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Thinking about microcolonies as phage targets

Stephen T. Abedon
Department of Microbiology; The Ohio State University; Mansfield, OH USA

Phage targets for adsorption can include: (1) individual bacteria; (2) bacterial cellular arrangements such as streptococci; (3) microcolonies consisting of bacterial clones as can make up bacterial lawns and biofilms; and (4) bacterial biofilms themselves. While much effort has gone into considering category 1, and some into category 4, substantially less has been put into the question of how bacterial association into clonal arrangements or microcolonies might affect phage-bacterial interactions. Recently I have been exploring just this issue within a single-authored monograph published in 2011 and a theoretical article published in 2012 as part of a special issue of the journal, *Viruses*. For this commentary, I have been invited to summarize my thinking on how bacterial association into either cellular arrangements or microcolonies might affect their susceptibility to phages along with related issues of bacterial resistance to phages and phage propagation in the context of both plaques and biofilms.

Keywords: bacteriophages, biofilm, cellular arrangement, lysis inhibition, microcolony, phage, phage ecology, plaque formation, T-even phages

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Correspondence to: Stephen T. Abedon;
Email: abedon.1@osu.edu

At the level of whole organisms—by which I mean whole cells and individual virus particles—the common perception of phage-bacterial interactions is based on what physicists call mass action.[1] Specifically, this is in terms of the likelihood of an encounter of freely diffusing particles, such as virions and bacteria.[2] Alternatively, a fair amount of effort has gone into characterizing phage-bacterial interactions under circumstances where only one of the two are freely diffusing, that is, where bacteria instead are surface bound, particularly forming into biofilms.[3] Between these two extremes exists a little explored, though perhaps highly relevant, "middle ground," one that is represented by clonal bacterial arrangements along with bacterial microcolonies. These latter forms of bacterial existence together may be involved in a substantial quantity of phage interactions in nature as well as during phage therapy, and this is particularly so to the extent that bacterial species can be present as individual microcolonies within biofilms.[3–5] In this commentary I consider bacterial arrangements and microcolonies as targets for phage adsorption along with subsequent infection.[6] In addition, I discuss related issues of bacterial resistance to phages,[6–8] formation of phage plaques[3,9] and associated aspects of the ecology of phage exploitation of biofilms.[3]

At the simplest level of consideration, once a phage has productively infected one bacterium then, due to subsequent release of virion progeny, all of the bacteria found in the immediate vicinity should be more vulnerable to phage encounter.[6,10] When bacteria are massed into physically associated clonal groupings, such as cellular arrangements or microcolonies, what has occurred is a more or less locking of these cells into each other's immediate vicinity. Such groupings, in particular, may locally exceed those minimum bacterial densities sufficient to support ongoing phage replication and persistence, what has been described as a phage proliferation threshold.[4] Closely associated bacteria furthermore might locally exceed "winner" densities,[11] that is, concentrations of bacteria which are sufficient to support phage propagation to levels that can result in substantial bacterial eradication. Given environmental spatial structure in the form of impediments to extensive mixing, such winner densities could be locally

present even if bacteria, more globally, are not found at similarly high densities.

Once phage infection within a bacterial grouping has occurred, then there can be greater local loss of bacteria than would be the case given phage infection of a single, isolated bacterium. Indeed, the cells making up entire bacterial arrangements or microcolonies could potentially be sequentially lost to a single phage lineage, one that is propagating exclusively at the expense of such a bacterial grouping. Arrangements or microcolonies also can serve, dimensionally, as larger targets for phage encounter than individual bacteria, e.g., up to twice as large for two bacteria found in association vs. a single bacterium found alone. The overall result can be a greater vulnerability to phage infection by bacteria that are found in arrangements or microcolonies relative to identical but otherwise isolated bacteria. This, in turn, suggests that the bacteria making up these groupings might be ecologically successful only when phage predation pressure is lower, either due to lack of phage presence or because bacteria display resistance to those phages that are present.[6]

Close physical association resulting in increased susceptibility to destruction equivalently serves as one of the bases of nuclear chain reactions. For example, ^{235}U that is held at a critical mass—as equivalent to a proliferation threshold density for bacteria and phage infection—can sustain the propagation of ^{235}U-destroying neutrons.[6,12] This is the case also with exergonic chemical reactions, where the activation energy required to initiate one reaction can be supplied by the energy released as a product of another reaction. Such couplings can be sustained, though, only if reactions are sufficiently spatially associated so that, for example, energy can be efficiently transferred prior to its dilution into the environment.

