



MICROBIOLOGY

Essential phage component induces resistance of bacterial community

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Despite extensive knowledge on phage resistance at bacterium level, the resistance of bacterial communities is still not well-understood. Given its ubiquity, it is essential to understand resistance at the community level. We performed quantitative investigations on the dynamics of phage infection in *Klebsiella pneumoniae* biofilms. We found that the biofilms quickly developed resistance and resumed growth. Instead of mutations, the resistance was caused by unassembled phage tail fibers released by the phage-lysed bacteria. The tail fibers degraded the bacterial capsule essential for infection and induced spreading of capsule loss in the biofilm, and tuning tail fiber and capsule levels altered the resistance. Latent infections sustained in the biofilm despite resistance, allowing stable phage-bacteria coexistence. Last, we showed that the resistance exposed vulnerabilities in the biofilm. Our findings indicate that phage lysate plays important roles in shaping phage-biofilm interactions and open more dimensions for the rational design of strategies to counter bacteria with phage.

INTRODUCTION

Because of the widespread occurrence of antibiotic resistance, there has been increased interest in eradicating pathogens using bacteriophage (1, 2). A major obstacle in the application of phages is resistance, as bacteria can develop resistance to phages within a short period of time (3, 4). Various resistance mechanisms have been uncovered, including mutating the receptors for phage binding (4), using restriction modification (5), and evolving CRISPR-Cas systems (6); more recently, multiple families of antiphage systems were discovered (7–9). These are all cellular-level mechanisms. However, bacteria usually live in communities (10, 11), and community-level resistance is much less understood. Different from cellular-level resistance, community-level resistance is often phenotypic (12). They do not usually come with the fitness cost that often accompanies genotypic resistance (1, 12); they are hard to circumvent, while genotypic resistance can often be overcome by the phage through rapid evolution (13, 14). Therefore, it is important to understand the mechanisms of community-level resistance.

Now known community-level resistance mechanisms include reducing phage receptor expression through quorum sensing (15, 16), limiting phage access through dense packing of the bacteria and secretion of extracellular matrix (17–20), reducing phage replication through forming metabolically dormant regions (21, 22), and avoiding phage predation through refuge sites in spatially heterogeneous environments (12, 23, 24). More recently, it was shown that some bacteria could become resistant by shedding their cell wall using the endolysin released by the phage-lysed bacteria in the community (25). Although this phenomenon is limited to Gram-positive bacteria under certain osmotic pressure, it points to an overlooked direction that the content of bacterial lysate could be a source of resistance, and it may play important roles in shaping a broad range of bacteria across a diverse spectrum of environments.

Bacterial lysate contains newly replicated phage progenies. Those phages are usually assembled in a modular fashion during replication. For tailed phages, their major parts—capsid, tail, and tail fiber—are first assembled separately and then joined together (26). It was found that some phages produce disproportional amount of tail fibers relative to other structural components (27), which may result in free tail fibers in the lysate and potentially affect phage-bacteria interaction (28). Many pathogenic bacteria have dense layer of capsules on their surface, which are polysaccharides consisted of repeating oligosaccharide units that may be either linear or branched (29). The capsules usually mask the molecules on the membrane of the bacteria, which prevents direct binding of those potential receptors by phages (30). Therefore, many phages have evolved the ability to use the capsule as their primary receptor; they first bind to the capsule and then degrade the capsule, which enables the phages to access the secondary receptors on the cell membrane and to inject their genetic material into the bacteria (31). The binding of the capsule is usually carried out by phage tail fibers; in addition, the tail fibers often also function as depolymerases that hydrolyze the capsule (28). Here, we showed that the free tail fiber protein gp46 in the lysate played important roles in shaping *Klebsiella pneumoniae* biofilms and caused resistance of the biofilm to phage. The resistance provides a mechanism for the coexistence of bacteria with phage. We also showed that the resistance exposes vulnerabilities in the biofilm, which could be exploited to counter bacteria with phage.

RESULTS

***K. pneumoniae* biofilm developed phenotypic resistance to phage infection**

We cultivated *K. pneumoniae* biofilm in a microfluidic system developed in our lab (Fig. 1A and Materials and Methods) (32). Briefly, we loaded the bacteria to a designated location in the microfluidic chip, and they formed a stable and densely packed community in the growth chamber. The growth chamber has a length of 10 mm, width of 2.4 mm, and thickness of 6 μ m. The large lateral dimension allows the formation of big communities containing millions of bacteria while maintaining steady medium flow for extended durations;

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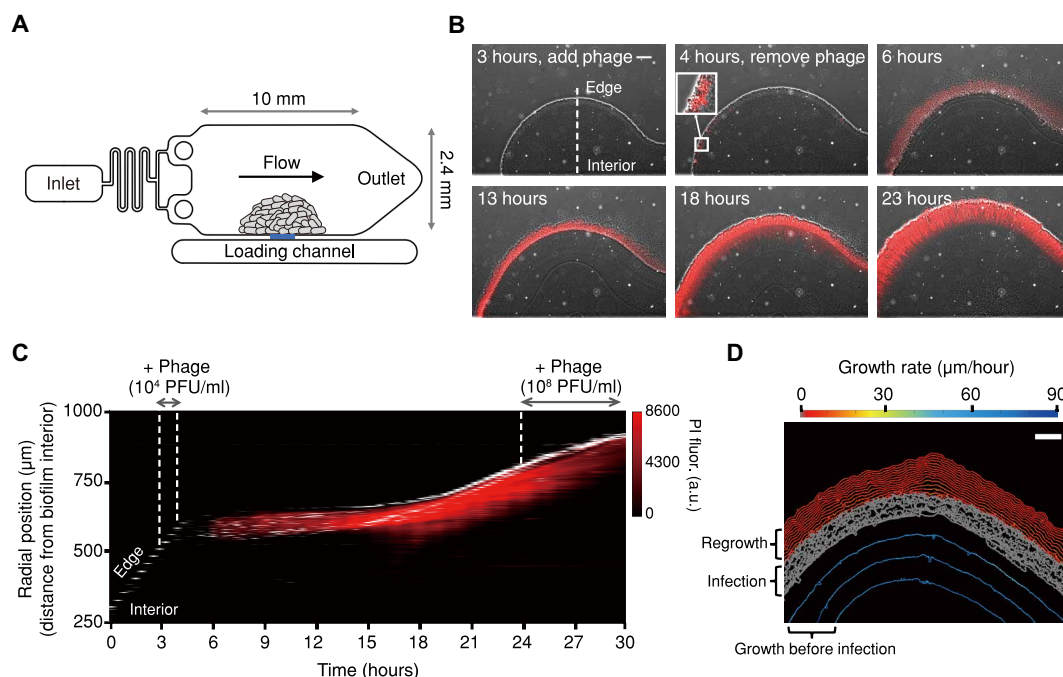


Fig. 1. Spatial-temporal dynamics of phage infection of *K. pneumoniae* biofilm. (A) Schematic diagram (top view) of the microfluidic system used in this study. Growth medium is injected in the inlet, flow through the growth chamber, and flow out of the outlet. At the beginning of each experiment, a small number of bacteria are injected through the loading channel to the seeding zone (marked in blue); those bacteria later grow into a large densely packed community in the growth chamber. (B) Representative images of the biofilm during different stages of phage infection. Composite of phase contrast and PI (a cell death indicator) fluorescence (shown in red). Scale bar, 100 μm. (C) Kymograph (spatial-temporal profile) of the biofilm. The y dimension indicates the spatial profile of PI fluorescence along the dashed line shown in (B). The x dimension indicates how the spatial profile evolved over time. The white outline indicates the location of biofilm edge. PI fluorescence is shown in red. The dashed lines indicate the timing of phage treatments. (D) Contour plot of biofilm edge. The lines illustrate the location of biofilm edge at 1-hour intervals. The colors indicate growth rate of biofilm at corresponding moments. Scale bar, 100 μm. Results are representative of more than three biological replicates. a.u., arbitrary units.

