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Bacteriophage Migration via Nematode Vectors: Host-Parasite-Consumer Interactions in Laboratory Microcosms

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Pathogens vectored by nematodes pose serious agricultural, economic, and health threats; however, little is known of the ecological and evolutionary aspects of pathogen transmission by nematodes. Here we describe a novel model system with two trophic levels, bacteriophages and nematodes, each of which competes for bacteria. We demonstrate for the first time that nematodes are capable of transmitting phages between spatially distinct patches of bacteria. This model system has considerable advantages, including the ease of maintenance and manipulation at the laboratory bench, the ability to observe many generations in short periods, and the capacity to freeze evolved strains for later comparison to their ancestors. More generally, experimental studies of complex multispecies interactions, host-pathogen coevolution, disease dynamics, and the evolution of virulence may benefit from this model system because current models (e.g., chickens, mosquitoes, and malaria parasites) are costly to maintain, are difficult to manipulate, and require considerable space. Our initial explorations centered on independently assessing the impacts of nematode, bacterium, and phage population densities on virus migration between host patches. Our results indicated that virus transmission increases with worm density and host bacterial abundance; however, transmission decreases with initial phage abundance, perhaps because viruses eliminate available hosts before migration can occur. We discuss the microbial growth dynamics that underlie these results, suggest mechanistic explanations for nematode transmission of phages, and propose intriguing possibilities for future research.

It is increasingly recognized that ecology and evolution factor heavily in epidemiology (e.g., that of cholera and avian flu) (10, 11, 34), but traditional disease models often do not capture the complexity of ecological interactions or coevolutionary responses. In this study, we establish the tractability of a three-species model ecosystem for the study of a rapidly evolving vectored pathogen. Spatially discrete bacterial populations act as resources for nematodes and as hosts for bacteriophages. The nematode acts as a vector for the phage, facilitating the spread of its own competitor.

In natural and agricultural systems, nematode vectors can foster the migration of viruses between individual host plants. DNA virus examples include pea early-browning virus (41, 42) and tobacco rattle virus (19, 20); these tobraviruses are generally ingested by worms foraging on infected root cells, can reside in the worm pharyngeal lumen, and are either posteriorly released or evolved for anterior release (transmitted to a new host), depending on the nematode species (18, 20). Grapevine fanleaf virus (family *Comoviridae*) is a segmented RNA virus that is similarly carried by a nematode vector, and it causes the most severe viral disease of grapevines worldwide, reducing fruit yields up to 80% (1, 43).

Nematode-vectored pathogens can also directly impact human health; commercially available fruits and vegetables can be contaminated by opportunistic pathogenic bacteria transmitted by nematodes, including *Salmonella* spp., *Listeria* spp., and *Escherichia coli* (3, 4, 21). Strikingly, the pig lungworm nematode has been implicated in directly transmitting the virus responsible for the 1918 influenza pandemic (30–33), but the potential role of nematode vectors in future flu or other viral disease epidemics is seldom discussed.

Despite the importance of interactions between viruses and their nematode vectors, little research has explored the ecological or evolutionary dynamics of nematode-assisted virus transmission and the ensuing effects on final hosts. This shortcoming is perhaps due to the lack of a tractable model system. Current models of the ecology and evolution of vector-borne diseases include chickens and mosquitoes to study avian malaria (28) and three-spined sticklebacks and bird vectors to examine cestode parasites of fish (27). However, such systems may be limited by constraints of laboratory space and monetary costs of feeding and caring for macroorganisms. In contrast, strictly microbial models offer the benefits of inexpensive culturing, large population sizes, and rapid generation times. Perhaps their greatest strength is indefinite freezer storage, allowing direct comparisons between ancestral genotypes and derived descendants (23, 38, 39, 40). For these reasons, microbial models have greatly contributed to our general understanding of ecology, evolution, and molecular biology (6, 9). A similarly tractable petri dish-sized model for nematode-vectored viruses would be highly valuable for studying complex multispecies interactions, host-pathogen coevolution, disease dynamics, and the evolution of virulence.

