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Report

Phage-Mediated Selection on Microbiota of a Long-Lived Host

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Summary

It is increasingly apparent that the dynamic microbial communities of long-lived hosts affect their phenotype, including resistance to disease [1–3]. The host microbiota will change over time due to immigration of new species [4, 5], interaction with the host immune system [6, 7], and selection by bacteriophage viruses (phages) [8], but the relative roles of each process are unclear. Previous metagenomic approaches confirm the presence of phages infecting host microbiota [8, 9], and experimental coevolution of bacteria and phage populations in the laboratory has demonstrated rapid reciprocal change over time [10, 11]. The key challenge is to determine whether phages influence host-associated bacterial communities in nature, in the face of other selection pressures. I use a tree-bacteria-phage system to measure reciprocal changes in phage infectivity and bacterial resistance within microbial communities of tree hosts over one season. An experimental time shift shows that bacterial isolates are most resistant to lytic phages from the prior month and least resistant to those from the future month, providing clear evidence for both phage-mediated selection on bacterial communities and bacterial-mediated selection on phage communities in nature. These reciprocal changes suggest that phages indeed play a key role in shaping the microbiota of their eukaryotic hosts.

Results and Discussion

Recent results from a natural bacteria-phage interaction within a long-lived eukaryotic host (the horse chestnut tree) demonstrate that phages are highly prevalent within tree leaves and are locally adapted to bacterial hosts collected from the same host tree, relative to neighboring trees of the same species [12]. These lytic bacteriophages are obligate killers of their bacterial host cells and can therefore impose strong selection on bacterial populations and communities for resistance [10]. This selection can lead to a temporary advantage for resistant bacterial mutants and/or immigrant bacterial strains that happen to be resistant to local phages, and it therefore has great potential to maintain diversity at both the population and community levels [13–15]. To determine whether phages impose selection upon the microbiota of the host tree, I measured changes in phage infectivity (i.e., the ability to infect and lyse bacterial host cells) and bacterial resistance to infection over the course of a season. Coevolutionary dynamics of multiple host-parasite systems have been successfully demonstrated using a "time-shift approach" [16], in which hosts and parasites are sampled from multiple points in time and then each is crossed against antagonists from past, contemporary, and future time points [17, 18]. This approach has been used successfully to document pairwise coevolution between bacteria and phages in the laboratory; bacteria were found to be relatively more resistant to phages from previous time points (suggesting that the bacteria had evolved resistance) and relatively less resistant to phages from future time points (suggesting that the phages had counteradapted to overcome the evolved resistance) [10, 19]. However, given the complexity of microbial communities in nature and the high dispersal rate of both bacteria and phages, it is unclear whether the dynamics observed in the lab are relevant to natural systems. I therefore applied this "time-shift" approach to test for reciprocal changes in phage infectivity and bacterial resistance of microbial communities living within leaves of the horse chestnut tree.

Culturable bacterial isolates from the leaf interior of the same branches of eight horse chestnut trees were collected every month for 6 months. In eight fully reciprocal cross-inoculation experiments (one per tree), I measured both whether phage populations from a given time point were able to infect each of 24 bacteria from past, contemporary, and future time points (by measuring the presence of plaques—localized absence of bacterial growth—in which a given phage inoculum had been spotted onto a lawn of a given bacterial isolate) and how successful that phage inoculum was (the number of plaques formed). I first examined bacterial resistance to time-shifted phages and, for the three bacterial time points in which there were phage samples from 1 month prior and 1 month later (June, July, and August), found that bacteria differed in their resistance to phages from the past, contemporary, and future points in the season (Friedman test for effect of time shift, with tree as a blocking factor: $n = 24$, $\chi^2 = 9.640$, $p = 0.008$), with no effect of bacterial sampling day on proportion infected (Kruskal Wallis tests: $p > 0.05$ across phage time points). This result remains significant ($n = 21$, $\chi^2 = 6.205$, $p = 0.045$) after removal of tree 4, for which future phages do particularly well. Hosts were most resistant to phages from one month earlier (1.74% susceptible, with 95% confidence limits of 0.84, 3.17) and least resistant to phages from one month in the future (13.10% susceptible, with 95% confidence limits of 10.54, 16.23; Wilcoxon signed rank test with Bonferoni correction, $p = 0.004$), with resistance to contemporary phages intermediate (7.18% susceptible, with 95% confidence limits of 5.16, 9.53), suggesting that phages are shaping change in the bacterial community over very short timescales (Figure 1).

