Sex and the Evolution of Intrahost Competition in RNA Virus ϕ6

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ABSTRACT

Sex allows beneficial mutations that occur in separate lineages to be fixed in the same genome. For this reason, the Fisher-Muller model predicts that adaptation to the environment is more rapid in a large sexual population than in an equally large asexual population. Sexual reproduction occurs in populations of the RNA virus ϕ6 when multiple bacteriophages coinfect the same host cell. Here, we tested the model's predictions by determining whether sex favors more rapid adaptation of ϕ6 to a bacterial host, *Pseudomonas phaseolicola*. Replicate populations of ϕ6 were allowed to evolve in either the presence or absence of sex for 250 generations. All experimental populations showed a significant increase in fitness relative to the ancestor, but sex did not increase the rate of adaptation. Rather, we found that the sexual and asexual treatments also differ because intense intrahost competition between viruses occurs during coinfection. Results showed that the derived sexual viruses were selectively favored *only* when coinfection is common, indicating that within-host competition detracts from the ability of viruses to exploit the host. Thus, sex was not advantageous because the cost created by intrahost competition was too strong. Our findings indicate that high levels of coinfection exceed an optimum where sex may be beneficial to populations of ϕ6, and suggest that genetic conflicts can evolve in RNA viruses.

IF sex is defined as the exchange of genetic material between organisms (Michod and Levin 1988), then sexual reproduction is found to be extremely widespread in nature. This is surprising because sex has certain costs associated with it. For example, the production of males leads to a twofold cost of sex (Williams 1975; Maynard Smith 1978; Seger and Hamilton 1988). Another consequence of sex is that it tends to break apart well-adapted combinations of genes (coadapted gene complexes). Thus, whenever favorable combinations of genes are brought together into single individuals via mutation, recombination, and/or syngamy, sex has the potential to immediately tear them apart at its next occurrence (Shields 1988). For these reasons one would expect asexuality to be selectively favored; therefore, the prevalence of sex in natural populations of organisms remains an intriguing question in evolutionary biology (Michod and Levin 1988; Hurst and Peck 1996).

Two general hypotheses have been suggested for the evolution of sex. Positive selection models propose that sex may be advantageous because it generates beneficial variation in novel or changing environments. On the other hand, purifying selection models argue that sex may have evolved because it reduces or prevents the buildup of deleterious mutations (mutational load). Both hypotheses are similar in that they ascribe to sex the role of promoting linkage equilibrium (Felsenstein 1974). In the first case, the environment is variable and sex brings together novel and genetic combinations favored by positive selection. In the second case, the environment may be constant, but the genome is changing because the rate of deleterious mutations is high. Sex brings together parts of genomes that have not been destroyed by mutations, and selection then acts to purify them through the removal of deleterious mutations.

A model of positive selection developed by Fisher (1930) and Muller (1932; see also Maynard Smith 1988) proposes that adaptation is more rapid in a large finite population of sexual organisms than in an equally large, but asexual, population evolving in the same environment. Suppose that two favorable mutations, *A* and *B*, can arise in separate individuals in the same population and that each mutation can increase under selection. In an asexual population, these mutations can at best compete with each other until one or the other spreads to fixation [see Gerrish and Lenski (1998) for theoretical treatment of this process]. Thus, an *AB* individual can arise only if the two mutations appear sequentially in a single evolving lineage (*e.g.*, *A* occurs and increases to fixation, but *B* can be fixed only if it occurs in an individual that is already *A*). In contrast, if *A* and *B* occur in different individuals in a sexual population, genetic exchange allows the two mutations to be combined in a single descendant. For this reason, the Fisher-Muller hypothesis predicts that sex has the potential to accelerate the pace of adaptive evolution. Note that this argument applies only in large finite populations. An infinite population is so large that the ge-

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netic combination *AB* can arise spontaneously, whereas, in a small population, the advantage is offset because any beneficial mutation that occurs is likely to be fixed (or lost) before the next one appears.

Sexual reproduction in RNA viruses is analogous to that in eukaryotes (Chao 1994). When two or more viruses coinfect the same host cell, hybrid progeny are produced through genetic exchange between the parent genomes. In some RNA viruses, the exchange is by recombination (Jarvis and Kirkegaard 1991), but in others it is achieved by segmenting the virus into several smaller RNA molecules, and hybrid progeny are reassortants containing segments descending from the various coinfecting parents. Examples of the latter include certain viruses that infect humans (*e.g.*, Hantavirus and influenza; Ramig 1991). Because recombination between segments is rare or nonexistent in segmented RNA viruses (Horiuchi 1975; Mindich *et al.* 1976; Holland *et al.* 1982), this suggests that reassortment evolved as an alternative to recombination for the purpose of promoting sex (Pressing and Reanney 1984; Chao 1988). If so, sex in RNA viruses and eukaryotes may be independent evolutionary events, and segmentation in these viruses becomes a particularly instructive model for testing theories for the evolution of sex as a general phenomenon.

Previous experiments have shown that fitness of RNA virus ϕ6 decreases when viral lineages are subjected to a succession of population bottlenecks (Chao 1990; Chao *et al.* 1992). The fitness decline results because the intensified genetic drift produced by small bottlenecks leads to the buildup of deleterious mutations, a phenomenon termed Muller's ratchet (Muller 1964). Sex in ϕ6 may be advantageous in combating Muller's ratchet because segment reassortment presumably recreates (from mutated individuals) progeny with no or fewer mutations (Chao *et al.* 1992, 1997). These combined results suggest that the conditions favoring the evolution of sex through Muller's ratchet may be easily satisfied in an RNA virus such as ϕ6. However, to assess the generality of these results, alternative hypotheses for the evolution of sex must also be evaluated.

