nine. Without these factors that promote replication slippage and unequal crossovers, the introns exhibit less divergence than the repeated exons and tend to require fewer alignment gaps (Figs. 2B and 3).

The greater divergence of the *Flag* repetitive exons relative to the introns could be due to different directional selection regimes operating on the Flag fibers of *N.c.* versus *N.m.* If so, then a unique sequence element that is advantageous to one species is expected to be present in all of its repeated exons and absent from the repeated exons of another species. Given that there are very few fixed differences among the ensemble repeats within *N.c.* or *N.m.*, directional selection does not seem to account for rapid sequence divergence relative to the repeated introns. Also, purifying selection on the silk protein structure is reflected in the strict maintenance of the ensemble organization (Fig. 2A). Instead of speeding up evolution, functional constraints should decrease sequence divergence of the exons. Thus, the greater exonic divergence still can be best explained by the molecular architecture of the *Flag* gene.

Flag was known to be a modular protein with three basic motifs composing a large ensemble repeat. The genomic organization of the *Flag* gene suggests a new hierarchical level of modularity. Not only are the ensemble repeats encoded by repeated exons, but the intervening introns are also iterated copies. This molecular architecture results in efficient within-gene concerted evolution. Probably through some combination of gene conversion and unequal crossing-over at repetitive exons, *Flag* remains fairly homogenized over its entire 15,500-bp coding sequence. However, this same highly repetitive architecture apparently prevents the coding sequences of *Flag* from completely homogenizing. The evolution of the *Flag* gene represents a case in which homogenization of repeats through purifying selection and recombination is offset by mutational mechanisms inherent in the basic structure of the DNA sequences (*12*). Thus, the repetitive genetic architecture of spider silk encourages sequence homogenization as well as rapid sequence divergence. This conflict has implications for the interpretation of high-performance silks as optimally adapted supermolecules (*1*, *13*).

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Effects of Environment on Compensatory Mutations to Ameliorate Costs of Antibiotic Resistance

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Most types of antibiotic resistance impose a biological cost on bacterial fitness. These costs can be compensated, usually without loss of resistance, by second-site mutations during the evolution of the resistant bacteria in an experimental host or in a laboratory medium. Different fitness-compensating mutations were selected depending on whether the bacteria evolved through serial passage in mice or in a laboratory medium. This difference in mutation spectra was caused by either a growth condition–specific formation or selection of the compensated mutants. These results suggest that bacterial evolution to reduce the costs of antibiotic resistance can take different trajectories within and outside a host.

Among the major factors determining the frequency of resistance in a bacterial population are (i) the volume of antibiotic use, (ii) the costs of resistance to bacterial fitness, and (iii) the ability of bacteria to genetically compensate for such costs (*1*, *2*). Generally, both plasmid- and chromosomally conferred resistances cause fitness losses, even though exceptions are known. When resistance has a cost, compensatory mutations can ameliorate these costs, commonly without loss of resistance (*3*, *4*).

To determine whether the costs of resistance are compensated by different mutations under different growth conditions, we examined two

types of antibiotic resistance in *Salmonella typhimurium* strain LT2: streptomycin resistance (SmR) caused by mutations in the *rpsL* gene, which encodes ribosomal protein S12 (*5*), and fusidic acid resistance (FusR) caused by mutations in the *fusA* gene, which encodes elongation factor G (EF-G) (*6*). All resistant mutants studied grow slowly in laboratory media because of a decreased rate of protein synthesis (*5*, *6*). As shown here for the FusR mutants and previously for the SmR mutants (*4*), they were also slow-growing in mice. Independent lines of the resistant bacteria were evolved by serial passages in a laboratory medium (LB, Luria Bertani broth) or in mice in the absence of antibiotic. By this procedure, spontaneous mutants were selected by virtue of their faster growth rates (*7*). The occurrence of compensated mutants was determined by plating bacteria from each cycle on agar plates; when fast-growing compensated mutants became the dominant population, the experiments were terminated. One random fast-growing clone from each lineage was examined. For the SmR mutants, the number of generations of growth and the population sizes were similar in mice and in LB. Thus, the opportunity for compensated mutants to arise and evolve was similar under the two growth conditions. For the FusR mutants, the number of generations of growth was fewer in mice than in LB (*7*). The compensatory mutations were located by sequencing of the gene with the resistance mutation and of extragenic targets that have previously been implicated in compensation (*8*).

