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Pervasive compensatory adaptation in *Escherichia coli*

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To investigate compensatory adaptation (CA), we used genotypes of *Escherichia coli* which were identical except for one or two deleterious mutations. We compared CA for (i) deleterious mutations with large versus small effects, (ii) genotypes carrying one versus two mutations, and (iii) pairs of deleterious mutations which interact in a multiplicative versus synergistic fashion. In all, we studied 14 different genotypes, plus a control strain which was not mutated. Most genotypes showed CA during 200 generations of experimental evolution, where we define CA as a fitness increase which is disproportionately large relative to that in evolving control lines, coupled with retention of the original deleterious mutation(s). We observed greater CA for mutations of large effect than for those of small effect, which can be explained by the greater benefit to recovery in severely handicapped genotypes given the dynamics of selection. The rates of CA were similar for double and single mutants whose initial fitnesses were approximately equal. CA was faster for synergistic than for multiplicative pairs, presumably because the marginal gain which results from CA for one of the component mutations is greater in that case. The most surprising result in our view, is that compensation should be so readily achieved in an organism which is haploid and has little genetic redundancy. This finding suggests a degree of versatility in the *E. coli* genome which demands further study from both genetic and physiological perspectives.

Keywords: bacteria; compensatory adaptation; deleterious mutations; epistasis; fitness; natural selection

We all were sea-swallow'd, though some cast again,
And by that destiny to perform an act
Whereof what's past is prologue, what to come
In yours and my discharge.
(William Shakespeare, *The Tempest* (Act 2, Scene 1))

1. INTRODUCTION

Deleterious mutations in a population can be substituted by random drift (particularly in small populations), by hitchhiking with a beneficial mutation (particularly in asexual populations) or as a consequence of changing environments (such that a formerly beneficial mutation becomes deleterious). A deleterious substitution has several possible fates: it may (i) cause extinction of the population in which it resides, (ii) persist indefinitely, (iii) revert to its former state, or (iv) be compensated for by another mutation elsewhere in the genome which ameliorates its deleterious effect. The focus of this paper is on the last possibility, which we call compensatory adaptation (CA).

In the field of molecular evolution, compensatory mutations have been defined as two alleles which are independently deleterious but neutral when they occur together (Kimura 1985, 1990). However, the concept of compensation is both older and more general than this usage. Wright (1964, 1977, 1982) invoked CA in promoting the spread of major mutations by means of modifier alleles which diminish the deleterious side-effects of the major mutations. In this context, a compensatory mutation is any mutation which masks the deleterious effect of another mutation. As a consequence of the potential for compensation, genotypes which carry deleterious mutations should show larger and faster gains in relative fitness than genotypes which are at or near a fitness peak. We use this property as a means of operationally defining CA, as shown in figure 1. The relative fitness of an unmutated parent strain is 1.0, while deleterious mutations reduce fitness to lower values. Following a period of genetic adaptation, the final fitness of an evolving lineage will generally be higher than its initial fitness. If the fitness values of all lineages increase to the same relative extent, regardless of their initial fitness, then that is merely proportional adaptation. However, if lineages that were initially less fit experience relatively larger fitness gains, then that indicates CA (provided that no reversion has occurred).

CA provides evidence for epistasis, which underlies such diverse evolutionary theories as fitness landscapes with multiple peaks (Wright 1932) and the mutational deterministic hypothesis for the evolution of sex (Kondrashov 1988). Yet, despite this importance, surprisingly few data exist on the frequency and form of epistatic interactions (Whitlock *et al.* 1995; Fenster *et al.* 1997). Several experiments have demonstrated CA by selecting epistatic modifiers which reduce the costs of resistance to insecticides, parasites or antibiotics. In the Australian sheep blowfly, resistance to diazinon engendered substantial fitness costs to the flies when it first evolved, but the costs were compensated for by modifier mutations in later generations (McKenzie *et al.* 1982). Lenski (1988) demonstrated large fitness costs of mutations which confer the resistance of *Escherichia coli* to the virus T4, but after 400 generations in the absence of T4, these costs were much reduced even though resistance remained. Several experiments with bacteria have shown that CA can quickly overcome the pleiotropic costs which arise from resistance to various antibiotics (Bouma & Lenski 1988; Cohan *et al.* 1994; Lenski *et al.*

