Formation of biofilms under phage predation: considerations concerning a biofilm increase

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(Received 4 December 2012; final version received 11 February 2013)

Bacteriophages are emerging as strong candidates for combating bacterial biofilms. However, reports indicating that host populations can, in some cases, respond to phage predation by an increase in biofilm formation are of concern. This study investigates whether phage predation can enhance the formation of biofilm and if so, if this phenomenon is governed by the emergence of phage-resistance or by non-evolutionary mechanisms (eg spatial refuge). Single-species biofilms of three bacterial pathogens (Pseudomonas aeruginosa, Salmonella enterica serotype Typhimurium, and Staphylococcus aureus) were pretreated and post-treated with species-specific phages. Some of the phage treatments resulted in an increase in the levels of biofilm of their host. It is proposed that the phenotypic change brought about by acquiring phage resistance is the main reason for the increase in the level of biofilm of P. aeruginosa. For biofilms of S. aureus and S. enterica Typhimurium, although resistance was detected, increased formation of biofilm appeared to be a result of non-evolutionary mechanisms.

Keywords: biofilm; spatial refuge; host-parasite; bacteriophage therapy; coevolution; spatial heterogeneity

Introduction

Biofilms are complex microbial communities established on a wide range of surfaces, and are generally encapsulated by an extracellular protective matrix composed of various types of biopolymers (Abbe et al. 2011). Microscopic investigations have revealed that biofilms develop not only on the inert surfaces of medical devices or on dead tissues, but that they can also form on living tissues, as in the case of endocarditis (Kirketerp-Møller et al. 2008). The formation of biofilms can have important clinical implications particularly in chronic infections (Davies 2003). Biofilms may be considered to be a cellular survival mechanism; cells within a biofilm are more resistant to environmental threats than their planktonic counterparts (Kirketerp-Møller et al. 2008; Donlan 2009). The doses of antibiotic used effectively against planktonic cells are usually not enough to tackle biofilms, leading to resistant subpopulations remaining in the biofilm and recurring infections (Bedi et al. 2009).

Bacteriophages (phages), obligate parasites of bacteria, have been claimed to be effective at inhibiting the formation of biofilms and in dispersing mature biofilms. The ability of phages to inhibit and/or eradicate biofilms has been demonstrated for biofilms of Pseudomonas aeruginosa (Hanlon et al. 2001; Knezevic & Petrovic 2008; Ahiwale et al. 2011; Knezevic et al. 2011; Pires et al. 2011), Klebsiella pneumonia (Bedi et al. 2009), Escherichia coli (Carson et al. 2010), Proteus mirabilis (Carson et al. 2010), and Staphylococcus epidermidis (Curtin & Donlan 2006) amongst others (Lévesque et al. 2005; Cornelissen et al. 2011; Kelly et al. 2012). Despite the positive results, there is evidence in the literature that phage predation can lead to an increase in bacterial biofilm levels under certain conditions (Schrag & Mittler 1996; Lacqua et al. 2006; Gödeke et al. 2011; Heilmann et al. 2012), a phenomenon also reported for some antibiotics (Hoffman et al. 2005).

Different mechanisms are proposed for this increase in the formation of biofilm. The strong selective pressure that virulent phages exert on their host community will eventually lead to the emergence of resistant mutants. These mutants may have altered phenotypes, increasing their tendency to form biofilms (Lacqua et al. 2006). Therefore, whether or not the emergence of phage resistance (evolutionary mechanisms) will result in biofilm increase is context-dependent and governed by the phenotypic change resulting from resistance mutations in the host bacteria against a specific phage. Non-evolutionary mechanisms are also believed to play a role in increasing the tendency of bacteria to form biofilms. The establishment of coexistence by the presence of a permanent wall population, or a wall effect, is a well-known phenomenon which allows the co-occurrence of bacterial species with an unequal fitness (Chao & Ramsdell 1985). There are also studies which indicate that forming a wall population stabilizes populations of phage-sensitive bacteria with their virulent phage (Schrag & Mittler 1996; Weiss et al. 2009; Heilmann et al. 2012); thus, lytic phage predation can result in elevated levels of formation of a biofilm in a
bacterial community. Heilmann et al. (2012) developed a rigorous numerical model which predicts that the wall effect will be a governing factor in stabilizing communities of resistant and sensitive host bacteria with their virulent phage. This suggests that the wall effect will affect the formation of biofilms in any phage–host system. Therefore, it is hypothesized that the ability of a phage to control the formation of a biofilm is governed by the additive, synergistic, antagonistic, or suppressive interaction of evolutionary and non-evolutionary mechanisms; virulent phages cannot be deemed suitable for the control of biofilms based solely on their lytic ability towards the host.

