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EPISTASIS BETWEEN NEW MUTATIONS AND GENETIC BACKGROUND AND A TEST OF GENETIC CANALIZATION

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Abstract.—The importance for fitness of epistatic interactions among mutations is poorly known, yet epistasis can exert important effects on the dynamics of evolving populations. We showed previously that epistatic interactions are common between pairs of random insertion mutations in the bacterium *Escherichia coli.* In this paper, we examine interactions between these mutations and other mutations by transducing each of twelve insertion mutations into two genetic backgrounds, one ancestral and the other having evolved in, and adapted to, a defined laboratory environment for 10,000 generations. To assess the effect of the mutation on fitness, we allowed each mutant to compete against its unmutated counterpart in that same environment. Overall, there was a strong positive correlation between the mutational effects on the two genetic backgrounds. Nonetheless, three of the twelve mutations had significantly different effects on the two backgrounds, indicating epistasis. There was no significant tendency for the mutations to be less harmful on the derived background. Thus, there is no evidence supporting the hypothesis that the derived bacteria had adapted, in part, by becoming buffered against the harmful effects of mutations.

Key words.—Epistasis, *Escherichia coli,* fitness, genetic canalization, mutation.

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Epistatic interactions between mutations are important for many evolutionary theories, including several that seek to explain the origin and maintenance of genetic systems such as sexual recombination, diploidy, and reproductive isolation (Hill 1982; Kondrashov 1982, 1985; Kondrashov and Crow 1991; Coyne 1992; Whitlock et al. 1995; Gavrilets 1999; Peck and Waxman 2000). Perhaps the most prominent evolutionary theory in which epistasis plays a major role is the shifting balance theory of Wright (1931), in which nonadditive gene interactions are necessary to produce multiple fitness peaks and intervening valleys. There has recently been debate about this theory, much of it focused on the likelihood of peak-shifts (Coyne et al. 1997, 2000; Peck et al. 1998; Wade and Goodnight 1998; Whitlock and Phillips 2000). Such events presume the existence of nearby peaks on the fitness surface and hence they require epistasis (Whitlock et al. 1995). In our view, an even more fundamental issue is this presumption and associated questions: How common is epistasis? How often are mutations deleterious on one genetic background but beneficial on another? How widely spaced are multiple fitness peaks?

Much effort has gone into finding and characterizing epistasis among genes. Quantitative genetics experiments are, in principle, able to detect epistatic interactions as a component of genetic variance for quantitative traits (Bauman 1959; Sprague et al. 1962; Cockerham 1984; Goodnight 1987; Whitlock et al. 1995; Cheverud and Routman 1996; Shaw et al. 1997). However, this approach often generates ambiguous results because it lacks statistical power and provides a composite of numerous interactions between ill-defined loci throughout the genome. Moreover, even if there is a strong physiological interaction between alternative alleles at two loci, the resulting epistatic variance will be small if the relevant alleles are at low frequency. Other studies have shown compensation for deleterious mutations or for harmful pleiotropic effects of beneficial mutations (McKenzie et al. 1982; Lenski 1988; Cohan et al. 1994; Lenski et al. 1994; Schrag et al. 1997; Björkman et al. 1998; Burch and Chao 1999; Moore et al. 2000). All these studies provide evidence, direct or circumstantial, that the compensatory alleles do not produce the same beneficial effect in the absence of the mutation for which they compensate; hence, they all indicate epistasis. However, in these experiments, selection ''finds'' compensatory mutations among a large set of mutations, and thus it is unclear whether mutations with epistatic effects are rare or common in absolute terms. Mutation accumulation experiments have also been used to look for epistasis (Kitigawa 1966; Mukai 1969; De Visser et al. 1996, 1997; Shabalina et al. 1997), but these kinds of experiments suffer from two limitations: (1) a lack of precise knowledge of the number of mutations accumulated in each genotype; and (2) the fact that a log-linear decline in fitness may indicate an absence of epistasis or, alternatively, widespread epistasis in which antagonistic and synergistic interactions offset one another (Elena and Lenski 1997).

