

Deciphering in vivo efficacy of virulent phages in the mammalian gut

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Unité de Biologie Moleculaire du Géne chez les Extrêmophiles Groupe Interactions Bacteriophages-Bacterie chez l'Animal

Deciphering in vivo efficacy of virulent phages

in the mammalian gut

Thèse de doctorat de Microbiologie

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Abstract

The mammalian gut is a complex and heterogeneous environment inhabited by a large and diverse microbial community, including bacteria and their viral predators, bacteriophages (phages). Several studies have shown that changes in the abundance and diversity of these two communities can be linked to health and disease, indicating that both play crucial and likely synergistic roles in homeostasis. However, the interactions between virulent phages and bacteria in the gut are still poorly understood, which is also an obstacle for the design of successful therapeutic interventions based on phages. Independent experiments have shown that, while efficient at reducing bacteria in the gut, in spite of phage amplification. These observations suggest that there are unknown factors in the gut environment that modulate the interactions between phage and bacterial populations. This thesis aims to uncover some of these factors, opening avenues to better understand the interactions between these two antagonistic entities.

First, in order to investigate the possible influence of the bacterial physiology modulation in the gut, we performed a comparative genome-wide RNA-sequencing analysis of an *Escherichia coli* strain (55989) in different growth conditions, for which some phages displayed differential efficiencies. These included exponential and stationary growth phases *in vitro*, as well as samples from different gut sections of colonized mice. Results showed major physiological changes between *in vivo* and *in vitro* conditions, including the differential expression of several genes and pathways previously associated to phage infection, as well as others that were previously undescribed.

Second, taking advantage of a new murine model of controlled microbiota (with 12 microbial strains), we monitored the population dynamics and the spatial distribution of a cocktail of three virulent phages targeting an additional strain of *Escherichia coli* (Mt1B1). We observed the coexistence of both populations, with no phage resistant clones being detected over several weeks. The spatial distribution of the populations showed a severe reduction in phage particles in the ileum mucosal section. This suggests a possible ecological scenario of source-sink dynamics, where a reservoir of uninfected sensitive bacteria seeds other regions of the gut, where phage infection and amplification is more efficient.

Finally, we studied how the addition of a pathogenicity island, which are often horizontally transmitted in natural communities, can affect the susceptibility of a strain to phages, by modulating the expression of chromosomal genes. We find that the pks pathogenicity island changes the expression of tRNAs and genes involved in restriction-modification, ultimately resulting in an increased susceptibility of the strain to certain phages.

Overall, this work demonstrates that phage and bacteria interactions in the gut are not mainly determined by evolutionary arms-race dynamics as they can be influenced both by structure and by physiological or phenotypic changes that occur in this environment. These results uncover some of the complexities of phage-bacteria interactions in their natural environments, highlighting the need for further research to decipher the role of phages in health and disease.

Keywords: Phage-Bacteria interactions, Microbiota, Bacterial ecology, Bacterial physiology

Resumé

Le système digestif des mammifères est un environnement hétérogène habité par une communauté microbienne nombreuse et diverse, qui inclut notamment des bactéries et leurs prédateurs viraux, les bactériophages (phages). L'étude de multiples échantillons humains a montré que des changements d'abondance et de diversité de ces deux communautés peuvent être liés à l'état de santé (Lozupone et al., 2012) (Manrique et al., 2017). Malgré cela et le renouveau de l'utilisation des phages comme agents thérapeutiques, les interactions entre phages virulents et bactéries dans le système digestif sont encore mal comprises.

Bien que les phages virulents soient capables de réduire significativement la densité bactérienne *in vitro*, ils n'ont pas la plupart du temps d'impact important sur leurs hôtes bactériens dans l'intestin, malgré leur amplification. De plus, le tractus gastro-intestinal des mammifères est divisé en plusieurs sections contiguës (Donaldson et al., 2016) qui génèrent une gamme de micro-environnements différents. Ces environnements sont caractérisés par des fluctuations de plusieurs paramètres (pH, nutriments, eau, oxygène ouconsistence) qui ont un impact important sur la physiologie et l'écologie des bactéries (He et al., 1999; Koziolek et al., 2015; Wang et al., 2007).

Ensemble, ces observations suggèrent que des facteurs présents dans l'environnement intestinal modulent les interactions entre les populations antagonistes de phages et de bactéries. Cette thèse a pour objectif d'étudier ces facteurs afin d'améliorer notre connaissance de la dynamique de ces interactions.

Tout d'abord, pour élucider l'influence de la physiologie bactérienne, nous avons réalisé une analyse comparative des ARN messagers de la bactérie *Escherichia coli* (souche 55989) extraits de diverses conditions de croissance y compris des cultures *in vitro*, pendant les phases exponentielle et stationnaire, ainsi que dans différentes sections de l'intestin de souris, dans lesquelles certains phages montraient des efficacités différentes. Nous avons pu ainsi mettre en évidence une expression différentielle de nombreux gènes et voies métaboliques comme l'absorption du fer, la nutrition et la consommation d'oxygène, étayant les résultats qui avaient précédemment identifiés ces fonctions comme étant essentielles à la colonisation de l'intestin des mammifères par *E. coli* (Conway and Cohen, 2015).

En prenant en compte l'efficacité différentielle de multiplication des phages observée lors d'expériences *ex vivo*, nous avons également trouvé plusieurs gènes et voies liés à l'infection par les phages. Nous avons identifié une différence d'expression de gènes codant des fonctions liées à des récepteurs de phage ou à l'augmentation de la formation de biofilm, qui jouent un rôle important dans la défense bactérienne contre les infections par les phages (Lourenco et al., 2018).

Nous avons ensuite confirmé expérimentalement que la délétion d'un de ces gènes (*rfaL*), codant pour la ligase O-antigène, provoque effectivement une réduction importante de l'efficacité d'infection par un phage. L'antigène O étant un récepteur utilisé par plusieurs phages, nos résultats montrent que l'environnement intestinal peut influencer l'efficacité d'infection d'un phage en agissant sur l'expression de certains gènes. Le même raisonnement peut s'appliquer au gène *fliA* (dont l'expression est aussi modifiée. En effet, ce gène code un régulateur de la synthèse du flagelle qui est une structure souvent utilisée comme récepteur par certains phages.

Ces résultats ont permis de confirmer la validité de notre approche expérimentale basée sur la caractérisation du profil transcriptomique des bactéries au sein même de l'environnement intestinal afin d'identifier les facteurs clés susceptibles de moduler la coexistence entre les populations antagonistes de phages et de bactéries.

Puis, grâce à un nouveau modèle murin au microbiote contrôlé (contenant 12 souches), nous avons suivi la dynamique des populations ainsi que la distribution spatiale d'un cocktail de trois phages virulents ciblant une souche Mt1B1 d'*E. coli*. Nous avons pu observer la coexistence de ces deux populations ainsi que l'absence de clones résistants aux phages pendant plusieurs semaines. La distribution spatiale des populations a mis en évidence une importante réduction de la quantité de phages présente dans la partie mucosale de l'iléum.

Fait intéressant, nos données ont également montré que parmi les phages testés, un seul possédait un motif semblable à celui de l'immunoglobuline, précédemment impliqué dans l'affinité des phages pour le mucus (Barr et al., 2013; Fraser et al., 2006). Ceci est cohérent avec nos observations *in vivo* montrant les phages étudiés sont moins abondants dans les sections muqueuses. Ensemble, nos résultats suggèrent que les interactions entre phages et bactéries obéissent à une dynamique écologique dite « *source-sink* ».

Dans ce modèle, les bactéries localisées dans le mucus intestinal sont protégées des phages et forment un réservoir (« *source* »), tandis que dans la fraction luminale du tube digestif les phages, plus abondants, peuvent infecter leurs bactéries cibles et ainsi se multiplier (« *sink* »).

Ensuite, nous avons étudié l'impact d'un ilot de pathogénicité sur la sensibilité d'une bactérie à des phages, ayant découvert de manière fortuite que la souche MG1655 d'*E. coli* portant l'ilot « *pks* » devenait plus sensible à certains phages. Nous avons d'abord montré que la production de la toxine en soi, codée par l'opéron *pks*, n'était pas responsable du phénotype. Puis, nous avons démontré que cet ilot de pathogénicité provoque une expression différentielle de plusieurs gènes chromosomiques potentiellement impliqués dans la sensibilité accrue à certains phages.

