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RESEARCH ARTICLE

Bacteriophages ϕMR299-2 and ϕNH-4 Can Eliminate *Pseudomonas aeruginosa* in the Murine Lung and on Cystic Fibrosis Lung Airway Cells

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ABSTRACT ***Pseudomonas aeruginosa*** **is a common cause of infection in the lungs of patients with cystic fibrosis (CF). In addition, biofilm formation and antibiotic resistance of *Pseudomonas* are major problems that can complicate antibiotic therapy. We evaluated the efficacy of using bacteriophages to kill the pathogen in both biofilms and in the murine lung. We isolated and characterized two phages from a local wastewater treatment plant, a myovirus (ϕNH-4) and a podovirus (ϕMR299-2). Both phages were active against clinical isolates of *P. aeruginosa*. Together, the two phages killed all 9 clinical isolate strains tested, including both mucoid and nonmucoid strains. An equal mixture of the two phages was effective in killing *P. aeruginosa* NH57388A (mucoid) and *P. aeruginosa* MR299 (nonmucoid) strains when growing as a biofilm on a cystic fibrosis bronchial epithelial CFBE41o- cell line. Phage titers increased almost 100-fold over a 24-h period, confirming replication of the phage. Furthermore, the phage mix was also effective in killing the pathogen in murine lungs containing 1×10^7 to 2×10^7 *P. aeruginosa*. *Pseudomonas* was effectively cleared (reduced by a magnitude of at least 3 to 4 log units) from murine lungs in 6 h. Our study demonstrates the efficacy of these two phages in killing clinical *Pseudomonas* isolates in the murine lung or as a biofilm on a pulmonary cell line and supports the growing interest in using phage therapy for the control and treatment of multidrug-resistant *Pseudomonas* lung infections in CF patients.**

IMPORTANCE **Given the rise in antibiotic resistance, nonantibiotic therapies are required for the treatment of infection. This is particularly true for the treatment of *Pseudomonas* infection in patients with cystic fibrosis. We have identified two bacterial viruses (bacteriophages) that can kill *Pseudomonas* growing on human lung cells and in an animal model of lung infection. The use of bacteriophages is particularly appropriate because the killing agent can replicate on the target cell, generating fresh copies of the bacteriophage. Thus, in the presence of a target, the killing agent multiplies. By using two bacteriophages we can reduce the risk of resistant colonies developing at the site of infection. Bacteriophage therapy is an exciting field, and this study represents an important demonstration of efficacy in validated infection models.**

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Cystic fibrosis (CF) is an inherited genetic disorder that chronically affects the lungs and digestive system (pancreas and intestine) of children and adults worldwide. CF also affects the mucus and glands of the liver, sinuses, and sex organs causing progressive disability due to multiorgan system failure. CF results from mutations in the transmembrane conductance regulator gene (1–3). The defective enzyme leads to the production of unusually thick and sticky mucus and high levels of chloride containing secretions into ducts and body cavities. These secretions clog the lungs, leading to life-threatening infections (4, 5).

The lungs of CF patients are often colonized at infancy or in early childhood with *Pseudomonas aeruginosa* that may damage the epithelial surface, resulting in altered airway physiology and impairment of mucocillary clearance. This chronic infection is one of the main causes of lung function decline and mortality in CF patients (6, 7). Indeed, 80 to 95% of patients with CF succumb to respiratory failure brought about by chronic bacterial infection and concomitant airway inflammation (7). *P. aeruginosa* is particularly persistent in the lungs due to its aerobic nature and its ability to form biofilms in the lungs of CF patients. Another significant factor is the inherent resistance of *P. aeruginosa* to many antibiotics due to membrane impermeability (8–12). Other acquired mechanisms of resistance include production of β-lactamases and carbapenemases (13) and multidrug efflux pumps (14). It has also been noted that the most prevalent and severe chronic lung infections in CF patients are caused by mucoid *P. aeruginosa* strains (4, 11, 15).

Reports have shown that organisms in biofilms are able to tol-

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erate 10- to 1,000-fold-higher levels of antibiotics than planktonic bacteria (16, 17), and this can mean that the antibiotic concentration needed to eradicate a biofilm is higher than the peak serum concentration (18), rendering it ineffective. The continued emergence and reemergence of biofilm-forming *Pseudomonas* resistant to one or more antibiotics pose a continuous challenge in the treatment of lung infections in CF patients. As a result, there is a need for alternative, nonantibiotic approaches such as phage therapy (19, 20). Although phage therapy has been practiced in Eastern European countries for decades, it has been neglected by the Western world for many years. However, there is now a growing interest in the use of phage therapy for the control and treatment of multidrug-resistant bacterial infections in general, and for *Pseudomonas* lung infections in CF patients in particular. Studies on phage efficacy in clearing of biofilms formed on abiotic surfaces (catheters and microtiter plates) by *P. aeruginosa* confirmed that phage can reduce the bacterial load in these biofilms by 50 to 99% (21, 22). A recent *in vitro* study showed that *Pseudomonas* phage PT-6 was able to reduce the viscosity of alginate polymers extracted from *P. aeruginosa* by almost 65%, a mechanism used by phages to attack the exopolysaccharide matrix of mucoid *Pseudomonas* to gain access to the host cell (23). Recent reports show the promising role phage therapy could play in the treatment of acute lung infections in an *in vivo* murine lung model with *P. aeruginosa* (24, 25) and *Burkholderia cenocepacia* (26). The effectiveness of phage therapy in rescuing larvae (an invertebrate infection model) infected with *Burkholderia cepacia* complex from death was reported by Seed and Dennis (27).

Currently, there is little information concerning the effect of bacteriophage on biofilms growing on a lung tissue model. In this report, we describe how a mixture of two newly isolated phages can kill and clear *lux*-tagged *Pseudomonas* from the lungs of infected mice and in biofilms growing on the surface of a cystic fibrosis bronchial epithelial (CFBE41o-) monolayer. In this study, we used bioluminescence imaging—a powerful tool for studying bacterial infections in small-animal models, since it allows accurate real-time *in vivo* temporal and spatial tracking of tagged bacteria in living animals (25, 28). Monitoring the light emitted by tagged *Pseudomonas* cells *in vivo* was a valuable tool in verifying the effectiveness of the phage mix to kill the pathogen. *Pseudomonas* cell numbers were reduced by a magnitude of 3 to 4 log units when the phage mix was tested in both *in vivo* and *in vitro* systems.

