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On the interactions between virulent bacteriophages and bacteria in the gut

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We recently described the targeting of O104:H4 *Escherichia coli* in mouse gut by several virulent bacteriophages, highlighting several issues relating to virus-host interactions, which we discuss further in this addendum to the original publication.

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Background

Recent metagenomic studies have confirmed that the digestive tract is a complex environment in which bacteria and bacteriophages coexist.[1] However, little is known about the nature of the interactions between these two antagonist populations in this very particular environment. Given the constant exposure to bacteriophages resulting from food consumption, the overall effect of bacteriophages in the human gut might be thought to be negligible as, to our knowledge, no effect of bacteriophages has ever been reported. However, temperate bacteriophages have long been known to play an important role in the transfer of genetic information between hosts, and it has been shown that, as expected, such transfer can occur in vivo in mouse gut.[2] These findings confirmed that prophages, like other mobile genetic elements, participate in genomic shuffling within the gut microflora.[3–5] However, what do we know about the impact of virulent bacteriophages on the gut micobiota? Evidence for an active role of virulent bacteriophages dates back to the first paper to coin the term "bacteriophage," published by Félix d'Herelle, who isolated the bacteriophages from patients recovering from dysentery and suggested that these organisms played an active role in disease recovery. This observation formed the cornerstone of a new field, phage therapy, which is now returning to the fore as a possible solution for treating bacterial infections due to multidrug-resistant pathogens.[6,7] Several studies have investigated the use of virulent bacteriophages to treat intestinal infections.[8,9] Possibly the most surprising result of these studies was the finding that no common pattern could be deciphered for either bacteria or bacteriophages. Differences in bacteria, bacteriophages and colonization or infection models may account for this, but this situation contrasts strongly with observations in other animal models of infection (in the skin, lungs, etc.) for which different bacteria and bacteriophages have also been evaluated but for which highly effective curative action has frequently been reported.[10] It is therefore possible that interactions between bacteriophages and bacteria are much more complex in the gut than in other organs. Our recent article provides a detailed illustration of this view, and goes one step further, by investigating the limiting factors for these virus-host interactions in the intestine.[11]

The Bacteriophages

We isolated three bacteriophages, each belonging to one of the three families of the Caudovirales order, from sewage. These bacteriophages (CLB_P1, CLB_P2 and CLB_P3) were individually

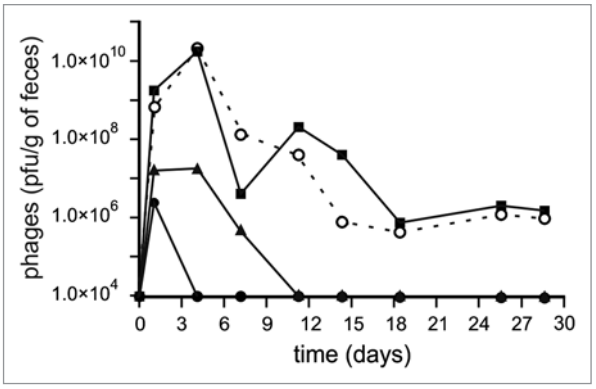

Figure 1. Changes in fecal bacteriophage concentration over time in mice colonized with the O104:H4 55989 *E. coli* strain. Four groups of 4 to 5 mice were colonized with *E. coli* 55989Str on day 0. Three days later, they received normal drinking water or drinking water containing one of the bacteriophages studied (closed circles, CLB_P1; squares, CLB_P2; triangles, CLB_P3) or a cocktail of the three bacteriophages (dashed line) at a concentration of 1×10^5 pfu/ml each. On day 30, 20 individual colonies from feces were individually assessed for sensitivity to bacteriophages. The concentration of bacteria in the feces over the 30-d period was stable at about 1×10^8 cfu/ml.

