Remote extracellular attacks on bacteriophage

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Bacteriophages and their hosts co-evolve while exploiting and defending against the other, respectively. Known anti-phage defences prevent attachment to the cell or target phage nucleic acids within the cell; variation in such defences shapes phage host range. While investigating the host range of the virulent myxophage Mx1 among natural isolates of the social bacterium *Myxococcus xanthus,* we found not only that Mx1's host range is very limited, but also that Mx1 is itself antagonised by most isolates. These antagonisms inactivate many phages and appear mediated by both cell-bound compounds and diffusive molecules. In harming phage before infection, anti-phage public goods represent a new category of defence. While some isolates secrete anti-phage compounds only constitutively, others do so facultatively in response to Mx1, behaviour suggestive of anti-phage adaptation. *M. xanthus* also alters the heat-stress-tolerance of phage surviving initial interaction. Such effects vary greatly across genotypes; some genotypes failing to antagonise Mx1 in direct encounters nonetheless reduce phage tolerance of later heat stress. Collectively, our results suggest that *M. xanthus* may have adapted to produce extracellular anti-phage agents that inactivate some phages and weaken others. Secreted anti-phage agents raise intriguing questions regarding the costs and benefits of extracellular versus cell-associated anti-phage defences.

Introduction

Bacteriophages are perhaps the most abundant type of viruses on our planet[1]. They pervasively threaten but also often benefit their bacterial hosts[2–5]. Phage-bacteria interactions and their evolution over billions of years cascade outward to impact bacterial social interactions[6], bacterial diversification[7], microbial-community interactions[8], many microbe-macrobe interactions[9], and global nutrient cycling[10]. The range of bacterial species and strains that can support replication by a given phage type – its host range – determines its ecological impact and evolutionary potential. Some phage types can broadly infect multiple bacterial species, whereas others can replicate only within very closely related sublineages of a single species, and phage host ranges often overlap[11]. Host range is likely to evolve in concert with other traits such as maximum reproductive rate[12], but the evolutionary causes and effects of host-range evolution remain under extensive investigation.

A major form of coevolution between bacteria and phage is often described as an arms race[13]. On one side, phages are selected to become more efficient at invading and/or replicating within bacterial cells. On the other, bacterial cells evolve diverse defence mechanisms to survive phage attacks, targeting multiple stages in the phage life cycle.

Focusing on virulent DNA phage, successful lytic life cycles involve several stages; bacteria have evolved defences targeting each. Upon encountering a bacterial host, a phage particle attaches to a cell-surface receptor and inserts its genetic material into the cell[14,15]. Some bacterial defences prevent phage adsorption by modifying[16] or masking[17] potential receptors. If phage genetic material is successfully inserted, it hijacks host replication machinery to proliferate. However, phage DNA is often recognized and destroyed by a bacterial restriction-modification system (RM system)[18] or a CRISPR-Cas immune system[19]. If such systems that directly target phage DNA are absent or fail to stop phage replication, phage proteins are synthesized and new particles are formed, up to hundreds of which per cell can be released when an infected cell bursts. Some bacteria can inhibit phage propagation through self-induced death using abortive infection systems (Abi systems) or by inhibiting DNA synthesis with nucleotide depletion mechanisms[20–23].

While the above-mentioned defence mechanisms against phage, by preventing replication, all have group-level consequences, they operate on or within the individual defending host cell. Other mechanisms, however, such as the secretion of defensive diffusible secondary metabolites, have greater potential to generate protection against phages at broader community levels. Such secreted metabolites were first described in the 1950s and have recently regained attention, but their molecular modes of action remain unclear[24]. Such metabolites are mostly anthracycline[25] and aminoglycoside[26] antibiotics, both commonly produced by Streptomycetes, that inhibit replication by diverse DNA phages after introduction of phage genetic material into a prospective host cell. That such metabolites might have the potential to benefit cells other than their producers, and therefore function as anti-phage public

goods (hereafter APPGs), raises many fascinating questions regarding the potential costs and benefits of shared *vs* private anti-phage mechanisms at individual, population, and community levels.

Like Streptomycetes, myxobacteria produce a vast array of secondary metabolites[27], some of which might, like anthracyclines and aminoglycosides, have anti-phage properties. However, soil-dwelling myxobacteria are perhaps best known for their highly social life cycles, which involve many forms of intercellular communication and coordination, including during social motility[28], group predation[29], aggregative multicellular fruiting body development[30] and social spore germination[31]. Because phages adhere to cell-surface molecules that may be involved in bacterial sociality, interactions with phages may have played important roles in shaping the complex cooperative behaviours of myxobacteria[32].

Although phage capable of infecting *M. xanthus* were first isolated many decades ago[33], relatively few have been isolated since and very little is known about their host ranges[34]. We initially sought to characterize the host range of a virulent myxophage, specifically the range of *M. xanthus* genotypes susceptible to being exploited by the virulent myxophage Mx1 to fuel phage population growth. To this end, we introduced phage Mx1 into cultures of *M. xanthus* strains isolated worldwide and of known phylogenetic relatedness. Upon finding that many of the bacterial genotypes actually antagonize the phage rather than *vice versa*, we tested whether the antagonizing compounds produced by *M. xanthus* are diffusible or cell-bound, and whether they are produced constitutively or facultatively in response to interaction with phage. As a function of *M. xanthus* genotype, we then characterized the heat sensitivity of both diffusible antagonistic compounds produced by *M. xanthus* and of Mx1 phage that had interacted with *M. xanthus*.

Methods

Strains and culture conditions

M. xanthus natural isolates examined in Rajagopalan *et al*. 2015 were selected to include, in most cases, two independent isolates from each of eight globally distributed sampling locations/regions, thus encompassing a wide range of phylogenetic distances among strains[35]. Strain GJV1, a derivative of the reference strain DK1622[36], was included also. Frozen stocks of bacterial isolates were inoculated on CTT hard-agar plates (CTT HA; 10 mM Tris pH 8.0, 8 mM $MgSO_2$, 10 $g.l^{-1}$ casitone, 1 mM KPO_2, pH 7.6; 1.5% agar[37]) and incubated at 32 °C with 90% rH until sufficiently grown. Samples of growing plate cultures were transferred to CTT liquid medium (same as CTT-HA without agar) and incubated at 32 °C, 300 rpm until mid-log-phase. To initiate each experiment, cultures were centrifuged (3 min at 12000 rpm) and resuspended to ~2 x 10^8 cells ml^{-1} in CTT liquid.

Here we studied the virulent myxophage Mx1, a double-stranded DNA phage[33]. The Mx1 stock was generated by infecting the lab strain GJV1 growing in liquid CTT. Phage particles were isolated using 10% chloroform followed by filtration (0.22 μm).

Infection assays

Phage infection was initiated with density-adjusted bacterial cultures (~2 x 10^8 cells ml^{-1}) and diluted phage (MOI ~0.01) in 4 ml CTT liquid supplemented with $CaCl_2$ (0.5 mM). Cocultures were incubated shaken at 300 rpm, 32 °C for 24 h. To determine final phage numbers, 10% chloroform was added to each sample. Samples were incubated shaking and vortexed to ensure the release of phage particles from bacterial cells. After centrifugation (3 min at 12000 rpm) and removal of the supernatant containing all phage particles, viable phage particles were determined in double-agar-overlay plaque assays. To do so, we used *M. xanthus* strain DZ1 as an indicator strain for all phage population-size counts, as it is highly susceptible to infection by Mx1 and allows clear plaque formation[38]. 10 μl of DZ1 suspension at 10^{10} cells ml^{-1} and 10 μl of serial diluted phage were mixed with 1 ml CTT soft agar (CTT SA; CTT liquid with 0.5% agar) in the liquid state cooled to 50 °C and mixture was then poured on top of solidified CTT HA plates. After 24 h of incubation at 32 °C, we counted PFU numbers.

