

PERSPECTIVE OPEN

Piggyback-the-Winner in host-associated microbial communities

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Phages can exploit their bacterial hosts by lytic infection, when many viral particles are released at cell lysis, or by lysogeny, when phages integrate into the host's genome. We recently proposed a new dynamic model of bacteria–phage interactions in which lysogeny predominates at high microbial abundance and growth rates. This model, named Piggyback-the-Winner (PtW), contrasts to current accepted models on the frequency of lysis and lysogeny and predicts that phages integrate into their hosts' genomes as prophages when microbial abundances and growth rates are high. According to PtW, switching to the temperate life cycle reduces phage predation control on bacterial abundance and confers superinfection exclusion, preventing that a closely-related phage infects the same bacterial cell. Here we examine how PtW is important for metazoans. Specifically, we postulate that PtW and the recently described bacteriophage adherence to mucus (BAM) model are strongly interrelated and have an important role in the development of the microbiome. In BAM, phage produced by the microbiome attach to mucins and protect underlying epithelial cells from invading bacteria. Spatial structuring of the mucus creates a gradient of phage replication strategies consistent with PtW. We predict that lysogeny is favored at the top mucosal layer and lytic predation predominates in the bacteria-sparse intermediary layers. The lysogeny confers competitive advantage to commensals against niche invasion and the lytic infection eliminates potential pathogens from deeper mucus layers.

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INTRODUCTION

Lytic and temperate phage infections have markedly different outcomes for the bacterial host. In the former, phage act as predators and control bacterial abundance via cell lysis. Lytic predation has been modelled according to classical predator–prey Kill-the-Winner dynamics, in which a fast-growing bacterial strain is killed by its phage until another strain rises, leading to control of bacterial community abundance.¹ In contrast, during a temperate infection, the phage integrates into the host chromosome, replicating with their host as a prophage (some phages can replicate as plasmids). Prophage protect the host cell from infection by closely related phage via superinfection exclusion, a process in which various proteins block other phage from establishing a productive infection. Prophage also confer fitness improvement via lysogenic conversion, i.e., prophage expresses genes that alter the lysogen's physiology.² The lytic to lysogenic switch has been characterised in a small number of host-phage models including *E. coli* and lambda phage, however, the factors determining the fate of phage infections in complex microbial communities remains poorly understood.

The oceans are currently the most extensively explored biome in regards to phage–bacteria interactions.³ Studies based on prophage induction by DNA-damaging agents suggest that lytic infections are more common in high nutrient availability conditions, whereas lysogeny is more frequent in oligotrophic conditions.^{4,5} Recently, using a combination of approaches including the analysis of bacterial and viral counts, metagenomics and experimental manipulation of host growth rates we demonstrated that lysogeny is in fact a successful strategy when bacteria are in high abundance and growing rapidly.⁶ Host availability and metabolic status are important factors that determine whether

phage infection occurs via lytic dynamics or lysogeny. Coral reefs worldwide are currently experiencing microbialization, a phenomenon defined as the increased abundance and energetic demands of microbes in relation to that of macrobes (e.g., fish) as a result of anthropogenic disturbance.^{7,8} Therefore, coral reefs represent an exceptionally useful natural system for the study of lysis/lysogeny decisions in complex microbial communities. We observed a negative relationship between the virus-to-microbe ratio (VMR) and bacterial abundance in coral reefs across the Pacific and Atlantic Oceans spanning a gradient of human impact, showing that at high bacterial abundances there were relatively fewer phage. High VMR is observed when lytic predation is high, thus the negative relationship we observed was the first indication of a decreased importance of lysis in high bacterial abundance environments. Metagenomic analysis showed that the rise of resistant bacterial strains does not explain the decrease in lytic production, a mechanism proposed based on Red Queen co-evolutionary dynamics.⁹ Instead, viral metagenomes isolated when bacterial abundance was high showed an increased representation of integrase and excisionase genes, hallmarks of temperate phage, along with prophage-like sequences, demonstrating that lysogeny is more predominant at high bacterial abundances. By experimentally manipulating bacterial community growth rates in laboratory conditions, we demonstrated that high bacterial growth is the driver of the switch to lysogeny. This transition was later corroborated by the analysis of 2110 bacterial genomes, showing that minimal doubling time is the life-history trait mostly correlated with the frequency of prophages.¹⁰

