

## Hybrid Frequencies Confirm Limit to Coinfection in the RNA Bacteriophage $\phi 6$

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**Coinfection of the same host cell by multiple viruses may lead to increased competition for limited cellular resources, thus reducing the fitness of an individual virus. Selection should favor viruses that can limit or prevent coinfection, and it is not surprising that many viruses have evolved mechanisms to do so. Here we explore whether coinfection is limited in the RNA bacteriophage  $\phi 6$  that infects *Pseudomonas phaseolicola*. We estimated the limit to coinfection in  $\phi 6$  by comparing the frequency of hybrids produced by two marked phage strains to that predicted by a mathematical model based on differing limits to coinfection. Our results provide an alternative method for estimating the limit to coinfection and confirm a previous estimate between two to three phages per host cell. In addition, our data reveal that the rate of coinfection at low phage densities may exceed that expected through random Poisson sampling. We discuss whether phage  $\phi 6$  has evolved an optimal limit that balances the costly and beneficial fitness effects associated with multiple infections.**

When multiple virus genotypes coinfect the same host cell, an individual virus may experience decreased fitness due to heightened intracellular competition (3, 11, 25). A consequence of such competition is the evolution of defective interfering (DI) particles. DI particles are effectively viral genomes that become deleted for protein coding regions but retain and duplicate the target sequences recognized by the replication and encapsidation machinery (9, 10). By virtue of their smaller size and multiple targets, DI particles gain an intracellular replicative and competitive advantage over ordinary viruses during coinfection. Although DI particles generally evolve during very high multiplicities of infection (the ratio of phage to host cells in a given mixture), recent work has shown that even moderate coinfection can be costly to a virus (23). Here evolution does not lead to DI particles but to intact viruses with adaptations that enhance their intracellular fitness at the expense of their ability to exploit the host. Thus, viruses that can limit or prevent coinfection should possess a selective advantage, and it is not surprising that numerous viruses have evolved mechanisms to do so (19, 20, 26).

In this study, we tested whether and to what number of viruses coinfection is limited in bacteriophage  $\phi 6$ . When multiple  $\phi 6$  parent phages coinfect the same host cell, hybrid progeny are generated that possess genetic markers from more than one parent (13). Because coinfection is necessary for the creation of hybrids, we noted that the limit to coinfection (the maximum number of viruses that enter a single host cell) can be estimated from the frequency of hybrids observed at different multiplicities of infection. For any given limit, increasing the multiplicity of infection above the limit should have a diminishing effect on hybrid production. Our estimate is derived by comparing observed frequencies of hybrids to a series of theoretical distributions based on differing limits to coinfection.

Although a limit was established previously by Olkkonen and Bamford (16), who used the amount of incorporated  $^{14}\text{C}$  label as a measure of the number of phage entering a cell, we have reestimated the limit through development and application of a genetic approach. Aside from providing a test of the previous estimate, our motivation was to develop a simpler method that could be easily adapted to a variety of viruses and culture conditions. More-readily-obtained estimates of the limit to coinfection have recently become important because many theoretical models and studies of viral evolution require knowledge of the rate of coinfection (2, 4, 5, 13, 17, 23). Intracellular competition clearly creates a cost to coinfection, but it has been argued that coinfection is advantageous because the replication of more than one virus within a cell allows for sexual reproduction (3). Thus, a testable expectation is that viruses may benefit by evolving a limit, but the limit should not be as low as one virus per cell.

**Experimental design.** Because the genome of  $\phi 6$  is comprised of three double-stranded RNA segments denoted small, medium, and large (8, 12, 14, 18), and recombination in  $\phi 6$  is lacking or occurs at an extremely low rate (13), the generation of hybrid progeny during coinfection is strictly through segment reassortment. To monitor the frequency of hybrids produced, we crossed two  $\phi 6$  strains, MX and LX, which were genetically marked on the middle and large segments, respectively. Letting X denote the marked segment and + denote the unmarked or wild-type segment, the genotypes of MX and LX are therefore (+ X +) and (++ X).