the small vertical dimension shapes the biofilm into semi-two-dimensional with uniform thickness (6 μm). Because of the dense packing of the bacteria in the vertical dimension, only the biofilm periphery is in direct contact with the flowing medium, and nutrients in the medium could only reach the biofilm interior through diffusion. This setting allows us to mimic the spatial heterogeneity commonly observed in natural biofilms and, at the same time, facilitates quantitative measurements of the entire community (32, 33). When the biofilm reached a size of ~500 μm, we treated the biofilm with a low titer of phage Kp11 (GenBank: ON148528.1), a lytic *Podoviridae* phage that targets *K. pneumoniae* with K2 capsule (34). Specifically, we switched to medium containing 10^4 plaque-forming units (PFU)/ml of Kp11 and then switched back to normal medium after 1 hour, and the flow rate was kept constant throughout the process. At the end of the 1-hour treatment, we found that a few local regions at the biofilm periphery were infected by the phage, as shown by the fluorescent cell death indicator propidium iodide (PI) (Fig. 1B). As a control, there is only low level of cell death that happens sporadically in the biofilm without phage treatment (fig. S1). Approximately 2 hours later, the infection spread to the entire periphery of the biofilm, substantial lyses of the biofilm was observed, and biofilm growth stopped (Fig. 1, B and C, and movie S1). Since we had removed the external supply of phage, the spreading of infection in the biofilm was driven by the replication of the phage. Unexpectedly, the biofilm resumed growth at ~10 hours after the phage treatment, although with significantly slower growth rate

than before (Fig. 1C, 10 versus 70 μm/hour before infection). We then switched to medium containing high titer (10^8 PFU/ml) of Kp11 but found that the growth rate of the biofilm was not affected, showing that the biofilm acquired resistance to Kp11 (Fig. 1C). This resumption of growth was also evident when biofilms of other *K. pneumoniae* strains were infected with their corresponding phages (fig. S2, A to C).

When the biofilm resumed growth after phage infection, the growth was uniform throughout the periphery of the biofilm (Fig. 1D). This suggests that the resistance developed by the biofilm was phenotypic, as genotypic resistance caused by mutations is stochastic and should only result in local resumption of growth. Therefore, we measured whether the bacteria generated during the uniform regrowth of the biofilm were resistant to the phage. Specifically, we treated the biofilms with Kp11 until they showed uniform regrowth, collected all the bacteria from the microfluidic chamber, and counted the percentage of cells that are resistant to the phage by culturing them on agar plates containing high titer of Kp11 (Fig. 2A and Materials and Methods). We found that a small percentage (<3%) of the isolated bacteria were resistant to Kp11 (Fig. 2B and fig. S3A). Those resistant bacteria are likely mutants generated spontaneously, as we also isolated a small percentage of resistant bacteria from biofilms that were never treated with phage (fig. S3A). Since the percentage of resistant bacteria was far lower than the percentage of bacteria generated during the uniform regrowth (16.61 to 58.49%, depending on duration of regrowth)

(Fig. 2B and fig. S3B), we concluded that the uniform regrowth was due to phenotypic resistance formed by the biofilm.

Phage tail fiber rendered the biofilm resistant to phage infection

Our *K. pneumoniae* strain was predicted to have multiple phage defense systems (fig. S2D) (35), which could potentially inactivate the invading phages. However, knocking out those defense systems did not abolish the resistance of the biofilm to Kp11 (fig. S2, E to L), suggesting that the resistance was due to other mechanism. We noticed that a bright band appeared in the phase contrast images of the biofilm after the phage infection, and the band moved toward biofilm interior as infection progressed (Fig. 2C, indicated by red arrows). To see whether it was related with the resistance of the biofilm, we

investigated the cause of the band. We first tested whether it was caused by diffusion of phage into the biofilm. Specifically, we labeled Kp11 with the fluorescent dye fluorescein isothiocyanate (FITC) and treated the biofilm with the labeled phage. We found that FITC fluorescence could only penetrate ~60 μm into the biofilm periphery (Fig. 2, D and E, and fig. S4, A and B); we confirmed that the labeled phage infected the bacteria normally (fig. S4C). This result shows that phage penetration was limited to the periphery region of the biofilm, and the phase band was due to diffusion of other molecules. We wondered whether it was caused by unassembled components from phage replication. We found that the purified phage tail fiber protein gp46 could induce the same band in the biofilm (Fig. 2F and fig. S5A). After 3 hours of treatment with gp46, the biofilm became resistant to high titer (10^7 PFU/ml) of phage (Fig. 2G). These results

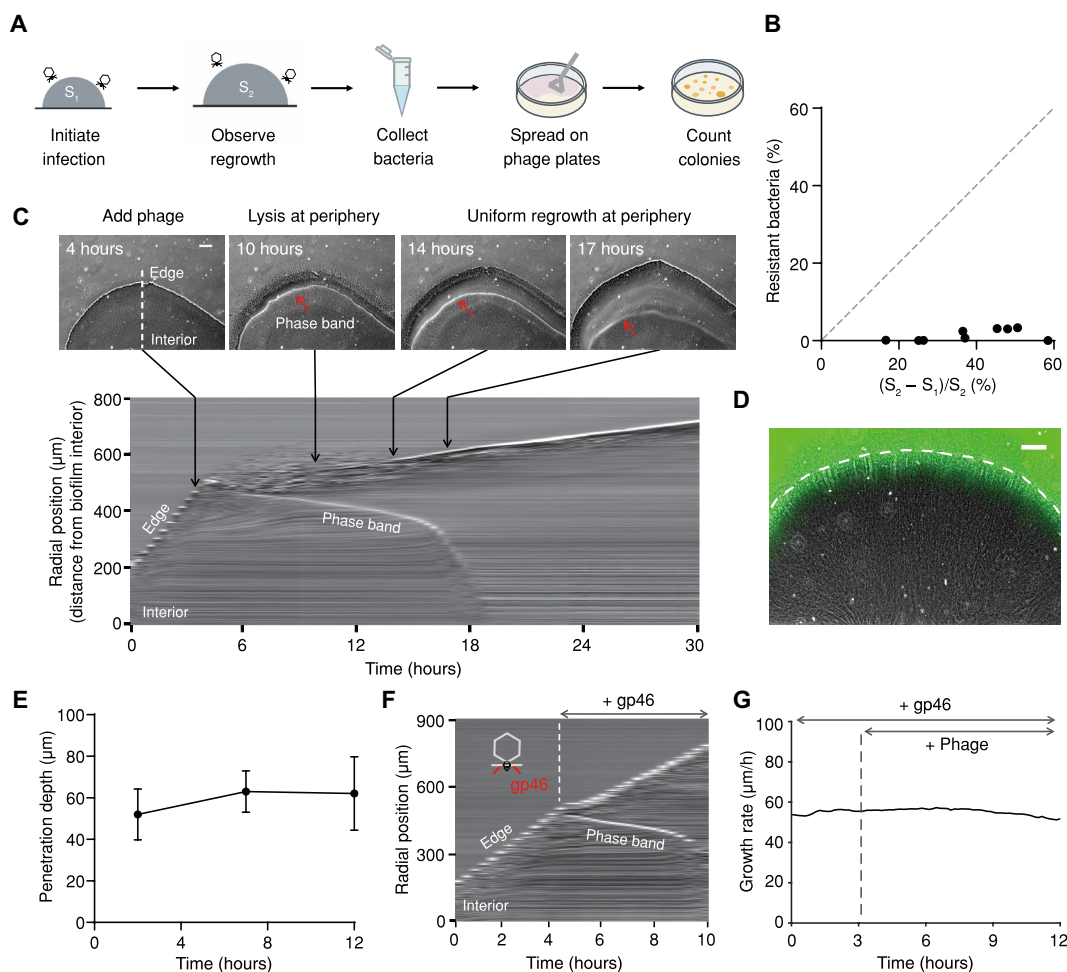


Fig. 2. Phage tail fiber protein renders the biofilm resistant to phage infection. (A) Workflow for measuring percentage of resistant bacteria in the biofilm (Materials and Methods). S₁, area of the biofilm right before phage treatment; S₂, area of the biofilm right before collecting bacteria. (B) Percentage of resistant bacteria in the biofilm. The dashed line indicates the expected relationship between the percentage of resistant bacteria and the percentage of biofilm size increase, if the resistance was caused by mutation. (C) Kymograph of the biofilm upon phage infection. The y dimension indicates the spatial profile of phase contrast intensity along the dashed line shown in the snapshot. The x dimension indicates how the spatial profile evolved over time. The black arrows indicate the time of the representative phase contrast images shown above the kymograph. The red arrows indicate the phase band induced by phage treatment. Scale bar, 100 μm . (D) Composite of phase contrast (gray) and fluorescent (green, indicates concentration of FITC-labeled phage particles) images of the biofilm. The dashed line indicates the edge of the biofilm. Scale bar, 100 μm . (E) Penetration depth of FITC-labeled phage in the biofilm. Error bar, SD; five biological replicates. (F) Kymograph of the biofilm upon treatment with purified phage tail fiber protein gp46 (shown in red in the phage cartoon, 10 $\mu\text{g}/\text{ml}$). The y axis indicates the spatial profile of phase contrast intensity along the dashed line shown in fig. S5A. (G) Growth rate of the biofilm during gp46 pretreatment (20 $\mu\text{g}/\text{ml}$) and phage infection. Representative of three biological replicates.

suggest that the appearance of uniform regrowth after phage infection was because of the free gp46 released by the lysed bacteria.