To investigate this hypothesis, single-species biofilms of three bacterial pathogens (Gram-negative and Gram-positive) were established and challenged with species-specific phages (two dosing levels) under two settings. Exposure to phages either occurred during biofilm growth (pretreatment) or after the development of a mature biofilm (post-treatment). To assess the effect of phage-resistance on the population dynamics of the bacterial biofilms, two different single phage treatments and a mixture of the two phages were used; the phages were chosen from different families so that their mixture would decrease the frequency of emergence of phage resistance, as confirmed empirically. The biofilm was sampled at various time points to verify the presence of phage-resistant mutants.

Materials and methods

Culture of bacteria and phage propagation

The strains used in the study are listed in Table 1. P. aeruginosa PAO1 was provided by M. Elimelech (Yale, USA), Staphylococcus aureus was purchased from ATCC (CEDARLANE Laboratories Ltd, Canada), Salmonella enterica and all the phages were purchased from Félix d’Hérelle Reference center for Bacterial Viruses (Université Laval, Canada).

To initiate a bacterial culture, an inoculum from a frozen glycerol stock was streaked onto a tryptase soy agar plate and incubated overnight at 37°C. A single colony from the plate was inoculated into 10 ml of trypticase soy broth (TSB), and incubated overnight (37°C, 120 rpm) from which a 200 μl aliquot was diluted 1:100 in fresh TSB and grown to an OD₆₀₀ of 0.2–0.3. A soft agar overlay technique was then used to propagate all phages used in this study as described elsewhere (Kropinski et al. 2009).

Quantifying the frequency of emergence of phage resistance

The decrease in the emergence of resistant mutants was confirmed by quantifying the frequency of formation of resistant bacteria for pure phage and phage mixture treatments using the method described by Carlson (2004). Briefly, the phage was mixed with its host (phage: bacteria = 10⁵–10⁶) and spread onto an LB agar plate. The number of bacterial colonies that grew after incubation for 24 h at 37°C was divided by the number of colonies on the control (bacteria not mixed with phage) to calculate the resistance frequency.

An in vitro model for biofilm development

A microtiter plate assay (Merritt et al. 2011) was used to grow and study the biofilms. Each bacterial species was grown overnight in TSB (37°C, 120 rpm) from a single colony picked from an agar plate of no more than three days old. This culture was diluted 1:100 in TSB and loaded into the wells of five untreated 96-well polystyrene flat-bottomed microtiter plates (Costar; Corning Inc, Corning, NY, USA) in two rows. The plates were then incubated at 37°C and shaken gently to limit the effect of chemical and phage gradients on the system. The biofilms were pretreated or post-treated with phage (see below). At 8, 12, 14, 48, and 72 h one multiwell plate was destructively treated for biofilm and phage quantification and phage resistance testing. Firstly, the planktonic bacteria in the wells were transferred to a fresh microtiter plate and their OD₅₇₀ value was measured. The wells were then washed twice with phosphate-buffered saline (PBS) and either sampled for resistance testing or stained with 1% crystal violet for quantification of the total attached biomass. The OD₅₇₀ value of the anhydrous ethanol subsequently used to

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| Host bacteriophage | S. enterica serotype Typhimurium (HER1023), clinical isolate | P. aeruginosa PAO1, clinical isolate | S. aureus ATCC 25923, clinical isolate |
|-------------------|-------------------------------------------------|---------------------------------|---------------------------------|
| Bacteriophage     | 1 – PRDI (HER23)                                | 1 – E79 (HER359)                | 1 – K (HER474)                  |
| Phage family      | Tectiviridae                                    | Myoviridae                      | Myoviridae                      |
| Forward primer    | TGTTGTGTTAAATACCGCA                             | GGGGGATCTTCGGAACCTCA            | AACCGCTTACCAAGGCAACGAT          |
| Reverse primer    | CACAAAATCCATCTCTGGGA                            | TCCTTAGAGTTGCCACCACCG           | TGCGCATTTACCAGCTACACAT          |
| Primer reference  | This study                                      | (Spilker et al. 2004)           | This study                     |
dissolve the crystal violet was used as a quantitative measure of the biofilm. The titer of phage in the supernatant was determined, at each time point, using the soft agar overlay technique. The experiments described below were all performed in triplicate. Every 24 h half of the medium in each well was replaced with fresh TSB without disturbing or washing the biofilm to limit the effect of resource limitation.

**Choosing phage to bacteria ratios**

To select appropriate phage: bacteria ratios (multiplicity of infection (MOI)), phage was added to a bacterial inoculum with MOIs ranging from $10^{-7}$ to $10^5$ (Figure S1a) and the biofilm was grown for 12 h. [Supplementary material is available via a multimedia link on the online article webpage.]. The MOI at which the biofilm was inhibited by 50% or more after 12 h was chosen as the 50% inhibitory phage dose. For comparison, an MOI 1/100 of this 50% inhibitory dose was also tested.

