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Disruption of *Mycobacterium smegmatis* Biofilms Using Bacteriophages Alone or in Combination with Mechanical Stress

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Abstract

Environmental mycobacteria are capable of forming biofilms in low-nutrient environments, and these biofilms may act as reservoirs for opportunistic infections. The purpose of this study was to determine if bacteriophages could disrupt existing biofilms of acid-fast staining *Mycobacterium smegmatis*. Using the MBEC 96-well plastic peg assay system, *M. smegmatis* biofilms were created and then tested for their stability in the presence of mycobacteriophages isolated from a Minnesota sphagnum peat bog. All phages tested were lytic and were observed to have weak, intermediate, and strong abilities to disrupt *M. smegmatis* biofilms. The formation of biofilms was severely impaired in the presence of mycobacteriophages. Phage treatment was also shown to augment *M. smegmatis* biofilm disruption by mechanical forces of sonication or water flow. Our study shows that, as with biofilms of Gram-positive and Gram-negative bacteria, mycobacterial biofilms are also susceptible to destruction by bacteriophages.

Keywords

***Mycobacterium smegmatis*, Biofilm, Mycobacteriophage, Bacteriophage**

1. Introduction

The genus *Mycobacterium* contains over 120 species, including saprophytic and pathogenic bacteria [1]. Mycobacteria can be classified into two groups: obligate pathogens such as *Mycobacterium tuberculosis* and *Mycobacterium leprae*, and environmental mycobacteria (EM), such as *Mycobacterium avium* complex. While the

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majority of human infections are attributed to the tuberculosis complex of mycobacteria, EM are increasingly relevant in clinical settings as the cause of opportunistic infections that include skin lesions, pulmonary infections, lymphadenitis in children, endocarditis, meningitis, and disseminated disease [2]-[4]. EM infections are expected to increase due to expanding groups of the elderly, HIV-infected individuals, and those on immunosuppressive therapy [5]. EM are transmitted from aquatic and environmental sources primarily by ingestion, inhalation, and inoculation [6].

Bacteria typically inhabit their environments by forming biofilms, surface-attached communities of bacteria that are embedded in an extracellular polymeric substance (EPS) [7]. The composition of EPS varies based on the bacterial species and can contain polysaccharides, proteins, nucleic acids, and lipids [8]-[11]. Numerous mycobacteria, including *Mycobacterium smegmatis*, have been observed to grow in biofilms [12]-[16]. However, it is unclear if biofilm formation plays a role in pathogenic mycobacteria like *M. bovis* [17], *M. mariunum* [18], and *M. lulcerans* [19]. EM are highly enriched in showerheads, and a source of *Mycobacterium avium* has been traced to home showers of those infected [20]-[22]. Biofilm-borne bacteria exhibit a unique physiology compared to their planktonic (free-floating) counterparts. Biofilm cells may have increased resistance to environmental stresses like low pH, UV exposure, salinity, and dehydration [23]-[25]. Physiological heterogeneity and reduced growth rates inside biofilms confer resistance to disinfectants and antibiotics [12] [13] [26] [27]. The ability to adhere to surfaces combined with a high resistance to chlorine-based disinfectants enables mycobacteria to colonize municipal water systems [28]-[31]. This highlights the need for alternative biofilm intervention strategies for EM.

Since the discovery of bacteriophages over 100 years ago, the ability of phages to sanitize surfaces and treat human diseases has been examined. More recently, the ability of bacteriophages to reduce biofilm populations was demonstrated in a number of clinically relevant species: *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus epidermidis*, *Pseudomonas fluorescens*, and *Staphylococcus aureus* [26] [32]-[36]. No previous studies have examined the interaction of bacteriophages in mycobacterial biofilms. Here we show that environmental mycobacteriophages can disperse existing *M. smegmatis* biofilms and can prevent these biofilms from forming initially.

2. Materials and Methods

2.1. Bacterial Strains and Growth Condition

Biofilms were generated using *Mycobacterium smegmatis* mc^2 155. Cells were grown on 7H11 agar (Difco) for 3 - 4 days at 37˚C before inoculating into 7H9 broth supplemented with 0.05% (v/v) Tween 80 and growing to logarithmic phase (OD_{600} 0.75) at 37˚C prior to use in biofilm assays.