Phage tail fiber penetrated into the biofilm and degraded bacterial capsule

Many pathogenic bacteria express capsules (36), which mask potential phage receptors on the cell membrane (5, 37). Therefore, many phages have evolved to use their tail fibers to first bind to the capsule and then degrade the capsule, and both capsule binding and degradation are essential for the initiation of infection (Fig. 3A) (38, 39). To find out whether Kp11 functions in the same way, we fused mCherry to the N terminus of its tail fiber protein gp46. We found that mCherry-gp46 accumulated on the surface of wild-type bacteria but not on the capsule-defective strain $\Delta wcaJ$ (encodes the initial glucosyltransferase for capsule synthesis) (40) (Fig. 3B and fig. S6A). This showed that gp46 bound specifically to the capsules on the surface of the bacteria. We also found that the fluorescence gradually decreased over time and disappeared within 25 min (Fig. 3C),

suggesting that gp46 degraded the capsule. Using Alcian blue-stained SDS-polyacrylamide gel electrophoresis (SDS-PAGE) gel analysis, we confirmed that gp46 did have depolymerase activity (fig. S6C and Materials and Methods). Last, we constructed a mutant gp46 (gp46^{mut}) without depolymerase activity (fig. S6C and Materials and Methods) and found that it accumulated stably on the surface of encapsulated bacteria (Fig. 3C) but not on the $\Delta wcaJ$ strain (fig. S6B). These results show that gp46 specifically binds to the capsule on the surface of the bacteria, degrades the capsule, and then is released from the bacteria after the degradation.

To visualize the penetration of gp46 into the biofilm, we treated the biofilm with mCherry-gp46. First of all, mCherry-gp46^{mut} could not penetrate into the biofilm and were mainly enriched at the edge of the biofilm (Fig. 3D and fig. S6D). In contrast, mCherry-gp46 concentrated in a band that coincided with the phase band and gradually moved toward biofilm interior (Fig. 3E and fig. S6, E and F). This shows that the depolymerase activity of mCherry-gp46 facilitated its penetration into the biofilm, and the phase band indicates the

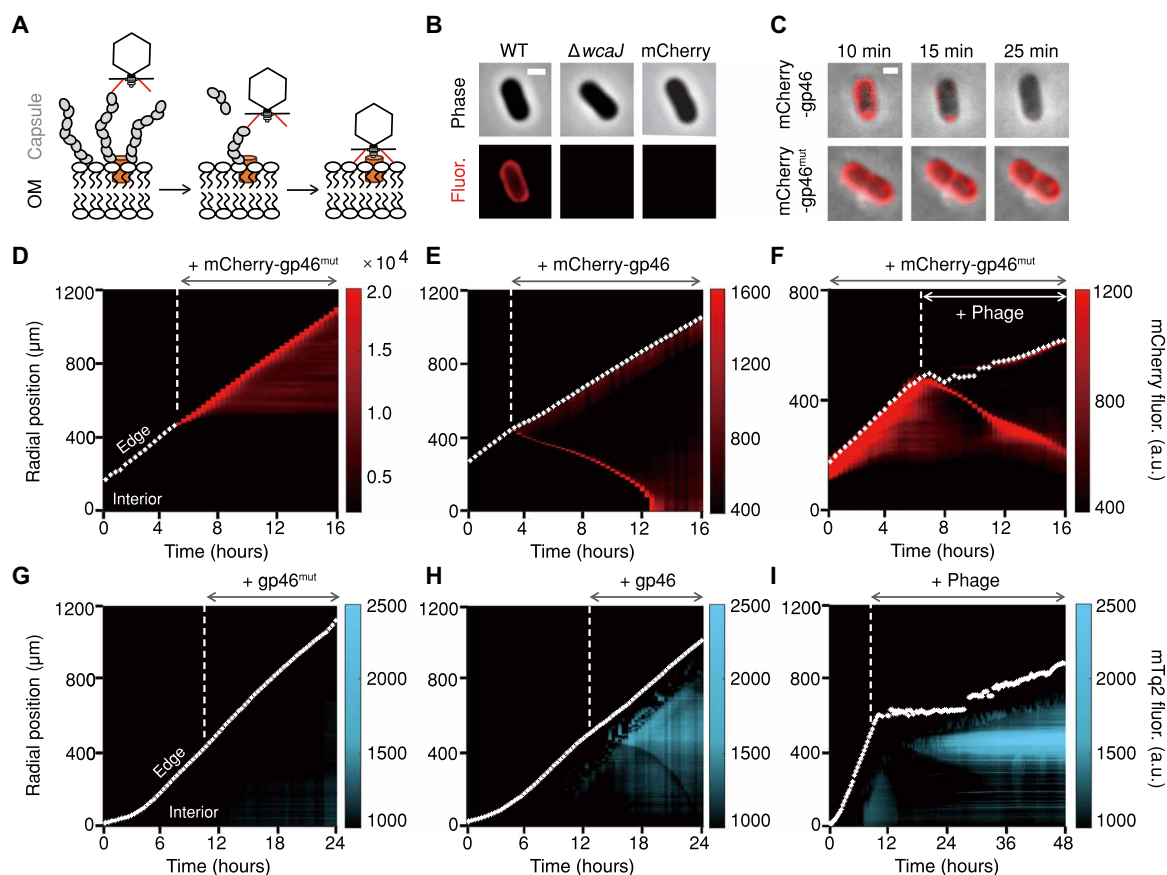


Fig. 3. Action of phage tail fiber protein in the biofilm. (A) Schematic diagram showing the process of phage infecting encapsulated bacteria. Step 1: specific binding of phage to bacterial capsule by the tail fiber; step 2: degradation of capsule by the tail fiber; step 3: specific binding of phage to secondary receptor on bacterial outer membrane (OM). (B) Phase contrast and fluorescent images of bacteria. Wild-type (WT; left) and capsule-deficient bacteria (middle) were incubated with mCherry-gp46, respectively, and wild-type bacteria (right) were incubated with mCherry. Imaged at 10 min after incubation. Scale bar, 1 μ m. (C) Composite images of bacteria incubated with gp46 and gp46^{mut}, respectively. Captured at the indicated time after incubation. Scale bar, 1 μ m. (D to F) Kymographs showing biofilms treated with mCherry-gp46^{mut} (left), mCherry-gp46 (middle), mCherry-gp46^{mut}, and then plus phage (right). The y dimension indicates the spatial profile of mCherry fluorescence along the dashed lines shown in fig. S6 (D, E, and G), respectively. The x dimension indicates how the spatial profiles evolved over time. (G to I) Kymographs showing biofilms treated with gp46^{mut} (left), gp46 (middle), and phage (right). The y dimension indicates the spatial profile of mTq2 fluorescence (expressed under the *ompk26* promoter) along the dashed lines shown in fig. S7 (C to E), respectively. The white marks indicate the edge of the biofilm. All the results are representative of three biological replicates. gp46 concentrations are 10 μ g/ml, except for (B) and (C) (100 μ g/ml) and (F) (1 μ g/ml).

location where mCherry-gp46 has penetrated to. Given that we continuously supply mCherry-gp46 in the flowing medium outside of the biofilm, the lack of fluorescence outside of the band indicates that the capsules in that region have been degraded.

Then, we verified whether the abovementioned process also applied to infection of the biofilm by phage. To this end, we first labeled the capsule in the biofilm using mCherry-gp46^{mut} and then treated the biofilm with phage (Fig. 3F). We found that the phage caused gradual penetration of mCherry-gp46^{mut} into the biofilm (Fig. 3F and fig. S6G), and the penetration front coincided with that of the phase contrast band (fig. S6H). Behind the penetration front, the fluorescence stayed low, suggesting that the capsules had been degraded by the gp46 released from the lysed cells at biofilm periphery.