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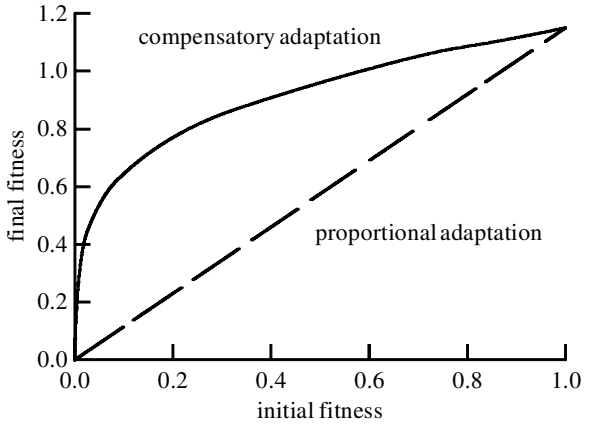

Figure 1. Schematic illustration of CA. The fitness of a parental strain is 1.0, while deleterious mutations reduce the fitness to lower initial values. Following a period of genetic adaptation, the final fitness of an evolving line will generally be higher than its initial fitness. If the fitness of all lines increases to the same degree, regardless of their initial fitness, then that is simply proportional adaptation. However, if lines that were initially less fit exhibit relatively larger fitness gains, then that indicates CA.

1994; Schrag & Perrot 1996; Schrag *et al.* 1997; Björkman *et al.* 1998; for a review, see Lenski 1998). In a recent study which did not involve any resistance mutations, Burch & Chao (1999) found that one particular deleterious mutation in an RNA virus could be compensated for to varying degrees by several different mutations.

In this study, we examine CA for deleterious mutations in *E. coli*, with the overall aim of determining whether it is a pervasive phenomenon. We do not know, for example, whether other past studies may have tested for CA, but produced negative results and gone unpublished. In addition, most previous studies which have reported compensation have concerned resistance mutations (see above) or other specific circumstances, such as RNA secondary structures (Kirby *et al.* 1995; Stephan 1996). Here, we investigate compensation for random deleterious mutations in many different genes and we extend the process to allow simultaneous compensation for multiple deleterious mutations. We first compare the patterns of CA in lines which carry single mutations of large versus small deleterious effect. We then examine CA in lines which carry either one or two mutations. Finally, we compare CA in lines carrying two mutations with multiplicative versus synergistic effects on fitness.

2. MATERIAL AND METHODS

(a) *Bacterial genotypes and culture conditions*

All the genotypes used in this study were derived from a single parental clone of *E. coli* B. This clone (REL4548) was isolated from a population which evolved for 10 000 generations in a serial transfer regime in Davis minimal medium supplemented with glucose (25 μg ml^{-1}) at 37 °C, during which time its rate of fitness increase slowed substantially after an initial period of rapid adaptation (Lenski & Travisano 1994). All of the experiments and assays in this study were performed under the same culture conditions as used for the long-term experimental evolution which produced the parental clone (see Lenski *et al.* (1991) for details), except that we used unshaken tubes instead of shaken flasks for ease of handling.

In a previous study, mini-Tn*10* mutagenesis was used to put random insertion mutations into this well-adapted background and some combinations of these random mutations were then constructed using P1 transduction (Elena & Lenski 1997). The fitness effects caused by these insertions are mostly a function of the site of insertion and not the resistance marker used to screen for the insertion (Elena *et al.* 1998). The 14 mutant genotypes used in the present study were chosen from those previously constructed using the criteria described below. Mini-Tn*10* insertions are usually very stable, i.e. they can neither precisely excise nor undergo secondary transposition owing to the features engineered into the mini-Tn*10* system (Kleckner *et al.* 1991).

(b) *Experimental designs*

To examine the generality of CA and the possible influences of certain factors on this process, we performed four sets of experiments. Each experimental set involved first allowing evolution to proceed in lines founded by different genotypes and then measuring the changes in fitness which occurred during that evolution. All four of the evolution experiments lasted 200 generations (30 days), after which each selected population was frozen in 10% glycerol at −80 °C for later analyses.

