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Tracing the interaction of bacteriophage with bacterial biofilms using fluorescent and chromogenic probes

MM Doolittle[1], JJ Cooney[1] and DE Caldwell[2]

[1]*Environmental Sciences Program, University of Massachusetts, Boston, MA, USA;* [2]*Department of Applied Microbiology and Food Science, University of Saskatchewan, Saskatoon, S7N OWO, Canada*

Phages T4 and E79 were fluorescently-labeled with rhodamine isothiocyanate (RITC), fluoroscein isothiocyanate (FITC), and by the addition of 4′6-diamidino-2-phenylindole (DAPI) to phage-infected host cells of *Escherichia coli* and *Pseudomonas aeruginosa*. Comparisons of electron micrographs with scanning confocal laser microscope (SCLM) images indicated that single RITC-labeled phage particles could be visualized. Biofilms of each bacterium were infected by labeled phage. SCLM and epifluorescence microscopy were used to observe adsorption of phage to single-layer surface-attached bacteria and thicker biofilms. The spread of the recombinant T4 phage, YZA1 (containing an rII-LacZ fusion), within a *lac*⁻ *E. coli* biofilm could be detected in the presence of chromogenic and fluorogenic homologs of galactose. Infected cells exhibited blue pigmentation and fluorescence from the cleavage products produced by the phage-encoded β-galactosidase activity. Fluorescent antibodies were used to detect non-labeled progeny phage. Phage T4 infected both surface-attached and surface-associated *E. coli* while phage E79 adsorbed to *P. aeruginosa* cells on the surface of the biofilm, but access to cells deep in biofilms was somewhat restricted. Temperature and nutrient concentration did not affect susceptibility to phage infection, but lower temperature and low nutrients extended the time-to-lysis and slowed the spread of infection within the biofilm.

Keywords: biofilms; bacteriophages T4 and E79; *Escherichia coli*; *Pseudomonas aeruginosa*; SCLM; fluorescence

Introduction

Bacteriophage infection of bacterial cells has been employed to garner knowledge about bacterial physiological processes, chromosomal and cell membrane structure and organization, genetic control of phage and host biochemical pathways, as well as ecological processes [20,25,27,33,34]. Such studies have been conducted almost exclusively using free-living, planktonic bacterial cells. Exceptions are studies focused on phage that infect bacteria growing in natural soil samples [22], soil reactors [26], or adsorbed to stainless steel surfaces [37]. Despite the realization that aquatic bacteria exist *in situ* predominantly in biofilms, and the increased emphasis on the importance of biofilms to ecological processes [13], little attention has been focused on the ability of phage to infect biofilms. Many studies with phage have utilized bacteria growing on an agar surface as a lawn, but not on biofilms growing on submerged surfaces. Phage resistance exhibited by mucoid *Pseudomonas* strains grown on agar plates [32] has been cited as an example of biofilms' enhanced phage resistance [14] but extrapolations to submerged biofilms have not been validated in the refereed literature. The inference that all biofilms are resistant to phage infection was disproven by the demonstration [17] that an *E. coli* biofilm growing on the submerged surfaces of a Modified Robbins Device was susceptible to infection by somatic coliphage T4.

It is not known whether surface-attached cells in a biofilm are more or less susceptible to phage infection than surface-associated or planktonic cells. The nature of how phage infection spreads within a biofilm (eg as plaques or in plumes) also remains uncharacterized. To focus some light on these issues, fluorometric and chromogenic tracers were employed to visualize how phage infection manifests itself within *E. coli* and *P. aeruginosa* biofilms.

Correspondence: JJ Cooney, Environmental Sciences Program, University of Massachusetts at Boston, MA 02125-3393, USA
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Materials and methods

Strains

Escherichia coli and *Pseudomonas aeruginosa* strains used to colonize flow cells to form biofilms and the bacteriophages used to infect them are described in Table 1.

Table 1 Bacterial hosts and lytic bacteriophages

	References
Escherichia coli:	
strain B (prototroph)	[10]
strain ECCB, adherent variant of strain B	isolated in present work
strain 3000 XIII Genotype: HfrΔ(gpt-lac)5, relA, spoT1, thi-1 (deleted for lac I-Z)	[4]
Coliphage:	
T4D⁺ (Doermann wildtype)	[15]
T4-lacZ (Sa delta 9,YZA1) Genotype: rIIB-LacZ fusion	[39]
Pseudomonas aeruginosa:	
strain PAO-1 (prototroph)	[38]
Pseudomonas phage:	
E79	[38]

Fluorescent labeling of phage coats

Titers of concentrated bacteriophages T4 and E79 were obtained by infection of *E. coli* B and *P. aeruginosa* PAO-1 cultures, respectively, as described by Doolittle and Cooney [16]. Phage lysates were filtered (0.45-μm pore-size GFA, Millipore, Bedford, MA, USA) and concentrated by centrifugation but not CsCl-purified prior to conjugation with rhodamine isothiocyanate (RITC, Sigma Chemical Co, St Louis, MO, USA) or fluorescein isothiocyanate (FITC; Sigma) by the method of Cherry [11]. A 100-μl portion of 0.2 M Na_2HPO_4, pH 9.0, was added dropwise to a 400-μl phage concentrate stirred by a small stir bar in a 1.5-ml Eppendorf tube. Two hundred micrograms of RITC were dissolved in 1.0 ml 0.1 M Na_2HPO_4, pH 9.0, in another Eppendorf tube. Undissolved precipitate was pelleted by brief centrifugation in a microfuge. A 200-μl portion of the RITC solution was added dropwise to the phage concentrate while the concentrate was being stirred. One drop (*ca* 40 μl) of 0.1 M Na_3PO_4 was added immediately to increase the pH and the mixture was incubated at 25°C.

After 4 h the mixtures were loaded on 20–70% CsCl step-gradients in 5-ml tubes and centrifuged for 20 min at 150000 × *g* in a SW 50.1 rotor at 20°C. This facilitated the separation of intact phage, which formed a bright red band at the 50%/60% CsCl interface, from ghost phage which comprised a faint, diffuse pink band at the 20%/30% CsCl interface. Intact phage were collected from the gradient and dialyzed in Spectra/Por® 12000–14000 molecular weight-cutoff membrane tubing (Spectrum Medical Industries, Los Angeles, CA, USA), first against 2 M, then 1 M, 0.5 M, 0.25 M, and 0.1 M NaCl and finally two changes of SM buffer [31]. The titers of the purified fluorescently-labeled phage solutions were determined by soft agar overlay on their respective bacterial hosts [16]. RITC- and FITC-labeled phage were denoted by the suffixes -RH and -FL, respectively, following their names.