RESULTS

Isolation of phage from a sewage treatment plant. The overall aim of this study was to assess the potential of bacteriophage therapy to treat *Pseudomonas* lung infections. We initially isolated two phages, ϕNH-4 and ϕMR299-2, from sewage obtained from a water treatment plant. Both phages were demonstrated to be virulent to *P. aeruginosa*. Scanning electron microscopy revealed that phage ϕMR299-2 virions have isometric capsids of 40 to 60 nm in diameter and very short tails measuring 10 to 20 nm (Fig. 1A). Morphologically, phage ϕMR299-2 shows similarity to *Pseudomonas* ϕPap3 (29), a podovirus that has an isometric capsid and short tail. We have assigned ϕMR299-2 to type species coliphage T7 and to the family *Podoviridae* (Report of the International Committee on Taxonomy of Viruses [30, 31]). Phage ϕNH-4 possessed an isometric capsid of 50 to 60 nm in diameter and a contractile nonflexible tail with cross striations (noncontracted tail length of 150 nm and contracted tail length of 85 nm; tail diameter

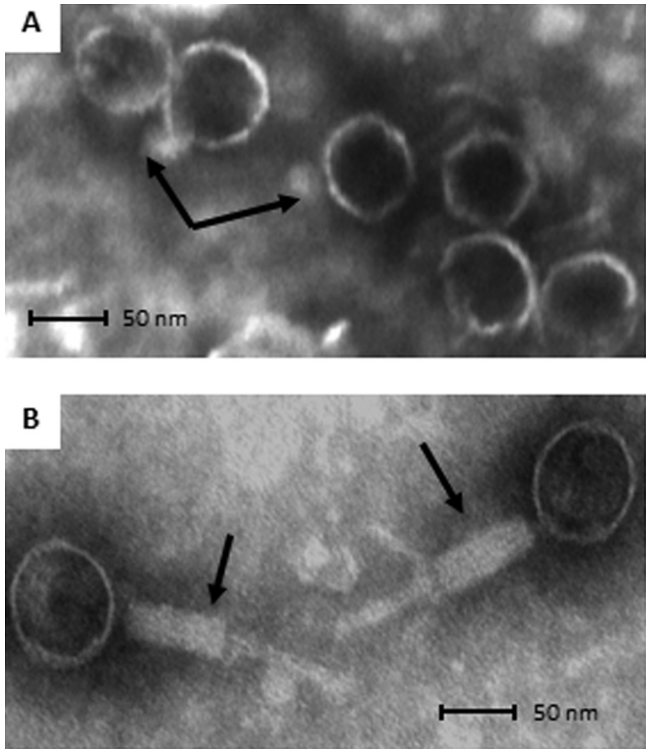


FIG 1 Scanning electron microscopy images of phage ϕ229-2, a podophage (A) and phage ϕNH-4, a myophage (B), stained with 0.2% phosphotungstic acid. The arrows point to the short tail (10 to 20 nm long) of the podophage in panel A and to the contracted tail sheath of the myovirus in panel B.

of 20 nm when contracted). In addition, a putative DNA injecting structure of 70 nm in length (narrower than the contracted tail sheath) and tail fibers were observed (Fig. 1B). Morphologically, ϕNH-4 shows similarity to ϕPB1, ϕLBL3, and ϕSN that are classified into the T4 morphological group of the *Myoviridae* (32). On the basis of structural characteristics obtained from the microscopic analysis, we assign ϕNH-4 as a member of the *Myoviridae* family according to the International Committee on Taxonomy of Viruses (30, 31).

An equal ratio of ϕNH-4 and ϕMR299-2 was used in all experiments to assess their ability to kill *Pseudomonas*. The host range of ϕNH-4 and ϕMR299-2 was determined by exposing different CF *Pseudomonas* isolates to each individual phage using a plaque assay. Of the ten *Pseudomonas* isolates tested, eight were sensitive to both phages, while all were sensitive to at least one phage (Table 1). When the ϕ299-2 and ϕNH-4 phages were added to *Pseudomonas* in LB broth either separately or in combination, the individual phage resulted in reduction of 3 to 4 log units while the combination of the two resulted in a reduction of about 4.5 log units (see Table S1 in the supplemental material).

Phage genome overview. Before phage can be used in human therapy, it is important to assess any potential risk associated with the phage genomes such as the presence of genetic determinants for toxins or other virulence factors or for the capability to integrate into the host genome. The complete sequences of phages ϕ299-2 and ϕNH-4 were determined using 454 pyrosequencing. Phage ϕ299-2 consists of a double-stranded DNA (dsDNA) molecule of 44,789 bp with a GC content of 52%, significantly lower

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TABLE 1 Strains and plasmids used in this study

Strain or plasmid	Description[a]	Source[b] or reference	Sensitivity[c] of strain to the following phage:	
			*ϕ*NH-4	*ϕ*MR299-2
Pseudomonas aeruginosa strains				
MR299	Human CF sputum isolate	CUH	+++	+++
MR299::p16S*lux*	*lux*-tagged MR299	This study	+++	+++
NH57388A	Stable mucoid CF mouse sputum isolate	20	+++	++
NH57388A::p16S*lux*	*lux*-tagged NH57388A	This study	+++	++
MR300	Human CF sputum isolate	CUH	++	−
MR325	Human CF sputum isolate	CUH	−	+
MR326	Human CF sputum isolate	CUH	+++	++
MR327	Human CF sputum isolate	CUH	++	++
MR330	Human CF sputum isolate	CUH	+++	++
MR331	Human CF sputum isolate	CUH	++	+
CH001	Human CF sputum isolate	AH	+++	+++
POA1	UCC culture collection	UCC	+++	++
Plasmid p16S*lux*	*lux*-tagged plasmid vector	19		

