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CONSTRAINTS ON THE COEVOLUTION OF BACTERIA AND VIRULENT PHAGE: A MODEL, SOME EXPERIMENTS, AND PREDICTIONS FOR NATURAL COMMUNITIES

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> The coexistence of bacteria and bacterial viruses is . . . sustained by a delicate mutational equilibrium that saves both protagonists from total extinction. (Stent 1963, p. 181)

One prevailing view of host-parasite (and prey-predator) coevolution is that of an endless "arms race" between host defenses and parasite counterdefenses, as embodied in the Red Queen hypothesis (Van Valen 1973; Schaffer and Rosenzweig 1978; Dawkins and Krebs 1979). The perpetuation of this arms race depends on at least two conditions, exclusive antagonism and genetic complementarity. Whether host-parasite coevolution is exclusively antagonistic depends strongly on the mode of parasite transmission (May and Anderson 1983). If successful reproduction by the parasite is in any way dependent on the survival of the host (or vice versa), then selection may favor hosts and parasites with mutually beneficial characteristics. Genetic complementarity requires that for each gene (or set of genes) conferring host resistance, there exists some corresponding gene (or set of genes) in the parasite restoring exploitative ability (Flor 1956). While there is evidence for such "gene-for-gene" relationships among crop plants and their fungal parasites (Flor 1956; Van der Plank 1968; Day 1974; Barrett 1983), the generality of this genetic complementarity remains unclear.

Microbial populations provide unique opportunities for direct experimental testing of evolutionary hypotheses because of their high densities and short generation times (see references in Dykhuizen and Hartl 1983). A gene-for-gene arms race is frequently assumed for bacteria and the viruses, or phage, that exploit them (Stent 1963; Rodin and Ratner 1983*a*, 1983*b*). Because reproduction by virulent phage necessarily entails the death of the bacterial host, the coevolution of bacteria and virulent phage is probably exclusively antagonistic (Levin and Lenski 1983).

In this paper, we expand the formal theory of interactions between virulent

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phage and bacteria (Levin et al. 1977) by incorporating mutational events into the dynamics. Theoretical considerations lead to specific predictions about the time course of evolutionary changes in the relationship between bacteria and virulent phage, and to more general predictions about the consequences of evolutionary constraints for their coexistence. We present the results of experiments with *Escherichia coli* and virulent phage which agree closely with predicted ecological and evolutionary dynamics, and which indicate the existence of constraints that preclude endless arms races. Our discussion focuses on the nature of these constraints, and we use these constraints to generate falsifiable hypotheses concerning the structure of natural communities of bacteria and virulent phage.

THEORY

The Ecological Model

The theory developed in this paper is an extension of that presented by Levin et al. (1977; see also Campbell 1961) which we summarize below. A resource enters a habitat of volume V ml at a constant rate ρ, which is expressed as turnovers of that volume per hour. Cells (uninfected and infected) and phage are washed out of the habitat at the same rate ρ, along with unutilized primary resources, metabolic wastes, and dead cell debris. The resource enters the habitat at a concentration C μg per ml, and has a concentration r μg per ml within the habitat. There are n uninfected bacteria per ml, m infected bacteria per ml, and p phage per ml in the habitat.

Uninfected bacteria multiply via binary fission at a per capita rate, $\psi(r)$ per h, which is some increasing function of the resource concentration. Each production of a new bacterium uses up ϵ μg of the resource. The liquid habitat is thoroughly mixed, so that bacteria and phage encounter one another at random at a frequency that is proportional to the product of their densities. The adsorption rate parameter δ ml per h governs the rate at which phage encounter and irreversibly infect bacteria. During a latent period of duration θ h, phage multiply in their host bacterium, after which time the fated cell lyses, releasing β phage particles. The concentrations of primary resource, uninfected and infected bacteria, and phage are related by the following time-delay differential equations:

$$\dot{r} = \rho(C - r) - \psi(r)\epsilon n \tag{1}$$

$$\dot{n} = \psi(r)n - \rho n - \delta np \tag{2}$$

$$\dot{m} = \delta np - \rho m - e^{-\rho\theta}\delta n'p' \tag{3}$$

$$\dot{p} = \beta e^{-\rho\theta}\delta n'p' - \delta np - \rho p \tag{4}$$

where n' and p' are evaluated at time $t - \theta$, and the dot notation indicates differentiation with respect to time. $e^{-\rho\theta}$ is the fraction of bacteria infected θ h ago that has not washed out of the system. These equations assume that infected cells are not reinfected, and that infected cells neither use resources nor grow; these assumptions can be modified without significantly affecting the general properties of the model.

Equations (1)–(4) differ from the Lotka-Volterra predator-prey equations in two respects: (1) the prey's growth rate is a function of a potentially limiting resource; and (2) there exists a time lag associated with the predator's numerical response. The Lotka-Volterra equations are neutrally stable, while (1) is stabilizing and (2) is destabilizing. Levin et al. (1977) discuss the theoretical conditions for stability in equations (1)–(4), while Levin et al. (1977) and Levin and Lenski (in press) argue from empirical results that experimental communities of bacteria and virulent phage are more stable than anticipated from theory.