To estimate the limit of coinfection, crosses were made at various multiplicities of infection. As the multiplicity of infection is increased, it is expected that the frequency of hybrids increases to a maximum that is determined by the limit of coinfection. In a cross between MX and LX, six possible hybrid genotypes can be produced. However only the wild-type reassortant (++++) was monitored, because it is the only genotype that is distinguishable from the parental genotypes (see below). In theory, if phage fitnesses are equal (but see below) a maximum frequency of (++++) progeny will be reached when individual cells are infected with an equal number of MX and LX phage [frequency (MX) = frequency (LX) = 0.5]. In this case, the probability that progeny will acquire both wild-type segments is obtained simply by multiplying the frequencies of

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the two segments as follows:  $0.5 \times 0.5 = 0.25$ . Only if the limit to coinfection is infinitely large (i.e., no limit) will the ratio of MX to LX within a given cell approach 1:1. As the limit is reduced, this ratio will deviate from 1:1 within most cells. Thus, as the limit to coinfection decreases, so will the maximum frequency of (+++) progeny. If the limit is one, no hybrids are formed regardless of the multiplicity of infection. To determine an estimate of the limit, our experimentally derived frequencies of (+++) hybrids were then compared to expected frequencies based on a theoretical model.

**The model. (i) Expected frequency of hybrids.** To generate the expected values, we created a model that predicts the frequency of (+++) hybrids over a range of multiplicities of infection in crosses between MX and LX phages. Five assumptions are implicit in the model: (i) phages attach randomly and irreversibly to cells; (ii) phages enter and infect a cell up to the designated limit to coinfection; (iii) in a host cell infected by either MX or LX phages alone, the number of progeny phage produced depends on the replicative ability of each parental phage and is independent of the number of infecting parental phage; (iv) in a host cell infected by both MX and LX, the wild-type markers are dominant and the number of progeny produced is equal to that of a cell infected by only a wild-type (+++) phage (7); and (v) hybrids are created by random reassortment of segments.

Assuming first that there is no limit to coinfection, let the frequency of host cells infected with  $n$  phage be Poisson distributed (21) and represented as

$$p(n) = \frac{e^{-m} m^n}{n!} \tag{1}$$

where  $m$  is the multiplicity of infection. Among the subset of host cells infected with  $n$  phage, let the frequency of cells with  $i$  MX phage and  $j$  LX phage be binomially distributed and denoted

$$b(n,i) = \binom{n}{i} q^i (1 - q)^j \tag{2}$$

where  $i + j = n$ , and  $q$  is the frequency of MX at the start of the cross. This model corresponds exactly to independent Poisson infection by MX and LX, where the Poisson parameter ( $m$  in the above model) for each phage may differ.

Assuming also that no intracellular replicative differences exist between the marked X and the wild-type segments, the following ensues within a cell with  $i + j = n$  phage. The frequencies of marked medium and large segments following replication in the infected cell are, respectively,

$$g_M(n,i) = \frac{i}{n} \tag{3}$$

$$g_L(n,i) = \frac{j}{n} = \frac{n - i}{n} \tag{3a}$$

The frequency of medium and large + segments in the same cell is then  $1 - g_M(n,i)$  and  $1 - g_L(n,i)$ , and the frequency of (+++) hybrid progeny produced by a cell infected with  $i + j = n$  phage (for  $i \geq 1, j \geq 1$ ) is

$$f(n,i) = [1 - g_M(n,i)][1 - g_L(n,i)] \tag{4}$$

The total (+++) hybrid progeny produced by cells infected with  $n \geq 2$  phage is then

$$\sum_{n=2}^{\infty} \sum_{i=1}^{n-1} p(n) b(n,i) f(n,i) \tag{5}$$

and assuming further that cells infected with only MX or only LX phage produce the same number of progeny, the total phage progeny produced by all infected cells ( $n \geq 1$ ) is

$$\sum_{n=1}^{\infty} p(n) \tag{6}$$

Thus, the final frequency of (+++) hybrids when there is no limit to coinfection and no fitness differences among the phage is

$$H = \frac{\text{equation 5}}{\text{equation 6}} \tag{7}$$

To incorporate a limit to coinfection, it is necessary to adjust the summations in equation 5. Let  $N$  be a constant representing the limit to the number of phage that can enter and infect a cell. Summations involving values of  $n < N$  are not affected, but those requiring  $n \geq N$  are affected because  $n$  cannot increase above  $N$ . Thus, equation 5 changes to

$$\sum_{n=2}^{N-1} \sum_{i=1}^{n-1} p(n) b(n,i) f(n,i) + \sum_{n=N}^{\infty} \sum_{i=1}^{N-1} p(n) b(N,i) f(N,i) \tag{8}$$