Supernatant assays

We investigated whether the observed antagonisms were mediated by secreted diffusible compounds. To recover cell-free supernatants (Fig. S1), cultures with bacteria and phage were centrifuged (5 min at 12000 rpm) 24 h after infection and the phage-containing supernatant was carefully removed and titered (stage 1). 3 ml of the supernatant was transferred to a new flask, to which ~3.7 x 10^6 phage particles ml^{-1} were added, followed by incubation for another 24 h (stage 2). The viable phage population size was determined using the double-agar-overlay assay. Control assays in which bacteria were grown in the absence of phage were performed as above, except phage particles were not added to the bacterial culture for the first 24 h of incubation, only to the supernatant for the second 24 h incubation period.

Temperature assays

We tested whether high temperature eliminates the negative effect on phage of exposure to *M. xanthus* supernatant (Fig. S2). To do so, we grew Mx1 with all bacterial isolates and titered phages in the resulting supernatants. Supernatant aliquots were then heated at 45, 55, 65 or 75 °C for 30 min and cooled down 10 min on ice. The number of viable phages was determined a second time for each heat-treated supernatant. We then exposed phages to the heat-treated supernatants and samples were handled as noted previously (stage 2 of Fig. S2). For control treatments without phage interaction, phages were not added to bacterial cultures, only to the purified supernatant.

Test for supernatant effects on phages T4 and DMS3vir

To test whether *M. xanthus* compounds that inactivate Mx1 phage also harm bacteriophages that infect non-myxobacterial species, we exposed phage T4[39], a virulent phage of *Escherichia coli* and DMS3vir[40], a virulent phage of *Pseudomonas aeruginosa*, to supernatants from co-cultures of *M.*

xanthus isolates grown with Mx1 phage. We grew Mx1 with each of the five bacterial isolates that previously showed the biggest supernatant effect on Mx1 and extracted the supernatant as described previously. Subsequently, ~2 x 10^6 phage particles ml^{-1} of T4 and DMS3vir were added to separate supernatants of each bacterial genotype. After 24 h of incubation, PFUs were quantified using the double-agar-overlay assay with the respective host strain of either phage. We used *E. coli* strain MG1655 as host for T4 and *P. aeruginosa* strain UCBPP-PA14 csy3::LacZ [40] as host for DMS3vir. To be sure that Mx1 particles remaining in the supernatant have no lytic effect on *E. coli* or *P. aeruginosa,* a control treatment was included that tested for possible plaques of Mx1 on both bacterial strains in absence of T4 or DMS3vir. No such plaques were observed at any plating dilution.

Test for phage reduction resulting from attachment to cell debris

To test whether reductions in Mx1 populations caused by some *M. xanthus* strains might be due to adsorption to floating cell debris that renders some phage particles inactive, we monitored the number of phage particles during the bacterial growth cycle. If adsorption to debris contributes significantly to phage decline, we expect very low, if any, reduction in phage numbers during the bacterial exponential phase, while phage reductions should increase after transition to stationary phase when most cell death occurs. We adopted this approach rather than testing whether addition of artificially generated cell debris would decrease phage counts, as the latter approach would not control for any effects of the debris-generation process on interaction with phage. The character of artificially generated cell debris might differ from that of debris resulting from spontaneous cell death.

To initiate the experiment, we allowed four *M. xanthus* strains to grow for 0, 12, 24, 36, 48, and 60 hours, respectively (after initiating cultures at ~2 x 10^8 cells ml^{-1} by transfer from prior log-phase cultures), before harvesting their supernatant. At each time point, bacterial culture luminescence was measured with BacTiter-Glow™ microbial cell viability assay (Promega G8230) to characterise the timing of growth phase and the transition to stationary phase; 25 μl of culture were mixed gently with the same amount of BacTiter reagent in a white 384-well plate and incubated for five min before measuring the luminescence. The rest of the culture was used to test for effects of supernatant exposure on Mx1 viable-population size as described above. Briefly, phage-free bacterial cultures were centrifuged (five min at 12000 rpm) and the supernatant was carefully removed. 3 ml of the supernatant were transferred to a new flask, to which phage particles were added to a final density of ~3.7 x 10^6 phages/ml, followed by incubation for another 24 hours. To determine the number of viable phage particles after 24 hours, a double-agar-overlay assay was performed, as described above. Control phage populations not exposed to bacterial supernatant were incubated in CTT liquid for 24 hr before plating.

Statistical analysis

All experiments were performed in three or four temporally separate, independent biological replicates. Analyses were performed using R version 4.1.2 and Rstudio version 2021.09.0.351[41,42]. Strain-specific effects were tested for with one-way ANOVA followed by two-tailed Dunnett tests (package multcomp[43]) to compare against single treatments. For supernatant experiments, we first calculated ratios of observed *vs* expected phage numbers and pre *vs* post heat-treatment values and tested for between-strain differences with one-way ANOVA. Upon detection of significant effects of the strain identity, differences between individual observed and expected values were tested for with multiple one- or two-sample paired *t* tests with correction for multiple testing (Benjamini & Hochberg). Alternatively, a single *t* test was performed combining the data for all strains. For the luminescence and phage titer temporal datasets, we used linear models with bacterial strains, time and their interaction as explanatory variables followed type III ANOVA as implemented in the car[44] package and posthoc tests with emmeans[45] package. All figures were created using the package ggplot2[46]. Raw data and representative code are available from the Dryad Digital Repository: https://doi.org/10.5061/dryad.3tx95x6kj

Results

Mixtures of Mx1 phage with liquid cultures of 16 *M. xanthus* natural isolates and the reference strain GJV1 were incubated for 24 h (MOI ~ 0.01) before the number of phage particles capable of forming plaques on the *M. xanthus* indicator strain DZ1 (hereafter referred as 'viable phage' for all population-size assays) from each culture was determined. The *M. xanthus* genotypes varied greatly in their effects on viable phage numbers (Fig. 1; linear model, $F_{17,36} = 854.33$, $p < 0.001$). However, our results indicated that Mx1 has a very narrow host range, as only two of the 17 bacterial strains allowed Mx1 to increase in viable-population size, namely GJV1 and Sulawesi 05 (posthoc Dunnett contrasts, both $p < 0.001$). These strains are positioned in different subclades of a whole-genome based phylogeny (Fig. 1).

Surprisingly, among the remaining 15 strains in which Mx1 is unable to complete its lytic life cycle, ten caused large decreases in viable-phage population sizes (reductions of 97.6-99.9%), while five had no significant effect on phage numbers. This is an intriguing observation, since phage numbers were expected to remain constant over short periods when bacterial cells cannot be utilized as a suitable host. No clear relationship between phylogenetic position and the three categories of effects on phage number - neutral, successful phage replication, and antagonism of phage - is evident.

Phage inactivation is partially caused by diffusible public goods

Reductions of viable phage numbers upon interaction with bacteria might result from diverse mechanisms. These include intracellular mechanisms such as integration into host DNA to form prophage, prevention of phage genome replication or particle assembly, as well as extracellular

mechanisms such as diffusible or cell-surface-bound compounds that alter the phage in a manner preventing successful growth on strain DZ1, or cell debris that triggers non-infective ejection of phage DNA. We began investigating these alternatives by testing whether diffusible compounds derived from the phage-antagonistic *M. xanthus* strains might be partly or fully responsible for the observed reductions in phage numbers.