2.2. MBEC Biofilm Assay

Log-phase cultures of *M. smegmatis* were pelleted and suspended in 7H9 broth without Tween 80 before bacteria were allowed to attach to plastic pegs of MBEC assay plates (MBEC™ Biofilm Technologies Ltd., Calgary, Alberta). MBEC plates were gently rocked at room temperature (RT) with fresh media changes every 3 days. Biofilms were quantified using two different assays: 1) crystal violet (CV) staining of attached biofilms, and 2) serial dilutions to count colony-forming units (CFUs) following biofilm disruption. Quantification of *M. smegmatis* biofilms by CV staining closely approximates quantification by determining viable cell counts in a biofilm (**Figure 1**). Serial dilution plate counting was performed by aseptically removing pegs containing biofilms from MBEC plates with sterile pliers and placing them in individual microcentrifuge tubes containing 200-µl aliquots of 7H9broth without Tween 80. The tubes were gently vortexed to remove unbound cells and the fluid was discarded. Remaining biofilm cells were recovered from the pegs by adding them to 200-µl aliquots of 7H9 broth with Tween80 and sonicating them in a water bath sonicator (FS20, Fisher Scientific) for 5 min. CFUs were enumerated by serial dilutions and plate counting on 7H11 agar plates.

CV staining was performed, as previously described [37]. Briefly, MBEC plates containing biofilms were rinsed in phosphate buffered saline (PBS) to remove planktonic cells before fixing biofilms in methanol for 15 min and allowing them to dry. The plates were then stainined in a CV solution (0.5%, w/v) for 30 min before rinsing with PBS. Stained MBEC plates were placed into a 96-wellmicrotitre plate wells containing 150 µl of 33%

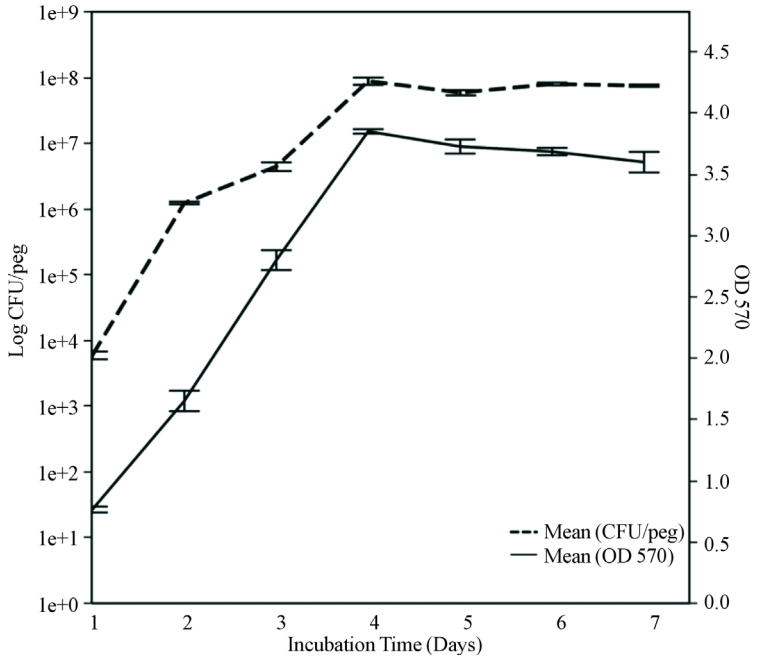

Figure 1. Growth of *M. smegmatis* biofilm in 7H9 broth in the MBEC device. Bacterial counts were performed by removing three pegs and extracting bacteria by water bath sonication. Viable counts were determined by serial dilution. Crystal violet (CV) staining was performed and the retained stain was quantified. The solid line represents the mean CFU/peg of three independent experiments (n = 9). The bars represent standard error (SE) and the dashed line represents the mean absorbance of dye extracted from pegs of three independent experiments (n = 9).

(v/v) glacial acetic acid for 20 min. Eluted stain was quantified with a microtitre plate reader at 570 nm (SpectraMax M3, Molecular Devices).

2.3. Isolation and Production of Phages

Liquid samples were collected from the aqueous layers of a peat bog in the Big Bog State Recreation Area (Waskish, MN). These environmental samples were first enriched for phages by adding *M. smegmatis* and incubating at RT for 7 days. Samples were then centrifuged at 3000 ×g for 10 min at RT, and the supernatants were passed through 0.2-µm filters. Individual phages were isolated using an agar overlay technique. Briefly, filtered bog supernatants were incubated with log-phase *M. smegmatis* overnight in phages buffer (10 mM Tris, pH 7.5, 10 mM $MgSO_4$, 68 mM NaCl, and 1 mM $CaCl_2$). These mixtures were then suspended in 5 ml of 7H9 top agar (0.35% agar w/v), supplemented with 1mM of $CaCl_2$, and poured onto 7H11top agar plates. These plates were incubated at RT for 2 days and an additional1day at 28˚C before scoring for plaques. Individual plaques were picked and purified through 3 repeated rounds of infection of *M. smegmatis*.

Working phage lyates were prepared by selecting a plate from the final plaque purification process that contained enough phages to clear the entire petri plate. Phage buffer was added to this plate and allowed to stand for 3 h at RT. Liquid was collected and filtered through 0.2-µm syringe filters to produce sterile stocks of purified phages. Phage titer counts were determined by serial dilutions, and lysate stocks were equilibrated to a final concentration of 1×10^7 plaque-forming units (PFUs)/ml.