Equation 6 is not changed by a limit to coinfection, but it is now more correctly written as

$$\sum_{n=2}^{N-1} p(n) + \sum_{n=N}^{\infty} p(n) \tag{9}$$

which is presented because the format of equation 9 is more easily interpreted when fitness differences are added.

To incorporate any fitness differences, the effects of both progeny number per infected cell (burst size) and intracellular replication must be considered. Dealing first with intracellular fitness, let the replicative abilities of the marked segments in MX and LX be  $d_M$  and  $d_L$  and that of their respective + segments be  $1 - d_M$  and  $1 - d_L$ . Thus, the frequency of progeny carrying the marked middle segment and that carrying the marked large segment in a cell infected with  $i + j = n$  phage are modified from equations 3 and 3a to become, respectively,

$$g_M^*(n,i) = \frac{\frac{i}{n} d_M}{\frac{i}{n} d_M + \frac{j}{n} (1 - d_M)} \tag{10}$$

$$g_L^*(n,i) = \frac{\frac{j}{n} d_L}{\frac{i}{n} (1 - d_L) + \frac{j}{n} d_L} \tag{10a}$$

From equations 4, 10, and 10a, the frequency of (+++) hybrid progeny produced by a cell infected with  $i + j = n$  phage (for  $i \geq 1, j \geq 1$ ) when intracellular fitness differs, becomes

$$f^*(n,i) = [1 - g_M^*(n,i)][1 - g_L^*(n,i)] \tag{11}$$

To deal with any differences in progeny number, simply let  $W_M$  and  $W_L$  be the progeny number produced by a cell infected with either only MX or only LX phage, respectively, where the progeny number by a cell infected with only + phage is standardized to have a value of  $W_+ = 1$ .

Combining  $W_+$  and equations 8 and 11, the total number of (+++) hybrids is then expected to be

$$\sum_{n=2}^{N-1} \sum_{i=1}^{n-1} p(n)b(n,i)f^*(n,i)W_+ + \sum_{n=N}^{\infty} \sum_{i=1}^{N-1} p(n)b(N,i)f^*(N,i)W_+ \quad (12)$$

where  $W_+$  is number of progeny produced by a cell infected by  $i + j = n$  phage for  $i \geq 1, j \geq 1$  (at least one MX and one LX phage) because of assumption iv above.

To translate equation 12 into a frequency value, it must be divided by the total number of progeny phage, but equation 9 must be modified before it can be used. Adding the fitness values  $W_M, W_L$ , and  $W_+$  changes equation 9 because there are progeny that are produced by cells infected with either only MX or only LX phage. Thus, equation 9 can be decomposed into two sets of terms. The first is progeny produced by pure infections or

$$\sum_{n=1}^{N-1} p(n)[q^n W_M + (1 - q)^n W_L] + \sum_{n=N}^{\infty} p(n)[q^N W_M + (1 - q)^N W_L] \quad (13)$$

The second is progeny produced by mixed infections or

$$\sum_{n=2}^{N-1} p(n)[1 - q^n - (1 - q)^n]W_+ + \sum_{n=N}^{\infty} p(n)[1 - q^N - (1 - q)^N]W_+ \quad (14)$$

With these changes, the frequency of (+++) hybrids under a limit to coinfection and fitness differences is finally derived to be

$$H = \frac{\text{equation 12}}{\text{equation 13} + \text{equation 14}} \quad (15)$$

**(ii) Parameter estimation.** Using equation 15 to measure the limit to coinfection requires estimates of the values of the fitness parameters  $d_M, d_L, W_M$ , and  $W_L$ .