(i) *Control for non-compensatory adaptation*

As defined in §1, CA refers to the fitness gains in a line which carries one or more deleterious mutations which are greater than those that would occur in an otherwise identical line which did not carry any mutations. Therefore, to serve as a control, we founded six replicate populations using the unmutated parent clone from which all of the mutant genotypes used in the other three experiments were derived.

(ii) *Compensation for mutations with large versus small effects*

We chose two genotypes with large deleterious effects ($W \sim 0.6$) and two with smaller effects ($W > 0.8$) from the single mutants that were available (Elena *et al.* 1998). Three replicate lines were founded with each of these four genotypes.

(iii) *Compensation for single versus double mutations*

We chose three additional genotypes with single mutations and three genotypes that carried two mutations from those available (Elena & Lenski 1997; Elena *et al.* 1998). Because the rate of CA may depend on initial fitness, we used single and double mutants with similar fitness values. The double mutants used in this experiment were produced by successive rounds of insertion mutagenesis and so the form of interaction between the mutations is unknown. Three replicate lines were established using each of the six genotypes.

(iv) *Compensation for double mutants with synergistic versus multiplicative effects*

We chose four genotypes with two mutations from those that were produced by combining individual mutations of known effect (Elena & Lenski 1997). Two of the four genotypes had synergistic interactions, in which the combined effect of the two mutations was significantly more harmful than predicted from the separate effects. The other two genotypes had pairs of mutations which were approximately multiplicative in their effects. Again, the overall fitness of the two classes of genotype was

similar. In this experiment, six replicate lines were founded from each of the four genotypes.

(c) *Southern blots*

We ran Southern blots on each of the ancestral genotypes and derived lines following standard procedures (Elena *et al.* 1998). This procedure will detect possible cross-contamination of the evolving lines, because the ancestral genotypes have different insertions and, hence, unique 'fingerprints'. We saw no evidence of cross-contamination. This procedure also allowed us to check the evolutionary stability of the insertion mutations. Loss of an insertion mutation may indicate genetic reversion, which does not qualify as CA. In the vast majority of lines (50 out of 54 in the three experiments with mutated founders), there was no change in fingerprint in the 200 generations. However, in four of the evolving lines the fingerprint was altered, but the resistance markers associated with the transposon insertions were retained; these observations may indicate some rearrangement other than elimination of a transposon insertion. In any case, the exclusion of these anomalous lines has no qualitative effect on the main statistical interpretations, as described in § 3.

(d) *Fitness assays*

The parental clone (REL4548) and all 14 mutant genotypes are unable to use arabinose (Ara^-). We isolated a spontaneous Ara^+ mutant of the parental clone; we used this mutant as a common competitor in all fitness assays. Ara^- and Ara^+ strains produce red and white colonies, respectively, on tetrazolium-arabinose (TA) agar (Lenski *et al.* 1991). Fitness was measured in culture conditions identical to those used for the evolution experiments and both competitors were separately acclimatized to those conditions prior to being mixed. The assay procedures follow the same protocol described in detail elsewhere (Lenski *et al.* 1991). Relative fitness is defined as the ratio of the population growth rates realized by two competitors as they compete with one another for a finite pool of resources (Lenski *et al.* 1991).

Depending on the particular experiment, at least three and as many as 12 replicate fitness assays were performed for each ancestral genotype or evolved line. Nine assays were run with the unmutated Ara^- parent and its Ara^+ counterpart in order to test the neutrality of the arabinose marker. The Ara marker is neutral within the limits of resolution ($t = 0.1893$, d.f. $= 8$ and $p = 0.8546$) and is not considered further.

(e) *Statistical analyses*

For each experiment, we performed three nested analyses of variance: (i) the initial fitness values, (ii) the final fitness values, and (iii) the relative change in fitness between the initial and final values. For (i), there are two effects that one can test: the difference between class means and heterogeneity between genotypes within a class. For (ii) and (iii), there are three testable effects: the differences between classes, heterogeneity due to the founding genotype within a class and heterogeneity between the replicate evolving lines founded from the same genotype. For (iii), we used the mean estimate of initial fitness for each founding genotype in order to compute the relative change associated with each final estimate for the corresponding derived lines. We generally performed *F*-tests of the mean square associated with the effect of interest relative to the mean square of the effect at the level immediately below. However, when the *F*-ratio for the denominator effect was itself very low, we conservatively used the mean-square error to test the effect of interest.