**Inhibition of biofilm formation using phage (pre-treatment)**

To investigate the ability of phage to inhibit the formation of biofilms, phage was added to the wells of the multiwell plate simultaneously with the bacterial inoculum at $t=0$ using 2 MOIs (see above). Each phage treatment was repeated in triplicate. Phage buffer (saline–magnesium–gelatin buffer: NaCl 5.8 g l$^{-1}$, MgSO$_4$ 0.96 g l$^{-1}$, 1 M Tris 50 ml, gelatin 0.1 g l$^{-1}$, pH 7.5) was added to the control wells ($n=3$). For each bacterial species, 3 phage treatments were used, 2 single-phage treatments (phage 1 and phage 2 in Table 1) and 1 mixed phage treatment (1:1 mixture of both phages). Each treatment was applied to 3 wells in each row. The biofilm was allowed to develop in the presence of phage for a specified period of time (8, 12, 14, 48, or 72 h), after which the biofilm was quantified as above.

**Challenging mature biofilms (post-treatment)**

The efficacy of phage at eradicating a mature biofilm was investigated by challenging a biofilm grown for 48 h with single and mixed phage treatments. The biofilm was prepared as described above and allowed to develop for 48 h, after which the planktonic bacteria were removed, the wells were washed ($\times 3$) with PBS and phage was added to the wells with 2 MOIs (see above); 3 wells were used for each phage treatment. The addition of phage was chosen as time zero for phage post-treatment. The 3 control wells in each row contained phage buffer alone. The biofilm was challenged with the phages for a specified period of time (8, 12, 14, 48, and 72 h), after which the biofilm level was quantified. To calculate the amount of phage required to obtain the target MOI for post-treatment of the biofilm, the extent of biofilm development was quantified for each species of bacteria after 48 h; biomass was removed from the wells and the number of CFUs was determined.

**The characterization of phage-resistant isolates from the biofilm and verifying for the presence of phage host-range mutants**

Several colonies of bacteria were recovered from the biofilms at each of the specified time points (8, 12, 14, 48, and 72 h) during the course of the study. Samples of the biofilms were collected using an inoculating needle after the washing step but prior to quantification using crystal violet. The samples were streaked onto LB agar plates and 10 single isolated colonies were chosen from each biofilm. The susceptibility of each isolate to the phage with which it was challenged was determined using the spot test. A 1 μl aliquot from the liquid culture of each variant (OD$_{600}$ 0.2–0.3) was mixed with 2 ml of 0.5% LB agar and spread onto a 5 cm 1.5% LB agar plate. The phage (10$^6$ pfu ml$^{-1}$) was spotted (1 μl) on the soft agar layer, incubated at 37 °C overnight and inspected for plaque formation. Colonies that were found to be resistant to the ancestral form of their corresponding phage were further verified for contamination via PCR with species primer pairs directed against 16S rDNA (Table 1). The primers were designed according to the conserved regions reported in the literature for each species (Gürtler & Barrie 1995; Lin & Tsen 1996; Spilker et al. 2004). Furthermore, at each time point, 100 μl of cell-free (filter sterilized) supernatant from the wells were collected. This supernatant was examined for its ability to form plaques on the resistant bacteria recovered at the same time point, using the spot-test assay described above. The formation of plaques indicated the presence of phage host-range mutants in the supernatant.

**Statistical analysis**

All assays were repeated a minimum of three times with triplicates for each sample in each experiment. Results are reported as means ±95% confidence intervals. The significance of the difference between levels of biofilm was analyzed using the Student’s *t*-test, (Statistica 8.0, Stat Soft. Inc., San Jose, CA) and *p*-values < 0.05 were considered significant.

**Results**

The appropriate MOIs for the treatment of biofilms were chosen (via the protocol described above) to be 10 (50% inhibitory MOI) and 0.1. Data presented in Figure S1b are representative of those obtained for the MOI selection experiment. Furthermore, the frequency of resistance
for the phages used in this work were determined and compared to the frequency of resistance for the phage mixture. Using a phage mixture decreased the resistance by 1/100 for *P. aeruginosa* and *S. enterica* Typhimurium and 1/10 for *S. aureus* thus confirming that the phage mixtures chosen can decrease the frequency of resistance.

**Pretreatment of biofilms, inhibition of formation**

The effect of phage on the load of planktonic bacteria (as indicated by OD<sub>600</sub>) and the total sessile biomass (as indicated by OD<sub>570</sub>) is presented in Figure 1 for MOI=10 and Figure S2 for MOI=0.1. The OD<sub>570</sub> values for all the biofilms were divided by the planktonic growth level (OD<sub>600</sub>) to compensate for differences in the growth rate as described in the literature (Dunne 1990; Deighton & Borland 1993; Yang et al. 2010; Pompilio et al. 2011; Sayem et al. 2011). Furthermore, all the values for planktonic and biofilm levels were presented as the fold change relative to the level of the control. For reference, the unnormalized values for OD<sub>570</sub> and OD<sub>600</sub> are presented in Figures S3 and S4 for phage pretreatment and Figure S5 for phage post-treatment. Samples from the biofilm were taken at each time point and tested for phage susceptibility using the spot test. The percentages of resistant biofilm isolates are summarized in Table 2. The resistant cells were further verified for contamination using PCR and spot tested with an aliquot of the bacteria-free supernatants from the planktonic culture in the wells. This was to check for the presence of phage host-range mutants that could infect the resistant cells. Phage titer counts were performed at each time point for planktonic bacteria in the wells. Representative phage titers are presented for phage mixture treatments at MOI=10 in Figure S6. Other phage treatments followed the same trend and are therefore not included.