2.4. Screening Phages for Biofilm Inhibition

Phage stocks were individually screened for their abilities to destroy existing *M. smegmatis* biofilms on MBEC pegs. Two-day-old biofilms of *M. smegmatis* on pegs were submerged into individual wells of 96-well polystyrene plates containing 7H9 broth containing 1 mM $CaCl_2$ and varying strains of phages. After a 3-day exposure to the phages, CV staining was used to quantify remaining biofilms. The data was expressed as a mean percen-

tage of the biofilm growth on non-phage treated controls and represents three independent experiments.

2.5. Prevention of Biofilm Formation

MBEC assay plates were simultaneously incubated with a combination of planktonic *M. smegmatis* and phages before staining with CV to determine if phages reduce attachment of bacteria to surfaces. Each peg was exposed to *M. smegmatis* (5×10^6 CFUs/ml) with increasing concentrations of phages from 1×10^2 - 1×10^6 PFUs/ml. MBEC plates lowered into the wells were allowed to incubate at RT with gentle shaking (130 rpm) for 4 days before assaying biofilm formation by CV staining. The data was expressed as a mean percentage of the biofilm growth observed on non-phage treated controls. Three independent experiments were performed with 12 replicates for each treatment, producing a total n = 36.

2.6. Phage and Antibiotic Biofilm Challenge

Disruption of existing 2-day-old biofilms was measuredin the presence of the antibiotic isoniazid (INH), bacteriophages, or a combination of INH and phages. MBEC plates with biofilms were submerged into wells of a 96-well plate containing either INH (10 μg/ml) and/or phages (1×10^3 PFUs/ml) for durations of 24, 48, or 72 h. Two pegs for each treatment were removed, water-bath sonicated, and serially diluted to quantify surviving bacteria.

2.7. Phage Effects on Dispersal of Biofilm Bells by Mechanical Forces

Two-day-old biofilms on MBEC plates were exposed to phages for 3 days before the MBEC plates were cut in half. One half of the plate was rinsed by gentle submersion in PBS prior to CV staining and served as a control treatment. The other half was subjected to either sonication or to flowing water prior to CV staining to examine how phage treatment affects subsequent mechanical disruption of biofilms. For the sonication assay, the MBEC plate was placed in microtiter plates containing 7H9 and sonicated for 5 min using a water bath sonicator. Sonication for this length of time caused no change in the viability of *M. smegmatis* cells (data not shown). Data was expressed as a percentage of the CV staining on non-sonicated control pegs of the corresponding treatment.

The effect of running water on phage treated biofilms was examined by anchoring the MBEC plate with the pegs facing upward in a 4 × 8 cm trough. The trough was then flooded with deionized water before 25 liters of deionized water was dispensed from a carboy at an average flow of 8.33 L/min to ensure consistency between trials (**Figure 2**). Data was expressed as a percentage of the absorbance of non-water exposed control pegs of the corresponding treatment. Two independent experiments were performed with 12 replicates of each treatment, for a total n = 24 for in both sonication and water exposure assays.

2.8. Effects of Biofilm Age on Phage Mediated Disruption

Biofilms were grown using the MBEC system for 2 days or 6 days prior to infection. These two durations represent actively growing and steady-state biofilms, respectively (**Figure 1**). Biofilms on pegs were subjected to destruction by phages for 1 - 3 days as described above, and the effects of phage disrupting biofilms were determined by CV staining. Data was expressed as a percentage of the biofilm growth on the corresponding 2 or 6 day non-phage controls. Two independent experiments were performed with 24 replicates of each treatment for a total n = 48.

2.9. Scanning Electron Microscopy

Pegs containing existing biofilms with and without phage treatment were aseptically removed and fixed in 5% (v/v) gluteraldehyde/0.1 M cacodylate buffer (pH 7.2) for 24 h at 4˚C. Pegs were then air dried for 7 days and sputter-coated with gold-palladium prior to being visualized by using a JEOL JSM-6490LV scanning electron microscope, as previously described [38].