In a previous study, we avoided the problems described above by employing a direct approach in which we constructed *Escherichia coli* genotypes containing random mini-Tn*10* insertion mutations alone and in combination (Elena and Lenski 1997). We found that, on average, fitness declined as a roughly log-linear function of the number of mutations. However, we also showed that this relationship masked frequent epistatic interactions among mutations, some of which were antagonistic and others synergistic.

The primary goal of the present work is to determine whether these mutations also interact epistatically with mutations that were fixed during 10,000 generations of experimental evolution. If there is frequent epistasis between the insertion mutations and the two genetic backgrounds, ancestral and derived, then a secondary goal of this study is to

evaluate whether the interactions tend towards genetic canalization. That is, the derived background may have evolved, either directly or indirectly, to be better buffered against the harmful effects of random mutations (Waddington 1959; Scharloo 1991; Stearns and Kawecki 1994; Barkai and Leibler 1997; Wagner et al. 1997; Rutherford and Lindquist 1998; Wagner and Stadler 1999; Gibson and Wagner 2000; Kawecki 2000; Wilke et al. 2001). To pursue both these goals, we placed 12 insertion mutations individually into the derived and ancestral backgrounds, and we then measured the effects of each mutation on fitness in each background.

Materials and Methods

Genetic Backgrounds

All the genotypes employed in this study were constructed in two genetic backgrounds, designated REL4548 and REL606. REL4548 is an evolutionarily derived clone sampled after 10,000 generations (=1500 days) at 37°C in Davis minimal (DM) medium supplemented with glucose at 25 μg/mL (Lenski et al. 1991; Lenski and Travisano 1994). During this period, the population substituted a number of beneficial mutations, which improved its competitive fitness by approximately 50% relative to the ancestral clone, REL606 (Lenski and Travisano 1994). The particular evolving population that was used is designated Ara-1, and it retained the low ancestral mutation rate throughout the 10,000 generations (unlike some populations: see Sniegowski et al. 1997).

Both the ancestral and derived clones are unable to metabolize the sugar arabinose. We obtained a spontaneous Ara^+ mutant from each clone (REL607 from REL606, and SFE4548+ from REL4548) by plating billions of cells onto minimal agar that contained arabinose as the only source of carbon. We used these Ara^+ clones as competitors in the fitness assays described below. Ara^+ and Ara^- cells are distinguishable by their production of red and white colonies, respectively, on tetrazolium arabinose (TA) indicator agar (Lenski et al. 1991).

Transposon Mutagenesis

The methods for producing random insertion mutations, and molecular confirmation of the insertions, are described fully in Elena et al. (1998). Briefly, insertion mutations were generated using mini-Tn*10* constructs, which have certain properties that make them useful for mutagenesis of *E. coli* (Kleckner et al. 1991). (1) The transposase genes are not carried by the mini-Tn*10* but are encoded by the delivery vector (a defective λ phage that is itself unable to integrate into the chromosome), such that secondary transpositions cannot occur. (2) The transposase genes have been engineered to yield insertions that are essentially random with respect to the target DNA sequence. (3) Various mini-Tn*10* constructs encode resistance to chloramphenicol, kanamycin, or tetracycline, making it easy to find mutants by plating cells on agar supplemented with the appropriate antibiotic. The site of the insertion mutation, and not the resistance marker, is primarily responsible for the resulting effect on fitness (Elena et al. 1998). The 12 insertion mutations used in the present study were all originally generated on the REL4548 background using the mini-Tn*10* that carries the tetracycline-resistance (Tet^R) marker, the expression of which is phenotypically repressed in the absence of antibiotic.

These 12 mutations were chosen because their effects on the derived genetic background ranged from nearly neutral to effectively lethal in the DM medium used for the evolutionary derivation of REL4548 and fitness assays. In comparison with the full set of random mutations previously generated, the 12 mutations in this study are substantially overdispersed with respect to their fitness effects. Six of the 12 mutations reduce relative fitness in the derived background by 0.03 or less, whereas the other six reduce fitness by at least 0.09. The full set is described well by a compound distribution, in which these two modes represent about 97% and 3%, respectively, of the overall weighting (Elena et al. 1998). The possible relevance of the over-representation of mutations with severe effects will be addressed in the Results and Discussion.