[a] UCC, University College Cork.
[b] CUH, Cork University Hospital (Cork, Ireland); AH, Alimentary Health Ltd. (Cork, Ireland).
[c] Symbols: +++, very strong lysis; ++, strong lysis; +, moderate lysis; −, no lysis.

than the 66.6% of *P. aeruginosa*. Both the genome size and GC content of this phage are similar to the closely related *Pseudomonas* phage *ϕ*PaP3 (29). A total of 68 open reading frames (ORFs) are predicted, 21 of which have a leftward orientation and 47 of which are transcribed to the right (Fig. 2). Three tRNA genes ($tRNA^{Asn}$, $tRNA^{Asp}$, and $tRNA^{Pro}$) were also identified in phage *ϕ*299-2, clustered at the 5' end of the genome. While *ϕ*299-2 is a lytic phage, comparative genomic analysis revealed that its closest homolog is the temperate *Pseudomonas* phage, *ϕ*PaP3 (29), isolated from hospital sewage. Interestingly, a recent study calls into question the temperate nature of *ϕ*PaP3, since no site-specific recombinase is encoded up- or downstream of the *ϕ*PaP3 *attP* site, and immunity or reactivation of the integrated *ϕ*PaP3 DNA was not demonstrated (33). Of the 68 predicted protein products of *ϕ*299-2, 56 display the highest amino acid identity to proteins from *ϕ*PaP3 (see Table S3 in the supplemental material). Among these are proteins involved in particle formation (ORF03, ORF04, ORF06, and ORF07), genome replication (ORF29, ORF33/40, encoding DNA polymerase I subunits and ORF41, a putative primase/helicase), a putative lysozyme-like endolysin (ORF02) and 47 proteins of hypothetical function. A conserved genomic organization is also evident on comparing *ϕ*299-2 to *ϕ*PaP3. The remaining 12 ORFs show significant identity to proteins from the lytic *Pseudomonas* phage LUZ24 (33). According to Ceyssens et al., *ϕ*PaP3 and *ϕ*LUZ24 represent a new genus within the *Podoviridae* family (33). Considering the close relationship between *ϕ*299-2 and *ϕ*PaP3 and *ϕ*LUZ24, it is likely that *ϕ*299-2 also belongs to this genus.

Phage *ϕ*NH-4 is a member of the widespread and conserved PB1-like viruses, with a genome of 66,116 bp and a GC content of 55.5%, also significantly lower than that of its host. A total of 94 ORFs were identified in the sequence; the predicted protein products of 56 of these ORFs have the highest amino acid identity to *ϕ*LMA2 (ST4), isolated from a river in Maastricht, Holland, in 2007 (32). Other *ϕ*NH-4 proteins show significant identity to predicted proteins from PB1-like viruses such as *ϕ*14-1, *ϕ*SN, *ϕ*LBL3,

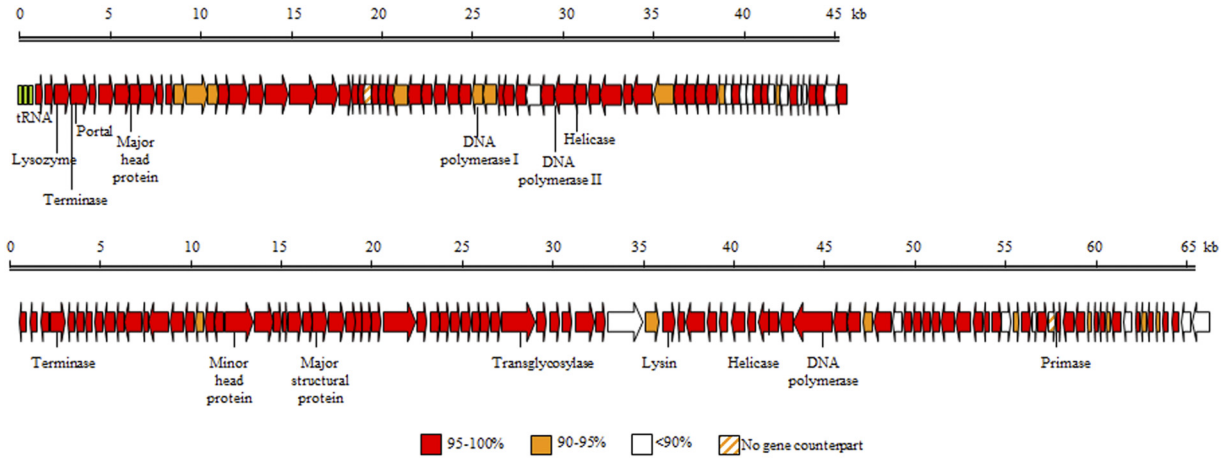


FIG 2 Genome organization of phage *ϕ*MR299-2 (top) and *ϕ*NH-4 (bottom). The predicted open reading frames are indicated by the thick arrows, which are shaded to show the level of protein identity to the corresponding regions of the closest *P. aeruginosa* (phage PaP3 for *ϕ*MR299-2) or phage LMA2 (for *ϕ*NH-4).

