A Virus Booster for Game Theory

Experiments with a selfish genotype of RNA bacteriophage ϕ6—a parasite of a parasite—provide evidence for prisoner's dilemma

Paul E. Turner

Name me somebody that is not a parasite, and I'll go out and say a prayer for him.

Bob Dylan, 1966

Parasites travel seemingly hazardous paths, relying on other organisms to complete their life cycles. Then again, all organisms depend ecologically on other species for survival, however tangentially. An imposing stand of giant redwoods, for example, very much depends on neighboring microbial species to rid the forest floor of debris. Although species differ in their degree of interconnectedness with other organisms, parasites appear to have forged the most intimate of those relationships.

Viruses exist at an extreme of this continuum of interconnectedness: they are obligate parasites that depend on host cells to reproduce and to furnish them with metabolic activity. Although viruses replicate, mutate, and adapt to their environment in ways similar to other biological entities, their extreme reliance on host cells challenges how we define life. Should we pity the viruses? I think not. Despite their overwhelming dependence on other organisms, viruses factor significantly in natural ecosystems, where their abundance often exceeds that of available hosts.

This teeming multitude creates opportunities for mixed infections, where multiple viral genotypes or species ecologically interact within varied hosts. What are the consequences for virus evolution? Mixed infections can profoundly affect the fitness, or reproductive success, of an individual virus. Differing viruses within a mix compete with one another for limited intracellular resources, and one or a few of them may stimulate generalized immune responses that combat some or all the viral types within the mix. Counterbalancing these disadvantages, viruses within such mixes may share beneficial genes through recombination, and they may also enhance one another's pathogenic effects by weakening the host. Thus, coinfections can positively and negatively influence viral pathogenesis, transmission success, and exchanges of genetic information.

Despite abundant clinical data and models demonstrating the importance of virus interactions, relatively few empirical studies address the evolutionary ecology of mixed infections in viruses. Examining virus coinfections in controlled experiments helps to bridge this intellectual gap.

Virus Interactions as Sociology

Certain terms used in sociology can be applied to describe viral interactions. During reproduction, for example, viral gene products diffuse within a host cell, meaning no individual virus has "exclusive access" to its own products. This sharing of wares creates a conflict of interest, leading either to cooperation or defection (selfishness) strategies. A virus that induces the host cell to make excessive amounts of a shared product can be defined as a cooperator because its actions benefit other coinfecting genotypes. In contrast, a virus that induces cells to synthesize less, but appropriates a larger share, of specialized products can be called a defector.

Defection is epitomized by emergence of defective-interfering (DI) particles—viruses that are "defective" because they lack essential genes and thus rely on functional proteins synthesized at the behest of cooperator (helper) viruses (Fig. 1). Full-length viruses may also depend on other viruses during a particular stage of their infectious cycles. For example, umbraviruses can

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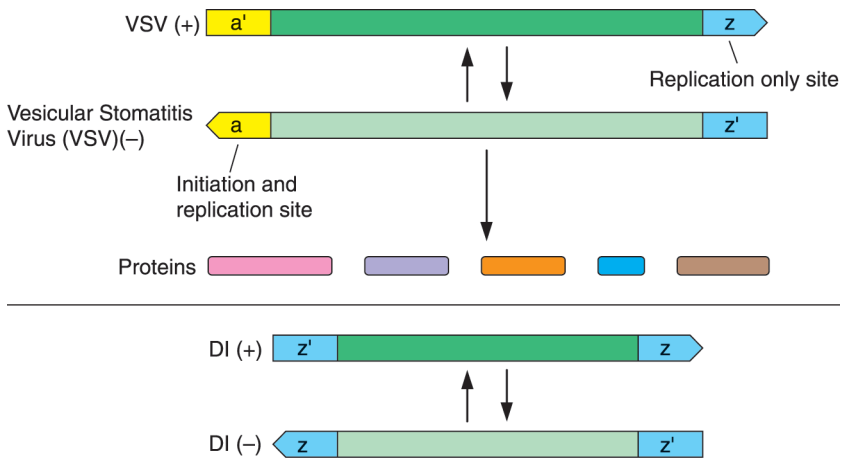
FIGURE 1



