TWO-STEP RESISTANCE BY *ESCHERICHIA COLI* B TO BACTERIOPHAGE T2

RICHARD E. LENSKI

Department of Zoology, University of Massachusetts, Amherst, Massachusetts 01003

Manuscript received November 10, 1983
Revised copy accepted January 6, 1984

ABSTRACT

Numerous authors have noted the difficulty in obtaining mutants of *E. coli* B that are resistant to bacteriophage T2 using standard procedures of plating large numbers of cells in the presence of excess phage. Yet, T2-resistant mutants appear in continuous culture at rates inconsistent with this difficulty. This paradoxical result derives from the fact that resistance to T2 usually arises as a consequence of two nonindependent mutations. Mutant bacteria resistant to phage T4 are very common and increase rapidly in continuous culture with phage T2 owing to an approximate halving of the rate at which T2 adsorbs to and kills these partially resistant mutants. The rate at which these partially resistant mutants then give rise to fully resistant mutants is approximately two orders of magnitude higher than the rate obtained by direct selection. These results are consistent with biochemical evidence that T2 adsorption to *E. coli* B involves both the bacterial lipopolysaccharide (to which phage T4 adsorbs) and a bacterial surface protein. However, this genetic evidence suggests that T2 can adsorb to either receptor type alone, whereas the biochemical evidence suggests that T2 requires a complex of the two receptors for adsorption to *E. coli* B. These results also indicate that the effects of genetic background can influence not only the selective advantage associated with particular mutations but also the rate at which certain selectively defined characteristics arise via mutation.

A number of researchers have noted the difficulty in obtaining T2-resistant mutants of *Escherichia coli* B using the standard procedure of plating large numbers of cells in the presence of excess phage (*DEMEREC and FANO 1945; LURIA 1946; HERSHEY 1946; LEVIN, STEWART and CHAO 1977; HANTKE 1978*). This contrasts with the ease of obtaining mutants resistant to most other virulent phage (*e.g., DEMEREC and FANO 1945*). However, T2-resistant mutants appear in continuous culture (*LEVIN, STEWART and CHAO 1977*), at rates that are inconsistent with the low rate of mutation observed from direct plating (personal observations). The purpose of this paper is to demonstrate experimentally that this apparent paradox occurs because T2 resistance can derive from either a single rare mutation or a pair of common mutations. In the latter case, the intermediates are partially resistant, which causes them to become rapidly fixed in continuous culture; hence, the net rate at which T2 resistance is evolved is accelerated.
MATERIALS AND METHODS

Experimental strains: The bacterial strains used in this experiment were all derived via spontaneous mutations from an E. coli B strain given to B. LEVIN by S. LEDERBERG. The characteristics of these strains are given in Table 1, and I use the "bar" notation to indicate successive selections for phage resistance (see DEMEREC and FANO 1945). The inability of the parental strain to utilize arabinose or xylose and its resistance to streptomycin and phage T6 served as useful markers for distinguishing mutants from contaminants in the estimation of mutation rates, described in the following data. Wild-type phages T2, T4, T5, T6 and T7 were given to B. LEVIN by C. THORNE.

Estimation of mutation rates: The procedure for estimating rates of mutation followed that of LURIA and DELBRUCK'S (1943) classic fluctuation test. Independent cultures of phage-sensitive bacteria were started from approximately 100 cells each, making it unlikely that any culture initially contained phage-resistant cells. These cultures were grown to resource saturation in minimal medium [7 g of K2HPO4, 2 g of KH2PO4, 1 g of (NH4)2SO4, 0.1 g of MgSO4·7H2O, 0.002 g of Thiamine·HCl, 0.5 g of Na3C6H5O7·2H2O and 0.007 g of CaCl2·2H2O] plus glucose. The entire contents of most cultures were plated in soft agar along with about $1 \times 10^9$ of the selecting phage; a few cultures were serially diluted and plated in soft agar to estimate the total number of cells present in each culture before selection. It was possible to obtain total cell numbers such that some, but not all, of 40 replicate cultures contained cells resistant to the particular selecting phage by adjusting the volume and the glucose concentration of cultures in preliminary experiments. The mutation rate, $\mu$, could then be estimated from the Poisson distribution without any assumptions, e.g., the relative growth rates of resistant and sensitive cells. Thus: $\mu = -\log p_0 / N$, where $N$ is the total number of cells per culture, and $p_0$ is the proportion of cultures yielding zero resistant mutants.