Here we present results of experiments initiated to examine whether the conditions favoring an advantage of sex by the Fisher-Muller hypothesis could be similarly satisfied in ϕ6.

Experimental system—RNA virus ϕ6: The RNA virus used in this study is the bacteriophage ϕ6. Although its natural bacterial host is unknown, ϕ6 can be grown in the laboratory on *Pseudomonas phaseolicola*, the phytopathogen responsible for bean blight (Vidaver *et al.* 1973). Phage ϕ6 has a genome that is divided into three double-stranded RNA molecules (Semancik *et al.* 1973). Total genome size in ϕ6 is 13,379 nucleotides, and the three segments comprise 22, 30, and 48% of the genome (McGraw *et al.* 1986; Gottlieb *et al.* 1988; Mindich *et al.* 1988); thus, the relative segments are referred to as small, medium, and large, respectively. Because a single phage contains all three segments (Day and Mindich 1980), a lone phage infecting a host cell can reproduce, but reproduction is then asexual. In contrast, genetic exchange (sex) occurs when multiple ϕ6 viruses coinfect the same host cell and generate reassortant (hybrid) progeny (Mindich *et al.* 1976).

RNA virus ϕ6 provides a powerful system to explore the evolution and advantage of sex (Chao 1990; Chao *et al.* 1992, 1997). Its characteristics include short generation times and extremely high rates of spontaneous mutation: on the order of 10^{-3} to 10^{-5} errors per nucleotide replication (Chao 1988). These features allow ϕ6 to be easily propagated in the laboratory for hundreds of generations, permitting evolutionary processes to be studied in detail.

Experimental overview: Sex in viruses is easily manipulated by controlling the multiplicity of infection (moi), or ratio of viruses to bacterial cells. We chose to examine the effect of sex at moi's of 0.002 and 5. At both moi's, and assuming Poisson sampling (Sokal and Rohlf 1981), the proportion of cells infected with 0, 1, and ≥2 phages is, respectively, $P(0) = e^{-\text{moi}}$, $P(1) = (e^{-\text{moi}} \times \text{moi}) / 1$, and $P(\geq 2) = 1 - P(0) - P(1)$. Thus, only $P(\geq 2) / (1 - P(0))$ or 0.1% of all infected cells contain two or more viruses at an moi of 0.002, and reproduction is primarily asexual. By the same logic, at an moi of 5 coinfection by two or more viruses is common and 97% of cells should experience multiple infections.

A single clone of bacteriophage ϕ6 was divided into three sexual (moi = 5) and three asexual (moi = 0.002) populations, and then allowed to evolve through propagation on the bacterial host *P. phaseolicola*. Presence or absence of sex in experimental populations was imposed for 50 consecutive days, which is equivalent to 250 generations of viral evolution. Throughout the study, a daily sample from each population of evolving viruses was stored in the freezer for later study. At the end of the 50-day experiment, samples from each population (taken at discrete time intervals) were competed against a common competitor of the ancestral genotype to measure changes in fitness. In this way, we determined whether phage adaptation was more rapid in sexual populations than in asexual populations.

The Fisher-Muller model predicts that sex allows more rapid evolution in a sexual population than in an asexual population *of equal size*. Equal size is an important criterion because, all else being equal, any population of large *N* should evolve faster than a population of small *N*. This is simply because beneficial mutations are expected to appear more often in a larger population (*i.e.*, more individuals are present where these mutations occur at random). Thus, a crucial component of our experimental design was to eliminate differences in population size among the sexual and asexual treatments. We did so by controlling the number of viral progeny harvested in each treatment population. When

one or more phages infect a cell, the resultant viral progeny form a visible plaque on the surface of the bacterial lawn. Each plaque in the asexual treatment was produced through infection of one phage (on average), and every day we harvested 500 plaques to propagate each asexual population ($N = 500$). In contrast, each plaque in the sexual treatment was produced through coinfection of five phages (on average), and here we harvested only 100 plaques for daily propagation ($N = 500$).

MATERIALS AND METHODS

Phage and bacteria: All viruses were originally derived from a single clone of bacteriophage φ6, previously described by Chao *et al.* (1992). We also obtained a spontaneous host-range mutant of φ6, referred to as φ6*h*. Host-range ability occurs through a point mutation on the medium segment, and a previous study showed that the *h* marker imposed a 5% fitness cost (Chao *et al.* 1992). Preliminary experiments confirmed that φ6*h* carries a 7% fitness cost under our experimental conditions (data not shown). All fitness measurements relative to φ6*h* reported below are adjusted to reflect the cost of the *h* marker.

The *P. phaseolicola* host strain used in all experiments was purchased from the American Type Culture Collection (ATCC No. 21781). An additional host strain, *P. pseudocaligenes ERA*, was obtained from the laboratory of L. Mindich (Public Health Research Institute, New York). φ6*h* forms clear plaques when plated on a mixed lawn containing both *P. phaseolicola* and *P. pseudocaligenes*. In contrast, non-host-range phages form turbid plaques on a mixed lawn because they do not kill the *P. pseudocaligenes* cells present.

Culture conditions and media: All phages and bacteria were grown, plated, incubated, and diluted at 25° in LC medium, a modification of Luria broth (Mindich *et al.* 1976). Liquid LC medium allows a stationary-phase bacterial density of $\sim 4 \times 10^9$ cells/ml for *P. phaseolicola*, and $\sim 5 \times 10^{10}$ cells/ml for *P. pseudocaligenes ERA*. All bacterial cultures were inoculated by a single bacterial colony placed into 10 ml LC medium in a sterile flask. Culture flasks were grown for 24 hr in a shaking incubator at 25° and 120 rpm. During this 24-hr period, bacterial cultures attained stationary-phase densities. All bacterial stocks were stored in a 4:6 glycerol/LC (v/v) solution at −20°.