Nous avons identifié une surexpression de deux ARNt différents (asparagine et aspartate). Une analyse de biais d'utilisation des codons a montré que ces ARNt sont préférentiellement requis par le phage par comparaison à la souche hôte MG1655 utilisée dans ces expériences. Cependant, la surexpression de chacun de ces ARNt n'a pas permis de leur attribuer une implication majeure dans le phénotype observé.

Nous avons aussi remarqué une réduction de l'expression d'un gène (*hsdS*) codant la protéine de spécificité impliquée dans le système de restriction-modification EcoKI. Ce système a été décrit comme jouant un rôle de barrière contre le transfert horizontal de gènes qui est la méthode usuelle de transfert des ilots de pathogénicité entre bactéries. Nous émettons l'hypothèse que la réduction d'expression du gène *hsdS* permettrait à l'ADN du phage d'être plus stable pour qu'il puisse terminer son cycle infectieux sans être détruit par les défenses bactériennes. Des études complémentaires seront nécessaires pour vérifier cette hypothèse.

Suite à des travaux concomitants à cette thèse et qui ont mis en évidence que le microbiote intestinal facilitait l'évolution des phages à infecter plusieurs hôtes (« host-jump »), nous avons réalisé une caractérisation phénotypique et génomique de clones résistants aux phages ainsi que des phages ayant évolués au cours de ces expériences Nos résultats montrent que dans la situation où phages et bactéries débutent de nouvelles interactions, la dynamique par « course à l'armement » prend place, en opposition aux résultats observés lorsque les interactions entre phages et bactéries sont plus anciennes.

Au cours de ce travail, nous avons révèlé que les interactions entre phages et bactéries au sein du tube digestif ne reposent pas principalement sur une dynamique de « course à l'armement », mais plus subtilement sur des changements phénotypiques imposés par l'environnement du tube digestif, influençant la physiologie bactérienne et la distribution spatiale des populations. Le choix d'études transcriptomiques s'est avérée être payant pour l'identification de facteurs influençant l'infection par un phage *in vivo*, que ce soit des différences physiologiques par rapport aux environnements *in vitro*, ou encore des différences provoquées par l'interférence d'ADN étranger (sous forme d'ilots de pathogénicité).

Ce travail contribue à la levée du voile sur les nombreux facteurs qui influencent les interactions entre phages et bactéries dans un environnement aussi complexe que l'intestin.

Ainsi, cette thèse révèle que les interactions entre phages et bactéries ne reposent pas sur une dynamique de « course à l'armement », mais plus subtilement sur des changements phénotypiques imposés par l'environnement du tube digestif. En conclusion, nos résultats montrent que la complexité de l'écosystème microbien intestinal doit être plus profondément étudiée pour améliorer notre compréhension du rôle des phages dans la santé et leur utilisation en phagothérapie.

Mot de clés : interactions bacteriophage-bactérie, microbiote, écologie, physiologie bactérie

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Chapter 1

Introduction

The review of the literature presented hereafter led to the publication of two review articles that can be found in the Annexes

Chapter 1

Introduction

Microbiota

In nature, bacteria are typically found in large mixed communities that create networks of interactions. These communities can be more or less diverse, depending on multiple environmental factors, such as the type of structure or the availability resources (Gibbons and Gilbert, 2015). Specific communities have been shown to inhabit particular ecosystems. These bacterial populations together with several other microorganisms are called microbiota.

One community that has received particular attention on the last years is the human microbiota. Resident bacteria can be found inhabiting different locations throughout the human body, from the mouth, to the skin and many others including some that were initially considered as sterile such as the lungs (Human Microbiome Project, 2012) (fig1).



Figure 1. Human Microbiome diversity map, represented by a phylogenetic tree and abundances (outside bars) of the organism present in the human microbiome. Extracted from (Morgan et al., 2013)

The gut microbiota is drawing much attention, since it has been shown to play an important role in nutrient and drug metabolism, protection against pathogens (colonization resistance) and modulation of the immune and nervous system (Belkaid and Hand, 2014; Sharon et al., 2016; Sonnenburg et al., 2005). Recent studies have shown how the interactions between the gut microbiota and the human host influence health and disease (Lozupone et al., 2012) (fig2). Alterations of the gut bacterial community play an important role in diseases like obesity (Turnbaugh et al., 2006), diabetes (Qin et al., 2012) and inflammatory bowel diseases (Frank et al., 2007). Moreover, this microbial community can not only influence gut related diseases but also play an important role in several mental disorders like autism or depression showing intricate interactions with the brain (Zhu et al., 2017). In line with these findings a recently new concept – the pathobiome - has emerged, which postulates that a disease is not caused by a single pathogen but by a general change on the resident microbiota (Vayssier-Taussat et al., 2014). Despite the growing number of studies, much remains unknown regarding how mechanistically these communities are regulated and how they impact their human hosts (Bull and Plummer, 2014; Shreiner et al., 2015).

The human gut is a heterogeneous environment divided in several contiguous sections (Donaldson et al., 2016). It is also characterized by a range of different micro-environments throughout its course, exposing its microbial inhabitants to fluctuating parameters like pH, nutrients, water, oxygen or density (from liquid to semi-solid or solid), which in turn have been shown to create fluctuations in the diversity of gut bacteria (Minot et al., 2011). Therefore, unveiling the functions of these microbial communities is of high importance for the understanding of human health.



Figure 2. A schematic diagram of the compositional transitions between healthy and disturbed microbiota extracted from (Lozupone et al., 2012)

The gut bacterial community of healthy humans is represented by 6 phyla: approximately 90% belong to the Firmicutes and Bacteroidetes and the other 10% represent Proteobacteria, Actinobacteria, Fusobacteria and Verrucomicrobia (Mirzaei and Maurice, 2017). This community has been shown to be stable at the taxonomic level but highly variable at the species level, with diet playing an important role on the composition of these communities (David et al., 2014a; David et al., 2014b; Faith et al., 2013; Ley et al., 2008; Lozupone et al., 2012).

Although bacteria are typically the focus of microbiota studies, other organisms cohabit in this community, notably protozoans, fungi and viruses (Huseyin et al., 2017; Lukes et al., 2015; Ogilvie and Jones, 2015; Parfrey et al., 2014). Recent studies focused on the viral component of several microbiota, describe the virome (viral community) of the skin (Hannigan et al., 2015), oral cavity (Abeles et al., 2014) and the intestines. The gut virome is highly variable between individuals, including between twins and their mothers, where bacterial composition was found to be highly similar (Reyes et al., 2010). This suggests that the virome composition is specific to each individual and has no correlation to individual genetic proximity (Minot et al., 2013; Minot et al., 2011; Reyes et al., 2010). Nevertheless, some viruses are shared by the majority of humans (fig3). Bacteriophages, which will be called phages through this thesis, have been observed to be the most abundant viruses present in the human gut (Dutilh et al., 2014; Lim et al., 2015).



Figure 3. Human gut phageome A. Viral abundance detected in enfants in the first days after birth. **B.** Phageome analysis of the healthy human gut. Adapted from (Lim et al., 2015; Manrique et al., 2016)

As highly efficient bacterial predators, phages are postulated to have a significant impact in shaping the bacterial composition of the human gut. Phages can be temperate or virulent, with integration on the bacterial chromosome or not, respectively (for more information see section 3). However, and despite their abundance, this impact is still poorly understood. A recent study has revealed that a core phageome (phage community) is shared by the majority of healthy humans but almost 50% of the phages are subject specific (Manrique et al., 2016; Stern et al., 2012) (fig3). Several metagenomic studies suggest that the majority of the phages show evidence for a temperate lifestyle, due to the abundance of integrases sequences on the analysis (Minot et al., 2011; Reyes et al., 2010). However, it has also been described that one of the most prevalent phage families in the gut, which is present in at least 50% of human population, is the Crassphage family (Dutilh et al., 2014; Yutin et al., 2018). The Crassphage family was suggested to be composed of virulent phages, but recent studies showed that some may have a temperate lifecycle (Yutin et al., 2018). This family has been predicted to infect bacteria of the phylum Bacteroidetes, and one of the phages was recently isolated and shown to infect the gut symbiont *Bacteroides intestinalis* (Shkoporov et al., 2018).

Phages are most likely to play an important role on the gut ecosystem, with major consequences for human health. For instance, dramatics changes in the richness and diversity of the phage community has been shown in conditions like inflammatory bowel disease, ulcerative colitis or Crohn's disease (Manrique et al., 2016; Norman et al., 2015). This association with disease phenotypes may be related to increase of phage diversity which can be correlated with the modulation of the bacterial communities, as suggested by the work of Zuo et al and OTT et al.