To further confirm this claim, we examined the response of the bacteria upon encountering gp46. We first measured the response of planktonic bacteria using RNA sequencing (RNA-seq) and compared the transcriptional changes of the bacteria upon gp46 treatment. Our analysis showed that *ompK26*, which encodes the outer membrane protein porin, was notably up-regulated by gp46 treatment (fig. S7A). We constructed a fluorescent reporter strain to monitor the promoter activity of *ompK26* (fig. S7B). As a control, we found that treatment with gp46^{mut} caused no notable change in *ompK26* expression in the biofilm (Fig. 3G and fig. S7C). In contrast, treatment of the biofilm with gp46 caused notable up-regulation of *ompK26* in the region behind the penetration front (Fig. 3H and fig. S7D). Last, treatment with phage also caused notable up-regulation of *ompK26* in the region behind the penetration front (Fig. 3I and fig. S7E). We confirmed this observation with three additional representative genes (fig. S7F). These results show that phage infection resulted in penetration of free gp46 into the biofilm and degradation of capsule along the process.

Tuning tail fiber or capsule-level altered biofilm resistance to phage

On the basis of the results above, we propose the following mechanism of how resistance emerged in the biofilm (Fig. 4A). Because of the dense packing of the bacteria, phage has limited diffusion into the biofilm. Therefore, phage infection initiates from biofilm periphery and gradually progresses toward biofilm interior. During phage replication, unassembled tail fiber proteins are released into the biofilm upon lysis of the bacteria. Those tail fiber proteins attach on the surface of encapsulated bacteria, degrade the capsule, detach from the bacteria after the degradation, and then attach again on other encapsulated bacteria. Through this process, the tail fiber proteins facilitate their own penetration into the biofilm, resulting in spreading of capsule loss in the biofilm. Since the initiation of phage infection requires specific binding of the capsule, the capsule loss resulted in resistance to phage and uniform regrowth of the biofilm.

This mechanism suggests that altering the level of free gp46 would affect biofilm resistance. Specifically, increasing/decreasing the level of free gp46 would lead to faster/slower degradation of capsule and therefore faster/slower recovery of growth after phage infection. To validate this prediction, we treated the biofilm with various concentrations of gp46 3 hours after switching to medium containing phage (10^7 PFU/ml, 3 hours is the time when the phase band normally appears). Consistent with the prediction, we found that higher concentration of gp46 led to faster recovery of biofilm growth (Fig. 4B). We also tested the effect of reducing the level of

free gp46 in the biofilm. To this end, we engineered the bacteria to knock down the expression of gp46, using dCas9 to inhibit the transcription of the *gp46* gene (fig. S8). Consistent with our expectation, we found that the knockdown led to significant increase of recovery time for biofilm regrowth (Fig. 4C).

We noticed that, after the biofilms recovered from phage infection, their growth rates were much slower than before the infection (Fig. 4D). To find out why, we engineered the phage by inserting the *sfGFP* gene after the capsid gene on the phage genome, so that the fluorescent protein sfGFP (superfolder green fluorescent protein) is expressed when the phage replicates (Fig. 4E and fig. S9). We found that a fraction of the bacteria at biofilm periphery were continuously infected even after the biofilm recovered its growth (Fig. 4F). At biofilm periphery, the bacteria constantly synthesis new capsules during proliferation (Fig. 3, E and F, and fig. S6, F and H; weak capsule staining at biofilm periphery), which makes them sensitive to the phage in the biofilm. However, there are also free gp46 released from lysed cells. Whether a particular bacterium is infected by the phage depends on whether its capsule is degraded by the free gp46 before it encounters phage. This predicts that we could increase the biofilm regrowth rate by increasing the degradation of their capsule, as it would reduce the fraction of infected bacteria in the biofilm during regrowth. To validate this prediction, we treated the biofilm with purified gp46 3 hours after infecting the biofilm with phage. Consistent with our prediction, we found that the treatment led to faster regrowth of the biofilm, and the regrowth rate increased with the concentration of gp46 (Fig. 4G). These results show that the biofilm regrowth after phage infection is the net outcome of constant bacterial proliferation and phage lysis, and proliferation exceeded lysis.

The proposed mechanism also suggests that altering the level of capsule would also affect biofilm resistance. Specifically, increasing the level of capsule would lead to slower formation of the low capsule zone and therefore slower recovery of biofilm growth after phage infection. To validate this prediction, we constructed a bacterial strain by replacing the native *wcaJ* gene with isopropyl- β -D-thiogalactopyranoside (IPTG)-induced *wcaJ* (Materials and Methods). Using the classical uronic acid assay for capsule quantification, we verified that higher concentration of IPTG led to higher level of capsule (fig. S10). We then measured how increasing capsule affected the recovery of biofilm growth upon phage infection. Specifically, we treated the biofilm with different concentrations of IPTG 3 hours after switching to medium containing phage (10^7 PFU/ml). As expected, we found that the recovery of biofilm growth increased with IPTG concentration (Fig. 4H). We also measured the growth rate of the biofilm after the recovery. As expected, we found that the capsule induction led to slower regrowth of the biofilm, and the regrowth rate decreased with the strength of the induction (Fig. 4I). These results suggest that, if we keep increasing the strength of capsule induction, we may finally avoid the development of resistance. When we induced capsule with 100 μ M IPTG from the beginning of cultivating the biofilm, we found that the entire biofilm could be lysed by the phage (Fig. 4J and movie S2).

Effect of tail fiber is important in developing antibiofilm strategies

Biofilms are major causes of infections and are often resistant to antibiotics and to host immunity (41, 42). Our findings here could provide guidance for developing strategies to counter biofilms with