After exposing bacterial cells to phage (or to media only in no-phage controls) in stage 1 of this experiment (Fig. S1), cell-free supernatant (or media only in no-bacteria controls) was harvested and tested for potential effects on phage viability after new phages were added at the start of stage 2. We calculated observed and expected phage numbers for each bacterial interaction treatment. The expected number in each case was the sum of the number of viable phages remaining in the supernatant at the end of stage 1 and the number of new phages added at the start of stage 2. The observed number was the count of viable phages remaining after exposure to the harvested supernatant during stage 2. The difference between observed and expected phage counts varied as a function of bacterial-strain identity (ANOVA, $F_{17,54} = 101.63$, $p < 0.001$ followed by posthoc contrasts).

Supernatants from the same ten strains that antagonized phage when they could interact directly with bacterial cells (Fig. 1) were found to reduce viable phage counts below expected values (Fig. 2, one-sided paired *t*-tests with Benjamini-Hochberg correction, $p < 0.05$). This outcome indicates that diffusible compounds – whether actively secreted or cell debris - are at least partially responsible for the anti-phage antagonisms observed in our original experiments. Among those ten strains, the diffusible phage-antagonistic compounds present in supernatant accounted for 40-96% of the total antagonistic effects of these strains observed in our original experiment (Fig. 2).

Anti-phage compounds are produced during vegetative growth and disappear in stationary phase

One hypothesis that might explain the observed antagonisms of Mx1 by some *M. xanthus* strains is that many phages might become inactive by attaching to cell-membrane debris from dead *M. xanthus* cells and then releasing their DNA while failing to infect a live cell. Under this hypothesis, the degree of cell debris due to cell lysis available to remove viable phage would have to vary greatly across *M. xanthus* strains given the highly divergent effects of different *M. xanthus* genotypes on Mx1 (Figs. 1 and 2).

Under the cell-debris hypothesis, because cell death in bacterial cultures increases in stationary phase[47], we expect that anti-phage effects of *M. xanthus* supernatant from stationary-phase cultures would be stronger than effects of supernatants from lower-density exponential-phase cultures. To test this hypothesis, we used four *M. xanthus* strains to examine effects on Mx1 viable-population size of supernatants from *M. xanthus* cultures that varied in age since culture initiation. In our earlier experiments, one of these strains supported Mx1 growth (DK1622_GJV1, Fig. 1), one had no effect on Mx1 population size (MC3.5.9c15) and two strongly reduced Mx1 populations (Chihaya 01 and New

Jersey 06). Mx1 was exposed to supernatants from cultures still far from (0 and 12 hours), approaching (24 hours) and clearly in (36, 48 and 60 hours) stationary phase (Fig. S3A). No growth differences between the four bacterial strains were detected (Fig. S3A; $F_{3,76} = 1.93, p = 0.13$).

As expected from our prior results, none of the culture supernatants from DK1622_GJV1 or MC3.5.9c15 reduced Mx1 population size relative to control phage populations (not exposed to culture supernatant), irrespective of culture age (Fig. S3B, bacterial strain x time point interaction, $F_{15,48} = 76.8$, $p < 0.001$, all one-sided *t*-tests on marginal means against phage control $p > 0.05$). In contrast to the effects of DK1622_GJV1 and MC3.5.9c15, supernatants from 12-, 24- and 36-hour cultures of Chihaya 01 and New Jersey 06 greatly reduced Mx1 populations ($t_{48} = -3.93, p < 0.001$) whereas 0-, 48- and 60-hour cultures did not (except for New Jersey 06 after 60 hours). The non-effect of 0-hour cultures was likely due to low concentrations of anti-phage compounds in the freshly inoculated, low-density cultures. Under the cell-debris hypothesis, we would have expected antagonisms of Mx1 by Chihaya 01 and New Jersey 06 supernatants to remain low during exponential growth, increase with transition into stationary phase and remain high or increase further with increasing time in stationary phase. Instead, the antagonisms increased already in exponential phase, when we expect cell death to be minimal, and peaked just after entry into stationary phase. However, they disappeared in supernatants from older cultures (48 and 60 hours) that had been in stationary phase for an extended period, when we expect the highest amounts of cell debris. These patterns are not expected under the cell-debris hypothesis. Rather, they strongly suggest that viable Chihaya 01 and New Jersey 06 cells secrete anti-phage compounds during exponential growth and cease producing these compounds upon entry into stationary phase. They also indicate that the anti-phage efficacy of these compounds lasts less than 12 hours under our stationary-phase culture conditions, given that anti-phage effects of 36-hour culture supernatants are not observed in supernatants from 48-hour cultures.

Anti-phage compounds are expressed both constitutively and facultatively

Secretion of phage-inactivating compounds might be either constitutive or rather facultatively expressed upon interaction with phages. To examine these hypotheses, we compared effects of supernatants of bacteria that either had or had not previously interacted with Mx1 phage (experiment stage 1, Fig. S1) on the survival of phage freshly exposed to the supernatants (experiment stage 2, Fig. S1).

In one treatment of this experiment, the ten bacterial strains that significantly reduced viable phage numbers in the Fig. 2 experiment were infected with phage during stage 1 and culture supernatant was harvested after bacteria-phage interaction. As in the Fig. 2 experiment, a known number of new phage particles was then exposed to the harvested supernatant in stage 2. Nine of the ten strains again significantly reduced viable phage counts (Fig. 3, one-sided paired *t*-tests with Benjamini and Hochberg

correction, $p < 0.05$), the sole exception being Sulawesi 08, which was therefore excluded from additional analysis in this experiment.

In a second treatment, the bacteria were not exposed to Mx1 phage in stage 1; harvested supernatant therefore contained only diffusible compounds secreted in the absence of phage. Of the nine strains still under consideration, supernatant from all but one (Chihaya 20) caused a decline in phage number (one-sided paired *t*-test with Benjamini and Hochberg correction, $p < 0.05$), indicating that they produce compounds harmful to phage even in the absence of the viruses.

However, for several bacterial strains, the degree of phage inactivation by supernatant was found to differ between supernatant from phage-exposed *vs* non-phage-exposed bacteria. Specifically, the supernatants of strains Columbia 01, Columbia 03, Nei 05 and Nei 10 showed greater negative effects on phage when the bacteria had been previously exposed to phage than when they had not (Fig. 3, one-sided paired *t*-tests with Benjamini and Hochberg correction, $p < 0.05$); a difference for strain Chihaya 20 was nearly significant as well ($p = 0.056$). No effect of bacteria-phage interaction on supernatant potency was detected for the remaining four strains.

These results indicate that some *M. xanthus* isolates secrete some anti-phage compounds constitutively while secreting other compounds only facultatively in response to interacting with phage, whereas other phage-antagonistic isolates appear to secrete their antagonistic compounds only constitutively. Thus, distinct *M. xanthus* strains appear to differ qualitatively in the behavioural modes by which they neutralize phage threats.