Estimation of T2 adsorption rates: The rates at which T2 adsorbs to E. coli B and its derivatives were estimated using a modification of the basic procedure of SCHLESINGER (1932). Phage were added to exponentially growing cultures of bacteria (the cell density of which had just been estimated via several independent serial dilutions) in the same minimal medium plus glucose at 37°C and 100 rpm. Every 2 min, for a total of 10 min, a sample was taken from this culture, diluted 2500-fold and chloroformed. The dilution effectively stops the cell density-dependent process of phage adsorption, whereas the chloroform kills bacteria and phage that have adsorbed to the bacteria but leaves free (i.e., unadsorbed) phage unaffected. The duration of the experiment is limited to 10 min, in order that significant cell growth is precluded (their doubling time in these conditions is about 1 hr); more importantly, intracellular production of complete phage progeny, which would be released by chloroforming, has not commenced (the duration of this "eclipse" period is about 14 min). The adsorption coefficient, $\delta$, is then estimated from the slope, $s$, of the exponential decay in the concentration of free phage estimated by regression of log, free phage against time, corrected for the density of cells, $N$, on which the adsorption occurs: $\delta = s / N$.

Chemostat experiments: The design of the chemostats used is shown in the appendix to CHAO, LEVIN and STEWART (1977). The same minimal medium was used as for estimating mutation and adsorption rates, with 300 mg/liter of glucose at 37°C. The volume was adjusted to about 15 ml, and the flow rate was adjusted to about 0.25 turnover per hr. Cell populations were initiated from exponentially growing cultures in the same medium; phage T2 were added simultaneously. Initial densities of the populations are shown in Figure 1. Cells and phage were sampled directly from the chemostat vessel, serially diluted and plated. Phage were plated in soft agar on a lawn of B/6; cells were plated on tetrazolium lactose plates (see recipes in LEVIN, STEWART and CHAO 1977). Cell populations were differentiated by parallel plating in the presence of about $1 \times 10^9$ phage.

RESULTS

Consistent with the references cited in the introduction, the rate of mutation for E. coli B to T2 resistance is low, almost three orders of magnitude lower than the rate of mutation to T4 resistance (Table 2). This rate is not affected by prior selection for T5 resistance but is increased about two orders of magnitude by prior selection for T4 resistance. The two major classes of T4-
RESISTANCE TO PHAGE T2

FIGURE 1.—Chemostat dynamics of phage T2 (Δ—Δ) and component E. coli B populations, as determined by selective plating: T5 resistant (○—○); T4 resistant (○—○); and T2 resistant (●—●).

TABLE 1

Bacterial strains

<table>
<thead>
<tr>
<th>Strain designation</th>
<th>Relevant characteristics</th>
<th>Source or derivation</th>
</tr>
</thead>
<tbody>
<tr>
<td>B/6</td>
<td>ara&lt;sup&gt;-&lt;/sup&gt; xyl&lt;sup&gt;-&lt;/sup&gt; str&lt;sup&gt;'&lt;/sup&gt; T6&lt;sup&gt;'&lt;/sup&gt;</td>
<td>S. Lederberg (to B. Levin)</td>
</tr>
<tr>
<td>B/6/5</td>
<td>As for B/6, plus T5&lt;sup&gt;'&lt;/sup&gt;</td>
<td>Selection with phage T5</td>
</tr>
<tr>
<td>B/6/4</td>
<td>As for B/6, plus T4&lt;sup&gt;'&lt;/sup&gt;</td>
<td>Selection with phage T4</td>
</tr>
<tr>
<td>B/6/4, 7</td>
<td>As for B/6, plus T4&lt;sup&gt;'&lt;/sup&gt; T7&lt;sup&gt;'&lt;/sup&gt;</td>
<td>Selection with phage T4</td>
</tr>
</tbody>
</table>

resistant strains (those that are and are not also resistant to phage T7: see Demerec and Fano 1945) both exhibit this greatly enhanced rate of mutation to T2 resistance. This result indicates that the evolution of T2 resistance by E. coli B can be accelerated by prior selection for resistance to phage T4. Note, however, that the rate of one-step resistance to T2 (about 10<sup>-10</sup>) is nonetheless much greater than the product of the two-step process (about 10<sup>-15</sup>).

