MiniReview

The interaction of phage and biofilms

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Abstract

Biofilms present complex assemblies of micro-organisms attached to surfaces. They are dynamic structures in which various metabolic activities and interactions between the component cells occur. When phage come in contact with biofilms, further interactions occur dependent on the susceptibility of the biofilm bacteria to phage and to the availability of receptor sites. If the phage also possess polysaccharide-degrading enzymes, or if considerable cell lysis is effected by the phage, the integrity of the biofilm may rapidly be destroyed. Alternatively, coexistence between phage and host bacteria within the biofilm may develop. Although phage have been proposed as a means of destroying or controlling biofilms, the technology for this has not yet been successfully developed.
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1. Introduction

Although relatively large numbers of phage are found in many of the environments in which biofilms are formed, and despite the increasing volume of studies on microbial biofilms, relatively few have examined the effects of bacteriophage on the bacterial components of these mixed microbial communities. Some reports are now emerging on the interactions in vitro between phage and experimental single species and mixed species biofilms. In any examination of these interactions, the question of access of the viral particles to the surface of their host cells must also be considered. *Bdellovibrio* can reach the bacterial cell even when the cell is surrounded by a discrete capsule [1], but phage lack the propulsion mechanisms of these predatory bacteria. In this paper we review the current state of knowledge on the interactions of phage and biofilms.

2. Biofilm composition and structure

Numerous definitions of biofilms exist, but in general they can be regarded as agglomerations of microbial cells and their excreted products attached to inert surfaces. In natural environments, these are mixed microbial cultures normally consisting predominantly of prokaryotes with some eukaryotes. Thus, as well as microbial cells, the surrounding milieu contains a range of macromolecular products in which exopolysaccharide (EPS) secreted by the cells is the dominant macromolecular component, while the water content is probably about 90–97% [2]. Further secreted products include enzymes and other proteins, bacteriocins, and low mass solutes and nucleic acid released through cell lysis. The lysis may occur either naturally with cell ageing or through the action of phage and bacteriocins. Pronounced lysis may also be found in *Pseudomonas aeruginosa* strains that overproduce quinolone signals [3]. In some biofilms, large amounts of protein may be found in the extracellular matrix [4]. The structure of the biofilm is dependent on many factors: the component micro-organisms, their physiological state, the physical environment including turbulent or linear flow conditions and the surface to which the cells are attached [5,6]. A biofilm presents a dynamic environment inside which the microbial cells generally remain viable and metabolically active,

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although this depends on their position within the biofilm. Consequently, any 'snapshot' of composition and structure represents a single view from a series of constantly changing microcosms. Accurate analysis of composition is therefore difficult to achieve. It is also potentially misleading to extrapolate data from a limited number of laboratory experiments, usually performed using rich culture media, to other environments which often contain a diverse range of different component microbial cells. Nielsen et al. [7] also noted that changes in substrate caused marked alterations to the spatial configurations of commensal bacterial mixtures, leading in some circumstances to formation of separate microcolonies. They suggested that bacteria or biofilm component cells could reorganise structurally in response to the physiological conditions prevailing. Cells expressing specific functional genes within specialised microenvironments in the biofilm could then ensure optimal use of nutrient sources [8].

The use of specific methods such as labelling with green fluorescent protein (GFP) [9,10] permits identification of cell types, while labelled lectins may also identify the presence and location of some monosaccharides and carbohydrate structures [11].

3. Viral access to the bacterial surface

Various structures including extracellular polymers and heterologous microbial cells may impede viral access to the bacterial cell surface. Many phage, but not all, may produce polysaccharases or polysaccharide lyases as described below. Some phage are also known that produce enzymes that degrade the poly-γ-glutamic acid capsule of *Bacillus* spp. [12]. In the absence of degradative enzymes, there may be few physical barriers to the surface of at least a portion of the potential host cells. Many biofilms possess an open architecture with water-filled channels, which would allow the phage access to the biofilm interior. Improved confocal laser scanning microscopy and similar techniques have also demonstrated the microheterogeneity of biofilms with variable distribution of cells, matrix and fluid-filled channels and pores [13]. As biofilms age and cells die and slough off, potential new viral receptor sites may become available. Flood and Ashbolt [14] observed that wetland biofilms could entrap viral-sized particles and concentrate them over 100-fold, compared with the number in the surrounding water column. Nevertheless, the persistence of these inert particles for over 7 months does not necessarily imply such prolonged persistence for actual bacteriophage. The authors did, however, suggest that viruses could be released when portions of the biofilms sloughed off with ageing. The location of the particles within the biofilms was also observed to alter with time. Phage release would also be expected for the bacteriophage as they achieved local lysis of susceptible cells and as associated enzymes degraded EPS within the bio-

film. As bacteria excel at adapting to differing nutrient conditions [15], changes to the host cell surface could be expected with either loss or gain of possible phage receptors. A further factor which might influence phage retention within biofilms lies in the role of hydrophobic and electrostatic interactions. In the interaction of a coliphage with both hydrophobic and hydrophilic membranes, a critical factor in the retention of the phage was its iso-electric point [16]. On hydrophilic membranes, hydrophobic interactions were dominant in viral retention, which was further increased by salt.