Agar concentrations in plates were 1.5 and 0.7% for bottom and top LC agar, respectively. The volume of top agar was 3 ml/plate, and that of bacterial lawns was 200 μl. Plates used in all evolution experiments contained lawns made from overnight bacterial cultures of *P. phaseolicola*. P/E plates used in some assays contained a mixture of *P. phaseolicola* and *P. pseudocaligenes ERA* at a 200:1 volumetric ratio; ordinary and host-range phages produce turbid and clear plaques, respectively, on P/E plates.

Phage lysates were prepared by plating plaque-purified phage with top agar and a *P. phaseolicola* lawn. After 24 hr, plaques in the top agar were resuspended in 3 ml of LC broth and centrifuged at 3000 rpm for 10 min. Supernatant containing the phage lysate was filtered (0.22 μm, Durapore; Millipore, Bedford, MA) to remove bacteria. Phage lysates were stored at −20° in a 4:6 glycerol/LC (v/v) solution.

Sexual treatment: A single clone of φ6 was used to prepare a phage lysate as described above. At the start of the experiment this lysate was used to found three replicate populations in the sexual treatment, designated "S." Each S population was then mixed with an overnight culture of *P. phaseolicola* at moi = 5 (*i.e.*, 1×10^{10} phage/ml to 2×10^9 bacteria/ml). These mixtures were placed in a nonshaking incubator for 40 min to allow phage adsorption. Following adsorption, 500 phages from each mixture were plated on a *P. phaseolicola* lawn for 24 hr incubation. The next day, the propagation cycle was completed when 100 of the resultant plaques from each population were harvested to prepare a new phage lysate; because each plaque contained the progeny of five viruses (on average), *N* equaled 500 in each S population. The propagation cycle was then repeated using the new lysate, and a total of 50 cycles was conducted for each population. The *P. phaseolicola* hosts used in propagation were grown daily from a frozen stock. This prevented evolution of phage resistance by the host bacteria and eliminated the possibility that bacteria and phage would coevolve. As each cycle represents approximately 5 generations of viral evolution, 250 generations occurred during the experiment. Following daily propagation, a sample from each population's lysate was stored in a −20° freezer for future study.

During the first five cycles of the experiment (and periodically thereafter), lysates were titered to gauge the exact concentration of phage per milliliter. These data were used to ensure the accuracy of moi during the subsequent propagation cycle. Because the titer of phage lysates was not highly variable (data not shown), cycles propagated without titering were based on the mean titer in the initial five cycles ($\sim 2 \times 10^{10}$ phage/ml).

Asexual treatment: The same φ6 lysate described above was used to found the three replicate populations in the asexual treatment, designated "A." Each A population was mixed with an overnight culture of *P. phaseolicola* at moi = 0.002 (*i.e.*, 4×10^6 phage/ml to 2×10^9 bacteria/ml). Adsorption followed by plating was identical to that described in the S treatment. The next day, the propagation cycle was completed when 500 of the resultant plaques from each population were harvested to prepare a new phage lysate; because each plaque contained the progeny of only one phage (on average), *N* equaled 500 in each A population. As in the S treatment, later propagation cycles were based upon the mean titer of lysates in the first five cycles ($\sim 1 \times 10^{11}$ phage/ml). Lysates were stored in a −20° freezer as previously described. Aside from possible phage interactions during adsorption, the asexual treatment was designed to minimize interactions between viruses. Figure 1 depicts major features of the propagation cycle in each experimental treatment.

Paired-growth experiments: After the method of Chao (1990), fitness was measured by comparing the growth rate of a test phage (or mixed population of phages) relative to that of the ancestral phage bearing an *h* marker (φ6*h*). Competitors were mixed at a 1:1 volumetric ratio, and ~400 viruses were plated with top agar on a lawn containing 200 μl of overnight *P. phaseolicola* culture ($\sim 8 \times 10^8$ cells). Because no preadsorption occurred before plating, every virus in the lawn infected a cell alone. After 24 hr incubation, the resulting 400 plaques were then harvested and filtered to produce a lysate. The ratio of test phage to φ6*h* in the starting mixture (R_0) and in the harvested lysate (R_1) was estimated by plaques formed on P/E plates, where the ratio of the two phages was based on the *h* marker. Thus, fitness was assayed on a *P. phaseolicola* lawn, but the starting and final ratios were assayed on a mixed lawn of hosts. The number of plaques per paired-growth plate and mixed lawn plate was maximized at 400 because this minimized plaque overlap and, hence, interaction between phages. Fitness (W) is defined as $W = R_1/R_0$. If $W = 1$, then the test phage has the same fitness as the reference phage (φ6*h*); if $W < 1$, it has a lower fitness and accordingly for $W > 1$. For increased sensitivity when fitness differences were small, our protocol was repeated, in which case $W^t = R_t/R_0$,