The interaction between vesicular stomatitis virus (VSV) and a DI particle provides an example of virus selfishness. VSV is a single-stranded negative-sense RNA virus. When VSV enters the cell it transcribes mRNA to make proteins. However, VSV also replicates by serving as the template for the complementary positive-strand genome, VSV(+), which is used to make more VSV(-). The 3′ terminus of VSV(-) contains an initiation site (**a**) for both transcription and replication, whereas the 3′ terminus of VSV(+) has a site (**z**) for only replication. The complementary sequences of **a** and **z** on the opposite strands are **a′** and **z′**. Also depicted is one form of a DI particle of VSV that is shorter and lacks any protein-coding sequences. More importantly, it has a **z** replication site at the 3′ terminus of both its negative and positive strands. Whereas VSV(-) allocates time for transcription, DI(-) does not and is effectively equal to DI(+). Thus, the DI RNA relies on the complete virus to provide proteins, but it has a higher replicative fitness than the VSV genome. (Adapted from Chao et al., Q. Rev. Biol. **75**:261–275, 2000.)

spread from cell to cell within the tissues of a host plant, but these viruses count on mixed infections with luteoviruses to provide protective capsid proteins required for their transmission via aphid vectors between plants.

Virus interactions such as those between DI particles and their helpers can be described using complex mathematics. However, much simpler models can be equally illuminating, especially a group of mathematical models known collectively as game theory.

Virus Interactions as Game Theory

Game theory describes interactions between individuals that use conflicting strategies, and the goal is to use mathematics to describe the rules of the game and to determine which strategy will prevail. Evolutionary game theory, popularized by John Maynard Smith of the University of Sussex, England, holds that if all members of a population adopt the ideal fitness strategy within a niche, no mutant using an alternative strategy can take over the niche. However, if neither strategy can entirely displace the other, both strategies will coexist in a polymorphism.

A 2×2 payoff matrix is a convenient way to summarize a contest between cooperators and defectors (Fig. 2). The general model holds that if both players cooperate, they are given a reward, $R = 1$, which is larger than the punishment if both defect, $P = (1 - c)$. When a defector and cooperator interact, the defector benefits from the temptation to cheat, $T = (1 + s_2)$, whereas the cooperator suffers the sucker's payoff, $S = (1 - s_1)$.

The key to evolutionary game theory is that an individual's fitness payoff as a cooperator or defector can change, depending on how many players in the population are using a particular strategy and the rate at which that individual encounters them. That is, fitness can be frequency-dependent. DI particles provide a good illustration. Imagine a population of viruses composed entirely of cooperator (helper) genotypes, replicating in an environment where mixed infections are common. If a mutant DI particle appears in the population, it has a very large fitness advantage ($T \gg 1$) because it is surrounded by cooperators that provide essential proteins.

Selfish DI particles will thus become increasingly common in the population. However, as the selfish genotypes increase in frequency, their fitness will decline because fewer cooperators are present. In fact, if DI particles entirely take over the population, their fitness, and hence that of the population, falls to zero ($P = 0$) because these genotypes cannot reproduce on their own. For this reason, the only stable strategy for a DI particle is to have a genetic polymorphism involving a helper virus, where $T > R > S > P$ (Fig. 2). Stable associations between ordinary and helper-dependent viruses exist in natural populations, and the phenomenon is particularly well described among plant viruses.

A famously intriguing result from game theory is the prisoner's dilemma, where the single unbeatable strategy is to defect, even though both players would be better off if they cooperated. For a population to be composed entirely

Studying the Basics of Viral Evolution, Mentoring, and All That Jazz

Paul Turner grew up just after the most explosive period of the civil rights era. But his father, a Presbyterian minister who marched on Washington with the Rev. Martin Luther King Jr., never allowed his three children to forget what came before them. "My father has a very strong social conscience," Turner says. "He and my mother both have roots in the South. He wanted to make sure we didn't lose sight of the times where there were very deep racial divisions. To be sure, there still are— but when we were growing up, much of the good fight had been fought."