characterized in vitro on planktonic cultures and on biofilms.[12] The T4-like bacteriophage CLB_P2 had the best characteristics in vitro, in terms of the lack of bacteriophage-resistant clones in planktonic conditions and efficacy on biofilms. However, a cocktail of the three bacteriophages was more effective against biofilms than individual bacteriophages, suggesting a synergistic effect potentially due to the combined action of multiple cell wall-degrading enzymes or a capacity to infect multiple physiological stages of the bacterium within the biofilm. Surprisingly, CLB_P1, a T7-like bacteriophage, was the least efficient at reducing biofilms, despite the bacteriophage T7 having previously been identified as a good candidate for therapeutic applications on the basis of in vivo experiments.[13] It strongly indicated that given the high genomic variability of bacteriophages generalization of their in vivo efficacy cannot be drawn only from bacteriophage morphology/classification. Finally CLB_P3, a close relative of bacteriophage T1, is, to our knowledge, the first T1-like bacteriophage ever to be tested in an in vivo model.

The Bacteria

A colonization model based on the enteroaggregative *E. coli* strain 55989 was the starting point of our study. This serotype O104:H4 strain was initially isolated from an AIDS patient suffering from diarrhea. A very closely related clone of the 55989 strain recently acquired a prophage encoding the Shiga toxin stx2, leading to the emergence of highly virulent O104:H4 *stx2*+ clones, which caused an outbreak of diarrhea and hemolytic uremic syndrome affecting 4000 people in Western Europe in June 2011.[14] Interestingly, CLB_P1 and CLB_P2 but not CLB_P3 bacteriophages isolated with 55989 strain also infected efficiently several of these epidemic clones.[12]

The Animal Model

E. coli strains are not normally considered to be resident in the mouse intestinal microbiota, although there have been sporadic reports of their presence.[9,15] We used an intestinal carriage model in which high levels of *E. coli* are maintained over several weeks through the addition of an antibiotic to drinking water. This antibiotic eliminates part of the natural flora, allowing stable colonization by *E. coli*. In the absence of antibiotic pressure, *E. coli* levels decrease over the course of a few hours to a few days, depending on the strain used.[16]

Histological analyses of the gut tissues of mice after three days of colonization with strain 55989 revealed no damage, demonstrating that this model mimics, in both the small and large intestines, the state of asymptomatic carriage found in humans for enteroaggregative *E. coli*.

The Interactions of Bacteriophages and Bacteria in Animals

Contact between virulent bacteriophages and their bacterial hosts triggers an "arms race" between the two species. It has been reported, in both in vitro and field studies, that bacteriophage-resistant mutants emerge, at various rates, and that variants of the bacteriophages subsequently infect these new clones.[17,18] However, no such long-term study has yet been performed in the gut environment. Our model, involving the addition of bacteriophages to the drinking water of colonized mice for a period of 24 h, leading to the continuous replication of bacteriophages within the gut over a period of several weeks, provides us with an opportunity to address such questions in vivo, within the digestive tract. No bacteriophage resistant mutants were observed after three weeks in mice treated with the cocktail of three bacteriophages.[12] We also analyzed the three bacteriophages individually: CLB_P1 was rapidly cleared (< 4 d), CLB_P3 persisted for more than twice as long (< 11 d) and CLB_P2 persisted in the gut for at least 30 d without selecting for the growth of predominantly bacteriophage-resistant mutants (**Fig. 1**). The absence of such mutants in the gut was surprising, as we know that bacteriophages infect bacteria in this organ, exerting a selective pressure on their hosts that should lead to the selection of such mutants. However, the gut environment may not be favorable to the selection of bacteriophage-resistant mutants, as such mutants may have no selective advantage and may be rapidly outcompeted by the wild-type strain. This would result in the mutants being eliminated from the gut as soon as they appeared.