The biofilms of *P. aeruginosa* pretreated with phage E79 maintained levels significantly (p<0.05) lower than the control for up to 24 h (MOI=10) and 12 h (MOI=0.1), but there was no statistically significant difference from the control at other time points (Figures 1a and S2a). The planktonic growth for this system remained significantly below the planktonic growth of the control for up to 48 h (MOI=10) and 24 h (MOI=0.1) (Figures 1b and S2b). Phage PP7 did not slow planktonic growth at both the high and low MOIs for more than 8 h (Figures 1b and S1b), however, biofilms pretreated with this phage maintained levels significantly below the control at most time points (Figures 1a and S2a). The mixed phage treatment maintained lower levels of biofilm than the control for up to 24 h for both MOIs, but the planktonic growth in the same system was maintained markedly below the level of the control for up to 72 h (MOI=10). On average, 90% of the colonies isolated from the biofilm of *P. aeru-

*ginosa* pretreated with E79 and 70% of those isolated from biofilms pretreated with the phage mixture were resistant to the ancestral forms of the phages with which they had been treated (Table 2). This figure dropped to less than half for biofilms treated with PP7. The phage titer decreased initially for all systems but did not decrease significantly (p>0.05) throughout the experiment (Figure S6a). Cell-free supernatants formed plaques on lawns of all resistant colonies indicating the presence of phage host-range mutants capable of lysing host cells resistant to the ancestral form of the phage. These mutants were not isolated or quantified.

For *S. aureus* (a representative Gram-positive bacteria), the phage mixture and bacteriophage K maintained the planktonic bacterial load below the level of the control at MOI=10 (Figure 1d). However, none of the treatments were found to be effective in decreasing the biofilm load for *S. aureus*; the biofilms pretreated with phage reached 4–6 times the control levels between 12 and 48 h for different phage treatments (Figure 1c). This rise was more gradual for the mixed phage treatment, and ultimately at 72 h, the biofilm levels of all three systems were comparable to that of the control (Figure 1c). For MOI=10, the OD<sub>570</sub> value was relatively low for all samples at 72 h (Figure S2c), whereas for MOI=0.1 (Figure S2d), the mixture of phages resulted in enhanced biofilm levels at 72 h. Figure S2 also indicates that *S. aureus* formed less biofilms at all time points compared to *P. aeruginosa*. The biofilms treated with phage at MOI=0.1 also showed a significant increase above the control levels (Figure S2c), except biofilms pretreated with bacteriophage K (MOI=0.1), which did not attain levels significantly different from the control (p>0.05). In the low phage treatment (MOI=0.1), the planktonic load was only significantly less than in the control for 8 h (Figure S2d) after which the variability in these data led to an increase in p-values above the significance threshold. Less than 50% of the colonies isolated from treated biofilms of *S. aureus* were resistant to the ancestral form of phages with which they had been treated (Table 2). Phage titer decreased by 2 logs when the maximum levels of biofilm were observed for each treatment, and eventually increased (Figure S6b).

The biofilms of *S. enterica* Typhimurium pretreated with phage (MOI=10) maintained levels significantly less than the control for 12 h only (when treated with P22) and 24 h (when treated with PRD1), as shown in Figure 1e, after which the difference was not statistically significant. The exception to this pattern were biofilms treated with phage P22; at 72 h the levels of biofilm were significantly (p<0.05) higher than the control (Figures 1e and S2e). P22 had no significant effect on the level of planktonic growth after 8 h (Figure 1f) but PRD1 and the phage mixture maintained a significant difference from
the control for up to 48 h (PRD1) and 72 h (mixed phage treatment). The phage mixture did not seem to affect the levels of biofilm for this system (Figure 1e). For MOI = 0.1, the level of biofilm was only significantly less than the control at 12 h (Figure S2e). Less than 50% of the colonies isolated from the treated biofilms of S. enterica Typhimurium were resistant to the ancestral forms of the phages with which they had been treated (Table 2). Phage titers decreased at 24 h for all treatments then increased at 48 h and eventually reached values $\sim$1 log less than the initial values at 72 h (Figure S6c). The presence of phage host-range mutants was confirmed by the
ability of cell-free supernatants to form plaques on lawns of all the isolated colonies at all time points for both S. aureus and S. enterica Typhimurium.