Transduction of Mutations

To move each mutation from the evolved background (REL4548) to the ancestral background (REL606), we employed generalized transduction using P1*vir* and the protocol given by Zyskind and Bernstein (1989). In brief, each donor clone bearing an insertion mutation was infected with P1*vir* to produce a library of encapsidated bacterial chromosome fragments. Each library was then used separately to infect the recipient clone. Recombinants bearing the insertion mutation were selected on TA agar containing 10 μg/mL of tetracycline.

The average size of a transduced fragment is only about 2% of the total genome size of *E. coli* (Masters 1996). Based on the kinetics of genetic adaptation, it appears that about three beneficial mutations were fixed in the first 2000 generations in the population that gave rise to REL4548, and the rate of substitution was much less in later generations as the pace of adaptation greatly decelerated (Lenski and Travisano 1994; Elena et al. 1996). Thus, it seems unlikely a priori that any of the beneficial mutations in REL4548 would be co-transduced with an insertion mutation of interest. However, to reduce even further the unlikely possibility of linkage, we performed three successive rounds of transduction for each mutation, using as donor the recipient from the previous transduction and as recipient the ancestral REL606 clone. After the three rounds of transduction, southern hybridizations were performed (as described in Elena et al. 1998), and these confirmed that the insertion mutations were in the same chromosomal position in both genetic backgrounds (data not shown).

Fitness Assays

The fitness of each mutant genotype was measured in competition against the Ara^+ variant in the same genetic background and under the same culture conditions in which the experimental evolution took place (see above). Before each fitness assay, the two competitors were separately grown under those same conditions to acclimate them to the competition environment. They were then each diluted 200-fold into fresh DM medium, and a sample was immediately plated on

TABLE 1. Two-way analysis of variance. Genetic background is a fixed effect, whereas insertion mutation is a random factor.

Source	SS	df	MS	*F*	*P*
Genetic background	0.0167	1	0.0167	0.2960	0.5973
Insertion mutation	10.0334	11	0.9121	750.6168	<0.0001
Interaction	0.6222	11	0.0566	46.5509	<0.0001
Residual	0.1167	96	0.0012		

TA agar to estimate their initial densities. Every day, for six days, the mixed population was diluted 100-fold into fresh DM medium. After six days, a sample was plated on TA agar to obtain the final density of each competitor. Each daily dilution allowed the population to grow 100-fold, which is 6.64 cell generations ($=\log_2 100$) per day. One mutation, in both genetic backgrounds, rendered the bacteria unable to grow in DM medium; in that case, relative fitness was, by definition, zero. A few other genotypes had such low fitness that they produced few or no colonies after six days. For those, we reran the competitions for one or three days to ensure sufficient numbers in the final sample to calculate accurately their relative fitness. For each competitor, we computed its net growth rate as $m = \ln(100^t \cdot N_t/N_0)/t$, where N_0 and N_t are initial and final densities, respectively, and t is the number of days of competition. The fitness of the mutant relative to its Ara$^+$ competitor is defined simply as the ratio of their net growth rates.

An independent control competition between the unmutated strain and its Ara$^+$ variant was run in parallel with each and every experimental competition between a mutant and the Ara$^+$ variant. Thus, the effect, if any, of the Ara marker state on fitness was eliminated by dividing the experimental value by the control value prior to statistical analysis. All fitness assays were replicated fivefold, for a total of 120 ($=12$ mutations $\times$ 2 backgrounds $\times$ 5 replicates) estimates of relative fitness, with each one obtained from the paired experimental and control competitions.

