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Suppression of Enteric Bacteria by Bacteriophages: Importance of Phage Polyvalence in the Presence of Soil Bacteria

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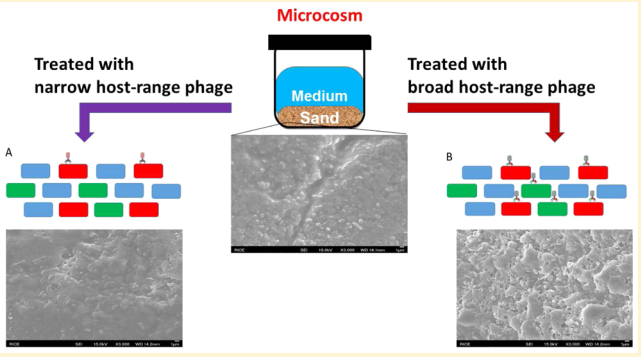
Supporting Information

ABSTRACT: Bacteriophages are widely recognized for their importance in microbial ecology and bacterial control. However, little is known about how phage polyvalence (i.e., broad host range) affects bacterial suppression and interspecies competition in environments harboring enteric pathogens and soil bacteria. Here we compare the efficacy of polyvalent phage PEf1 versus coliphage T4 in suppressing a model enteric bacterium (*E. coli* K-12) in mixtures with soil bacteria (*Pseudomonas putida* F1 and *Bacillus subtilis* 168). Although T4 was more effective than PEf1 in infecting *E. coli* K-12 in pure cultures, PEf1 was 20-fold more effective in suppressing *E. coli* under simulated multispecies biofilm conditions because polyvalence enhanced PEf1 propagation in *P. putida*. In contrast, soil bacteria do not propagate coliphages and hindered T4 diffusion through the biofilm. Similar tests were also conducted under planktonic conditions to discern how interspecies competition contributes to *E. coli* suppression without the confounding effects of restricted phage diffusion. Significant synergistic suppression was observed by the combined effects of phages plus competing bacteria. T4 was slightly more effective in suppressing *E. coli* in these planktonic mixed cultures, even though PEf1 reached higher concentrations by reproducing also in *P. putida* (7.2 ± 0.4 vs 6.0 ± 1.0 $\log_{10}$PFU/mL). Apparently, enhanced suppression by higher PEf1 propagation was offset by *P. putida* lysis, which decreased stress from interspecies competition relative to incubations with T4. In similar planktonic tests with more competing soil bacteria species, *P. putida* lysis was less critical in mitigating interspecies competition and PEf1 eliminated *E. coli* faster than T4 (36 vs 42 h). Overall, this study shows that polyvalent phages can propagate in soil bacteria and significantly enhance suppression of co-occurring enteric species.



INTRODUCTION

Bacteriophages, which are viruses that infect and replicate within bacteria, are the most abundant and diverse living entities in the biosphere.[1] Estimates place their numbers in the range of 10^{31}, with a common environmental virus-to-prokaryote ratio of about 10:1.[2,3] As antibacterial agents and important vectors for horizontal gene transfer,[4] phages are significant drivers of bacterial evolution and community structure. Consequently, phages can also significantly influence biogeochemical cycles and energy flows in various ecosystems.[5] Given their ecological importance and potential applications for controlling problematic bacteria,[6] there is a need to advance fundamental understanding of phage−host interactions in complex microbial communities. This includes discerning how phage host range affects their propagation dynamics and microbial community structure in both planktonic (i.e., suspended) and biofilm systems.

Phages alter their hosts' metabolic repertoire, fitness, and competitive capability or directly eliminate the most susceptible bacteria.[7,8] Interspecific bacterial competition in the presence of phages has been studied theoretically[8,9] and empirically.[10,11] Depending on the nature of phage-host associations and environmental conditions, phages can facilitate competitive exclusion[9,12] or enhance the bacterial hosts' coexistence.[11,13] However, most these studies have only considered phages that typically infect only one species and cannot reproduce by using hosts from other genera in the microbial community. No previous publications have considered how (broad host range) polyvalent phages (i.e., phages capable of intergeneric infection[14]) affect bacterial suppression and interspecific competition. This is a critical knowledge gap given the recent recognition of the prevalence of polyvalent phages in the environment[15−17] and their potential to exhibit more complex propagation dynamics. Specifically, whereas polyvalent phages use a wider variety of bacteria to reproduce, they generally experience a lower efficiency of infection (i.e., the ratio between the infectious titer for a given host and the maximum observed titer[18]) and slower growth,[17,19] which may offset their ability to influence microbial community structure.

This study addresses how the phage host range affects interspecific bacterial competition and enteric bacteria

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suppression. We compare the effect of coliphage T4 versus polyvalent phage PEf1 on three competing bacteria: (1) *Escherichia coli*, representing an enteric bacterium commonly associated with fecal pollution and (for some *E. coli* strains) infectious disease,[20] (2) *Pseudomonas putida* (alternative host to PEf1 but not to T4), and (3) nonhost *Bacillus subtilis*, which cannot be infected by these phages. The latter two represent indigenous soil bacteria commonly associated with biodegradation of pollutants.[21] *E. coli* suppression is quantified under both planktonic conditions and model biofilms, which offer potential resistance to phage diffusion and to propagation of phage-resistant mutants. We discern the contributions to *E. coli* suppression from phages versus competition by soil bacteria (demonstrating synergism) and highlight the importance of polyvalence in enhancing phage propagation in alternative hosts (e.g., soil bacteria) and significantly boosting suppression of potential enteric pathogens.