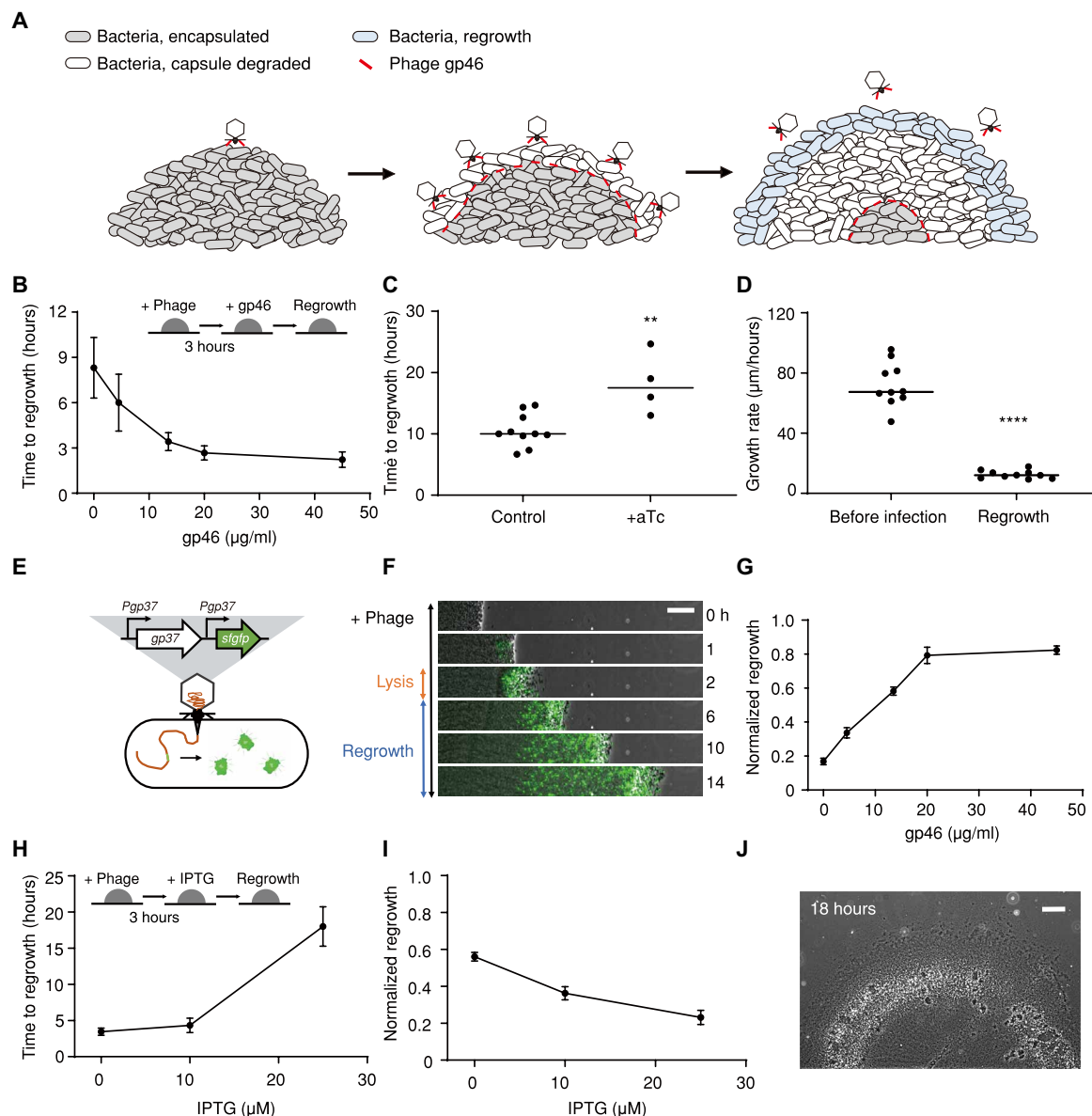


Fig. 4. Mechanism of biofilm resistance to phage. (A) Schematic diagram showing the process of phage infecting bacterial biofilm and the formation of resistance. Left: Biofilm periphery encounters phage. Middle: Biofilm periphery infected and lysed by phage, releasing more phages and unassembled tail fibers. Right: Unassembled tail fibers diffuse ahead of the phages and degrade bacterial capsule, causing formation of low-capsule zone that expands into the biofilm. (B) Effect of gp46 treatment on the recovery time of the biofilm. The inset shows the experimental workflow. Error bar, SD; three biological replicates. (C) Effect of inhibiting gp46 expression (fig. S8) on biofilm recovery time. [anhydrotetracycline] = 1 ng/ml. Ten biological replicates for control, four for inhibition. (D) Biofilm growth rate before phage infection and during regrowth. Ten biological replicates. (E) Schematic of the reporter phage Kp11::sfgfp used to visualize infection in individual bacteria. (F) Film strip of biofilm periphery after phage infection. The sGFP fluorescence (green) indicates active phage replication in the bacteria. Scale bar, 100 μm. (G) Effect of gp46 treatment on the growth rate of the biofilm during regrowth. Same experimental workflow as in (B). Growth rate is normalized by the rate before phage infection. Error bar, SD; three biological replicates. (H and I) Effect of tuning capsule level (fig. S10) on the recovery time and the regrowth rate of the biofilm. The inset shows the experimental workflow. Three biological replicates. (J) Effect of overexpressing capsule on the outcome of phage infection. Capsule was overexpressed under 100 μM IPTG starting from the very beginning of biofilm cultivation. Scale bar, 100 μm. Representative of three biological replicates. All statistical data [(B), (C), (D), (G), (H), and (I)] are represented as the mean ± SD; statistical significance was determined by two-sided Student's *t* test: ***P* = 0.00145 and ****P* = 2.595 × 10⁻⁰⁷.

phages. Specifically, the degradation of capsule by the tail fiber could have important implications and may even open more possibilities.

A common practice in phage therapies is to combine phages with antibiotics. Since capsule may affect the permeation of antibiotics into the bacteria (43), we tested the effect of capsule on the efficacy of various antibiotics. We found that knocking out *wcaJ* decreased

the MIC (minimum inhibitory concentration) of kanamycin, gentamicin, and tobramycin, increased the MIC of polymyxin B and E, and did not cause notable change for ciprofloxacin and neomycin (Table 1). We then tested whether this would affect the efficacy of phage-antibiotic combination. Consistent with the change of MIC, we found that Kp11 promoted the inhibition of the biofilm with

Table 1. Minimum inhibitory concentration of antibiotics in *K. pneumoniae* ATCC43816 and $\Delta wcaJ$.

Antibiotics	Minimum inhibitory concentration ($\mu\text{g/ml}$)	
	Wild type	$\Delta wcaJ$
Kanamycin (Kan)	12.5	6.25
Gentamycin (Gen)	6.25	1.5625
Tobramycin (Tob)	6.25	3.125
Ciprofloxacin (CIP)	0.05	0.05
Neomycin (Neo)	6.25	6.25
Polymyxin E (PE)	50	100
Polymyxin B (PB)	25	50

kanamycin, gentamicin, and tobramycin (Fig. 5, A and B, and fig. S11, A and B)—fast inhibition of the biofilm could be achieved with low antibiotic concentration ($0.5\times$ MIC), and no growth recovery was observed. In contrast, combination of Kp11 with the other antibiotics ($0.5\times$ MIC) could not inhibit biofilm growth (Fig. 5B and fig. S11, C to F). More notably, Kp11 notably reduced the efficacy of polymyxin: Polymyxin B ($6.25\times$ MIC) could cause substantial disruption of the biofilm (Fig. 5, C and D); however, combination with Kp11 abolished the disruption (Fig. 5, C and E). We confirmed that this antagonistic effect was due to the degradation of capsule, as gp46 alone is sufficient to abolish the disruptive effect of polymyxin B (Fig. 5F). We observed similar effect with polymyxin E (fig. S11, G to J). These results show that it is important to consider the effects of bacterial lysate when combining phages with antibiotics.

Because of the dense packing of the bacteria, the penetration of many molecules in biofilms is limited. We found that mCherry-gp46^{mut} could not effectively penetrate the biofilm, while mCherry-gp46 could (Fig. 3, D and E). This suggests that the capsule is a major factor limiting the diffusion of proteins in the biofilm. We found that mCherry could not effectively penetrate the biofilm either (Fig. 5G); however, after we treated the biofilm with gp46, mCherry readily diffused into the biofilm (Fig. 5G). This opens the possibility of combining phages with other macromolecules. For example, dispersion is an effective strategy to eradicate biofilms (44, 45). We found that cellulase could disperse the biofilm (Fig. 5H and fig. S11K), possibly by degrading the extracellular matrix essential for adhesion (46). However, unrealistically high cellulase concentration is needed (160 U/ml, 20% of stock concentration). We reasoned that facilitating the diffusion of cellulase into the biofilm may increase its efficacy. Consistent with our speculation, we found that by combining with phage, the entire biofilm could be dispersed with low concentration (8 U/ml) of cellulase (Fig. 5, I and J, and movie S3).

DISCUSSION

Here, we uncovered a type of resistance, which is caused by a vulnerability inherent in how phages infect encapsulated bacteria—they need to first bind to the capsule and then degrade the capsule, and both capsule binding and degradation are essential for the initiation of infection. When infected bacteria are lysed to release fully assembled phage particles, they also release some unassembled phage

components. In the community setting, the unassembled tail fibers caused premature degradation of the capsule, rendering the bacteria insensitive to phage infection. In principle, in addition to biofilms, this type of phage resistance may also happen in dense planktonic culture. It would be interesting to find the conditions under which notable effects could be observed in the latter scenario. We would like to emphasize that there are important differences between biofilms and planktonic culture. In planktonic culture, everything is well-mixed. For each bacterium, whether its capsule is degraded by unassembled tail fiber before it encounters phage is stochastic. Therefore, its resistance to phage is also stochastic. In contrast, in biofilms, the dense packing of the bacteria causes spatial gradients. Specifically, unassembled tail fibers always diffuse ahead of the phages due to their smaller size and degrade the capsules ahead of the phages. Therefore, the resistance of most bacteria in the biofilm should be deterministic (see exception below).

After the biofilm developed resistance, we still observed infections continuously happening at the periphery region of the biofilm. This is because bacteria at biofilm periphery continuously synthesize new capsules, and the resistance is the net outcome of the balance between degradation of capsule and synthesis of new capsule. Specifically, whether a particular bacterium at biofilm periphery is infected depends on whether its capsule is degraded by the free tail fiber before it encounters phage. This provides a mechanism for the coexistence of lytic phages and bacteria. Since phages rely on the availability of hosts to proliferate, complete eradication of the host would be an undesirable outcome. The mechanism reported here enables the survival and proliferation of both the phage and the host. Since community living is ubiquitous, this type of coexistence may play an important role in the ecology of phage-bacteria interactions in nature.

For therapeutic use of phages, resistance is a major challenge. It is crucial to carefully chose phages and therapeutic strategies to mitigate bacterial resistance. The resistance mechanism uncovered here raises the need for alternative strategies. We found that promoting capsule synthesis led to better elimination of the biofilm. However, capsules could help bacteria to evade host immunity (47). Therefore, it is unclear whether promoting capsule synthesis could be an applicable strategy. Phage-antibiotic combination is commonly used. However, it is often unclear what governs the outcome of a particular combination. We showed that the degradation of capsule by phage tail fiber could affect the efficacy of antibiotics. Therefore, it is important to consider the bacterial lysate when combining phages with antibiotics. In addition, the order of phage and antibiotic treatments also matters. We treated the biofilm with phage before using antibiotics, so that the degradation of capsule occurs before the bacteria encounter antibiotics, and changing the order may lead to different outcomes. Thus, further systematic investigations are warranted.