Although nematode transmission of viruses between macroorganisms is well established (14, 15, 36), no study has shown that they can similarly foster the migration of bacteriophages between bacterial populations. The notion that nematodes are likely to act as vectors to transfer phages stems directly from their natural biology. Many free-living nematodes are readily

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TABLE 1. Input numbers of microbes used in phage migration experiments

Expt	No. of nematodes (worms)	No. of bacteria (cells)	No. of phage (plaque forming units)	No. of replicates
Nematode density assays	0	2.35×10^8	82	10
	300	2.35×10^8	82	10
	900	2.35×10^8	82	10
	2,700	2.35×10^8	82	10
Host density assays	2,000	2.5×10^8	78	5
	2,000	5.0×10^8	78	5
	2,000	2.5×10^9	78	5
Phage density assays	2,000	2.6×10^8	97	8
	2,000	2.6×10^8	970	8
	2,000	2.6×10^8	9,700	8
	2,000	2.6×10^8	97,000	8
	2,000	2.6×10^8	970,000	8
	2,000	2.6×10^8	9,700,000	8

found alongside their bacterial prey in terrestrial and aquatic environments. Here, phages can outnumber bacteria up to 10-fold (16), providing ample opportunity for worms to mechanically transfer phages between bacterial populations as they forage. It is conceivable that certain phages may even feature traits promoting nematode transmission (e.g., similar to tobravirus specificity for worm pharynxes) because worms are mobile and exhibit chemotaxis to bacteria.

Here we show that *Caenorhabditis elegans* nematodes are able to transmit phage from infected to uninfected bacterial populations, demonstrating a tractable model for studying pathogen-host-vector biology. We employed the segmented RNA phage ϕ6 and *Pseudomonas syringae* pv. *phaseolicola*, a powerful and increasingly popular model system for addressing ecological and evolutionary hypotheses (2, 7, 8, 39, 40). These experiments took place in the ecological arena of a single petri dish, a simple laboratory microcosm. We show that virus migration between spatially discrete bacterial populations increases with both worm density and bacterial abundance but is negatively correlated with initial phage inocula. We discuss the microbial population dynamics underlying these results, suggest mechanistic explanations for nematode transmission of phages, and propose future applications for this tractable model.

MATERIALS AND METHODS

Study organisms. Phage ϕ6 (ATCC no. 21781-B1) is a lipid-coated, double-stranded RNA virus of the family *Cystoviridae* (13, 25, 26). The virus causes lytic infection in plant pathogenic *Pseudomonas* spp. In the laboratory, the virus is typically grown on *P. syringae* pv. *phaseolicola* (ATCC 21781), which causes halo blight of beans. *C. elegans* var. Bristol (N2; obtained from the *Caenorhabditis* Genetics Center) is a 1-mm-long, free-living, soil-dwelling nematode common to the temperate regions of the world. *C. elegans* is a self-fertilizing hermaphrodite that feeds, somewhat indiscriminately, on bacteria.

Culture conditions and media. Phage and bacteria were grown, plated, incubated, and diluted at 25°C in LC medium, a modification of Luria broth, using published methods (8). All bacterial cultures were grown from a single colony placed in 10 ml LC medium with shaking (120 rpm). After 24 h, *P. syringae* pv. *phaseolicola* cultures attained stationary-phase density ($\sim 4 \times 10^9$ cells/ml). Bacterial stocks were stored in a 4:6 glycerol-LC medium (vol/vol) solution at −80°C.

Phage lysates were prepared by plating plaque-purified phage with 3 ml top agar and 200 μl *P. syringae* pv. *phaseolicola* culture. After 24 h, plaques in the top agar were resuspended in 4 ml of LC broth and centrifuged at 3,000 rpm for 10 min. The supernatant containing the phage lysate was filtered (0.22-μm Durapore filter; Millipore, Bedford, Mass.) to remove bacteria. Phage lysates were stored at −20°C in a 4:6 glycerol-LC medium (vol/vol) solution.

C. elegans worms were maintained at 20°C on agar plates inoculated with 200 μl *P. syringae* pv. *phaseolicola* in LC medium, following published methods (17, 29, 44). Plates seeded with immature *C. elegans* were incubated for 4 to 5 days until sufficient numbers of adults were present for experiments.