Turner's was the only African-American family in his suburban Syracuse, N.Y., neighborhood, and most of the other black students attending classes with him were bused in from the city. "I definitely felt isolated at times," he recalls. "Fortunately, I had my brother and sister."

His father and mother, an elementary school teacher, stressed the importance of an education. "If you are educated, it opens doors for you," he recalls them telling him. Even today, "I'm in a field right now were there aren't a lot of black faces," he says. "I often go to scientific meetings where mine is the only African-American face there. You'd have to be incredibly naive to think you don't stand out."

That continuing sense of relative isolation helps to account for why Turner, an assistant professor of ecology and evolutionary biology at Yale University, devotes energy to supporting and creating scientific educational opportunities for young minority students. Recently, for example, he has served as a mentor for underrepresented minorities in science at Yale in its Science, Technology and Research Scholars (STARS) Program, which sponsors laboratory research experience for women, blacks, and other minorities who are studying science. And this year Turner was named one of the top 10 emerging scholars of color by *Black Issues in Higher Education* magazine (see *ASM News*, April 2003, p. 200).

As a postdoc in 1998, Turner participated in a program at Arizona State University, where he met with African-American, Native American, and Hispanic science undergraduates to share his experiences as a researcher in evolutionary biology. In 1995, he participated in a workshop sponsored by the American Association for the Advancement of Science organized to address the public's response to such publications as *The Bell Curve*, which "misrepresent scientific data in order to associate human intelligence with racial categories," he says.

As a graduate student, during the summers of 1990 and 1991, Turner worked as a lab coordinator and instructor for the Howard Hughes Medical Institute's summer science academy research program at the University of Cali-

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of defectors would be surprising because of the irrationality of selfish behavior and the inherently low fitness payoff that results when all individuals cheat. However, prisoner's dilemma can easily occur if the punishment of cheating is a fitness value greater than the sucker's payoff (cost of cooperating). That is, $T > R > P > S$, allowing cooperators to be driven from the population.

This result is paradoxical according to the tenets of Darwinism, which emphasize "survival of the fittest," because ancestral cooperators are replaced by evolved defectors that lower the overall fitness of the population. Although prisoner's dilemma is relatively easy to demonstrate mathematically, it is notoriously difficult to prove in biological systems because it requires measuring the fitness of pair-wise interactions between two slightly different organisms, or players, for which the outcome is extinction for one of them. However, our recent experiments with bacteriophage ϕ6 show that prisoner's dilemma does apply to viral populations.

Mixed Infections in Bacteriophage ϕ6

The RNA bacteriophage (phage) ϕ6 has been cloned, sequenced, and extensively studied. A ϕ6 particle contains roughly 13.3 kb of double-stranded RNA divided into three segments. Phage ϕ6 is a lytic virus in the family *Cystoviridae* and features a typical life cycle of infection, replication, and burst. Its natural bacterial host

fornia at Irvine. The program provided for more than 100 socioeconomically disadvantaged high school students to live on campus for a month and to experience computer, classroom, and lab training in several areas of biology. The goal was to better prepare them for their first year of undergraduate work.

"First, I believe my responsibility is to lead by example," Turner says, explaining why he tries to reach out to black youth. "If I can be perceived as a role model, it shows younger people that they can achieve the same things—that it is possible for an African-American to achieve these things." He adds "But I've also been fortunate enough to be able to go the extra mile and mentor. A lot of underrepresented kids are more comfortable being mentored by someone with the same background."

Turner, 36, was born in Philadelphia, and moved to Oakland, Calif., when he was about 4, and by third grade moved back east with his family to Syracuse where Turner remained through high school, before heading to the University of Rochester to study biomedical engineering. "I found the electives I was taking in biology much more interesting than the engineering courses, so I switched my major," he recalls. "I was much happier."