Several hypotheses may be put forward to explain why the infection of bacteria with bacteriophages did not lead to the growth of resistant bacterial clones. First, random collisions between bacteriophages and bacteria can be artificially increased in test tubes, by shaking, whereas such collisions are probably much less frequent in vivo. Nevertheless, we showed that a dose-dependent decrease in the number of bacteria in the gut could be obtained in vivo, suggesting that the vast majority

(over 90%) of bacteria could be infected by bacteriophages. However, this was not enough to trigger the complete elimination of the bacterial host, suggesting that some bacteria may be physically protected against bacteriophage infection. Such protection might result from the localization of bacteria in particular sites inaccessible to bacteriophages, such as crypts or villi. This population never coming into contact with bacteriophages may be the source of the wild-type bacteria responsible for the renewal of the sensitive bacterial population. In contrast to bacteriophages that rely on passive diffusion to move from one site to another, bacteria actively move within the intestine and colonize inaccessible niches giving them the possibility to escape bacteriophage predation.[19] Alternatively, the bacteria may be physiologically protected against bacteriophage infection. Our ex vivo experiments on homogenized colonized gut samples revealed that, in this case, the bacteria were less permissive to bacteriophage infection than bacteria grown in LB and subsequently added to homogenized non colonized gut samples. This was true for bacterial cells isolated from the mouse colon, but not from the ileum, reflecting the removal of *E. coli* by bacteriophages in the ileum. The metabolism of bacterial cells clearly differs between the small and the large intestine, due mainly to differences in nutrient availability, and we confirmed in vitro that this *E. coli* strain had a lower permissivity to bacteriophage infection in stationary phase than in the exponential growth phase. The antibiotic used to maintain the level of bacterial colonization in the gut may counteract the effect of the bacteriophages. It is possible that the selection pressure imposed by the antibiotic favors colonization with this *E. coli* strain not only directly, due to its resistance, but also indirectly due to a lack of metabolic competitors eliminated by the antibiotic.

Overall, the low permissivity to bacteriophage infection and lack of bacteriophage-resistant mutants are consistent with the hypothesis put forward by Reyes et al. that predator-prey relationships are scarce in the intestine.[2] These authors suggested that the low coevolution dynamics in the gut might be due to the predominance of prophages in this organ. Our work suggests that bacteria with a low permissivity to virulent bacteriophage infections may also play a role in this phenomenon. There may be several reasons for the low permissivity to infection observed. For example, the localization or expression of the bacteriophage receptor at the cell surface may be modified in the gut. This has been shown for bacteriophages infecting the intestinal pathogen *Vibrio cholereae*, as the pattern of expression of the CTXφ and JF9 bacteriophage receptors, TCP and CRP, respectively, is modified by environmental conditions along the length of the intestine.[20–22] Phase variation has also been implicated in bacteriophage receptor availability at the cell surface.[23]

In addition to host responses, the viral cycle in the gut environment may itself be affected. As suggested by several authors, pseudolysogeny may play a significant role in virus-host interactions in specific environmental conditions.[24,25] Pseudolysogeny is characterized by a "pause" in the classical lytic cycle. The viral genome is maintained in the bacterial cytoplasm in a plasmid-like form until favorable conditions for the resumption of the lytic cycle occur. Unlike lysogenic phages, pseudolysogenic phages do not integrate into the bacterial chromosome.[26] Pseudolysogeny seems to be entirely dependent on host physiology, as no viral gene has yet been shown to be involved. This hypothesis is supported by our findings that the infection of *E. coli* in vivo in the intestine could not be inhibited (with DNA damaging agents, bacteriophage washout and phage-receptor interaction competitor agents) whereas such inhibition was possible in vitro when the strain was grown in LB. Indeed, the presence of pseudolysogens in the bacterial cytoplasm, where they have access to the host DNA repair system, may enable them to survive dsDNA breaks (caused by damaging agents). Moreover, decreasing extracellular bacteriophage concentration, either by washout or through the use of competitor agents, would not change the fate of host cells harboring these viral pseudolysogens, as they would by lysed when favorable conditions for lytic cycle resumption were encountered, such as during the plating of samples in Petri dishes. This led us to develop a molecular approach (qPCR) to quantifying bacteria from samples containing excess bacteriophages (note that a DNase treatment step was added prior to DNA extraction to eliminate DNA originating from lysed cells). This excess may have been reached because the bacteria contained pseudolysogens, making it difficult to obtain accurate counts by direct plating methods. This suggests that some published studies may have overestimated bacteriophage efficacy, due to the absence of bacterial colonies on plates for samples containing bacteriophages.