MATERIALS AND METHODS

Bacteria, Bacteriophage, and Culture Conditions. The bacterial strains in this study included *E. coli* K-12 (ATCC 700926), *P. putida* F1 (ATCC 700007), and *B. subtilis* 168 (ATCC 23857). Culturing was conducted in M63 glucose medium at 30 °C [12 g of KH_2PO_4, 28 g of K_2HPO_4, 8 g of $(NH_4)_2SO_4$ per liter water supplemented with 1 mM of $MgSO_4$, 0.2% glucose, and 0.5% casamino acids].[22] Coliphage T4 (Carolina Biological 12-4330), which was reported to infect only *E. coli* and closely related *Shigella* species,[23] infected *E. coli* K-12 but none of other tested bacterial strains. The polyvalent phage PEf1, which can infect both *E. coli* and *P. putida* (Figure S1) but not *B. subtilis*, was previously isolated by a sequential multihost approach.[19] Bacteria in exponential phase were used for phage treatment, phage enumeration, and phage characterization. Double-layer plaque assays[24] used tryptone agar plates containing 0.7% agar for the soft agar and 1.1% for the base layer. Phage titration was performed using *E. coli* K-12 as the host and expressed as plaque forming units (PFU) per milliter. The samples were centrifuged to obtain supernatant for extracellular phage titration. Phages were enumerated after direct dilution using the double-layer plaque assay. Quantitative phage abundance analysis was performed by observation of virus-like-particles (VLP) via fluorescence microscopy after staining with SYBR Green I according to the standard protocol.[25]

Bacteriophage Growth Parameter Characterization. The fraction of free phages was measured as a function of time in adsorption tests, and phage adsorption rate constants were determined based on the first-order kinetics: $kB = (\ln(P_0) - \ln(P_t))/t$, where k is the adsorption rate constant (mL/min), B is bacterial density (CFU/mL), P_0 is number of free phages at time 0 (PFU/mL), and P_t is number of free phages at time t (PFU/mL).[26] One-step growth curve experiments were conducted to measure the burst size and latent time of each phage.[27] Efficiency of plating (EOP) was quantified by calculating the ratio of phage plaque titers obtained with a given host to the phage plaque titers obtained with a reference strain[18] (i.e., *E. coli* K-12 in this case). All parameters were determined with bacteria in exponential phase in M63 medium at 30 °C (Table S1).

Bacterial Challenge Test in Planktonic State. To compare the inhibitory efficiency of phage T4 and PEf1 in planktonic states, 10 mL of 10^5 CFU/mL *E. coli* K-12 were subjected to the following treatments: (i) 10^6 PFU/mL phage T4, (ii) 10^6 PFU/mL phage PEf1, (iii) soil bacteria (1 *P. putida* and 1 *B. subtilis* per *E. coli*), (iv) soil bacteria phage mixture I (1 *P. putida*, 1 *B. subtilis* and 10 T4 per *E. coli*), and (v) soil bacteria phage mixture II (1 *P. putida*, 1 *B. subtilis*, and 10 PEf1 per *E. coli*). During the 72 h experiment, to avoid nutrient depletion and the accumulation of phage particles (which confounds analysis), samples were collected at 12 h intervals, and 0.1 mL of culture was transferred into 9.9 mL of fresh medium. The samples were centrifuged to obtain supernatant for extracellular phage titration and cell pellets for bacterial DNA extraction. Since cultures were diluted 100-fold each step, bacteria were considered completely eliminated when densities were below 100 CFU/mL.

To avoid interference by dead cells, ethidium bromide monoazide (EMA) was used to bind the DNA from dead cells.[20] Briefly, cell suspensions were stained with 20 μg/mL EMA (Sigma-Aldrich) in the dark for 5 min, placed on ice for 1 min, and exposed to bright visible light for 10 min prior to DNA extraction. Bacterial genomic DNA was then extracted using an UltraClean Microbial DNA Isolation Kit (MoBio, Carlsbad, CA) according to the manufacturer's instructions.

Challenge Tests with Bacteria Embedded in Soft Agar. The two-species mixture (10^5 CFU *E. coli* K-12 and *B. subtilis* 168, or 10^5 CFU *E. coli* K-12 and *P. putida* F1) and three-species mixture (10^5 CFU *E. coli* K-12, *B. subtilis* 168 and *P. putida* F1) were inoculated with coliphage T4 or polyvalent phage PEf1 (10^5 PFU) in the soft agar of double-layer plates. The plates were incubated for 12 h at 30 °C, and then three disk-shaped samples (2 cm in diameter) were randomly taken from the soft agar of each plate. The samples were physically ground, and bacterial DNA was extracted using a PowerSoil DNA Isolation Kit (MoBio, Carlsbad, CA). Phage λ DNA was used as an internal standard to calculate the recovery rate of all DNA extractions.[28]

Isolation and Characterization of BIMs. Bacteriophage-insensitive mutants (BIMs) resistant to phage PEf1 and T4 were isolated as follows.[29] The soft agar of double-layer plates was seeded with 10^7 CFU hosts and 10^9 PFU phage (multiplicity of infection, MOI = 100) and then incubated for 24 h at 30 °C. Surviving colonies were picked from the confluent lysis assay plate and streaked onto a nutrient agar. Bacterial contamination was excluded by using selective agar for plating (EMB agar for *E. coli* and *Pseudomonas* selective agar for *P. putida*) and verifying purity by colony PCR with specific biomarkers (*E. coli*-specific universal stress protein gene A[30] and *P. putida*-specific toluene dioxygenase gene *todC1*[31]). The ratio between the number of surviving colonies and the number of initially inoculated bacteria (10^7 CFU) was calculated as the BIM frequency.