The equilibrium density for the bacterial population is found by setting equation (4) equal to zero:

$$\hat{n} = \rho/[\delta(\beta e^{-\rho\theta} - 1)]. \tag{5}$$

The equilibrium density for the phage population is found by setting equation (2) equal to zero:

$$\hat{p} = [\psi(\hat{r}) - \rho]/\delta. \tag{6}$$

The equilibrium concentration of resources, $\hat{r}$, depends on the characteristics of the bacterial growth function $\psi(r)$, as well as on the equilibrium density of bacteria, $\hat{n}$. However, experiments with *Escherichia coli* and virulent phage in continuous culture indicate that phage-limited bacterial populations can exist at densities orders of magnitude below that obtained in the absence of phage (Paynter and Bungay 1969; Horne 1970; Levin et al. 1977; Chao et al. 1977). When this is the case, $\hat{r}$ should be very near C, the concentration at which resources enter the habitat, and $\psi(\hat{r})$ should be very near the intrinsic maximal growth rate for the bacteria in that medium.

The equilibrium density of a phage-limited bacterial population is independent of its rate of binary fission, and consequently independent of the availability of primary resources, provided that there are sufficient resources to support the bacteria in the absence of phage. Thus, the equilibrium density of a phage-sensitive bacterial population is unaffected by the presence of a phage-resistant bacterial population, provided the former has a higher rate of binary fission (than the latter) when the latter is at resource-limited equilibrium. In contrast, the equilibrium density of the phage does depend on its host's rate of binary fission; this equilibrium is lowered by the presence of a resistant population which saps some of the resources otherwise available to its host.

Incorporating (a Little) Genetics

Selection can act on inherited variation in any of the parameters governing the dynamics of bacteria and phage. Our concern here is with the evolution of resistant bacteria and host-range phage. Resistant bacteria have a phage adsorption parameter equal to zero, and typically arise spontaneously via single mutations (e.g., Luria and Delbruck 1943; but see Lenski [1984*c*] for an example of resistance arising via 2 mutations). Host-range phage that can infect these (formerly) resistant bacteria also appear via spontaneous mutations (e.g., Luria 1945). In general, these phage mutants have an extended, not an altered, host range, as

they can infect both bacteria that are resistant to the wild-type phage and bacteria that are sensitive to the wild-type phage (Schwartz 1980).

In the analysis that follows, we assume that all variation in the bacterial and phage populations can be ignored except resistance and host range. This is justified on the grounds that the effects of these mutations on fitness are likely to be much larger than the effects of other mutations. We also assume that neither bacteria nor phage are subject to recombination; our concern is with the sequential incorporation of single mutations into bacterial and phage populations. Finally, we assume for mathematical convenience that bacteria and phage populations are at their equilibrium densities until mutations become established which change those equilibria.

The evolution of resistant bacteria.—We use subscripts 0 and 1 to denote phage-sensitive and phage-resistant bacteria, respectively. The rate at which resistant mutants are expected to appear is proportional to the volume of the habitat, V; the density of bacteria, $\hat{n}_0$; the mutation rate, μ; and the rate of cell multiplication, $\psi_0(\hat{r})$:

$$A = V \cdot \hat{n}_0 \cdot \psi_0(\hat{r}) \cdot \mu. \tag{7}$$

(See Kubitschek [1970, pp. 126–134] for a discussion of cases in which mutants appear at a rate not strictly dependent on the rate of cell multiplication.)

Resistant mutants have a net selective advantage provided only that their growth rate is sufficient to offset losses to washout, as they are not subject to phage infection: $\psi_1(\hat{r}) > \rho$. However, there is a certain probability that a favored mutant (or both of its daughter cells, etc.) will be washed out of the habitat prior to replication. This situation is formally analogous to the classical problem of Gambler's Ruin. At any instant, the resistant cell population is subject to the loss of an individual via washout, or the gain of an individual via binary fission. (A slightly different formulation applies if one views bacterial reproduction as a discrete, not continuous, process.) Although the absolute rate of gains and losses increases with population size, their relative likelihood remains constant (until, of course, the resistant cells approach resource limitation). The probability of the next demographic event being a gain, G, is equal to $\psi_1(\hat{r})/[\psi_1(\hat{r}) + \rho]$, while the probability of a loss, L, is equal to $\rho/[\psi_1(\hat{r}) + \rho]$. There are two possible outcomes; the resistant mutant may establish a resource-limited population, or the resistant population may become extinct. The probability of extinction is given by

$$X = [(L/G)^Y - (L/G)^Z]/[(L/G)^Y - 1] \tag{8a}$$

where Y and 0 are the possible outcomes, and Z is the starting point (Feller 1957; assuming $L \neq G$). For our purposes, the starting point is a single resistant cell, and the nonzero outcome is the number of resistant cells in a resource-limited population. With $Z = 1$ and Y very, very large, equation (8a) simplifies; if $\psi_1(\hat{r}) > \rho$, then

$$X = \rho/\psi_1(\hat{r}) \tag{8b}$$

while if $\rho > \psi_1(\hat{r})$, then $X = 1$.

The resistant population initially grows exponentially at a rate $\psi_1(\hat{r}) - \rho$. This rate becomes appreciably diminished only as the resistant cells approach their

resource-limited density, $\hat{n}_1$. We can approximate the time taken for a single resistant cell to give rise to a resource-limited population by:

$$S = [\log_e (V \cdot \hat{n}_1)]/[\psi_1(\hat{r}) - \rho]. \quad (9)$$

Note that the effect of increasing the mutation rate diminishes as the growth of the resistant cells (i.e., S) replaces their appearance and establishment (i.e., $A[1 - X]$) as the time-limiting factor in their evolution.

