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EXPERIMENTAL STUDIES OF PLEIOTROPY AND EPISTASIS IN *ESCHERICHIA COLI*. I. VARIATION IN COMPETITIVE FITNESS AMONG MUTANTS RESISTANT TO VIRUS T4

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Abstract.—Mutants selected for novel phenotypes frequently exhibit maladaptive pleiotropic effects. One may reasonably ask whether these effects are properties of the novel phenotypes per se, or whether these effects depend upon the particular genotypes conferring the novel phenotypes. To address this issue, I examined an array of independent mutants, derived from *Escherichia coli* B, that were all completely resistant to the virus T4. Each resistant mutant had maladaptive pleiotropic effects, but there was highly significant variation in competitive fitness among mutants. The degree of reduction in competitive fitness was strongly associated with cross-resistance to virus T7 and with the inferred position of the mutated gene in a complex metabolic pathway. This variation in competitive fitness permits refinement of the resistant phenotype by selection among resistant genotypes. This mechanism complements refinement of the resistant phenotype by selection for epistatic modifiers of maladaptive pleiotropic effects.

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The response to selection acting on one character may be constrained by genetic correlations with other characters that are subject to opposing selection pressures (Falconer, 1981; Maynard Smith et al., 1985). A major cause of genetic correlations is pleiotropy, whereby a gene substitution simultaneously affects two or more characters. Mutants selected for novel phenotypes frequently have maladaptive pleiotropic effects (Caspari, 1952; Wright, 1968). Antagonistic pleiotropic effects of genes can produce trade-offs between components of fitness or between measures of fitness across environments (Dykhuizen and Davies, 1980; Rose, 1982; Service and Lenski, 1982; Via, 1984; Lenski and Levin, 1985; but see also Futuyma and Philippi [1987] and Luckinbill [1984]).

There are at least two distinct mechanisms by which a constraint caused by antagonistic pleiotropic effects could be broken. First, genes having a similar effect with respect to one phenotypic trait may vary in their effect on some other trait (Hall, 1983; Cohan and Hoffmann, 1986). For example, selection acting on alternative alleles conferring equal levels of pesticide resistance would be expected to fix that one allele which had the least adverse effect on growth rate. Alternatively, epistatic modifiers of pleiotropic genes that alter the strength of the antagonistic pleiotropy might be selected (Caspari, 1952; Fisher, 1958; Uyenoyama, 1986). For example, genotypes that are resistant to pesticides may be refined by selection for modifiers that ameliorate maladaptive pleiotropic effects (McKenzie et al., 1982; Clarke and McKenzie, 1987).

The ease of culture, large populations, and short generations of *Escherichia coli* permit an experimental approach to the study of

evolutionary processes (Dykhuizen and Hartl, 1983; Hall, 1983; Levin and Lenski, 1985). Because *E. coli* and its viruses have served as model systems in microbial genetics and molecular biology, the genetic and molecular bases of their interactions are generally well understood (Lenski, 1987).

Resistance of *E. coli* B to virus T4 arises via mutations that cause defects in the lipopolysaccharide (LPS) core of the cell envelope (Weidel, 1958; Wilson et al., 1970; Tamaki and Matsuhashi, 1973; Prehm et al., 1976; Wright et al., 1980). Several pleiotropic effects have been associated with mutations causing defects in the LPS core, including increased permeability to hydrophobic compounds (Tamaki and Matsuhashi, 1971; Havekes et al., 1976; Nikaido, 1979) and reduced competitiveness (Ames et al., 1974; Lenski and Levin, 1985). The reduced competitiveness of resistant bacteria facilitates a virus-mediated, frequency-dependent coexistence of sensitive and resistant genotypes (Lenski and Levin, 1985).

One may ask whether this reduced competitiveness is a property of the resistant phenotype per se, or whether the effect depends upon the particular genotype conferring resistance. In this paper, I examine an array of independent mutants, derived from *E. coli* B, that are all completely resistant to the virus T4 to determine whether they vary in competitive fitness. In a companion paper (Lenski, 1988), I examine changes in fitness that occur in evolving populations of resistant and sensitive bacteria for evidence of epistatic modifiers of the maladaptive pleiotropic effects.

Materials and Methods

Bacterial and Viral Strains.—Twenty independent mutants resistant to the virulent bacteriophage T4 were isolated from *Escherichia coli* B (Lenski and Levin, 1985). Each mutant was selected by plating bacteria and excess viruses in soft agar, isolating a single colony, and purifying the resistant population by restreaking the cells. All of the mutants thus obtained were completely resistant to T4, as determined by testing concentrated lysates of T4 against lawns of the resistant strains.