BIMs were streaked once to avoid resensitizing and remove potentially coevolving phages.[32] Spot tests were conducted to confirm the phage resistance of new isolated BIMs.[18] To determine potential fitness costs associated with phage resistance development, growth kinetics and biofilm formation were measured. Bacterial specific growth rates were determined based on OD_{600} measurements during the 12 h exponential growth phase (all tested bacteria reached stationary phase after 12 h). The biofilm formed on the surface of 96-well tissue culture plates after a 24 h incubation was measured according to a microtiter dish biofilm formation assay.[33]

Inhibition of *E. coli* K-12 in Newly Established Biofilm. *E. coli* K-12, *P. putida* F1, and *B. subtilis* 168 (10^5 CFU/mL for each bacterium) were incubated in glass vials (Kimble 20 mL

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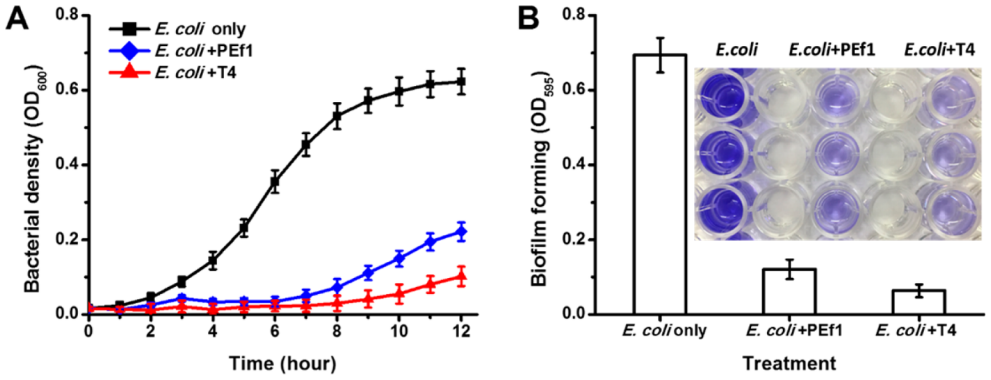

Figure 1. *E. coli* K-12 growth curve (A) and biofilm formation (B) in the presence of phage PEf1 or T4. The initial MOI was 1 for both tests. The biofilm formation was measured after 24 h of incubation. Coliphage T4 was more effective than polyvalent phage PEf1 at suppressing *E. coli* in pure culture. Error bars indicate ± one standard deviation from the mean of triplicate independent experiments.

Disposable Scintillation Vials) containing 5 mL of medium and 1.0 g of quartz sand (Figure S3). The microcosms were shaken at 240 rpm and 30 °C using a Multi-Therm shaker (Benchmark Scientific Inc. Edison, NJ). After 24 h incubation, the bulk solution was replaced with 5 mL of fresh medium inoculated with bacteria (10^5 CFU/mL for each strain) and phages (T4 or PEf1 at 10^6 PFU/mL) to adjust the initial MOI in the bulk solution to the same value used in the prior liquid culture experiments in order to facilitate comparison. The time point when phages were added was considered as time zero. No phages were added in the control group. During the 5 day experiment, 4.9 mL of liquid culture was replaced with an equal volume of fresh medium at 24 h intervals.

The bulk solution was collected and centrifuged to obtain supernatant for phage titration and cell pellets for bacteria enumeration. The sand was gently rinsed three times with 0.2 mL of PBS. Sand sampled was suspended in 0.2 mL of PBS supplemented with 0.05% (v/v) Tween-20 and gently sonicated at 40 kHz for 20 min in a 4 °C bath sonicator (Branson, Danbury, CT) to disrupt the biofilm matrix and disperse the bacterial cells and entrapped phages.[34] The 0.2 mL suspension was centrifuged to obtain the supernatant for phage titration and the cell pellet for bacteria quantification.

Primer Design and Real-Time Quantitative PCR Analysis. The *E. coli*-specific universal stress protein gene A (*uspA*) and toluene dioxygenase gene *todC1* were chosen as specific biomarkers to assess the abundance of *E. coli* K-12 and *P. putida* F1, respectively. Both genes were quantified by qPCR using SYBR Green. The bacterial 16S rRNA gene, quantified by Taqman qPCR, was used for the total bacteria enumeration.[35] The extracted genomic DNA from *E. coli* K-12 and *P. putida* F1 with different bacterial densities were used to establish the standard curve between threshold cycle (Ct) value and $\log_{10}$ CFU for each bacteria individually. Detailed information on SYBR Green qPCR and Taqman qPCR on primers and probes, reaction reagents, and temperature programs is included in the Supporting Information (Table S2).

Scanning Electron Microscopy of Biofilm on Sand Surface. The washed sand samples were fixed in 4% glutaraldehyde 0.1 M phosphate buffer solution (PBS, pH = 7.4) overnight at 4 °C. The samples were dehydrated in a series of ethanol−water solutions (50, 60, 70, 80, 90, 95, and 100%, vol/vol) for 30 min at 4 °C. The sand was placed on the carbon tape pasted stub and then sputter-coated with ∼7 nm gold film under vacuum using the Denton Desk V Sputter System. The microstructure of biofilm on the sand surface was observed using JEOL 6500 scanning electron microscopy. Duplicates were performed for samples from both control and phage treatment microcosms, and more than 10 representative images were captured for each sample.

Statistical Analysis. Student's *t* test (two-tailed) was used to determine the significance of the differences between treatments. Differences were considered to be significant at the 95% level ($p < 0.05$). The synergy quantification of combination treatments was performed using the Bliss independence model.[36] Combination indices (CI) were calculated as follows:

$$CI = (E_{Competitors} + E_{Phages} - E_{Competitors} \times E_{Phages}) / E_{Competitors+Phages} \quad (1)$$

where $E_{Competitors}$, E_{Phages}, and $E_{Competitors+Phages}$ represent suppression factors for *E. coli* caused by interspecific competition, phage infection, and the combination of interspecific competition and phage infection, respectively.[37] The Bliss model was selected because the modes of action for bacterial inhibition by interspecies competition and phage infection are independent. Each suppression factor was calculated as the ratio of *E. coli* density in the presence of a specific stressor (phages or competitors) to the density of unexposed *E. coli*.