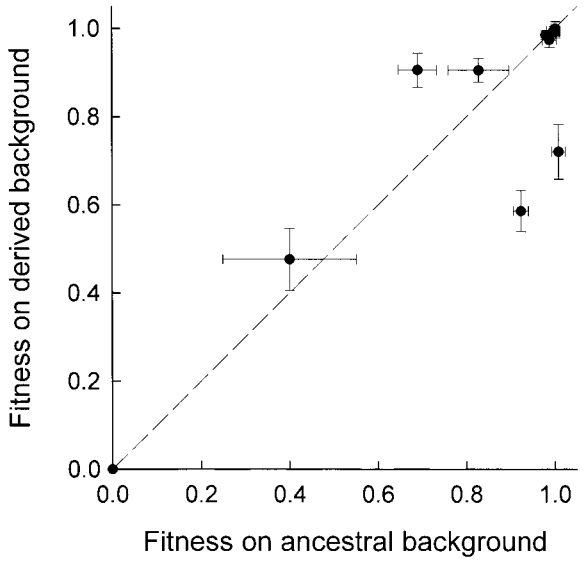

FIG. 1. Fitness of each insertion mutation in the two genetic backgrounds, ancestral and derived. Vertical and horizontal error bars show the 95% confidence limits on each background, based on replicated fitness assays for each genotype. The dashed line is the isocline that corresponds to equal fitness on both backgrounds.

RESULTS

Twelve insertion mutations were each introduced into two different genetic backgrounds, one ancestral and the other derived by 10,000 generations of propagation in minimal glucose medium. The effects of these mutations on fitness in that same medium were then measured in each background with fivefold replication. Table 1 shows the two-way analysis of variance. In this analysis, genetic background is a fixed effect and insertion mutation is a random factor. The underlying data were heteroscedastic (Levene's test: $F_{23,96} = 7.2585$, $P < 0.0001$), with the error variance tending to increase at lower fitness values. This effect occurred because, at low fitness, fewer counts were obtained for the losing competitor and, moreover, it was necessary to shorten the competitions to obtain satisfactory counts. We reran the analysis using both exponential and rank transformations, which reduced but did not eliminate the heteroscedasticity; none of the conclusions were substantively changed by these transformations. We also calculated selection rates (differences in growth rates: see Travisano and Lenski 1996) instead of relative fitnesses (ratios of growth rates), again with the same substantive results. Furthermore, as described below, analyses that do not assume homoscedasticity confirm the biological effects of interest.

The two backgrounds did not differ significantly in the average effect of the mutations on fitness ($P = 0.5973$), whereas there was highly significant variation among mutations in their fitness effects ($P < 0.0001$). That is, some of the mutations were more harmful than others, regardless of genetic background. Figure 1 shows the significant positive correlation between relative fitness values measured in the two backgrounds (Spearman $r = 0.6503$, $n = 12$, one-tailed $P = 0.0110$); this nonparametric test does not depend on the variance structure of the underlying data. The primary focus of this experiment is the interaction between the insertion mutations and genetic backgrounds, which is highly significant ($P < 0.0001$) and indicates that the fitness effect of at least some mutations depended on the genetic background. This interaction implies epistasis between the deliberately introduced insertion mutations and one or more alleles that distinguish the ancestral and derived backgrounds. Given that the main effect of mutation and the interaction between mutation and background were both significant, it is also appropriate to compare the corresponding variance components, that is, their respective contributions to the overall variation in fitness. Using maximum-likelihood estimates (Lynch and Walsh 1998, chapter 27), the variance of mutation effects

TABLE 2. Mean fitness (± standard error) for each mutation on the derived and ancestral genetic backgrounds. Also shown are the paired difference (± standard error) of the fitness estimates on two backgrounds, the corresponding *t*-statistics, and the associated two-tailed significance level (all tests with 4 df). Asterisks denote those cases that were significant at $P < 0.05$ even after correcting the significance level for multiple tests (Rice 1989). Mutations are listed from highest to lowest fitness in the derived background.