**Post-treatment with phage: eradication of biofilms**

To assess the effect of phage treatments on fully developed biofilms, 48 h old biofilms of each bacterial species were challenged with phage, and the planktonic and sessile bacterial loads were monitored for 72 h. The changes in the level of the biofilms are presented in Figure 2 and the corresponding planktonic bacterial load is presented in Figure S7. The biofilms of P. aeruginosa challenged with phage PP7 exhibited significantly lower levels of biofilm after phage challenge for 48 h (Figure 2a and b). The biofilms challenged with E79 attained values 3–4 times that of the control after phage challenge for 24 h, after which the level of biofilm remained slightly below (but not significantly different from) the level of the control. Biofilms challenged with the mixed phage treatment maintained levels significantly lower than the control for 8 h only, after which there were no statistically significant differences from the control. The only exception to this pattern was for the biofilm challenged with the mixed phage for 24 h (MOI = 10), which exhibited values larger than the control (Figure 2a). It is of note that the planktonic load was kept below the level of the control for up to 12 h by all phage treatments, but did not show a significant difference to the control beyond this point (Figure S7c and d). As before, most of the colonies isolated from biofilms of P. aeruginosa treated with E79 or with the mixed phage were resistant to the ancestral forms of the phages with which they had been treated. This figure dropped to less than half for biofilms treated with PP7 (Table 2).

For biofilms of S. aureus, single phage treatments (MOI = 10) resulted in biofilm levels 5–6 times higher than that of the control at 24 h, after which the difference from the control was not statistically significant (Figure 2c). Although the same trend was observed for MOI = 0.1 (Figure 2d), the high variability of the results meant that the p-values exceeded the significance threshold. The planktonic bacterial load in contact with these biofilms did not change significantly from that of the control in most cases (Figure S7c and d). It is noteworthy (Figure S5) that the control biofilm of S. aureus had an OD$_{570}$ of ~2 after phage treatment for 24 h (biofilm growth for 72 h), but these values had dropped to ~0.5 after 72 h (Figure S4). The biofilms grown for 48 h were completely washed to remove waste products and planktonic cells and new medium was added to the wells (see Materials and methods). This additional treatment could have resulted in the increase observed in Figure S5 compared to Figure S4 as reported previously in the literature (Jackson et al. 2002). The other explanation could be the effect of the phage challenge; the sensitive host popula-

| Bacterial strain | Pre-treatment with phage | Post-treatment with phage |
|------------------|--------------------------|---------------------------|
| P. aeruginosa    | PP7 23 (5)               | 15 (3)                    |
|                  | E79 96 (10)              | 92 (5)                    |
|                  | Mix 75 (8)               | 82 (9)                    |
| S. enterica      | P22 45 (2)               | 40 (6)                    |
| Typhimurium      | PRD1 34 (1)              | 31 (7)                    |
|                  | Mix 28 (5)               | 36 (5)                    |
| S. aureus        | K 43 (5)                 | 35 (5)                    |
|                  | 44AHJD 26 (5)            | 30 (5)                    |
|                  | Mix 33 (5)               | 27 (5)                    |

Table 2. Summary of phage resistance data for biofilm samples.

The number of samples used for each data point is: 5 time points x 3 replicates x 3 biofilm samples per replicate x 10 colonies from each biofilm sample = 450. The numbers in parenthesis represent the 95% confidence intervals. All numbers are rounded to the nearest digit.

Discussion

This study investigated the effect of phage predation on biofilms of 3 different bacterial species. The effect of single phages and a phage mixture on the inhibition of biofilm formation and the eradication of developed biofilms were investigated. Most of the systems studied were effective in inhibiting/removing biofilm to levels significantly below the control for only 12–24 h; the PP7 – P. aeruginosa system was effective beyond this time for up to 72 h. The principal observation in this study was that
for some systems, the level of biofilm increased above that of the control (e.g., S. aureus pretreated and post-treated) even though the level of planktonic bacteria remained less than or equal to the level of the control at all times. Thus, it appeared that predation by phages increased the tendency of bacteria to form biofilms for certain phage–host systems. Furthermore, the use of a phage mixture generally did not prove to be superior for the control of biofilms. Since the phage mixture was shown to decrease the frequency of resistance in the bacterial host, this suggests that the emergence of phage resistance was not the main phenomenon limiting the efficiency of phage treatment for controlling the biofilms in the systems under study. Moreover, phages can mutate to produce phage

Figure 2. The level of biofilm for a 48 h biofilm post-treated with phage. (a) P. aeruginosa (MOI=10); (b) P. aeruginosa (MOI=0.1); (c) S. aureus (MOI=10); (d) S. aureus (MOI=0.1); (e) S. enterica Typhimurium (MOI=10); (f) S. enterica Typhimurium (MOI=0.1). Biofilm load is presented in terms of fold change in the value of normalized biofilm level (OD570/OD600) compared to the control (which = 1). *Statistically significant difference from the control (p < 0.05). Time zero is chosen as the time of addition of phage to the system.
strains that are lytic towards the resistant host cells (phage host-range mutants). When the bacterial host mutates, selection will favor the phages that are capable of killing and replicating in these bacteria and thus phage host-range mutants will have a selective advantage (Comeau & Krisch 2005). Phage host-range mutants were present in all the bacteria/phage combinations under study. This is indicated by the ability of bacteria-free supernatants to form plaques on phage-resistant biofilm isolates. This also suggests that phage-resistance may not be the main factor limiting phage efficiency.