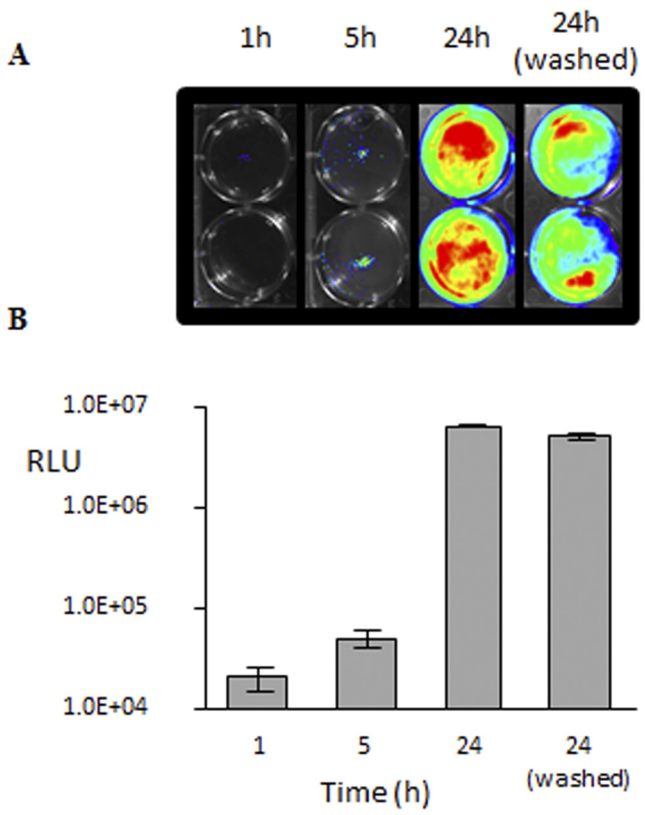


FIG 3 (A) Growth of *lux*-tagged *Pseudomonas* biofilms on the surface of the CFBE410- cell monolayer. Light was measured 1, 5, and 24 h (before and after the monolayer was washed with MEM). (B) Readings from 6 wells are shown. Values are shown as means ± standard deviations (SD) (error bars).

ϕJG024, and ϕPB1 itself (32). As is the case with other PB1-like viruses, the genes in ϕNH-4 are arranged in a compact manner and appear to be organized into at least 7 transcriptional blocks, alternating on both strands (Fig. 2). Based primarily on sequence similarity to ORFs in the genomes of phages LMA2 and JG024, genomic regions encoding phage particle formation (ORF19 to -47) and phage DNA replication (ORF55 to -70) could be identified. ORF48 encodes a putative endolysin with 100% identity to the endolysin of phage ϕLMA2 and belongs to a lysozyme-like superfamily.

Biofilm growth by *lux*-tagged *Pseudomonas* on CFBE41o- cell monolayer. We studied the ability of phage to penetrate and attack *Pseudomonas* cells growing as biofilms on the surface of a layer of human lung epithelial cells. CFBE41o- cells (CFBE stands for cystic fibrosis bronchial epithelial) were grown in standard 6-well tissue culture plates, and a confluent culture with a tight junction between cells was achieved after 8 to 10 days of incubation (12). The confluent culture was inoculated with *lux*-tagged *Pseudomonas* strains NH57388A::p16S*lux* or MR299::p16S*lux*, and growth was monitored in this static system for 24 h (Fig. 3A). The addition of arginine in the minimal essential medium (MEM) enhanced the formation of biofilms and helped preserve the integrity of the CFBE41o- cells, as suggested by Anderson et al. (12). The amount of luminescence recorded for the growing biofilms increased by 2 log units during the 24-h incubation (Fig. 3B). Washing the biofilm monolayer culture twice with MEM removed all planktonic *Pseudomonas* cells. The absence of motile cells and the presence of only adhered clusters of microcolonies of various sizes scattered across the epithelial cell monolayer were confirmed by phase-contrast microscopy (see Fig. S2 in the supplemental material). The amount of bioluminescence recorded after removing planktonic cells was only 25% lower than that obtained before washing (Fig. 3A), which confirmed that the majority of *Pseudomonas* cells were adhered to the epithelial monolayer. We determined the number of CFU after washing the monolayer at 2.6×10^7 to 3.8×10^7 CFU/well and 4.2 to 5.4×10^7 CFU/ well (well area of 9.5 cm^2) for strains NH57388A and MR299, respectively.

Calcofluor white, a fluorescence enhancer that binds to the $\beta(1–3)$ and $\beta(1–4)$ polysaccharide linkages found in biofilm matrices produced by exopolysaccharide-producing organisms (12, 34, 35) was used to stain biofilms. The staining revealed that the *Pseudomonas* cells were contained within a polysaccharide matrix (Fig. 4A and B). The structures formed by the two *Pseudomonas* strains NH57388A and MR299 measured on average 20 to 30 μm by 30 to 40 μm in diameter. The presence of abundant numbers of *Pseudomonas* cells packed in polysaccharide matrices and attached to the cell line led us to conclude that the microcolony structures formed on the monolayer fit the definition of biofilms.

Clearing of biofilms by phage. We examined the ability of the mixture of phages to clear 24-h-old biofilms of *P. aeruginosa* NH57388A or MR299. Calcofluor white staining revealed a change to open and weak matrices in the presence of the ϕNH-4 and ϕMR299-2 phages, indicating considerable destruction to the biofilm structure (Fig. 4C and D). At the same time, phage titers increased almost 2 log units during the 24-h period, confirming significant phage replication (Fig. 4E). The amount of luminescence recorded for both *Pseudomonas* biofilms also decreased by 2 log units over a 24-h period (Fig. 5). In contrast, the light level remained high and unchanged in the control biofilms (with no phage added) over the same period. Direct plating results also confirm that the significant reduction in light was a direct result of the destruction of the *Pseudomonas* cells by the phage. The numbers of CFU estimated for the biofilms before phage added were 2.6×10^7 to 3.8×10^7 CFU/well and 4.2×10^7 to 5.4×10^7 CFU/ well (well area of 9.5 cm^2) for strains NH57388A and MR299, respectively, and the amount of phage added was 0.5×10^8 to 1.0×10^8/well (multiplicity of infection [MOI] of 2 to 5). During the 24-h incubation, the number of *Pseudomonas* cells in the biofilms was reduced by 3 to 4 log units in the presence of phage.

Clearing of *Pseudomonas* from murine lungs. The ability of phage to kill *Pseudomonas in situ* in the lungs of infected 8-week-old female BALB/c mice ($n = 16$) was also examined (Fig. 6). In this regard, *lux* tagging of *Pseudomonas* cells was very useful in monitoring the fate of these cells in the lungs of infected mice. The presence of *Pseudomonas* was evident in the lungs of both test and control mice 2 h after infection. The amount of light recorded in the control mice (without phage) increased 3-fold and reached its maximum level after 6 h. The amount of light recorded in mice treated with phage decreased significantly during the same period.