The observed change of leaf microbiota toward increased resistance to past phages and the subsequent response of the phage to these changes is likely a combination of population- and community-level processes. First, it could be driven by mutation-based coevolutionary change of associated bacterial and phage lineages over time, as has been demonstrated in the lab [10, 19]. This is in line with previous results where phages were found to be more infective to bacterial populations from their local tree relative to the

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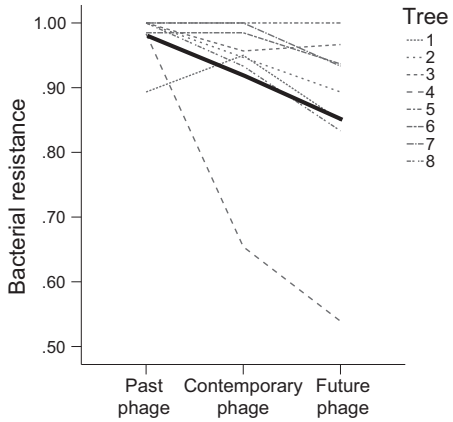

Figure 1. Bacterial Resistance to Time-Shifted Phage from Past, Contemporary, or Future Points in the Season

Resistance is represented by the proportion of bacteria (out of 24) from leaves sampled in June, July, or August that are resistant to phages from 1 month prior (past), that same month (contemporary), or the following month (future). Means for each of the eight trees are represented with gray dashed lines, and overall mean resistance is shown with a solid black line.

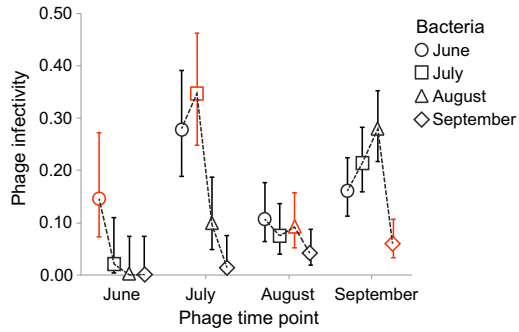

Figure 2. Infectivity of Phage Inocula to Time-Shifted Bacteria from Each Time Point

Infectivity was tested on all bacterial isolates from each time point, measured as the proportion of bacteria (out of the 24 tested per time point) that were susceptible to phages from each inoculum. Susceptibility was determined based on plaque formation (i.e., localized absence of bacterial growth) on bacterial lawns of each isolate. Phage and bacteria from the same time points are highlighted in red. Only those phage inocula for which plaques were observed on at least one of their 96 potential bacterial hosts tested (four time points, 24 hosts per time point) were used in the analyses. Error bars represent 95% confidence limits based on binary distribution. See also Figure S1.

same species of bacterial hosts from neighboring trees [12]. The pattern could also represent phage-mediated selection on bacterial community composition, whereby resistance to local phages influences the successful colonization of leaves by bacterial immigrants and therefore the successional dynamics of the bacterial community. Indeed, it is well known that the microbial composition of a leaf changes over the course of a season [3, 5] and that bacterial dispersal within forests is a key component of successional dynamics [20]. Finally, the pattern may be indicative of "diffuse coevolution," in which the pairwise interactions of two species are directly influenced by other species [21]. Previous work found that the culturable bacterial communities of horse chestnut leaves represent at least four genera [12], and phages from the horse chestnut phyllosphere have been shown to have broad host ranges, in which individual phages are capable of infecting multiple bacterial species and even multiple genera of these leaf-associated bacteria [22]. Therefore, a given phage genotype could select against multiple bacterial species simultaneously and multiple bacterial species could evolve resistance against a shared phage. As it is unclear what the coevolutionary units might be within these complex communities, a better understanding of both phage specificity and within-lineage evolution will help tease apart the relative importance of population and community level processes to the evolution of the microbiome.