These authors observed efficacy of filtrates of fecal microbiota transplantation (FMT) treatments, suggesting that phages, which persist in the filtered sample, can act as a modulation factor of the bacterial communities (Ott et al., 2017; Zuo et al., 2018). Very recently it has also been shown that, in a successful FMT treatment of recurrent *Clostridium difficile* infections, the composition of phage communities has been stably transmitted from donors to recipients during a long period (>12months) (Draper et al., 2018).

It is essential to take in consideration that both the microbial and viral communities are not static and can change over time, due to several factors like diet or stress, such as antibiotics ((Howe et al., 2016; Langdon et al., 2016)). The coexistence of these two antagonistic populations is suggestive of a major role played by phage-bacteria coevolution in maintaining gut community structure and equilibrium (Scanlan, 2017) (fig4). Ergo, shifted microbial ecosystems associated to disease (pathobiome), could themselves result from drifted coevolutionary processes.

Despite its importance, the interactions between phages and bacteria in the mammalian gut, are still poorly understood.



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Figure 4. Phage and bacteria communities in the gut extracted from (Mirzaei and Maurice, 2017)

On the next chapters will be introduced in more detail the two main characters of these microbial communities, bacteria and phages, and some of the already described mechanisms of interactions between them.

Bacteria

Bacteria are some of the most ubiquitous organisms on the planet and new metagenomics studies frequently uncover new bacterial species, further confirming their overwhelming abundance, estimated 5×10^{30} on Earth, and diversity (Oh et al., 2014; Signori et al., 2014; Venter et al., 2004; Whitman et al., 1998). They vary in shape and length and are found in almost all habitats on the planet, from oceans to deserts, soil, ice or even nuclear waste, demonstrating their great ability to thrive in different and sometimes extreme environments.

Several studies have shown that bacteria play an extremely important role on major global processes on our planet such as biogeochemical cycles of carbon, nitrogen, oxygen and many others (Falkowski et al., 2008). Bacterial diversity goes beyond their shapes and membranes (gram-positive vs gramnegative), as they differ in nutrients metabolism, respiration, virulence, genomic composition, plasmids and also antimicrobial resistances and phage susceptibility (Ferenci, 2016).

In the human body, the gastrointestinal tract is the organ with the highest bacterial colonization since the gut is rich in nutrients by these microbes. The bacterial composition of this microbiota has been assessed by several studies based on 16S ribosomal gene sequencing, showing a highly diverse community, being dominated by the two phyla Firmicutes and Bacteriodetes (Mirzaei and Maurice, 2017). This community is acquired through time since birth, with some strains remaining for months to years while others can be more transient and remain only for few days (Rodriguez et al., 2015). One of the early colonizing species is *Escherichia coli* which was shown to colonize the gastrointestinal tract of newborns in the first 40 hours after the birth, though the colonization can be influenced by the mode of delivery, infant diet, hygiene levels and medication ((Gronlund et al., 2000; Magne et al., 2005)). As a result of this early colonization, *E. coli* has been shown to be present in approximately 90% of humans (Lescat et al., 2013; Smati et al., 2013), at a frequency of less than 1% in healthy individuals (Eckburg et al., 2005).

Escherichia coli a model organism

Even within a single species, bacteria can display a high degree of phenotypic flexibility. A well-known example is the model bacterium *Escherichia coli*.

E. coli belongs to the phylum Proteobacteria, class Gammaproteobacteria, from the order Enterobacteriales and family Enterobacteriacea, being part of the genus Escherichia. The species *E. coli* can be divided in 4 major phylogenetic subgroups (A, B1, B2 and D). It is a gram-negative rod-shaped bacterium and a facultative anaerobe, being able to grow both in presence or absence of oxygen. It can also survive when exposed to temperatures between 4-45 °C. Despite being highly flexible, it is most commonly found in environments with a pH of 7 to 8, and the optimal temperature for its growth is 37°C. Despite its low abundance in the gut of healthy individuals, *E. coli* plays an important role particularly on initial microbiota colonization of new-borns by consuming the existing oxygen and therefore turning the environment more suitable for colonization by the microbiota anaerobic bacteria (Gronlund et al., 2000; Magne et al., 2005).

E. coli strains can be identified as commensals, some are classified as opportunistic pathogen, and fewer as strictly pathogenic (Leimbach et al., 2013). *E. coli* can thus be divided into six pathotypes: enterotoxigenic (ETEC), enteropathogenic (EPEC), entero-invasive (EIEC), enterohemorrhagic (EHEC), enteroaggragative (EAEC) and adherent-invasive (AIEC). These diverse pathogenic traits within the species of *E. coli* are linked to the plasticity of its genome.

The genome of an *E. coli* strain display on average 4700 genes, but the pan-genome from *E. coli* species is 4 times larger with approximately 18000 families of orthologous genes. Analysis of the pan-genome defined a core genome (gene shared by all the strains) of only around 2000 genes (Hendrickson, 2009; Touchon et al., 2009; van Elsas et al., 2011) (fig5).



Figure 5. A schematic diagram of *E. coli* species genomic flexibility extracted from (van Elsas et al., 2011)

E. coli versatility and ability to constantly adapt to diverse environments is driven by several mechanisms, such as spontaneous DNA mutations (insertion or deletions, movement of transposable elements, duplications or translocations, single nucleotide polymorphisms) or exchanges of DNA with other bacteria by horizontal gene transfer (eg, through prophages or plasmids). These events often lead to the emergence of pathogenic *E. coli* strains, as observed, for instance, in the evolution of pathogenicity islands (Gal-Mor and Finlay, 2006). Pathogenicity islands, are genomic islands encoding virulence genes, such as toxins like colibactin, that can be on the bacterial chromosome or extrachromosomal (eg. plasmids) and can be vertically and horizontally transferred between bacterial cells.

The work reported in this thesis will use three strains of *E. coli*, two described as commensal, strain Mt1B1, isolated from the mice gut microbiota and strain MG1655, a model laboratory strain, and the third strain, an enteroaggragative isolate, strain 55989, which was recovered from the diarrheagenic stools of an HIV-positive adult suffering from persistent watery diarrhea in the Central African Republic in 2002 (EAEC 0104:H4).

E. coli Mt1B1 is part of the "Mouse Intestinal Bacterial Collection" (miBC) (Lagkouvardos et al., 2016). It was shown to colonize the gut of the gnotobiotic mice carrying the synthetic microbiota OMM-12 providing colonization resistance to *Salmonella enterica* serovar Typhimurium (Brugiroux et al., 2016; Garzetti et al., 2018). The synthetic microbiota OMM-12 is a consortium of 12 bacterial species representing the 5 more prevalent phyla of the mice microbiota, inoculated to axenic mice and that stably colonize the gut of these animals for several mice generations (Brugiroux et al., 2016) (fig6).



Figure 6. Stability of the relative abundances of the 12 species in the oligo-MM mice through different generations. Extracted from (Brugiroux et al., 2016).

The EAEC, strain 55989 (EAEC O104:H4) belongs to phylogenetic group B1. EAEC strains are known to be opportunistic pathogens associated with acute and chronic diarrhea. It is more prevalent in paediatric diarrhea (Nataro et al., 2006) but some strains were also found in healthy adults (Cohen et al., 2005) and in HIV immunocompromised patients. EAEC was described as one of the major causes of travellers' diarrhea (Croxen and Finlay, 2010). It was also involved in for several outbreaks of diarrhea (Dallman et al., 2014; Estrada-Garcia et al., 2014), notably the recent 2011 outbreak in Germany, in which the identified *E. coli* strain was the strain 55989 that acquired the shiga-toxin gene (Muniesa et al., 2012). The pathogenicity of EAEC *E. coli* strains is associated with their adherence to the mucosal layer of the intestine, promoted by the production of aggregative adherence fimbriae (AAFs), related to Dr adhesins (Korotkova et al., 2006). The biosynthesis of fimbriae is encoded by genes in a plasmid natural to 55989 strain, belonging to the pAA plasmid family (Croxen and Finlay, 2010). After adherence they then release toxins (enterotoxins or cytotoxins) that will damage the mucosal layer and subsequently the epithelial cells, inducing inflammation that ultimately leads to diarrhea (Blanton et al., 2018).

Bacteriophages

Discovery and nature of bacteriophages

Bacteriophages (from the Greek phagein – to eat), are viruses that infect bacteria and their name translates literally to "bacterial eaters" (fig7). They are one of the most abundant entities on Earth with an estimate of 10^7 particles per millilitre of ocean water. This amounts to an estimate of 10^{10} phages in a single liter of ocean water, a number that surpasses the number of humans on Earth (7.6x10⁹) (Breitbart et al., 2018).