The evolution of host-range phage.—We must now consider the order in which the resistant bacteria and host-range phage appear. If host-range phage appear prior to the evolution of resistant bacteria, they have no advantage and may well be at a disadvantage (e.g., Chao et al. 1977) owing to their reduced specificity (Schwartz 1980). On the other hand, if host-range phage appear subsequent to the establishment of resistant bacteria, they are likely to have a profound selective advantage, with orders of magnitude more hosts than available to the wild-type phage.

At equilibrium, the rate of reproduction by wild-type phage must be equal to ρ, because washout is the only source of loss to the total phage population (i.e., including those in infected cells). Therefore, the rate at which host-range phage mutants are expected to appear is given by

$$H = V \cdot \hat{p} \cdot \rho \cdot \nu \quad (10)$$

where ν is the rate of mutation to extended host range. Recall from equation (6) that the equilibrium density of wild-type phage declines subsequent to the establishment of a phage-resistant, resource-limited bacterial population, and becomes zero if there is no reduction in the competitive ability of bacteria associated with resistance.

The likelihood of a host-range mutant surviving to reproduce is low (even assuming the same parameters of infection as for wild-type phage) prior to the evolution of resistant bacteria. This is true because, at equilibrium, only one phage in β can survive to produce progeny. Therefore, if resistance has no effect on the competitive ability of bacteria, then the persistence of the phage population requires a mutation rate sufficiently high to ensure that host-range phage are present during the exponential growth phase of the resistant bacteria, the duration of which is S h (eq. [9]).

The more general case, however, appears for bacterial resistance to engender some cost in terms of bacterial competitive ability; this issue is considered in the "Experiments" and the "Discussion" sections of this paper. In such cases, phage can persist even if host-range phage mutations do not appear during the rise of resistant bacteria. Should a host-range phage appear subsequent to the attainment of the resource-limited state by the resistant bacteria, the superabundance of available hosts makes the probability of establishment high and the time to establishment short. We do not need explicit equations for these processes, however, since experiments indicate that it is the appearance of host-range phage, and not their establishment, which limits their evolution.

Equations (5)–(10) will be used in interpreting the experiments to which we now turn our attention.

EXPERIMENTS

Materials and Methods

Our experiments have been carried out using *Escherichia coli* B and several of the virulent T-phage. The unselected bacterium (obtained from S. Lederberg, and used previously in the studies of Levin et al. [1977] and Chao et al. [1977]) carries no plasmids or restriction systems, but does possess a number of markers that permit us to distinguish between phage-resistant mutants and contaminating bacteria: $ara^{-}xyl^{-}str^{r}T6^{r}$. All of the phage-resistant clones in this paper have been checked for these markers, and can therefore be identified as derivatives of the B strain.

The phage we consider in this paper are wild-type T2, T4, T5, T7 (obtained from C. Thorne), and their host-range mutants generated in this study. It should be noted that "T" is an arbitrary designation and is indicative of neither taxonomic affinity nor temperance. In fact, these four virulent phages are phylogenetically and functionally quite diverse. T2 and T4 are clearly related on morphological and other grounds, but are unrelated to T5 or T7 which are themselves unrelated (Bradley 1967). T4 and T7, though unrelated, both adsorb directly onto some component of the lipopolysaccharide in the bacterial cell wall (Wright et al. 1980); T2 and T5 make use of two different bacterial surface proteins for their adsorption (Schwartz 1980), although T2 may also adsorb to the lipopolysaccharide (see Lenski 1984*c*).

Most of the experiments described in this paper were carried out in continuous culture devices, or chemostats, whose design is given in the Appendix to Chao et al. (1977). The volume of the chemostats was adjusted to about 15 ml, and the flow rate to about .3 turnovers per h. The medium used in these chemostats was a minimal salts solution (see recipe in Lenski 1984*c*) supplemented with 0.3 g glucose per liter; glucose is limiting in the absence of phage. Samples were taken directly from the chemostat vessel and serially diluted through a 0.85% NaCl solution. Bacterial densities were estimated from colony counts on tetrazolium lactose plates, phage densities from plaque counts on sensitive bacteria in layered agar plates (see recipes in Levin et al. 1977). Phage-resistant and sensitive cells were distinguished by plating in the presence of excess phage; wild-type and host-range phage were distinguished by plating on sensitive and resistant bacterial lawns.

In the same medium used for the chemostat experiments, we independently estimated all parameters critical to the interaction of *E. coli* B and phage T4. Our estimate of the rate of T4 adsorption followed Adams (1959, p. 468). Phage were added to a culture of sensitive cells of known density; the cells were growing exponentially, and hence in the same physiological state as would occur in a phage-limited chemostat. After several minutes (but prior to any intracellular formation of complete phage particles) the culture was chloroformed, killing cells and phage that had irreversibly adsorbed to cells, but leaving free phage unaffected. The adsorption parameter was then estimated from the exponential decline in the density of free phage corrected for the bacterial density. The burst size and latent period of phage infection were determined from the one-step growth experi-

TABLE 1

ESTIMATES OF PARAMETERS GOVERNING THE DYNAMICS OF THE INTERACTION BETWEEN *Escherichia coli* B AND VIRULENT PHAGE T4 IN CHEMOSTAT CULTURE