These various concepts of self-propagation are directly applicable to epidemiology, where a pathogen or parasite must possess a reproduction number of at least one to persist within a host population. Reproduction numbers of less than one imply that susceptible hosts are simply too far apart for parasite transmission to efficiently occur. Organism clumping, such as into households for humans, thus is expected to facilitate parasite transmission between host individuals. Given such clumping, the likelihood of subsequent epidemic propagation becomes dependent particularly on the between-clump basic reproduction number.[13] Indeed, one can contrast within- and between-clump reproduction numbers, with the parasite propagation between clumps—e.g., between households—potentially less efficient than parasite propagation within clumps, such as within households.

Increased vulnerability to parasites that can come with cell clumping, including the cells that make up bodies, is presumably an important reason that multicellular organisms have evolved sophisticated immune systems. Bacteria also possess mechanisms of resistance to, for example, phage attack.[14] These mechanisms include restriction-modification systems and the newly appreciated CRISPR-*cas* systems.[7] Many of these mechanisms, furthermore, are analogous in their functioning to immune system components in animals.[8] One group of phage-resistance mechanisms, abortive infections, are paradoxical as protectors of bacteria, however, because the individual bacteria that effect the actual resistance nonetheless die. That is, abortive infection systems can be viewed, among bacteria, as altruistic traits.[15]

Cellular self-sacrifice is not unusual in terms of resistance to pathogens as effected for example by animal immune systems, such as is seen with cytotoxic T-cell-mediated elimination of virus-infected cells[16] or in the short lifespans of neutrophilic leukocytes.[17] Within the context of bacterial cells that are associated into arrangements or microcolonies, the sacrifice of one cell consistently could possess utility for the sake of eliminating a potential microcolony invader, such as a phage. More generally, this is an argument of inclusive fitness, the idea that genes promote their own propagation rather than solely the propagation of the expressers of the phenotype associated with a gene.[18,19] Bacteria thus could display abortive infections so that clonal bacterial kin, as co-located within cellular arrangements or microcolonies, might live despite phage encounter.[3,6,20] As many abortive infection mechanisms have been discovered in association with lactic-acid bacteria employed in the food industry, particularly *Lactococcus lactis*,[21] one can speculate that the utility of abortive infection systems to these bacteria could be due to spatial structure developed during milk fermentation, in the course of lactation, or simply as these bacteria inherently form into arrangements or microcolonies.

This effect of microcolony survival despite phage encounter can be seen during variations on standard plate-based selection protocols for phage-resistance among bacteria. That is, unless very high phage densities are employed (e.g., > 10^7/ml), then bacterial growth into microcolonies may occur prior to phage-bacterial encounter, resulting in colony survival despite loss of phage-infected bacteria to abortive infections.[22] Phage-microcolony interactions nevertheless are most commonly observed in the laboratory during plaque formation as occurs within bacterial lawns. These bacterial lawns also can be sufficiently similar to bacterial biofilms, such as in terms of bacterial immobility as well as the related formation of microcolonies, that some have suggested their use as surrogates for certain aspects of biofilm study (reviewed in ref. 4).

Toward consideration of the phage-microcolony interactions as they may occur during the formation of phage plaques, and as extrapolated to phage exploitation of biofilms, I define the following terms regarding bacterial presence:

- Microcolony: Closely associated collection of especially clonally related bacteria that is sufficiently small so as to be not readily discernible individually by the naked eye; a highly localized, three-dimensional array of bacteria.
- Biofilm: Substantial grouping of bacteria including as consisting of multiple microcolonies.
- Lawn: Substantial grouping of bacteria including as consisting of multiple microcolonies but lacking the fluid over layer seen with biofilms.
- Biofilm-containing environments: Multiple bacteria-associated surfaces that, across fluid-filled volumes, are potentially in biological communication.

In addition, I consider various ways in which one might differentiate among those phages making up biofilm- or lawn-exploiting phage populations:

• Explorer phages: Phage particles that by chance exit a biofilm, entering the fluid over layer and, as a consequence, potentially diffusing substantial distances to infect new biofilms.

• Scout phages: Phages that by chance are limited in their diffusion to within the plane of a biofilm or bacterial lawn and that, also by chance, infect cells that are present in microcolonies other than the microcolony in which their parental infection was found.

• Settler phages: Phages, otherwise genetically identical to explorer and scout phages, that by chance infect cells present in the same microcolony as their parental infection.

Explicitly, explorer, scout and settler phages are all members of the same phage population. Indeed, as I will suggest, what I describe as explorer phages may, within the context of phage exploitation of biofilms, give rise to settler and scout phages; scout phages, in addition, can give rise to settler phages, and all three phage types can directly parent explorer phages. This perspective, furthermore, is simply a complication on the standard consideration of phage-infected bacteria in broth cultures giving rise to free phages that, in turn, give rise to phage-infected bacteria, with explorer phages serving explicitly as distant-bacteria-acquiring, free-phage equivalents.