Comparison of electron micrographs with scanning confocal laser microscope images of fluorescently-labeled phage

RITC-labeled phage were spotted on Formvar® (Monsanto Co, St Louis, MO, USA) -coated EM grids and negatively stained with 2% phosphotungstate [6], then visualized using a Phillips 600 transmission electron microscope at 100 kV. The same grids were placed in a dammed chamber on a glass slide, hydrated, covered with a 1-mm thick glass coverslip, and then viewed with a scanning confocal laser microscope (SCLM). The SCLM consisted of a Nikon Microphot-FXA microscope fitted with an argon laser (Ion Laser Technology, Salt Lake City, UT, USA) controlled by the BioRad MRC-600 computer software program, COMOS® (BIO-RAD Laboratories, Mississauga, Ont, Canada). Images obtained using Nikon 60× Fluor and 100× Planomat (1.4 NA) objective lenses were viewed on a color monitor and recorded on Panasonic optical disks using a Corel WORM drive. Color images were printed using a Sony color video printer or printed from Kodak 100HC Daylight Ektachrome film used to photograph images from the monitor.

DAPI-labeling of phage DNA

High titers ($\geq 10^9$ PFU μl^{-1}) of Bradley-type A phages T4 and E79 were labeled with 4′, 6-diamidino-2-phenyl-indole (DAPI) by a modified version of the procedure described by Furukawa *et al* [21]. Shake flask cultures of *E. coli* B or *P. aeruginosa* PAO-1 were grown in 25 ml of Luria-Bertani broth (LB) at 30°C to a log phase concentration of 10^8 cells ml^{-1}. A single plaque of phage T4 or E79 was added to its host. DAPI (125 μg, Sigma) was added to each infected culture to attain an empirical concentration of 5 mg L^{-1}. After shaking the suspension for 6 h, DAPI-labeled progeny phage were harvested as described above. Phage labeled with DAPI were denoted by the suffix -DP following their name.

Growth and infection of biofilm bacteria

Glass flow cells (Figure 1) were constructed by sandwiching two 50-mm lengths of 3.0-mm o.d. silastic medical-grade tubing (Dow Corning, Midland, MI, USA) between a 25-mm × 50-mm × 0.1-mm thick glass coverslip and a standard 25-mm × 75-mm × 1-mm thick glass microscope slide. The silastic tubing strips were glued with GE RTV 118 silicone glue to the glass parallel to one another to form a 4-mm wide channel. A 1.5-cm length of Masterflex 16 silicone tubing (Cole Parmer, Niles, IL, USA) inserted with a 90°-angle barb-fitting was glued to each end of the open channel. This was accomplished by removing a 1.1-mm thick half-circle section from the end of the tubing lacking the barbed fitting, leaving a notch in the tubing's end that was glued to the top of the coverslip, and an overhang that abutted the end of the channel and extended to (and was glued to) the surface of the glass slide that formed the bottom of the flow cell.

Flow cells were sterilized by perfusing them with 5% sodium hypochlorite. They were subsequently rinsed with

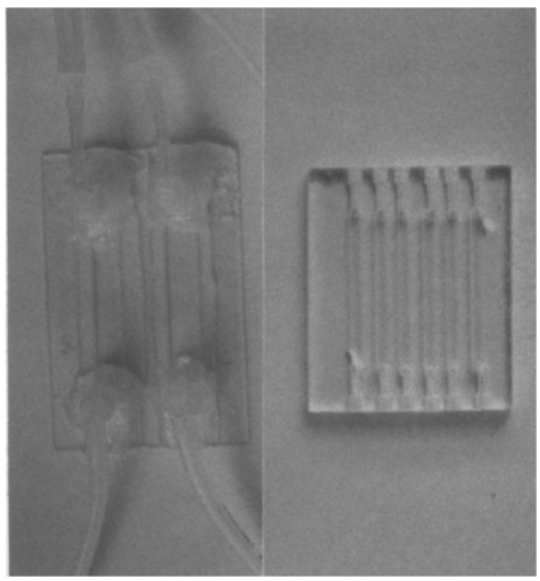


Figure 1 Flow cells. The top and base of the left flow cell is made of glass and the sides of silastic tubing. The right flow cell has a Lexan base with a glass top-cover. Silicone tubing is connected at both ends for the imput of medium at one end and the output of waste medium at the other end.

sterile distilled water, then pre-conditioned with LB/2 broth. Colonization was initiated by injecting log-phase *E. coli* B or *P. aeruginosa* PAO-1 cells from flask cultures into the flow cells. Pumping of LB broth through flow cells held at 37°C and 22°C was resumed only after a 20- and 60-min (respectively) cessation of flow. This lull in flow served to enhance bacterial colonization of the glass surfaces prior to their exposure to increased shear forces. Growth of biofilms was monitored by phase contrast microscopy. From 10^7 to 10^{10} labeled phage were injected into the flow cells after the biofilms had grown for 24–48 h. The extent of adsorption of RITC-labeled phage was observed using the Nikon Microphot-FXA fitted with epi-fluorescence illumination (EPI) provided by a 100-W mercury lamp, the light from which was passed through the H filter block. SCLM was also employed to visualize the adsorption pattern and record images. The photon-counting mode with the GSH (green sensitive high) filter in line with photomultiplier tube No. 1 (PMT-1) was used instead of 'normal' scanning to get high resolution images of RITC-labeled phage. FITC-labeled phage were detected using 'normal' scanning with the BSH (blue sensitive high) filter in line with PMT-2. Adsorption and DNA injection of DAPI-labeled phage were detected using EPI passed through the B filter block. ANS (8-anilino-1-naphthalene-sulfonate) was added to the medium at a final concentration of 60 μM to visualize the increased fluorescence of infected cells [23,36] over non-infected cells.

Detection of T4-LacZ phage infection of a lac⁻ E. coli biofilm

E. coli 3000 XIII cells grown at 37°C in continuous culture in LB/2 broth culture [17] or at 22°C in shake culture in M9 minimal salts medium plus glucose (M9-Glu, [30]) were used to colonize a Lexan® (General Electric, Mt Vernon, IN, USA) flow cell (Figure 1) covered with a 0.1-mm thick glass coverslip which was perfused with LB/2 or M9-Glu, respectively, at 22°C or 37°C. Lexan flow cells were constructed by milling multiple channels in rectangular blocks of Lexan, then drilling inlet and outlet ports from the ends of the block. Silicone tubing was glued into the port holes and a 0.1-mm thick glass coverslip was glued over the channels with silicone glue.

One hour prior to phage infection of the biofilms, both 4-chloro-5-bromo-β-D-galactopyranoside (X-GAL, Molecular Probes, Eugene, OR, USA) and 4-methyl-umberiferyl-β-D-galactopyranoside (MUGAL, Molecular Probes) were added to the LB/2 broth reservoir, each at a final concentration of 25 μM. Biofilms were infected by perfusion of T4-LacZ (YZA1) phage through the flow cell after injection upstream. In the presence of X-GAL, infected cells appeared blue using light-field or PC microscopy whereas in the presence of MUGAL their fluorescence was detected by SCLM using a BSH filter in line with PMT-2.