3. Results

3.1. Formation of *M. smegmatis* Biofilms Using MBEC Assay

The MBEC assay system (also known as the Calgary Biofilm Device) is a high-throughput device for testing

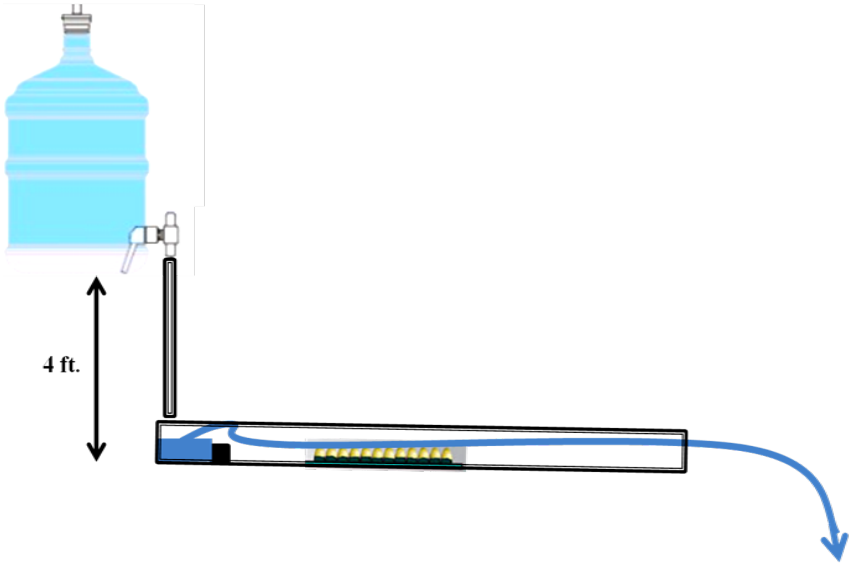

Figure 2. The experimental apparatus used for exposing *M. smegmatis* biofilms to the mechanical force of running water. MBEC top plates were anchored pegs facing up in the trough. A set volume of 25 L was used to flood the trough at an average flow of 8.33 L/min. Biofilms were then quantified using CV staining.

susceptibility of bacterial biofilms to antimicrobial substances [12] [13]. It consists of a plastic plate with 96 pegs that can be individually submerged into solutions in 96-well tissue culture plates. This assay system has previously been used to study mycobacterial biofilm formation and corresponding resistance to biocides [12] [13] [18]. We first determined if MBEC plates can be used to generate *M. smegmatis* biofilms that can then be exposed to antibiotics or mycobacteriophages. To determine the optimal time for phage treatment of biofilms, a growth curve for *M. smegmatis* biofilms was constructed. Biofilm exhibited a sigmoidal growth curve with a 2-log increase during the first 2 days and maximum growth of 9×10^7 CFU/peg occurring on day 4 (**Figure 3**). Similar results were obtained from crystal violet (CV) staining (**Figure 3**). To determine if the sonication of bacterial biofilms was detrimental to *M. smegmatis* survival, aliquots of planktonic *M. smegmatis* were sonicated in a water bath and surviving cells enumerated using dilution plate counting. No decrease in viable bacteria was observed during 5 min of sonication (data not shown). Based upon the observed growth of *M. smegmatis* on MBEC pegs, 2 and 6-day-old biofilms were selected for future phage treatments since these two time points represent actively growing and steady-state biofilms, respectively. This experiment also establishes the validity of using CV staining to quantify biofilms, as the CV stain data closely correlated with the number of viable cells released from biofilms by sonication (**Figure 1**).

3.2. Screening Phages for Biofilm Inhibition

Because sphagnum peat bogs are rich sources of environmental mycobacteria [39], these locations were utilized to obtain novel strains of mycobacteriophages. Isolation of environmental mycobacteriophages from a single bog in northern Minnesota resulted in over 40 different purified isolates, which produced clear plaques on *M. smegmatis* lawns that ranged in diameter from 1 - 4 mm. Thirty-eight phage isolates were screened for their ability to disperse 2-day old biofilms on MBEG pegs, and these isolates exhibited varying abilities to destroy biofilms was monitored by CV staining (**Figure 3**). The greatest destruction of *M. smegmatis* biofilms occurred with mycobacteriophage isolates CU 14A, CU 9, DL6, DL 12, and DL 16A that all resulted in CV staining that was 17% - 19% the level of non-phage treated biofilms. Isolates DL 9 and DL 11B were lytic phage that resulted in the smallest reductions of biofilm that was 67% the intensity of non-phage treated biofilms. The remaining 32 phage isolates destroyed existing biofilms in an intermediate range (**Figure 3**). The three phage isolates CU 14A, DL 2A, and DL 9 (**Figure 3**, arrowheads) represented strong, intermediate, and weak abilities to destroy biofilms, respectively, and they were chosen for further characterization of their interactions with *M. smegmatis* biofilms.