Diffusible anti-phage compounds are heat sensitive

If the diffusible antagonistic compounds revealed by our experiments are proteinaceous (or require polypeptides to function), heat treatment should inactivate those compounds and eliminate the antagonistic effects. To investigate this hypothesis, we heated supernatants at 45, 55, 65 and 75 °C before exposing a known number of added phage particles (Fig S2). Exposure to 55, 65 and 75 °C eliminated negative effects of supernatant on phage (Fig. S4, one-sided paired *t*-tests, $p > 0.05$), whereas in most cases exposure to 45 °C did not, suggesting that proteinaceous factors (or heat-sensitive secondary metabolites) are necessary for the observed phage inactivation.

The robustness of phage surviving antagonistic supernatant depends on the source bacterial genotype

Our experiment testing for heat inactivation of phage-antagonistic compounds in supernatant revealed an unexpected result from the 65 °C treatment, in which antagonistic activity of supernatant compounds was eliminated by the heat treatment. Comparison of plaque forming unit (PFU) values immediately before and after heat treatment of supernatant from cultures that included both bacteria and phage revealed a significant decline in phage number in supernatants from 13 strains (Fig. 4, one-sided paired *t*-tests with Benjamini-Hochberg correction, $p < 0.05$). The observation that phage-

antagonistic compounds in supernatant are fully deactivated by 65 °C heat (Fig. S4) suggests that these decreases in viable phage counts were due to the heat treatment. In contrast, in the phage-only control in which liquid was harvested from phage suspensions lacking bacteria, viable phage counts did not decrease upon exposure to 65 °C, indicating that Mx1-particle viability is not intrinsically sensitive to this level of heat stress. These results thus suggest that for phages that survived interacting with bacterial cells and diffusible compounds produced by cells prior to heat treatment, some of those interactions reduced the tolerance of the surviving phage particles to subsequent heat stress.

Such effects were not identical across bacterial strains. Four bacterial strains had no effect on post-interaction phage tolerance of heat stress (GJV1, Serengeti 21, Sulawesi 05 and Sulawesi 08). However, only two of those four strains (GJV1 and Sulawesi 05) were among the seven strains that failed to reduce viable phage population size in the Fig. 1 experiment. In contrast, Serengeti 21 and Sulawesi 08 both antagonized Mx1 directly by reducing particle counts in the Fig. 1 experiment but failed to negatively impact the heat-stress tolerance of those phage that did survive interacting with them.

Intriguingly, five *M. xanthus* strains that did not reduce viable Mx1 counts in our first experiment nonetheless reduced the heat tolerance of phage particles that survived interacting with them (Fig. 1; New Jersey 10, KF328c11, Tübingen C22, Tübingen C42 and MC359c15). For example, strain KF328c11 had no detectable effect on phage numbers immediately after Mx1 had been exposed to this strain, but phage populations that had interacted with this strain were reduced by more than 70% upon exposure to 65 °C heat stress. Thus, some *M. xanthus* strains harm Mx1 latently, reducing their future resilience under stress without immediately inactivating them.

Because viable phage counts varied at the start of our heat treatment (due to variable effects of different bacterial genotypes), we tested whether viable-phage density at the start of the 65 °C heat treatment (the last step of stage 1 of this experiment, Fig. S2) might have impacted the results shown in Fig. 4A. However, no correlation between stress tolerance and phage number immediately prior to heat treatment was detected (Fig. 4B, Pearson's correlation $r_s = 0.174$, $n = 17$, $p = 0.518$). Thus, the observed variation in heat-stress tolerance of Mx1 phage is caused by different effects of the distinct *M. xanthus* genotypes to which Mx1 was exposed. Bacteria had no effect on phage survival at lower temperatures of 45 and 55 °C (Fig. S5, one-sided paired *t*-test with Benjamini-Hochberg correction, $p > 0.05$).

We further tested whether bacterial-strain effects on Mx1 heat-stress tolerance depend on direct interaction between bacterial cells and phage or might rather be caused by diffusible compounds in supernatant derived from bacterial cultures that had not been exposed to phage (Fig. S2). We found that for almost all strains, supernatant from phage-free cultures reduced phage stress tolerance as much as did exposing phage to cultures still containing bacterial cells (Fig S6, two-sided paired *t*-tests with Benjamini-Hochberg correction, $p > 0.055$). This indicates both that i) the compounds that reduce phage heat-stress tolerance are generally diffusible secretions and ii) these compounds are generally secreted constitutively in the absence of phage. This result further suggests that the compounds that inactivate

phage upon bacteria-phage interaction, which in several cases are produced by bacteria facultatively in response to interaction with phage (Fig. 3), are often distinct from the compounds that reduce the heat-stress tolerance of phage. For example, interaction with Mx1 causes several strains (Colombia 01, Colombia 03, Nei 05, Nei 10, and Chihaya 20) to facultatively secrete (at least) one compound that directly inactivates many phage particles (Fig. 3), but in the absence of interaction with Mx1, these same strains also constitutively secrete at least one different compound that reduces Mx1 heat-stress tolerance (Fig. S6). Thus, individual *M. xanthus* strains appear to often produce multiple distinct compounds that exert different antagonistic effects on phage.

Anti-myxophage compounds do not exert generic anti-phage effects

Finally, we began exploring what range of phage the anti-myxophage compounds secreted by several *M. xanthus* strains might antagonize. To do so, we tested whether supernatants from five *M. xanthus* genotypes grown with Mx1 significantly reduce viable populations of the *Escherichia coli* phage T4[39] (another tailed, myophage) or the *Pseudomonas aeruginosa* phage DMS3vir [40] capable of infecting host strains of those species. In only one supernatant-phage pairing (DMS3vir with Nei 10 supernatant) were phage counts significantly reduced by exposure to *M. xanthus* supernatant (Fig. S7, posthoc Dunnett contrasts, $p < 0.001$). Given the structural similarities of T4 and Mx1, both of which are tailed myoviridae, our results indicate that *M. xanthus* APPGs have narrow target ranges; their activities even might be largely specific to phage that can infect myxobacteria (or a subset thereof).

Discussion

The persistent threat of death from virulent phage has inevitably selected for bacterial defences[13]. Previously known mechanisms that hinder phage infection exert their anti-phage effects within or on the bacterial cell[17,48–50]. We have shown that many *M. xanthus* isolates secrete diffusible public goods that inactivate, and in some cases latently injure, lytic phage extracellularly, before entry into the bacterial cell. In doing so, these myxobacteria greatly reduce extracellular viable phage population sizes. Effects of log- and stationary-phase bacterial supernatants on phage populations indicate that the diffusible anti-phage agents are secreted products of living cells rather than merely membrane debris from dead cells. *M. xanthus* strains that inactivated phage did so most effectively when direct cell-phage contact was possible, implicating cell-associated factors, but most of these strains also secreted APPGs. *M. xanthus* strains varied greatly in their anti-phage effects, including in the magnitude, production mode and character of those effects, implicating differences in the detailed mechanisms employed. APPGs were produced only constitutively by some strains, but in others were secreted facultatively in response to bacterial interaction with phage. Some strains only produced APPGs that immediately inactivate phage, while others only produced APPGs that latently injure the phage, compromising their ability to survive future environmental stress; yet other strains produced both categories of compounds.

The APPGs produced by *M. xanthus* appear to be polypeptides (or require polypeptides to function) because exposure to 55 °C heat eliminated all anti-phage activity of bacterial supernatants. These APPGs thus differ in molecular character from non-proteinaceous APPGs produced by some Streptomycetes, namely aminoglycoside and anthracyclines antibiotics[24]. Such APPG polypeptides might be secreted directly into extracellular space from the cell membrane. Alternatively, secreted outer-membrane vesicles (OMVs) might be involved[51]. OMVs can mediate interspecific killing[52] and have been hypothesized to play roles in cell-cell communication[53] and predation[54]. In some species, OMVs have been found to have act as phage traps, with phage attaching to receptor proteins on the OMV surface and introducing their DNA into OMVs rather than cells and thus failing to replicate[55,56]. OMVs secreted by myxobacteria might serve a similar role and/or might bear polypeptides that directly inactivate phage particles upon contact.