RESULTS

Coliphage T4 Suppressed *E. coli* Monocultures More Effectively than Polyvalent Phage PEf1. *E. coli* growth in suspended pure culture was inhibited to a greater extent by T4 than by PEf1 (Figure 1A). Relative to uninfected controls, *E. coli* viability (i.e., the ratio between *E. coli* abundance in the phage-treated group to that in the phage-free control) after 12 h of incubation in the presence of T4 at MOI = 1 (i.e., one phage initially present per *E. coli* cell) was 12 ± 4%, which is significantly lower ($p < 0.05$) than 28 ± 4% in the presence of PEf1 at the same MOI. Similar results were observed for biofilm formation; T4 was more effective than PEf1 (10 ± 3% versus 18 ± 4% *E. coli* viability after 24 h exposure, $n = 3$, $p < 0.05$) at inhibiting *E. coli* growth in microtiter dish biofilm formation assays (Figure 1B). These results are consistent with the higher adsorption rate constant (2.3×10^{-9} vs 0.6×10^{-9} mL/min), shorter latent time (35 vs 50 min), and larger burst size (117 ± 8 vs 99 ± 5 PFU/cell) for T4 than PEf1 (Table

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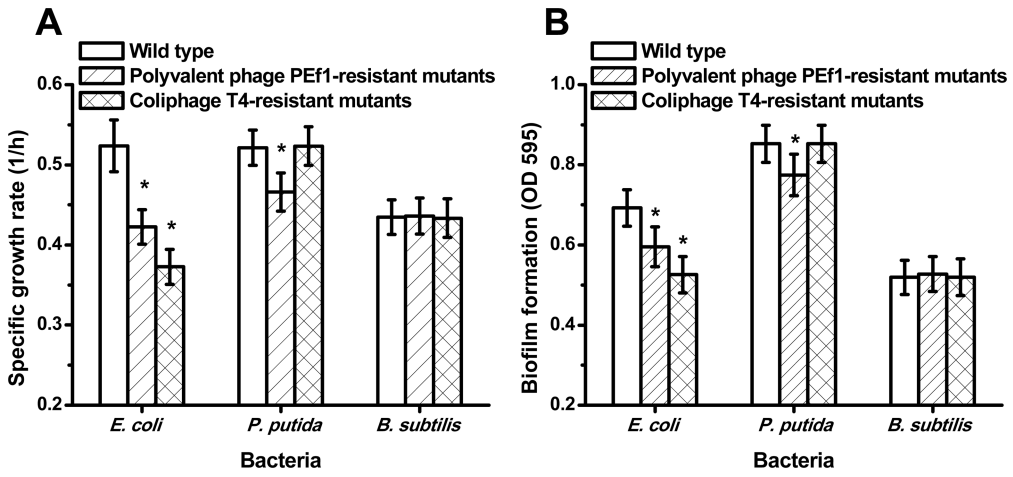

Figure 2. Fitness costs of bacterial hosts due to the development of phage resistance are reflected in the decreased specific growth rate (A) and biofilm formation capability (B). The specific growth rate was measured during the exponential growth phase (in the 12 h bacterial growth curve), and the biofilm formation was measured after 24 h incubation. *E. coli* is a host to both PEf1 and T4; *P. putida* is an alternative host to PEf1 but not to T4, and *B. subtilis* is not a host to either phage. Error bars represent ± one standard deviation from the mean of 10 replicates. The asterisks (*) represent a significant decrease ($p < 0.05$).

S1), which results in faster propagation and higher probability of infecting *E. coli* in monoculture.

***E. coli* Resistance to T4 Results in Higher Fitness Costs than Resistance to PEf1.** The development of resistance is a common bacterial response to phage infection, but a given host may experience different susceptibility and fitness costs for different phages. We assessed the BIM frequency of *E. coli* exposed to T4 or PEf1 (MOI = 100) and compared the resulting fitness costs associated with phage resistance. The BIM frequency of *E. coli* with T4 was $6.2 \pm 1.1 \times 10^{-7}$, which is significantly lower ($p < 0.05$) than that of *E. coli* toward PEf1 ($3.8 \pm 0.5 \times 10^{-6}$). The resistance to T4 decreased the (exponential phase) specific growth rate of *E. coli* by 28.8 ± 3.6% (from 0.52 ± 0.03 to 0.37 ± 0.02 h^{-1}), compared to a 19.3 ± 1.6% decrease (from 0.52 ± 0.03 to 0.42 ± 0.02 h^{-1}) associated with resistance to PEf1 (Figure 2A). Similarly, the 24 h biofilm formation of *E. coli* resistant to T4 decreased by 22.4 ± 2.1% (from 0.69 ± 0.04 to 0.53 ± 0.04 OD_{595}), while that of *E. coli* resistant to PEf1 decreased by only 14.3 ± 2.9% (from 0.69 ± 0.04 to 0.60 ± 0.05 OD_{595}) (Figure 2B). The higher fitness cost associated with resistance to T4 than to PEf1 is consistent with the more effective suppression of *E. coli* pure cultures by T4.

Polyvalent Phage PEf1 Proliferates to a Greater Extent and Is More Effective than T4 at Suppressing *E. coli* in Mixed Cultures under Simulated Biofilm and Sandy Slurry Conditions. Although PEf1 was less effective than T4 in suppressing *E. coli* in monoculture (Figure 1), it was significantly more effective than T4 under more realistic mixed cultures in both sandy slurry microcosms (Table 1, Figure 3, and Figure 4A) and in soft agar microtiter formation assays (Table S3 and Figure 4B). In these mixed cultures, polyvalence enhanced PEf1 propagation in other hosts (i.e., *P. putida* F1 serving as alternative host for PEf1[19]) and reached significantly higher concentrations than T4 in the bulk solution (7.88 ± 0.16 vs 6.83 ± 0.17 $\log_{10}$PFU/mL, $p < 0.05$), which enhanced *E. coli* suppression.

