analytical theory for sustained populations of bacteria in a population of hosts. Theor. Popul. Biol. **53**: 152–165.
- Vasi, F., M. Travisano and R. E. Lenski, 1994 Long-term experimental evolution in *Escherichia coli*. II. Changes in life-history traits during adaptation to a seasonal environment. Am. Nat. **144**: 432–456.
- Zengel, J. M., R. Young, R. R. Dennis and M. Nomura, 1977 Role of ribosomal protein S12 in peptide chain elongation: analysis of pleiotropic, streptomycin-resistant mutants of *Escherichia coli*. J. Bacteriol. **129**: 1320–1329.

Communicating editor: M. Slatkin