Multifunctionality. Diffusible bacterial secretions serve highly diverse functions; they can mediate cooperative interactions such as communicating information about cell density or nutritional status[57] or mediate antagonisms such as killing competitors[58] or prey[59]. We have found that bacterial secretions can also act extracellularly as anti-phage agents. One possible benefit of using public goods as anti-phage agents is multi-functionality; some diffusible secretions might mitigate phage threats while also serving some other function. For example, aminoglycoside and anthracycline antibiotics produced by some Streptomycetes have the potential to both kill competitor bacteria extracellularly and, as DNA-intercalating agents, intracellularly prevent replication of injected phage DNA. Once future work has defined the molecular identity of myxobacterial APPGs, it will be of interest to test whether they serve additional functions, for example in predation or conspecific interference competition.

The hypothesis of phage-targeted adaptation. In evolutionary biology, high degrees of trait-function specificity and trait complexity are often considered to strengthen arguments that a given trait evolved as a particular adaptation[60]. For example, the high complexity and specificity of CRISPR-Cas systems strongly suggest that they evolved primarily as adaptations for defence against phage. But there are often reasons why a given trait effect may not have evolved as an adaptation *per se*. For example, the multiple antagonistic effects of aminoglycoside and anthracycline antibiotics hinder clarity regarding their primary adaptive benefits. Their antagonism on some phages may have been a major selective contributor to their initial evolution and subsequent maintenance, but also might be largely a mechanistic byproduct of selection for their anti-bacterial effects. Contributing to this ambiguity, production of anthracycline or aminoglycoside antibiotics has not been shown (to our knowledge) to protect producing strains from phages capable of introducing DNA into producer cells or their conspecifics. Effects against phages with host ranges relevant to antibiotic-producing strains is a prerequisite for inferring that the anti-phage effects of these antibiotics are adaptations in their own right.

Our demonstration that many *M. xanthus* genotypes secrete public goods that inactivate a phage type – Mx1 – capable of killing producer conspecifics promotes the basic plausibility of the hypothesis that the anti-phage effects of these APPGs are adaptations *per se*. The additional demonstration of facultative specificity, namely that many of these strains secrete some APPGs only after direct exposure to Mx1, further strengthens the argument for this hypothesis. In turn, this also increases the plausibility of the hypothesis that the anti-phage effects of cell-associated (*i.e.* non-diffused) compounds (Figs. 1 and 2) and of APPGs produced constitutively (Fig. 3) are adaptive *per se*.

Susceptible-phage ranges. Once the molecular identities of *M. xanthus* APPGs are known, defining the ranges of phage genotypes that they each can inactivate or latently injure – their susceptible-phage ranges – will also be of interest. These ranges, together with the host-ranges of targeted phage, have large implications for understanding the costs and benefits of APPG production across variable community compositions. For example, the sociobiological and community-ecology implications of APPG production will differ greatly between broad (potentially including inter-species effects[24]) *vs* narrow susceptible-phage ranges. We have shown that the anti-myxophage compounds secreted by several *M. xanthus* strains do not generically harm all bacteriophages, *e.g.* phages of *E. coli* and *P. aeruginosa* (Fig. S7); defining their susceptible-phage ranges more precisely requires further study. Investigation of the mechanistic bases of variation in susceptibility to APPGs should improve understanding of functionally important differences in phage-particle composition and form across phage types.

Conspecific diversity in anti-phage mechanisms. As noted previously, *M. xanthus* strains vary greatly in their anti-phage effects, including in the magnitude (Figs. 1-3), production mode (Fig. 3) and character (Fig. 1 *vs* Fig. 4) of those effects. Differences in the magnitude of phage inactivation might be explained by either differences in the molecular character of anti-phage compounds produced by different strains (including possibly cocktails of multiple compounds by individual strains) or differences in production levels of the same compound(s). Similarly, facultative inactivation of phage in response to exposure to Mx1 might result from either increased production of anti-phage compounds already produced constitutively at a lower level or from production of compounds only secreted at all upon interaction with the phage.

Fitness implications of latent injury. Animal injury from physical violence between conspecifics or between predators and prey is common and can reduce the future fitness of injured parties[61]. Our experiments reveal that, in addition to extracellularly inactivating phage with public goods, some bacteria can latently injure phage particles without immediately inactivating them. Supernatants from several *M. xanthus* strains reduced the physical robustness of Mx1 phage that survived supernatant exposure, reducing their ability to survive future heat stress. This suggests that phage in natural populations that survive encounters with myxobacterial cells or their secretions may often be compromised in their ability to survive a variety of future environmental stresses.

Our results reveal a previously unknown category of bacterial defence against phage, namely compounds that actively harm phage particles prior to introduction of genetic material into the cell, rather than prevention of absorption or prevention of phage-DNA/RNA replication after successful entry. While UV radiation[62] and other abiotic factors[63] are known to physically weaken phage particles, less is known about compounds of biotic origin that do so. Future characterization of how *M. xanthus* APPGs physically debilitate and/or latently injure phage is thus of interest. The observations that some strains only inactivate Mx1, others only latently injure the phage, and yet others do both suggest that, at least in some cases, inactivation and latent injury are mediated by distinct compounds.

Social and community implications. Our findings raise intriguing questions regarding the relative costs and benefits of distinct anti-phage defence mechanisms, played out over multiple biological scales, as well as their relative contributions to shaping phage host ranges. All effective defence mechanisms deployed by any cell confer social benefits to other cells simply by reducing phage reproduction and thus the likelihood that others will be infected. Other cost/benefit features, however, differ across defence categories. Bacteria that undergo apoptosis in response to phage infection gain no self-benefit at the cellular level (even if there is a self-benefit at the genetic level)[64,65]. Cell-internal defences such as CRISPR-Cas systems that allow potential survival of infected cells can provide a cellular-level self-benefit, but at the risk of death if phage circumvent the internal defence[66]. Prevention of phage absorption without other harm to phage particles confers the self-benefit of protecting potential victim cells from infection but leaves unabsorbed phage at large to potentially infect other cells.

By reducing external viable-phage population sizes, however, extracellular inactivation of phage particles potentially benefits both APPG producers and other cells within relevant proximity that might otherwise be susceptible. Future research might investigate the relative effectiveness of extracellularly-acting APPGs *vs* other defence strategies at limiting phage epidemics across distinct ecological conditions. The sociobiological effects and evolutionary fate of any given form of APPG production will be determined by the relative degrees to which producer cells protect themselves and fellow producers *vs* non-producers from being harmed by phage. Production of APPGs is thus a social behaviour that should be incorporated into models of multi-trait social evolution[67], as well as models of microbial-community interaction networks.

Data accessibility

Raw data and representative code are available from the Dryad Digital Repository: https://doi.org/10.5061/dryad.3tx95x6kj.

Conflict of interest

The authors declare no conflict of interest.