We also showed that capsule hindered the diffusion of macromolecules such as proteins into the biofilm, and degradation of the capsule facilitated the diffusion. This opens the possibility of combining phages with a broad range of therapeutic macromolecules. Our combination of phage with cellulase demonstrated this potential. More strategies along this line are worth exploring. For example, during phage infection, many proteins released by the lysed bacteria could potentially diffuse into the biofilm. An especially interesting one is endolysin, which is used by the phage to lyse bacteria at the late stage of infection. The problem here is that for Gram-negative bacteria, such as *K. pneumoniae*, extracellular endolysin is ineffective

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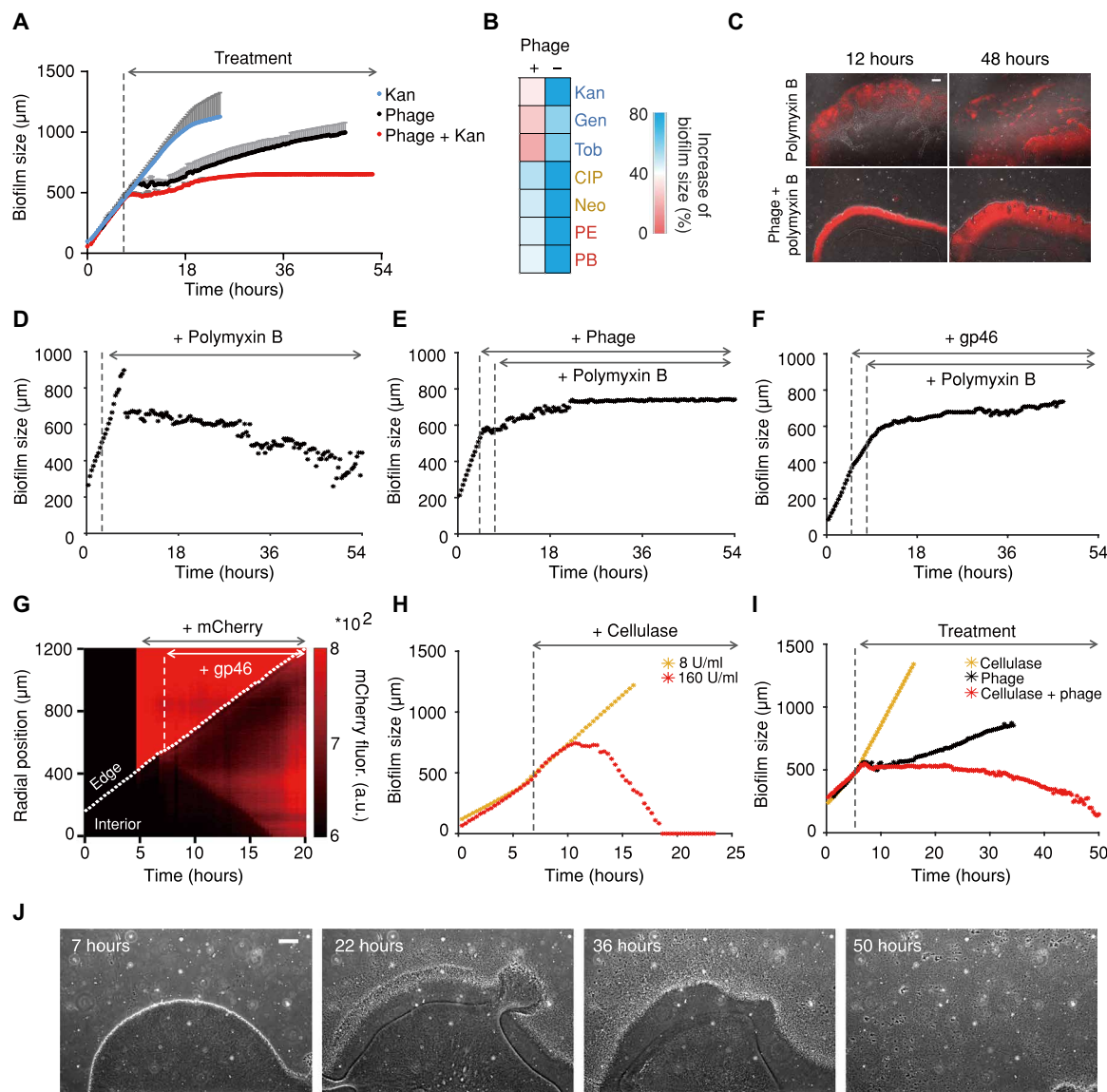


Fig. 5. Implications for countering biofilms with phage. (A) Growth dynamics of the biofilm treated with kanamycin (blue), phage (black), and phage-kanamycin combination (red), respectively. Kanamycin concentration was $6.25 \mu\text{g/ml}$ ($0.5\times \text{MIC}$). Phage concentration was 10^7 PFU/ml. Data are represented as the mean $\pm$ SD of three biological replicates. (B) Percentage increase of biofilm size after 24 hours of antibiotic and phage-antibiotic combination treatments, average over two biological replicates. Antibiotic concentrations are $0.5\times$ of their corresponding MICs: kanamycin (Kan), $6.25 \mu\text{g/ml}$; gentamycin (Gen), $3.125 \mu\text{g/ml}$; tobramycin (Tob), $3.125 \mu\text{g/ml}$; ciprofloxacin (CIP), $0.025 \mu\text{g/ml}$; neomycin (Neo), $3.125 \mu\text{g/ml}$; polymyxin E (PE), $25 \mu\text{g/ml}$; polymyxin B (PB), $12.5 \mu\text{g/ml}$. (C) Snapshots of biofilms treated with polymyxin B alone or in combination with phage. Composite of phase contrast and PI fluorescence (red). Time indicates duration of treatment. For the combination, phage treatment was started 3 hours before antibiotic. Polymyxin B, $156.25 \mu\text{g/ml}$ ($6.25\times \text{MIC}$). Phage, 10^7 PFU/ml. Scale bar, $100 \mu\text{m}$. (D and E) Growth dynamics of the biofilms shown in (C). (F) Growth dynamics of biofilm treated with gp46 ($10 \mu\text{g/ml}$) and polymyxin B. (G) Kymograph showing penetration of the mCherry protein in the biofilm before and after gp46 treatment. The white markers indicate the edge of the biofilm. Red indicates mCherry fluorescence. (H) Growth dynamics of the biofilm treated with different concentrations of cellulase. (I) Growth dynamic of the biofilm treated with cellulase, phage, and phage-cellulase combination, respectively. Cellulase, 8 U/ml ; phage, 10^7 PFU/ml. (J) Phase contrast images of the biofilm treated with phage-cellulase combination in (I). Scale bar, $100 \mu\text{m}$. All the results are representative of two biological replicates.

due to the protection of the outer membrane. Thus, a potential strategy is to use engineered phage to produce modified endolysins that could penetrate bacterial outer membrane; when infecting biofilms with this type of phage, the lysed bacteria would release both free tail fiber and modified endolysin, and the former would facilitate diffusion of the latter into the biofilm, potentially leading to lysis of the entire biofilm.

Last, phage cocktail is a commonly used strategy, and our findings suggest a counterintuitive strategy for the design of cocktails. Specifically, degradation of the capsule by the free tail fibers would expose potential receptors that are normally masked by the capsule. Therefore, while the bacteria become insensitive to the capsule-targeting phages, they become sensitive to phages that infect non-encapsulated strains of the bacteria. Since the latter could not infect encapsulated

bacteria, they are normally ruled out in therapies that target those bacteria. Our findings suggest that the counterintuitive combination of these two types of phages may perform better in the eradication of biofilms.

MATERIALS AND METHODS

Strains and cultivation conditions

The bacteria and phages used in this study are listed in table S1. Bacteria were grown in M63B1 medium [22 mM glucose, 100 mM KH_2PO_4 , 15 mM $(\text{NH}_4)_2\text{SO}_4$, 0.8 mM MgSO_4 , and 3 μM vitamin B1, adjust to pH 7.4 with KOH]. During strain construction, bacteria were grown in LB broth [tryptone (10 g/liter), NaCl (10 g/liter), and yeast extract (5 g/liter)]. The following antibiotics or chemicals were added as specified: apramycin (30 $\mu\text{g/ml}$), spectinomycin (300 $\mu\text{g/ml}$), L-arabinose (0.2%), chloramphenicol (30 $\mu\text{g/ml}$), ampicillin (100 $\mu\text{g/ml}$), sucrose (5%), and PI (1 $\mu\text{g/ml}$).