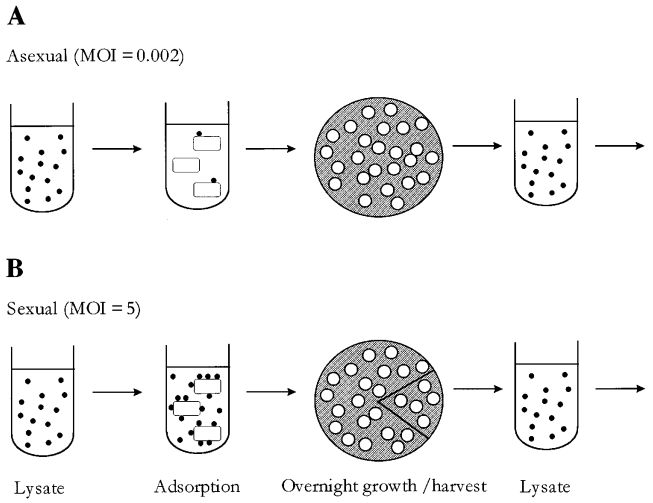

Figure 1.—Summary of the propagation schemes for the asexual and sexual treatment groups. Most aspects of propagation were identical in the two treatments. Phage (●) adsorbed to bacterial cells (□) at a given multiplicity-of-infection (moi), and this mixture was used to seed a bacterial lawn. During overnight growth, the viral progeny formed visible plaques (○). These plaques were harvested, and the bacteria were removed by filtration to create a new lysate. (A) The asexual treatment contained moi = 0.002, ensuring that each plaque produced was the result of a single infection, (B) whereas, the sexual treatment contained moi = 5, ensuring that each plaque was the result of coinfection by five viruses (on average). To control for differences in population size between the two groups, one-fifth as many plaques were harvested in the sexual treatment as in the asexual treatment. There were three replicate populations in each group, and all of the populations were propagated for 50 days. See text for details.

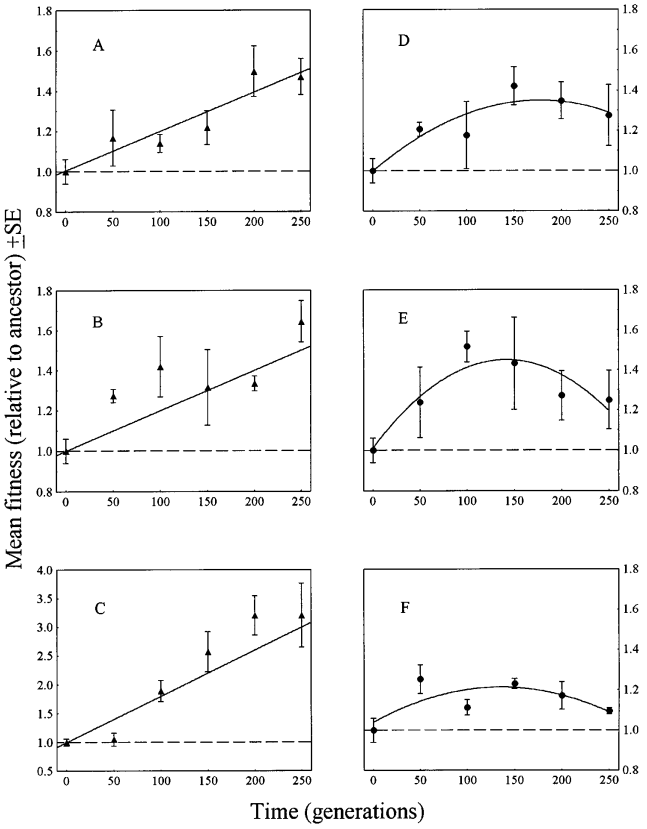

Figure 2.—Fitness improvement in terms of host exploitation (paired-growth) for each population in the two treatments. (A–C) Populations A1, A2, and A3, respectively, from the asexual treatment; (D–F) populations S1, S2, and S3 from the sexual treatment. Each point represents mean fitness (±SE) relative to the common competitor (ϕ6*h*) based on 3 replicate assays, except for the ancestor that is based on 10 assays. See text for statistical analyses.

where t is the number of repetitions, and R_t is the ratio after t repetitions (Chao 1990).

Modified fitness assays: Fitness was also estimated in the two evolutionary environments: moi = 5 and moi = 0.002. The above fitness assay was modified so that two competitors were mixed at an equal volumetric ratio, but were allowed to adsorb to *P. phaseolicola* for 40 min at a given moi. Following adsorption, the phages were plated with top agar on a *P. phaseolicola* lawn. To ensure that modified fitness assays matched the treatment conditions as closely as possible, the total number of phage per plate equaled that in the experiment proper (∼500 plaques per plate). W in the modified fitness assays was calculated as described above.

RESULTS

Fitness improvement in experimental populations: To address the Fisher-Muller model, we sought to determine whether the S and A treatment populations differed in their rates of fitness improvement. Paired-growth experiments (Chao 1990) measure fitness as the ability for an infecting virus to exploit its host in the absence of interaction with competing viruses (see materials and methods). We estimated fitness at 50 generation intervals for each population in the S and A treatments relative to the common competitor of the ancestral genotype, ϕ6*h*. Fitness assays were replicated ($n = 3$) for each population. As shown in Figure 2, the S and A treatment populations underwent very different fitness gains during the experiment. Population A3 showed a final fitness improvement of approximately twice that in any other experimental population (Figure 2C). More importantly, final mean values for fitness in the A populations exceeded those in the S populations, and a nonparametric test showed that this ranking of final fitness values in the two treatments was statistically significant (one-tailed Mann-Whitney rank test with $U_s = 9$, $n_1 = n_2 = 3$, $P = 0.05$). We then calculated the grand mean fitness for the three replicate populations at each time point. The A populations experienced a positive linear improvement in fitness over time (linear regression: slope = 0.0047, $t = 16.494$, d.f. = 4, $P < 0.001$). In contrast, the fitness trajectory in the S populations was concave; these populations appeared to quickly reach a selective plateau that was followed by a fitness decline. The regression model that best fit the experimental observations in the S treatment was a negative quadratic ($F_{(2,3)} = 20.768$, d.f. = 3, $P = 0.017$). Our results clearly indicated that the A populations experi-

enced a more rapid increase in fitness than the S populations, suggesting that sex is costly in this experimental system.