Self-organized bacterial refuges are one of several possible non-evolutionary mechanisms which stabilize systems of phage-sensitive and phage-resistant bacteria with their virulent phage (Schrag & Mittler 1996; Heilmann et al. 2012). The spatial refuge hypothesis predicts that physical heterogeneity in the environment protects some sensitive bacteria from phage infection, and thus is a factor in stabilizing predator–prey communities (Van den Ende 1973; Bohannan & Lenski 2000; Brockhurst et al. 2006). One of the implications of this theory is that the stability (coexistence without either species being driven to extinction) of a bacteria-phage community will increase as opportunities for the growth of a biofilm on a solid substratum increases. Contrary to initial assumptions, it has been demonstrated that being in a biofilm does not form a physical barrier against phage attack (Adams & Park 1956; Hughes, Sutherland, Clark et al. 1998; Hughes, Sutherland, Jones 1998; Hanlon et al. 2001; Briandet et al. 2008). Therefore, the spatial refuge theory could not fully explain the increase in the formation of biofilm, especially as the homogeneity of the system was maintained by constant shaking. However, the change in the level of metabolism brought about by the transition from planktonic to biofilm phenotype has been shown to decrease the rate of propagation of phages (Hanlon et al. 2001; Cerca et al. 2007). If bacterial cells in a biofilm are no longer able to support phage amplification to high densities, then acquiring a biofilm phenotype could be a means of ‘physiological refuge’ rather than just a means of spatial refuge for phage-sensitive host cells. Biofilms could also constitute a numerical refuge for cells; since biofilms occupy only a small fraction of the total environmental volume entering into a biofilm state would reduce the density of the host in the medium, thus potentially decreasing exposure to phages (Chao & Ramsdell 1985; Abedon 2012). Heilmann et al. (2012) developed a model to predict the effect of refuges on the stability of phage–host systems. The authors proposed bacterial density-dependent, or quorum-sensing, mechanisms such as the formation of biofilms to produce the refuges required for sensitive bacteria to achieve coexistence with the phage in the short term, giving them time to evolve resistance which would guarantee their stability in the long term (Heilmann et al. 2012). A somewhat different view on this subject was recently presented (Abedon 2012). It was argued that the cost of being exposed to a phage would be higher if the bacterial cells are physically associated to each other, since the progeny of the phage from one infected host cell could immediately infect many other bacteria. In this model, it is predicted that entering into multicellular bacterial communities is only beneficial for the host if the pressure from phage predation is low (Abedon 2012). Although this model does not take into account the reduced level of metabolic activity of the cells in a biofilm, it correlates well with some of the results from these studies, and could explain situations where phages enhanced the formation of the biofilms and then the biofilms subsequently decrease significantly, as was the case with S. aureus. In most cases where the level of the biofilm increased above the level of the control, phage titers were observed to have decreased (Figure S3). It is hypothesized that this decrease in the titer is a result of the emergence of resistant cells which means that the host population could not maintain high phage titers. According to the model of Abedon (2012), this decrease in the phage titer would result in an increase in the formation of biofilm to stabilize the host populations. As phages coevolve, and phage host-range mutants develop, the phage titers rise and the levels of the biofilm decrease, which is in accordance with the model of Abedon for high phage pressure scenarios. In the following section, each biofilm system is evaluated separately with the aim of explaining the observed response to phage predation based on one or a combination of the models presented above.

The interaction of biofilms of P. aeruginosa with phages
For biofilms of P. aeruginosa, phage PP7 failed to inhibit planktonic growth but led to a significant decrease in the level of biofilm. It is known that one of the main mechanisms of phage resistance is envelope modification by blocking or modifying the phage receptors on the host cell (Labrie et al. 2010). PP7 is a pili-specific phage (Bradley 1972), and thus it is hypothesized that cells resistant to this phage may have either lower levels of pili synthesis or mutations affecting the pilin protein such that it cannot be bound by the phage. Twitching motility is a mode of surface translocation completely dependent on the pili (Bardy et al. 2003). PP7-resistant cells have been observed to have lower twitching motility (Hosseinidoust et al. 2013); although these resistant cells were not obtained from the same biofilm model, this observation nonetheless confirms the hypothesis. Pili are also the principal adhesions mediating the adherence of cells to surfaces (Bardy et al. 2003) and they play an important role in the formation of biofilms of P. aeruginosa (Barken et al. 2008). If the pili are deactivated by any mechanism it can be predicted
that the formation of biofilm will decrease. Therefore, it is possible that the significant efficacy of PP7 for the inhibition and eradication of biofilms is a result of bacteria developing resistance to this phage. The phage resistant cells would still persist in the planktonic form, which explains why a reduction in the OD600 value was not observed. The observation that less than half of the isolates from the biofilms treated with PP7 were resistant to this phage further confirms that PP7-resistant cells do not favor biofilm formation.