4. The interaction of phage and bacterial polysaccharides

Phage may carry on their surface enzymes that degrade bacterial polysaccharides. These enzymes are very specific and seldom act on more than a few closely related polysaccharide structures [17,18]. Numerous phage have been isolated which induce enzymes capable of degrading the EPS of various Gram-negative bacterial genera. These include phage for biofilm-forming bacteria. On plating out, such phages are typified by haloes of varying size surrounding plaques obtained from single infected bacterial cells. The haloes represent susceptible bacteria from which the associated EPS has been removed by excess soluble enzyme released on lysis of each infected bacterium (Fig. 1). A study using strains of *Enterobacter agglomerans* indicated that bacteriophage with polysaccharide depolymerases for these strains were common in the environments tested [19,20]. In particular, phage possessing depolymerases specific for linkages found in the EPS of *E. agglomerans* strains 53b and Ent were especially common. The polysaccharide depolymerase of phage SF153b was highly active over a wide range of environmental conditions. This was vital if the phage was to gain access to receptors on the cell wall and infect the bacteria under as many conditions as possible. Phage with associated enzyme can readily reach the cell surface by digesting their way through capsular polysaccharide as elegantly indicated in the studies of Bayer et al. [21].

In a study of phage acting on *Lactococcus lactis*, Deveau et al. [22] reported that differences in EPS composition did not affect phage sensitivity and that EPS was not required for phage infection, nor did EPS confer significant resistance to phage. However, the various phage used did not induce polysaccharide-degrading enzymes and phage sensitivities were thus derived solely from the ability of the phage particles to access the surface of the bacterial hosts. Regardless of whether or not the phage produce polysaccharases, the outcome of viral adsorption and entry to the host bacterium will depend on many factors associated with the physiological state of the host. One can assume that many bacteria within the biofilm will present sub-optimal environments for phage infection and growth, an aspect that has been
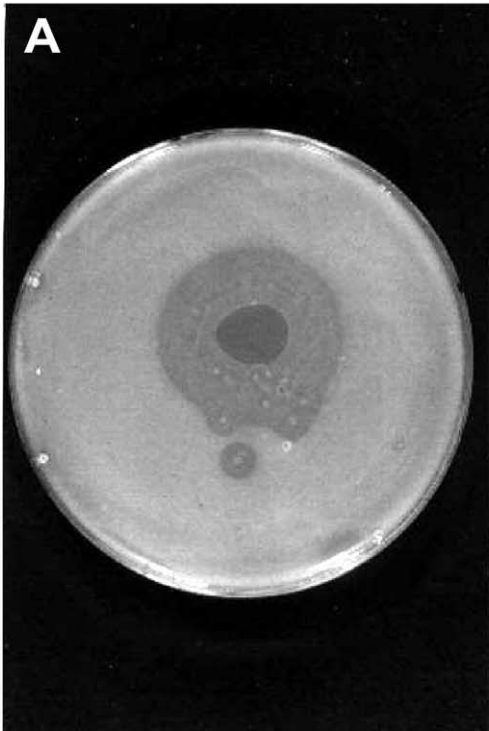
Fig. 1. A: A typical polysaccharase-inducing phage – confluent lysis showing zone of clearing surrounded by halo of viable bacteria from which polysaccharide has been removed. B: A phage for biofilm-forming *E. agglomerans*.

examined by Hadas et al. [23]. These workers demonstrated that bacterial growth rate affected several parameters of T4 adsorption and growth in *Escherichia coli*, and similar results can be expected for other bacteria. Although many phage studies relate to Gram-negative bacteria, *Staphylococcus epidermidis* biofilms also show susceptibility to phage [24].