Plasmid construction

All knockout strains of *K. pneumoniae* were generated by a CRISPR-Cas9-mediated genome-editing method (48). Briefly, we cloned single guide RNAs (sgRNAs) into the pSGKP plasmid through T4 ligation and amplified the homologous arms of the genes of interest from the genomic DNA of the wild-type strain using Q5 Hot Start High-Fidelity 2X Master Mix (New England Biolabs). We then electrotransformed the pSGKP-sgRNA plasmid and the corresponding homologous arms into pCAS9-harboring *K. pneumoniae*-competent cells and cultured them in LB supplemented with 0.2% L-arabinose. Subsequently, we selected the transformants using plates containing spectinomycin and apramycin at 30°C and verified them by DNA sequencing. Last, we cured plasmids with 5% sucrose at 37°C. The plasmids and primers are listed in tables S2 and S3, respectively.

Phage purification

For phage propagation and purification, we followed the Phage On Tap protocol (49) with minor modifications. Briefly, we cultured *K. pneumoniae* ATCC43816 in LB broth with shaking at 220 rpm and 37°C until it reached an optical density at 600 nm (OD_{600}) of 0.6 to 0.8. Then, we added the phage Kp11 and incubated the culture for 1 hour to allow phage amplification. Subsequently, we added deoxyribonuclease I (DNase I) to the culture and incubated it at 37°C for 1 hour. Then, we centrifuged the culture at 4000g and 4°C for 20 min. We further filtered the supernatant using Millex Sterile Filter (Millipore, 0.22- μm pore size) and concentrated the lysate with ultrafiltration tubes (Millipore, Amicon Ultra-15, 100 kDa). Last, we washed the lysate with TMS buffer [50 mM tris-HCl, 100 mM NaCl, and 10 mM MgCl_2 (pH 7.5)] and filtered the phage preparation (0.22- μm pore size). The resulting purified phage was stored at 4°C.

Plaque assay

We performed phage titration using the double-layer plaque assay (50). We mixed 100 μl of bacterial culture ($\text{OD} \sim 0.6$) and serially diluted phage samples with 3 ml of LB broth containing 0.7% soft agar and spread the mixture over 1.5% LB agar plates at room temperature for 30 min. Then, we inverted and incubated the plates at 37°C overnight. Last, we determined the PFU by counting the visible plaques.

Biofilm culturing and medium switching

We cultured the biofilms using a microfluidic system described in our previous work (32). Briefly, we picked a single colony from LB agar plate, inoculated it into 10 ml of LB broth in a 50-ml centrifuge tube, and then cultivated the bacteria for 4 hours at 37°C in a shaker at 220 rpm. After cultivation, we centrifuged the culture at 7000g for 3 min, washed the pellet three times with 5 ml of phosphate-buffered saline (PBS) buffer, and lastly resuspended the pellet with 10 ml of PBS buffer and used it as the loading culture. Before loading the bacteria, we coated the inner surface of the microfluidic chamber with 5% bovine serum albumin (BSA) for 1 hour to minimize random bacterial adhesion and then washed out the unbound BSA with M63B1 medium. Subsequently, we loaded the abovementioned culture to the seeding zone (shown in blue in Fig. 1A) until approximately 100 bacteria cells were trapped. Throughout biofilm culturing, we maintained constant medium flow using the Elveflow OB1Mk3 Pressure Controller (Elvesys) under constant pressure of 20684.2719 Pa (corresponding to a flow rate of approximately 500 $\mu\text{m/s}$), and we kept the cultivation temperature of 37°C.

To switch medium, we opened the medium exchange port for 30 to 60 s to discharge the previous medium (approximately 1 ml) and then introduce the fresh medium into the growth chamber. We infected the biofilms with medium containing 10^7 PFU/ml of phage, unless specified otherwise.

Time-lapse microscopy and image acquisition

Biofilms were observed with phase contrast and fluorescent microscopy. The microscope used was Olympus IX73 (Japan) with Andor's Zyla 4.2 sCMOS camera (UK). Objective lens (10 $\times$) was used, unless specified otherwise. CellSens Dimension software (Olympus, version 1.18) was used to monitor the dynamics of biofilms, and images were taken every 20 min.

Extraction of bacteria from the microfluidic system and bacteria counting

We took images of the biofilm right before phage infection and after phage infection at defined time points using microscopy. Then, we cut open the microfluidic chip and washed the growth chamber using 100 μl of PBS buffer to collect all the bacteria. We used 10 μl of the suspension, counted the number of bacteria using Petroff-Hausser counting chambers, and spread the remaining suspension on LB agar plates containing 10^{10} PFU of phages. Subsequently, we incubated the plates at 37°C for 12 hours and then counted the number of colony-forming units. Last, we recovered these survivors for further experiments, including phage susceptibility assay and biofilm culturing.

Phage susceptibility assay

We cultured the bacteria in LB broth under shaking (220 rpm) at 37°C until reaching an OD_{600} of 0.6. Then, we mixed 100 μl of the culture with 3 ml of LB broth containing 0.7% soft agar and spread it over 1.5% LB agar plates at room temperature for 30 min. Next, we dropped 10 μl of phage suspension (10^{10} PFU/ml) on the bacterial layer. After the droplets had dried, we inverted and incubated the plates at 37°C overnight to inspect the lysis zone.

Phage labeling with fluorescent dye

To label the phage particles, we mixed 250 μl of purified Kp11 phage particles (10^{11} PFU/ml) with 250 μl of sodium carbonate (0.2 M) and 100 μl of FITC (5 mg/ml, dissolved in dimethyl sulfoxide)

(Sigma-Aldrich, #45950). Then, we incubated the mixture at 24°C with continuous shaking at 400 rpm for 1 hour. Subsequently, we dialyzed the reaction mixture against the PBS buffer (4°C) using dialysis membrane (Solarbio, YA1051) to separate the FITC-labeled phage particles and the unbound FITC. Last, we filtered the FITC-labeled phage (0.22- μ m pore size) and stored them at 4°C.

Fluorescent phage modification

We genetically modified Kp11 using the CRISPR-Cas9 system. Briefly, to insert the *sfgfp* gene under the control of the promoter for the major capsid protein gene *gp37*, we cloned sgRNA targeting *gp37* into the pCAS9-SGKP plasmid through T4 ligation and cloned the homologous arms into the pSGKP derivative plasmid by Gibson assembly. Then, we electrotransformed both plasmids into *K. pneumoniae*-competent cells and selected the transformants using plates containing spectinomycin and apramycin. Subsequently, we cultured the bacteria carrying these plasmids in LB broth (supplemented with spectinomycin and apramycin) until $OD_{600} \sim 2$. Next, we mixed 200 μ l of the bacterial culture and 10 μ l of properly diluted phage suspensions into 3 ml of LB broth containing 0.7% soft agar (supplemented with spectinomycin and apramycin) and spread them on 1.5% LB agar plates. The plates were inverted and incubated at 37°C for 12 hours. Then, we picked well-isolated plaques from the plates and verified them via DNA sequencing. Last, we repeated this procedure at least three times to purify the recombinant phage.

We used high titer ($\sim 10^{10}$ PFU/ml) phage lysates for DNA extraction. Specifically, we treated 200 μ l of the phage lysate with DNase I (Solarbio, 100 μ g/ml) and incubated it at 37°C for 1 hour to remove bacterial DNA. Then, we extracted phage genomic DNA using the TIANamp Virus DNA/RNA Kit (catalog no. DP315-F).

Phage infection experiments in plate reader

We diluted (1:100) overnight seed cultures into 3 ml of fresh M63B1 medium in a 15-ml centrifuge tube and incubated at 37°C with shaking at 220 rpm for 2.5 hours (reached exponential phase, $OD_{600} \sim 0.6$). Then, we diluted the culture to OD_{600} of 0.1 ($\sim 10^8$ bacteria per ml, for fig. S4C) or 0.01 ($\sim 10^7$ bacterial per ml, for figs. S8B and S9C) and added phage at the defined multiplicity of infection (MOI). Then, we monitored optical density and fluorescence intensity of the culture in microplate reader (Tecan Spark, performed measurements every 5 min). The growth temperature was set at 37°C.