For the SmR mutants, all LB-selected compensated mutants contained extragenic suppressor mutations in either the *rpsD* or *rpsE* gene, whereas all mouse-selected mutants had one specific intragenic compensatory mutation. For the FusR mutants, compensation was preferentially by intragenic suppression for bacteria grown in LB and mainly by true reversion for bacteria evolved in mice (Table 1). Compensated mutants commonly retained resistance. Thus, 17 of the 18 types of compensated FusR mutants and all of the compensated SmR mutants kept their resistance. Two tests were performed to ensure that the compensatory mutations were specific adaptations. When control lines of the sensitive parent were serially passaged under both growth conditions, no compensatory mutations were selected, which shows that these mutations are not generally beneficial. Furthermore, when *rpsD* compensatory mutations were placed in the sensitive background, they did not increase fitness (*4*).

We considered two models to explain the different spectra of compensatory mutations: The difference was either in the rate of formation of the compensatory mutations or in the selection of the compensated mutants. For the FusR mutants, selection accounted for the difference in compensatory mutations. Full compensation of the growth defect in LB can be conferred either by reversion (which is rare) or by intragenic compensatory mutations (which are common because they can occur by many different substitutions). Thus, intragenic mutants were preferentially found in LB because they were more common. In contrast, when the relative fitness of the compensated bacteria was determined by competition assays against the wild-type strain in mice, the revertants were fully compensated, whereas the intragenically compensated mutants showed only partial compensation. The 15 different compensated mutants evolved in LB showed relative fitness values from 0.33 to 0.94, whereas the true revertants had a relative fitness of 1.0 (Table 2). Thus, even though the revertants (because of their smaller genetic target size) were rarer than the second-site suppressors, they were predominantly selected in mice because they had fully restored fitness.

Why were certain compensatory mutations able to ameliorate fitness losses for bacteria

Table 1. Spectra of compensated mutants isolated after evolution in LB and in mice.

Resistance	Resistance mutation*	Growth condition during compensation	Compensatory mutation†			Number of independently evolved mutations examined
			Extragenic	Intragenic	Reversion	
Fusidic acid	P413L	LB	0	26 (see Table 2)	2	28
	P413L	Mice	0	11 (see Table 2)	14	25
Streptomycin	K42N	LB	14 (see Table 3)	0	0	14
	K42N	Mice	0	10 (see Table 3)	0	10

*Amino acid changes (*11*). †The type and number of compensatory mutations or revertants found. The significance of the difference in compensatory mutations found was determined by Fisher's exact test (*18*). For both resistances, P values were $<10^{-4}$.

Table 2. Fitness in mice and in LB of FusR and compensated mutants.

Strain	Mutation (number of independent isolates found)	Compensation selection condition†	Relative fitness in mice‡	Relative fitness in LB‡
	EF-G*			
JB124	wt	NA	1.0	1.0
JB393	P413L	NA	No growth	0.41
JB2080	wt (revertant) (2)	LB	1.0	1.0
JB2124	P413L, G13C (3)	LB	0.94	1.0
JB2111	P413L, L413Q (3)	LB	0.85	1.0
JB2105	P413L, R407G (1)	LB	0.82	0.90
JB2117	P413L, A378V (3)	LB	0.81	1.0
JB2115	P413L, G13A (1)	LB	0.79	1.0
JB2108	P413L, V363F (1)	LB	0.74	0.96
JB2119	P413L, L413V (1)	LB	0.68	1.0
JB2104	P413L, A66V (2)	LB	0.66	1.0
JB2112	P413L, I294S (1)	LB	0.64	1.0
JB2122	P413L, V376A (3)	LB	0.63	1.0
JB2114	P413L, F444L (3)	LB	0.59	0.96
JB2109	P413L, A378T (1)	LB	0.54	0.87
JB2113	P413L, L387P (1)	LB	0.42	0.93
JB2120	P413L, V291E (1)	LB	0.36	1.0
JB2110	P413L, T423I (1)	LB	0.33	0.90
JB2153	wt (revertant) (14)	Mice	1.0	1.0
JB2180	P413L, F334L (7)	Mice	0.72	ND
JB1777	P413L, I294D (3)	Mice	0.52	1.0
JB1744	P413L, P683L (1)	Mice	0.29	0.96

*Amino acid changes in EF-G (*11*). †Growth condition under which the compensated mutants were selected (*7*). ‡The relative fitness is defined as the generation time of the wild type divided by the generation time of the mutant (*19*). wt, wild type; NA, not applicable; ND, not determined.