Experimental protocols. Nematode, bacterial, and phage densities were independently manipulated to examine the influence of each organism on phage migration. Common features of these experiments are described below, whereas specific details are given in Table 1. For all experiments, phage-infected and phage-free bacterial solutions were made from overnight bacterial cultures and purified phage lysates. Petri dishes (100 × 15 mm) containing LC agar were seeded with infected and uninfected bacteria at diametrically opposite locations on the agar surface, roughly 8 cm apart (Fig. 1). Nematodes were obtained from 5-day-old plates containing adult populations. M9 buffer (29) was used to wash nematodes into a 15-ml centrifuge tube. Subsequently, the nematodes were cleaned by sucrose flotation (see the Comprehensive Protocols Collection at http://cobweb.dartmouth.edu/~ambros/index.html). Cleaned nematodes were placed on the agar surface between bacterial patches (Fig. 1), followed by incubation in the dark at 20°C (Table 1). After 48 h, initially phage-free patches were removed by use of a plug tool (sterilized metal 18- by 150-mm test tube cap) and placed into a centrifuge tube containing 4 ml LC; the plug tool was flame sterilized between samples. After 30 s of vortex mixing, lysates were prepared as described above. Titers of phage lysates were determined on *P. syringae* pv. *phaseolicola* cells to quantify transferred phage abundance.

In all experiments, the response variable was the phage titer in the initially uninfected bacterial population. This number resulted from migration of phage particles to the host patch and their subsequent reproduction within the patch. Throughout this paper, we will refer to this viral abundance as a transmission index. Because of the difficulties involved, we made no effort to separate the processes of migration and population growth, which can be confounded by time.

RESULTS

Nematode density and phage migration. A crucial test for the impact of nematode density on phage migration is to determine whether worm presence in the environment is even necessary for phage spread between spatially separated bacterial populations. Our replicated ($n = 10$) controls showed that in the absence of worms, a 48-h incubation was insufficient for phage to spread from a productively infected population of hosts to an uninfected population roughly 8 cm away (Fig. 1). These data strongly suggest that the phage is incapable of aerosol transmission between distinct host patches, even over very short distances.

We then tested whether phage migration increased with the input density of nematode vectors. These data were analyzed

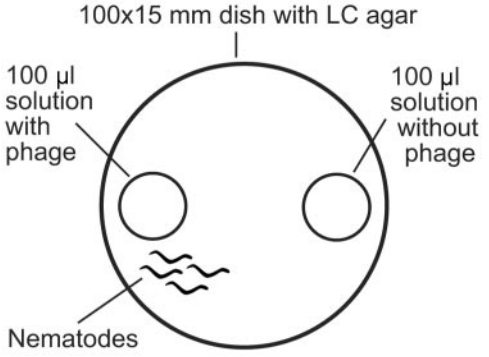

FIG. 1. Basic experimental protocol for phage-bacterium-nematode ecosystem on agar. Phage-free and phage-containing patches of bacteria are placed on opposite sides of a petri dish. Nematodes are placed on the plate and allowed to forage on bacteria. Due to nematode movement, phage are transferred from phage-containing patches to phage-free patches.

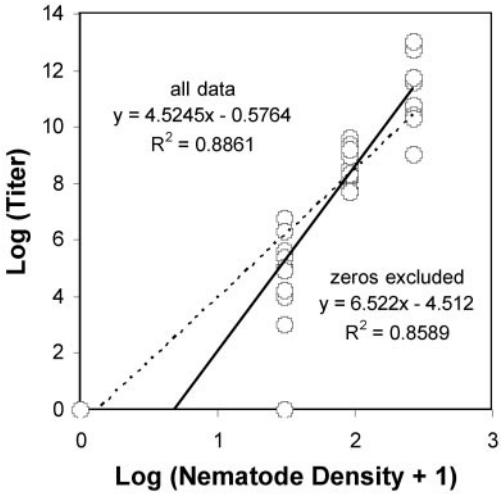

FIG. 2. Regression lines for log (solid line) and log + 1 (broken line) transforms of phage titers in initially phage-free patches as a function of nematode density. The solid line excludes zeros from analysis.