Because the observed decrease in bacterial resistance to phages from the future could be accounted for by changes in phage density or diversity over time, I also examined the success of each phage inoculum on time-shifted bacterial hosts from past, present, and future time points. For the 18 leaf-generated inocula that were found to contain phage, infection success was highest on bacteria from the past (18.1%, with 95% confidence limits of 15.6, 20.9) than on contemporary bacteria (11.8%, with 95% confidence limits of 9.1, 15.2), and was lowest on bacteria from the future (3.6%, with 95% confidence limits of 2.3, 5.5) (Figure 2; Table 1, contrast of contemporary versus future bacteria, $p = 0.003$; past versus future, $p = 0.01$). These results were qualitatively similar when phage success was measured as the number of infective phage particles per inoculum (see Table S1 and Figure S1 available online). The decreased success of phages on bacterial hosts from future time points confirms that the bacterial community responded to phage-mediated selection, and the relatively higher infectivity of phages on bacteria from contemporary or recently past time points suggests that phages are adapting to common bacterial genotypes and/or species within the leaf. The success of phages on bacterial hosts from the past may be relatively short lived, however, as phages were most infective to either contemporary bacterial hosts or those from the recent past. In particular, phages from the end of the season were somewhat less infective to bacterial hosts from much earlier in the season (Figure 2). Although not statistically significant, this pattern is suggestive of frequency-dependent dynamics rather than arms-race coevolutionary dynamics [13] and contrasts with patterns observed in experimental microcosms, in which phage host range typically increases over coevolutionary time [23, 24]. However, it is consistent with data from nonmicrobial host-parasite systems [17, 18] and with experimental coevolution of bacteria and phages in soil microcosms [19].

The observed infection success of phages on their contemporary bacteria or bacteria from the past again complements previous evidence that phages are locally adapted to bacteria from the same tree, relative to bacteria from other trees [12]. The results of the cross-tree inoculation from the present study also show evidence for phage local adaptation across space; phages were found to be on average 8% (±3.0%, 95% confidence interval) more infective to bacteria from the same tree than on those from different trees (see Figure S2). This consistent phage local adaptation suggests that the phages have the evolutionary advantage in this arms race, as might be predicted based on their shorter generation times and larger population sizes [25]. It also further emphasizes that the spatial scale at which phages are adapting to their bacterial hosts is likely the tree environment. There exist very

Table 1. Results from GLM of Phage Infection Success

	df	MS	*F*	p
Tree	7	0.181	2.704	0.017
Phage time point	4	0.056	0.838	0.507
Time shift	2	0.252	3.76	0.029
Phage time point × time shift	4	0.999	1.48	0.22
Error	57	0.067		

Success of phage inocula from each leaflet on bacterial hosts from past, contemporary, or future points in the season (time shift). Due to low prevalence, phages from the first time point (May) were excluded from this analysis. Proportion infected variable was arcsine square-root transformed. See also Table S1.

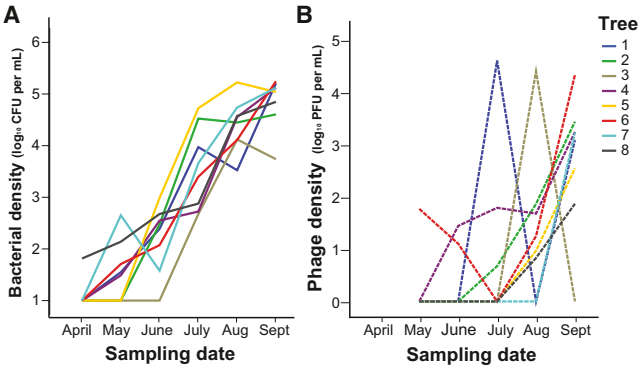

Figure 3. Bacterial and Phage Dynamics over Time

(A) Bacterial density increased over the course of the season (repeated-measures ANOVA on log10 transformed colony-forming units [cfu] for each of two leaflets per tree over time; main effect of time $F_{5,4}$ = 446.79, p < 0.001, but no effect of tree host $F_{7,8}$ = 1.86, p = 0.202, or tree host by time interaction $F_{35,40}$ = 1.07, p = 0.415). Whole leaflets were homogenized in buffer, and estimates were made from replicate colony counts of serially diluted homogenate.