Use of fluorescent antibodies to detect non-labeled phage

A 24-h biofilm of *E. coli* 3000 XIII, grown in a Lexan flow cell (Figure 1) perfused at 37°C with LB/2, was infected by injection of 10^8 PFU T4 YZA1 ($LacZ^+$) phage into the flow cell. Perfusion with LB/2 broth was continued for an additional 24 h. At 24 h post-infection the flow cell was perfused with phosphate-buffered saline (PBS; pH 7.6) for 30 min, then injected with rabbit anti-T4 polyclonal antibodies diluted 1 : 1000 and incubated at room temperature (22.5°C) for 45 min. After flushing out primary antibodies with PBS perfusion for 10 min, de-complemented goat 'blocking' serum (Sigma) diluted 1 : 200 in PBS was injected and incubated at 22.5°C for 10 min. Following another 10-min PBS flush, the secondary antibody FITC-conjugated goat anti-rabbit IgG (Sigma) diluted 1 : 100 in PBS was injected and incubated at 22.5°C for 90 min. After flushing the flow cell with PBS for another 30 min the antibody-treated biofilm was observed using the SCLM with the BSH filter aligned with PMT-2.

Results and discussion

Fluorescent-labeling

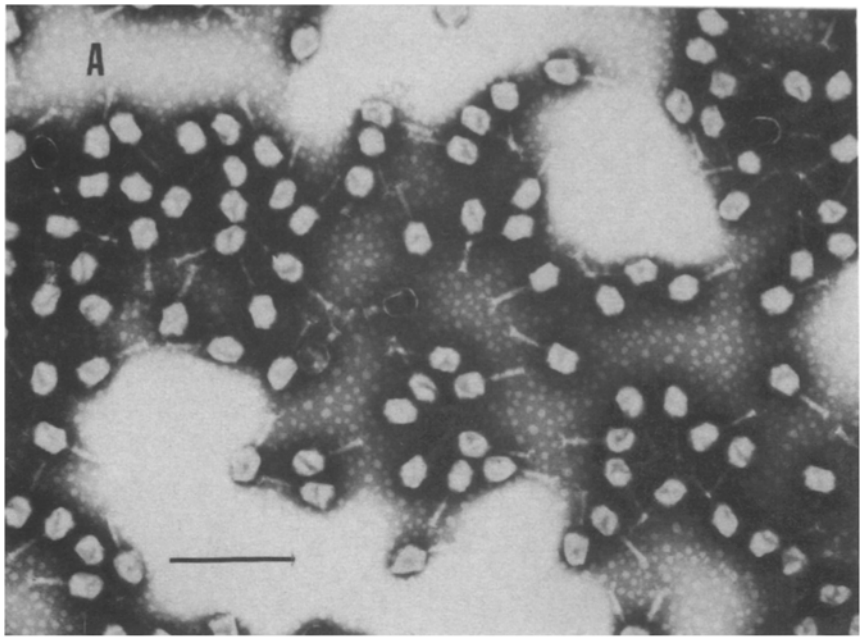
The fluorescent labeling of phages T4 and E79 facilitated visualization of their adsorption to host cells and detection of DNA injection. Fluorescein and rhodamine conjugate with proteins, whereas DAPI intercalates DNA and has been used to visualize and quantify attached bacteria [43] as well as phage [21] and viruses from marine waters [24]. The RITC- and FITC-labeling reactions caused slight losses in phage infectivity based on losses of titer (Table 2) but these losses are typical of those that occur during purification by centrifugation and dialysis. Forty-two and nineteen percent of the RITC- and FITC-labeled phage, respectively, were recovered as infectious plaque-forming-units (PFU) in soft agar overlays of bacterial host lawns. RITC-labeled phage titers were stable when stored at 4°C in SM buffer containing trace amounts of chloroform added as a preservative. When stored at 4°C, the RITC-labeled phage titers remained infective for $\geq$ 3 months as opposed to $\leq$ 1 month for the FITC-labeled phage.

Single point sources of fluorescence that are smaller than 100 nm (eg an FITC-conjugated IgG molecule) appear as a 100-nm fluorescent flare according to the point-spread function [9]. A T4D+ phage has dimensions of 85 × 150 nm and would be expected to appear as a fluorescent spot with a diameter of 100 nm if it were labeled with a single RITC molecule. Comparison of the size and distribution of T4D-RH phage in electron micrographs (Figure 2 A and B) with that of fluorescent spots ($\geq$ 150 nm in size) observed in SCLM images (Figure 3) of the same phage-covered grid suggested that the fluorescent spots are phage conjugated with numerous RITC molecules. Differences in the number of RITC molecules conjugated to phage particles could be

Table 2 Recovery of viable RITC- and FITC-labeled phage

Phage	Total PFU prior to reaction	Label	Total PFU after reaction	Infective phage (%)
T4D+	9.6×10^{11}	RITC	4.0×10^{11}	42
E79	1.0×10^{12}	RITC	4.2×10^{11}	42
YZA1[a]	6.4×10^{11}	FITC	1.2×10^{11}	19

[a]T4 rIIB-LacZ phage.



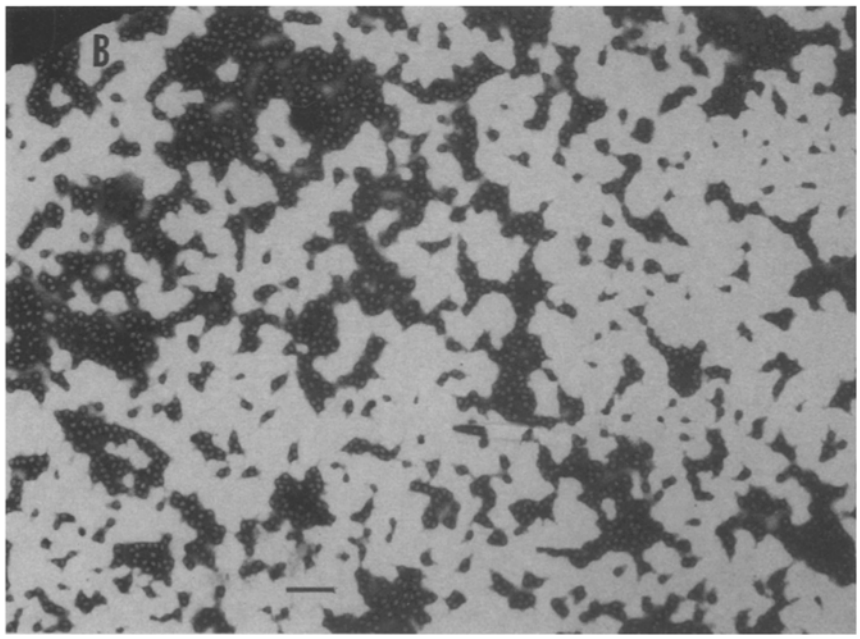


Figure 2 Transmission electron micrographs of RITC-labeled bacteriophage T4-RH spotted on Formvar-coated grids, dried, and negatively-stained with 2% phosphotungstate (PTA). (A) Size bar = 0.25 μm. (B) Size bar = 0.5 μm.

responsible for differences in the intensity of fluorescence among spots.