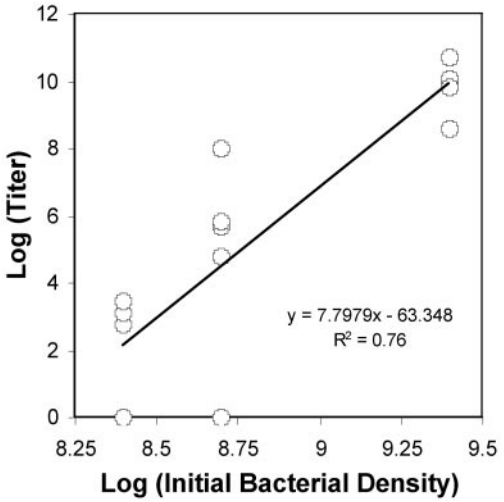

FIG. 3. Regression line of log phage titers in initially phage-free patches as a function of initial bacterial density.

as a replicated regression (5) of log-transformed titers as a function of the log of nematode density. The log of phage titer recovered from the initially uninfected host population increased linearly as a function of log + 1 nematode density. The slope and intercept of the relationship were highly sensitive to our treatment of zeros in the data set. We present regressions of log + 1 values (which include the zero data [$R^2 = 0.89$; $F_{1,38} = 296$; $P < 0.0001$]) and pure log transformations ($R^2 = 0.86$; $F_{1,27} = 164$; $P < 0.0001$) of phage titers in Fig. 2, which shows the regression parameters. This experiment demonstrates unequivocally that nematodes can be vectors for phage ϕ6 and that phage vectoring is a function of nematode density.

Host density and phage migration. A key aim of the current study was to establish the best conditions for future experiments on the ecology and evolution of nematode-vectored viruses; therefore, we explored whether manipulation of the host density in the infected patch impacted virus transmission between patches. Our data supported the expectation that greater densities of the local host population would lead to increased titers of vectored phage. These data were analyzed as a replicated regression of log-transformed titers as a function of the log of bacterial host density. We found that the log titer of phage in the initially uninfected host population increased linearly as a function of the log of initial host density ($R^2 = 0.76$; $F_{1,13} = 41$; $P < 0.0001$) (Fig. 3). This result implies a bottom-up effect on the spatial structure of the community. The data also suggest that phage might undergo migration while infecting a bacterial host cell that is carried from a source population to an uninfected population.

Phage density and subsequent transfer. We explored whether the number of phage that infect the source population impacts the density of phage recovered from an initially uninfected patch. A naïve expectation would be that increased phage inocula should lead to larger numbers of recovered migrants. However, it is important that the host bacteria are a living, expanding resource for the infecting phage; premature decimation of the resource reduces phage yield, probably by precluding resource growth (8). Thus, an increased phage inoculum could reduce the number of phage available to the vector. Our data were consistent with the latter interpretation. These data were analyzed as a replicated regression of log-transformed titers as a function of the log of phage inoculum size. An increase in the initial density of phage in the colonized resource patch reduced the number of phage recovered from the initially uninfected resource patch. The log titer decreased linearly as a function of log inoculum size ($R^2 = 0.68$; $F_{1,58} = 121$; $P < 0.0001$) (Fig. 4). Like its growth rate (8), the phage's transmission rate is negatively density dependent.

DISCUSSION

We describe a novel laboratory model for examining complex ecological and evolutionary interactions comprising three species and two trophic levels. In this system, nematodes, bacteria, and bacteriophage can be grown simultaneously in a

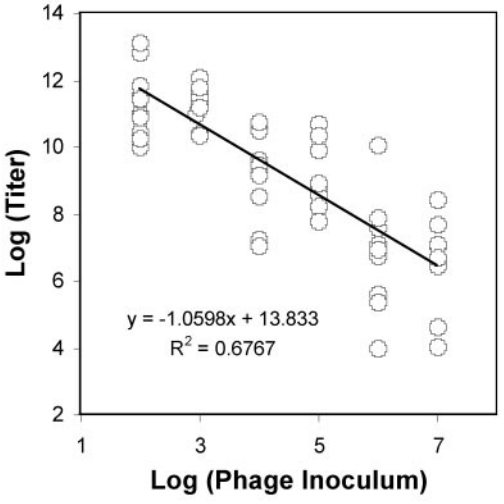

FIG. 4. Regression line of log phage titers in initially phage-free patches as a function of the initial phage inoculum in the source patch.