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grown in LB but not in mice? It has been shown that the FusR mutants have alterations in their levels of the nucleotides (p)ppGpp (*9*). Because these nucleotides are pleiotropic regulators of gene expression (*10*), alterations in their concentrations could affect the expression of virulence-related genes and thereby cause fitness differences in mice, without necessarily affecting growth in a laboratory medium.

For the SmR mutants, growth selection could not account for the different compensated mutants. From the competition experiments in mice, we did not detect any substantial difference in the competitive ability of the *rpsD* extragenic compensated mutants found after evolution in LB and the intragenic *rpsL* compensated mutants (AAC_{42} → AGA mutation) found after evolution in mice (Table 3). The *rpsD-rpsL* double mutants found showed relative fitness values from 0.91 to 1.0, and the intragenically compensated *rpsL* mutant showed a relative fitness of 1.0. Thus, the absence of *rpsD* compensatory mutations in the *rpsL* mutant evolved in mice could not be explained by poor fitness compensation. An alternative explanation was that the ability of the *rpsD-rpsL* double mutants to compete with the *rpsL* mutant in the evolution experiments was low when the mutant was present at a low frequency. Thus, in the competition experiments to examine the fitness of the evolved strains, the competing strains were mixed at a 1:1 ratio, whereas the compensated *rpsD-rpsL* mutants appearing in the evolution experiments initially represented a minor type among the majority noncompensated *rpsL* mutants. To test whether the competitive ability of the compensated mutants was decreased when they were present at a lower frequency than the competitor, one compensated *rpsD-rpsL* mutant [Gln^{53} → Leu^{53} (Q53L), K42N (*11*) (Table 3)] was mixed with the *rpsL* mutant (K42N) at a low ratio ($1:10^4$) for competition in mice. Under these conditions also, the *rpsD-rpsL* mutant was able to compete out the *rpsL* mutant.

These results suggest that the different spectra of SmR compensatory mutations were caused by condition-dependent generation of the mutations. Considering the population size, the number of generations that the evolving bacteria spent in either mice or LB, and the fitness of the mutants, we calculated that the relative rate of the intragenic AAC → AGA mutation versus the extragenic *rpsD* mutations had to be several hundred times greater in mice than in LB in order to account for the difference in compensatory mutations (*12*). The specificity of the selected mutations in mice; i.e., only AAC_{42} → AGA mutations in preference to AAC_{42} → AAA reversion or extragenic *rpsD* compensatory mutations, suggests the existence of a mutational mechanism that limits mutations to a particular nucleotide change within the *rpsL* gene. An increased repair of mismatched DNA heteroduplexes (template-induced mutations), as described in the *rIIB* gene in bacteriophage T4, could explain the occurrence of the particular AAC_{42} → AGA mutation found in mice (*13*). Compatible with this idea was the finding that the mutation frequency in the *codA*, *codB*, or *upp* genes was higher for bacteria grown in mice than for those grown in laboratory medium. Thus, the median mutation frequency was about 13 to 28 times greater in bacteria recovered from mice than in bacteria grown in laboratory medium (*14*).

These findings about the specificity of the types of compensatory mutations found have several implications. First, they substantiate previous findings that reduced drug use might not result in a reduction of the frequency of resistant bacteria because of compensatory evolution and the maintenance of resistance in the compensated clones (*2*). Second, growth in mice and in laboratory medium imposes different constraints on the translation machinery (*15*). Third, and most important, the nature of the evolved compensatory changes is environment-dependent, which suggests that evolution to compensate for fitness losses caused by resistance mutations or other alterations, such as those associated with colonization and virulence, might occur by different mechanisms within and outside a host. In general terms, the rates and directions of molecular evolution may follow different trajectories because of the specific environment and its influence on mutation formation or selection. Finally, our data show that determinations of the amelioration of the costs of resistance should be performed in experimental hosts to allow a relevant assessment of the mechanism of compensation in resistant bacteria.

Table 3. Fitness in mice and in LB of SmR and compensated mutants.