Parameter	Estimate
Chemostat volume	$V = 15$ ml
Chemostat flow rate	$\rho = .3$ per h
Phage adsorption rate	$\delta = 3 \times 10^{-7}$ ml per h
Phage burst size	$\beta = 80$
Latent period of infection	$\theta = .6$ h
Intrinsic growth rate: sensitive bacteria	$\psi_0(\hat{r}) = .7$ per h
Mutation rate to phage resistance	$\mu = 8 \times 10^{-8}$
Intrinsic growth rate: resistant bacteria	$\psi_1(\hat{r}) = .7$ per h

NOTE.—Intrinsic growth rates assume glucose is superabundant, which is the case prior to the establishment of a resource-limited population of resistant bacteria.

ment of Ellis and Delbruck (1939), again using exponentially growing bacteria. The maximum growth rates of T4-sensitive and T4-resistant bacteria were estimated directly from spectrophotometric growth curves. The rate of mutation to resistance was estimated from the Poisson using the proportion of independent cultures, each containing a known number of bacteria, which contained zero mutants (i.e., the fluctuation test of Luria and Delbruck [1943]). The estimates of these parameters are given in table 1.

The other medium used in this work was LB broth (see recipe in Miller 1972) supplemented with 2.5 g $MgSO_4 \cdot 7H_2O$ per liter. This medium was used to obtain maximum phage concentrations in order to search more efficiently for rare host-range mutants of phage.

Specifics of the design and analysis of our experiments are, for clarity, presented along with the results.

Results

Chemostat observations with Escherichia coli *B and phage T4.*—Figure 1 illustrates the dynamics of a chemostat inoculated with approximately equilibrium densities of *Escherichia coli* B and phage T4. Bacteria that are resistant to the phage have appeared and attained their resource-limited equilibrium density by about 108 h. Phage T4 persists, but no host-range mutants are detected.

The parameters governing the chemostat dynamics of *E. coli* B and phage T4 are presented in table 1. Using equations (5) and (6), we can calculate the predicted equilibrium densities for sensitive bacteria and wild-type phage prior to the evolution of resistant bacteria. The theoretical equilibrium of sensitive cells is 1.5×10^4 cells/ml, in reasonable agreement with that observed in figure 1. Formally demonstrating the persistence of the sensitive population subsequent to the evolution of resistant bacteria is problematic, because sensitive cells are anticipated to be a very small minority and cannot be detected by selective plating without special markers (see Chao et al. 1977). We will show later, however, that (1) the phage-sensitive bacteria have a clear growth-rate advantage under re-

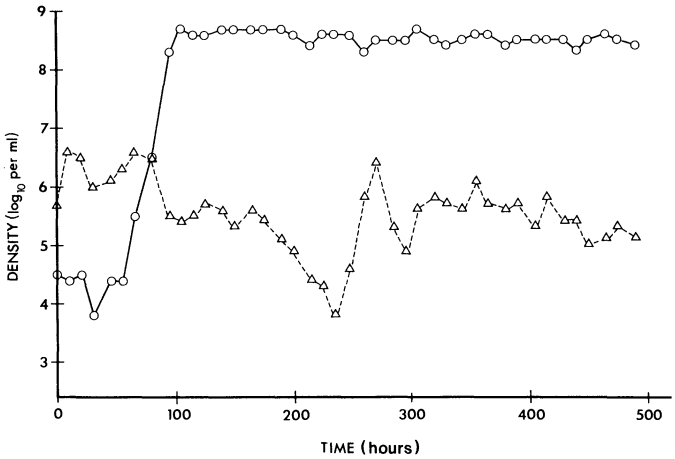

FIG. 1.—Densities of *Escherichia coli* B (——○——) and phage T4 (– – △ – –) in chemostat culture. See table 1 for parameters governing the dynamics of this interaction.

source-limiting conditions; and (2) phage T4 have no detectable capacity for growth on the resistant bacteria. Together these observations clearly imply the persistence of a minority sensitive population which supports the phage population. (In the absence of sensitive bacteria, and with a flow rate of about .3 per h, phage would decline an order of magnitude in about 8 h.) The theoretical equilibrium density of phage prior to the attainment of resource limitation by the resistant bacteria is 1.3×10^6 phage/ml, again reasonably consistent with observation. Furthermore, we clearly see the anticipated reduction in phage density subsequent to the evolution of resistant bacteria, as the resistant cells sap some (but not all) of the resources previously available to the phage's sensitive hosts.

In seven such chemostats, we have observed the T4-resistant bacteria becoming resource-limited after approximately 84, 84, 96, 108, 132, 144, and 144 h, while in three such chemostats the phage drove the bacteria to extinction prior to the appearance of resistant bacteria. (These extinctions call into question our assumption of equilibrium densities for sensitive bacteria and phage, but actually strengthen our claim that an arms race is not a necessary outcome of the coevolution of bacteria and phage.) In none of the 10 chemostats did we observe any host-range phage.