DISCUSSION

There has been increased interest in phage therapy as a means of combating bacterial lung infections. However, there is little information about the efficacy of bacteriophage in clearing *Pseudomonas* growing on CF lung tissue or in an animal model. It has been well documented that the CF lung environment causes normally

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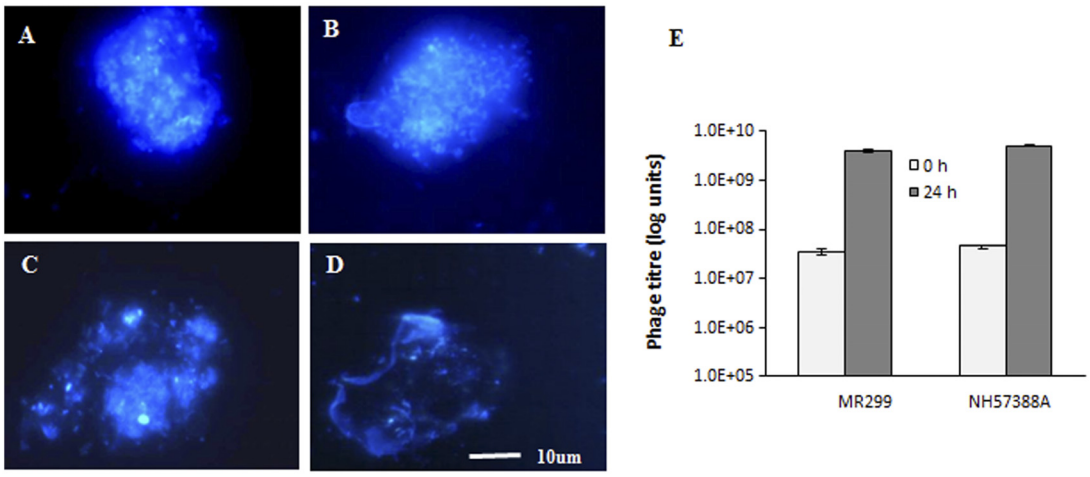


FIG 4 Fluorescent image of 24-h-old culture of *P. aeruginosa* cells grown on a CFBE41o- cell monolayer after Calcofluor white (fluorescent enhancer) staining. Staining confirms that *P. aeruginosa* NH57388A (A) and MR299 (B) are embedded in an exopolysaccharide structure prior to phage exposure. After 24-h incubation in the presence of mixed phages, staining indicates open and weak matrices with reduced numbers of cells for both NH57388A (C) and MR299 (D). An increase in phage titer was observed over the 24-h incubation period for both MR299 and NH57388A strains (E).

motile, planktonic *P. aeruginosa* to form mucoid biofilms (36–38). Our data show that 1 h after the addition of *lux*-tagged *Pseudomonas* cells to a CF epithelial monolayer, only a very low level of localized light was detected. The signal then increased over 100-fold in 24 h (Fig. 3). Washing the 24-h-old biofilm with MEM medium resulted in 25% reduction in the amount of light, indicating that 75% (2.6×10^7 to 5.4×10^7 CFU/well) of the *Pseudomonas* adhered as microcolonies over the entire surface of the CF cell monolayer. It is well documented in the literature that microcolony dispersal in *Pseudomonas* is a feature of biofilm maturation (39–42).

To be effective, phage must be able to penetrate the biofilm exopolysaccharide. This may account for the fact that it took more time (22 to 24 h) for phage (at similar MOI) to clear *Pseudomonas* growing on CFBE41o- cells than the 5 to 6 h required to clear recently introduced planktonic cells from the lungs of infected mice. We observed proliferation of *Pseudomonas* cells in the lungs of mice in the absence of phage, whereas phage treatment pre-

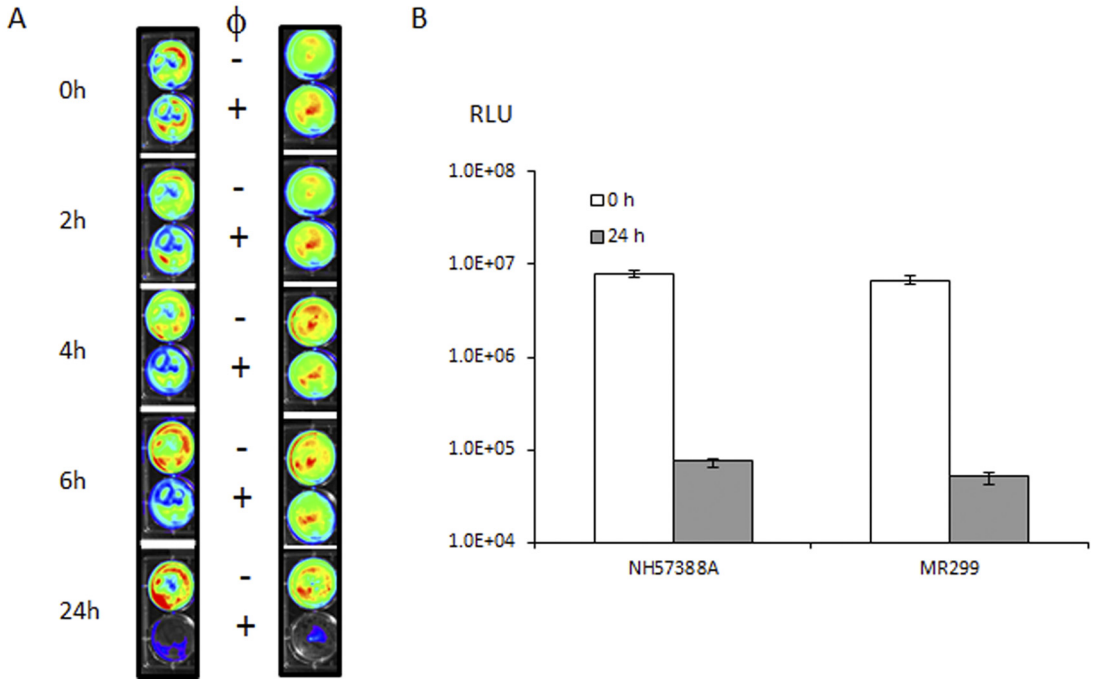


FIG 5 (A) Light emitted from nonmucoid *P. aeruginosa* MR299 strain and mucoid NH57388A strain grown on a CFBE410- cell monolayer for 24 h in the presence (+) and absence (−) of phage mix. (B) The RLU values are mean ± SD readings from 3 wells.