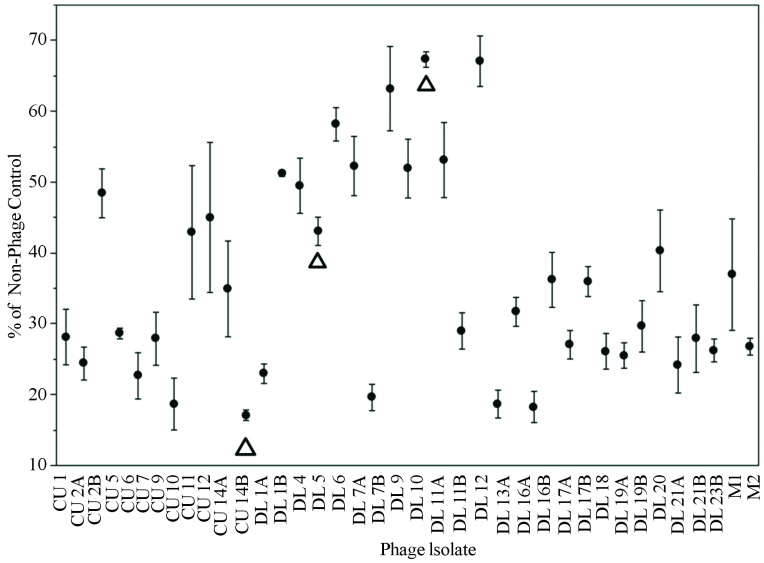

Figure 3. Screening of purified mycobacteriophage isolates for destruction of *M. smegmatis* biofilms. Data was obtained by CV staining. The bars represent SE and the points represent staining as a mean percentage of biofilm growth on non-phage treated controls for three independent experiments (n = 3). The arrowheads indicate the relative destructive capabilities of the three phage species chosen for further analysis.

3.3. Prevention of Biofilm Formation

While mycobacteriophages can be shown to destroy existing *M. smegmatis* biofilms (**Figure 3**), we wished to determine if they could also prevent the initial formation of these biofilms. MBEC assay plates were incubated in the presence of a combination of planktonic *M. smegmatis* and phages to determine if bacterial attachment and subsequent biofilm formation would be prevented. Biofilms grown individually in the presence of all three phage species tested showed a decrease in amounts relative to biofilms formed in the absence of phages (**Figure 4**). The greatest inhibition of biofilm formation (96% reduction compared to non-phage control pegs) occurred with phage DL 9 using a titer of 1×10^6 PFU/well. Prevention of biofilm formation directly correlated with increasing phage titers for DL 2A and DL 9, but phage CU 14A showed equal inhibition of biofilms regardless of concentrations used (**Figure 4**).

3.4. Phage Effects on Mechanical Biofilm Dispersal

Based upon CV staining of phage-treated biofilms, phages alone are not sufficient to completely eliminate *M. smegmatis* biofilms (**Figure 3** and **Figure 4**). However, it is possible that in addition to lysing *M. smegmatis* cells existing in a biofilm that the phages can also disrupt the structures of biofilms and make them more susceptible to dispersion by mechanical disruption. To test this idea, 2-day-old biofilms were treated with phages for 3 days prior to exposing them to disruption by either sonication or flowing water. Following phage treatment and mechanical disruption, pegs were stained with CV and amounts of retained stain were quantified (**Figure 5**). Sonication produced a 17% reduction in mass of biofilms that had not been treated with phages (**Figure 5(a)**, black bar). However, sonication of biofilms pretreated with phage CU 14A or DL 2A resulted in 54% and 40% reduction in biofilms, respectively, compared to no phage treatment (**Figure 5(a)**). Phage isolate DL 9 did not elicit significantly greater sonication dispersal compared to non-phage controls. CV staining of the fluid from sonicated biofilms that had been pre-treated with CU 14A and DL 2A showed detached multicellular aggregates in the fluid (data not shown). Such cell aggregates were not seen in fluid from sonicated biofilms that had not been treated with phages. This shows that some phage species have the ability to loosen the matrixes of *M. smegmatis* biofilms.

Instead of sonication, an experiment was performed to measure biofilm dispersal by flowing water following phage treatment. Flowing water is a more natural condition that mycobacterial biofilms encounter in municipal