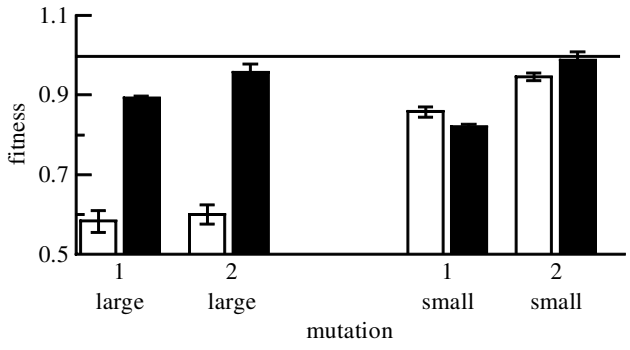

Figure 2. Compensation for mutations with large versus small effects. The open and filled bars show the mean fitnesses before and after 200 generations of experimental evolution, respectively. Two genotypes carry deleterious mutations of large effect and two carry deleterious mutations with smaller effects. The error bars show the standard errors based on independent replicate lines. See § 3(b) for the statistical analyses.

3. RESULTS

(a) *Control for non-compensatory adaptation*

On average, the six control lines evolved had a mean fitness of 1.036 relative to their ancestral state. This evolutionary improvement in the unmutated genotypes was significant ($t = 3.342$, d.f. $= 5$ and $p = 0.0205$). However, as will become clear, the fitness gains in the control group were quite small in magnitude relative to the gains we observed in most mutated lines and so the latter were compensatory.

(b) *Compensation for mutations with large versus small effects*

Figure 2 shows the fitness of each genotype in this experiment both before and after the experimental evolution. Prior to the evolution, there was a significant difference between these two classes ($p = 0.0203$). There was also significant heterogeneity between genotypes within a class ($p = 0.0107$).

After 200 generations, the difference between the two classes had been eliminated ($p = 0.8338$), indicating that the more harmful effects in one class were ameliorated by CA. There remained significant heterogeneity between founding genotypes within a class ($p = 0.0001$). However, there was no discernible heterogeneity between lines founded from the same genotype ($p = 0.5529$), indicating that the adaptation was consistent across independently evolving replicate populations.

The analysis of variance based on the final fitness values is, from a statistical perspective, a negative result. It could in principle indicate either CA or mere statistical noise. We can test for CA more directly by running the analysis of variance on the relative change in fitness between the start and finish of the experimental evolution. There was a highly significant difference between the two classes ($p = 0.0090$). Both genotypes which carry mutations with large effects experienced much greater relative gains than did either genotype with mutations of smaller effect (figure 2). There was also heterogeneity between genotypes within a class in their relative gains ($p = 0.0251$), which indicates that some deleterious mutations were more easily

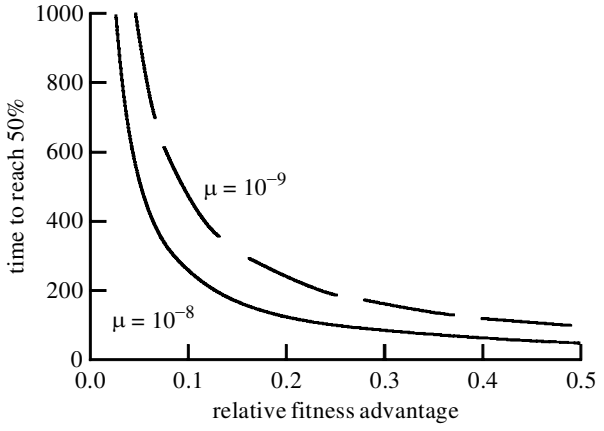

Figure 3. The time required for a beneficial mutation to reach 50% in a population as a function of its relative fitness advantage and mutation rate μ. The time required is the expected waiting time for a mutation to appear, corrected for losses due to drift, plus the substitution time required for the mutation to increase from $1/N_e$ to $N_e/2$ given its relative fitness (Lenski *et al.* 1991; Gerrish & Lenski 1998). Here, $N_e = 3 \times 10^7$, which is the effective population size in our experiments given the serial transfer regime (Lenski *et al.* 1991).

compensated for than others in the same class, but there was again no heterogeneity between lines founded from the same genotype ($p = 0.4567$).