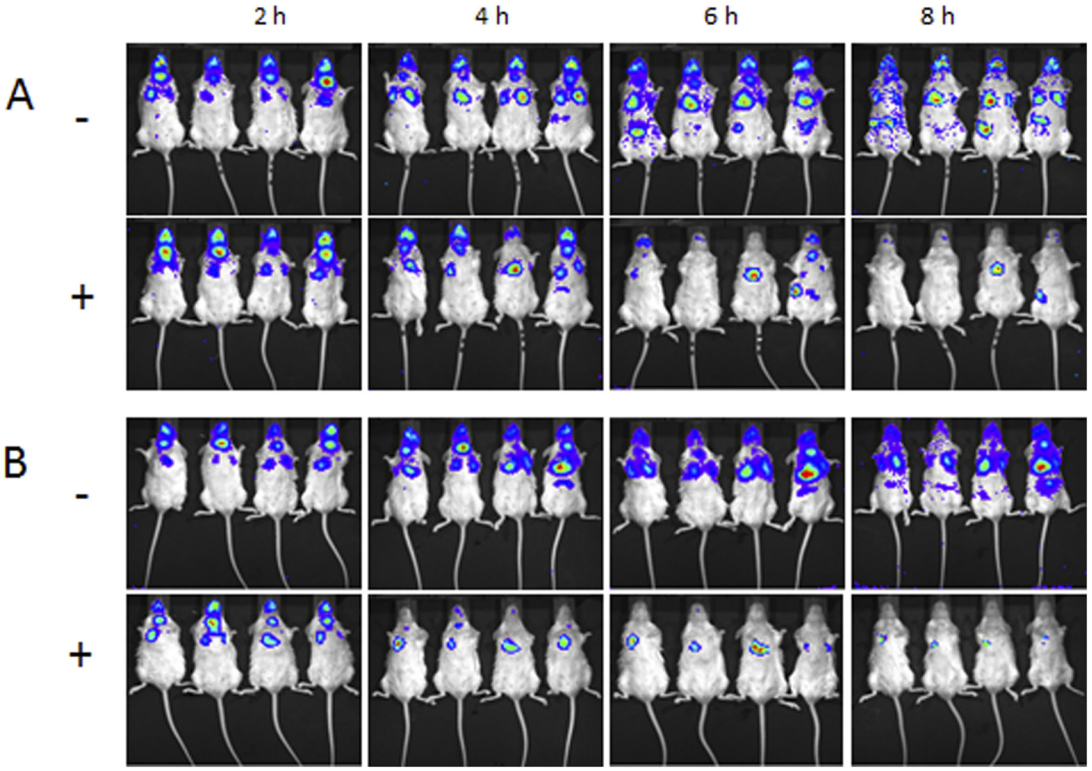


FIG 6 Mice ($n = 8$) were infected with nonmucoid *P. aeruginosa* MR299 (A) and mucoid NH57388A (mucoid strain) (B). Test mice (+) were treated with the phage mix (ϕMR299-2 and ϕNH-4B). Phage was given 2 h after the mice were infected with *Pseudomonas*. Control mice (−) did not receive the phage mix.

vented growth and reduced the bacterial load to a nondetectable level during the 6-h period. A recent study reported that the amount of light measured in mice infected with a *lux*-tagged *Pseudomonas* PAK strain also decreased over 6 h when phage PAK-P1 was administered 2 h after infection (24, 25). The amount of light increased in the control mice and in the mice that received a delayed phage treatment. The authors concluded that administering phage 2 h after infection was critical to resolving infection in the mouse model system and suggested that the efficacy of phage needed to be tested against biofilms.

One advantage of using phage to control bacterial infection is that they can replicate at the infection site (24). In addition to being a very effective way of clearing *Pseudomonas*, the use of a phage mix has another advantage over the use of a single phage in that it reduces the likelihood of a phage resistance population emerging. Different phages often use different bacterial receptors and therefore will require independent mutations to generate resistance to each phage (43). Consequently, unless mutants with generalized resistance mechanism evolve, a mix of different phages for which there is no cross-resistance should be able to prevent multiple phage resistance and provide indefinite control of bacterial population.

In conclusion, this study demonstrates that *Pseudomonas* growing on a CF airway tissue monolayer could be killed by phage. Since chronic lung infections in CF patients are associated with *Pseudomonas* biofilms rather than planktonic cells, we anticipate that biofilm clearing from lungs of CF patients by bacteriophage might take place in a similar manner to that observed with the exopolysaccharide-producing microcolonies growing on an epithelial cell monolayer. Moreover, we subsequently demonstrate that the same phage mix was effective in killing the pathogen in the lungs of infected mice. Our study reinforces the growing interest in using phage therapy as a means of attacking multidrug-resistant CF infections.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *Pseudomonas* strains isolated from CF patients and used in this study are shown in Table 1. LB medium was used throughout this study to culture *Pseudomonas* strains. A double-strength LB medium was made for phage isolation by doubling the weight of dry ingredients required to prepare single-strength LB broth. Cultures were grown at 37°C under aerobic conditions and shaking at 180 rpm. Solid media and soft agar overlays contained 1.5% and 0.7% agar (BD Difco, Oxford, United Kingdom), respectively.