In sandy slurry microcosms, PEf1 could reproduce in both *E. coli* and *P. putida* and reached higher (10-fold) concentrations than T4, which could reproduce only in *E. coli* (Figure 3 and Figure S5). Higher PEf1 concentrations in mixed cultures

Table 1. Mean Bacterial and Viral Densities[a] in the Microcosms over the 5 day Incubation Period

phase	component	treated with T4	treated with PEf1
bulk solution	phage (T4 or PEf1)	6.8 ± 0.3	7.9 ± 0.3
	E. coli K-12	5.7 ± 0.3	4.8 ± 0.2
	P. putida F1	8.3 ± 0.1	7.5 ± 0.3
sand surface	*E. coli* K-12	5.4 ± 0.3	4.5 ± 0.2
	P. putida F1	6.9 ± 0.4	5.9 ± 0.4

[a]Units are $\log_{10}$ CFU/mL for bacteria in bulk solution, $\log_{10}$ CFU/mg for bacteria on sand surface, and $\log_{10}$ PFU/mL for phages.

facilitated *E. coli* infection and suppression (Figure 3 and Figure 4A). *E. coli* concentrations in the bulk liquid stabilized after 3 days at 4.7 ± 0.1 $\log_{10}$CFU/mL in the presence of PEf1, which is 1.3 orders of magnitude lower than in microcosms with T4 (Figure 3). PEf1 also suppressed *E. coli* to a greater extent and for a longer duration than T4 in the mixed-culture biofilm attached on the sand surface (Figure 4A). Prior to phage amendment, the initial densities of *E. coli* in newly formed mixed-species biofilm were 5.64 ± 0.15 $\log_{10}$ CFU/mg sand (Figure 4A). After PEf1 amendment, the attached 5 day *E. coli* density decreased by 93 ± 2% to 4.51 ± 0.21 $\log_{10}$ CFU/mg sand, while that in microcosms amended with T4 it increased by 44 ± 26% to 5.80 ± 0.23 $\log_{10}$ CFU/mg sand (Figure 4A). Compared to controls without phage, PEf1 reduced attached *E. coli* viability by 2.4 orders of magnitude, while T4 achieved only 1.1 order of magnitude suppression. Furthermore, scanning electron microscopy (SEM) of the sand biofilm revealed significantly greater disruption in microcosms treated with PEf1 than those treated with T4 (Figure 5).

Additional experiments were conducted with bacteria embedded in soft agar plates to assess the effect of phages under biofilm-like conditions without confounding effects such as phage reproduction in suspended hosts or recolonization from the bulk solution. Whereas soft agar tests do not represent the complexity of biofilms commonly encountered in natural and engineered systems, they offer a simple system to investigate factors that influence bacterial attachment and biofilm formation in reductionist experiments that exclude such confounding factors.[38,39] When *E. coli* and *B. subtilis* were

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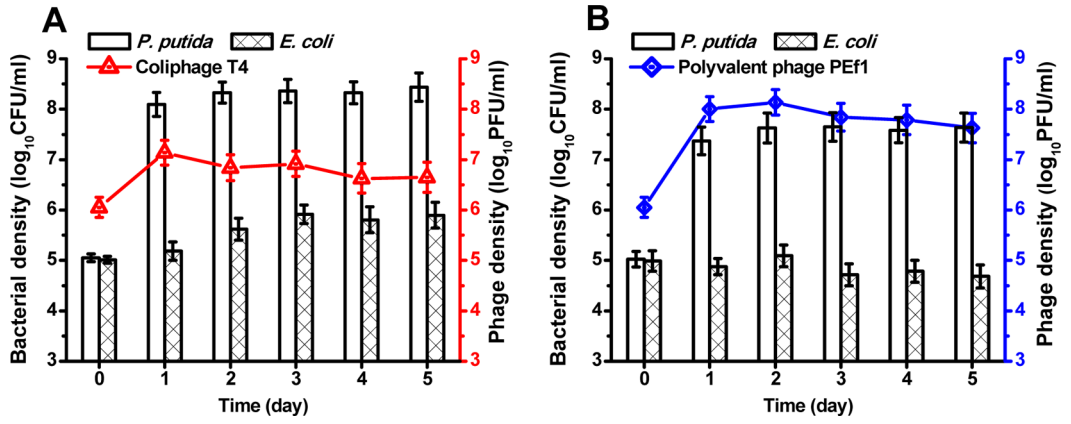

Figure 3. Bacterial and phage densities in the bulk solution when coliphage T4 (A) or polyvalent phage PEf1 (B) was introduced to the mixed-species sand slurry microcosms. PEf1 was able to reproduce in both *E. coli* and *P. putida*, reaching higher densities than T4. Error bars indicate ± one standard deviation from the mean of triplicate independent experiments.

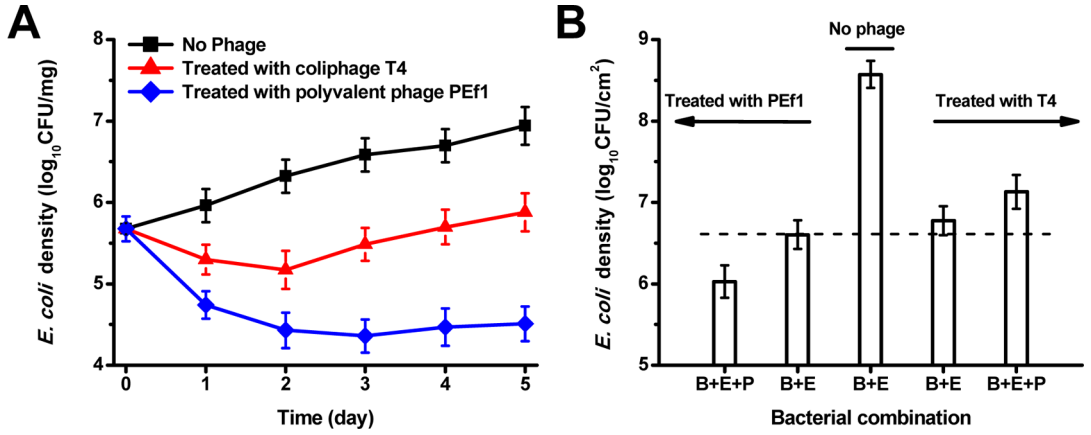

Figure 4. Comparison of PEf1 and T4 efficacy to suppress *E. coli* K-12 in mixed-species sand slurry microcosms (A) and in soft agar (B). *E. coli* K-12 densities in soft agar were quantified after 12 h of incubation. B, E, and P represent *B. subtilis* 168, *E. coli* K-12, and *P. putida* F1, respectively. PEf1 could infect both *E. coli* and *P. putida*, suppressing *E. coli* to a greater extent, even though T4 was a more effective suppressor in pure culture (Figure 1). Error bars indicate ± one standard deviation from the mean of triplicate independent experiments.