If the absence of direct selection for T4 resistance is assumed, how might such T4-resistant cells become established in continuous culture with phage
T2? Two possibilities exist: (1) the T4-resistant cells might have a higher intrinsic growth rate, or (2) the T4-resistant cells might have a lower susceptibility to infection by phage T2. Previous experiments (R. E. LENSKI and B. R. LEVIN, unpublished results) have shown that T4 resistance confers no intrinsic growth rate advantage to E. coli and, in fact, engenders a competitive disadvantage under steady-state (i.e., resource-limiting) conditions. Thus, if the prior fixation of T4-resistant mutants is to explain the rapid evolution of T2 resistance in continuous culture, then one must be able to demonstrate a lower rate of infection (i.e., adsorption) of phage T2 to T4-resistant than to T4-sensitive bacteria.

Just such a result is seen in Table 3; the rate at which T2 adsorbs to T4-resistant cells is approximately half the rate at which T2 adsorbs to T4-sensitive cells. Note that T5 resistance has little or no effect on the T2 adsorption process, whereas the T4 resistance effect applies to both classes of mutants.

This reduction in the adsorption rate provides a strong selective advantage for T4-resistant bacteria over T4-sensitive bacteria in continuous culture with phage T2, as seen in Figure 1. (Four such chemostat experiments were performed, all yielding the same qualitative results). The T4-sensitive population used in this particular replication was T5 resistant, whereas the T4-resistant population was T5 sensitive. T5 resistance provided a convenient neutral marker; by neutral, it is meant that neither does it affect sensitivity to phage T2 (Table 2) nor does it affect competitive ability (R. E. LENSKI and B. R. LEVIN, unpublished results; see also DYKHUIZEN and HARTL 1983 and references therein). Note the increase of the T4-resistant population from an initially small minority and the subsequent appearance of a T2-resistant population and its rise to resource limitation. This T2-resistant population is T4 resistant and T5 sensitive, indicating that it was derived via mutation from the T4-resistant population and not from the T4-sensitive population. Thus, resistance to T2 in continuous culture has evolved not as a result of direct selection for a single mutation conferring T2 resistance, but rather as the result of indirect selection for partially resistant mutants that are predisposed toward T2 resistance.

Added support for this interpretation is that those T5-resistant mutants isolated after 20 hr were also T4 resistant. These T5-resistant, T4-resistant cells
TABLE 3

Estimation of T2 adsorption rates

<table>
<thead>
<tr>
<th>Bacterial strain</th>
<th>Cells/ml</th>
<th>Exponential rate of phage decline</th>
<th>Correlation coefficient</th>
<th>Adsorption rate constant (ml/hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>B/6</td>
<td>5.5 x 10^7</td>
<td>9.56</td>
<td>0.987</td>
<td>1.74 x 10^-7</td>
</tr>
<tr>
<td>B/6</td>
<td>6.5 x 10^7</td>
<td>7.68</td>
<td>0.78</td>
<td>1.48 x 10^-7</td>
</tr>
<tr>
<td>B/6/5</td>
<td>9.0 x 10^7</td>
<td>7.68</td>
<td>0.977</td>
<td>1.97 x 10^-7</td>
</tr>
<tr>
<td>B/6/4</td>
<td>5.9 x 10^7</td>
<td>5.07</td>
<td>0.955</td>
<td>1.03 x 10^-7</td>
</tr>
<tr>
<td>B/6/4</td>
<td>8.3 x 10^7</td>
<td>6.09</td>
<td>0.966</td>
<td>7.54 x 10^-8</td>
</tr>
<tr>
<td>B/6/4, 7</td>
<td>6.3 x 10^7</td>
<td>5.73</td>
<td>0.992</td>
<td>1.10 x 10^-7</td>
</tr>
<tr>
<td>B/6/4, 7</td>
<td>6.3 x 10^7</td>
<td>4.93</td>
<td>0.981</td>
<td>7.83 x 10^-7</td>
</tr>
</tbody>
</table>