Equations (7)–(9) enable us to determine the rate of mutation to phage resistance which would be consistent with the experimental observations. From equation (9), the time required for a single resistant cell to give rise to a resource-limited population of about 3×10^8 cells per ml (see fig. 1) is about 56 h. This leaves about 52 h for the appearance of the mutant which gave rise to a resource-limited population after 108 h (the median of the 7 observed times given above). From equation (8b), the probability that a single phage-resistant mutant establishes a resource-limited population is about $1 - .43 = .57$. Together, these values imply a rate of mutation for the entire population of about $1/(52 \times .57) =$

0.03 per h. Using equation (7) and the cell density estimated from equation (5), we estimate a mutation rate per replication event of about 2×10^{-7}. This agrees reasonably well with the independently estimated mutation rate of 8×10^{-8} (table 1), especially given the opportunities for error. Equally important is the observed variation in time to establishment of a resource-limited population, which is clearly consistent with the stochastic nature of the mutational process.

Chemostat studies with other phages.—We also monitored less intensively two chemostats for at least 300 h for each of the phages T2, T5, and T7 with *E. coli* B. We observed cells resistant to each of the phages T2, T5, and T7. We also observed at least one class of host-range mutant for phages T2 (1 replicate) and T7 (both replicates), but not phage T5. For T2 (both replicates) and T7 (1 replicate), we further observed bacteria that were resistant to these host-range phage. Phage T5 became extinct subsequent to the establishment of resistant bacteria, while phages T2 and T7 persisted even after the appearance of the second-order resistant bacteria.

These observations are qualitatively consistent with the results of previous studies for phages T2 (Levin et al. 1977) and T7 (Chao et al. 1977) under quite similar culture conditions, as well as for phages T2 (Paynter and Bungay 1969) and T4 (Horne 1970) under rather different culture conditions.

Constraints on the bacteria.—From table 1, we see that there is no detectable difference in the growth rates of T4-sensitive and T4-resistant *E. coli* B when glucose is superabundant. The phage-mediated coexistence of sensitive and resistant bacteria, however, requires only that the sensitive cells have a growth-rate advantage when the resistant bacteria are at their resource-limited equilibrium. Therefore, a more appropriate test is to allow the sensitive and resistant strains to compete in the absence of phage. In figure 2*a*, we have plotted the proportion of bacteria resistant to T4 in a chemostat with flow rate of approximately .3 per h. Exponentially growing bacteria were added to the chemostat in densities about one order of magnitude below resource-limited equilibrium. Thus, for several hours the cells continued to grow exponentially, while subsequently we observed a rapid reduction in the frequency of T4-resistant bacteria. It is possible to estimate the differential in the growth rates of the sensitive and resistant bacteria, d, according to the following equation (e.g., Dykhuizen and Hartl 1983):

$$d = [\log_e R(t_1) - \log_e R(t_2)]/(t_2 - t_1) \tag{11}$$

where $R(t)$ is the ratio of resistant to sensitive bacteria at time t. (Note that the proportion of resistant bacteria, and not this ratio, has been presented in fig. 2.) We calculate that between the second and fourth samples, T4-resistant bacteria are declining at a net rate of $d = .16$ per h. The growth rate of the sensitive bacteria must be equal to the flow rate ρ at the resource-limited equilibrium. Therefore, the growth rate of the resistant bacteria must be equal to $\rho - d$, or approximately .14 per h, indicating a selective disadvantage of about 50%.

We performed similar experiments with *E. coli* B resistant to T2, T5, and T7, each allowed to compete against the unselected B strain. T2-resistance and T7-resistance both engendered severe competitive disadvantages, while there was no observable competitive disadvantage associated with T5-resistance (fig. 2*b*). Re-

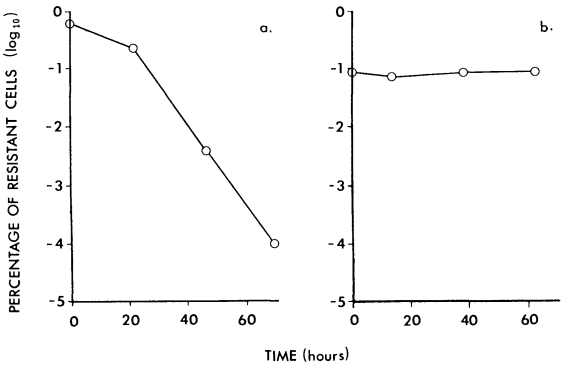

FIG. 2.—Competition in absence of phage between phage-sensitive and phage-resistant clones of *Escherichia coli* B. *a*, T4-resistant; *b*, T5-resistant.

call that we observed extinction of only phage T5 subsequent to the establishment of resistant bacteria. Thus, the results of the competition experiments are in all cases consistent with the theoretical prediction that nonevolving phage can persist if and only if resistant bacteria are at a competitive disadvantage to sensitive bacteria under conditions of resource limitation.

Constraints on the phage.—We have seen that while T4-resistant mutants appear in chemostat culture, corresponding host-range mutants do not. The difficulty with using chemostats to explore the limits of coevolution of bacteria and phage is that population sizes are much lower than those which are obtained in batch culture. Therefore, rather than waiting to see if we ever observe host-range T4 in chemostats, we have grown very large numbers of phage and searched for these mutants. The standard procedure for isolating host-range mutants is to plate large numbers of phage on a lawn of resistant bacteria and look for plaques indicative of host-range mutants. We have chosen an alternative procedure of selective enrichment, however, for the following reason: the direct plating approach does not exclude the possibility that either the wild-type phage or some host-range mutant is capable of growth on the resistant bacteria, but with such meager infective capacity that no plaque formation is observed on the resistant lawn.