On coral reefs, phage lysogenic switches enhance ecosystem microbialisation modifying microbial interactions in the food web.¹¹ In this scenario, phage control of bacterial abundance

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through lytic infection is impaired, releasing phage predation control of bacteria. Added to the growth stimulus of high dissolved organic carbon release from algae in degraded reefs, PtW dynamics contributes to increased bacterial biomass and energetic requirements.⁷ As water-column microbes are implicated in particulate matter formation and benthic pelagic coupling, microbialization increases the flux of carbon back to the benthic community, reshaping trophic relationships.^{11,12}

HOW DOES PIGGYBACK-THE-WINNER WORK IN MUCOSAL SURFACES?

Metazoans harbour complex microbial communities that function together forming holobionts.¹³ Mucosal surfaces are hotspots of microbial colonisation in metazoan hosts such as in coral mucus and the human gut and lungs.^{14,15} Microbial abundance in coral mucus can be as high as 1.8×10^6 cells per ml, which is almost an order of magnitude higher than the overlying water.¹⁶ Phages are diverse and abundant members of metazoan mucosal communities^{17,18} and exhibit shifts in community composition during environmental perturbations or the rise of diseased states in their host.^{19,20} Few studies have tried to determine the abundance of phages in mucosal surfaces. Barr *et al.*²¹ showed that VMR is in average four times higher in metazoan-associated mucosal surfaces when compared with the surrounding environment. Phages can directly interact with metazoan mucins through the expression of proteins with immunoglobulin-like domain on their capsids.²¹ By attaching to mucins, phage display sub-diffusive hunting behaviour (i.e., move slower than would be expected by diffusion), enhancing their abilities to find, infect and kill bacteria in the mucus environment by increasing the rate of phage–bacteria encounters.²² This model of bacteria–phage interaction in holobionts is called bacteriophage adherence to mucus (BAM) and highlights the existence of a phage-mediated form of immunity.²³ High rates of lytic infection in bacteria-rich mucosal surfaces contrast to predictions of PtW, since lysis is expected to be reduced in these conditions. Metagenomic analysis of mucosal viral communities from the human gut and lungs show these communities have strong lysogenic signatures.^{24,25} It is estimated that up to 50% of viral particles in the human gut are temperate, though the BAM model lacks

predictions about the role of lysogeny in mucus. Although lysogenic infections may fit into the BAM model as a form of memory against invasive bacterial strains, high microbial abundances and VMR in these environments makes it challenging to reconcile these two well-characterised phage–bacteria interaction dynamics in holobionts. While it is known that these two dynamics occur, their conditions and outcomes are remarkably different. Here we reconcile the two models based on mucus spatial structure.

SPATIAL STRUCTURE DETERMINES LYSIS/LYSOGENY SWITCHES IN HOLOBIONTS

Metazoan mucosal surfaces display well-defined spatial organisation. Constant secretion of mucin molecules by epithelial cells creates a mucin concentration gradient, with high concentrations of mucins in the inner layers close to the epithelium and lower concentrations in the outer layers, where mucus is continually sloughed out.²⁶ The dynamic nature and tridimensional complexity of mucus are reflected in gradients of microbial colonisation found in vertical profiles of mucus layers. Bacterial abundance is higher in the outer layer, whereas the inner layer is virtually deprived of bacterial cells.^{27,28} As a result, bacterial densities display the opposite gradient of that observed for mucins. Phage distribution in mucosal surfaces is predicted to be positively correlated with mucin concentration in outer and mid mucus layers, given the direct interaction between phage capsids and mucin molecules, and then decreases closer to the epithelium.^{21,22} Thus, VMR is higher at intermediate mucin concentration found in the middle of the mucus layer (Figure 1).