single petri dish, presenting opportunities to study vector-host-parasite interactions using methods not previously attempted or described. Advantages of the system include its ease of propagation, short generation times, high mutation rates, potential for rapid evolution, and ability to handle large population sizes in a controlled laboratory setting. The system not only has relevance as a model for economically and medically important nematode-pathogen interactions (3, 4, 14, 15, 36) but also demonstrates that nematodes can foster the migration of phages between bacterial populations.

Our initial explorations were centered on a model for vector dispersal of a disease organism, where we were especially interested in ecological conditions that maximize disease transmission. To this end, we separately explored the influences of nematode density, bacterial density, and initial phage inoculum size on the migration of phage from infected to uninfected patches of their hosts. This is analogous to studying the transmission of a pathogen within a host metapopulation.

By manipulating the nematode density and treating the host and phage densities as constants, we first demonstrated that *C. elegans* vectors were necessary for migration of the RNA phage ϕ6 between spatially separated patches of *P. syringae* pv. *phaseolicola* bacteria. Furthermore, and not surprisingly, increasing the number of nematodes in the system had the effect of enhancing the transmission of phage between patches. The slope of the log-log linear effect of nematode density on phage transmission was sensitive to our treatment of zeros in the data set (Fig. 2). If we treat the absence of transmission as a zero rate, then we assume that some transmission will occur even when there are very few worms. Conversely, if we treat the absence of transmission as missing or undefined data, then the regression indicates that transmission should only occur above a threshold nematode density. As a consequence of a vector density threshold for transmission, small nematode populations could escape competition with phage. Note that the ability of *C. elegans* to vector phage in this way is not restricted to ϕ6; in other experiments, we observed that the DNA phage T2, a typical T-even phage of *E. coli*, can similarly migrate between populations of its host bacteria via worm vectors (Y. W. Yang, J. J. Dennehy, N. A. Friedenberg and P. E. Turner, unpublished data). The mechanism of transmission in these cases is still unclear (see below).

By manipulating the host density and holding the nematode and phage densities constant, we showed that increased numbers of hosts in the infected patch promoted the transmission of phage to the uninfected habitat. Several factors could contribute to this result. First, the greater host availability in the initially infected patch expectedly translated to greater phage production during the 48-h experiment. Regardless of the transmission mechanism, a high host abundance should lead to larger numbers of phage progeny produced by the relatively small number of viruses in the initial inoculum (i.e., multiplicity of infection of $\ll$1.0 in our assays) (Table 1). If the mechanism of phage transmission is through direct attachment to or ingestion by nematodes, then increasing the number of phage produced in a patch should result in increased phage transmission. This scenario assumes that virus binding to the worm exterior is reversible (i.e., allowing viruses to adsorb later to hosts in the uninfected patch) or that the phage can survive within the worm for later anterior or posterior release to undergo productive infection. However, our preliminary data suggest that this mode of transmission is highly unlikely. We conducted a replicated experiment where phage suspended in LC medium were streaked across the agar surface of a plate, separating a population of nematodes and a patch of *P. syringae* pv. *phaseolicola* cells. After 48 h, no phage were recovered from the bacterial patch (data not shown). Alternatively, the mechanism of phage transmission may be entry of phage within the host cell in the infected patch followed by a cellular association (attachment or ingestion) with the nematode such that the host and its parasite are transmitted jointly to the uninfected patch. Thus, if phage are transmitted inside bacteria, this could easily account for the higher transmission frequency we observed as the host density increased in the system.