At first glance it may seem surprising that compensation was greater for more harmful mutations than for less harmful ones. However, this outcome is not unexpected when viewed from a dynamic perspective. Figure 3 shows the time required for a beneficial mutation to reach 50% in a population as a function of its relative fitness advantage and the rate at which it occurs in a population whose size is 3×10^7 (which is equal to the effective size in our experimental populations given the serial transfer regime) (see Lenski *et al.* 1991). The time required is the expected waiting time for a mutation to appear, corrected for the beneficial mutations lost due to drift (Haldane 1927), plus the substitution time calculated from the relative fitness (Lenski *et al.* 1991; Gerrish & Lenski 1998). Consider first a genotype carrying a deleterious mutation of large effect which has initial fitness of 0.6. Let us assume that mutations exist which can compensate for 75% of the deleterious effect which would have a 50% relative fitness advantage, that is $(0.6 + 0.75\ (1 - 0.6))/0.6 = 1.5$. Such a compensatory mutation would approach fixation within 200 generations even if it occurs at a mutation rate of only 10^{-9} (figure 3). Now consider a genotype carrying a deleterious mutation of smaller effect, which has an initial fitness of 0.9. A mutation which compensates for 75% of the deleterious effect would have only an 8% advantage. Even if the compensatory change occurs at a mutation rate an order of magnitude higher, i.e. 10^{-8}, it would take many more than 200 generations to approach fixation in the population (figure 3). Thus, compensation is expected to be faster in severely handicapped genotypes than in those which are fitter, provided mutations which can compensate for a comparable fraction of the initial deleterious effect are accessible.

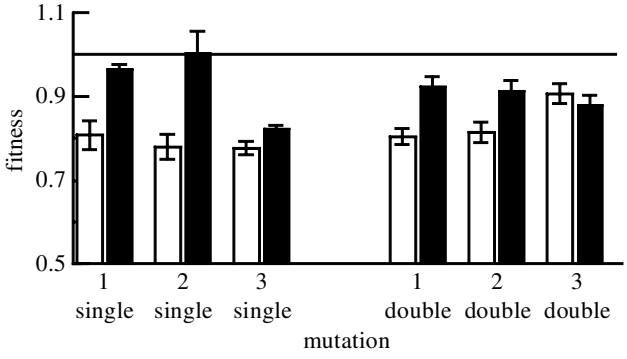

Figure 4. Compensation for single versus double mutations. The open and filled bars show the mean fitnesses before and after the experimental evolution, respectively. Three genotypes carry single deleterious mutations and three genotypes carry two deleterious mutations. The error bars show the standard errors based on independent replicate lines. See § 3(c) for the statistical analyses.

(c) *Compensation for single versus double mutations*

Figure 4 shows the relative fitnesses of each genotype in this experiment before and after the 200 generations of experimental evolution. Initially, there was no significant difference in fitness between these two classes ($p = 0.2031$), indicating that the genotypes with one and two mutations were well matched. However, there was significant heterogeneity between genotypes within a class ($p = 0.0343$). The final fitness data support the same general pattern: there was no significant difference between the single and double mutants ($p = 0.6775$), although there remained significant heterogeneity between the genotypes within a class ($p = 0.0087$). As in the previous experiment, there was no detectable heterogeneity between lines derived from the same genotype ($p = 0.3029$), indicating consistent adaptation in replicate populations. Expressed in terms of the change in relative fitness during the evolution experiment, the effects due to class ($p = 0.3134$), genotype within class ($p = 0.0017$) and replicate line within the founding genotype ($p = 0.2823$) were all qualitatively the same as those based on the final fitness values. In addition, the effect of class on the final fitness ($p = 0.6502$) and on the change in relative fitness ($p = 0.3005$) remained non-significant if we excluded three lines whose insertion fingerprints changed during the evolution experiment.