Transformation of *Pseudomonas* with p16*lux* plasmid. *P. aeruginosa* strains NH57388A (mucoid) and MR299 (nonmucoid) were transformed with p16S*lux* plasmid (44) by the method of Shen et al. (45). In brief, *Pseudomonas* strains were grown in LB medium at 37°C until an optical density (OD) at 600 nm of 0.8 is reached. To facilitate electroporation, *Pseudomonas* exopolysaccharide was digested by adding alginate-lyase (catalog no. A1603; Sigma, Japan) to a final concentration of 2 U ml^{-1}. The cell enzyme mixture was incubated at 37°C for 30 min. The cells were centrifuged at 10,000 × *g* (4°C) for 10 min. The resultant pellet was washed twice with chilled electroporation buffer (containing 300 mM glucose, 5 mm $CaCl_2$, and 25 mM HEPES in distilled water [pH 7.0]) and resuspended in 0.1 ml of buffer (1×10^9 to 1×10^{10} CFU/ml). These electrocompetent cells were mixed with 10 μl of p16S*lux*-tagged plasmid DNA (10 μg) and incubated on ice for 10 to 15 min. The mixture was immediately transferred to a chilled electroporation cuvette (0.2-cm electrode gap Gene-pulser cuvette; Bio-Rad, Hercules, CA) and subjected to a

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single voltage shock by applying a pulse with an ECM 630 BTX Harvard precision pulse apparatus (Holliston, MA) at the following settings: capacitor, 25 μF; resistor, 200 Ω; and voltage, 2.5 kV. Immediately after the electric shock, 900 μl of chilled SOC medium (catalog no. S1797; Sigma-Aldrich) was added to the mixture and incubated on ice for 10 min, followed by incubation at 30°C for 2 to 3 h. The cells were concentrated by centrifugation at 10,000 $\times$ *g* (room temperature), and transformants were obtained by plating cells on LB agar containing erythromycin (800 μg/ml) and incubating at a permissive temperature (30°C) for 24 to 48 h. Ery^r colonies were checked for light emission using a VivoVision IVIS100 imaging system (Xenogen, Alameda, CA), luminescence was measured in relative light units (RLU) (in photons second^{-1}), and the presence of p16S*lux* was confirmed by miniprep and restriction analyses. A standard curve was generated to determine the relationship between light measurements and CFU (see Fig. S3 and Table S2 in the supplemental material).

Isolation of phage from sewage. *Pseudomonas* phages were isolated from fresh sewage obtained from a local sewage treatment plant as described previously (46) with some modifications. Sewage samples were centrifuged at 3,200 $\times$ *g* value (Heraeus Labofuge 400 centrifuge; Thermo Fisher Scientific, Inc., MA) for 12 min, and the supernatant was filtered using a 0.45-μm-pore-size filter (Sarstedt, Actiengeselischaft and Co., Germany). The sewage filtrate (5 ml) was mixed with an equal volume of double-strength LB broth, supplemented with 10 mM $CaCl_2$, and inoculated with a mixture of ten CF *P. aeruginosa* cultures (50 μl each of 1 $\times$ 10^7 to 2 $\times$ 10^7/ml). The samples were incubated overnight aerobically (37°C) with slow shaking (20 to 30 rpm). Following overnight incubation, the cultures were centrifuged at 3,200 $\times$ *g* value for 12 min to remove bacterial cells and debris, and the supernatant was filtered through a sterile 0.45-μm-pore-size filter. A double-layer LB agar plate containing a lawn of individual host culture supplemented with 10 mM $CaCl_2$ was prepared, and 10 μl of cell-free filtrate containing phage was applied to the plate. The plates were examined for the presence of plaques after incubating aerobically for 18 to 24 h at 37°C.

Plaque purification and bacteriophage titers. Phages were purified by successive single plaque isolation and propagation. In general, a single plaque was picked from a plate using a sterile capillary tube and added to a mid-log-phase *Pseudomonas* culture (10^8 CFU/ml) supplemented with 10 mM $CaCl_2$. The culture mixture and phage mixture were incubated at 37°C overnight. The lysate was filtered through a 0.45-μm-pore-size sterile filter, serial dilutions were made, and plaques were allowed to form on a lawn of the same host culture. Single plaques were purified through 3 successive rounds of plaquing and repeated three additional times after which purified phages were obtained. Phage titer was determined as the number of PFU/ml by plaque assay as previously described (47).

DNA extraction and restriction digestion analysis. High-titer-purified phage suspension was prepared by concentration of phage particles from 400 ml cell lysate in LB medium to a final volume of 1 ml in sterile ice-cold ammonium acetate (0.1 M, pH 7.2) as described before (46), and DNA was extracted from the high-titer-purified phage as previously described (46, 48, 49). Phage DNA was digested with restriction endonuclease EcoRI (New England Biolabs, MA) according to the supplier's recommendation, and digested samples were analyzed by gel electrophoresis using agarose gel (0.7%) containing ethidium bromide (SF1).

Genome sequencing and annotation. The genomes of phages ϕNH-4 and ϕ299-2 were sequenced by Beckman Coulter Genomics (Sanger sequencing services; Beckman Coulter, Takeley, United Kingdom) on a 454 GS-FLX sequencer, and sequences were assembled into contigs using the Newbler program (Roche Applied Sciences). The quality of the sequence was assessed using Hawkeye (Amos) (50). To confirm phage genome structure, primers were designed at contig ends for PCR amplification using Platinum PCR SuperMix (Invitrogen) followed by direct sequencing of the PCR products, and full phage genome assembly was performed using the Phred-Phrap-Consed package (51, 52). ORFs were predicted using GLIMMER 3.02 (53). The resulting gene models were fed into GAMOLA (54) for annotation. Complementary annotation was provided using the RAST annotation server (55). Data were manually curated using Artemis version 11 (56) where additional programs were then used, including BLASTp (57), GATU (58) and RBS finder (53). Comparative genomics with reference phages (ϕPaP3 and ϕLMA2) were analyzed using the Artemis comparison tool (ACT) (7) and the Mauve alignment tool (59).