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Figures

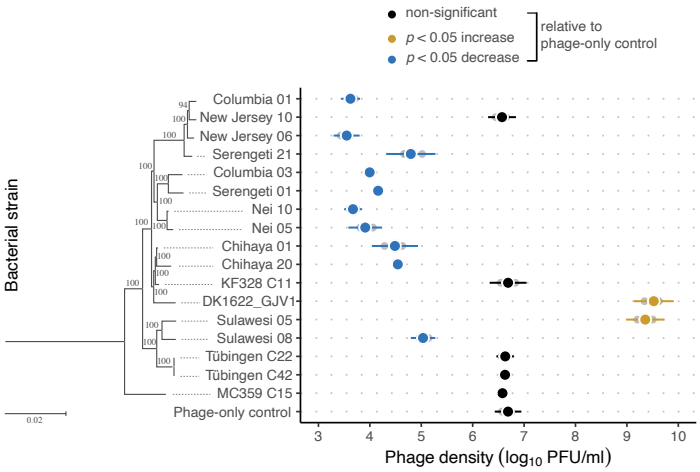


Figure 1. Most *M. xanthus* natural isolates antagonize bacteriophage Mx1, which has a very narrow host range. Phage density 24 h after infection of bacterial strains and in the absence of bacteria (control, black circle). Circles are mean $\log_{10}$-transformed PFU values across three biological replicates (light grey dots) and error bars represent 95% confidence intervals. Colours show outcomes of tests for differences between phage numbers for phage populations that interacted with *M. xanthus vs* control populations that did not (black: no significant difference, yellow: significant increase in phage number due to interaction with *M. xanthus*, blue: significant reduction; ANOVA and subsequent Dunnett contrasts, $p < 0.05$). The phylogeny is a maximum-likelihood tree reproduced from Rajagopalan *et al.* 2015 inferred from ~4.5 Mbp of orthologous genome sequence using *M. xanthus* strain *M. fulvus* HW-1 [68] as an outgroup. Bootstrap values supporting branch inferences are shown.

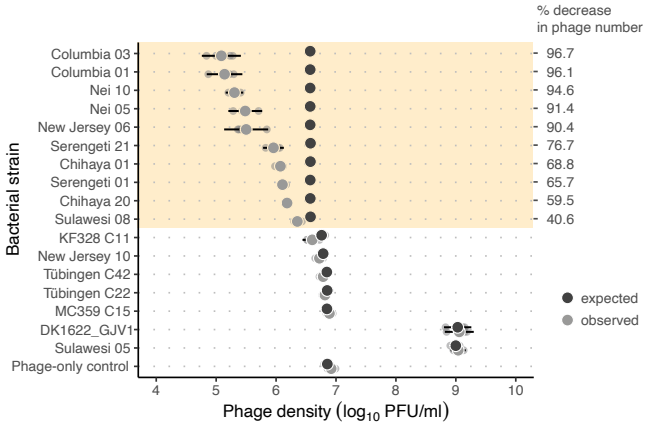


Figure 2. Antagonism of phage is mediated predominantly by diffusible bacterial secretions. Phage density after 24 h exposure to supernatant extracted from cultures in which bacteria and phage interacted over the previous 24 h. Expected numbers represent the sum of PFU counts immediately after 24 h of stage 1 bacteria-phage interaction (Fig. S1) and the number of phage particles added at the start of experiment stage 2 (to determine the effect of supernatant on newly added phage, Fig. S1). Observed numbers are PFU counts after incubation with supernatant at the end of stage 2. Means of $\log_{10}$-transformed PFU values and 95% confidence intervals are shown. Light grey dots represent biological replicates ($n = 4$). Percentage values indicate what proportion of decreases in phage number caused by *M. xanthus* shown in Fig. 1 can be attributed to diffusible supernatant compounds based on this assay. Significant differences between observed and expected phage numbers are indicated by coloured background (one-way ANOVA followed by two-sample paired *t* tests with Benjamini & Hochberg correction, $p < 0.05$).

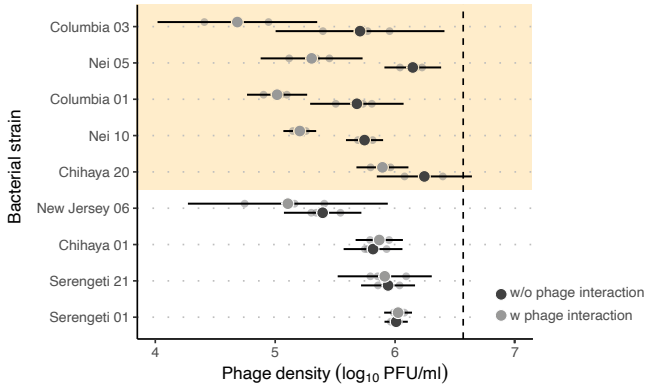


Figure 3. Secretion of phage-inactivating compounds by *M. xanthus* is often a facultative response to interaction with phage. Mean values of $\log_{10}$-transformed PFU counts for Mx1 phage exposed to supernatant from cultures containing both bacteria and phage (grey) or from cultures with bacteria only (dark grey). Error bars represent 95% confidence intervals. Light grey dots indicate biological replicates (n = 3). The dashed line indicates the number of phages initially added to the supernatant. Coloured shading indicates significant differences between treatments with bacteria+phage supernatant and bacteria-only supernatant (one-way ANOVA followed by two-sample paired t tests with Benjamini & Hochberg correction, $p < 0.05$).