The strategy used to measure  $W_M$  and  $W_L$  was to cross MX phage with wild-type  $\phi 6$ , and similarly for LX phage, at multiplicity of infection equal to 0.02. At any multiplicity, the proportion of infected cells is from equation 1 equal to  $1 - p(0)$ , and the proportion of cells infected with only one phage is  $p(1)$ . At a multiplicity of 0.02, the frequency of cells infected with one phage among all infected cells is  $p(1)/[1 - p(0)] = 0.990$ . Thus, following the relative number of MX (or LX) in a cross with wild-type  $\phi 6$  at a multiplicity of 0.02 offers a good estimate of  $W_M$  (or  $W_L$ ) because 99% of the reproduction will be infected by cells with either one MX (or LX) phage or one wild-type phage. As the procedure for estimating  $W_M$  and  $W_L$  is identical, the analysis to follow considers only  $W_M$ .  $W_M$  and  $W_L$  are measured relative to wild-type phage in separate experiments.

Let  $q$  be the frequency of MX before reproduction in a cross with wild-type  $\phi 6$  at a multiplicity of infection of  $m = 0.02$ . If  $q'$  is the frequency of MX phage after reproduction, then (see reference 6)

$$q' = \frac{qW_M}{qW_M + (1 - q)W_+} \quad (16)$$

Because  $W_+ = 1$  as defined above,  $W_M$  can be solved from equation 16 by measuring  $q$  and  $q'$ .

To determine  $d_M$  and  $d_L$ , we noted that each parameter could be estimated by crossing MX or LX with wild-type phage at a higher multiplicity of infection and measuring the frequency of (+++) phage in the progeny. A multiplicity of 5 was

chosen. The strategy used is again presented only for MX because  $d_M$  and  $d_L$  were estimated in separate experiments, and the procedure for LX is equivalent.

Unlike the previous cross between MX and LX, a cross between MX and wild-type phage produces (+++) progeny that are hybrids (from mixed infections) and nonhybrids (from infection of wild-type alone). Thus, the estimate of  $d_M$  is based on the total frequency of (+++) phage and not simply that of hybrids. The number of (+++) progeny produced during pure infections involving the wild type is

$$\sum_{n=1}^{N-1} p(n)[(1 - q)^n W_+] + \sum_{n=N}^{\infty} p(n)[(1 - q)^N W_+] \quad (17)$$

Within mixed infections, the probability of obtaining a (+++) phage depends only on the sampling of one segment, and equation 11 is simplified to

$$h(n,i) = [1 - g_M^*(n,i)] \quad (18)$$

Thus, the number of (+++) progeny produced by mixed infections is derived by modifying equation 12 and replacing equation 11 with equation 18 to obtain

$$\sum_{n=2}^{N-1} \sum_{i=1}^{n-1} p(n)b(n,i)h(n,i)W_+ + \sum_{n=N}^{\infty} \sum_{i=1}^{N-1} p(n)b(N,i)h(N,i)W_+ \quad (19)$$

To convert the number of (+++) progeny into a frequency, it must be divided by the total number of progeny, which in equation 15 corresponded to the sum of equations 13 and 14. For the present estimate, equation 14 is unchanged, but equation 13, which describes the contribution of pure infections, is changed because the cross is now between MX and wild-type phage. Thus, equation 13 becomes

$$\sum_{n=1}^{N-1} p(n)[q^n W_M + (1 - q)^n W_+] + \sum_{n=N}^{\infty} p(n)[q^N W_M + (1 - q)^N W_+] \quad (20)$$

Combining these changes, the expected frequency of (+++) phage produced in a cross between MX and wild-type phage is therefore

$$P = \frac{\text{equation 17} + \text{equation 19}}{\text{equation 14} + \text{equation 20}} \quad (21)$$

The above derivations allowed  $W_M$  and  $W_L$  to be estimated directly from equation 16 and used immediately in any other equation. However, although  $P$  in equation 21 could be measured experimentally, it was not possible to solve directly for  $d_M$  because  $N$  was still an unknown. Thus, equation 21 had to be solved by assuming a series of possible values of  $N$ . For each assumed value of  $N$ , equation 21 was iterated with increasing values of  $d_M$  to yield a value of  $P$  that differed by less than  $10^{-4}$  from an experimentally determined value of  $P$ . An estimate of  $d_L$  was then similarly derived for the same series of assumed values of  $N$ .