Electron microscopy and phage characterization. Electron microscopy image of phages was obtained by applying a drop of high-titer phage suspension (1 $\times$ 10^9 to 2 $\times$ 10^9 PFU/ml) deposited on carbon-coated copper grids, negatively stained with 2% (wt/vol) potassium phosphotungstate (pH 7.2) and examined with Zeiss Supra 40VP scanning electron microscope (Carl Zeiss SMT Ltd., Cambridge, United Kingdom) fitted with a scanning transmission electron microscope detector (STEM) operating in bright-field mode at an accelerating voltage of 25 kV (Moorepark National Food Imaging Centre, TEAGASC Food Research Centre [TFRC], and Advanced Microscope Research Facility, University College Cork, Cork, Ireland).

Cell culture and *Pseudomonas* biofilm formation on the surface of a monolayer of human bronchial epithelial cells (CFBE41o- cells). Cystic fibrosis bronchial epithelial (CFBE41o-) cell (60, 61) cultures were grown by the method of Anderson et al. (12). In general, CFBE41o- cells were seeded in sterile 6-well, flat-bottom tissue culture plates (Sarstedt, Newton, NC) at a concentration of 10^6 cells/well and maintained in minimal essential medium (MEM) containing 10% fetal bovine serum, 2 mM l-glutamate, 100 μg/ml penicillin, and 100 μg/ml streptomycin (all from Invitrogen GIBCO, United Kingdom). The cells were grown at 37°C and 5% CO_2 using a Jouan IGO150 cell life incubator (Jouan, St. Herblain, France) for 8 to 10 days until cells form a confluent monolayer and tight junctions. The medium (MEM) was changed every 2 or 3 days until confluent growth was achieved.

For biofilm formation, *lux*-tagged *P. aeruginosa* cells were grown on the confluent CFBE41o- cell monolayer using a coculture model system (12). Once the monolayer growth was achieved (between 8 and 10 days), the medium was replaced with 1.5 ml fresh MEM (without fetal bovine serum, penicillin, and streptomycin), and *lux*-tagged *Pseudomonas* cells were inoculated (1 $\times$ 10^7 to 2 $\times$ 10^7 CFU/well). The plates were incubated at 37°C and 5% CO_2 for 1 h. The medium containing the unattached (planktonic) *Pseudomonas* cells was then removed using a sterile serological pipette and replaced with fresh MEM supplemented with 0.4% arginine and incubated for 24 h. Planktonic *Pseudomonas* cells were removed, and the biofilm culture was washed twice using MEM supplemented with 0.4% arginine. The integrity of the epithelial cell monolayer and the presence of growing *Pseudomonas* microcolonies were assessed by phase-contrast microscopy (Olympus IX50 inverted system microscope; Olympus Co., Tokyo, Japan). Luminescence from biofilms was monitored by Vivo Vision IVIS100 imaging system (Xenogen, Alameda, CA). Biofilm CFU was estimated by the method of Wirtanen et al. (62) with some modifications. A 24-h biofilm growing on an epithelial cell monolayer was washed twice with MEM and then scraped off using a tissue culture scrapper and transferred to a 2-ml Eppendorf tube containing 1 ml phosphate buffer. The tube was then vortexed thoroughly for 1 to 2 min to release the cells. After this, the samples were then serially diluted, and a plate count was made on a LB plate incubated at 37°C overnight.

Applying phages to biofilms and lung infections in mice. Fifty microliters of the phage mixture (1 $\times$ 10^9 to 2 $\times$ 10^9 PFU/ml of defined phage [containing ϕNH-4 and ϕMR299-2] in a 1:1 mixture) was applied to wells containing 24-h-old biofilms on a CFBE41o- cell monolayer, and the plates were incubated at 37°C and 5% CO_2 for 24 h. Biofilm clearing was monitored by measuring light and images taken for times indicated, using the IVIS100 imaging system. The lungs of 6- to 8-week-old conventional female BALB/c mice (n = 16) were infected with *Pseudomonas* by the method of Riedel et al. (28). The mice were infected intranasally with 50 μl *lux*-tagged *Pseudomonas* NH57388A or MR299 in phosphate buffer (2 $\times$ 10^8 to 5 $\times$ 10^8 CFU/ml). Two hours following infection, 50 μl of a phage mix suspension in phosphate buffer containing *Pseudomonas*

phages ϕNH-4 and ϕMR299-2 (2×10^9 to 5×10^9 PFU/ml for a multiplicity of infection [MOI] of 10) were given intranasally. Fifty microliters of phosphate buffer was given to the control groups. Animals were anesthetized with isoflurane, light was monitored, and images were taken using the IVIS 100 system at the time points indicated. Animals were kept in an animal colony, and all experiments were approved by the animal ethics committee of University College Cork.

Biofilm staining. *Pseudomonas* biofilms were stained with the fluorescent enhancer Calcofluor white (fluorescent brightener 28, catalog no. F3543; Sigma Aldrich, China). In brief, the monolayers of CFBE41o- cells containing biofilms were removed from wells using a sterile cell scraper (Sarstedt, Actiengeselischaft and Co., Germany) and mixed with a drop of Calcofluor white (0.1%) on a microscope slide. A coverslip was applied to the slide; the edges were sealed with paraffin oil, and the microscope slide was incubated for 1 h (37°C). Stained biofilm preparations were assessed by using an Olympus BX51 fluorescence microscope fitted with a U-RFL-T fluorescent power supply unit, and images were taken with a DP50 integrated camera (all from Olympus Optical Co., Japan).

Nucleotide sequence accession numbers. The completed phage genome sequences of phages NH-4 and MR299-2 were deposited in GenBank database and assigned accession numbers JN254800 and JN254801, respectively.

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SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.00029-12/-/DCSupplemental.

Figure S1, PDF file, 0.1 MB.
Figure S2, PDF file, 0.1 MB.
Figure S3, PDF file, 0.1 MB.
Table S1, DOC file, 0.1 MB.
Table S2, DOC file, 2.9 MB.
Table S3, DOC file, 0.1 MB.
Table S4, DOC file, 0.1 MB.

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