To cement his burgeoning interest in evolutionary biology research, he spent half a year before graduate school working as an intern at the National Audubon Society in Maine, and later obtained his doctorate in microbial ecology and evolution at Michigan State University in East Lansing. Turner's research focuses on virus ecology and evolution, host-parasite interactions, and the exchange of genetic information between viruses.

"I'm interested in the nuts and bolts of what allows viruses to be more successful—to better understand the basic biology of viruses and evolution," he says. "My work doesn't focus on combating viruses, or viral diseases per se. What has gotten little attention is the fact that viruses feature the ability to interact with one another, with repercussions for their evolution. We often don't focus on this when studying viruses. Mostly, when studying viral diseases, we look at a virulent strain in isolation in the laboratory. We just look at its genetic makeup and how it differs from viruses out there—which takes it out of the context of the particular ecology from which it evolved."

When Turner is not consumed with the mechanisms of how viruses evolve, he collects musical recordings, favoring the jazz of John Coltrane and Dizzy Gillespie. He boasts of his extensive collection of "vinyl" (long-playing records), in addition to CDs. He is married to another Yale researcher, marine biologist Mary Beth Decker. They have a two-year-old daughter, Claire, and a newborn daughter, Sadie.

Marlene Cimons
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is unknown, but $\phi 6$ readily grows within the plant pathogen *Pseudomonas phaseolicola*.

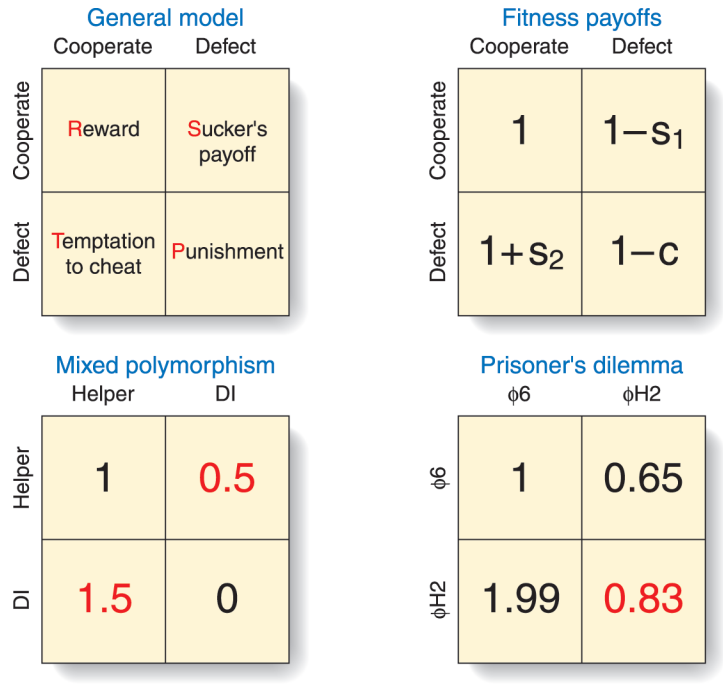
Phage $\phi 6$ offers a powerful means for studying the evolutionary consequences of mixed virus infections. As with any RNA virus, $\phi 6$ features an extremely high rate of spontaneous mutations (on the order of 10^{-3} to 10^{-5} errors per nucleotide replication). Coupled with its short generation time (about five generations per day), this high mutation rate allows investigators to propagate the virus for hundreds of generations and to study adaptive processes in detail. More importantly, coinfection in $\phi 6$ may be easily manipulated by controlling the multiplicity of infection (MOI), or ratio of viruses to bacterial cells.

Lin Chao of University of California, San Diego, and I recently examined competitive interactions among phage $\phi 6$ variants. To do so, we divided a single clone of wild-type $\phi 6$ into three high-multiplicity (MOI = 5) and three low multiplicity (MOI = 0.002) populations, and allowed each of them to infect *P. phaseolicola* over many replication cycles. When the MOI = 5, coinfection is common and 97% of cells should experience multiple infections, whereas only 0.1% of infected cells contain two or more viruses when MOI = 0.002.