Evidently, compensation can occur in genotypes which carry two mutations as well as in those which carry only one mutation. However, in this experiment we do not know how much of the overall fitness handicap of the double mutants is attributable to each mutation alone and their interaction. The next experiment addresses this issue using genotypes in which we know the separate effects of each mutation as well as the form of their interaction.

(d) *Compensation for double mutants with synergistic versus multiplicative effects*

Figure 5 shows the relative fitness of genotypes with two mutations which interact in either a multiplicative or synergistic manner before and after the experimental evolution. There was no significant difference in initial fitness between these classes ($p = 0.2524$), so that representatives of the classes were well matched. There was also

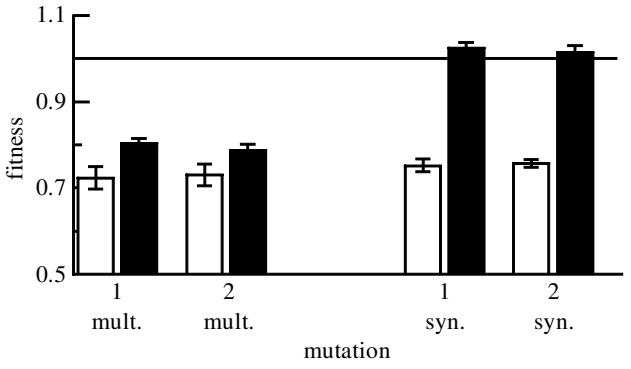

Figure 5. Compensation for double mutants with synergistic (syn.) versus multiplicative (mult.) effects. The open and filled bars show the mean fitnesses before and after the experimental evolution, respectively. Two genotypes each possess two mutations the interactions of which are approximately multiplicative, and two genotypes each possess two deleterious mutations the interactions of which are synergistic (i.e. the combined effect is worse than expected from the separate effects of the component mutations). The error bars show the standard errors based on independent replicate lines. See § 3(d) for the statistical analyses.

no discernible heterogeneity between genotypes within a class prior to evolution ($p = 0.9597$).

After 200 generations of experimental evolution, genotypes with synergistic interactions between their mutations evolved significantly higher fitness than those with multiplicative effects ($p = 0.0016$). In this experiment, there was no detectable heterogeneity between the genotypes in a class ($p = 0.7045$), nor among replicate lines founded from the same genotype ($p = 0.8183$).

The same conclusions were supported by analyses of the change in relative fitness during the evolution experiment. That is, the effects due to class ($p = 0.0059$), genotype within class ($p = 0.3938$) and replicate lines within genotypes ($p = 0.8333$) were all qualitatively the same as those based on the final fitness values. In addition, the effects of class on final fitness ($p = 0.0009$) and on the change in relative fitness ($p = 0.0043$) remained highly significant if we excluded one line whose insertion fingerprint changed during its evolution. This experiment indicates that double mutants with synergistic interactions undergo faster compensation than those with multiplicative effects.

As with mutations of large versus small effect, there is a plausible explanation for the faster CA in genotypes with synergy between two deleterious mutations. Table 1 contrasts the fitness surfaces for two pairs of mutations with the same combined fitness, where one pair is multiplicative and the other includes a large synergistic effect. The marginal gain that can be achieved by compensating for either component mutation is larger when the interaction is synergistic, because such compensation overcomes not only the individual effect of that mutation but also the harmful interaction. Hence, the gain in relative fitness is larger and the rate of adaptation is therefore faster (figure 3).

(e) *General trend to compensation*

The three experiments summarized in figures 2, 4 and 5 included 14 different genotypes which carried one or two deleterious mutations. Of this total, nine had relative fitness increases of greater than 10% (an average of three or six independent lines for each). In contrast, none of the six control lines improved that much. A one-tailed Fisher's exact test indicated this difference was highly significant ($p = 0.0071$).

Table 1. *Hypothetical fitness surfaces for two pairs of mutations in which the double mutants have the same fitness but one pair is multiplicative whereas the other pair is synergistic*

(The marginal fitness gain achieved by compensating for either component mutation is larger for the synergistic pair because compensation overcomes the harmful interaction as well as the effect of one mutation.)