To test the specificity of the labeled phage, RITC- or DAPI-labeled phage were mixed with their host bacteria and with non-host bacteria. *P. aeruginosa* and *Staphylococcus aureus* served as non-host bacteria for phage T4D+ (-RH and -DP) and *E. coli* and *S. aureus* for phage E79 (-RH and -DP). This resulted in an enhanced fluorescence of host cells but not non-host cells. Matching pairs of phase contrast and epifluorescent images of *E. coli* and *P. aeruginosa* cells exposed to T4-DP and E79-DP phage (Figure 4A–H) demonstrate this specificity. Only the *E. coli* B:T4-DP (Figure 4B) and *P. aeruginosa* PAO-1 : E79-DP (Figure 4F) mixtures of bacterial host : labeled phage exhibited epifluorescence. The other two non-host : phage mixtures were non-fluorescent (invisible) upon exposure to ultraviolet light.

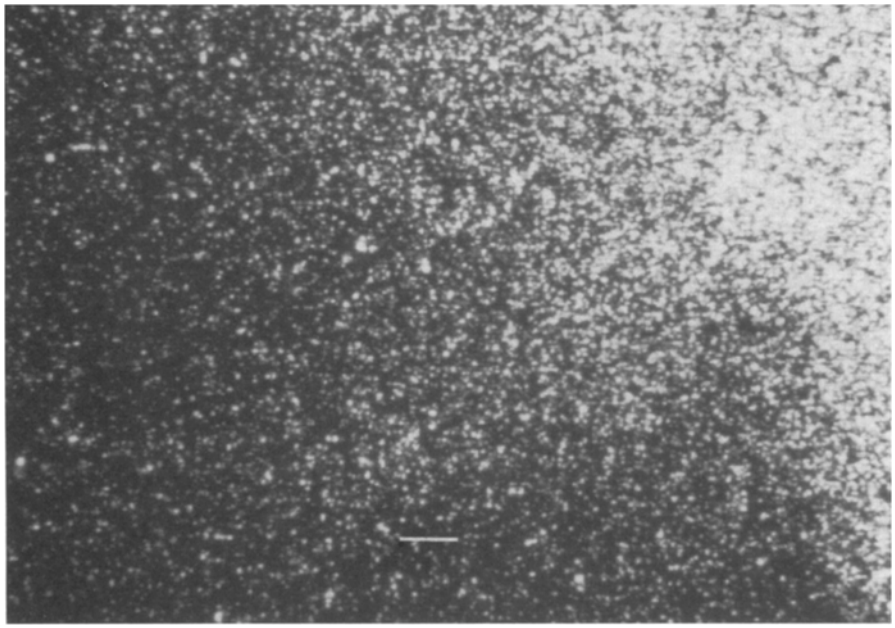


Figure 3 SCLM image of RITC-labeled T4 phage on the PTA-stained grid. The grid was glued to a glass slide with cyanoacrylate. A shimmed glass coverslip was glued on top of the grid and a small volume of water was injected into the compartment to hydrate the grid. Hydration was necessary for the RITC-labeled T4-RH phage to fluoresce. The white areas are the fluorescent emissions from the RITC-labeled phage. Size bar = 1.0 μm.

Infection of biofilms

The isolate of *E. coli* B that was used at the onset of experimentation colonized the surfaces of the glass flow cells poorly. A variant of *E. coli* B, ECBB, which exhibited enhanced colonization of glass flow cell surfaces was isolated. ECBB colonized the glass surfaces in a manner similar to the packing maneuver [28] exhibited by *Pseudomonas* species and formed biofilms of 15–30 μM thickness. ECBB was susceptible to T4 phage infection and formed plaques when infected at a low MOI (multiplicity of infection), 10^{-5} phage cell^{-1}, using soft agar overlay [2]. When T4D-RH phage were injected into a flow cell containing an *E. coli* ECCB biofilm, they bound to cells at a rate proportional to the concentration of phage added. This was gauged by the observation that biofilms exposed to greater numbers of labeled phage exhibited greater fluorescence (10^7 phage $< 10^8$ phage $< 10^9$ phage $< 10^{10}$ phage).

The rate of phage adsorption to biofilm cells was not quantified due to inherent difficulties associated with measuring the rapid adsorption of 100-nm fluorescent phage particles to cells throughout the entire thickness of the biofilm. The background autofluorescence of *P. aeruginosa* biofilms was greater than that of the *E. coli* biofilms but was negated by lowering the background sensitivity of the photomultiplier. Lowering the sensitivity often made detection of cells infected by single, labeled phage more difficult. Cells with multiple phage particles bound were more easily detected. Rhodamine fluoresces more weakly than fluoroscein but is not as prone to quenching or photo-bleaching [19]. When T4D+ phage were labeled with FITC (T4D-FL), their adsorption to single cells at a relatively low MOI was observed more readily (Figure 5) than that of RITC-labeled phage (T4D-RH) at the same laser power and aperture settings.

When a *P. aeruginosa* PAO-1 biofilm was exposed to E79-RH phage, the phage adsorbed to cells on the outer surface of thick biofilms but appeared unable to penetrate to all of the innermost cell layers of the biofilm (Figure 6). When labeled E79 phage were added to a flow cell containing a thin biofilm of *P. aeruginosa* PAO-1, the phage adsorbed to the majority of cells. 'Cloud-like' masses of what appeared to be EPS obscured the outline of cells within or proximal to them suggesting that phage penetration was inhibited. When DAPI-labeled T4 phage (T4-DP) were adsorbed to *E. coli* B strain ECBB biofilms, many cells were bright white when observed with EPI. The diffusion of DAPI-labeled phage around cells illuminated by EPI was videotaped. Putative infected cells became enlarged and spheroplast-like with white dots encircling their periphery (Figure 7). Many of these bright dots seemed to traverse the outer cell membrane, which may represent injection of phage DNA into the periplasm that occurs during super-infection at a high MOI [2]. Instances of diffuse fluorescence in the cytoplasm of infected cells were observed as described by Furukawa *et al* [21]. The fluorescence of infected cells was accentuated when ANS was added to the medium. These observations are in agreement with those of Hantke and Braun [23] who observed that increased accessibility of ANS to cell membranes mediated by phage infection increased ANS's fluorescence. T4D-RH phage appeared to adsorb to all *E. coli* ECCB cells in the biofilm depicted in Figure 8. Apparent differences in accessibility of *E. coli* and *P. aeruginosa* biofilms to their phages may be due to inherent structural differences of the biofilms (eg exopolymeric matrix) or phages or possibly an artifact in that the fluorescence of E79-RH phage which have penetrated thick layers of the PAO-1 biofilms may be quenched by overlying layers giving the appearance of phage inaccessibility.