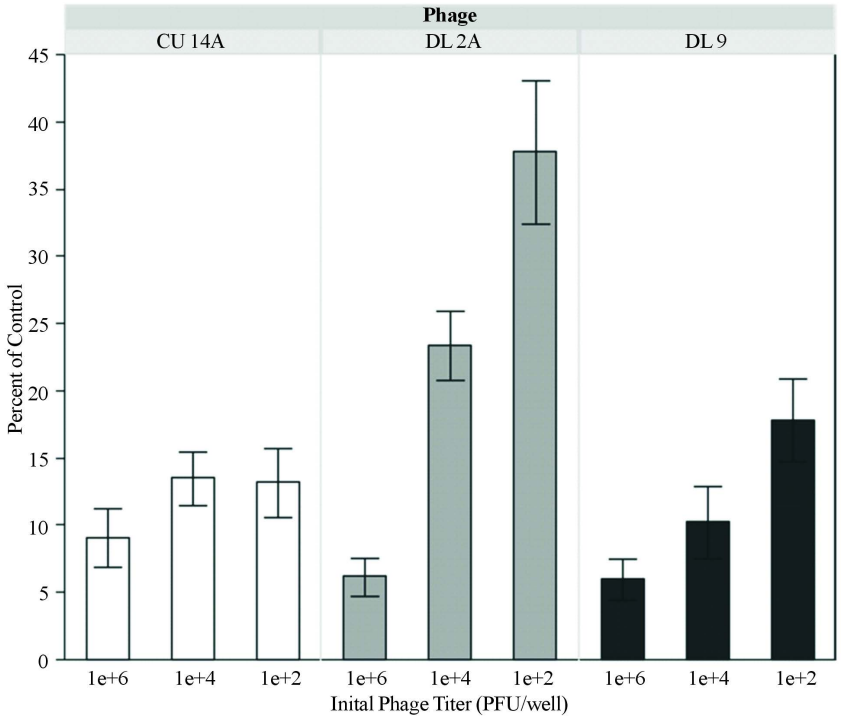

Figure 4. Presence of phage prevents *M. smegmatis* biofilm formation. MBEC plates were incubated 4 days in the presence of *M. smegmatis* and of 10^2, 10^4 or 10^6 PFU/well of phage before quantifying biofilm formation by CV staining. Bars represent staining as a mean percentage of non-phage control biofilms. Error bars represent the SE of three independent experiments with 12 replicates (n = 36).

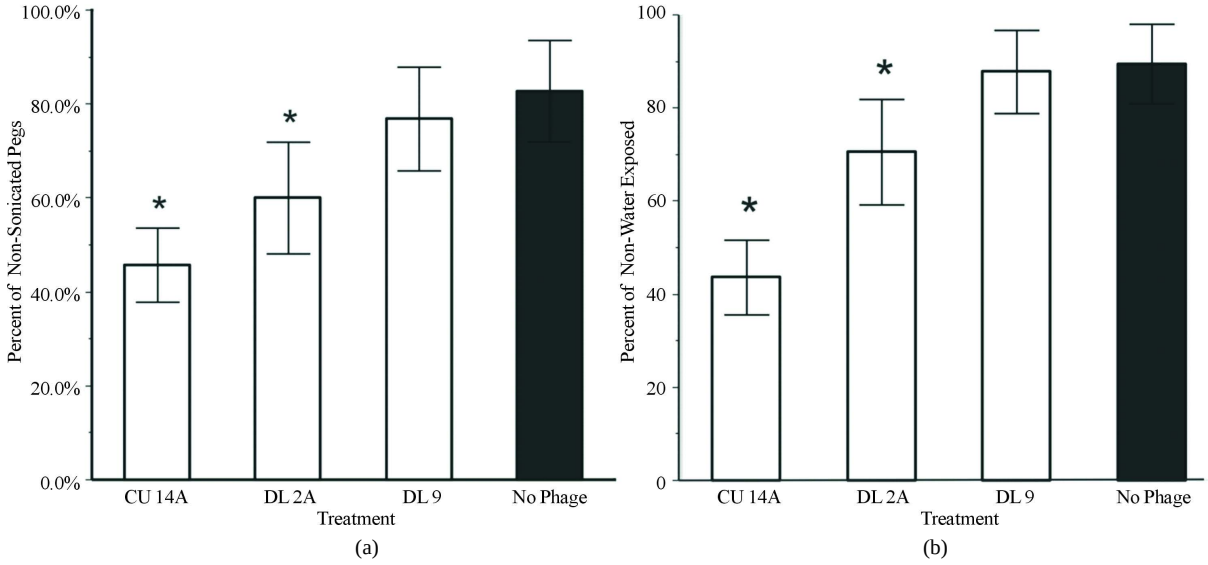

Figure 5. Phage treatment of biofilms increases *M. smegmatis* dispersal by mechanical disruption. (a) Dispersal of biofilms in response to sonication. Attached bacteria were quantified using CV staining and data expressed as a percentage of staining on non-sonicated pegs for the corresponding treatment. Error bars represent SE of two independent experiment with 12 replicates (n = 48). *Significant reduction in biomass compared to the non-phage treatment (Tukey's HSD test, $P < 0.001$). (b) Dispersal of biofilms in response to flowing water. Attached bacteria were quantified using CV staining and data expressed as a percentage of staining on non-water exposed pegs for the corresponding treatment. Error bars represent SE of two independent experiment with 12 replicates (n = 48). *Significant reduction in biomass compared to the non-phage treatment (Tukey's HSD test, $P < 0.001$).

water sources. Exposure of biofilms to phages CU 14A and DL 2A produced a greater reduction in biomass (51% and 30%, respectively) compared to only a 11% reduction of biofilms by water flow if the biofilms were not exposed to phages ($P < 0.001$, **Figure 5(b)**). Exposure to phage DL 9 resulted in no significant increase in water dispersal compared to non-phage controls.