The parental strain possesses several phenotypic markers that allow mutants to be distinguished unambiguously from contaminants. These include inability to utilize L(+) arabinose (*ara*), sensitivity to phage T5 (*fhuA*$^+$), resistance to phage T6 (*tsx*), and resistance to streptomycin (*rpsL*). Each of the mutants described in this paper has been checked for these markers. All mutants, like the parental strain, are prototrophic.

A mutant of the T4-sensitive parental strain capable of growing on arabinose was isolated by plating a large number of cells on minimal arabinose agar. The arabinose marker is extremely useful in distinguishing competing strains on the basis of their colony color on tetrazolium arabinose indicator plates (Levin et al., 1977). This mutation will be shown to be effectively neutral in the medium used in this study.

Demerec and Fano (1945) and several more recent studies have shown that mutations conferring resistance to T4 in *E. coli* B often, but not always, generate cross-resistance to another virulent phage, T7. The sensitivity to T7 of all 20 T4-resistant mutants was determined by titering a concentrated lysate of T7 on bacterial lawns in soft agar.

As noted earlier, mutations producing defects in the LPS core cause cells to be more permeable to hydrophobic compounds, including certain growth-inhibitors like the antibiotic novobiocin (Tamaki and Matsuhashi, 1971; Havekes et al., 1976). Nineteen of the 20 T4-resistant mutants used in this study were sensitive to 100 μg/ml of novobiocin, whereas the parental T4-sensitive strain was resistant to 400 μg/ml of this antibiotic, as determined by the appearance of colonies on broth plates within 24 hours. One T4-resistant mutant had an intermediate degree of novobiocin sensitivity. These observations indicate that these mutations confer resistance to T4 by altering the LPS core, thereby preventing adsorption of the virus, rather than by blocking the subsequent course of the viral infection. The novobiocin-sensitive phenotype of the T4-resistant strains also proved useful in mapping 19 of these mutations, as described below.

Rough Mapping of Resistance Mutations.—The most recent genetic map for *E. coli* lists three loci (or closely linked sets of

loci) as being involved in synthesis of the LPS core (Bachmann, 1983). These are *lpcA,* which maps at approximately 6 minutes; *lpcB,* which maps very roughly to 65 minutes; and *rfa,* a set of loci, which map to 81 minutes. Precise mapping of mutations affecting the LPS core is especially difficult for two reasons. First, the transducing virus P1, like T4, adsorbs to the LPS core, so that P1 is not useful for mapping mutations that cause defects in the LPS core (Tamaki and Matsuhashi, 1971; Wright et al., 1980). Second, mutants defective in LPS core are inherently poor recipients in conjugative matings (Tamaki and Matsuhashi, 1971; Havekes et al., 1976).

Despite these obstacles, approximate map positions for 19 of the 20 mutations conferring T4-resistance were determined as follows. Each mutant strain was mated to a series of Hfr donors. Overnight cultures of donor and recipient were grown in broth, then each was diluted 1:200 into fresh broth. After four hours of uninterrupted mating, mixtures were plated onto broth plates supplemented with 50 μg/ml of streptomycin and 100 μg/ml of novobiocin. Streptomycin provided selection against each of the donors used in this study, while novobiocin selected against the T4-resistant recipients, as described above. The numbers of recombinants for each pair of donor and recipient were counted, and a subset of the recombinants produced in each mating were then scored for additional markers to characterize linkage relationships further. The donors used in this study and their essential properties are shown in Table 1.

Measurement of Relative Fitness.—Relative fitness was measured in the absence of phage and, thus, provides an indicator of competitiveness under the specified culture conditions. Measurements were made in batch culture at 37°C and 120 rpm, using 10 ml of a minimal-salts medium that contains 7 g dibasic potassium phosphate (trihydrate), 2 g monobasic potassium phosphate, 1 g ammonium sulfate, 0.5 g sodium citrate (dihydrate), 0.1 g magnesium sulfate (heptahydrate), 0.002 g thiamine hydrochloride, and 0.025 g glucose per liter of deionized water.

In each experiment, the Ara^+ derivative of the T4-sensitive parental strain competed against one of the twenty Ara^- T4-resistant mutants, or against the Ara^- T4-sensitive parental strain. Each competitor was preconditioned in the experimental medium by growing a small inoculum to saturation density. This ensured that the physiological states of the two competitors were equivalent and that any differences in fitness were characteristic of the experimental environment and not of a transition between two environments. After preconditioning, 0.05 ml of the Ara^+ competitor and 0.05 ml of the Ara^- competitor were mixed in 10 ml of fresh medium, and an initial sample was removed, serially diluted, and plated on tetrazolium arabinose indicator agar. These samples were then scored the following day on the basis of colony color. The mixed culture was incubated for one day, during which time saturation density was achieved, before a final sample was removed, diluted, and plated. Relative fitness was calculated as the ratio of the number of doublings between initial and final samples for the two competing strains. Differences in relative fitness may reflect differences in lag phase, growth rate, survival at saturation density, or some combination of these.