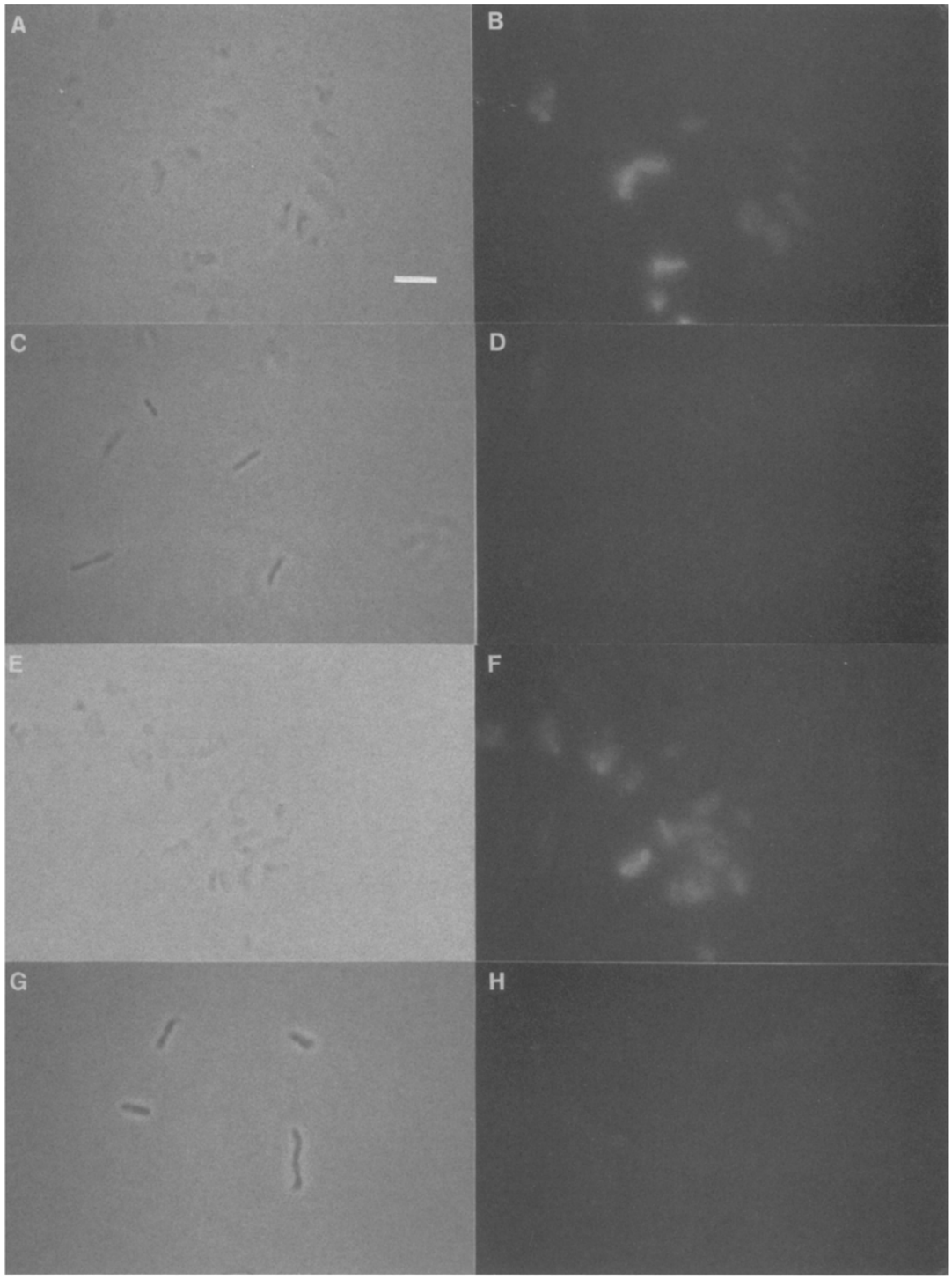


Figure 4 Matching pairs of phase contrast (A,C,E,G) and epifluorescent (B,D,F,H) images of DAPI-labeled phage mixed with host and non-host bacterial cells. (A,B) *E. coli* B + T4-DP; (C,D) *P. aeruginosa* PAO-1 + T4-DP; (E,F) *P. aeruginosa* PAO-1 + E79-DP; (G,H) *E. coli* B + E79-DP. Photographs of epifluorescent images (B,D,F,H) were made at the same magnification and exposure settings in order to compare relative magnitudes of florescence. Size bar = 10 μm.

Monitoring the spread of phage T4 infection in a biofilm

Whereas the use of RITC- and DAPI-labeled phage permitted visualization of patterns of adsorption and phage DNA injection at primary sites of infection, it did not permit detection of progeny phage and the spread of infection to secondary sites in the biofilm. The use of T4 LacZ (YZA1) phage and *E. coli* 3000 XIII facilitated tracking the spread of infection by progeny phage and time-to-lysis of cells infected at a relatively low MOI. By adding X-GAL to the medium supplied to a flow cell, the formation and spread of blue patches of infected cells could be observed by phase

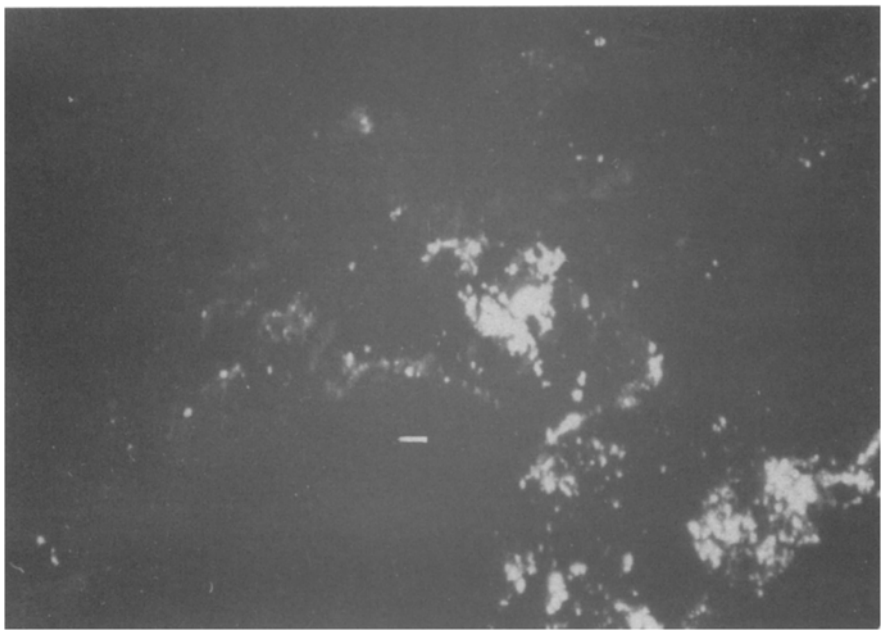


Figure 5 SCLM composite image of *E. coli* ECCB biofilm to which FITC-labeled T4 phage (T4-FL) were adsorbed at a low MOI. This composite is a projection of 24 images collected at 0.5-μm intervals spanning a depth (z-axis) of 12 μm through the biofilm. The bright spots are the phage adsorbed to the cells of the biofilm. Size bar = 5 μm.