The bacterial lawns—within which phage plaques develop—exist as confluences of bacterial microcolonies.[23] Freely diffusing phage particles within these lawns may be more likely to first encounter the exterior of microcolonies, which may be physiologically better equipped to support robust phage infections than microcolony interiors.[24] Upon lysis of infected bacteria, some phage progeny, what I call settler phages, may then penetrate to underlying cells found within the same microcolony. Other phage progeny, also settler phages, may encounter cells along the surface of the same "parental" microcolony. Still other phage progeny may diffuse to adjacent microcolonies (what I here call "near" settler phages) and, indeed, some virions may diffuse to even more distantly located microcolonies that nevertheless are found within the same bacterial lawn or biofilm ("far" settler phages). With phages infecting bacterial biofilms, phage diffusion also can be out of the biofilm entirely, that is, into the overlying fluid—forming what I call explorer phages—and thereby initiating phage dissemination toward distant biofilms.

Phage propagation in association with microcolonies thus could be split between enhancing productivity during exploitation of obtained microcolonies (**Fig. 1**, **Step 2**, "Settler" phages) and enhancing rates of outward dissemination toward new microcolonies that may or may not be immediately locally present (**Fig. 1, Steps 3 and 4**, "Scout" phages). Infections by both phage types produce virions, some fraction of which could disseminate away from parental biofilms entirely (**Fig. 1, Step 5**, "Explorer" phages) and, ideally for the phage's involved, acquire bacteria found in new biofilms and/or new microcolonies (**Fig. 1**, **Step 1**). Explorer phages in particular are potentially able to acquire distantly located microcolonies, effecting a long-distance dissemination step. The more explorer phages that are produced, per focus of phage infection of a bacterial biofilm, then the more likely that new, distantly located microcolonies/biofilms may be acquired by phage populations, that is, by phage populations consisting at various times, and in different locations, of combinations of explorer, scout and settler phages.[3]

Given especially a utility associated with producing greater numbers of virion progeny, then those phages that penetrate further into microcolonies, settler phages, could benefit from being more-complete exploiters of individual microcolonies. At least some phage accessory proteins therefore could exist that serve to enhance this ability. What form or forms might such enhancement take? As I have suggested elsewhere,[3] the T-even phage lysis inhibition phenotype[25–27] could provide just such augmentation. With these phages, infected bacteria that have been adsorbed by additional phages (secondary adsorption) display longer latent periods along with larger burst sizes (lysis inhibition) while other infections, ones that have not been secondary adsorbed, display more-rapid infection turn-around but smaller burst sizes (rapid lysis). Furthermore, it is particularly those bacteria that are infected within regions of high densities of bacterial infections, and resulting high free phage densities, that would be expected to display lysis inhibition.[25]

One can envisage the initial, low multiplicity infection of a grouping of bacteria leading to rapid lysis and thereby relatively rapid subsequent acquisition of neighboring bacteria, dynamics that might be prevalent or important particularly at plaque peripheries during plaque development. Within the immediate vicinity of phage release from infected bacteria, however, densities of free phages may be especially high, such as is seen within the interior of plaques as well as, perhaps, during phage exploitation of individual microcolonies (**Fig. 1**, **Step 2**). It is due to these higher densities of free phages and associated higher multiplicities of phage adsorption that lysis inhibition would be induced.[25–27] The resulting overall enhancement of local phage productivity—where more infections × more phages produced per infection due to lysis inhibition = more phages produced—could have the result, within a biofilm context, of enhancing the production of explorer phages and thereby elevating the likelihood of eventual phage acquisition of bacteria found in distant biofilms.

While this is a description of lysis inhibition as one means by which group productivity may be enhanced, note that from a purely selfish perspective one can view these settler phages as existing within microenvironments in which shorter latent periods, which otherwise could shorten phage generation times, nevertheless would possess little utility due to a local dearth of uninfected, phage-susceptible bacteria. Within the vicinity of lysis-inhibited bacterial infections, the result thus could be "selfish" selection among phages for larger individual burst sizes[25] that, at the same time, has the effect of enhancing group productivity. For additional consideration of lysis inhibition from the perspective of phage selfishness, see reference 28.