Mutation designation	Derived background	Ancestral background	Difference	Paired *t*	*P*
Tet-34	0.9999 ± 0.0058	1.0013 ± 0.0032	−0.0014 ± 0.0072	0.1930	0.8564
Tet-163	0.9944 ± 0.0036	0.9978 ± 0.0055	−0.0034 ± 0.0061	0.5556	0.6081
Tet-19	0.9934 ± 0.0030	1.0006 ± 0.0038	−0.0072 ± 0.0032	2.2553	0.0871
Tet-64	0.9922 ± 0.0049	1.0015 ± 0.0032	−0.0093 ± 0.0055	1.6795	0.1683
Tet-8	0.9855 ± 0.0036	0.9795 ± 0.0024	0.0060 ± 0.0032	1.8907	0.1316
Tet-41	0.9743 ± 0.0061	0.9881 ± 0.0056	−0.0138 ± 0.0027	5.1769	0.0066
Tet-154	0.9060 ± 0.0139	0.6898 ± 0.0156	0.2162 ± 0.0285	7.5881	0.0016*
Tet-16	0.9055 ± 0.0098	0.8277 ± 0.0245	0.0778 ± 0.0303	2.5659	0.0623
Tet-44	0.7208 ± 0.0244	1.0088 ± 0.0055	−0.2879 ± 0.0209	13.7542	0.0002*
Tet-90	0.5861 ± 0.0168	0.9232 ± 0.0061	−0.3371 ± 0.0212	15.8846	<0.0001*
Tet-116	0.4767 ± 0.0255	0.4001 ± 0.0543	0.0766 ± 0.0667	1.1483	0.3148
Tet-130	0.0000	0.0000	0.0000	—	1.0000

was 0.0784, whereas the variance associated with the interaction was 0.0101. Although the epistatic mutation-by-background interaction was highly significant (Table 1), the main effect of mutation contributed more to the overall fitness pattern (Fig. 1). However, the particular mutations used in this study had fitness effects that were more variable than the distribution from which they were taken (see Materials and Methods); this sampling bias inflates the variance of mutation effects and might also inflate that associated with the interaction between mutation and background.

To identify which insertion mutations exhibit epistasis with the genetic backgrounds, we ran a series of paired *t*-tests (Table 2). Four of the 12 mutations (Tet-41, -44, -90, and -154) had significantly different fitness effects on the two backgrounds at $P < 0.05$. Three of these cases remained significant even after we applied a Bonferroni correction to reflect multiple tests of the same hypothesis (Rice 1989). The paired *t*-tests, unlike the analysis of variance, do not assume homoscedasticity; these cases confirm the inference of epistatic interactions between mutation and genetic background. One mutation (Tet-154) had a significantly less harmful effect on the derived background than in the ancestor, a pattern that is consistent with the buffering predicted by the canalization hypothesis. However, two mutations (Tet-44 and -90) had more severe effects on the derived background, a pattern that contradicts this hypothesis. Using the average fitness difference between the two backgrounds for all 12 mutations (Table 2), we calculated the mean effect of the genetic background as −0.0236 (±0.0434 SEM). This value indicates a slight, but nonsignificant, tendency for these mutations to be more harmful in the derived background than in the ancestor ($t_{11} = 0.5441$, $P = 0.5972$; Wilcoxon's signed-ranks test, $T_S = 27$, $P = 0.6352$).

All three mutations showing significant epistasis with the genetic background come from the group of six mutations that exhibited the most deleterious effects in the derived background; none of the six mutations that had much weaker effects on fitness indicated significant epistasis after the Bonferroni correction. These data suggest that epistasis may be more common among genes with larger effects. However, this pattern is not statistically significant (Fisher's exact test, two-tailed $P = 0.1818$) in this sample of only 12 mutations.

DISCUSSION

Our results bear on two general issues in evolutionary genetics, epistasis and canalization. We showed previously that many pairs of random insertion mutations interact epistatically in the bacterium *E. coli*, with some of these interactions antagonistic and others synergistic (Elena and Lenski 1997). In this study, we extend the evidence for widespread epistasis by demonstrating that many of these mutations also interact epistatically with mutations that were fixed during the adaptation of an evolving population to its experimental environment (Table 1). In particular, three of the 12 insertion mutations that we examined have significantly different fitness effects in these two genetic backgrounds (Table 2). In one case, a mutation that reduced fitness by almost 30% in the derived background was effectively neutral in the ancestor, whereas in another case a mutation that lowered fitness by more than 30% in the ancestral background had only about a 10% effect in the derived background (Fig. 1). Several other studies have demonstrated that the deleterious effects of many mutations can be compensated by epistatic mutations that occur elsewhere in the genome (McKenzie et al. 1982; Lenski 1988; Cohan et al. 1994; Lenski et al. 1994; Schrag et al. 1997; Björkman et al. 1998; Burch and Chao 1999; Moore et al. 2000). In the case of compensation, however, selection *found* the epistatic mutations among many mutations that arose in the populations. Therefore, it is unclear from studies of compensation whether epistatic interactions are common in absolute frequency, or whether such interactions are rare but become important when there is intense selection to ameliorate deleterious effects.