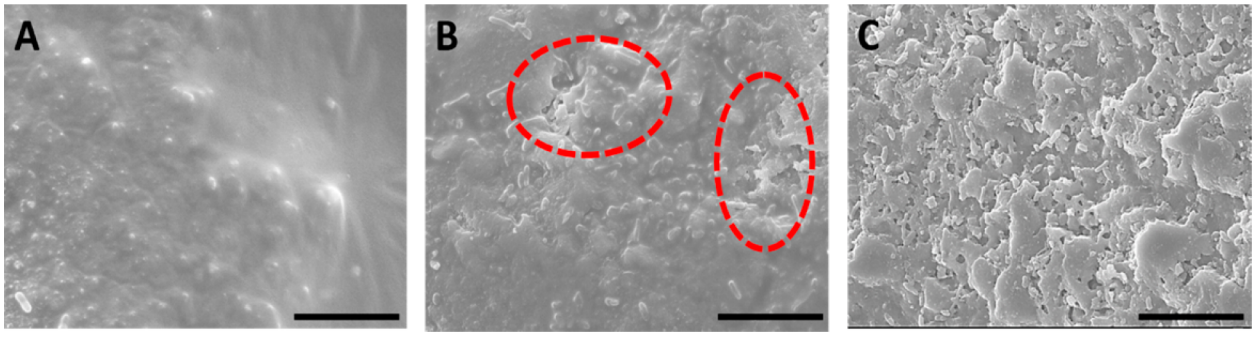

Figure 5. SEM images of intact biofilm without phage treatment (A) and damaged biofilm treated with T4 (B) and PEf1 (C). Sand samples were taken at the end of the 5 day microcosms tests. The biofilm (*E. coli*, *P. putida*, and *B. subtilis*) treated with T4 was partially damaged (red circles), while that treated with PEf1 was damaged to a greater extent. The scale bar represents 10 μm.

cocultured without phages, the *E. coli* density reached 8.57 ± 0.17 $\log_{10}$ CFU/cm^2 after 12 h (Figure 4B). *E. coli* densities decreased dramatically when the bacteria were challenged with either PEf1 (6.60 ± 0.18 $\log_{10}$ CFU/cm^2) or T4 (6.77 ± 0.18 $\log_{10}$ CFU/cm^2) (Figure 4B, $p < 0.05$). Notably, when *P. putida* was also present, a lower *E. coli* density (6.06 ± 0.20 $\log_{10}$ CFU/cm^2) was observed for the community challenged with PEf1, while the *E. coli* density increased to 7.15 ± 0.21 $\log_{10}$ CFU/cm^2 in the community challenged with T4 (Figure 4B). Hypothetically, *P. putida* served as a physical barrier for T4 diffusion through static hindrance and off-target adsorption (T4 adsorption constant to *P. putida* was 0.1 × 10^{-9} mL/min), protecting *E. coli* from phage infection. In contrast, *P. putida* enhanced PEf1 proliferation, facilitating its propagation through the biofilm.

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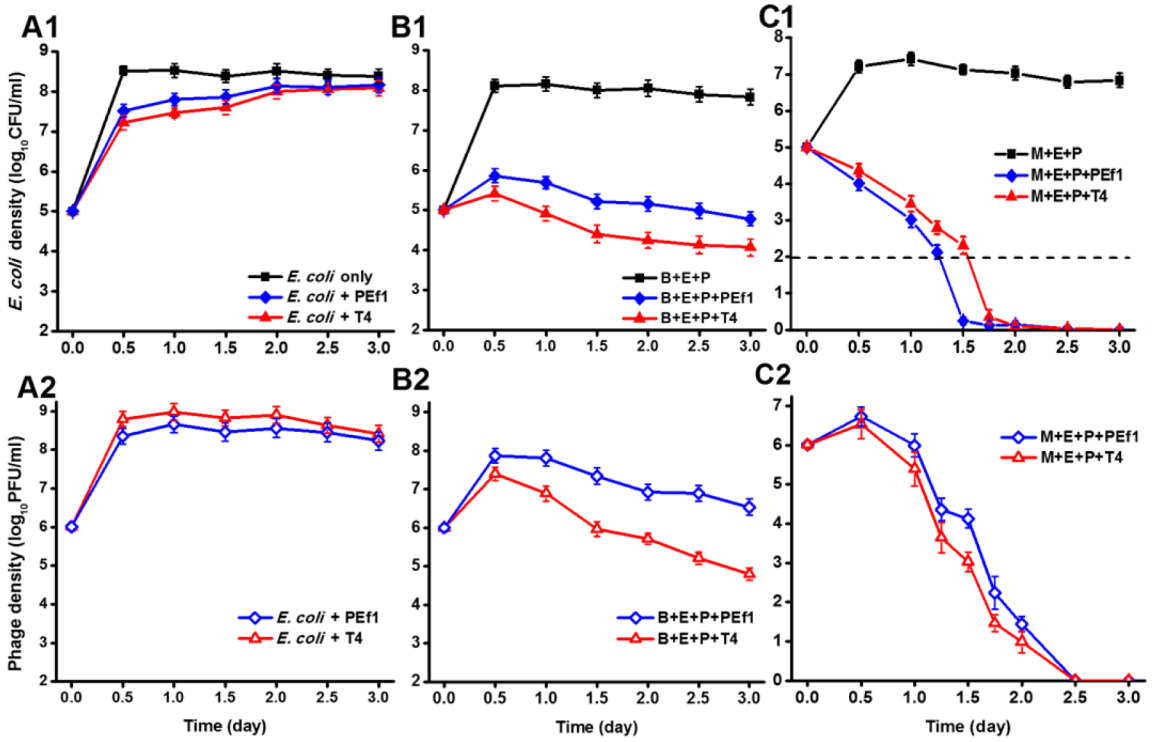