3.5. Effects of Biofilm Age on Phage-Mediated Dispersal

Initial screening of mycobacteriophages was performed using actively developing *M. smegamatis* biofilms with a single exposure length to phages (**Figure 3**). Biofilms will exhibit multiple phenotypes over the course of development, including differential regulation of genes and expression of proteins [40]. We tested if different stages of biofilm development had different susceptibilities to phage-mediated dispersal. To determine the effect of biofilm maturation on phage susceptibility, biofilms were grown for either 2 or 6 days prior to infection. Two and 6 days produce actively growing biofilms and steady state biofilms, respectively (**Figure 1**). For phage species CU 14A and DL 2A, the greatest biofilm reduction was seen for 2-day-old biofilms instead of for the more mature 6 day-old biofilms ($P < 0.05$, **Figure 6**).

Scanning electron microscopy (SEM) was used to directly visualize the effect of phages on *M. smegmatis* biofilms on the MBEC pegs (**Figure 7**). Greater cell confluence can be observed in non-phage controls (**Figure 7(a)** and **Figure 7(c)**) compared to phage-treated pegs (**Figure 7(b)** and **Figure 7(d)**). Extensive EPS can be seen in the 6-day-old biofilms showing a uniform mat of cells (**Figure 7(c)**). The 2-day-old biofilm showed EPS production, but the biofilm structure is much more irregular than the 6-day-old biofilm and contains fluid-filled voids (compare **Figure 7(a)** with **Figure 7(c)**). Therefore, SEM analysis confirms that the age of *M. smegmatis* biofilms matters with regards to surface area that is potentially exposed to attack by lytic phages.

4. Discussion

Recent studies have shown bacteriophages can disrupt biofilms of a variety of different bacteria in which the extracellular matrixes are typically polysaccharide in nature [41]-[46]. However, it was previously unknown if phages could disrupt mycobacterial biofilms with lipid-based extracellular matrixes [47]. Here we show myco-

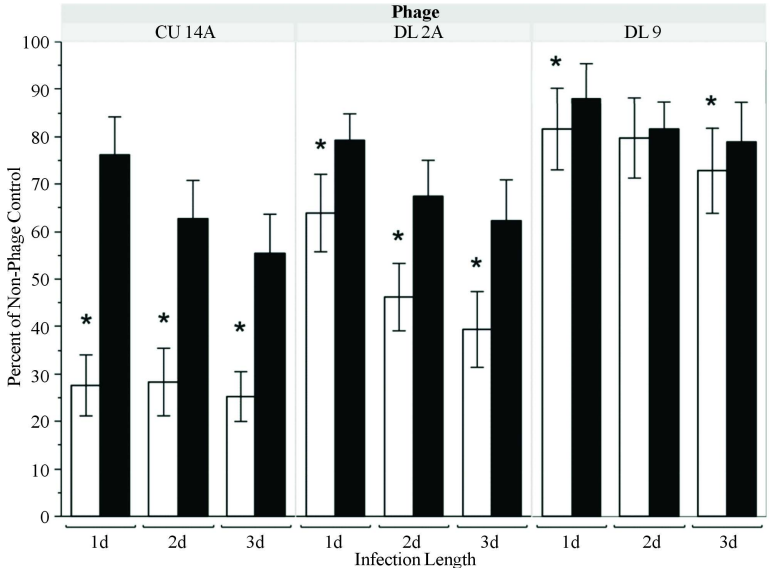

Figure 6. Effects of biofilm age on biomass reduction. MBEC devices were incubated either two or six days prior to infection, and biofilms were measured using CV staining. White bars represent staining as mean percentage of biofilm growth on non-phage treated controls for 2-day-old biofilms and black bars represent the mean percentage growth on non-phage treated 6-day-old biofilms. Error bars represent the standard deviation of two independent experiments with 24 replicates (n = 48). *Significantly greater reduction in biomass compared to 6 day biofilms (light bands; Tukey's HSD test, $P < 0.05$).