This lysis inhibition "thought experiment" helps to illustrate the inherent conflict that many phages could experience during their exploitation of bacteria within biofilms. The conflict, in particular, is between optimizing the rapidity

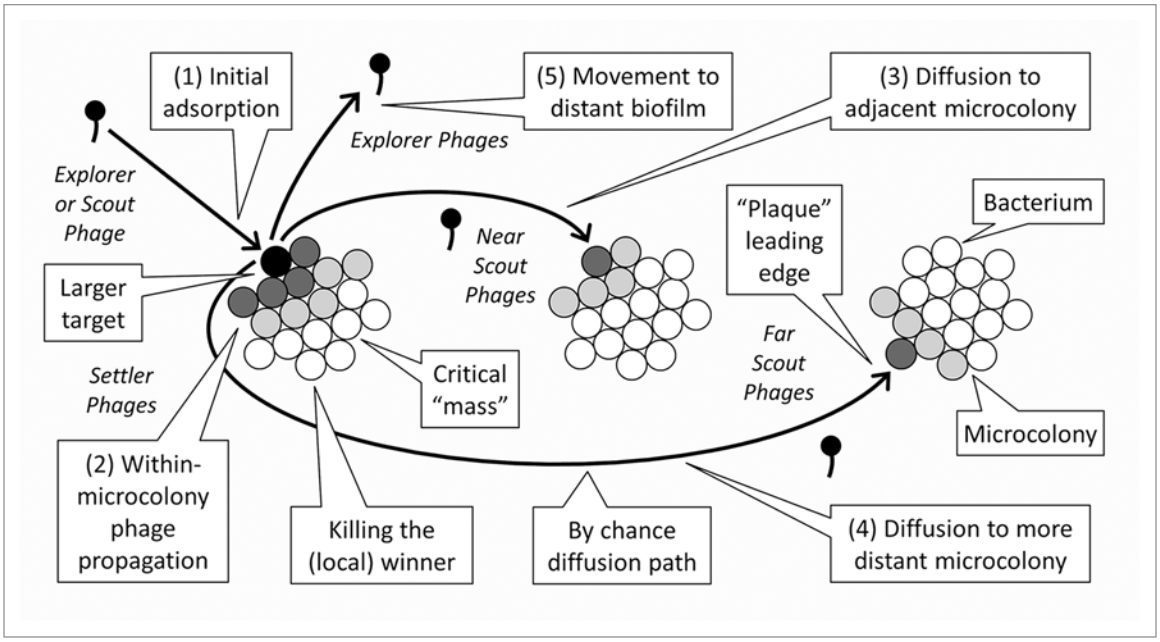

Figure 1. Phage propagation within as well as between spatially associated and well separated microcolonies. (**1**) The initial microcolony adsorption, by what can be described as either explorer or scout phages, may preferentially occur at microcolony peripheries, which is where bacteria also may be physiologically best able to support robust phage infections. (**2**) Penetration into and subsequent exploitation of individual microcolonies, by what are indicated as settler phages in the figure, could select for more effective microcolony exploitation particularly toward burst size enhancement of cells making up individual microcolonies. (**3**) Acquisition of adjacent microcolonies is mediated by what are indicated as near scout phages, phages that by chance diffuse away from the parental microcolony and toward nearby microcolonies. (**4**) Diffusion to more distant microcolonies found within the plane of the same biofilm or bacterial lawn may also occur, as mediated in the figure by far scout phages, and this occurs either by chance or instead if virions delay initiation of their adsorption abilities following release from parental infections. Regardless of the mechanism, such phages—simply by diffusing farther—would help to define a plaque's leading edge. (**5**) Movement to more distant microcolonies and biofilms is achieved by what are labeled as explorer phages in the figure, that is, phages that by chance happen to diffuse out of biofilms into the overlying fluid. Robust phage production, perhaps particularly by settler phages, and also subsequent virion durability could be crucial to phage acquisition of these other biofilms if they are quite distant and/or if the potential for virion survival during transit is otherwise relatively low. All phage movement away from the indicated infected bacterium—black arrows pointing away from the black circle—is post lysis. Subsequently acquired bacteria are found in increasingly lighter shades of gray. Settler, scout and explorer designations are further discussed in ref. 3 as well as the main text.

of local bacterial acquisition, such as via shorter phage latent periods, and producing larger numbers of phage progeny through greater burst sizes,[29,30] with both mechanisms contributing in different ways toward the overall production of explorer phages. Such enhancement especially of local productivity could be useful to the extent that a phage's ecology consists predominantly of occasional exploitation of rich "veins" of biofilm-associated microcolonies (**Fig. 1**, **Steps 2–4**) that alternates with long, dangerous journeys (**Fig. 1**, **Step 5**) by virions toward distant microcolonies to infect (**Fig. 1**, **Step 1**).

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