In order to make predictions about where lysis and lysogeny will be favoured in the tridimensional structure of the mucus, it is also important to consider the distribution of bacterial growth rates in the mucosal layers. There is evidence that bacterial growth rates are higher in top layers of biofilm, where we also find higher bacterial abundance.^{29,30} In contrast to free-living communities, nutrients are not a limiting factor in the rich-nutrient environment of metazoan mucus. In internal mucosal surfaces, such as human lungs and gut, bacterial growth rates in deeper mucosal layers are primarily limited by oxygen availability, similar to tridimensional profiles of biofilm communities growing on non-biological

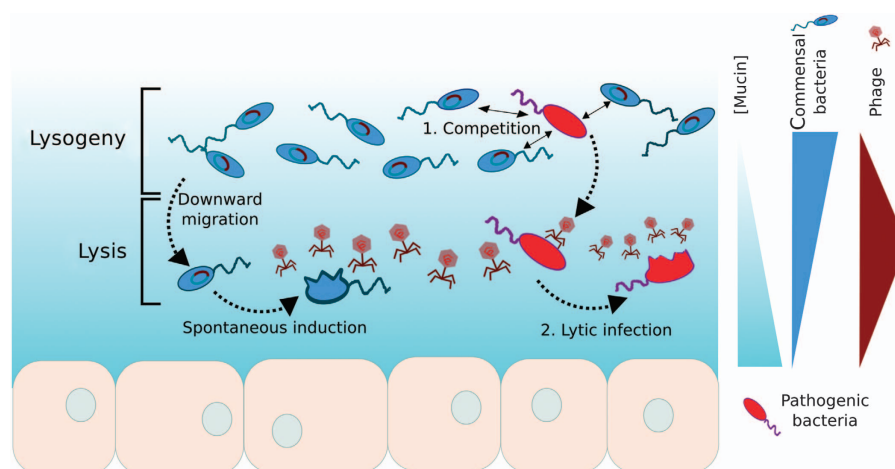


Figure 1. Spatially structured lytic to lysogenic switches in microbial communities associated to metazoan mucosal surfaces. The bottom nucleated cell layer represents the metazoan host epithelium. A mucin concentration gradient is formed from the epithelium to the top. Commensal bacteria abundance (blue flagellated cells) is higher at the top layer where mucin concentration is low, whereas phage abundance is higher at intermediate layers as a result to direct adherence to mucus. High bacterial abundance and growth at the top layers favours lysogeny, while lysis is favoured at the intermediate layer. An invading pathogen (red flagellated cell) encounter two steps of phage-mediated immunity: (1) competition with commensal lysogens displaying high fitness resulting from the integrated phage genome; (2) lytic infection at intermediate layers. An invasive lysogen containing a prophage that confers high fitness gain could outcompete commensals and escape lytic infection by maintaining high growth rates, leading to the establishment of infection.

surfaces.^{31,32} Independent of electron acceptor availability, dehydration of deep mucosal layers because of high mucin concentration is a strong growth limitation, making this area virtually microbial-free.^{29,33,34} High mucin concentration creates nanoscale pores and increases mucus viscoelasticity, impairing bacterial penetration and growth.³⁵ In these conditions, bacteria tend to form aggregates on the top layers the mucosal surface and the resulting gradient in bacterial growth rate profiles is similar to that of bacterial abundance (Figure 1).

Piggyback-the-Winner dynamics predict that lysogeny will be favoured in the top of the mucus layer, where bacterial abundance and growth rates are higher. Therefore, most of the commensal bacteria would host a prophage in their genomes and be protected against superinfection.² As a lysogenic bacteria moves downward in the mucin gradient and approaches the epithelium, bacterial growth rates and density decreases, favouring prophage induction and cell burst. Cell lysis and release of phage progeny in turn contributes to the maintenance of high VMR in intermediary mucin layers. From this dynamic emerges a spatial organisation of phage replication strategies in which lysogeny is dominant on the top mucus layers while lysis occurs mainly at the intermediary mucus layers (Figure 1). This model of lysis/lysogeny switch reconciles the two recently proposed mechanisms of virus–bacteria interaction in holobionts.

By situating phage replication strategies in the complex tridimensional structures of mucosal surfaces we are able to further develop the proposed phage-mediated immunity mechanism.²³ An invasive bacterium arriving to a host-associated microbial community would face two consecutive steps of protection by the commensal community: the first is the competition with the commensal microbes for resources at the top layer, where commensal bacteria fitness is high as a result of high rates of lateral gene transfer by transduction in phage integration. If this invasive bacterium passes this first line of defense, then high phage abundance at the deeper layers of the mucin gradient will increase the chance of viral infection. Assuming this microbe has no previous immunity to the commensal phage strains and infection occurs, growth conditions at the deeper mucin layer will favour lytic infection, eliminating the invasive bacterium. Two levels of phage-mediated immunity arise from this model: (1) widespread lysogeny at the top mucus layers protects commensal microbes from superinfection exclusion and increases their fitness; and (2) phage infection eliminates the invasive pathogens in deeper mucus through lysis, protecting the metazoan host against invasive pathogenic bacteria.