A high-titer phage T4 lysate was combined with an exponentially growing broth culture of T4-resistant bacteria (for a total volume of 10 ml). After 24 h, the culture was chloroformed, killing the bacteria but not the phage. One ml of these phage was added to nine ml of fresh broth. This culture was sampled and the titer of phage determined by plating on phage-sensitive bacteria. Any class of phage which failed to reproduce on the resistant bacteria would have declined 10-fold; any phage that did reproduce would yield progeny, which themselves would reproduce, ensuring their representation and enrichment in the subsequent culture. To this second culture were then added exponentially growing resistant bacteria, and this process was repeated daily. The observed reduction in phage concentration was compared with the 10-fold daily reduction expected if no phage reproduction occurred.

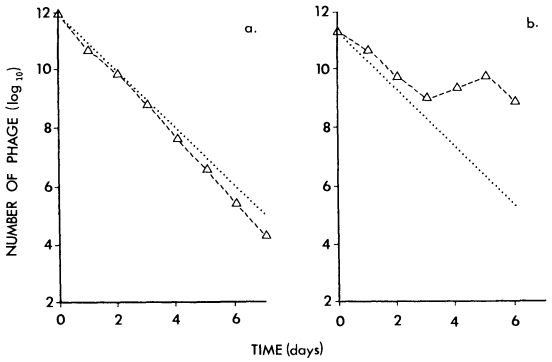

FIG. 3.—Search for host-range phage active against first-order resistant clones of *Escherichia coli* B. Dotted diagonal lines indicate anticipated rate of decline in phage numbers if no host-range phage are present. *a*, Phage T4; *b*, phage T2.

In figure 3*a*, we see that the 10-fold daily reduction in T4 titer did occur, indicating (1) the wild-type T4 had no capacity for growth on the T4-resistant bacteria; and (2) none of the initial number of T4 phage was a host-range mutant. We can therefore put an upper limit on the mutation rate which is consistent with not having observed host-range mutants among the 8.6×10^{11} T4 used to initiate this experiment. The probability, P, of observing no mutations in N replications (each phage representing a genomic replication) with a mutation rate of ν is given by

$$P = e^{-\nu N}. \tag{12a}$$

We wish to choose an acceptable probability, say .05, and solve for the maximum ν consistent with observing no host-range mutants; that is,

$$\nu = -(\log_e P)/N. \tag{12b}$$

For T4, we can say with 95% confidence that the rate of host-range mutations infective on this T4-resistant clone of B is less than 3.5×10^{-12}.

Given this maximum rate of mutation, we now ask, How long would we have to observe our chemostats to detect these host-range mutants? Assuming an equilibrium density for T4 of no more than 1×10^6 phage/ml subsequent to the establishment of the resistant bacterial population (see fig. 1), then from equation (10) we estimate a mutation rate for the entire population of about 1.6×10^{-5} per h. The first host-range mutation could not be expected to occur for at least $1/(1.6 \times 10^{-5}) = 63{,}000$ h (> 7 yr)!

We were similarly unable to detect host-range mutants of T5. From equation (12b), the rate of mutation to host-range T5 must be less than 8.6×10^{-12} (at the .05 level). In contrast, the enrichment experiments did yield host-range mutants for phages T2 (fig. 3*b*) and T7, including a second-order host-range mutant of T2 that had not been observed in the chemostats. Even with T2 and T7, however, we could not select indefinitely for host-range extensions (figs. 4*a*, 4*b*), although we could select for higher-order bacterial resistance; we initiated separate cultures as

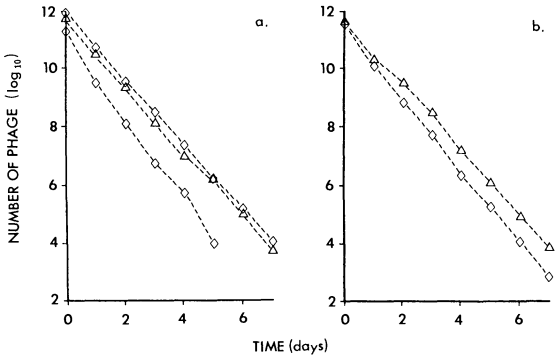

FIG. 4.—Search for further host-range phage active against higher-order resistant clones of *Escherichia coli* B. *a*, Phage T2 wild-type and two host-range mutants; *b*, phage T7 wild-type and host-range mutant. Wild types indicated by triangles, host-range mutants by diamonds.

before for wild-type and host-range phage, but observed no further host-range mutations. Pooling wild-type and host-range phage, we can say that the rates of mutation are less than 2.0×10^{-12} and 4.3×10^{-12} for T2 and T7, respectively (at the .05 level). We also note that the host-range T7 and one of the two host-range T2s declined more rapidly than the wild-type phage, indicating some additional mortality; we shall return to this phenomenon in the "Discussion."

DISCUSSION

Nature of the Constraints

We have demonstrated that a gene-for-gene arms race is neither a necessary condition for the coexistence of bacteria and virulent phage nor a general feature of their coevolution. Rather, our empirical results suggest that a resource-limited bacterial population becomes established, sooner or later, which persists indefinitely. The formal possibility remains, however, that very rare host-range mutations sustain more sporadic arms races on larger spatiotemporal scales, although these would be difficult to demonstrate.