The sexual and asexual treatments in this study also differ because intrahost competition between viruses occurs during coinfection. One possible explanation for our unexpected results is that sexual viruses evolved traits favoring within-host competition, rather than traits that improve host exploitation (as measured by paired-growth assays). To explore this hypothesis, we sought to determine whether the S and A treatment populations experienced fitness trajectories in their respective environments that differed from the paired-growth results (Figure 2).

Rate of adaptation to treatment conditions: We measured the fitness relative to ϕ6*h* for each population at 50 generation intervals using a fitness assay that was modified to match the population's evolutionary environment (*i.e.*, moi = 5 or moi = 0.002; see materials and methods). Fitness assays were replicated ($n = 2$) for each population, and the grand mean fitness of the three populations in each treatment group was calculated at each time point. The results are presented in Figure 3; for comparison, Figure 3 includes the grand mean data for paired-growth assays described above. Regression analysis shows that the A populations (Figure 3A) experienced a positive linear improvement in fitness in both their own environment (slope = 0.0043, $t = 10.418$, d.f. = 4, $P < 0.001$), and in terms of paired growth (see above). This general result held for each A population analyzed separately (data not shown), and the high variance observed in Figure 3A was due to inflated fitness values for population A3. We compared the two regression lines in Figure 3A for equality of slopes using a small-sample two-tailed *t*-test for parallelism (Kleinbaum and Kupper 1978). This test shows that the regression coefficients are not significantly different at the $\alpha = 0.05$ level ($T = 0.802$, $t_{0.05[8]} = 2.306$, d.f. = 8, $P > 0.4$). We concluded that the A populations showed an equally rapid rate of improvement in their own environment as that predicted by changes in paired-growth. This result was not unexpected because both assay environments provide little opportunity for interaction among competing phages.

In marked contrast, we observed that the S populations (Figure 3B) showed a very different fitness trajectory in their own environment when compared to changes in paired-growth. Regression analysis indicates that these populations experienced a positive linear improvement in fitness at moi = 5 (slope = 0.0040, $t = 5.395$, d.f. = 4, $P = 0.006$), unlike the fitness results from paired-growth assays (see above). This result held when each population was analyzed separately (data not shown). Thus, the S populations showed a rapid rate of improvement in their own environment, but these adaptive changes did not translate to rapid improvement in terms of paired-growth. We concluded that the sexual phages are evolved to be strong intrahost competitors, but are poorly adapted to conditions where coinfection is uncommon.

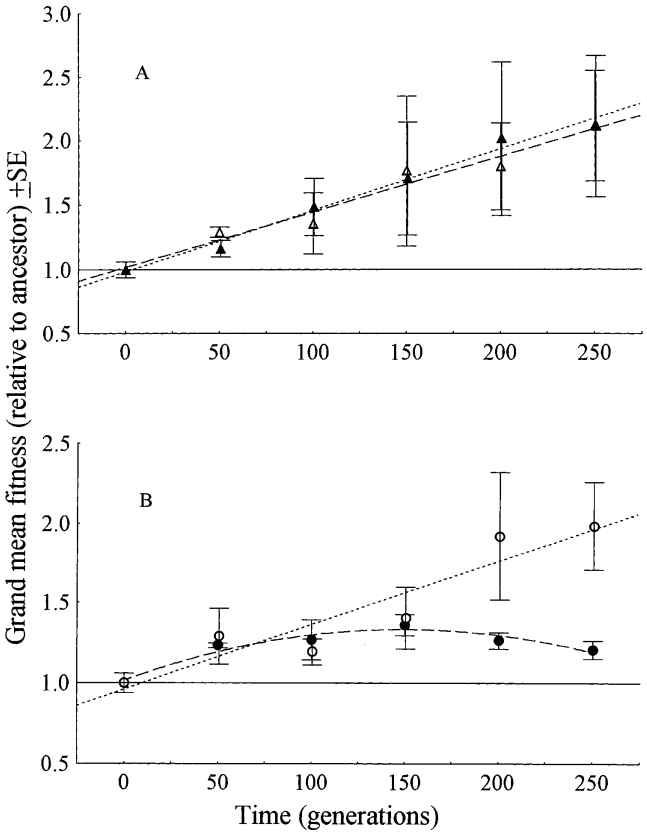

Figure 3.—Fitness improvement under treatment conditions compared to fitness measured through paired-growth assays. (A) Fitness improvement for the asexual populations measured at moi = 0.002 (△), compared to their paired-growth trajectory (▲). Both fitness trajectories are positive and linear, and do not differ statistically (see text). (B) Fitness improvement for the sexual populations measured at moi = 5 (○), and their paired-growth trajectory (●). Data show that fitness improvement in sexual populations is positive and linear only when coinfection is common, conditions similar to those in their evolutionary environment. Each point represents the grand mean (±SE) of three populations, except for the ancestor that is based on 10 assays. See text for statistical analyses.