LYSOGENY AND THE CO-EVOLUTION OF BACTERIA AND PHAGE IN MUCOSAL SURFACES

Although integration of viral genome into the bacterial chromosome has beneficial effects for the host through superinfection exclusion and lateral gene transfer, it is also argued that it represents an extra energetic expense, reducing host fitness.³⁶ Energetic costs resulting from resistance to infection through CRISPRs and mutation of membrane receptors lead to a trade-off between competition and resistance.³⁷ In models of virus–bacteria interaction this trade-off predicts that resistant bacterial strains display lower growth rates compared with sensitive ones.^{9,38} However, in nutrient-rich conditions, such as the mucosal surface environment, energetic costs of carrying a prophage would be reduced or insignificant, and host fitness can actually be improved through acquisition of useful genes.^{2,39} Superinfection exclusion is also a more efficient mechanism for escaping lysis than resistance through mutation, thereby avoiding Muller ratchet-like declines in fitness.⁴⁰ Red Queen dynamics predicts that antagonistic evolution of lytic phages and bacteria increases molecular evolution in both predator and prey.⁴¹ In a PtW scenario, gain of genomic islands formed by prophages would be a more important

diversification mechanism than the molecular evolution of CRISPRs elements, membrane receptors and competence genes.^{41,42}

In the spatially structured environment of mucosal surfaces, high lytic activity in intermediary layers, where phage display sub-diffusive motion increasing chances of host encounter, is predicted to increase diversification through antagonistic evolutionary mechanisms.^{22,43} Conversely, the main diversification mechanisms in the outer layers, where lysogeny is more prevalent, are lateral gene transfers and genomic islands created by phage integration.² In contrast to homogenous environments, spatial gradients in lysis and lysogeny in mucosal surfaces are predicted to contribute to maintenance of steady-state community assembly through spatial refuges and coexistence stabilization.^{44,45}

IMPLICATIONS OF PIGGYBACK-THE-WINNER FOR POLYMICROBIAL DISEASES

Many metazoan diseases are characterised by the colonisation of mucosal surfaces by complex and dense microbial communities that differ from the healthy microbiome.^{46,47} Cystic fibrosis (CF) is a the genetic disease caused by mutations in an ion chloride channel that leads to cationic influx and dehydration, compromising the ability of cells to clear (i.e., remove) mucus.^{48,49} In CF lungs, the thick, persistent mucus layer facilitates chronic microbial colonisation that have been extensively studied and will be explored here as a model for PtW dynamics in polymicrobial infections.^{50–52} Viruses are abundant members of the CF microbial community and encode genes related to clinically important microbial phenotypes, including small colony variants and antibiotic resistance.^{53,54} High microbial abundances and growth rates in CF mucus are predicted to stimulate PtW dynamics. In extreme, advanced CF states phages can be virtually absent from microbial biofilms, indicating bacterial escape from top–down control through lysogeny.⁵³

In chronic infections, PtW dynamics have the potential to benefit the long-term opportunistic pathogens instead of commensal microbes, representing a threat for the human host. The relative abundance of free temperate phages capable of lysogenic conversion of the liverpool epidemic strain of *Pseudomonas aeruginosa*, a common CF opportunistic pathogen, decreases with increasing *P. aeruginosa* abundance in the lung.⁵⁵ This negative relationship observed though quantitative PCR targeting specific phage strains is identical to that observed for whole community analysis in marine environments, soils, freshwater and so on.⁶ VRM reduction during *P. aeruginosa* blooms indicates higher integration rates during periods of high bacterial growth rates, consistent with PtW dynamics. From these observations, we predict that increased lateral gene transfer of virulence factors and antibiotic resistance genes resulting from high integration rates will enhance pathogen fitness. Lysogenic conversion of liverpool epidemic strain *P. aeruginosa* by PAO1 phages results in high *P. aeruginosa* competitive advantage in CF mouse model, and transposon mutagenesis of liverpool epidemic strain prophages reduces bacterial competitiveness, corroborating our predictions.^{56,57}