The potential role of pseudolysogeny in the maintenance of bacteriophages in the gut ecosystem raises many questions about interactions of bacteriophages and bacteria in animals. If bacterial host cells can divide while harboring a pseudolysogen, as suggested by Los et al., it would be interesting to determine whether the pseudolysogen persists only in the parental cell or whether it is transmitted to the daughter cells.[27] If such transmission occurs, does pseudolysogen replication involve host factors only, or is a subset of viral proteins involved? If viral genes are expressed during the pseudolysogenic stage, do they confer a selective advantage on the bacterial host, leading to pseudolysogen maintenance? Conversely, if the presence of the pseudolysogen is a disadvantage how does the host cell eliminate it? This might result in abortive infections, which are thought to play a role in CRISPR evolution, by providing viral spacer sequences.[28] During pseudolysogeny, allelic exchanges might also occur between pseudolysogens and bacterial genomes via homologous recombination. Extending this hypothesis to the level of the intestinal microbiota highlights the potential role of pseudolysogeny in horizontal gene transfer, which may contribute to microbiota dynamics. Undoubtedly, experimental assessments of the role of pseudolysogeny should now be further developed.

The Lessons to be Learned

First, our results highlight the need to develop molecular approaches for the accurate quantification of bacteria. Even the simplest determination of bacterial counts on Petri dishes was less simple

than initially anticipated, revealing unexpected facets to the gut environment. As in all scientific disciplines, reproducibility is essential for appropriate comparisons and the techniques used must therefore be reliable. The development of molecular approaches, as demonstrated by the modest efforts made in our study, would certainly increase the reliability of results.

Second, we provided evidence that not all bacteriophages active in vitro performed as well in vivo. This will undoubtedly lead to future studies, but it raises the question of how to select the best bacteriophages for therapeutic applications? Our results clearly indicate that CLB_P2 was the most suitable of the three bacteriophages tested for therapeutic usage, but the molecular properties underlying the better performance of this bacteriophage remain to be determined. We found that activities in vitro on biofilms and in vivo in animals were well correlated. For this particular model, the use of in vitro biofilms before in vivo testing might improve predictions of bacteriophage efficacy. However, other in vitro models may be required to mimic different settings in vivo. As it will not be possible to test all therapeutic candidates in a pertinent in vivo system on a large scale, the search for molecular determinants involved in virus-host interactions in vivo remains essential. Such determinants common to all bacteriophages are not likely to be identified however, we may expect some of them to be conserved among genetically closely related bacteriophages.

In recent years, microbiology has moved from single-celled model organisms to more complex environmental systems (e.g., lakes, oceans and sites of infection), but we need to bear in mind that bacteria do not live alone, there are always bacteriophages around… and that is as good as it gets. Many aspects remain to be elucidated to increase our understanding of the interactions between bacteriophages and bacteria in the gut. Studies of these aspects will improve our ecological understanding of this complex environment, potentially enhancing the use of bacteriophages to treat gut infections, which is dependent on such knowledge but also on academic and industrial supports as recently highlighted by Harald Brüssow.[29] However, so far laboratory studies focused on the tip of an iceberg, the submerged part corresponding to what is likely to be discovered in studying bacteriophages in a relevant ecological niche. In conclusion, even when taking everything we know about interactions between the well-known bacterium *E. coli* and its bacteriophages into account, attempts to study these interactions in the digestive tract, the real environment of *E. coli*, are likely to modify our current knowledge of these interactions, reflecting how much we still have to learn about the gut microbiome and the complex interactions among its components.

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