Populations in the two treatment groups were propagated for 50 consecutive days, which is equivalent to roughly 250 generations of viral evolution. Population samples (stored in the

freezer) were then competed against the ancestor to measure changes in fitness, in terms of their growth on *P. phaseolicola*. The phage cultured at high multiplicity gained an added advantage only during coinfection, suggesting that these viruses are defectors. However, because the viruses retain an ability to infect cells alone, they are not simply selfish DI particles.

FIGURE 2



Payoff matrices for a contest between cooperators and defectors; payoff is to an individual on the left, when it encounters the above opponent. The general model (upper left) verbally describes the outcome (abbreviated in red) of each pair-wise interaction, whereas the fitness payoffs (upper right) are the numerical unknowns. The lower left shows a hypothetical matrix for a contest between DI particles and helper viruses; $s_1 = s_2 = 0.5$ in this example. DI particles gain a replicative advantage over helper viruses, but the DI's cannot exist on their own, $(1 - c) = 0$. Therefore, the only long-term strategy for a DI is a mixed polymorphism (highlighted in red) involving a helper virus. The lower right shows the realized matrix for a contest between the ancestral cooperator (ϕ6), and an evolved full-length selfish virus (ϕH2). Because the defector replaces the cooperator while simultaneously lowering the mean fitness of the population (highlighted in red), these data are consistent with the prisoner's dilemma. (Data from P. Turner and L. Chao, Nature **398**:441–443, 1999.)

Evidence for Prisoner's Dilemma

The emergence of mutant phage ϕ6 defectors in our experiments provides us an opportunity to examine whether these viruses are caught in a prisoner's dilemma. We expect such variant viruses to meet two conditions. First, the fitness of the defectors relative to the ancestral cooperator must be frequency dependent because the general model predicts that defectors gain their greatest fitness advantage when rare (Fig. 2). Second, defectors should completely supplant the ancestral cooperator, resulting in reduced mean fitness of the population. In collaboration with Lin Chao, I conducted experiments to address these two hypotheses.

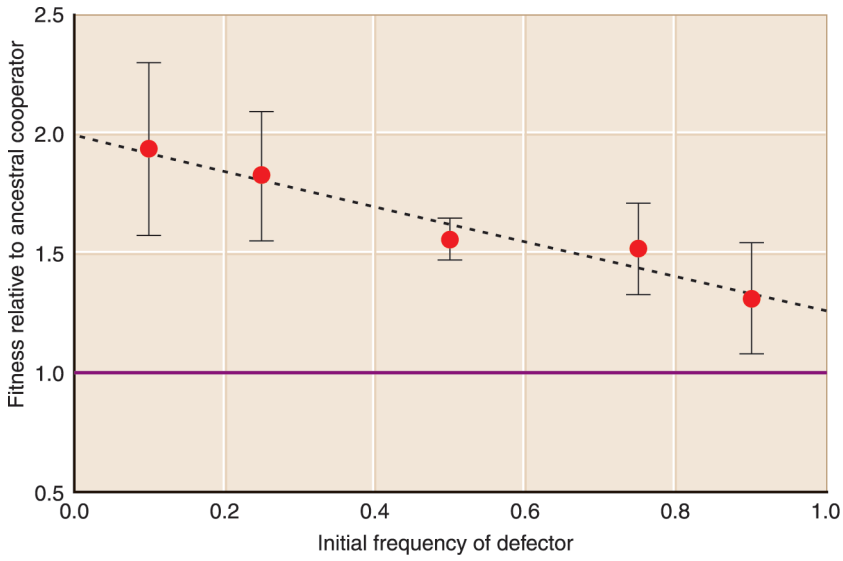
A selfish clone was isolated from one of the high-multiplicity populations at generation 200. We then measured the fitness of this virus relative to the ancestral cooperator at different initial frequencies in competitive environments containing frequent coinfections. Results show that the fitness of the defector decreases as its initial frequency increases in competition, verifying the first prediction (Fig. 3). When the defectors are rare, they mostly coinfect cells with cooperators and gain a large fitness advantage, whereas when common, they coinfect cells with other defectors and are less advantaged.