Figure 7. Scanning electron microscopy of a bacterial cell (Acinetobacter baumannii) being lysed by phage (Roach and Debarbieux, 2017).

The discovery of phages is typically attributed to Frederick Twort and Felix d'Herelle in the early 20th century (Abedon et al., 2011), even though several previous studies described observations that could possibly be attributed to phages (Abedon et al., 2011). In 1915, while attempting to propagate vaccinia virus, the primary component of the smallpox vaccine, on agar plates Frederick Twort noticed that contamination colonies were growing after 24h post-inoculation but had some "transparent points". He showed that this phenomenon occurred faster in younger cells and could be transmissible, ie, clear spots would originate on other plates even if diluted or filtrated. Despite its description Twort assumed that this agent could be a possible small bacteria or a small amoeba (Keen, 2015; Twort, 1936).

Two years later, in 1917, Felix d'Herelle was working with filtrated stool samples from Shigella dysentery patients when he observed the presence of an "antagonistic agent" against the pathogen. He described it as easy to cultivate and transmissible from one culture to another, and he hypothesized that it could be a parasitic microbe (Keen, 2015). He further continued studying these agents, that he called bacteriophages due to their properties in eliminating bacteria. Felix d'Herelle was also the first to hypothesize and use phages as therapy to treat infections, being also one of the first scientists supporting the work developed at the Eliava Institute in Georgia, where infectious diseases are still nowadays treated with naturally isolated phages (Kutateladze and Adamia, 2008).

Morphology and characterization

At least 11 families of phages have been described. These families include several different morphologies and genetic contents, which can be either single-strand (ss) or double-strand (ds) DNA or RNA. From these 11 families, the most abundant ones belong to the order Caudovirales, which include the Myoviridae, Siphoviridae and Podoviridae families. All Caudovirales possess dsDNA enclosed into an icosahedral head. This head is attached to a tail (which can vary in length and width), which has at its terminus the baseplate and fibers proteins that allow the phage to adsorb to the host cell. The subdivision between the 3 families is based on the different tail morphologies (fig 8). The Myoviridae family is characterized by a long contractile tail, where the contraction is needed for DNA injection. The other 2 families possess non-contractile tails, with the Siphoviridae family having long and flexible tails, while phages from the Podoviridae family are characterized by their short tails. The Podoviridae family has been also described to have the smaller genomes (40 to 60kb) compared to the other 2 families. However this notion has been recently changed, by the discovery of podoviridae crassphage, described with a genome sizes ranging from 90 to 105 kb (Guerin et al., 2018). All the phages isolated and used during the work described in this manuscript belong to these 3 families.





Phages lifecycles

Phages can undertake two different lifestyles, the virulent and the temperate (fig9). In one hand, the virulent life cycle is strictly lytic, with bacterial lysis at the end. On the other, temperate phages can choose between lytic or lysogenic cycles. During the latter, the phage DNA integrates into the bacterial chromosome. Both cycles start with the adsorption of the phage to the bacterial cell. This adsorption is normally mediated by the phage tail proteins that interact with specific receptors on the bacterial cell surface. These receptors can be the lipopolysaccharide, the peptidoglycan, flagellum proteins or outer membrane specific proteins.

These interactions are typically highly specific, which reduces the number of hosts targeted by the phage (i.e., they have a narrow host range, eg. infecting few strains from one species). However, certain phages are able to infect a broader range of hosts (eg. infecting multi-species). Afterwards, the viral DNA is injected into the host cell. During the lytic cycle, some phages shut-down the bacterial gene expression and hijack the transcriptional and translational machineries in order to replicate. After DNA replication and protein assembly the viral DNA is packaged in the capsid and with the help of some proteins encoded by the phage (holins, endolysis, lysis) the bacterial cell membrane is disrupted and the phage progeny is released into the environment, ready to infect new bacterial cells. Virulent phages are also able to enter into a pseudolysogeny state (described below) to delay lysis. It was also reported for phage T4, a dormant state when it infects *E. coli* cells are experiencing starvation, with the possibility to resume its cycle as soon as the cell restarts its replication cycle (Bryan et al., 2016).

On the other hand, during the lysogenic cycle, the phage DNA is integrated in the host chromosome (as a prophage) or kept as an extrachromosomal element (pseudolysogeny or carrier state). The process of lysogeny is mediated by integrases encoded by the phage genome. The lysis-lysogeny decision has been described by several mechanisms, but are thought to depend on the environmental phage density, and the corresponding number of available host cells (Shao et al., 2018). A novel recently described mechanism showed that phages can communicate via peptides encoded by their genome that will be released upon cell lysis (arbitrium). After lysis the free-phages will measure the concentration of this peptide and when reaching a threshold the phage will then engage on the lysogenic cycle instead (Erez et al., 2017).

In its prophage state, the virus replicates vertically with the bacterial cells, and consequently will be subjected to evolutionary changes and adaptations to its genomic environment (Bobay et al., 2013). These phages can be induced and resume a lytic cycle. Stimuli include: external or internal stresses, such as pH, temperature, antibiotic exposure, nutrients (Howard-Varona et al., 2017). Prophage induction can also occur due to the presence of other phages, showing the complexity of the interactions between not only phages and bacteria but also between phages themselves (Banks et al., 2003; Casjens and Hendrix, 2015; Christie and Dokland, 2012; Matos et al., 2013). The induction can also be triggered depending on the environment (Czyz et al., 2001). For example, *E. coli* phage lambda has been shown to have a 50-fold higher induction rate when colonizing the mice gut compared to *in vitro* growth conditions (De Paepe et al., 2016). Upon resuming their lytic cycles, it is possible that these phages encapsidate not only their DNA but also fragments of bacterial DNA, a process that is named transduction.

Specialised transduction can occur when the phage genome erroneously excises from the host genome and encapsidate fragments of adjacent bacterial DNA (fig9) (Touchon et al., 2017). Generalized transduction occurs when the DNA packaged in the phage capsid belongs exclusively to the bacterial cell (fig9) (Touchon et al., 2017). This latter type of transduction is associated with phages whose endonucleolytic enzymes randomly cut the bacterial chromosome. Recently another DNA transduction process has been described in *Staphylococcus aureus*. The phage replicates in situ, without excision from the bacterial chromosome, and viral capsids are packaged immediately after, sometimes including the flanking regions of the prophages as the capsids are filled using headful mechanism (DNA is encapsidated and cut only when the capsid is full). This process was named lateral transduction, and it was shown to be able to transmit genes that were several hundreds of kilobases afar from the integration site of the prophage (Chen et al., 2018).



Figure 9. Phage lifecycles and mechanisms of gene transfer extracted from (Touchon et al., 2017)

Recently, the role of temperate phages in the gut has been experimentally investigated revealing their ability to excise from bacterial chromosome, modulate the microbiome, acquire genetic information or even transfer between bacteria in response to inflammation (Cornuault et al., 2018; De Paepe et al., 2014; De Paepe et al., 2016; Diard et al., 2017). The work performed during this thesis will focus on virulent phages only.

Phages as tools and therapeutics

Phages have long been studied not only as a biological entity but also as an extremely useful toolkit for molecular biology. Phages have been used for genetic manipulation, detection of bacterial infections, also for the development of new technologies in drug discovery and delivery and other nanotechnological applications (O'Sullivan et al., 2016). Furthermore, phages have been used as a therapy against infectious diseases since its discovery (D'Herelle, F. (1917)). However after the discovery of antibiotics, which was shown to be more efficient towards a broader range of infections, the use of phages as therapeutics declined. Today, antibiotics are still one of our most useful therapies, but the growing concern regarding the surge of antibiotic resistant superbugs, has justified the renewed interest in alternative therapies. Phage therapy in particular has recently enjoyed a renaissance period, and the clinical use of phages has restarted in the western world, including compassionate treatments and few clinical trials (Kortright et al., 2019). However, these treatments are initiated with the single evidence that phages are infecting bacteria in vitro, without any additional insight on how this process could be translated in vivo.