Our results indicate that strong epistatic interactions between particular mutations and their genetic background are common, at least among those mutations that have large effects on fitness. This qualification arises because all three cases of significant epistasis in this study involved mutations with highly deleterious effects on the derived background. Such mutations represent only a few percent of a much larger set of insertion mutations that we previously studied (Elena et al. 1998). However, the association between epistasis and strength of independent effect was not statistically significant, owing to the small sample size; even if it were real, it

might indicate a scaling issue, rather than a genuine absence of epistasis involving mutations of smaller effect. Also, in this study we used one particular class of mutations—insertions of transposable elements—among the many types of mutation that occur in evolving genomes. We used transposon-insertion mutations because they are especially amenable to experimental construction, manipulation, and analysis. However, it is noteworthy that similar mutations, involving indigenous transposable elements called insertion sequences, occurred during the evolution between the ancestral and derived backgrounds (Papadopoulos et al. 1999; Schneider et al. 2000; Cooper et al. 2001). Of course, it would be interesting to repeat our experiments with other classes of mutations, but that is beyond the scope of this study.

A potential methodological caveat with respect to our finding of widespread epistasis in this study is that the transduction procedure used to move insertion mutations from the derived to the ancestral background might have brought along with it a physically linked mutation. In fact, however, this potential problem is unlikely for the following reasons. The number of beneficial mutations that are responsible for the adaptation of the evolved line seems to be small. Based on the fitness trajectory for this population, there appear to have been three selective sweeps in the first 2000 generations of the evolution experiment, none of which individually accounted for more than about a 10% fitness gain (Lenski and Travisano 1994). The rate of such sweeps became progressively much slower over time (Lenski and Travisano 1994), and it is therefore likely that fewer than 10 beneficial substitutions distinguish the ancestral and derived genetic backgrounds. P1 transduction moves about 2% of the *E. coli* chromosome; the probability that one or more of 10 beneficial mutations would be cotransduced is thus $1–0.98^{10} = 0.183$. As described in the Materials and Methods, we in fact transduced each insertion mutation into the ancestral background three successive times; the likelihood that a mutation in the derived background that was cotransduced in the first round would again be cotransduced in any successive round should be 0.5, on average. Thus, there is only a small chance ($0.183 \times 0.5 \times 0.5 = 0.046$) that a beneficial mutation would be cotransduced with the insertion mutation through all three rounds of transduction. Given that we transduced 12 mutations, one might still imagine that, with bad luck, there were one or two cases in which a beneficial mutation was inadvertently cotransduced to the ancestor. But in no case should the cotransducing mutation's effect on fitness be more than about 10%, nor should it yield a lower relative fitness in the ancestor than in the derived background. Yet, in all three cases in which an insertion mutation had significantly different effects in the two backgrounds, that difference was greater than 20%; and in one case, the insertion reduced the ancestor's fitness much more than it did in the derived background. There is also a theoretical possibility of cotransduction of neutral or slightly deleterious mutations that might have hitchhiked with the beneficial mutations during the evolution experiment. However, such mutations would necessarily have small fitness effects, and therefore they also could not account for the very large differences in the effects of these three insertion mutations on the two backgrounds. Thus, it seems very unlikely that any artifact can account for the three observed cases of strong epistasis between insertion mutations and genetic background.