5. Phage and single species biofilms

In studying *E. coli* biofilms in a modified Robbins device, Doolittle et al. [25] observed that the extracellular matrix of the biofilms did not protect the bacterial cells from infection with phage T4. Under the experimental conditions, either insufficient polymer was present on portions of the cell aggregates in the biofilm to inhibit the phage, or the matrix was sufficiently diffuse to allow the viral particles to penetrate unless the phage carried a hitherto unidentified depolymerase. However, as the rich growth medium used would not normally favour EPS production and no measurements of polysaccharide were made, the nature and extent of the matrix remains unclear. As most biofilms are now considered to contain 'pores' or water channels, these too would permit access for the phage. The apparent phage multiplicity of infection (MOI) was relatively low, but would possibly have been enhanced by lysis of planktonic cells. The results indicate that contrary to earlier hypotheses [26] even in non-EPS-producing strains, the location and arrangement of the cell matrix on the substratum failed to protect against phage infection. In a study in which phage were labelled with fluorescent and chromogenic probes [27] it appeared that phage T4-infected host cells of *E. coli* attached to or associated with the biofilm surface and were not inhibited by any extracellular macromolecules present. Corbin et al. [28] demonstrated that the MOI with T4 affected the outcome in a glucose-limited *E. coli* biofilm; 90 min post phage infection, biofilm disruption was greater with a MOI of 100 as opposed to 10. However, more prolonged incubation yielded a decrease in phage titre and an establishment of equilibrium between virus and host, with stable bacterial and viral numbers. This phenomenon is peculiar to biofilms, as in studies with planktonic cells, lysis of the bacteria is almost complete and no similar equilibrium occurs. This appears to be similar to the co-existence of bacteriocin-producing and bacteriocin-sensitive enteric bacterial strains seen in dual species biofilms [29]. In single species *Enterobacter cloacae* biofilms, combinations of three phage were required to achieve complete eradication of the biofilm [30]. Phage E79 was more successful at infecting the surface cells of thick *Pseudomonas aeruginosa* biofilms than cells at depth in the biofilm [27]. Although temperature and nutrient concentration did not affect susceptibility to phage infection, spread of infection within the biofilms was slower at lower temperatures or in defined

low-nutrient media. None of the phages induced polysaccharases or polysaccharide lyases [17] although, as with many phages, bacteriolytic enzymes might have been released from lysing cells. As Doolittle et al. [27] pointed out, differences in the biofilms formed by the Gram-negative species *E. cloacae* and *P. aeruginosa* might have caused differences in accessibility to phage. In removal of bacteria from stainless steel surfaces with listeriaphage, combinations of two phage or a phage in combination with quaternary ammonium compounds produced synergistic effects [31]. In another study using cell wall-deficient L-forms of *Listeria monocytogenes*, phage specific for these bacteria prevented them from forming biofilms [32].

In our studies on *E. agglomerans* biofilms [19,20], the cells were readily lysed and the biofilm degraded by addition of bacteriophage if certain criteria were met. The bacteria had to be susceptible to the phage, and the phage polysaccharide depolymerase had to be able to degrade the biofilm EPS. The phage then lysed the biofilm cells, the depolymerase enzyme degraded the EPS and caused the biofilm to slough off. If only one of these criteria was met, there was still a substantial degree of biofilm degradation. Addition of phage to phage-resistant biofilms showed the major role that the EPS depolymerase played in biofilm removal. This was confirmed when addition of phage-free depolymerase enzyme to the phage-susceptible biofilm resulted in rapid biofilm degradation. If neither criterion was met, the biofilm remained relatively unaffected. Electron micrograph images of biofilm degradation by phage revealed that the depolymerase enzyme played the major role in biofilm degradation. Most of the biofilm bacteria had been removed from the substratum by the action of the depolymerase long before the phage-infected bacteria had lysed. This also suggested that the EPS was not biofilm-specific, as the polysaccharide depolymerase degraded EPS produced by both planktonic and biofilm bacteria. This result agreed with earlier work that showed little difference between the chemical composition of EPS produced by biofilm and planktonic bacteria. The situation in natural environments must be considerably more complex.

The depolymerase may play a dual role as it may be crucial for phage release from the biofilm as well as the initial penetration and subsequent infection of cells. If a phage infects bacteria within a 'young' biofilm and enters a lysogenic state, the maturation of the biofilm may inhibit later release of phage. Alternatively, if the cells are effectively starved, pseudolysogeny may develop [33]. In this phage–bacterial host cell interaction, the phage nucleic acid is located within the host cell in an unstable, inactive state. Increased thickness of the biofilm, through addition of other species and their associated EPS, may result in the phage becoming trapped in the basal layers without the enzymatic capacity to liberate itself from the EPS mass that surrounds it. Phage release

and biofilm destruction may be very localised, especially when low phage numbers are present as may occur in natural biofilms. Multispecies biofilms could be expected to be more resistant to phage than single species, and this was confirmed by Tait et al. [29] who were unable to completely eliminate a phage-susceptible mixed species biofilm. This might be due to structural heterogeneity within the biofilm producing foci of bacteria inaccessible to the phage.