In vitro vs in vivo efficiency

The efficiency of virulent phages relies largely on the ability to recognize and connect to the specific receptors on the host cells. It seems a simple and reliable approach that turns into a highly complex event when taking in account the intricacy of its hosts and its interaction with the environment. For example some phages have been shown to have a differential (lower) efficacy in natural environments when compared to their incredible anti-bacterial activity *in vitro* (Chibani-Chennoufi et al., 2004; Maura et al., 2012; Weiss et al., 2009). Another peculiar observation was reported by several laboratories when studying the impact of phage on bacteria residing in the gut. When administered to these animals, phages can efficiently replicate from days to weeks, without a major decrease on the abundance of their targeted bacteria. In addition, during this pervasiveness period, the emergence of phage resistant clones have never showed, so far, to be the major cause of this balanced coexistence (De Sordi et al., 2017; Maura et al., 2012; Weiss et al., 2002).

Additionally, when different phages are tested for their ability to infect and replicate in an *ex vivo* GIT environment, it was shown that all phages replicate in the small intestine (ileum) section, while some displayed a reduced replication in the large intestine (colon) section as well as in the feces (Galtier et al., 2017; Maura et al., 2012).

Many factors can be responsible for the modulation of the interactions between phage and bacterial populations interfering with the efficiency of phage infection according to the conditions encountered. Some of these factors are discussed below.

This information demonstrates that the understanding the balancing dynamics between virulent phages and bacteria in natural ecosystems is needed for the development of various phage applications in the gut.

Phage-Bacteria interactions

Phage and bacteria coevolution

Populations dynamics

Phages have an important role in shaping bacterial communities and consequently shape most of the biological processes on the planet. Their interactions with the bacterial community can influence major biogeochemical cycles (like nutrient cycling and respiration) (Fuhrman, 1999; Suttle, 2007) and the global evolution of the biosphere as a whole, which benefits from all the genetic variation introduced by these interactions (Comeau and Krisch, 2005).

As mentioned earlier, phages carry metabolic genes expanding the capacity of the host to adapt to environmental changes and/or their virulence (role mostly assumed by temperate phages) which can confer an enhanced fitness to the bacteria (Bille et al., 2017; Brussow et al., 2004; Harrison and Brockhurst, 2017; Obeng et al., 2016). Such physiological modulations have been shown to influence the competition between different bacteria in microbial communities (Duerkop et al., 2012). For example, prophages can be used has competitive weapons against competitor phage sensitive strains. This was observed both *in silico* and in laboratory conditions for *Bordetella* p20 phages (Joo et al., 2006) and also for an intestinal commensal bacterium, *Enterococcus feacalis*, which encodes two different, but incomplete prophages, that when excised together form a completely functional phage particle that is able to eradicate competitor strains of *Enterococcus* in the gut (Duerkop et al., 2012).

The frequent interactions between phages and bacteria lead to the evolution of diverse mechanisms of resistance and counter-resistance by both populations driving to an evolutionary arms-race for coexistence. Several studies of phage-bacteria interactions *in vitro* have shown a limited coevolution between the two populations, with rapid short timescale evolution of defense and counter-defense dynamics, at the end of which bacterial populations typically evolve to a state in which they cannot be infected by the phage population, that is driven to extinction (Dennehy, 2012).

Extinction of the phage populations in nature has been shown to be likely influenced by the environmental context of their hosts, particularly regarding conditions of low resources (Wright et al., 2016). On the other hand, several other studies report that it is possible to sustain a long-term coevolution between phage and bacteria populations with an arms-race dynamics (Weitz et al., 2005). The hypothesis accounting for the selection towards adaptation of the host (bacteria) and counteradaptation of the parasite (phage) is mostly known as the Red Queen Hypothesis (Van Valen L. A new evolutionary law. Evol Theor. 1973;1:1-30). Several studies have been performed in order to understand its role on phage-bacteria interactions. Large part of these studies have been done in silico, but some have been performed in vitro (Buckling and Rainey, 2002a, b; Mizoguchi et al., 2003; Poullain et al., 2008; Weitz et al., 2005). Despite of the high selection imposed by the phages, the phagebacteria arm-race dynamics were observed to decrease in intensity over time (Hall et al., 2011). Genetic factors like the carriage of plasmids can also decrease the pace of these coevolutionary dynamics (Harrison et al., 2015). Fluctuating or spatial heterogeneous environments influence the rates of phage dispersal, and, as such, also play an important role in limiting the antagonistic coevolutionary dynamics. These environments generate refuges for sensitive bacteria, where they cannot be easily reached by the phages, promoting coexistence between these antagonistic populations (Brockhurst et al., 2006; Brockhurst et al., 2004; Harrison et al., 2013; Vogwill et al., 2008). Long-term interaction studies on phage-bacteria interactions have shown that the arms-race dynamics tend to be driven by frequency-dependent selection, where the bacteria present at higher frequency are more likely to be predated. The phage population will then increase in numbers and cause a decrease in the targeted bacterial population. When other bacterial species increase in frequency due to the niche left vacant by the phage predated populations, phages that infect these newly successful species will have an advantage, while the phages infecting the previous host will decrease making a cyclic renovation of the dominant species. This scenario was named kill-the-winner, has been described as predominant in the oceans (Breitbart et al., 2018; Hall et al., 2011; Lennon et al., 2007; Middelboe et al., 2009).

The coevolution between phage and bacterial populations gives rise to structured networks between phage and bacteria populations. These networks can be nested and modular. The interactions networks can be considered nested when phages and bacteria populations are ranked by the susceptibility or resistance of bacteria and infectivity of the phages, specialist (infecting few strains) or generalist (infecting many strains). On the other hand the interactions networks are considered modular, when the interactions take place within groups of phages and bacteria that are distinct from those present in other modules, with hardly no connection (Weitz et al., 2013). These two types of interactions can also coexist in nested-modular networks, which can be observed in complex ecosystems, including the mice gut where generalist phages are shown to be prevalent (De Sordi et al., 2017; Kim and Bae, 2018).

The complexity of the interactions between phages and bacteria can be influenced by many different factors, directly, as the defence mechanisms, or indirectly as environmental changes, which are discussed in detail below.

Bacteria and phage defence/counter-defence systems

Different mechanisms of bacterial defence against phages (and vice-versa) are currently known (Rostol and Marraffini, 2019). Many molecular details remain undetermined and, importantly, the role of these mechanisms in natural environments remains poorly understood. Therefore, new studies, particularly *in vivo*, are required to better understand their impact, for instance during phage therapy. Thereafter, the most common bacterial defence mechanisms are described below, with a focus on how they can play a critical role on survival of both bacterial and phage populations (fig10).

Prevention of adsorption by mutation or binding site concealing

The prevention of phage adsorption by the bacteria is the most prevalent defence mechanism in a Red Queen arms-race dynamics scenario. It consists on the emergence of *de novo* mutations that allow bacteria to modify or change the phage receptors preventing the phages to attach to the cells. These include: i) cell-surface modifications (mutations) that inhibit phage entry (Clement et al., 1983; Yu and Mizushima, 1982), ii) the production of proteins which are anchored on the membrane masking the receptors impairing or preventing phage attachment as shown in *E. coli* and *S. aureus* (Labrie et al., 2010; Nordstrom and Forsgren, 1974; Riede and Eschbach, 1986), iii) the production of an extracellular matrix as shown for *E. coli* K1's, which capsule is a barrier to phage T7 infection (Scholl et al., 2005), iV) release of outer membrane vesicles that contain the phage receptor, which sequester the phages (Manning and Kuehn, 2011).

These modifications, can be circumvented by the phages through the production of polysacharidesdegrading enzymes (Pires et al., 2016). In some cases, phages have also been shown to use these proteins as receptors (Roach et al., 2013).

Phage resistant clones with modified receptors were observed in vivo after phage treatments, such as on chicken infected by *Campylobacter jejuni* or calves infected by *Escherichia coli* (Holst Sorensen et al., 2012; Seed et al., 2014; Smith and Huggins, 1983), but were also detected in *Vibrio cholerea* infected patients which had not been treated by phages (Seed et al., 2014).

Furthermore, in complex microbial ecosystems, metagenomics analysis of samples from different individuals have revealed a high variability in bacterial membrane epitopes, including possible phage receptors, within the same bacterial species, which suggests that receptors are an active site of coevolution on this arms race between phages and bacteria (Zhu et al., 2015). Another example of this defence system was observed in the antagonistic coevolution between multiple wild marine T7-like cyanophages with their targeted bacteria, *Prochlorococcus*, which was characterised by genomic mutations responsible for bacterial resistance and phage re-infectivity, host range expansion (host-jump) and fitness effects (Enav et al., 2018). The host range expansion provides to phages the ability to infect alternative bacteria, host-jumps that have also been reported in a mouse model of coevolution in the gastrointestinal tract (GIT) (De Sordi et al., 2017) revealing that phage adaptation to bacterial defence cannot only lead to re-infectivity but broader range of hosts.