Figure 6. Batch growth experiment with *E. coli* K-12 exposed to polyvalent phage PEf1 or coliphage T4 in different bacterial mixtures. Panels A1 and A2 depict the densities of *E. coli* and phages in monoculture. Panels B1 and B2 depict densities in mixed culture with *B. subtilis* 168 (B), *E. coli* K-12 (E) and PEf1 host *P. putida* F1 (P). Panels C1 and C2 depict densities in more complex culture; M represents a mixture of soil bacteria: *B. subtilis* 168, *B. subtilis* subsp. *subtilis*, *Pseudomonas* subsp. CF600, *Pseudomonas nitroreducens*, *Shewanella oneidensis* MR-1, and *Serratia marcescens*. All strains were added at equal initial concentrations as *E. coli*. Error bars indicate ± one standard deviation from the mean of six independent experimental replicates.

Interspecies Competition Synergistically Contributes to *E. coli* Suppression. Additional bacterial challenge tests were conducted under planktonic conditions to discern how interspecies competition contributes to *E. coli* suppression without the confounding effects of restricted phage diffusion. In the absence of phages, the presence of bacterial competitors (1 *P. putida* and 1 *B. subtilis* per *E. coli*) decreased *E. coli* density by 0.55 ± 0.03 $\log_{10}$ CFU/mL after 3 days (Figure 6B1). Thus, interspecies competition alone suppressed *E. coli* by 72.1 ± 3.0%. Significantly higher suppression of *E. coli* was observed by the combined effects of phages plus competing soil bacteria (Figure 6A1 and B1). For example, after 3 days we observed 99.990 ± 0.002% *E. coli* suppression for the combined stress of T4 plus *P. putida* and *B. subtilis* versus 50.3 ± 3.4% individually for T4 alone. The corresponding numbers for PEf1 were 99.974 ± 0.003% for combined versus 43.8 ± 2.8% for phage alone. The Bliss independence model was used to determine whether this combined effect of competing bacteria and phages was synergistic. The combination indices were below 1.0 for all time points (Table 2), indicating significant synergistic interactions.

Individual competition by *P. putida* or *B. subtilis* in two-species mixed cultures decreased the 12 h *E. coli* abundance by 51 ± 6% and 32 ± 4%, respectively (from 8.52 ± 0.11 $\log_{10}$ CFU/mL for pure *E. coli* cultures to 8.21 ± 0.10 CFU/mL for cultures with *P. putida* and 8.35 ± 0.11 $\log_{10}$ CFU/mL for cultures with *B. subtilis*) (Figure S6,). Thus, *P. putida* was a more effective competitor than *B. subtilis* under the tested conditions. When both *P. putida* and *B. subtilis* were used as competitors in the planktonic bacterial challenge tests, PEf1 reached significantly higher concentrations (3- to 45-fold higher) than T4 (Figure 6B2) due to its ability to use *P. putida* as an alternative host. Surprisingly, the less abundant T4 exerted higher *E. coli* suppression (Figure 6B1). Specifically, the mean *E. coli* density treated with T4 (4.53 ± 0.45 $\log_{10}$ CFU/mL) was significantly lower ($p < 0.05$) than that treated with PEf1 (5.28 ± 0.35 $\log_{10}$ CFU/mL). Apparently, higher *E. coli* due to enhanced PEf1 propagation was offset by *P. putida* lysis (i.e., as shown in Figure S6A, its concentration decreased from 8.37 ± 0.09 $\log_{10}$ CFU/mL for cultures with T4 to 7.61 ± 0.14 CFU/mL for cultures with PEf1), which attenuated interspecies competition.

As the number of competing soil species increased (equal initial concentrations of *B. subtilis* 168, *P. putida* F1, *B. subtilis* subsp. *subtilis*, *Pseudomonas* sp. CF600, *Pseudomonas nitroreducens*, *Shewanella oneidensis*, *Serratia marcescens*, and *E. coli*), the suppression of *E. coli* was more pronounced for both T4 and PEf1, resulting in the complete elimination of *E. coli*

Table 2. Synergistic Suppression of *E. coli* by Interspecific Competition and Phage Infection

time (day)	competitors[a] + T4 (CI[b])	competitors + PEf1 (CI)
0.5	0.98 ± 0.01*	0.96 ± 0.02*
1.0	0.97 ± 0.01*	0.92 ± 0.01*
1.5	0.92 ± 0.02*	0.87 ± 0.04*
2.0	0.90 ± 0.02*	0.85 ± 0.02*
2.5	0.86 ± 0.03*	0.86 ± 0.03*
3.0	0.85 ± 0.02*	0.85 ± 0.02*

[a]*B. subtilis* 168 and *P. putida* F1 were used as competitors. [b]Combination indices (CI) (eq 1). [c]The asterisks (*) represent significantly smaller than 1.0 ($n = 6$, $p < 0.05$).

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(Figure 6C1). After 12 h, both T4- and PEf1-treated cultures displayed continually decreasing *E. coli* concentrations in tandem with decreasing phage titers, a pattern indicative of successful bacterial control.[40] In such more complex cultures, competition is exacerbated, and suppression of a single competitor (e.g., *P. putida*) is less critical in mitigating interspecies competition. Accordingly, in contrast to the simpler three-species culture, *E. coli* suppression was faster in the presence of PEf1 than T4, with complete elimination occurring in 36 vs 42 h (Figure 6C1 and 6C2).

DISCUSSION

Phages have been described as the "dark matter of the biosphere" because, though they are the most dominant biological entities in terms of numbers, diversity, and ubiquity, we still know relatively little about them.[5,41] Viral metagenomic studies have begun to shed light on bacteriophage diversity in various environments.[42−44] However, how phages behave in and influence such complex communities is still largely unknown. Historically, most phages have been considered to have relatively narrow host ranges, with polyvalent phages being the exception.[3] Nevertheless, recent studies have shown that polyvalent phages may be more widespread than previously assumed,[15,16,45−48] a finding that has important implications for their potential impact on horizontal gene transfer and nutrient cycling, and as drivers of microbial diversity. A major goal of this study was to determine how phage dynamics are influenced by polyvalence in both liquid culture and biofilms and whether this information could be applied to enhance the suppression of problematic enteric bacteria in microbial communities.