(B) Phage density, measured as the mean number of plaque forming units per ml of leaf homogenate across all susceptible bacteria, also increased over time, but this was dependent on tree (repeated-measures ANOVA on log10 transformed plaque-forming units [pfu] for each inoculum over time: tree host by time interaction, $F_{28,92}$ = 2.830, p < 0.001).

little data to date regarding the spatial structure of bacterium-phage interactions in nature [12, 26, 27]. Indeed, there is a long-standing debate about whether, given the great dispersal capabilities and ability of bacteria to rapidly adapt to new environments, "everything is everywhere" but selection by the local environment acts to sort which bacterial genotypes or species colonize [28, 29]. The present results indicate that phages are one such environmental factor imposing strong selection on bacterial communities within the phyllosphere; this selection might be (1) driving mutational change within bacterial lineages, (2) acting on standing genetic variation at both the bacterial population and community levels, or (3) shaping the success of new bacterial immigrants. A key future direction of research will be to differentiate these possibilities, as the speed and dynamics of coevolution of bacteria and phages within long-lived hosts is central to our understanding of bacterial pathogenicity and spread.

Finally, bacterial and phage populations were remarkably dynamic among leaves across both time and space (Figure 3). Densities of culturable bacteria within single leaflets increased over the season within all trees (Figure 3A), which likely represents either increasing bacterial colonization of the leaves or decreasing plant defenses in early July, as the tree moves from vegetative growth to storage and reproduction [30]. Phage density, as measured on susceptible hosts from each point in time, also increased over the season, although this change differed significantly from tree to tree (Figure 3B). The densities of phages and bacteria within a tree over the season were positively correlated (n = 40, Spearman's *rho* = 0.619, p < 0.001), suggesting that host availability is a key determinant of local phage density. The proportion of bacteria that were susceptible to their local phages within a given time point varied considerably from month to month, but no overall effect of time was seen (repeated-measures ANOVA on arcsine square-root transformed proportion infected for each host population over time: $F_{3,5}$ = 1.087, p = 0.435). This is particularly important, as it suggests that the pattern of increased bacterial resistance to past phages (Figure 1) is unlikely to be explained by a general increase in phage success over time, via either continual increase in density or diversity. This is further confirmed by the results of the phage time shift (Figure 2), as each inoculum was challenged against bacteria from multiple time points and therefore any overall changes in phage density or diversity would be accounted for.

In conclusion, time-shift experiments have previously shown rapid coevolution in a number of laboratory systems [10, 19] and over multiple years in water flea hosts and their bacterial parasites [17]. These data are particularly influential because antagonistic coevolution is known to maintain polymorphism within populations [31–34], drive divergence among populations [14, 35], shape community-level diversity [13, 36, 37], and, for host-parasite interactions, determine the success of infectious disease management [38]. The key advance of this study is that the dynamic changes seen in the laboratory and in free-living host-parasite populations are also occurring at a rapid rate among microbial communities living within a long-lived host. Given the lifespan of the tree host and the speed of change observed, these data support the possible role of phages in shaping the emergence and spread of disease as well as a general role in shaping microbial communities in other long-lived hosts, such as the microbiota of humans. Thus we will no longer be able to ignore the potential importance of phages in shaping microbiota, influencing the success, spread, and evolution of bacterial pathogens, and driving divergence of natural populations over time.