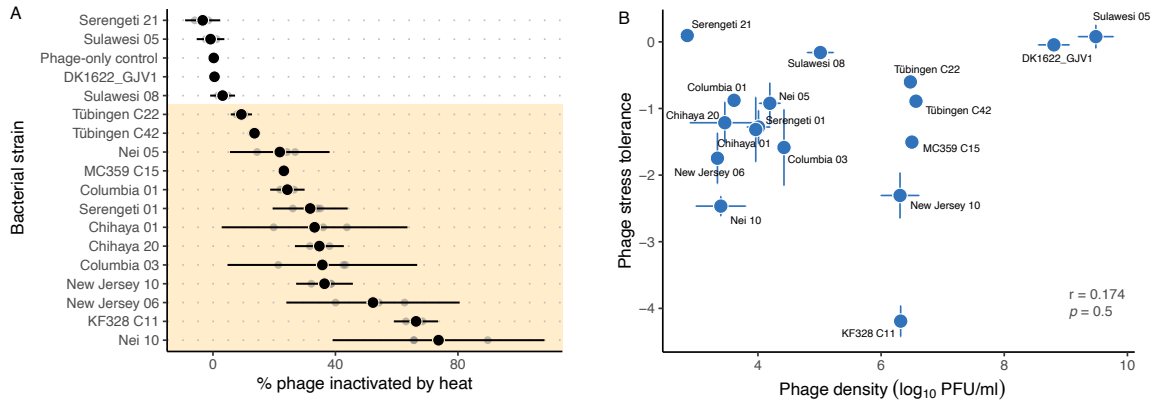
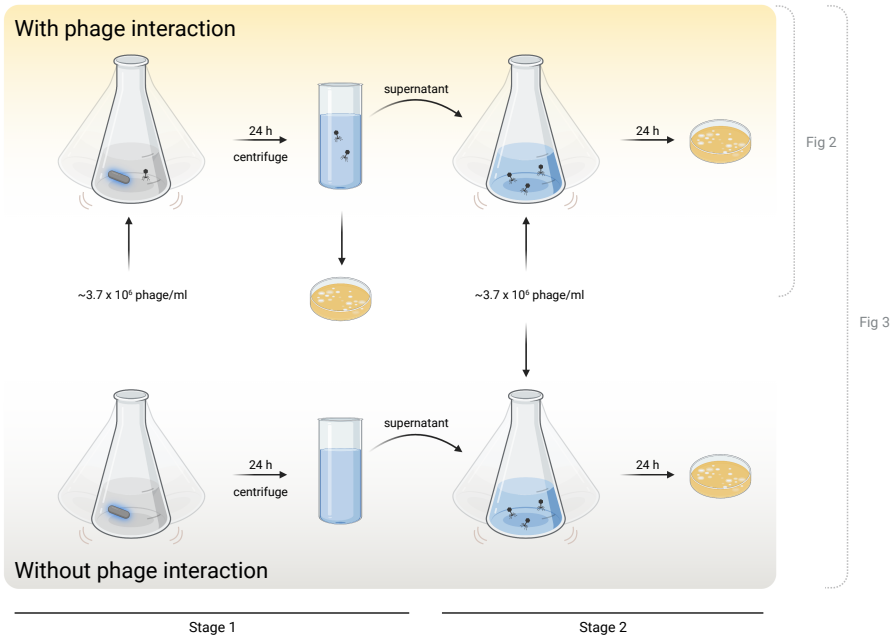
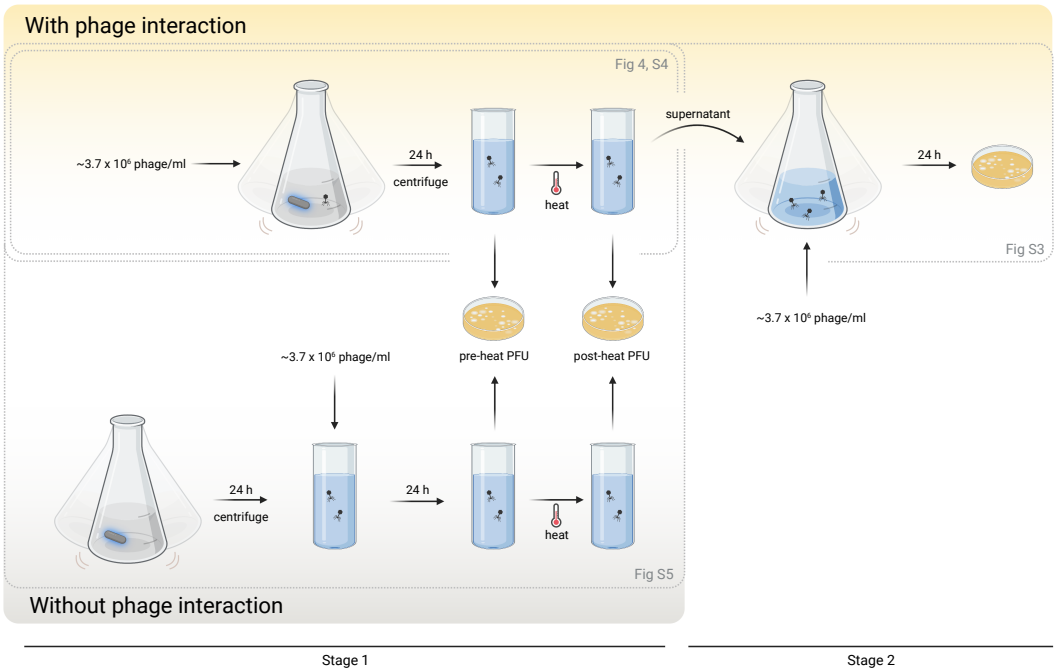


Figure 4. Interaction with bacteria reduces the heat-tolerance of phage to varying degrees as a function of bacterial genotype. A) Percentage of previously bacteria-exposed phage inactivated by exposure to 65 °C heat stress. Mean values and 95% confidence intervals are shown. Light grey dots indicate replicate values (n = 3). Coloured shading highlights significant differences between phage PFU before *vs* after heat exposure (one-way ANOVA followed by two-sample paired t tests with Benjamini & Hochberg correction, $p < 0.05$). B) Heat-stress tolerance does not correlate with phage density after interaction with bacteria and prior to the onset of heat stress. Mean values ± SME of $\log_{10}$-transformed PFU counts (Spearman correlation).

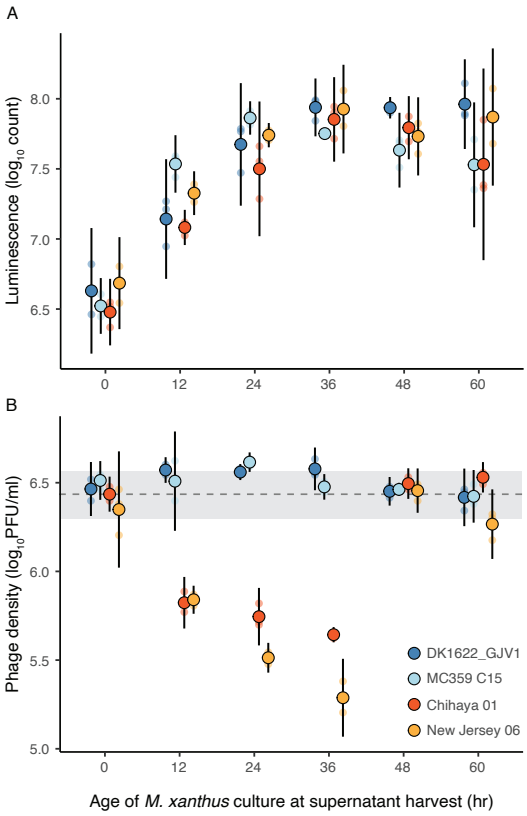
Supplementary Figures



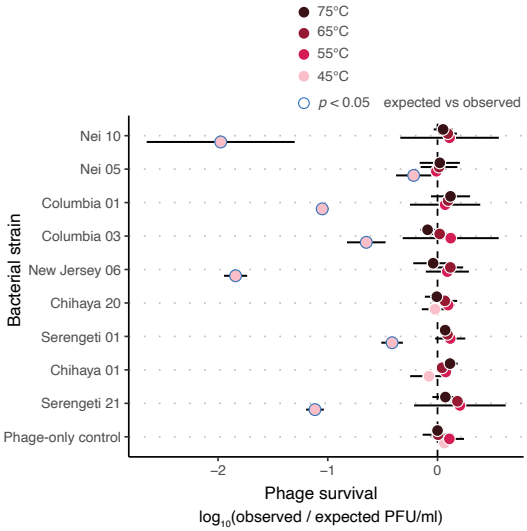
Supplementary figure 1. Design of experiments for Figs. 2 and 3. The top part of the diagram depicts the treatment in which direct contact between bacteria and phage was possible. Mx1 and *M. xanthus* natural isolates were mixed to initiate stage 1 of this treatment. After 24 h of incubation, cultures were centrifuged and viable-phage population sizes in supernatants were determined by dilution plating. To initiate stage 2, new phage particles were exposed to supernatant, effects of which on viable-phage population size were determined by dilution plating after another 24 h of incubation. The bottom part of the diagram shows the treatment in which direct contact between bacteria and phage was prevented. In this treatment, Mx1 was exposed to supernatants from phage-free bacterial cultures. Phage-only controls were also run for both the top and bottom protocols. Created with BioRender.com.