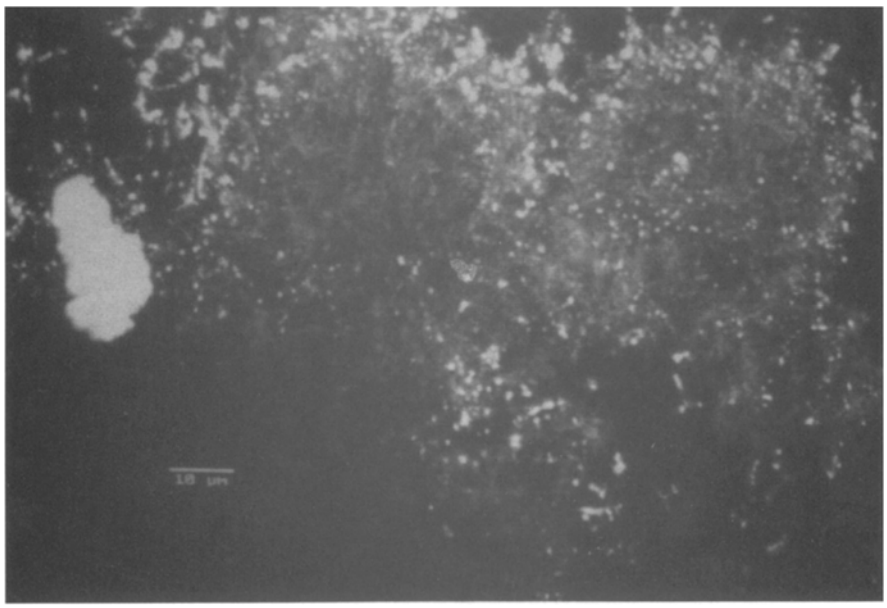


Figure 6 Infected biofilm of *P. aeruginosa*. SCLM composite projection of RITC-labeled E79 phage adsorbed to a *P. aeruginosa* PAO-1 biofilm. The image is a composite of 13 images collected at 5-μm intervals spanning a depth (z-axis) of 60 μm through the biofilm. The size bar = 10 μm. White spots represent fluorescing phage adsorbed to cells of the biofilm protruding from the surface of the coverslip. Direction of medium flow containing the injected phage was from top to bottom. The greatest adsorption of phage was on the upstream side of the biofilm's protrusions. Note that cells toward the center of the protrusions did not fluoresce significantly suggesting that phage access to these cells is restricted or the fluorescence emanating from these regions is quenched.

contrast microscopy (Figure 9A and B). Using a Panasonic color TV camera, videotape footage was obtained of the enlargement of infected cells to become blue spheroplast-like balloons prior to lysis. Infected cells on the biofilm surface (closest to the bulk flow) increased in size and burst prior to those cells lying deeper in the biofilm. Infected cells appeared to pull away from the extracellular matrix of the biofilm around them but did not detach from the biofilm. Patches of infected cells spread radially and downward into the biofilm to include cells attached to the glass surface. Infection of cells deeper within the biofilm was visualized better with SCLM detection of fluorescence generated by the cleavage of MUGAL by YZA1-encoded β-galactosidase synthesized in infected cells (Figure 10).

The spread of infection more closely resembled that of plaque formation (Figure 9B) than that of plume formation.

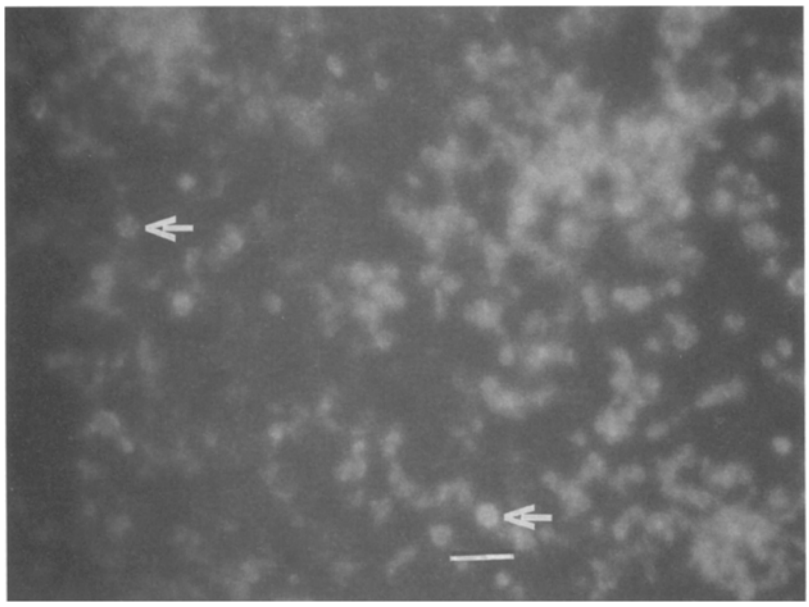


Figure 7 Epifluorescence of *E. coli* ECBB biofilm infected by DAPI-labeled T4 phage (T4-DP) in the presence of 60 μm ANS. The majority of cells were infected, rounded up, and fluoresced brightly with the appearance of a watch dial (arrows).

This observation indicates that radial diffusion (convective transport) rather than downstream flow (advective transport) of progeny phage away from lysing infected cells is predominant in the relatively stagnant boundary layer surrounding a biofilm. Our model of what happens during biofilm infection by phage assumes that during initial exposure to phage present in the bulk liquid the probability of being infected is greater for cells at the biofilm-liquid interface. Upon lysis of infected cells at the surface of the biofilm, the fluid boundary layer above the biofilm would tend to retain progeny phage. These progeny phage would in turn infect other biofilm cells. Hence, cells deeper in the biofilm would most likely become infected with greater frequency upon release of progeny phage from the initially infected cells. Some phage might escape to the bulk liquid where they could infect planktonic host cells.