Despite their selective advantage towards phage predation, these genomic events do not necessarily lead to the fixation of a dominant resistant bacterial population. For example, in several experimental phage-bacteria coevolution models in animals, phage resistant bacteria were never recovered. Moreover, metagenomics studies focused on the viral portion of the microbiome (virome) have failed to detect major signs of coevolution (De Sordi et al., 2018; Minot et al., 2013). One possible explanation might be the fitness cost of phage resistance. In 2011, Gomez and Buckling could observe that while evolving resistance growing in soil, bacteria presented a 36% decrease in fitness, contrary to what was recorded in vitro in which no fitness cost linked to resistance was observed (Gomez and Buckling, 2011). Studies have also shown that bacterial resistance evolved in an environment colonized by more than one phage, in comparison with selection against only one phage, leads to higher bacterial fitness cost when phage selective pressure is removed (Koskella et al., 2012). This fitness cost can also be related with the loss of virulence caused by the changes leading to phage resistance (Leon and Bastias, 2015). Additionally, it has also been described, that even when the phage selective pressure leads to a dominant population of resistant clones, a high rate of genetic transitions from resistance to susceptibility can work as a mechanism, for coexistence of phage and bacteria populations. This mechanism was named "leaky resistance" (Chaudhry et al., 2018).
Super-infection exclusion systems

Superinfection exclusion systems are used by bacterial cells to prevent infection by the phages. These systems, often encoded by prophage proteins predicted to be associated with the cell membrane, were observed to block the entry of the phage DNA on the host cells (Bondy-Denomy et al., 2016). This suggests that this mechanism plays an important role in the competition between phages). Examples of superinfection exclusion systems can be found in many different bacterial species, both Gramnegative or Gram-positive, like *E. coli* and *Salmonella* or *Lactococcus* and *Streptococcus*. One of the well-studied case are the imm and sp proteins from *E. coli* phage T4, which prevent re-infection of the cell by T4 or other T-phages. Protein imm is known to modify the phage injection site, while sp inhibits the activity of T4 lysozyme, preventing the degradation of the peptidoglycan to engage a super-infection. ((Labrie et al., 2010)).

Abortive infection systems

Abortive infection systems are "altruistic" systems that prevent the spread of the viral infection. They are considered altruistic because the system requires the premature death of the infected cell, in order to limit the replication of the phages and prevent the infection of the surrounding cells.

Abortive infection systems are probably widespread in bacteria, having already been described for several species, such as *E. coli*, *Lactococcus lactis*, *Bacillus subtilis* and several others (Chopin et al., 2005). A well-known example is the Rex two-component system from lambda lysogenic *E. coli* against lytic phage. This system is composed of two proteins, the first is RexA that is activated by the entry of foreigner viral DNA and further activates a second protein, RexB. The process ends with cell death after loss of active transport and the hydrolysis of ATP triggered by the protein RexB, leading to the termination of macromolecular synthesis (Molineux, 1991).

• Prevention of DNA replication

Bacteria have evolved defence mechanisms which act after phage attachment and DNA injection. These mechanisms include systems for cutting exogenous DNA (*restriction-modification* or *CRISPR-cas* systems) or for preventing phage DNA replication (*BREX* - BacteRiophage Exclusion (Goldfarb et al., 2015). The restriction-modification systems consist in a group of proteins whose function is to protect the cells from foreign DNA. When the phage unmethylated DNA enters the cells, it can be recognized and degraded by restriction enzymes or methylated by the cell methylases. Once the phage DNA is methylated its progeny will be protected from the R-M system, unless it infects bacterial cells with a different methylase protein. Different studies have shown the presence of these systems in several bacterial species (Labrie et al., 2010; Rostol and Marraffini, 2019).

But phages have already been shown to be able to circumvent Restriction-modification systems by evolving the ability to acquire methylase genes (McGrath et al., 1999), or by presenting altered DNA bases as hydroxymethylcytosines instead of cytosines, as in phage T4 (Labrie et al., 2010).

Another bacterial system involved on the prevention of phage DNA replication is the *CRISPR-cas* system (clustered regularly interspaced short palindromic repeats). This system consists in several loci composed of 21–48 bp direct repeats interspaced by non-repetitive spacers (26–72 bp) derived from foreign DNA sequences such as phages or plasmids. These spacers will then serve as guides for the *Cas* proteins which recognises possible invading DNA and cleave it. Metagenomic analysis of the *CRISPR-cas* locus in human gut microbiota have detected highly variable and rapidly evolving CRISPR sequences suggesting multiple attempts to escape phage predation in the gut (Stern et al., 2012). On the other side, phage have also evolved complex anti-defence mechanism against it, like the anti-

CRISPR (Acr) proteins, which are encoded by several phages (both lysogenic and lytic), and were shown to interfere with the bacteria CRISPR-systems, allowing the phage replication and assembly to proceed (Pawluk et al., 2018).





• Other anti-phage systems

There are other less conventional defence systems like the phage-inducible chromosomal islands (PICIs) of gram-positive bacteria which can interfere with the reproduction of certain phages. They do not interfere with the replication process because they need the phage proteins to be produced in order for the genomic sequence to be packaged in the phage capsid (Ram et al., 2012).

Another example of defence system has been recently described in Streptomyces. It was shown that three bacterial molecules can insert in the phage DNA and prevent its replication (Kronheim et al., 2018). Several other putative anti-phage systems have been also recently described by Doron S. et al 2018, demonstrating that many mechanisms underlying phage-bacteria interaction are still to be discovered.

• Phenotypic resistance

A stochastic differential gene expression, which is not necessarily induced by environmental conditions can also play a role on the balanced relationships between phages and bacteria. This stochasticity can lead to a phenotypically heterogeneous population of genetically susceptible bacteria in which, for example, a subpopulation of cells have a reduced production of receptors. This decreases the infection, making them persistent to phage with no acquired genetic resistance trait. This phenomenon, has been referred to in the literature as phenotypic resistance (Bull et al., 2014; Chapman-McQuiston and Wu, 2008; Levin et al., 2013) but in most cases the precise mechanism involved is poorly understood.

Environmental factors in –phage-bacteria interactions

Interactions between the phage populations and bacterial populations can also be modulated by external, environmental factors. These factors can regulate the spatial composition of populations, or their gene expression and physiology, which, in turn, can impact on the efficiency of phage infection, reproduction and amplification.

Bacterial physiology affects phage infection efficiency

Influence of temperature, pH, oxygen levels and medium

Phages are parasites, which by definition implies that they depend on their hosts in order to fulfil their life cycle. Phages are highly variable regarding to adsorption rates, eclipse times or even burst sizes, and all these kinetic parameters of infection can have a major influence in efficiency of phage predation. The physiology of the bacterial host cell can change depending on the growth phase which in turns is highly dependent However, the host itself can display various physiological states depending of several abiotic parameters, such as temperature, pH, oxygen, cell density or even nutrients fluctuations. These changes will thus influence the efficiency of phage attachment, replication or lysis. Most studies that attempted to understand the effect of the host physiology on the efficiency of phage infection have been performed in vitro. For example, in 2004, while studying interactions between phage US1 and its specific host, Pseudomonas fluorescens strain Migula, S. Sillankorva et al demonstrated how different temperatures could influence phage efficiency. They showed that the optimal infection occurred when cells were infected at 26°C, while changes the temperature to 4°C or 37°C had a major effect on phage infection, with a low infection rate at 4°C and none at 37°C. The same authors also observed successful phage infections in nutrient rich medium but not in glucose medium, demonstrating that not only temperature but also nutrient availability can modulate the efficiency of infection. Furthermore, when examining the outer membrane profiles of the cells growing at different conditions they observed two different proteins (17.5 and 99.0 kDa) with differential abundances. These proteins were not detected in bacteria growing at 37 °C or in a glucose medium, and the smaller protein was not detected at 4 °C, suggesting a possible role for these proteins as phage receptors. (Sillankorva et al). Similarly, in 1984, Bernard Labedan suggested that differences in growth temperatures may disturb the rigidity of the host cell membrane, which in turns affects phage T5 infection efficiency. Another factor with high impact on bacterial physiology is the presence of oxygen, which has been shown to have different effects on phage infection, although these effects differ between phages. For instance, studies in phage T4 showed that it can replicate as well in aerobically or anaerobically growing E. coli (Weiss et al., 2009), while different oxygen levels imposed on Bacillus thuringiensis could affect the duration of the infectious cycle of phage BAM35 (Daugelavicius et al., 2007).