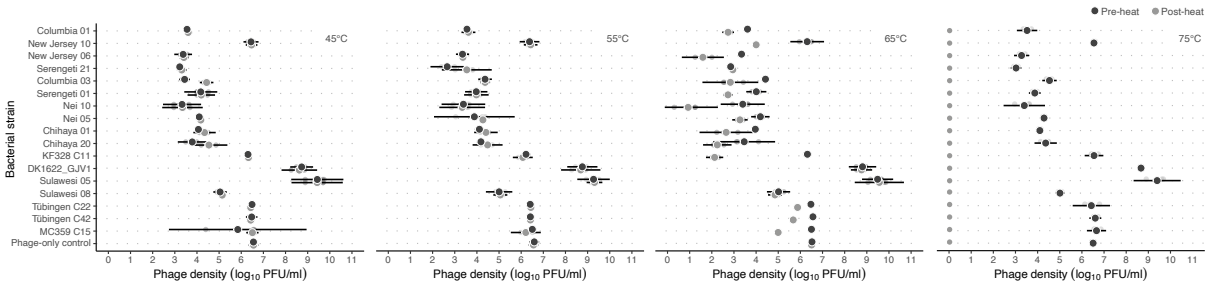
Supplementary figure 2. Design of experiments for Fig. 4 and supplementary figures 4-7. The top part of the diagram depicts the treatment in which direct contact between bacteria and phage was possible; Mx1 and *M. xanthus* natural isolates were mixed to initiate stage 1 of this treatment. After 24 h of incubation, cultures were centrifuged and viable-phage population sizes (pre-heat PFU counts) were determined by dilution plating, after which the phage suspensions were heated at 45, 55, 65 or 75 °C and viable-phage population counts were again assessed (post-heat PFU counts). To initiate stage 2, new phage particles were added to supernatant, effects of which on viable-phage population size were determined by dilution plating after another 24 h of incubation. The bottom part of the diagram shows the treatment in which direct contact between bacteria and phage was prevented. This part of the experiment was performed for stage 1 only. The supernatant of bacterial cells was harvested after 24 h of bacterial growth, after which phage were added and the suspensions then underwent the same protocol as in the top panel. Phage-only controls were also run for both the top and bottom protocols. Created with BioRender.com.



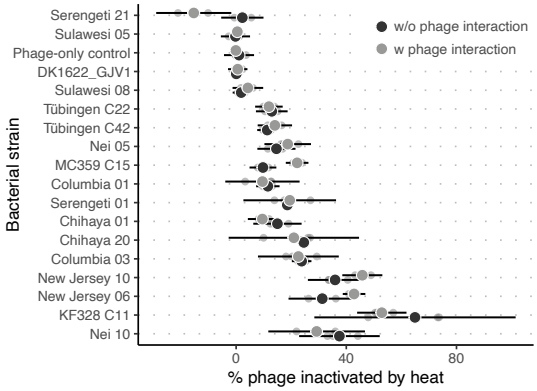
Supplementary figure 3. The stationary-phase cell-debris hypothesis for antagonism of Mx1 by many *M. xanthus* strains is not supported by patterns of culture-supernatant effects from two Mx1-antagonistic and two non-antagonistic strains. A. *M. xanthus* culture luminescence. Time-points represent the hours bacterial cultures were allowed to grow after inoculation at ~2 x 10^8 cells/ml until supernatant harvest. B. Phage counts after 24 hr of exposure to harvested bacterial supernatant. Phage-antagonistic strains Chihaya 01 and New Jersey 06 are shown in red and orange, respectively. Non-antagonistic strains DK1622_GJV1 (supports Mx1 growth) and MC359 C15 (does not support Mx1 growth) are shown in blue shading. The dashed line indicates the number of viable Mx1 after 24 h exposure to uninoculated culture medium. Error bars and grey shading are 95% confidence intervals.



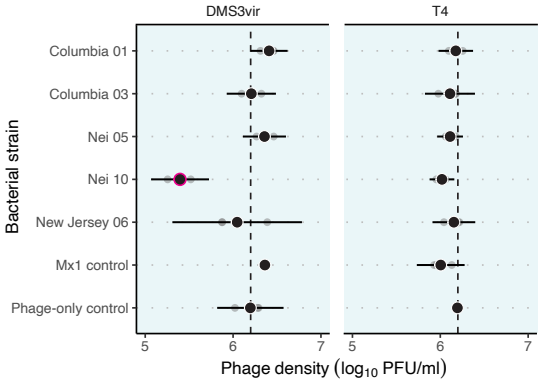
Supplementary figure 4. Heat deactivates diffusible phage-deactivating compounds produced by *M. xanthus*. 24h after phage exposure to nine bacterial strains that reduce viable phage population size, cell-free supernatant was treated at different temperatures and subsequently new phage particles were added to test for and quantify effects of heat-treated supernatant on viable phage population size. Plotted are mean values of the difference between expected and observed $\log_{10}$-transformed PFU counts. Expected numbers represent the sum of PFU counts in cell-free supernatant at the end of stage 1 of the experiment (see Fig. S2) and the number of phage particles added at the start of stage 2. Observed numbers represent PFU counts after incubation with heat-treated supernatants at the end of stage 2. Coloured dots indicate means at different temperatures. Error bars represent 95% confidence intervals. Significant differences between expected and observed phage numbers are highlighted by blue circles around dots (one-way ANOVA followed by two-sample paired *t* tests with Benjamini & Hochberg correction, $p < 0.05$). Observed numbers of each phage control replicate were used as expected numbers in the 75 °C treatment, since the number of phages that were added in stage 2 were lower than 3.7 x 10^6 phage particles ml^{-1}.



Supplementary figure 5. Differential stress tolerance of Mx1 as a function of bacterial-genotype interaction history is evident only under 65°C stress. Log_{10}-transformed PFU counts pre- and post-heat exposure (light grey and black, respectively) after coculture with different *M. xanthus* strains. Pre- and post-heat PFU counts do not significantly differ for phage treated with 45 or 55 °C (one-way ANOVA, $p > 0.05$) but do differ after 65 °C treatment (one-way ANOVA, $p < 0.05$). When the temperature is raised to 75 °C, all phage particles are inactivated irrespective of their interaction history. Light grey circles represent single replicate values ($n = 3$) and error bars are 95% confidence intervals. The lower error bar of Nei 10 in the 65°C treatment graph expands into the negative range and was shortened for visual purposes.



Supplementary figure 6. *M. xanthus* secretion of diffusible compounds that reduce Mx1 heat-stress tolerance is independent of exposure to phage. Percentage-means of phage particles inactivated at 65 °C after exposure to supernatant derived from bacteria previously exposed to phage (grey dots) or not (dark grey dots). 95% confidence intervals are shown. Small light grey circles indicate individual replicate values ($n = 3$). No significant effects of prior exposure to phage were detected (one-way ANOVA followed by two-sample paired *t* tests with Benjamini & Hochberg correction, $p > 0.05$).



Supplementary figure 7. Supernatants from *M. xanthus* isolates grown with Mx1 generally fail to harm the *E. coli* phage T4 or the *P. aeruginosa* phage DMS3vir. Right and left panels show PFU counts for the phages T4 and DMS3vir, respectively, on their respective indicator strains after exposure to supernatant from five *M. xanthus* strains cultured with Mx1 or from an Mx1-only control. The phage control shows the respective number of T4 or DMS3vir after 24 h incubation with no exposure to supernatant. The dashed vertical line shows the number of T4 or DMS3vir particles added. Red circle indicates treatment where phage count was significantly reduced by exposure to Mx1 treated supernatant (Dunnett contrasts, $p < 0.001$). Error bars are 95% confidence intervals.