Once they were obtained, the estimated and assumed values of  $d_M, d_L$ , and  $N$  were used with equation 15 to generate the expected values of  $H$  over a range of multiplicities of infection in crosses between MX and LX phages. Comparing these expected values to the experimentally observed values of  $H$  allowed the final estimate of  $N$ .

## MATERIALS AND METHODS

**Stocks and culture conditions.** All phages and bacteria were grown, plated, incubated, and diluted at 25°C in LC medium (13) by standard microbiological and previously described methods (4). The wild-type  $\phi 6$  and its host bacterium, *Pseudomonas phaseolicola*, were obtained from the American Type Culture Collection (no. 21781-B1 and 21781, respectively). From L. Mindich (Public Health Research Institute, New York City) we obtained LM1034, a *P. phaseolicola* host containing plasmid pLM746, which encodes the beta subunit of the  $\beta$ -galactosidase (B-Gal) gene (17); MX, the  $\phi 6$  phage with a  $\beta$ -Gal marker on the medium segment; and LX, the  $\phi 6$  phage with a  $\beta$ -Gal marker on the large segment.

**Genetic markers.** The  $\beta$ -Gal marker encodes the alpha subunit of the  $\beta$ -Gal gene. On selective plates containing 0.4% X-Gal (5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactopyranoside) and a 200- $\mu$ l lawn of overnight LM1034 culture, marked and unmarked phages form blue and white plaques, respectively. All phages in this study can be symbolized by their segment markers (small, medium, large), where + refers to wild-type and X refers to marked segments as follows: wild type (+++), MX (+X+), and LX (++X). The  $\beta$ -Gal marker was found to be extremely stable in MX and LX (data not shown), such that revertants could be ignored in our experiments.

**Crosses.** Two phages were crossed by single-burst experiments (22), which correspond to one cycle of infection and reproduction in a host cell. The phage were mixed at a 1:1 ratio and allowed 40 min adsorption to the host bacterium in LC broth at the desired multiplicity of infection. To obtain the desired multiplicity, phage numbers were adjusted by dilution and added to the bacteria that had been determined by spectrophotometer to be at a density of  $4 \times 10^8$  cells  $\text{ml}^{-1}$ . To remove free phage after the adsorption period, infected cells were washed three times by centrifugation for 1 min at 6,000 rpm in an Eppendorf microfuge, and the pellet was resuspended in LC broth, a derivative of Luria broth. After an additional 80 min, at which point most of the cells have burst (24), the lysate was filtered (0.22- $\mu$ m-pore-size; Durapore, Millipore) to remove surviving cells and to obtain the viral progeny containing the hybrid phage. The progeny were assayed by plating on X-Gal plates to determine the total number of phage and the frequency of (+++) hybrid phage, which do not carry any marked segments and therefore form white plaques.

**Computer modeling.** All iterations were by a Quick Basic program that is available upon request.

## RESULTS AND DISCUSSION

By applying equation 16, estimates of  $W_M$  and  $W_L$  were obtained by measuring  $q$  and  $q'$  at a multiplicity of infection of 0.02. On the basis of three independent replicates, it was determined that  $W_M = 0.228 \pm 0.029$  standard error and  $W_L = 0.599 \pm 0.086$  standard error. Following the described protocol (see (Parameter estimation),  $d_M$ ,  $d_L$ , and  $N$  were estimated by solving equations 15 and 21 simultaneously. For an assumed value of  $N$ , the corresponding values of  $d_M$  and  $d_L$  were substituted into equation 15 to generate a curve describing the relationship between the expected values of  $H$  for multiplicities of infection ranging from 1 to 25. A family of curves was then generated by assuming values of  $N$  from 1 to infinity (i.e.,  $N \geq 100$ ) (Fig. 1). As indicated earlier, experimental estimates of  $P$  were required for the initial solutions of equation 21, and values of  $0.865 \pm 0.017$  standard error and  $0.653 \pm 0.010$  standard error were obtained, respectively, from MX  $\times$  wild-type and LX  $\times$  wild-type crosses at a multiplicity of 5. As the multiplicity of infection is increased for the larger values of  $N$  in Fig. 1, the expected values of  $H$  asymptote above 0.25 because of the fitness disadvantage suffered by marked phage and segments. In the absence of any fitness difference, the asymptote should be 0.25 (see Experimental design).