were, moreover, T7 resistant, which (as noted earlier) is commonly associated with mutations to T4 resistance but which was not found in the T4-resistant population used to initiate this particular replication; therefore, these T5-resistant, T4-resistant mutants were derived from a secondary mutation in the T5-resistant population and not in the T4-resistant population. Thus, it appears that the T4-sensitive population was driven to extinction as a consequence of the increase in phage T2 density that accompanied the rise of the T4-resistant population (see LEVIN, STEWART and CHAO 1977 for a theoretical explanation of this counterintuitive phenomenon).

DISCUSSION

The results of these experiments indicate that resistance by E. coli B to virulent bacteriophage T2 may occur either as a direct (i.e., one-step) or an indirect (i.e., two-step) mutational process. The relative likelihood of observing direct and indirect mutations depends on the manner in which they are selected. If selection is "absolute," as occurs on a plate in the presence of excess phage, then even partially resistant mutants are destroyed, and only the direct mutants can be detected. If selection is "relative," as occurs in continuous culture where phage and bacteria reach a dynamic equilibrium, then partially resistant forms have an advantage, and both direct and indirect mutations can be detected.

The complexity of resistance by E. coli B to phage T2 is consistent with, and expands upon, suggestions of the early phage workers. LURIA (1946) hypothesized that resistance to T2 (and certain other relatively rare patterns of phage resistance) might actually be a combination of two or more mutations that result from a gross chromosomal change rather than point mutations. Although I have not determined the structural changes in the DNA associated with T2 resistance, the fact that direct resistance to T2 is significantly more common than the product of the indirect steps suggests structural changes other than simple point mutations. HERSHEY (1946) came even closer to an explanation for T2 resistance. He noted that T2 resistance could be selected by serial transfer with T2 in a salt-free medium, which limits the adsorptive ability of the phage and allows the survival of partially resistant mutants. HERSHEY fur-
ther noted that T2-resistant mutants thus obtained were usually also resistant to phage T4, but he did not show that T4-resistant mutants were partially resistant to T2 or that they were predisposed to evolving T2 resistance.

More recently, HANTKE (1978) has shown that inactivation of phage T2 in vitro requires both lipopolysaccharide (LPS) and a protein (Ia) from *E. coli* B; HANTKE postulated that T2 requires a complex of these two substances for adsorption in vivo. LPS is known to be the receptor for phage T4 on *E. coli* B (WILSON, LUFTIG and WOOD 1970); therefore, HANTKE'S results are consistent with the finding reported in this paper that T2 resistance is associated with T4 resistance (and hence the LPS). However, if both LPS and Ia are required for adsorption in vivo, then an appropriate mutation in either alone should be sufficient to cause resistance to T2. Yet, the genetic results presented in this paper show that two mutations may be required for T2 resistance, suggesting that T2 can adsorb to either the T4 adsorption site (LPS) or some other site (presumably Ia). I cannot offer an explanation for this apparent discrepancy between the biochemical and genetic evidence.

Finally, the enhanced evolution of T2 resistance by prior selection for T4 resistance is reminiscent of experiments demonstrating that the evolution of lactobionate utilization by lacZ-deleted *E. coli* requires prior selection for lactose, lactulose and/or galactosyl-arabinose utilizations (see review by HALL 1983). These indirect evolutionary pathways demonstrate that the effects of genetic background can subtly influence not only the selective advantage associated with particular mutations (see DYKHUIZEN and HARTL 1980; HARTL and DYKHUIZEN 1981) but also the rate at which particular selectively defined characteristics arise via mutation.

BARRY HALL, BRUCE LEVIN and an anonymous reviewer made a number of valuable suggestions, not all of which were incorporated. This work was supported by National Institutes of Health grant GM19848 to BRUCE LEVIN.

LITERATURE CITED


Corresponding editor: D. L. Hartl