Given that we observed epistasis, we could then ask whether the genetically adapted background is significantly better buffered than was its ancestor against the perturbations of new mutations (Waddington 1959; Scharloo 1991; Stearns and Kawecki 1994; Barkai and Leibler 1997; Wagner et al. 1997; Rutherford and Lindquist 1998; Wagner and Stadler 1999; Gibson and Wagner 2000; Kawecki 2000; Wilke et al. 2001). Such buffering could occur by selection for ''genetic canalization'' to suppress the phenotypic effects of deleterious mutations. However, the intensity of this selection is only proportional to the genomic mutation rate, and it may therefore be a rather weak force (Wagner et al. 1997; see also Nowak et al. 1997; Wagner 1999). In *E. coli,* the total genomic mutation rate for normal, DNA repair-proficient genotypes has been estimated to be about 0.003 mutations per generation (Drake 1991), and it is even less if only deleterious mutations are considered (Kibota and Lynch 1996). A few populations in our long-term evolution experiment evolved ''mutator'' phenotypes owing to defects in mismatch repair genes, which caused their genomic mutation rates to increase substantially (Sniegowski et al. 1997; Cooper and Lenski 2000). However, hypermutability did not evolve in the particular population used for this study. Therefore, given the low genomic mutation rate for *E. coli,* it seems doubtful that genetic canalization per se would have been a very potent force in the long-term evolution experiment. Buffering against the harmful phenotypic effects of deleterious mutations could also occur indirectly as a correlated response to ''environmental canalization'' (Wagner et al. 1997). That is, if environmental perturbations are more frequent than mutational ones, and if the harmful effects of mutational and environmental perturbations are ameliorated by the same physiological mechanisms, then it becomes more plausible that the genetically adapted, derived background would tend to be less harmed by new mutations than its ancestor.

However, we saw no compelling evidence for canalization. Some mutations were indeed less harmful in the derived background, but others showed the opposite pattern; on average, we saw no significant tendency in either direction. There are several possible explanations, both experimental and biological, for our failure to observe canalization with respect to deleterious mutations. (1) Our study had a rather small sample size, although it was easily sufficient to detect epistasis. The fact that epistatic effects were both strong and variable in direction indicates that a very large number of mutations would be necessary to detect canalization, because the effect of background is tested relative to the epistatic interaction variance. (2) Except for predictable (seasonal) fluctuations in resource concentration and population density caused by the serial transfer regime, we sought to maintain a constant environment during the evolution experiment. Any harmful effects of unpredictable environmental perturbations were therefore likely to be only a weak selective force for canalization. (3) Physiological mechanisms might not exist that can buffer against perturbations in general, whether genetic or environmental, as opposed to buffering against specific perturbations; if so, this would substantially limit the opportunity for canalization. (4) Canalization is usually pre-

dicted to evolve when there is a long history of strong stabilizing selection. The environment that produced the derived genotype was effectively novel to the ancestral genotype. The evolving population underwent rapid genetic adaptation during the first 2000 generations of the experiment, but it improved more slowly thereafter (Lenski and Travisano 1994; Cooper and Lenski 2000). Hence, it is difficult to specify the relative importance of directional and stabilizing selection, or even to be sure that there was selection for canalization.

In conclusion, our findings provide compelling support for widespread epistasis; our data do not demonstrate genetic canalization, nor do they offer a definitive rejection of this interesting hypothesis. However, several other recent studies have provided direct or indirect evidence for genetic canalization, including buffering against deleterious effects of mutations. Stearns and Kawecki (1994) found that life-history traits closely related to fitness in *Drosophila melanogaster* were better canalized than traits less related to fitness. Rutherford and Lindquist (1998) showed that *D. melanogaster* genotypes defective in the expression of a stress protein were phenotypically more variable owing to unmasking of otherwise cryptic genetic variation. Wagner and Stadler (1999) saw evidence for robustness to mutational effects in the secondary structures of RNA viral genomes. In a study with ''digital organisms''—computer programs that self-replicate and evolve—Wilke et al. (2001) provided direct evidence for genetic canalization, at least in that class of replicators. Populations of digital organisms were allowed to evolve in identical environments at either low or high mutation rates. Populations that evolved at the low mutation rate often achieved much faster replication, whereas those that evolved at the high mutation rate tended to be more robust to mutation. Owing to these evolved differences, the latter group prevailed in competition with the former group when experiments were run at high mutation rates, whereas the reciprocal was true in competition at low mutation rates.

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