We interpret these findings as evidence that the coevolutionary potential of virulent phage is less than that of their bacterial hosts, and we offer the following explanation. Bacteria may acquire resistance via mutations which either alter or block the synthesis of the particular phage receptor sites. Such mutations may occur not only as single base substitutions but also as insertions or deletions. Phage cannot acquire extended host range via mutations which block the synthesis of their adsorption organelle; rather, much more specific changes in either the organelle's configuration or the mechanism triggering the injection of the genetic material must occur. The best evidence for this interpretation is provided by the elegant work of Hofnung et al. (1976) with *Escherichia coli* K-12 and phage Lambda. These workers generated an array of phage-resistant bacteria using a

mutagenic substance causing single base substitutions. For some of these resistant clones, corresponding host-range mutants were isolated, while for others no such host-range mutants could be found. Mutations in the latter group of bacteria were demonstrated to represent nonsense mutations which yielded stop codons and, consequently, the failure to produce the specific phage receptor; the former class, in contrast, represented missense mutations and hence yielded a phage receptor, though one that was altered. Thus, bacterial resistance may occur by either the alteration or loss of function, with host-range mutations existing only to counter the former. Accordingly, we might expect that there exist T4 host-range mutants which can infect certain resistant mutants of *E. coli* B, even if they cannot infect the resistant mutants examined in this study. This is, in fact, the case; Crawford and Goldberg (1977) report a temperature-sensitive T4-resistant mutant of *E. coli* B for which there are corresponding host-range mutants.

In addition to mutational constraints (which are more severe for phage), the coevolution of bacteria and virulent phage is limited by selective constraints. Bacterial surface receptors exist, of course, for specific cellular functions which have been secondarily parasitized by phage. The loss or alteration of these surface receptors therefore changes the abilities of the bacterium to exploit its environment. If a particular surface receptor is required for the uptake of some substrate, and if that substrate is the sole source of an essential nutrient, then the loss of that receptor may be lethal (see also Schwartz 1980; Lenski 1984*a*). If, on the other hand, alternative pathways exist for obtaining or synthesizing the essential nutrient, then the loss of that surface receptor may be less disadvantageous than being sensitive to phage. Given the prototrophic nature of most coliform bacteria, we expect the latter situation generally may be applicable to them.

Although we did not explicitly look for selective constraints acting against host-range phage (but see Chao et al. 1977), we did detect one manifestation of such constraints. As noted earlier, host-range phage have an expanded, not an altered, host range. According to Schwartz (1980), this expanded host range is generally indicative of a trigger-happy mechanism for the injection of phage genetic material into its host, such that these mutants respond even to the imperfect adsorptions which occur on the surfaces of resistant bacteria. We interpret the mortality of host-range phage which we observed as imperfect adsorptions followed by unsuccessful attempts to inject their genetic material into these resistant bacteria.

Rosenzweig (1973) and Schaffer and Rosenzweig (1978) have suggested that predator-prey associations may reach a "coevolutionary steady state," where improvements in the predator's ability to exploit its prey or the prey's ability to escape its predator can be satisfied only at the expense of other "dissonant" functions. The constraints on bacteria that we have documented clearly derive from the dissonant selective pressures acting on bacterial surface receptors. But "trade-offs among competing selective demands" are not the only possible constraints on adaptive evolution (Gould and Lewontin 1979, p. 581). The failure to isolate host-range phage that are capable of infecting the "final" resistant bacteria cannot be readily explained by claiming no net selective advantage to these host-range mutants, given the superabundance of potential hosts. Instead, we believe

the absence of these host-range mutants results from severe architectural constraints on phages, which render them unable to counter the loss of particular receptor sites on the bacterial surface.

Consequences of the Constraints

If there exist mutations conferring bacterial resistance for which there are no (or only extremely rare) corresponding host-range phage mutations, how then do phage persist in nature? There are several alternative explanations (Lenski 1984*a*), of which we will argue one in this paper.

Phage can persist despite the evolution of resistant bacteria, provided that sensitive bacteria have some competitive advantage over resistant bacteria under conditions of resource limitation. We have seen that such selective constraints do exist (though not universally) for bacteria, and we have seen that mutational constraints are (at least generally) more severe for phage than for bacteria. We therefore anticipate that natural communities of bacteria and virulent phage will commonly exhibit phage-mediated coexistence of resistant and sensitive bacterial clones. Thus, we predict that (at least coliform) bacteria as a group will not be limited by phage. In particular, we expect that numerically dominant clones will be resistant to all co-occurring virulent phage; and that the appearance of virulent phage to which these dominant clones are sensitive will result either in their rapidly evolving resistance or in the rise of some co-occurring phage-resistant bacterial clones.

It might be argued that attempts to formulate hypotheses about natural communities of bacteria and virulent phage from our limited studies are useless; we have examined only a few strains under extremely artificial conditions. Yet this is precisely the value of laboratory studies; they suggest simple hypotheses which can be refuted by direct observation in the field. Given the dearth of information on these communities (Mason and Richardson [1981] have only 2 references to phage in their review of ecological considerations of human intestinal *Escherichia coli*), the difficulty of performing density manipulations on natural microbial populations, and the lack of explicitly stated hypotheses concerning the significance of phage in nature, this framework seems to us a plausible yet falsifiable representation of bacteria and virulent phage interactions in nature.