For completeness, we measured the fitness ($n = 2$) at 50 generation intervals for each population relative to ϕ6*h* in the *unevolved* treatment environment (*i.e.*, moi = 5 or moi = 0.002). The grand mean fitness of the three replicate populations in each treatment group was calculated at each time point. Regression analysis shows that the fitness improvement of asexual phages at moi = 5 was very rapid (slope = 0.0060, $t = 3.931$, d.f. = 4, $P = 0.017$). In fact, their rate of improvement was identical to that shown at moi = 0.002 (*t*-test for parallelism: $T = 1.069$, $t_{0.05[8]} = 2.306$, d.f. = 8, $P > 0.2$) and relative to changes in paired growth ($T = 0.833$, $t_{0.05[8]} = 2.306$, d.f. = 8, $P > 0.4$). At first, it may seem surprising that the A populations do equally well in

TABLE 1

Final mean fitness relative to the ancestor (ϕ6*h*) for each experimental population in three environments

		Treatment environment	
Population	Paired growth	moi = 0.002	moi = 5
A1	1.471	1.778	2.334
A2	1.645	1.581	1.824
A3	3.203	2.965	4.472
S1	1.276	1.366	1.438
S2	1.250	1.615	2.278
S3	1.095	1.197	2.243

Paired-growth and treatment environment values are means, $n = 3$ and 2, respectively.

environments that do and do not allow interactions between competing viruses. However, we emphasize that these fitness results are relative to ϕ6*h*. Because it is unknown whether this ancestral virus had ever experienced an environment similar to our sexual treatment, no prediction can be made regarding its performance. A valid set of predictions *could* be made if derived asexual phages were competed against derived sexual phages in the two evolutionary environments. However, we explore this scenario below and will reserve further comment until that set of data is presented.

A very different result was obtained in S populations. Regression analysis shows that the performance of sexual phages at moi = 0.002 was positive and linear, but not significant (slope = 0.0014, $t = 2.568$, d.f. = 4, $P = 0.062$). This rate of improvement at moi = 0.002 was less rapid than that shown by the sexual phages in their evolved environment at moi = 5 (small-sample two-tailed *t*-test for parallelism with $T = 2.784$, $t_{0.05[8]} = 2.306$, d.f. = 8, $P < 0.05$). Thus, the performance of sexual phages coincided very well with the degree of competitive interactions that occurred between viruses. That is, sexual phages did very well at moi = 5, worse at moi = 0.002 (where intrahost competition is rare, but phages may interact during adsorption), and very poorly in the complete absence of competitive interactions (paired growth). This relative ranking is emphasized in Table 1, where we list the final mean fitness at 250 generations for each experimental population in all environments. A one-way ANOVA confirms that the effect of assay environment on mean fitness is significant for the sexual phages ($MSE = 0.093$, d.f. = 2, $F_s = 5.337$, $P = 0.047$), but not for the asexual phages ($MSE = 1.148$, d.f. = 2, $F_s = 0.516$, $P = 0.621$). These data further suggest that the cost created by intrahost competition was so strong that it masked any advantage of sex in the S populations.

A tradeoff between intrahost competition and host exploitation explains the results shown in Figure 3B. The sexual phages do very well in an environment that allows for coinfection, but do poorly when intrahost interactions are minimized. It is possible to explore this hypothesis further by allowing single genotypes of sexual phages and asexual phages to compete directly in environments where levels of phage interaction differ. A switch to fitness assays involving head-to-head competition between evolved phages is desirable for two reasons. First, all fitness results reported thus far involved mixed populations of evolved viruses. Thus, the observed tradeoff must be a property of the majority of genotypes present, but competitions involving a pure clone of the majority genotype should serve only to magnify the apparent tradeoff. Second, all previous competitions assayed fitness relative to ϕ6*h*. This assumes that fitness is completely transitive in our system. Although most microbial studies show the magnitude of one derived genotype's advantage relative to another can be accurately predicted from each one's advantage relative to the ancestor (*e.g.*, Lenski *et al.* 1991; Travisano *et al.* 1995), nontransitivity and other complex selection dynamics have been demonstrated in some experiments (*e.g.*, Chao and Levin 1981; Paquin and Adams 1983; Turner *et al.* 1996; Souza *et al.* 1997). Thus, fitness assays involving direct competitions between clonal isolates would serve to eliminate any questions regarding nontransitivity in this study.

Tradeoff between intrahost competition and host exploitation in sexual phages: We sought evidence of whether the tradeoff shown by sexual phages would manifest in direct competitions between sexual phages and asexual phages. To explore this question, we randomly chose a single phage clone from one population in each treatment at a time-point where performance at moi = 5 exceeded that for paired growth (Figure 3). ϕS2 is a single clone isolated at 200 generations from population S2, whereas ϕA1 is that from population A1. We obtained a spontaneous host-range mutant of ϕA1, referred to as ϕA1*h*. Paired-growth fitness (±SE) of ϕA1 relative to ϕA1*h* was found to be 1.062 ± 0.070 ($n = 7$); all fitness results reported below are adjusted to account for the 6% fitness cost of the *h* marker. We then competed ϕS2 against ϕA1*h* at moi = 5 ($n = 5$) and at moi = 0.002 ($n = 5$). Results showed that mean fitness of ϕS2 relative to ϕA1*h* was 1.424 (±0.052 SE) at moi = 5, but 0.772 (±0.028 SE) at moi = 0.002. A *t*-test clearly indicates that the fitness of ϕS2 is dependent upon the amount of intrahost competition allowed ($t_s = 11.025$, d.f. = 8, $P < 0.001$). These results provide firm evidence that phages evolved in a sexual environment are selectively favored only when coinfection is common and, hence, the level of intrahost competition is intense.