These data also support the second prediction. Fitness of the ancestral (unevolved) cooperator is by definition equal to 1.0. Because the observed fitness of the defector exceeded 1.0 at each initial frequency, we predict that the evolved defector will always displace the ancestral cooperator. Furthermore, we used these data to estimate the temptation to cheat, $T = (1 + s_2)$. According to the general model (Fig. 2), fitness of defectors relative to cooperators is T/R when defectors are very rare in the population. Thus, the left *y*-intercept of the regression line corresponds to $T/R = (1 + s_2)/1$, or $s_2 = 0.99$ (Fig. 3).

By the same logic, the right *y*-intercept of the regression line is $P/S = (1 - c)/(1 - s_1)$. Because the intercept of 1.28 exceeds 1.0, $P > S$ as required by the prisoner's dilemma. But the ratio is not easily solved because it presents two unknowns; thus, additional experiments are needed to estimate *P* or *S*.

The above experiments measure fitness when different viral competitors simultaneously coinfect cells. We then modified these assays to mea-

FIGURE 3



The fitness of an evolved defector (ϕH2) relative to the ancestral cooperator (ϕ6) is frequency-dependent, when the phages are competed at MOI = 5 on *P. phaseolicola*. The dashed line indicates the best linear fit to the data (slope = −0.738, P = 0.0039, d.f. = 3). Standard errors are based upon *n*-1 = 3 degrees of freedom. (Data from P. Turner and L. Chao, Nature **398**:441–443, 1999.)

sure $P = (1 - c)$, the punishment that results when only defectors coinfect cells together. We did this by mixing cooperators and defectors with host cells in separate tubes, and then compared their growth rates on *P. phaseolicola*. We found that fitness of the defectors is less than 1.0 when cooperators are absent within the cell (Fig. 4), mimicking the situation when evolved defectors take over the population. By solving for $P = (1 - c) = 0.83$, we could estimate the other unknown parameter *S* and complete the payoff matrix (Fig. 2).

The evolution of prisoner's dilemma in our experiments likely occurs as follows. In the high-multiplicity populations, mutant defectors spontaneously appear and experience a large fitness advantage owing to their ability to sequester limited within-host products for replication. This competitive advantage allows the defectors to increase in number even though initially they are rare. As the defectors become more numerous, the cost of defection increases considerably because they encounter fewer cooperators per coinfection event. However, rare cooperators interacting with the defectors show the lowest fitness in the system. For this reason, defectors sweep through the population, despite a decrease in population fitness from an initial value of 1.0 to a value of $(1 - c)$.

Conclusions and Future Work

Our laboratory experiments provide the first empirical evidence for prisoner's dilemma in a biological system. But is it likely that phage ϕ6 can become trapped in a prisoner's dilemma in nature? Very little is known of the natural biology of ϕ6 and its recently discovered relatives in the family *Cystoviridae*, but the answer lies in the population structure of these viruses in the wild. If coinfection is extremely common, it is possible for mutant defectors or DI particles to appear and experience a competitive advantage. Sampling wild ϕ6 populations in one or more locales would provide information on the extent of mixed infections occurring in nature, and whether within-host competition or other ecological interactions among ϕ6 particles provide an impetus for natural selection.