In marine waters, Riemann and Middleboe [34] have suggested that viruses were responsible for much of the mortality occurring in bacterioplankton. They were thought to have a profound influence on the diversity of the bacterial community; their lytic products also provided a source of nutrients for non-susceptible bacteria. Algal viruses acting on eukaryotic algae such as *Emiliania huxleyi* also lyse the host cells and liberate potential nutrients for other micro-organisms [35]. Similar nutrient recycling through the action of bacteriophage would be expected in complex biofilms. In their review of phage applications, Marks and Sharp [36] reported proposals to control dental plaque- and caries-inducing *Lactobacillus* spp. with phage, but no successful reports of this type exist.

6. Phage and mixed species biofilms

As with single species biofilms, the physiological state of the host cells in mixed species biofilms and the availability of phage receptors on their surfaces will play a significant role in the outcome of phage attack. A further factor may be the role of bacterial cell co-aggregation. As pointed out by Rickard et al. [37], co-aggregation may be observed in biofilm communities from a variety of habitats and the tight cell–cell binding may occlude phage receptors. It is also possible that cell–cell binding may involve phage receptors directly. Various studies have suggested that biofilms may trap and accumulate virus-sized particles and produce a potential reservoir of human or bacterial pathogens [14]. Release of the particles was thought to be minimal except under conditions in which portions of biofilm sloughed off. Storey and Ashbolt [38] studied the persistence of phages for *Bacteroides fragilis* and *E. coli* in biofilms within an urban water distribution system. Upon initial addition, both phage were incorporated into biofilms formed on either stainless steel or polythene surfaces. However, after initial incorporation, loss of the phage was rapid but was then followed by a slower release of virus particles. Following the reported potential of these viruses to accumulate within the biofilms [14], it was suggested that problems might occur when large clusters of biofilm-associated phage became detached. Hydrodynamic forces or altered disinfection regimes might cause large-scale detachment. Ueda and Horan [39] have also reported the removal of enteric phage from domestic sewage by

biofilms in a membrane reactor, phage loss being greater in the laboratory bioreactor than in a full-scale sewage treatment plant. The membrane itself failed to remove phage efficiently. In both studies it would appear that the phage involved resembled T-even phages in appearance; in the absence of any evidence to the contrary, like them they lacked the ability to degrade any of the bacterial polysaccharides contributing to the biofilm structures.

The exact role of EPS in mixed biofilms is still not clear as component species may contribute different levels of EPS and subsequently affect phage depolymerase activity but the effects of phage-associated polysaccharases have been observed in studies using *E. agglomerans* labelled with GFP [40]. A 60-min treatment with a polysaccharase caused a 20–39% reduction in biofilm adhesion. EPS may also be involved in the initial adhesion of biofilm cells to surfaces.

7. Conclusions

While it has been suggested that phage might prove suitable for controlling biofilms containing enteric and other bacteria, evidence in support of this is still scanty [41]. Sklar and Joerger [42] attempted to use phage to control *Salmonella enterica* serovar *enteritidis* in chickens. Bacterial counts were reduced but not eliminated. Earlier studies suggesting the use of phage to control *E. coli* O157 were also unsuccessful even when cocktails of three different phage were employed in an attempt to ensure that bacteria resistant to one phage would be susceptible to one of the others [43]. Thus, under practical conditions, the failure of phage to completely eliminate the host bacteria, due either to failed access or to emergence of resistant strains, points to the impracticality of such methods. However, few biofilms behave identically. The addition of phage to biofilms in which antagonistic interactions are already occurring due to bacteriocin production may provide further stress and enhance the elimination process. In complex biofilms in natural environments, eukaryotic algae may also be present (e.g. [44]). Under these circumstances algal cell lysis through viral action is also possible as many virus for algal species have now been isolated and identified [35,44]. The differences in gene expression in biofilm cells as opposed to planktonic bacteria must also be taken into account. Stanley et al. [45], in a study of *Bacillus subtilis*, reported that approximately 6% of the genes were differentially expressed with a biofilm; these genes involved metabolism, motility and phage-related functions. It has even been suggested, although not confirmed experimentally, that some phage may be better adapted to grow in biofilms or in mixed cultures [46]. This is perhaps also an area that requires to be studied in parallel with further examination of the effects of phage on biofilms.

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