To determine an estimate of  $N$  by a fit of observed values of  $H$  to the curves in Fig. 1, MX and LX phage were crossed over a range of increasing multiplicities of infection. Crosses were replicated two times at each multiplicity, and the frequency of (+++) hybrids was measured by sampling the progeny population by three independent dilutions. The mean of the three samples provided the experimental estimates of  $H$  and are presented as a function of the multiplicity in Fig. 1. These data reveal that hybrid frequency increases with multiplicity but reaches a plateau of approximately  $H = 0.23$ . The observed maximum matches our mathematical model for a limit to coin-

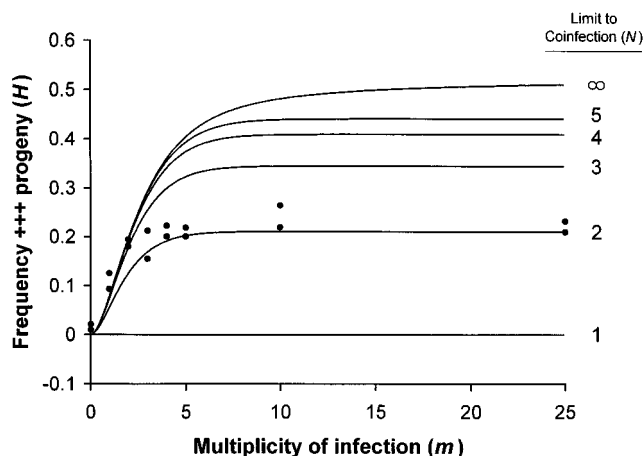


FIG. 1. Comparison between expected and observed values of  $H$ , frequency of (+++) hybrids generated in crosses between genotypes MX (+X+) and LX (++X). Curves were generated by a mathematical model that predicts  $H$  over a range of multiplicities of infection ( $m$ ), assuming a defined limit to coinfection ( $N$ ). Expected values for  $H$  are adjusted if marked segments experience a replicative disadvantage relative to wild type (see text for details). Observed values of  $H$  were generated in two replicate experimental crosses between phages MX and LX at eight multiplicities of infection (0.02, 1, 2, 3, 4, 5, 10, 25). Each point represents an independent estimate. Observed  $H$  saturates at a value corresponding to a limit of between two and three phages per cell.

fection between two and three phages per cell and Olkkonen and Bamford's (16) earlier estimate of three phages per cell.

Observed values are greater than expected at very low multiplicities of infection (Fig. 1), suggesting that coinfection at low multiplicities may be enhanced above the rate predicted by a random Poisson sampling. A possible explanation for the enhancement is the outer lipid membrane of  $\phi 6$  (1, 15). The stickiness of the membrane causes the phage to clump and may therefore enhance the entry of multiple phages into a cell. As a limit to coinfection may have evolved in viruses to prevent competition (see above), it is appealing to consider whether any enhancement, by clumping or an alternative mechanism, is either a passive consequence of the physiology of the phage or an evolved adaptation. Previous studies with  $\phi 6$  have shown that coinfection can be advantageous to the phage because it leads to sexual reproduction (segment reassortment); sex is advantageous because it combats the buildup of deleterious mutations by recreating (from mutated genomes) progeny with no or fewer mutations (2, 4, 5). Overall, our data suggest that  $\phi 6$  may have evolved mechanisms to enhance coinfection at low multiplicities of infection and to limit coinfection at high multiplicities. Such adaptations would serve to balance the costly and beneficial effects associated with viral coinfection.

It is hoped that the results of this study will encourage additional studies of the existence and mechanism for a limit to coinfection in viruses. Many issues clearly remain unanswered. How widespread is the phenomenon? To what extent is the limit controlled by viral or host genes? The model presented here potentially provides an easier method for detecting the limit in viruses capable of forming genetic hybrids. An immediately obvious advantage of the method is that the larger sample sizes it generates could be used to identify additional processes such as the enhancement of coinfection at low multiplicities.

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