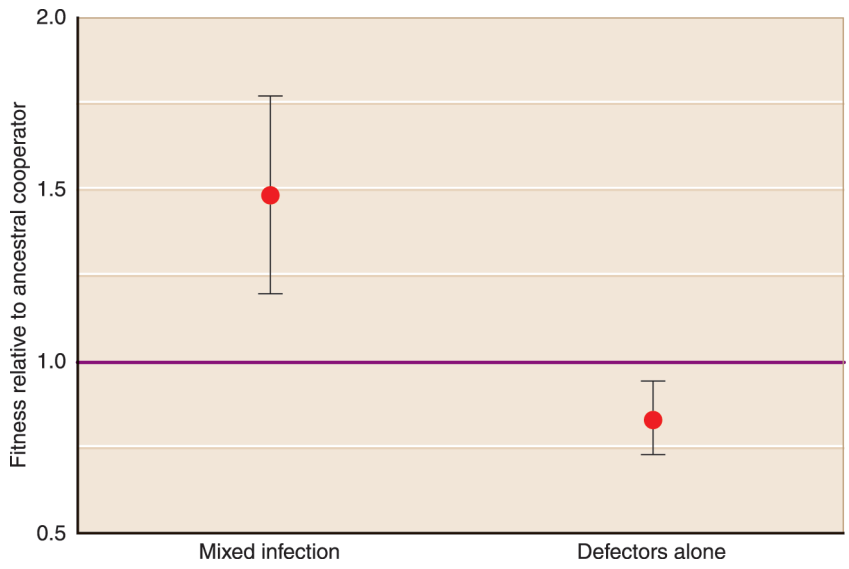
Although rates of coinfection for ϕ6 in the wild are unknown, the virus features an exclusion mechanism whereby only three viruses on average can infect a single cell, even though up to 50 viruses can attach to a cell. Therefore, our experiments where ϕ6 evolved at MOI = 5 occurred at the upper limit for virus entry. It is conceivable that such a high MOI creates an ideal condition for the evolution of selfish genotypes and that moderate levels of coinfection would be less favorable for their appearance. Further experiments are needed to address this possibility.

The mere existence of an exclusion mechanism in ϕ6 argues that this behavior reduces the level of within-host competition, suggesting that coinfection occurs readily enough in natural populations to be a target of selection. However, other explanations may account for the exclusion mechanism, such as structural limitations of the host cell in supporting entry of large numbers of infecting viruses. Thus, the importance of virus-cell interactions in the evolution of ϕ6 provides possibilities for future work.

The mechanism for selfishness in phage ϕ6 is unknown, but studies on other viruses suggest

several possibilities. One is preferential encapsidation. The ϕ6 genome is divided into three dsRNA segments. According to Leonard Mindich at the Public Health Research Institute in Newark, N.J., and his colleagues, each segment contains a *pac* (packaging) region, which is involved in the ordered entry of segments into viral procapsids during intracellular reproduction. In theory, selfishness could evolve if a selfish gene biased the entry of its resident segment into available capsids, a process analogous to meiotic drive in eukaryotes. A selfish gene could be as simple as a duplicated *pac* region if it allows one or more segments of a selfish virus to be preferentially packaged in the capsids produced by a cooperator. In turn, the added genetic material resulting from the duplication could explain why the selfish virus replicates more slowly when infecting the cell on its own. Certain DI particles can gain a replicative advantage through gene duplications involved in encapsidation.

FIGURE 4



When evolved defectors (ϕH2) co-infect cells on their own, their fitness relative to the ancestral cooperator (ϕ6) decreases below 1.0. Viruses were competed 1:1 at MOI = 5 on *P. phaseolicola*, but allowed to adsorb to cells separately. The difference between the fitness outcomes is statistically significant ($t = 5.056$, $P < 0.0001$, d.f. = 18). Standard errors are based upon $n - 1 = 9$ degrees of freedom. (Data from P. Turner and L. Chao, Nature **398:**441–443, 1999.)

For such seemingly simple entities without any metabolic capabilities, viruses can develop amazingly complex mechanisms of interaction. Selfish viruses are essentially parasites of parasites, but this does not appear to jeopardize their opportunities for evolutionary success in natural communities. It's a pity that it is difficult to readily observe the intimate relationships forged between selfish viruses and their helpers, and to interpret the importance of these associations in virus evolution and pathogenesis. Undoubtedly there are cryptic virus interactions yet to be discovered.

ACKNOWLEDGMENTS

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