Consistent with previous studies,[12] combining bacterial competitors with phages (PEf1 or T4) resulted in significantly greater inhibition of *E. coli* growth than separate treatments. The resulting Bliss combination indices were below 1.0 for all time points (increasing with incubation time), which indicates significant synergistic interactions (Table 2). One possible explanation for the observed synergy is that BIMs acquired resistance by losing or modifying genes coding for proteins that phages might use as receptors,[49] and this could incur fitness costs such as reduced motility, suppressed growth rate or decreased biofilm formation capability, which would make BIMs more susceptible to interspecies competition. Another possibility is that interspecies competition decreases nutrient and substrate availability to the target bacteria, thus delaying the development of phage resistance.[50] This would make the target bacteria more vulnerable to phage infection. Whereas further research is needed to elucidate the underlying mechanisms for the observed suppression synergism, these results suggest that phage therapy or biocontrol would be more effective in combination with compatible microbial control strategies.

In surveys of the natural environment, the most dominant species in any given community generally represents a mere 2−5% of the population.[51] Since most phages do not sustainably reproduce below a bacterial density of about 10^4 CFU/mL,[52] and many ecosystems contain greater than 100 different species of bacteria, a monovalent phage would only be expected to reproduce sustainably in well-mixed, planktonic environments (representative of most lab-scale studies) containing greater than 10^6 CFU/mL. However, the vast majority of bacteria in the environment exist embedded in a biofilm or in microcolonies,[53,54] where there is little mixing to increase the likelihood of a collision between a phage and its host. Since diffusion controls phage dispersion within a biofilm, phage properties (e.g., adsorption affinity and host range) as well as the attached microbial community structure might have a significant impact on phage propagation[55] and, therefore, the response of the bacterial host within the biofilm.[56] Indeed, high adsorption rates have been found to be detrimental to phage fitness in biofilm-like environments due to lower phage emigration after lysis.[38]

Our results demonstrate that phage efficacy to suppress target bacteria in a biofilm is also greatly affected by the composition of the microbial community. In two-species biofilms of *E. coli* and *B. subtilis*, we observed little difference between T4 and PEf1. However, the addition of *P. putida* (a PEf1 host) caused a decrease in T4 efficacy, while it improved the ability of PEf1 to suppress *E. coli* (Figure 4B). This is intuitive because *P. putida* not only facilitated PEf1 propagation, but also likely hindered T4 diffusion. Interestingly, PEf1 also suppressed *E. coli* more effectively than T4 in the bulk solution of microcosms containing the same three-species mixture (Figure 3A and B), but not in liquid batch culture without biofilm (Figure 6B1). Our data suggest that this occurred because the biofilm in the microcosm may have partly served as a protective bacterial refuge. Specifically, concurrently high phage and bacterial densities generally indicate poor bacterial suppression due to the presence of bacterial refuges (e.g., biofilms and microcolonies).[10,40] This was observed for microcosms with T4, where phage densities were over 100-fold higher in the bulk solution (Figure 3A) than in liquid batch cultures (Figure 6B2) at comparable time points. Apparently, biofilm-associated *E. coli* was not easily accessible to T4. In contrast, biofilm-associated *E. coli* was more accessible to PEf1 despite its slower proliferation than T4 in *E. coli* (Table S1), likely due to enhanced proliferation and propagation by the presence of additional hosts, and the resulting higher suppression is conducive to fewer *E. coli* cells repopulating the bulk solution (Figure 3A and B). Importantly, PEf1 genome sequencing did not reveal the presence of depolymerases that may have contributed to its ability to suppress biofilm growth; thus, the enhanced efficacy of PEf1 is putatively due to its expanded host range and lower adsorption rate constant.

The literature includes some contradictory results on the efficacy of phages to exert long-term suppression of target bacteria, possibly confounded by the development of phage resistance that facilitates coexistence. Interspecies competition is also an influential factor, often enhancing phage efficacy to suppress target bacteria. For example, some phages have been shown to exert long-term suppression of a target bacterial population in a natural soil community, but not in monocultures.[57] Both phage host range and bacterial growth conditions (planktonic vs biofilm) can also affect suppression of target bacteria. For instance, a study with narrow host-range phages reported long-term suppression of a planktonic, two-species bacterial community,[12] while similar tests did not result in significant suppression in either single or mixed-culture biofilms,[10] which agrees with our finding of the more difficult suppression of *E. coli* in biofilms.

An inability to exert long-term suppression of biofilm-associated target bacteria would pose a significant challenge to the use of phages for microbial control,[58] since most bacteria live within biofilms.[54] Promisingly, several recent studies (including this one) have demonstrated varying levels of success for phages in controlling target bacterial populations within biofilms.[59−62] However, unlike this work, previous publications did not consider polyvalent phages in mixed

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cultures, where both intra- and interspecies competition and complex phage propagation dynamics may substantially affect suppression efficacy. In contrast to prior studies that used narrow host-range phages, we find that polyvalent phages may provide a new approach for the long-term suppression of problematic bacteria in both planktonic and biofilm mixed-species systems.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.est.7b00529.

Phage plaques images (Figure S1), *P. putida* monoculture growth curves in the presence of different phages (Figure S2), SEM images of sand surface (Figure S3), fluorescence microscopic images of viral-like-particles (Figure S5), additional *E. coli* growth data in the presence of various combinations of competing bacteria and virus (Figure S4, Figure S6, Figure S7, and Figure S8), phage growth parameters (Table S1), primers and probes for qPCR analysis (Table S2), and *E. coli* growth data in soft agar (Table S3) (PDF)

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