Though it was expected that cells in a patch were infected by progeny phage (liberated by lysis of an initially-infected cell) and would lyse simultaneously, there did not seem to be any synchrony to their time of lysis. One explanation is that when a cell bursts amongst a patch of other infected cells they are showered by a multitude of progeny phage resulting in high MOIs. High MOIs increase the time-to-lysis of an infected cell [1]. In turn, reinfection of an already infected cell by phage liberated from an adjacent infected cell that lyses can also result in delay of lysis [2]. Alternatively, the EPS of the biofilm may serve to stabilize spheroplasts formed by phage-mediated synthesis of T4 lysozyme, or gradients (eg nutrients, oxygen) present within the biofilm may affect the rate of phage replication within infected cells. The relatively low rate of mass transfer of nutrients and oxygen mediated by molecular diffusion in a linear flow system can be responsible for the formation of concentration gradients within the biofilm and in the flow lamina above the biofilm [30].

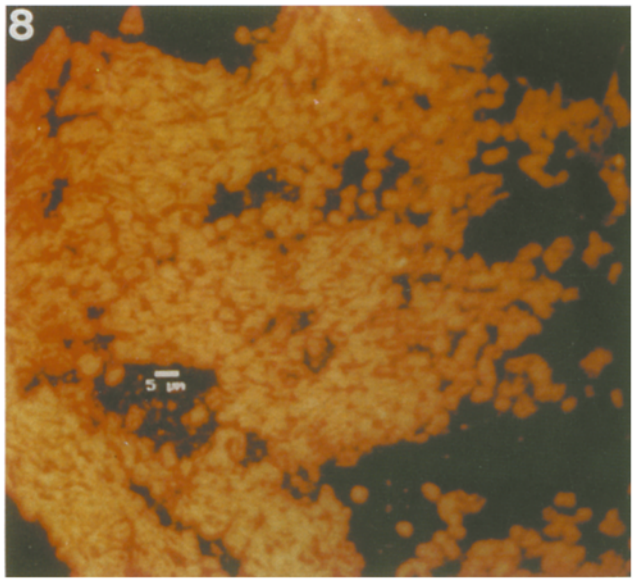
Biofilms grown and infected at 37°C formed blue patches that appeared to spread faster than those at 22°C. Time-to-lysis was apparently prolonged at 22°C. Biofilms grown on the defined M9-glucose medium also developed blue patches but at slower rates (observation not quantified) than those grown in the more nutrient-rich LB broth. These trends in the spread of phage infection in a bacterial biofilm population are similar to those observed for a population of planktonic bacterial cells [18].

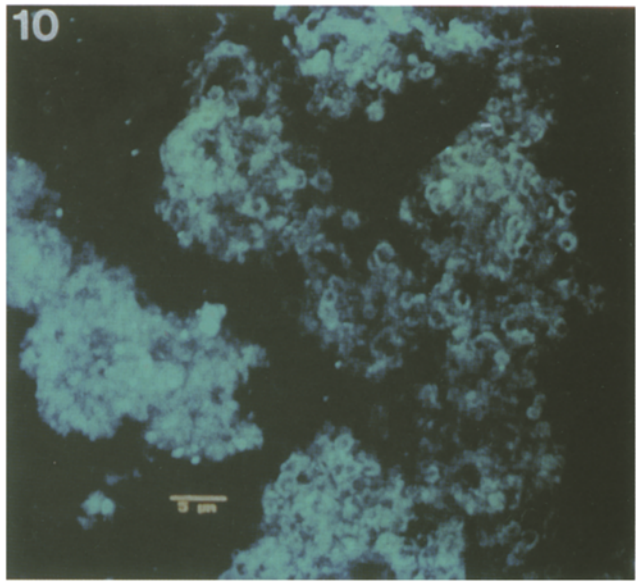
Use of antibodies to detect phage particles in biofilm

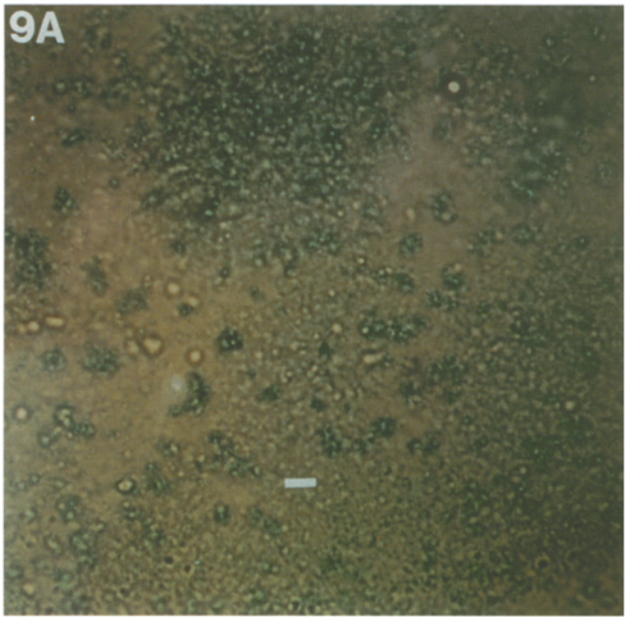
Preliminary results using FITC-conjugated secondary antibodies to detect primary anti-T4 antibodies bound to phage-infected cells indicated that cells in a biofilm became heavily covered with adsorbed phage as the infection progressed (Figure 11). Some cells fluoresced brightly suggesting that the antibodies penetrated spheroplast-like cells to bind progeny phage.

In summary, bacteriophages T4 and E79 can be fluorescently-labeled by conjugation with RITC and FITC or by addition of DAPI to phage-infected host cells. These processes generate labeled phage able to adsorb to and infect their hosts. *E. coli* and *P. aeruginosa* can attach to glass surfaces and form biofilms that are susceptible to infection by T4 phage. Both surface-attached and surface-associated (biofilm cells not directly attached to the surface) *E. coli* cells are susceptible to phage infection. While temperature and nutrient concentration did not affect susceptibility to phage infection, lower temperature and low nutrients extended the time to lysis and slowed the spread of infection. Phage E79 adsorbs to cells on the surface of biofilms of *P. aeruginosa* PAO-1 but access to cells deep in the biofilms or within protrusions of the biofilms may be restricted.