Several studies performed using the models phage T4 and its host *E. coli* have shown that when the latter grows at higher rates, phage T4 is absorbed and released more rapidly, its burst size increases and its eclipse and latent periods decrease (Bryan et al., 2016; Golec et al., 2014; Hadas et al., 1997; Nabergoj et al., 2018). Some of these observations led Hadas H. et al in 1997 to suggest that phage synthesis and assembly rates depend on the protein synthesis machinery of the host, whereas lysis time is correlated with cellular dimensions (Hadas et al., 1997). Other studies revealed that phages T4 and ms2 can enter a dormant state during the infection of cells in stationary phase. This dormant state has been referred to as "hibernation" and is a reversible state in which although some phage proteins are synthetized, the production and assembly of phages is paused until new nutrients available to the cells allowing the phage infection process to resume (Bryan et al., 2016; Propst-Ricciuti, 1976; Ricciuti, 1972). The stochasticity of other abiotic factors can also trigger the production of capsules by some bacteria, and these can decrease the likehood of phage infections by possibly masking their receptors (Ohshima et al., 1988). However, some phages can also use these capsules as preferential receptors (Roach et al., 2013), and thus the specific role of the capsules in the interaction between phage and bacteria is hard to predict.

Bacterial differential gene expression depending on growth conditions

The recent improvements in several techniques (membrane, chip, RNASeq) have facilitated the capture of the pool of mRNA from cells, in order to uncover the changes in the transcriptomic map of bacteria when growing in several conditions. Changes in the surrounding environment of a bacterial cell can lead to differential gene expression both in different *in vitro* conditions (Caglar et al., 2017; Feugeas et al., 2016; Gadgil et al., 2005; Houser et al., 2015) (Partridge J.D. et al 2006) and in complex systems, like the mammalian gut (Brathwaite et al., 2015; Denou et al., 2007; Janoir et al., 2013; Mobley, 2016).

The mammalian gut is a complex ecosystem with different sections that pose different environmental conditions for bacteria. The small intestine has a microaerobic environment with high levels of antimicrobial peptides and a semisolid state, whereas on the other hand the large intestine has been shown to be a more structured environment due to water absorption, is mostly anaerobic and present lower levels of antimicrobial peptides. Because these gut sections are markedly different, this suggests (and possibly requires) a highly dynamic gene expression in the bacterial cells. Denou E. et al in 2007, when comparing *Lactobacillus johnsonii* gene expression *in vitro* and *in vivo* (mice gut), have shown that not only there are major differences in gene expression between these two conditions, but also throughout the murine gastrointestinal tract (fig11) (Denou et al., 2007).



Figure 11. Comparison of *Lactobacillus johnsonii* gene expression profiles in different *in vitro* conditions and in different sections of the mammalian gut. Extracted from (Denou et al., 2007)

Another recent study has shown that bacterial species (*E. coli* and *B. thetaiotaomicron*) from either the mucusal or luminal contents of the mouse gut, exhibit differential transcriptional patterns. The authors have demonstrated a different utilization of resources by the two strains depending on their intestinal localization (Li et al., 2015).

All of these differences in the bacterial gene expression also affect how bacteria interact with phages. We can hypothesize that this bacterial differential gene expression, dependent on environment, can lead to repression of bacterial genes required for a successful phage infection, leading to a less susceptible host. However, during a successful infection, the phages can takeover the bacterial cells by manipulating its gene expression, for example by forcing degradation of its own RNA while hijacking the host metabolic machinery and RNA polymerase for production of phage transcripts (Blasdel et al., 2017; Chevallereau et al., 2016) (fig12).



Figure 12. Molecular mechanisms involved in phage PAK_P3 infection cycle in P. aeruginosa strain PAK Extracted from (Chevallereau et al., 2016)

Physical accessibility of bacteria (Bacterial ecological factors affecting phage infection)

In vitro studies of phage bacteria host interactions are typically performed in exponential phase cultures, and even the selection of phages itself is often performed in optimal growth conditions and with bacterial cells in planktonic state. However, this is a simplification of the natural conditions where phage and bacteria usually interact. In nature, the majority of bacterial populations tend to live not in a planktonic state, but instead they are usually found in multilayered aggregates of cells, which are adherent to each other and commonly adherent to surfaces by the production of a matrix of extracellular polymeric substances (EPSs) (Flemming et al., 2016).

Biofilms are extremely pervasive in natural environments and are the cause of many chronic bacterial infections (Costerton et al., 1999). These communities of pathogenic bacteria are also normally highly resistant to antimicrobial agents (Hoiby et al., 2010) and this makes the study of phage-bacteria interactions in biofilms a necessity if phage therapy is to be successfully applied in the clinical settings. Several studies have shown that the efficacy of phages against biofilms *in vitro* can be variable, and certain biofilm components may act as barriers against phage infection (Sutherland et al., 2004).

An example is the presence of an amyloid fiber network of CsgA (curli polymer) that was observed to protect the biofilms against the phage in two ways, by limiting phages penetration in biofilms and by preventing the phage attachment to the bacterial cell receptors (Vidakovic et al., 2018). However some phages have already been shown to efficiently target biofilms of different pathogenic strains like *Pseudomonas aeruginosa* (Fong et al., 2017; Shafique et al., 2017) and *Vibrio cholerae* ((Naser et al., 2017). Furthermore, some phages can produce certain enzymes that can degrade the polysaccharides produced by bacteria, thus facilitating the diffusion of viral particles in biofilms (Azeredo and Sutherland, 2008).



Figure 13. Phage mechanism against biofilms extracted from (Motlagh et al., 2016)

Nutrient availability and nutrient concentrations are highly heterogeneous within the biofilm structure, and can modify the efficiency of phage infections (Simmons et al., 2018). Moreover, the probability of infection changes with the capacity of the phages to diffuse in the biofilms.

Other studies have shown that when a phage treatment is applied to biofilms of *Campylobacter jejuni* (responsible for enteric human diseases) these phages can establish an association with the bacterial cells, assuming a carrier life cycle. These carrier state imposes some phenotypic modifications in the bacterial cells which, in one hand, gives them advantages to survive extra-intestinal environments but on the other hand prevents them to colonize chickens. Moreover, it was shown that bacterial cells in this state can function as an importer of viable phages to chickens already colonized with *C. jejuni* (Brathwaite et al., 2015; Siringan et al., 2014).

Biofilms or other similar heterogeneous environments can generate spatial refuges for the bacterial population, factors that reduce the probability of contact between bacteria and phage, promoting their coexistence, as shown both in silico and in vitro (Schmerer et al., 2014; Simmons et al., 2018). In silico, individual based stochastic spatial models have suggested that structured refuges can lead to coexistence without the emergence of resistant clones (Heilmann et al., 2012) (Sousa and Rocha, 2019). In vitro, experiments on the interactions between the populations of P. aeruginosa and phage PP7 in artificial heterogenic environment (static bacterial growth) showed reduced transmission and amplification, thus stabilizing the interactions between the two populations (Brockhurst et al., 2006). Similar mechanism was shown by Schrag and Mittler in 1996 with the coexistence of phage and bacterial populations in vitro where biofilm grew on the wall of a chemostat. Using a more structured environment, Eriksen RS. et al showed that although phage and bacteria populations can have a longterm survival when phages are applied to growing bacterial colonies, this phenomenon is dependent on bacterial numbers, with coexistence occurring only when bacterial colonies are composed of more than 50 000 cells (Eriksen et al., 2018). Furthermore, it has been observed for several strains (S. aureus, E. coli and B. subtilis) that phages would only actively replicate when the host cells would be higher than of 10⁴ CFU/ml known as "threshold for bacteriophage replication" (Wiggins and Alexander, 1985) or "proliferation threshold" (Payne et al., 2000). This data underline that density-dependent relationships can have a possible major role in modulating phage infections. The biofilms or structured environments and the bacterial density can often change depending on the bacterial growth conditions, highlighting the importance of studying them in natural conditions.