TABLE 1. Characteristics of Hfr donors used in rough mapping of 19 mutations conferring T4-resistance. Points of origin are given in minutes, on the *E. coli* linkage map; ↑ indicates markers transferred in order of increasing map position, and ↓ indicates markers transferred in order of decreasing map position. Additional markers: Ara^+ indicates ability to utilize arabinose; $T5^r$ indicates resistance to virulent phage T5; Tc^r indicates Tn*10*-encoded resistance to tetracycline. All five donors are St^s (73), which indicates streptomycin sensitivity. Strain X57 was obtained from B. Levin and then selected for T5-resistance; all other strains were obtained from B. Bachmann, Curator of the *E. coli* Genetic Stock Center, Yale University, New Haven, CT.

Strain designation	Point of origin	Additional markers (map positions)
X57	0↑	Ara^+ (1), $T5^r$ (4)
BW6156	7↓	Tc^r (84)
BW6159	67↑	Tc^r (85)
BW6175	80↑	Tc^r (90)
BW6169	84↓	Tc^r (61)

RESULTS

Approximate Map Positions of Mutations Conferring Resistance to T4.—From the

frequency of recombinants produced by the various matings and subsequent scoring of the recombinants for the additional markers indicated in Table 1, the following conclusions were drawn. Twelve of the mutations conferring T4-resistance occurred at (or near) the *lpcA* locus. These strains yielded recombinants most readily with donors X57 and BW6156, indicating a map position between 0 and 7 minutes. Linkage of Ara^+ and $T5^r$ markers in crosses with X57 further established that these 12 mutations all lay beyond 4 minutes. Six of the T4-resistance mutations occurred at (or near) the *rfa* loci. These strains yielded recombinants most readily with donors BW6169 and BW6175, indicating a position between 80 and 84 minutes. Observations on the linkage of Tc^r in the relevant crosses were consistent with this interpretation; linkage was strong for donors BW6156, BW6159, and BW6175, but very weak for donor BW6169, for which the Tc^r determinant lay beyond the selected St^r locus. One of the mutations conferring T4-resistance mapped at (or near) the *lpcB* locus. This strain yielded recombinants readily in matings with donors BW6159 and BW6169, but not in matings with the donor BW6175, indicating a map position between 67 and 80 minutes. Linkage of Tc^r in the relevant crosses further indicated that the mutation lay before the selected St^r locus at 73 minutes; linkage was strong for the donor BW6169, but very weak for the donor BW6159. Finally, one of the mutations conferring T4-resistance could not be reliably mapped owing to its intermediate degree of novobiocin sensitivity, as noted earlier.

Neutrality of the Arabinose Marker.—Twenty independent estimates of the fitness of the Ara^- T4-sensitive parental strain relative to its Ara^+ derivative were obtained. The mean estimate of the fitness of the Ara^- strain relative to the Ara^+ strain was 1.00, with a standard deviation of 0.02. Based on a t distribution with 19 degrees of freedom, the 95% confidence interval for this estimate of relative fitness ranges from 0.99 to 1.01. Thus, one can safely ignore any effects of selection acting on the arabinose marker when interpreting differences in fitness much greater than 1%.

Variation in Fitness Among Resistant Mutants.—Five measurements of relative fitness were obtained for each of the 20 T4-resistant mutants. Replicates were performed in sets on different days and are treated as a block effect. Friedman's nonparametric test was used instead of a parametric analysis of variance in order to avoid violating the assumption of homogeneity of variances (Sokal and Rohlf, 1981 p. 447). This test indicates highly significant variation in relative fitness among independent T4-resistant mutants (Table 2).