	+	*n*
multiplicative		
+	1.00	0.85
m	0.85	0.72
synergistic		
+	1.00	0.95
m	0.95	0.72

4. DISCUSSION

We have shown that CA for deleterious mutations is both common and rapid in evolving populations of *E. coli*. Most of the deleterious mutations that we investigated exhibited CA, which is defined as gains in fitness in mutated lines which are disproportionately large in comparison with those observed in unmutated control lines (figure 1) and which do not involve reversion of the original mutation. Moreover, CA was evident in only 200 generations and it is quite possible that even more cases would have been seen if the experimental recovery phase had lasted longer.

We also showed that more severely deleterious mutations are compensated for more quickly than are those that are less harmful (figure 2). This pattern is counterintuitive until one considers the issue from a quantitative and dynamic perspective; the selection coefficients that compensate for severely deleterious mutations will be larger than those that compensate for less deleterious ones and the resulting substitution process will therefore be faster (figure 3). Similar considerations could also explain our observation that CA was faster in genotypes with two deleterious alleles which interact synergistically than in genotypes with multiplicative effects (figure 5), because the largest marginal gain will be greater in the former case than in the latter, all else being equal (table 1). It is also possible that there are more genetic avenues of phenotypic compensation for mutations with larger effect than for those of smaller effect. As a hypothetical example, severely deleterious mutations may disrupt genes with widespread pleiotropic effects, which might provide more control points where compensatory changes can occur. We have no evidence to support this hypothesis. We emphasize the dynamic explanation (figure 3) because it is a simple mathematical consequence which requires no 'special pleading' with respect to the genetic basis of the effect.

It is reasonable to ask whether the dynamics we observed might be explained by some process other than CA. For example, perhaps a population with low initial fitness can improve by some generic form of adaptation rather than by a change which specifically compensates for the deleterious mutation which lowered its fitness. (One could argue, on semantic grounds, whether the term CA should apply only when the modifier is specific to a particular deleterious mutation; we prefer a more general usage.) There are three lines of evidence which indicate that the rapid improvements in populations carrying deleterious mutations are specific to the mutations rather than some generic response to low fitness *per se*. First, deleterious mutations with similar initial fitness differ considerably in the extent of their compensation (figure 4), indicating that the pattern of CA is specific to particular mutations. Second, the finding that compensation in double mutants depends on the form of mutational interaction (figure 5) further indicates that CA depends on the genotype and not only its fitness. Third, the severely disadvantaged mutants in this study (figure 2) have absolute fitness levels similar to the distant ancestor of the unmutated progenitor (Lenski & Travisano 1994). However, the severely disadvantaged mutants required only 200 generations to achieve 50% gains in fitness, whereas their distant ancestor required thousands of generations to achieve a comparable improvement (Lenski & Travisano 1994). All this evidence indicates that CA is specific to particular deleterious mutations and not merely a generic response to low fitness. In the future, we will try to move the various deleterious mutations between the evolved lines in order to test the specificity of the compensatory mutations directly.

The process of CA requires epistasis. No compensation would be observed if the marginal fitness gain associated with every beneficial mutation were simply proportional to the initial fitness. Thus, our study also implies that epistatic interactions are widespread and important in *E. coli*. We have not yet isolated the compensatory mutations and, therefore, we cannot test whether they are harmful, neutral or beneficial on the parental background (without the deleterious mutation). If a mutation which gives rise to CA is also beneficial on the parental background, then one might argue that it is not compensatory but rather that it is unconditionally beneficial. Nonetheless, the observation of a disproportionate gain implies compensation and, hence, epistasis. For example, if a beneficial mutation confers a 1% advantage on the parental background but provides a 10% gain in a background carrying a severely deleterious mutation, then the consequence is still CA.

This evidence for widespread epistasis adds generality to previous studies of epistasis in two respects. First, Elena & Lenski (1997) showed that many pairs of deleterious mutations are epistatic. Our findings indicate that epistasis is not confined to deleterious mutations which may help shape the evolution of genetic systems (e.g. Kondrashov 1988) but which are presumably not the main building blocks for most adaptations. Second, as noted in §1, most previous studies of CA have focused on genes which confer resistance. Our study used genotypes which carried random mutations and our results thus indicate that CA and its associated epistasis are widespread and not limited to specific pathways. However, not all deleterious mutations are equally amenable to compensation, even after one accounts for the effect of the magnitude of the deleterious mutation on the potential rapidity of CA (figure 3). Figure 4 shows three genotypes with single deleterious mutations having similar fitness effects; in two of them, all three evolving replicate lines largely overcame the handicaps within 200 generations, whereas none of the three lines founded by the third genotype did so.