Phage E79 binds to lipopolysaccharide (LPS) on the cell surface (Jarrell & Kropinski 1977). Functional LPS is required for the swarming motility of P. aeruginosa. There is evidence of a decrease in swarming motility for E79-resistant P. aeruginosa cells (Hosseindoust et al. 2013). Although these resistant cells were isolated from a different system, it is reasonable to hypothesize that such E79-resistant P. aeruginosa cells could have altered LPS. Furthermore, E79-resistant P. aeruginosa cells were shown to have a decreased swimming motility (Hosseindoust et al. 2013). Therefore, it follows that they could become sessile more readily. This could explain the observed increase in the formation of biofilm above the levels of the control for biofilms challenged with E79, despite the relatively effective control of planktonic growth by this phage. Most of the colonies isolated from biofilms of P. aeruginosa pretreated or post-treated with E79 were resistant to this phage which further confirms the hypothesis that E79 resistant cells have a higher tendency to form biofilms. The opposing selective pressures from phages PP7 and E79 on the phenotype of the resistant cells could explain why the phage mixture was not the best choice for inhibiting/eradicating biofilms of P. aeruginosa in this study.

It is also noteworthy that the levels of biofilm of P. aeruginosa increased above the levels of the control when challenged for 24 h with E79 and with the phage mixture (Figure 2a and b), but the biofilm levels decreased with further incubation (48 and 72 h). The observed decrease in the level of biofilm could in part be attributed to the usual biofilm dispersion being reached earlier because of the rapid increase in the biofilm and possibly greater accumulation of waste products. It could also be attributed to phage coevolution resulting in the emergence of host-range mutants that can lyse the E79-resistant cells within the biofilm.

**The interaction of biofilms of S. aureus with phages**

The biofilms of S. aureus pretreated or post-treated with phage did not exhibit levels of biofilm lower than the control; in many instances the levels of biofilm were significantly higher. However, it is important to note that the growth of biofilms of S. aureus under control conditions was generally quite low, which could be a result of the specific biofilm model chosen for this study. It appears that in the absence of phage, S. aureus did not exhibit the tendency to form biofilms, yet in the presence of phage the formation of biofilms was induced. The general pattern observed for the biofilms treated with phage was a significant increase above the level of the control, after which the level of the biofilm decreased eventually back to control levels. The increase in the level of the biofilm was accompanied by a decrease in the phage titers. Less than 50% of the colonies isolated from the biofilms were resistant to the ancestral form of the phages with which they had been treated. This suggests that the formation of S. aureus biofilms was not solely a result of phage resistance but rather a refuge for phage-sensitive cells. As the system is kept homogeneous, and assuming the biofilm matrix does not form a physical barrier against phage action, it is hypothesized that the apparent accumulation of S. aureus cells exhibiting the biofilm phenotype serves to protect sensitive cells by providing a physiological and/or numerical refuge.

Another observation from this system was the high efficacy of the bacteriophage K and the mixture of phages to decrease the growth of planktonic cells at MOI = 10 (Figure 1d), whereas the same system had significantly higher levels of biofilm compared to the control. Phage 44AHJD did not decrease the growth of planktonic cells compared to the control, but it had the same effect of increasing the level of biofilm as the 2 other phage treatments. An increase in the biofilm above the level of the control was also observed during pretreatment with MOI = 0.1, although this dose of phage had no significant effect on the growth of planktonic cells. Thus, the phage-to-bacteria ratio affects the growth of planktonic cells as expected but higher MOIs do not result in less biofilm for this system. Furthermore, using the mixture of phages did not appear to offer any advantage for inhibiting or eradicating biofilms of S. aureus.

The biofilms of S. enterica Typhimurium pretreated or post-treated with phage generally had a level of biofilm comparable to that of the control at 8 h. The level of biofilm then decreased (with the exception of post-treatment with MOI = 0.1) to levels below that of the control after which they increased (although not monotonically) to reach the same or higher levels than the control. The response of the biofilm to phage predation in this system could be explained by the evolutionary struggle between the phage and its host leading to the emergence of phage host-range mutants that can infect resistant cells and thereby decrease the level of the biofilm. The level of the biofilm increased with the emergence of resistance to the evolved phage which precipitated another round of phage coevolution and a concomitant decrease in biofilm levels. This theory is further confirmed by the phage titer, which
exhibits a zig-zag pattern comparable to that observed for the level of the biofilm (Figure S5c).