In order to gauge the magnitude of this variation and to examine possible correlates, a single "best" estimate of relative fitness was required for each of the 20 T4-resistant strains. Three possible estimators are: i) the mean of the five independent measurements; ii) the median of the five independent measurements; and iii) a composite obtained by pooling colony counts for the five replicate experiments. All three yield similar rankings of the relative fitnesses for the 20 resistant strains. In order that the magnitude of the variation among strains not be overestimated, the estimator chosen was the composite, which yielded the lowest variance in relative fitness among the T4-resistant strains. The estimated fitnesses are shown in Figure 1. Note that even the most fit T4-resistant mutants were much less fit than the T4-sensitive competitor. Note, too, the bimodal nature of the distribution of fitnesses of the T4-resistant mutants. Friedman's test was performed separately for the 16 mutants with estimated fitnesses between 0.48 and 0.65 and for the four mutants with estimated fitnesses between 0.84 and 0.87. There was no significant variation in competitive fitness within either of these modes (Table 2). Therefore, it is reasonable to conclude that there are two fundamentally distinct classes of mutations conferring resistance to T4; one class causes a relatively small reduction in fitness (about 15%), while the other engenders a much larger reduction (about 45%).

Associations of Fitness with Map Position and with Cross-Resistance to T7.—The map positions for 19 of the 20 mutations conferring T4-resistance are shown in Figure 1, along with the sensitivity or cross-resistance of each mutant to virus T7. The plating efficiency of T7 was at least 1,000-fold lower for all 16 of the mutants classified as cross-

TABLE 2. Tests for variation in competitive fitness among T4-resistant mutants, using Friedman's nonparametric test. If there is no variation, then the test statistic has an approximate chi-square distribution with $N - 1$ degrees of freedom.

Comparison	*d.f.*	Test statistic	Significance level
Among all 20 mutants	19	46.63	$P < 0.001$
Among 4 best mutants	3	1.32	$0.5 < P < 0.9$
Among 16 worst mutants	15	10.05	$0.5 < P < 0.9$

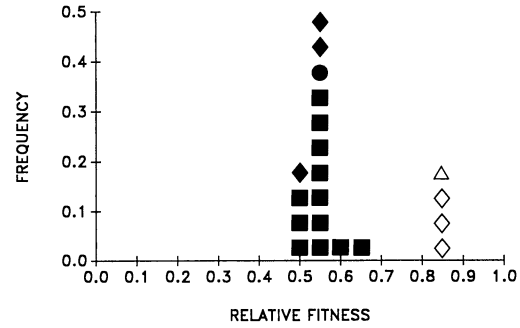

FIG. 1. Frequency distribution of relative fitnesses for 20 T4-resistant mutants. Analyses of independent replicates used to generate these data indicate highly significant variation in competitive fitness among the entire set of mutants, but not within either of the two modes. Fitnesses are calculated relative to an Ara$^+$ derivative of the T4-sensitive parental strain, which has a fitness of 1.00 ($\pm$0.01). Symbols indicate each mutant's sensitivity or cross-resistance to T7 and approximate map position: ■ = T7^r *lpcA*; ● = T7^r *lpcB*; ◆ = T7^r *rfa*; ◇ = T7^s *rfa*; △ = T7^s locus unknown. There is a marginally significant association between fitness and map position and a highly significant association between fitness and cross-resistance.

resistant than it was for any of the four mutants classified as sensitive to T7.

A two-tailed Fisher's exact test (Sokal and Rohlf, 1981 pp. 738–743) of the association between map position (*lpcA* and *rfa* only) and fitness (dichotomized as above) is marginally significant ($P = 0.049$). A two-tailed Fisher's exact test of the association between cross-resistance to T7 and fitness is highly significant ($P < 0.001$).

DISCUSSION

All 20 independent mutants of *Escherichia coli* B selected for complete resistance to virus T4 had maladaptive pleiotropic effects, as manifested by reduced fitness relative to the sensitive parental strain when competed in the absence of T4. However, these mutants varied greatly in the magnitude of their competitive disadvantages ($P < 0.001$). Thus, the reduction in competitiveness is more appropriately viewed as a property of the particular genotype conferring resistance to T4 than of the resistant phenotype per se.

There was a strong association ($P < 0.001$) between competitive fitness and cross-resistance to virus T7, which was not used in the isolation of the mutant strains. This observation permits some understanding of the functional relationship between genotype and phenotype. Although T4 and T7 are not closely related to one another (Bradley, 1967), both of these viruses adsorb to the LPS core. However, the particular moieties within the LPS core recognized by the viruses T4 and T7 are not identical (Fig. 2). T4 requires the first glucose residue for adsorption (Prehm et al., 1976; Wright et al., 1980), whereas T7 requires one or more heptose residues (Tamaki and Matsuhashi, 1971). Thus, any mutation that blocks synthesis of the LPS core prior to the incorporation of the first glucose residue causes resistance to T4, but only those mutations blocking the incorporation of heptose residues cause cross-resistance to T7 (Weidel, 1958; Tamaki and Matsuhashi, 1973; van Alphen et al., 1976; Havekes et al., 1976). The roughly three-fold greater reduction in competitive fitness for those mutants exhibiting cross-resistance indicates that maladaptive pleiotropic effects are much more severe for mutations that block steps occurring more centrally in the LPS core biosynthetic pathway than for those that block more peripheral steps. This result is consistent with the view expressed by Watt (1985) that the "visibility of genetic variation to selection" depends upon the "architecture" of metabolic pathways.