There are intrinsic differences between the *E.* coli B and *P. aeruginosa* PAO-1 biofilms. *E. coli* B and 3000 XIII are 'rough' (R) strains that lack the *O*-antigen side-chains of the lipopolysaccharide (LPS) which confer a smooth (S) colonial morphology [8]. These chains, when present, can







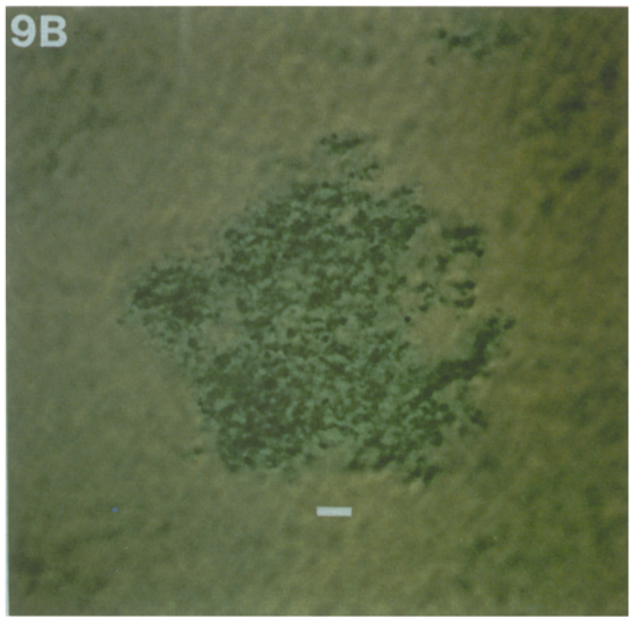


Figure 8 SCLM composite image of RITC-labeled T4 phage (T4-RH) adsorbed to an *E. coli* ECCB biofilm at a high MOI. This composite is a projection of five images collected at 2.5-μm intervals spanning a depth (*z*-axis) of 10 μm through the biofilm. Size bar = 5 μm.
Figure 9 Infection of *E. coli* 3000 XIII biofilm by T4 phage YZA1. (A) A flow cell colonized by *E. coli* 3000 XIII was perfused with half-strength LB broth (LB/2) containing 10 mg L^{-1} X-GAL. The biofilm was grown at 37°C and infected by injection of 10^{10} YZA1 phage into the silicon tubing at the channel's inlet. YZA1 contains an rII-LacZ fusion gene that upon expression in infected cells directs the synthesis of β-galactosidase which catalyzes the cleavage of X-GAL. The product of this reaction imparts blue pigmentation to the infected cells. (B) Infected 'patch' of blue *E. coli* 3000 XIII biofilm cells. The biofilm was grown in LB/2 broth at 22.5°C in an all-glass flow cell with XGAL added to a final concentration of 10 mg L^{-1} in the broth 1 h prior to injection of 10^{10} PFU YZA1 phage (T4-LacZ) into the flow cell. This photo was taken 25 h post-phage injection. Size bars = 10 μm.
Figure 10 SCLM composite image of YZA1-infected *E. coli* 3000 XIII biofilm grown at 37°C in LB broth containing 30 mg ml^{-1} MUGAL. Infected cells that produced β-galactosidase fluoresced from the cleavage of MUGAL. The blue hue is a pseudo-color endowed by the Biorad MRC-600 COMOS® computer software. Composite is a projection of 20 images collected at 1.5-μm intervals spanning a biofilm depth (*z*-axis) of 28.5 μm. Size bar = 5 μm.

restrict the access of bacteriocins and bacteriophage to their outer membrane protein (OMP) receptors, shielding the cells from their bactericidal effect [7,42]. While this shielding may block infection by some phages, other phages may still have accessibility to their receptors [12].

E. coli colonizes glass surfaces using the shedding maneuver which favors dispersion. *P. aeruginosa* PAO-1 uses a packing manuever [28] to colonize surfaces and forms microcolonies that eventually coalesce, or it forms a monolayer that develops many hanging protrusions (polyps) that contain pores (channels) through the biofilm. Structural differences between the biofilms of *P. aeruginosa* and *E. coli* may cause differences in accessibility to phage. Mucoid strains of *P. aeruginosa* produce alginate

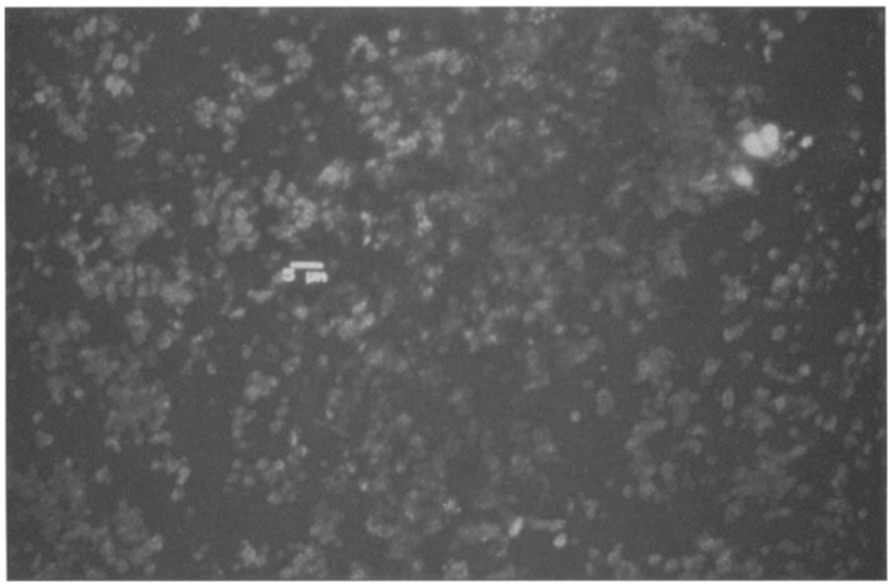


Figure 11 SCLM composite image of T4 phage-infected *E. coli* 3000 XIII biofilm treated with rabbit anti-T4 polyclonal antibodies then probed with FITC-labeled goat anti-rabbit secondary antibodies. This composite is a projection of 19 images collected at 1.0-μm intervals spanning a depth (z-axis) of 18 μm. The bright spots outlining the biofilm cells are the FITC-labeled antibodies. Size bar = 10 μm.

as part of their EPS that may limit accessibility of phage to their cellular receptors. Capsular polysaccharides present on *E. coli* [35] and *Pneumococcus* [5] grown in glucose-containing broth can inhibit phage infection partially or fully. In contrast, capsule-specific phages have been isolated that digest the capsular polysaccharides of strains of *E. coli* [40], *Klebsiella* [41], and *Pseudomonas* [3]. *P. aeruginosa* strain PAO-1 is a non-mucoid (non-alginate producing) strain that can form biofilms of considerable thickness (> 200 μm). Whether E79 phage can infect a *P. aeruginosa* PAO-1 biofilm and spread throughout it is unknown but, as demonstrated in this paper, E79 phage adsorb specifically to cells on the outer surface of the biofilm.

In conclusion, biofilms formed by some strains of bacteria are susceptible to phage infection when grown as a pure culture. Susceptibility to phage infection undoubtedly varies from strain to strain and species to species and will be affected by environmental conditions which affect the host cell's physiology and structure [8,29]. Conditions that are unfavorable to infection by one phage may favor infection by another phage. 'Biofilm-specific phage' may even exist that only infect cells growing as a biofilm. Most biofilms in nature are composed of multiple bacterial species whose spatial architecture and extracellular matrices may constrain the spread of a phage infection. Further studies are needed to understand what impact phage infection has on biofilms in the natural environment.

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