The dual role of PtW in mucosal surfaces, either creating a phage-mediated immune mechanism or enhancing disease states, as in CF, will be determined by initial mucosal colonisation and interference by external factors such as administration of antibiotics. Invasion of commensal communities by a pathogen harbouring a prophage with a large host spectrum would facilitate pathogenic take-over by (a) increasing the fitness and competitive advantage of the pathogen over resident microbes, allowing the pathogen to surpass the first level of immunity in Figure 1, and (b) killing through lytic infection the commensal bacteria that display lower growth rates compared with the pathogen. CF lung colonisation by opportunistic pathogens occur in early ages, when

patients are often receiving antibiotics.⁵⁸ These drugs suppress the resident microbial community, opening niches for pathogenic lysogens and leading to the establishment of their chronic infections.⁵⁹ *Staphylococcus aureus* infections have a pivotal role in early stages of CF lung injury, which lead to the proposal of prevention of *S. aureus* infection in children.⁶⁰ Although anti-staphylococcal prophylaxis in infants and young children with CF suppresses *S. aureus* colonisation, it increases the likelihood of *P. aeruginosa* infection over the course of a 5 to 7 years treatment.⁶¹ Early-life antibiotics treatment sets the stage for the long-term establishment of opportunistic pathogens carrying prophages encoding antibiotic resistance and virulence genes.⁶² Changes in antibiotics treatment and the addition of multiple drugs over the patient's life span have the potential to trigger expression of virulence factors and lead to CF exacerbation events.^{63,64}

FUTURE DIRECTIONS

Various culture-based and metagenomics studies cited here provide strong evidence for our model of PtW dynamics in spatially structured environments such as mucosal surfaces. However, further studies that test this model experimentally are necessary in order to fully understand its underlying controls and provide tools for how it may be applied in clinical settings. The first question to be answered is the profile of bacterial growth rates on mucus gradient, and if the growth rate profiles are consistent in various model systems. The use of single-cell resolution confocal microscopy, as applied before on *Vibrio cholera* biofilms, can show the architectural separation of bacterial growth, helping to understand growth rate constraints in mucosal surfaces⁶⁶. The next fundamental step is to quantify the frequency of lysogenic cells across the mucosal surface. To avoid the well-known bias of prophage induction assays, the application of high resolution microscopy and genome sequencing are the most advanced approaches available. The use of optical tweezers to isolate individual cells followed by large scale genome sequencing of single cells can provide reliable information on the frequency of lysogeny across mucus layers and the diversity of integrated prophages.^{67,68}

An intriguing aspect arising from the interplay between PtW and BAM is the dual role of PtW in shaping the healthy microbiome and, alternatively, facilitating the establishment of polymicrobial diseases. To understand these processes, the application of microfluidic devices can be scaled up to complex communities and assays with engineered lytic and temperate phages, shedding light on the success rates of lytic and lysogenic infections²². A microfluidic flow device applied to the study of coral micropropagates, called coral-on-a-chip, could allow researchers to track the onset of microbial colonization, fitness gain, and lateral gene transfer on coral mucosal surfaces⁶⁵. Finally, the role of PtW and BAM in the context of disease states will need to be addressed with the use of *in vivo* models. High-throughput quantification of microbial organization in the gut of gnotobiotic and human microbiota-colonized mice recently demonstrated that diet alters mucus thickness in the distal colon, increasing proximity of microbes to the epithelium²⁷. Immunofluorescence tracking of bacteria and phage using this pipeline can improve understanding of the effect of bacterial migration through the mucus framework on phage integration and induction.

CONCLUDING REMARKS

Here we reconcile two recently proposed models of bacteria-phage dynamics, PtW and BAM in host-associated microbiomes. We propose a spatially defined model where PtW is more prevalent in outer layers of mucosal surfaces where microbial abundance and growth rates are high. In intermediate layers, high virus-to-microbe ratios facilitated by BAM and phage sub-diffusive

motion favour lytic dynamics. A two-level phage-mediated immunity mechanism arises from this model: (1) bacterial lysogens at the top mucus layers have a competitive advantage against invasive pathogens; and (2) a pathogen that reaches deeper layers is attacked by lytic infection. Invasion by a lysogenic pathogen could, on the contrary, benefit the pathogen, enabling the onset of persistent infections.

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CONTRIBUTIONS

C.B.S. and F.L.R. conceptualised the model and wrote the paper.

COMPETING INTERESTS

The authors declare no conflict of interest.

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