For the biofilms, post-treated with phage at MOI = 0.1 (Figure 2f), a different pattern was observed; the level of biofilm initially increased above the level of the control (albeit with high variation) after which the level of the biofilm decreased significantly below the level of the control. This decrease could be attributed to the dispersal of the biofilm arising from an increase in the sessile biomass and an increase in waste products. As the phage titer did not increase significantly in this system, the decrease in the biofilm could not be attributed to phage action.

The levels of biofilm increased significantly above the levels of the control at 72 h for biofilms pretreated with P22 and post-treated with P22 and PRD1 (single-phage treatments, MOI = 10). It is not clear whether increasing the incubation time over 72 h would have resulted in a decrease in the level of the biofilm or not. The increase in the biofilm at 72 h cannot be explained by any of the theories cited previously. However, based on the model of Abedon (2012) a decrease in the phage titers would lead the system to form multicellular structures to stabilize itself in the presence of a phage. The phage titer was observed to decrease for these systems by 1 log at 72 h, thus these results correlate well with the predictions of the model. In this study the load of planktonic cells increased monotonically for all phage treatments and was always ≤ the level of the control. It has been suggested that DNA released as a result of lysis could increase the formation of biofilm (Gödeke et al. 2011). Thus, the increase in the level of biofilm could also be attributed to the accumulation of extracellular DNA as a result of phage-mediated cell lysis.

Conclusions

It has been proposed that lytic bacteriophages could become the new class of anti-biofilm agents (Donlan 2009). It was proposed that the biofilm controlling ability of a phage is governed by the additive, synergistic, antagonistic, or suppressive interaction of evolutionary and non-evolutionary mechanisms, and virulent phages cannot be deemed suitable for the control of biofilms based solely on their lytic ability towards the host. Based on the results of this study, the effect of phage treatment on biofilms is context-dependent and it is governed by a combination of evolutionary and non-evolutionary mechanisms. It is important to be aware that phages that show a plausible lytic effect against their host bacterium may not always be the best choice for the control of biofilms composed of that bacterium (eg E79, P. aeruginosa) since the phenotypic changes resulting from phage-resistance mutations in the host cell could result in an increased formation of biofilm. The same evolutionary mechanism (selection for phage-resis-
tant mutants) could prove advantageous if the right phage is chosen, as was observed for the PP7 and P. aeruginosa system; although phage-resistance developed readily, the resistant cells lacked the ability to form stable biofilms. Thus, phenotypic changes resulting from phage-resistance mutations are an important design parameter when choosing phages or mixtures of phages for the control of biofilms.

This study provides strong evidence suggesting that the formation of resistance is not the main factor limiting system efficiency (and in some cases increasing the level of the biofilm above the level of the control) for biofilms of S. aureus and S. enterica Typhimurium. In an attempt to decrease the effect of phage resistance, mixtures of phages, designed for this purpose, were employed. Phage host-range mutants were also found to be present in all the systems under study, yet neither seemed to affect the efficiency of the system. This suggested that non-evolutionary mechanisms played a governing role in phage-biofilm population dynamics in the study. Seeking refuge in the form of a biofilm decreases the metabolic activity of bacteria (and thus ability to support phage propagation) and also decreases their volume fraction in the medium. This is believed to be a means of securing coexistence for phage-sensitive bacteria. It should be noted that resource limitation as a result of using a static biofilm model, will undoubtedly affect the results of this study. Although replacing half of the medium in each well with fresh TSB every 24 h would limit this effect, it is acknowledged that the results obtained using this biofilm model may not be valid for other experimental designs. It would be interesting to confirm the effects in other biofilm models such as in continuous-culture flow cells.

This study also emphasizes that new techniques may be needed to reduce further the evolution of phage-resistant bacteria. These include using phages in combination with other antimicrobials, such as antibiotics, cycling through different phage mixtures, and engineering phages to directly target phage-resistance mechanisms (Labrie et al. 2010). An alternative approach would be the use of phage lysins for which the development of resistance has not been reported (Fenton et al. 2010). One question that also deserves more study is whether the evolution of phage resistance in vitro is relevant to in vivo conditions where bacteria may be replicating more slowly and are challenged with a greater set of environmental conditions (Capparelli et al. 2007; Lu & Koeris 2011). Further investigation is required to determine whether the trends observed in this study are reproducible in other biofilm models (eg chemostats). Finally, this work highlights the importance of a mechanistic understanding of the population and evolutionary biology of bacteria-phage interactions for the successful development of phages for the control of bacterial biofilms.
Acknowledgments
This work is supported by the Natural Sciences and Engineering Research Council of Canada (NSERC Strategic Research Network on Bioactive Paper-SENTINEL) and the Canada Research Chairs (CRC) program. M. Elimelech (Yale University) is acknowledged for providing P. aeruginosa PAO1, and C. O’May (McGill University) is thanked for technical advice and valuable feedback on the manuscript.

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