DISCUSSION

We examined the Fisher-Muller model for the evolutionary maintenance of sex (Fisher 1930; Muller

1932) using a bacteria-phage model system. This theory predicts that adaptation to the environment is more rapid in a sexual population, than in an equally large asexual population. Sex occurs in RNA virus ϕ6 when multiple viruses coinfect the same bacterial cell. The presence or absence of sex in viral populations can be easily manipulated in this system by controlling moi. A single clone of ϕ6 was used to found three S and three A populations. These experimental populations were then propagated on a *P. phaseolicola* host for 250 generations of viral evolution. At the end of the study, we compared the rate of fitness improvement, relative to a common competitor of the ancestral genotype, for populations in the two treatments.

Our study can be summarized by two major results. First, all experimental populations showed a significant increase in fitness relative to a common competitor of the ancestral genotype. However, we found no evidence that sex increased the rate of adaptation in terms of paired growth (competitive fitness in the absence of phage interactions). Rather, sexual populations of viruses adapted at a rate much slower than that of their asexual counterparts and even showed a fitness decline by the end of the study (Figure 2).

To explain our findings, we hypothesized that viral evolution was in response to a key difference between the two treatment environments: the level of intrahost competition experienced by viruses. When a virus is alone in infecting a host cell, its reproduction is strictly asexual, but selection is primarily for a virus that best exploits the host cell. Paired-growth (Chao 1990) measures fitness as the ability for an infecting virus to exploit its host in the absence of interactions with competing viruses (see materials and methods). In contrast, sexual or coinfecting viruses may be selected for host exploitation *and* for within-host competition of limited host resources. In the latter case, adaptation to intrahost competition may detract from the ability of the virus to exploit its host, and this within-host selection may then create a cost for coinfection. Lewontin (1970) suggested that when multiple viral genotypes coinfect the same host cell, strong intrahost competition may lead to the evolution of novel viral traits. Thus, we predicted that fitness trajectories from paired-growth assays should be more representative of fitness dynamics shown by asexual phages in their evolutionary environment than for those shown by sexual phages in their selective environment. To explore this hypothesis, we measured the fitness of populations in their respective treatment environments and compared these results to fitness changes observed in paired-growth assays.

Our second major result is that our observations can be explained by a systematic tradeoff between intrahost competition and host exploitation in sexual phages. That is, the derived sexual viruses are selectively favored only when coinfection and—hence—intrahost interactions are common (Table 1, Figure 3B). This is firm evidence that intense selection to compete for limited host resources lessens the ability of viruses to exploit the host. Although it was previously suggested that within-host competition may lead to the evolution of novel viral traits (Lewontin 1970), to our knowledge the present study is the first empirical evidence for this idea.

Further experiments are needed to elucidate the nature of the tradeoff shown here. In the meantime, we discuss three potential mechanisms that may be involved in the observed tradeoff. These include the evolution of defective viral genotypes that parasitize ordinary viruses, the evolution of genetic conflicts, and the impact of hard and soft selection on viral adaptation. The various mechanisms are not mutually exclusive.

Defective interfering particles: All viruses require living host cells to replicate. Certain viral genotypes are defective because they require helper activity from another virus genome or virus gene(s) to undergo replication (Holland 1991). In fact, defective viruses of this type have long been documented in association with many human and animal viruses (*e.g.*, Henle and Henle 1943; Bellet and Cooper 1959; see Holland 1991 for review). Huang and Baltimore (1970) coined the term "defective interfering (DI) particles" as a name for these viruses that lack essential RNA or DNA and that interfere specifically with helper phage by replicating at their expense. Essentially, DI particles are intracellular parasites that rely on functional proteins synthesized by coinfecting viruses. DI particles also have an intracellular replicative advantage over ordinary phage. For example, when DI and standard particles of RNA poliovirus coinfect the same host cell, the viral progeny is enriched by about 5% for particles containing DI RNAs (Cole and Baltimore 1973). DI particles vary in their mechanism of intrahost advantage. Because they typically contain fewer genes, the smaller size of DI particles may be sufficient for them to gain a replicative advantage (*e.g.*, DI particles of influenza A; Holland 1991). In other cases, DI particles have evolved more elaborate mechanisms to parasitize helper phages. For instance, some DI particles have retained replication initiation sites but have lost transcription initiation sites at the 3′ terminus, allowing a replicative advantage over virus genomes that must engage in transcription (Perrault and Semler 1970; Kailash *et al.* 1983).

The evolution or isolation of DI particles in association with RNA virus ϕ6 has never been documented. However, the derived sexual viruses in this study appear similar to DI particles because they experience a selective advantage only when intrahost competition is allowed (Figure 3B). One explanation is that a sexual environment allows these viruses to interfere, directly or indirectly, with the replication of other coinfecting viral genotypes. The derived sexual phages cannot be DI particles *per se* because they are able to undergo normal asexual reproduction without the aid of helper

viruses (see results). Furthermore, it has been argued that evolution and maintenance of DI particles would require an moi that is orders of magnitude higher than that imposed in our sexual treatment (Chao 1988). Still, the possibility exists that mechanisms similar to those employed by DI particles may allow the sexual phages in this study to gain a selective advantage during intrahost competition.