Experimental Procedures

Sampling of Bacterial Isolates and Phage Inocula

Single leaflets (second largest in the leaf) were collected from the same branch of each of eight trees, separated by between 25 and 450 m, within a park in Oxfordshire, UK. Sampling started on April 28, 2011, finished on September 29, 2011, and was performed every 3–4 weeks. Leaves were brought back to the laboratory, where they were surface sterilized with a 10% bleach, 0.01% Tween detergent solution, rinsed with sterile water, and placed in a 15 ml Falcon tube containing 0.1 M potassium phosphate (pH 7.2) and 20% glycerol buffer. Tubes were immediately frozen at −20°C and stored until the end of the season. To ensure there was representation for each tree over the course of the season, I sampled two leaflets per tree at each time point. Both samples were included in estimates of bacterial densities over time, but only one replicate (chosen at random) was used to test for bacterial susceptibility to phage. At the end of the season, leaves were rapidly thawed at 38°C and were homogenized with a Fast-Prep-24 instrument (MP Biomedicals) and three ceramic beads. Eight leaflets were processed at a time over the course of 4 months. The order in which leaflets were thawed and processed was chosen by randomization in order to disassociate day of collection from the field with day of bacterial isolation from the leaf in the laboratory. Because of this randomization, I could rule out the possibility that time postcollection (i.e., time in

the freezer) decreased bacterial survival. Day of processing was not correlated with bacterial cfu (log10 transformed, Pearson R = −0.072, p = 0.483).

I selected 24 bacterial isolates per leaflet sample by thawing buffer solutions, serially diluting them at 1:1, 1:10, and 1:100 in sterile buffer, and plating them on 1.2% Kings Broth agar plates for 24 hr at 28°C. Bacterial isolates were chosen by randomly assignment of a point on each plate and selection of the 24 colonies that were closest to the point, regardless of size or color. The remaining buffer (9 ml) was centrifuged at 550 g for 10 min and then used to generate phage inocula (one inoculum per leaflet at each time point) by filtration through a 0.45 μm filter, which was stored in the dark at 4°C. Phage communities from the two leaves were combined for each tree at every time point. Previous work has shown that phages are just as infective to bacteria from their sympatric leaf as they are to other leaves within the same tree [12], and therefore I bulked both phage inocula in order to have the quantities necessary for the fully reciprocal cross-innoculation.

Time-Shift Experiment

The time-shift experiment was performed with eight fully reciprocal cross-inoculations (one per tree). Only bacterial hosts from June, July, August, and September were used due to the low densities in April and May (Figure 3). First, each of the 768 bacterial isolates were screened for sensitivity to phages via the soft-agar overlay technique, in which a bacterial lawn is grown in soft agar and phage inocula are spotted onto the lawn prior to overnight incubation [7–9, 19, 20]. For this initial screen, one bulk inoculum of all phage isolates across all trees and time points was used, so as not to bias the results toward sympatric combinations (either spatially or temporally). Bacterial lawns were scored for plaque formation (i.e., localized absence of bacterial growth due to phage infection and spread), and only those bacteria for which plaques were observed were used in the full cross-inoculation. All 123 susceptible bacterial isolates were then examined for susceptibility to phages from the same tree, across all past, contemporary, and future time points, as well to phages from different trees (bulked across all time points). Again, a standard agar overlay was used; overnight cultures of each isolate were mixed into soft agar, poured into a square petri dish, and spotted with 10 μl of each of the 14 phage inocula (six sympatric across time, and eight bulked inocula from each tree) in a grid, in duplicate. Bacterial lawns were grown overnight at 24°C and scored for plaques, and phages were considered infective to a given bacterial host when both duplicates showed plaque formation. In the few cases in which individual plaques were not distinguishable (i.e., where the entire spot of inoculum was clear of bacterial growth), the inoculum was serially diluted to the point in which individual plaques were countable. This allowed me both to estimate density of phage and to confirm that the growth inhibition was due to phage infection, rather than, e.g., bacteriocins or antimicrobials in the homogenate. In addition, phage inocula from each tree and time point were used to inoculate previously characterized isolates of the causal agent of bleeding canker in horse chestnut trees, *Pseudomonas syringae* pathovar aesculi. Phages from two trees were found to infect one of the isolates (collected by Forest Research from Alice Holt Lodge, Farnham).

Supplemental Information

Supplemental Information includes Supplemental Experimental Procedures, two figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2013.05.038.

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