It should be emphasized that the "cost" associated with mutations conferring resistance to T4 is not strictly economic. That is, the T4-resistant strains do not divert material or energy resources to produce some shield against viral infection. (Some strains of *E. coli* can acquire partial protection against viruses via mutations that cause the

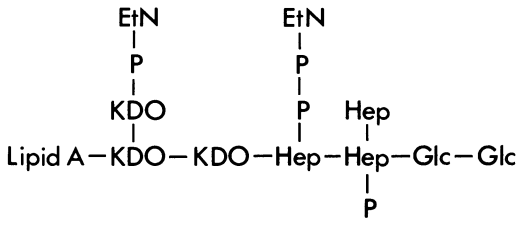

FIG. 2. Structure of the lipopolysaccharide (LPS) core in the cell envelope of *E. coli* B (after Wright et al., 1980; Prehm et al., 1975). Abbreviations for chemical structures are as follows: Glc = glucose; Hep = heptose; P = phosphate; EtN = ethanolamine; KDO = ketodeoxyoctonate. The core structure projects outward from Lipid A. Virus T4 adsorbs to the first glucose moiety in this structure, whereas adsorption of virus T7 requires one or more of the heptose moieties.

cell to produce a polysaccharide capsule [e.g., Paynter and Bungay, 1970]. These mutations cause colonies to take on a conspicuous "mucoid" appearance not observed in this study. In fact, *E. coli* B has been widely used to study cell-virus interactions precisely because it lacks the capacity to produce this capsule.)

It is possible to compute the average fitness of resistant mutants under a balance between mutation and selection for competitiveness. The overall rate of mutation to T4-resistance is approximately 8×10^{-8} (Lenski, 1984). One class of mutants has a fitness of about 0.55 relative to the sensitive genotype, implying a selection coefficient, s, of about 0.45. This class represents about 80% (16 of 20 in Fig. 1) of the mutations conferring T4-resistance, and so the corresponding mutation rate, μ, is about 6.4×10^{-8}. With a balance between selection and mutation (remember *E. coli* is a haploid organism), this class of resistant genotypes attains an equilibrium frequency, μ/s, of about 1.4×10^{-7}. The second class has a relative fitness of about 0.85 and makes up about 20% of the mutations conferring T4-resistance. The equilibrium frequency under a balance between mutation and selection is about 1.1×10^{-7} for this class. We can now compute the average selection coefficient associated with resistant genotypes that obtains at the equilibrium between mutation and selection:

$$\frac{(1.4 \times 10^{-7})(0.45) + (1.1 \times 10^{-7})(0.15)}{(1.4 \times 10^{-7}) + (1.1 \times 10^{-7})} = 0.32$$

This implies that the average fitness for the resistant genotypes is 0.68 relative to the sensitive genotype.

Now imagine that selection pressures change owing to the introduction of virus T4 into the environment. Assume that T4 does not drive the sensitive population extinct but, instead, mediates a frequency-dependent coexistence of sensitive and resistant genotypes (Lenski and Levin, 1985). If competitive interactions are transitive (so that the two classes of resistant genotypes can be ranked against one another as they can against the sensitive genotype), then the sensitive and less disadvantaged class of resistant genotypes attains an equilibrium subject to the opposing forces of selection for resistance and selection for competitiveness (Levin et al., 1977), while the more disadvantaged class of resistant genotypes is maintained only by mutation. Therefore, the vast majority of resistant genotypes belong to the less disadvantaged class, and the average competitive fitness of resistant genotypes increases to about 0.85 relative to the sensitive genotype.

Thus, variation in maladaptive pleiotropic effects permits refinement of the T4-resistant phenotype by selection among resistant genotypes. Empirical and theoretical research on refinement of novel phenotypes, including resistance to pesticides, has focused on selection for epistatic modifiers of maladaptive pleiotropic effects (Uyenoyama, 1986; Clarke and McKenzie, 1987). These two mechanisms are not mutually exclusive but, instead, are complementary. In the following paper (Lenski, 1988), I present evidence for epistatic modifiers of the maladaptive pleiotropic effects associated with mutations conferring resistance to T4.

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