Genetic conflicts: Sex requires that a genome expose itself to foreign genetic material, creating an opportunity for the evolution of genetic conflicts. Genetic conflicts occur during genetic exchange when a particular gene (or genes) promotes its own spread at the expense of other genes (Werren *et al.* 1988; Hurst *et al.* 1996). Well-described examples include the meiotic drive genes in *Drosophila melanogaster* and in mice (Lyttle 1993; Silver 1993). The advantage gained by DI particles during coinfection provides another example of genetic conflict (Chao 1994). DI particles experience a higher replication or encapsidation rate inside a coinfected cell, but this parasitism has a negative effect on the coinfection group (the group of viruses coinfecting a cell). For example, the total yield of polioviruses produced by an infected cell is inversely proportional to the frequency of DI particles in the coinfection group (Cole and Baltimore 1973). Although genic selection favors DI RNAs, selection at the level of coinfection groups opposes them, much the same way that selection on individuals may prevent deleterious meiotically driven alleles from becoming fixed (Lewontin 1970). We believe that the sexual phages in this study provide yet another example for the evolution of genetic conflicts. These viruses gain an obvious selective advantage during coinfection. As in the case of DI RNAs, there may be an opposing selective force that would stop these viruses from sweeping to fixation. If so, we can predict that the fitness advantage shown by sexual phages during coinfection should be dependent upon their initial frequency in competition and that an equilibrium may be reached, where sexual phages and asexual phages are able to stably coexist. This hypothesis is untested, but provides an intriguing possibility for future study.

Hard and soft selection: A final analogy may be drawn between our study and the concept of adaptation through hard and soft selection. Wallace (1970) defined hard selection as selection resulting from conditions that an organism must meet to function as a breeding individual. As an extreme example, consider a single-locus diploid system with dominant allele *A* and recessive allele *a*. If the homozygous combination *aa* is always lethal, then the failure of *aa* individuals to reproduce has little to do with conditions such as overcrowding in the population. In contrast, soft selection does not involve conditions necessary for reproduction; rather, it involves a fixed number of available positions that can be filled by any viable individual but, in reality, are filled by those individuals most fit to compete for the available positions (Wallace 1970). Thus, *W* can change depending on whether soft selection is at work. In ϕ6, intrahost competition is an environment where soft selection can act. The limited number of available "positions" is the burst size produced as a result of infection ($\sim$200–400 viruses). Whereas two competing viruses may be equally capable of producing progeny through asexual reproduction, the outcome may be very different when the two must compete for limited host resources (and hence, limited positions in the viral progeny). Here, it is the better intrahost competitor that contributes more progeny to the next generation.

Our paired-growth assay to measure fitness does not allow intrahost interactions between competing phage genotypes and it is here that hard selection should play a major role in the competitive outcome. On the other hand, coinfecting viruses must compete for limited host resources, and our sexual treatment is an environment where we would expect soft selection to be important. Thus, another way to visualize the genetic tradeoff apparent in the S populations is in terms of the relative contributions to phage adaptation of hard and soft selection. This concept is clearly illustrated when one compares the grand mean data shown in Figure 3B. For most of the experiment the sexual phages showed equivalent fitness improvement in the presence and absence of phage interactions (sex), indicating that hard and soft selection contributed equally to their total fitness gained. However, it was during the last 50 to 100 generations of viral evolution that the effects of soft selection became paramount in the sexual populations. Presumably, soft selection led to the evolution of viral traits that improve intrahost competition, but these phage adaptations seemed to occur at the expense of other traits molded by hard selection.

Relevance of findings to previous work, and concluding remarks: Several recent studies have empirically tested whether sex leads to an increase in fitness (Birdsell and Wills 1996; Da Silva and Bell 1996; Souza *et al.* 1997; Zeyl and Bell 1997). Souza *et al.* (1997) tested a model similar to that of Fisher-Muller, but using populations of the bacterium *Escherichia coli*. The rate of fitness improvement in these asexual populations had slowed considerably from an initially rapid pace (Lenski *et al.* 1991). The authors sought to determine whether sexual recombination with novel genotypes would reaccelerate the rate of adaptation in these populations. Although sexual recombination yielded dramatic increases in genetic variation, this variability did not translate to more rapid adaptive evolution. This surprising result can be explained by changes in the selective environment brought on by the recombinant genotypes themselves (Turner *et al.* 1996). That is, complex ecological interactions (such as bacterial cross-feeding) among recombinants led to unexpected environmental changes, leaving simple fitness estimates as an inadequate method for evaluating the Fisher-Muller type

model (Souza *et al.* 1997). The same analogy may be drawn for the empirical results in this study. We attempted to test the Fisher-Muller hypothesis by simply comparing rates of fitness improvement in paired-growth assays between the two treatment groups. However, this analysis was complicated by the evolution of a genetic tradeoff in the case of the sexual phages. We note also that the two studies may share an additional similarity. In the bacterial study, recombination led to unexpected *abiotic* changes in the environment that prompted an increase in biodiversity; secondary growth metabolites produced by recombinants allowed coexistence between bacterial competitors (Turner *et al.* 1996). In this study, we observed that sex caused unexpected changes in the *biotic* environment; intrahost competition led to the evolution of novel traits in viruses. As we alluded to above, the fitness advantage of these sexual viruses may be frequency dependent in nature. If so, we can expect that derived sexual and asexual phages would coexist in an environment that allows viral coinfection, leading again to an increase in biodiversity.

In strict terms, our study demonstrates a cost of intrahost competition rather than a cost of sex. However, because sex in all viruses requires coinfection, it cannot exist without the cost of intrahost competition. At the high moi we examined, sex was not advantageous because the cost created by intense intrahost competition was too strong. If an advantage of sex does exist in ϕ6, there must be an optimal moi that characterizes this advantage. Our study begins mapping the effect of moi on this presumed advantage. On the basis of our findings, we expect that the upper bound for the optimum is moi < 5. At low moi's, a viral population is asexual and would suffer whatever detriment results from not having sex; this indicates a lower bound of moi ≥ 2. Future experiments will be used to determine the optimal moi that leads to an advantage of sex in ϕ6.

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