Phage interaction with single cells

To visualize the fluorescent phage infection at the single-cell level, we diluted the bacteria at exponential phase to OD_{600} of 0.5 and added Kp11::sfgfp at MOI of 0.2. Then, we dropped the mixture on M63B1 agarose pad and incubated it at 37°C. Objective lens (100 $\times$) was used to take phase contrast and fluorescence images, with images captured every 2.5 min. Image analysis was performed in ImageJ.

Tail fiber protein engineering and purification

National Center for Biotechnology Information BLASTp analysis showed that the enzymatic region of the tail fiber protein gp46 was conserved with Dpk2 (51). Therefore, to generate the gp46^{mut}, we introduced D211A and E214A substitutions to the *gp46* gene. As for mCherry-gp46, we used (GGGS)₂ linker to fuse mCherry to the

N-terminal of gp46. In addition, all the purified proteins were fused with a 6 $\times$ histidine tag at the N terminus.

To purify proteins, we introduced pETDuet-1 derivative plasmids containing genes encoding His-tagged gp46, His-tagged gp46^{mut}, His-tagged mCherry, His-tagged mCherry-gp46, and His-tagged mCherry-gp46^{mut} into *Escherichia coli* BL21 (Invitrogen). Protein production was initiated by adding 2 ml of the overnight culture of the corresponding bacterial strains to fresh LB broth supplemented with ampicillin. The cultures were incubated at 37°C in a shaker at 220 rpm until reaching exponential phase ($OD_{600} = 0.6$ to 0.8). Subsequently, the cultures were further grown at 16°C in the presence of 1 mM IPTG for 16 hours. Then, we centrifuged the cultures at 4000 rpm for 15 min and resuspended the pellet by tris-NaCl buffer [50 mM tris and 150 mM NaCl (pH 7.5)]. After that, we added protein inhibitor phenylmethylsulfonyl fluoride (Thermo Fisher Scientific, catalog no. 36978, lastly 2 mM), DNase I (Thermo Fisher Scientific, catalog no. EN0521, 10 U), and ribonuclease A (Solarbio, R1030-1; 100 μ g) and lysed the cells by sonication. Then, we centrifuged the mixture at 15,000 rpm for 30 min (4°C) and applied the supernatant to cobalt beads (Takara, #635653). We washed the resin with 5 column volumes (CVs) of tris-NaCl buffer and subsequently washed the proteins with 10 CVs of tris-NaCl buffer containing 10 mM imidazole. Then, we eluted the protein by tris-NaCl buffer containing 200 mM imidazole. Then, we purified the protein containing fractions by size exclusion chromatography (Superdex 200 Increase 10/300 GL, GE Healthcare). The running buffer contains 50 mM tris and 150 mM NaCl. Last, we concentrated the proteins using the Amicon Ultra-4 100-kDa cutoff filter (Millipore).

Capsule extraction and quantification

We performed extraction and quantification of capsule by following a previous work (52). Briefly, we diluted overnight culture to OD_{600} of 0.2 and incubated in M63B1 medium for 5 hours at 37°C in a shaker at 220 rpm. Then, we mixed 500 μ l of the culture with 100 μ l of 1% Zwittergent 3-14 detergent (Sigma-Aldrich) in 100 mM citric acid (pH 2.0) and incubated the mixture at 50°C for 20 min. After centrifugation at 10,000 rpm for 5 min, we added 300 μ l of the supernatant to 1 ml of absolute ethanol and incubated at 4°C for 20 min. Next, we centrifuged the mixture at maximum speed for 5 min and dried the pellet. Subsequently, we resuspended the pellet in 200 μ l of distilled water, followed by addition of 1200 μ l of 12.5 mM borax (Sigma-Aldrich) in H₂SO₄, and boiled for 5 min. After incubation on ice for 10 min, we added 20 μ l of 0.15% 3-hydroxydiphenol (Sigma-Aldrich) and incubated the mixture at room temperature for 5 min. Last, we measured the absorbance of the mixture at 520 nm. The uronic acid concentration was determined using a standard curve of glucuronic acid (Sigma-Aldrich) and expressed in micrograms per OD_{600} .

SDS-PAGE and Alcian blue staining

To investigate the enzymatic activity of the purified gp46, we incubated 2 μ g of purified capsule with 10 ng of purified gp46, gp46^{mut}, mCherry-gp46, mCherry-gp46^{mut}, and protein buffer (50 mM tris and 150 mM NaCl), respectively, at 37°C for 1 hour. Following the addition of SDS-loading buffer (Takara) and boiling for 10 min, we analyzed the samples by 4 to 12% gradient SDS-PAGE and subsequently stained the gel with the cationic dye Alcian blue (53). Specifically, we fixed the gel in the fixing buffer (10% acetic acid and

25% ethanol in distilled water) for 30 min (50°C) and then transferred it to the staining buffer containing 0.125% Alcian blue (10% acetic acid and 25% ethanol in distilled water), followed by incubation at 50°C for 15 min in the dark. Last, we destained the gel with the fixing buffer at room temperature and visualized it.

Tail fiber protein interaction with single cells

To visualize the interaction between mCherry-gp46 and bacteria, we diluted the overnight culture to an OD₆₀₀ of 0.2 and cultured it in M63B1 medium at 37°C in a shaker at 220 rpm for 5 hours. The culture was then diluted to an OD₆₀₀ of 0.2. mCherry-gp46, mCherry-gp46^{mut}, and mCherry were added at a final concentration of 100 µg/ml, respectively. To identify the target of gp46 (Fig. 3B and fig. S6A), we dropped 10 µl of the mixture on a glass slide and covered it with a coverslip after incubation for 10 min at 37°C. To explore the function of gp46 (Fig. 3C), we dropped 10 µl of the mixture over M63B1 agarose pads immediately after mixing and took images every 2 min. The microscope settings were then consistent with those described above.

Tail fiber protein treatment and RNA extraction

To investigate the response of planktonic bacteria to gp46, we diluted (1:100) overnight culture into 200 µl of fresh M63B1 medium and cultured it in a 96-well plate using a microplate reader (Tecan, Spark) at 37°C. Once the culture reached OD₆₀₀ of 0.3, we added 0.2 µl of gp46 (final concentration, 10 µg/ml) and protein buffer (50 mM tris and 150 mM NaCl), respectively. After incubation for 3 hours, we used 100 µl of the culture for the total RNA extraction following MiniBac-seq (54). We used the extracted RNA for library construction and sequenced the samples using the Illumina NovaSeq 6000 system with 2 × 150 pair-end configuration.

RNA-seq analysis

We performed RNA-seq data analysis as previously described (54) with minor modifications. Briefly, we quality-checked the raw data and demultiplexed it by FastQC (v0.11.9) and cutadapt (v2.10). Then, we aligned the data to the reference genome of *K. pneumoniae* ATCC43816 (CPO64352.1) using bowtie2 (v2.4.1). Subsequently, we quantified the read count of each gene using the R package GenomicAlignments (v1.26.0) and converted them to transcripts per million. We performed differential expression analysis using DESeq (1.38.0).

Flow cytometry

To analyze phage-induced GFP fluorescence, we diluted exponential phase bacteria to OD₆₀₀ of 0.1 and added Kp11::sfgfp at MOI of 1 at 37°C. Then, we incubated the samples for 0, 20, 30, or 45 min, respectively, and immediately analyzed them without swirling at 4°C. For each sample, we measured 10,000 events using the BD FACS Calibur (Becton Dickinson) with the FITC channel (488-nm blue laser, plus a 505-nm long-pass filter and a 530/30-nm band-pass filter).

Image analysis

We performed image analysis using ImageJ (National Institutes of Health, USA) and MATLAB (MathWorks, USA). We used ImageJ to detect average fluorescence intensity of single cells, measure the area of the biofilms, and generate kymographs of the biofilms. We used MATLAB to calculate growth rates and fluorescence profiles within the biofilms.

Statistical analysis

We used two-tailed Student's *t* tests for statistical analysis in all figures, and the corresponding data are presented as mean ± SD unless otherwise specified. The number of replicates is indicated in the figure legends. For biofilms, biological replicates refer to biofilms that were cultivated in independent microfluidic chambers.

Supplementary Materials

The PDF file includes:

Figs. S1 to S11

Tables S1 to S3

Legends for datasets S1 to S14

Legends for movies S1 to S3

Other Supplementary Material for this manuscript includes the following:

Datasets S1 to S14

Movies S1 to S3

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