Let us briefly examine some observations which we believe support our views on natural communities of bacteria and virulent phage. (1) A review by Scarpino (1978) indicates that coliform bacteria greatly outnumber coliphage in sewage. This is similar to laboratory systems in which the dominant bacterial clone is resistant to all co-occurring phage, but is quite different from laboratory systems in which all bacteria are phage-sensitive (see fig. 1 in this paper; also Paynter and Bungay 1969; Horne 1970; Levin et al. 1977; Chao et al. 1977). (2) Evidence for the limited coevolutionary potential of phage is the great extent to which even unrelated phage share surface proteins and lipopolysaccharide regions as receptor sites in coliform bacteria (see Schwartz 1980; Wright et al. 1980). (3) Evidence for the trade-off between a bacterium's competitive ability and its resistance to phage is the finding that bacteria recently isolated from natural populations tend to be

resistant to most phage (B. R. Levin and R. E. Lenski, unpubl. observations), while laboratory strains (e.g., B, C, and K-12) cultured and stored for years in the absence of phage have apparently reverted to sensitivity.

Phage Therapy Revisited

With their discovery in the first quarter of this century, phage were advanced as agents for the treatment of infections by pathogenic bacteria. Numerous researchers isolated phage active against dysentery, cholera, diphtheria, gonorrhea, plague, and other dreaded diseases. Yet Stent (1963), Wilson and Miles (1964), and Peitzman (1969) have concluded that phage therapy was a failure. Stent (1963, pp. 8–9) has suggested that "the facility with which . . . bacteria . . . sport resistance against phages" was one factor limiting the application of phage therapy. The results presented in this paper support the contention that bacteria can rapidly evolve resistance to phage. The ability of phage to coevolve could, in principle, be a possible advantage over antibiotics; but constraints which we have documented severely limit the coevolutionary potential of virulent phage for such applications.

Williams Smith and Huggins (1982), however, successfully treated experimental *E. coli* infections in mice using phage. They were able to preclude the rise of a phage-resistant pathogen in the following way. First, pathogenicity of the target bacterium was shown to depend on the presence of a particular surface antigen (Williams Smith and Huggins 1980), a common situation. Second, they isolated phage whose adsorption site on the bacterial surface is that antigen. And third, they found that bacteria become resistant to the phage through the loss of that antigen, and hence their pathogenicity. In essence, they chose a phage for which the target bacterium suffered a nearly complete loss of its ability to utilize the "mouse substrate" as a consequence of resistance. We suggest that this "Achilles' heel" strategy be explored for other systems (e.g., Lenski 1984*b*) for which the evolution of resistance by pests or pathogens to agents of control is problematic.

SUMMARY

One view of the coevolution of parasites and their hosts is that of a gene-for-gene arms race between host defenses and parasite counterdefenses. We have incorporated mutations into a model of the ecological interactions between bacteria and virulent phage to determine rates of mutation that would be consistent with this scenario. The model assumes an open habitat (e.g., a chemostat) in which virulent phage and sensitive bacteria can coexist. Equilibrium densities of bacteria and phage are inversely proportional to the efficiency with which phage irreversibly adsorb to their hosts. The absolute rate at which mutations appear is proportional to the product of habitat size, population density, rate of increase, and mutation rate.

The bacterium *Escherichia coli* B readily evolved resistance to virulent phage T4 in our chemostat experiments. Approximately 100 h was required for the

appearance, establishment, and attainment of a resource-limited population of these T4-resistant mutants; this time period is close to that predicted from the model when the parameters of the model are estimated independently. No host-range phage T4 mutants appeared, yet the phage persisted even after the resistant bacteria had become resource-limited. We hypothesized that the failure to observe corresponding phage mutants indicates mutational constraints on the coevolutionary potential of this phage. We also hypothesized that the persistence of the wild-type phage indicates the presence of a minority population of sensitive bacteria that persists because of selective constraints which produce a competitive disadvantage for resistant bacteria under resource-limiting conditions. Both of these hypotheses were verified. Host-range T4 mutants occurred at a rate on the order of 10^{-12} or less, and could not be expected in the chemostats for several years. T4-sensitive and -resistant bacteria had very nearly the same exponential growth rates, but at steady state the latter had approximately a 50% disadvantage.

We also examined the interactions of *E. coli* B and virulent phages T2, T5, and T7 for evidence of selective and mutational constraints on the bacteria and phage, respectively. Under the conditions of our experiments, T2-resistant and T7-resistant (but not T5-resistant) bacteria also had clear competitive disadvantages to sensitive bacteria under resource-limiting conditions. We were able to isolate T2 and T7 (but not T5) host-range mutants. Even with T2 and T7, however, we could not select indefinitely for host-range mutants active against higher-order resistant bacteria. This general asymmetry in the coevolutionary potential of bacteria and phage occurs because mutations conferring resistance may arise by either the loss or alteration of gene function, while host-range mutations depend on specific alterations of gene function.

These constraints preclude observing endless arms races between bacteria and virulent phage. Instead, because of the asymmetry in coevolutionary potential of these hosts and parasites, we anticipate that natural communities of coliform bacteria and virulent coliphage are dominated by bacterial clones resistant to all co-occurring virulent phage. If virulent phage to which the dominant clones are sensitive should appear, then bacteria will either rapidly evolve resistance or be replaced by existing clones resistant to the phage. Thus, the role of virulent phage in structuring communities of bacteria is seen as important in determining clonal composition but unimportant in determining overall bacterial densities.

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