Strain	Mutation		Compensation selection condition†	Relative fitness in mice‡	Relative fitness in LB‡
	S4*	S12*			
JB124	wt	wt	NA	1.0	1.0
JB127	wt	K42N (AAC)	NA	0.50	0.79
JB2162	Q53L	wt	NA	0.62	0.68
TH5461	Q53L	K42N	LB	1.0	0.93
TH5664	K205N	K42N	LB	0.94	0.81
TH5604	Q53P	K42N	LB	0.91	0.96
TH5606	V200	K42N	LB	0.91	0.90
TH5516	I199N	K42N	LB	0.91	0.90
TH5667	UAG201	K42N	LB	0.91	0.84
JB1258	wt	K42R (AGA)	Mice	1.0	0.96

*Amino acid changes in ribosomal proteins S4 and S12 (*11*). The nucleotide sequence of codon 42 is shown in parentheses. †Growth condition under which the compensated mutants were selected (*7*). ‡The relative fitness is defined as the generation time of the wild type divided by the generation time of the mutant (*19*). wt, wild type; NA, not applicable.

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Parental Care and Clutch Sizes in North and South American Birds

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The evolutionary causes of small clutch sizes in tropical and Southern Hemisphere regions are poorly understood. Alexander Skutch proposed 50 years ago that higher nest predation in the south constrains the rate at which parent birds can deliver food to young and thereby constrains clutch size by limiting the number of young that parents can feed. This hypothesis for explaining differences in clutch size and parental behaviors between latitudes has remained untested. Here, a detailed study of bird species in Arizona and Argentina shows that Skutch's hypothesis explains clutch size variation within North and South America. However, neither Skutch's hypothesis nor two major alternatives explain differences between latitudes.

The dependent young of parents risk being eaten by predators. Variation in this predation risk may be an important source of natural selection on the behavior and life history strategies of parents (*1*), although it often has been overlooked. For example, the evolution of clutch size has long been attributed to food limitation rather than to nest predation in birds living in northern temperate climates (*2*). Yet nest predation may explain clutch size variation within (*3*) and among (*4*) latitudes. These contrasting views of food limitation versus nest predation could be resolved by an untested hypothesis proposed by Alexander Skutch in 1949 (*5*). Skutch argued that predators use parental activity to find nests, creating a predation cost that constrains the rate at which parents can visit nests to feed their young and thereby constrains clutch size by limiting food delivery rates.

Skutch's hypothesis is important for three reasons. First, it proposes a mechanism whereby nest predation may create food limitation by constraining the rate of food delivery. This mechanism could then resolve alternative findings that food limitation (*2*) and nest predation (*3*) influence clutch size evolution in north temperate latitudes (*1*). Second, it suggests that parental care tactics (food provisioning) may be shaped by nest predation, a selection pressure that has been largely overlooked even though parental care is widely studied (*6*). Finally, this hypothesis is a widely invoked explanation for the small clutch sizes of tropical and Southern Hemisphere birds (*4*), which commonly lay about half as many eggs as their north temperate counterparts (*2, 7*). Yet the theory underlying this hypothesis remains undeveloped and untested.

Here we develop the theory underlying Skutch's hypothesis and test it with extensive data and original sampling methods in large, intact, north temperate and subtropical Southern Hemisphere bird communities. In particular, we examine two questions: (i) Does nest predation influence parental care tactics and constrain food delivery? (ii) Do rates of food delivery and nest predation explain variation in clutch sizes among species within and between North and South America?

With respect to parental care, Skutch's hypothesis assumes that higher parental visitation rates incur a predation cost, so that individuals with greater activity suffer higher nest predation. This cost is expected to favor the evolution of reduced visitation rates in species or geographic regions where predation risk is higher. Parents should attempt to compensate for reduced visitation (*8*) by increasing the load of food brought per trip. However, as long as the food delivery rate (food load times visitation rate) is positively correlated with the visitation rate and the visitation rate is constrained by nest predation, then Skutch's hypothesis that nest predation constrains food delivery will be supported. Clutch size is expected to be determined by the rate at which food is delivered to the young. So if food delivery is constrained by nest predation and clutch size is determined by food delivery rate, then clutch size should decrease with nest predation.

We tested these predicted relationships by studying birds in large intact forests in Arizona and subtropical Argentina (*9*). We found and monitored 1331 nests in Argentina and 7284 nests in Arizona (*10*). Mean clutch size varied from 1.83 to 6.75 eggs among our study species and differed between latitudes; the clutch sizes of passerines at our Argentina site ($\bar{x} \pm$ SE = 2.58 ± 0.11, $n = 23$ species) were similar to those in the humid tropics [2.41 ± 0.06, $n = 217$ species (*11*)] but were much smaller than at our Arizona site (4.61 ± 0.24, $n = 21$ species). Smaller mean clutch sizes in southern latitudes could possibly reflect effects of taxa that are unique to these regions and that differ in their ecology from those of north temperate areas (*12*). To increase the strength of inference for our comparisons between latitudes, we con-

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