The hypothesis of transmission within hosts is supported by the results of our experiments where we manipulated virus inocula in the infected patch while holding the other factors constant. In this case, we found that increasing inocula led to fewer phage transmitted to the uninfected patch. At high phage densities, it is possible for the bacterial population to be lysed before large numbers of phage progeny are produced, that is, the phage deplete their resources (host cells) faster than the hosts can undergo multiple rounds of reproduction (i.e., faster than the resource replenishment rate). This idea is consistent with our previous finding that the phage population growth rate is negatively density dependent (8). If phage must be associated with a host cell for transfer between populations, then total lysis of the host population will certainly inhibit transmission. Even if phage do not depend on a host cell association for transmission, rapid depletion of the resource reduces the duration of contact between phage and worms, as nematodes will quickly abandon a depleted bacterial lawn. The net result may be that fewer phage are transmitted more quickly. This phenomenon would not be problematic for vector transmission if the viruses were able to migrate alone across the ecosystem. Rather, as asserted above, we hypothesize that phage transmission depends on viruses being transported within host bacteria. When higher densities of phage are present, it may be that all available bacteria are lysed before they can be transported by nematodes to phage-free populations. Therefore, phage transmission would depend on the survival of infected bacteria long enough that the phage-host associations are transmitted together to new uninfected host populations. We note that lysogeny (the transient stability of phage across host generations by incorporation into the bacterial genome) would likely enhance this type of transmission. However, unlike certain DNA phages, ϕ6 is an RNA virus that is thus incapable of experiencing a lysogenic life cycle. Although in vitro work shows that ϕ6 can enter a carrier state where it is transmitted vertically across host generations (35), this phenomenon occurs very rarely and has not been demonstrated under natural infection conditions; therefore, we concluded that carrier states for the virus are not an important factor in the current study.

The decrease in total transmission rate with increasing phage population sizes may have important implications for the temporal dynamics of the bacterium-nematode-phage system in natural soils. Negative density dependence is known to exert a strong stabilizing effect on population and community dynamics (24). In the case of our laboratory ecosystem, the

local dynamics are inherently unstable. Phage and nematodes compete for the common bacterial resource, and phage can deplete local resource populations in less than one worm generation. The worm's only advantage is its mobility. In a recapitulation of the competitor-versus-disperser paradigm of metacommunity models with implicit local dynamics (37), there may be a dynamic equilibrium at which the phage population size reduces transmission, leaving some patches in a large patch network free of phage. The long-term persistence of all three species would also require that new populations of consumer-free bacteria arise at some rate. Future work should address the effect of interpatch distance on the transmission rate. If phage are transported in the nematode gut, they may be cleared while the worm is traveling through an inhospitable matrix if patches of high bacterial activity are disparate. A decrease in transmission rate with increasing interpatch distances could provide an additional mechanism for worm persistence and could result in cycles of bacterial spatial structure driven by phage outbreaks if a large enough set of experimental patches could be created in the laboratory.

Our novel model system would be especially appropriate for studies of experimental evolution because the phage, host, and vector can all be maintained indefinitely in frozen stasis. This allows the power to evolve strains from a single ancestor and to later directly compare and contrast evolved traits of an ancestor and its derived descendants. In a previous study (8), we used serial passage experiments with phage ϕ6 to reveal that emerging viruses (those that mutate to infect a new host) featured a range of transmission rates for which the native host was a source and the novel host was a sink. Periodic exposure to the native host (via the manual transmission of phage between hosts) was sufficient for the maintenance of the viral population on the novel host. It would be intriguing to explore whether nematode vectors could similarly sustain emerging viruses by fostering phage migration between populations of different bacterial hosts. Multistrain populations of *C. elegans* have been shown to evolve higher dispersal rates in response to habitat instability in a metapopulation context (12). The maintenance of an emerging virus in this model system may therefore depend on the evolutionary response of vectors to the spatiotemporal structure of the environment. Other future experiments in source/sink dynamics could explore whether phages acquire traits that specifically enhance their transmission via worm vectors. Such a study would relate to the literature on the evolution of parasite virulence, where predictions are often made (but seldom tested) concerning the link between transmission efficiency and virulence (effect on host reproduction), defined here as the time to host lysis. Still other experiments could explore theory directly related to phage ecology and evolution, topics of growing interest in microbiology (22).

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