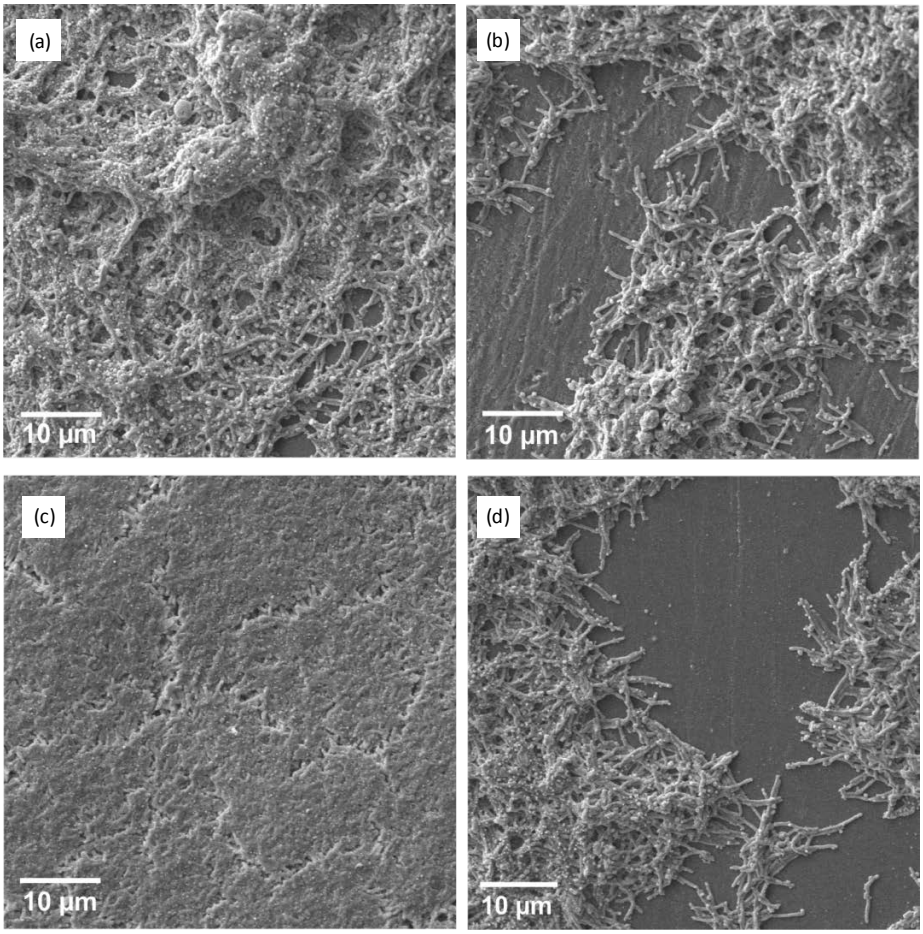

Figure 7. SEM of *M. smegmatis* (50,000× magnification) growing on an MBEC device after 2-day incubation and no phage treatment (a), 2-day incubation plus 3-day infection with CU 14A (b), 6-day incubation and no phage treatment (c), and 6-day incubation and 3-day infection with CU 14A (d).

bacteriophages isolated from a peat bog in northern Minnesota have a wide diversity of abilities to damage existing *M. smegmatis* biofilms. Bogs are known sites of mycobacterial diversity, and it is likely that phages and their host species have complex ecological relationships in these environments. The wide variety of biocidal activity of these phages on *M. smegmatis* biofilms (**Figure 3**) parallels the enormous genetic diversity found in different mycobacteriophages [48] [49]. Of the 39 mycobacteriophage species tested for biocidal activity on *M. smegmatis*, all exhibited at least some destructive potential on existing biofilms ranging from strong to intermediate to weak disruption activity as exemplified by the three phage isolates CU 14A, DL 2A, and DL 9, respectively. All three of these phage species produced clear plagues on *M. smegmatis* in soft agar (data not shown), but these three phage isolates had marked differences in their abilities to reduce existing biofilms (**Figure 3**, arrowheads). In addition to disrupting existing biofilms, these three mycobacteriophage species can also inhibit the initial formation of biofilms (**Figure 4**). Phage variables that may affect the varying amounts of biofilm disruption include phage size (smaller phages might penetrate matrixes of biofilms more easily), burst size (more phages might mean greater disruption of host cells), charge of phage particles (the matrixes of mycobacterial biofilms are believed to be hydrophobic) [47], host cell receptors (accessibilities to different receptors in biofilms), and phage genes (certain phages may have enzymes that disrupt biofilms differently).

While no phage species tested here could eliminate all attached *M. smegmatis* from MBEC pegs (**Figure 3**), at least two species showed an ability to disrupt biofilms and set them up for further disruption by the mechanical forces of sonication and flowing water (**Figure 5**). These results are relevant both to biofilm control strategies and to better understanding mycobacterial ecology. Efforts to control biofilms by mechanical measures could be

enhanced in the presence of phages that make cells easier to dislodge. When biofilms mature, they are capable of releasing planktonic cells into the environment to colonize new areas. Loosening of mycobacteria from biofilms by phages could enhance dispersal of live bacteria. Therefore, in addition to lysing bacteria it is possible that mycobacteriophages in nature help accelerate the spread of mycobacteria from stationary biofilms into the wider environment.

5. Conclusion

This study shows the potential for phages to prevent and to disrupt mycobacteria contamination on surfaces. Future studies will focus on the molecular characterization of phages, like CU 14A, which are highly effective in reducing biofilms. The approaches described here could also be applied to isolating phages to disrupt environmental biofilms of known human pathogens like *M. marinum* and *M. ulcerans* [18] [19].

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