We now turn to three broad implications of the findings that CA is pervasive and also potentially very rapid. First, there has long been interest in the role of historical contingency in adaptive evolution (Wright 1977; Gould & Lewontin 1979; Gould 1989; Travisano *et al*. 1995). For example, to what extent does a particular substitution (or sequence of substitutions) constrain or promote a population's subsequent evolution? CA is a historically contingent process because the potential for a population's future adaptation depends on its current state. Yet, paradoxically, CA also implies that 'what's past is prologue', that is, the fitness of an organism may revert to some ancestral level, but it does so by making additional substitutions rather than by reversion of the initial mutation. The fitness reversion may be the more important effect if the environment does not change but, if the environment should change, then the latent effects of the original and compensatory mutations may be more important (e.g. Travisano *et al*. 1994).

The second broad implication of CA concerns the accumulation of deleterious mutations in finite populations. Models of this process show that it can produce 'mutational meltdown' in small populations and it is therefore an important issue in conservation biology (Lande 1994, 1995; Lynch *et al*. 1995). However, CA will reduce the likelihood of population extinction due to the accumulation of deleterious mutations provided that the rates of the compensatory mutations are sufficiently high (Lande 1998; Whitlock & Otto 1999). Burch & Chao (1999) recently demonstrated that the rate of compensatory mutation in an RNA virus could be sufficiently high to operate even in very small populations ($N_e \leqslant 10^2$). However, their experiment involved only one handicapped genotype and so the generality of compensation across the genome remains open to question. In contrast, our study used 14 different genotypes bearing deleterious mutations and we showed that the majority were compensated, establishing the generality of the phenomenon at the genetic level. However, our experimental populations were large ($N_e > 10^7$) and we cannot estimate whether the rates of the compensatory mutations are high enough to prevent extinction of small populations. Perhaps a future study will evaluate both these issues in the same system. Another area for research is whether CA can be a potent force in sexual populations, given that recombination will tend to separate the compensatory and deleterious mutations.

The third broad issue concerns what CA may tell us about the genetic architecture and evolutionary versatility of an organism's genome. It is quite remarkable, in our view, that CA is so pervasive in an organism such as *E. coli*, particularly given the nature of the deleterious mutations that had to be overcome. All the deleterious

mutations that we used in this study were insertion mutations, which have been widely used in genetic studies because they produce 'knock outs' of gene function provided that they insert into a gene. The genome of *E. coli* consists largely of open reading frames (ORFs), whereas higher organisms typically contain far more non-coding DNA. Indeed, we have identified the sites of insertion for four mutations in this study, including two severe mutations that were substantially compensated, and all of these disrupted ORFs (D. E. Rozen, unpublished data). Moreover, *E. coli* is haploid and has very little genetic redundancy; with the exception of seven ribosomal operons, there are very few genes with multiple copies. Thus, it seems rather surprising that *E. coli* can so readily compensate for mutations that knock out gene functions which are obviously important based on their deleterious effects. Although *E. coli* has few genes with multiple copies, protein sequence analyses do suggest that many of its genes have resulted from the duplication of ancestral genes (Labedan & Riley 1995). Members of a particular gene family have different functions, yet the functions are typically related (Labedan & Riley 1995). Therefore, one plausible hypothesis for the widespread CA is that other members of the same gene family as the knocked out gene are recruited by structural or regulatory changes in order to perform the disrupted function (Mortlock 1984). This hypothesis remains to be tested in our evolved lines, but it illustrates the sort of process that may be involved. In any case, we believe that CA indicates a degree of versatility in the *E. coli* genome which demands further study from both genetic and physiological perspectives. In addition, the fact that so compact an organism as *E. coli* exhibits pervasive CA suggests that this phenomenon may be important in more complex organisms as well.

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