Mice models

Several studies have shown how the human gut microbiota can influence many aspects of our body. Most of this studies can link or correlate the microbiota with the diseases and disorders. However, in order to better understand the mechanisms behind these effect more analysis are needed with in vitro but also in vivo models, in which microbiota manipulation is enabled (Clavel et al., 2016). For many reasons mice is the predominant animal model for studies related to the intestinal microbiota. They are mammals that are easy to breed, to which several genetic tools were develop unabling the generation of mice lines that mimic several human conditions. Moreover, recently, mice lines have been derived without the presence of any microbiota, named germ-free or axenic mice. These models can then be used for studies on gut colonization of single to multiple strains consortiums. Given the complexity of the mammalian gut, where numerous components interact from the bacterial, viral or fungal populations to the immune system, germ-free mice have revealed to be a useful tool. Indeed, they have allowed the development of gnotobiotic animals in which known components of the microbiota can be studied in controlled conditions. One of the most recently developed model is the OMM-12, consisting in gnotobiotic mice colonized by 12 murine isolated strains, which can be passed from mothers to pups. These strains belong to the 5 most representative phyla of the natural mice microbiota and can be cultivated and followed by 16S qPCR throughout several mice generations. Gnotobiotic models can help by providing the control of certain parameters, like the microbiota composition, which would be impossible in a conventional mouse model. These models have been shown to be an indispensable tool towards disentangling dynamics and interactions in the gut (Reyes et al., 2013). For example, the OMM-12 model have been used to better understand the processes of colonization resistance as mechanisms of protection given by the microbiota against pathogens (Brugiroux et al., 2016). Mice models have been also used as models for microbiota studies due to similarities with the human microbiota (fig14). However despite sharing the most of the abundant genera (two most abundant phyla are Firmicutes and Bacteriodes), bacterial gene comparison between human and mice microbiota only shares 4% of similarities (Hugenholtz and de Vos, 2018).



Figure 14. Phylogenetic tree of mouse ceca-associated and human colon-associated 16S rRNA sequences adapted from (Ley et al., 2005).

This should be kept in mind when interpreting the data on ecology, evolution or physiology of bacterial isolates from humans.

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Thesis outline

In the environment, bacteria are highly exposed to viral predation exerted by phages. The dynamic interactions of these viral and bacterial antagonistic populations within the mammalian gut remains poorly studied. In contrast to the high efficiency of phage predation observed *in vitro*, which leads to the rapid growth of phage-resistant bacteria, *in vivo* studies have shown a long-term coexistence of both populations without detection of phage-resistant bacteria. These observations suggest that specific factors modulate virulent phage infections in the mammalian gut.

The main goal of this work is to identify some of these factors to understand how the gut environment influences the dynamics between bacterial and virulent phages populations.

We first explored how differential gene expression of bacteria grown in different conditions, as well as the presence of a pathogenicity island, can influence the susceptibility to phages. A comparative genome-wide RNA-sequencing analysis of an *E. coli* strain (*55989*), was performed in conditions where some phages displayed differential efficiencies is presented in chapter 2. These included *in vitro* cultures, during exponential and stationary growth phases as well as ileal and colonic sections of the mammalian gut. The analysis revealed major differences in gene expression between *in vitro* and *in vivo* growth. In parallel, we focused on phage CLB_P2, which displays an uniform efficacy in all tested conditions, to genetically decipher its complex interaction with its bacterial receptor.

In addition, in chapter 3, we uncovered that an *E. coli* strain carrying and expressing the *pks* pathogenicity displays an increased phage susceptibility. We studied the possible mechanisms involved by performing a comparative transcriptomics analysis between the strains carrying or not the *pks* pathogenicity island.

Second, in chapter 4 we used a new gnotobiotic murine model, in which microbiota is composed of 12 known different strains to identify the ecological spatial constrains that can protect bacteria from phage infection. We followed the dynamics between an *E. coli* strain (Mt1B1) and a cocktail of three newly isolated phages that we characterized and tested for the emergence of resistance (arms-race). We found that spatial distribution of both populations fits with the ecological model of source-sink dynamics.

Third, in chapter 5, taking advantage of concomitant work that highlighted that the gut microbiota diversity facilitates the evolution of phages, we characterized the phages and bacterial strains that coevolved. Phenotypic and genotypic variations that lead to arms-race dynamics between adapted virulent phages and its newly acquired host were determined.

Finally, in chapter 6, we integrate the results presented above into a general discussion including future perspectives deduced from this work.

Chapter 2

Bacterial gene expression modulation in the gut environment influences phage infection efficiency

Chapter 2

Bacterial gene expression modulation in the gut environment influences phage infection efficiency

Introduction

Virulent bacteriophages have been frequently proposed as therapeutics to target bacterial pathogens resistant to multiple antibiotics. However, despite several attempts at using different animal models, the use of virulent bacteriophages to efficiently reduce bacterial carriage in the gut have often been disappointing, achieving at best a moderate reduction. One of the models used in our laboratory is the gut colonization with the opportunistic enteroaggragative E. coli strain 55989 (EAEC O104:H4). This strain is associated with acute and chronic diarrhea (Cohen et al., 2005; Croxen and Finlay, 2010; Nataro et al., 2006). The pathogenicity of enteroaggregative *E. coli* strains involves adherence to the mucosal layer of the intestine where they release toxins (enterotoxins or cytotoxins) that damage the mucosal layer and, subsequently, the epithelial cells and induce inflammation causing diarrhea (Blanton et al., 2018). Its enteroaggreative adhesion phenotype is provided by the production of aggregative adherence fimbriae (AAFs) of which the biosynthesis is encoded by genes that can be found in plasmids (Croxen and Finlay, 2010). Previous work using a phage cocktail (phages CLB_P1, P2 and P3) against this strain, showed a decrease in ileal and fecal bacterial numbers on the first day after phage inoculation. However, the bacterial numbers returned to their original levels 3 days later (fig 1A) despite phage numbers remained high (fig1B). Moreover, no phage resistant clones were detected during the experiment (Maura et al., 2012). It was shown that the three phages used display differential ex vivo efficiencies on intestinal sections (ileum and feces) from conventional colonized with 55989 E. coli (fig1C). This differential replication in ex vivo conditions with homogenate sections of the gut has also been showed in other mice models (Galtier et al., 2017). Taking in account the complexity of the gut environment, the overall results led us to hypothesize that possible physiological differences between the cells colonizing the different intestinal sections may be modulating the phage infection in vivo. Indeed previous microarray studies in Lactobacillus johnsonii, showed a significant difference in gene expression when comparing the gut with in vitro cultures and also when comparing the gut sections (Denou et al., 2007). To test this hypothesis, we performed a genome-wide RNaseq experiment, to analyse the differential gene expression patterns from E. coli strain 55989 in the ileum and colon gut sections in comparison to lab conditions, exponential and stationary phase cultures. Furthermore, we tested how this differential gene expression can play a role in bacterial susceptibility to phages in the gut.

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Figure 1. The bacteriophage cocktail transiently reduces 55989Str concentrations in the mouse ileum. Ileal A) *E. Coli* 55989 numbers, B) phage numbers on day 4 and 7 after receiving, drinking free-phage water (white circles) or water containing a cocktail of bacteriophages at 3x 108 PFU/ml (gray circles) or 3 x 1010 PFU/ml (black circles) for 24 h only. C) *Ex vivo* experiments provide evidence for differential bacterial permissivity to virulent phages dependent on gut location (figures extracted from (Maura et al., 2012)).

Results and discussion

Phage differential activities along the gut

Previous studies have shown that phages P1, P2 and P3 display a differential replication in different sections of the gut of conventional mice (fig1C) (Maura et al., 2012). In order to better understand if these results depend on the microbiota composition of the mice gut, we assessed the ability of the same phages to replicate both in homogenized samples of the gut sections (ileum, colon and cecum) of monoaxenic mice (*ex vivo*) and in exponential and stationary phase cultures (*in vitro*), since phage infection has been shown to be influenced by bacterial growth. This allowed us to study if the differential ability to infect of the 3 phages was reproducible in germ-free mice gut and also to assess and correlate to phage replication in different growth state conditions.

When comparing the replication efficiency in the different sections of the gut of monocolonized mice, we observed that phage P1 showed a reduced replication in the colon and impaired replication in the cecum homogenates in comparison to ileum. These results are consistent with the ones observed in conventional mice when comparing ileum and feces samples (Maura et al., 2012). P2 also behaves in consistently between conventional and monoaxenic mice, with similar replication in all the section of the gut tested. On the other hand, we observed that replication of phage P3 varies between conventional or monoaxenic mice (fig2). Nevertheless, P3 shows differential replication between ileum and colon/cecum/feces.

Phages have been previously shown to also have a differential efficiency depending on the growth state of their hosts (Lourenco et al., 2018). It has also been shown that bacteria can experience differential growth states depending on the gut section they are colonizing (Li et al., 2015). Thus we decided to perform replication assays in different *in vitro* growth conditions, during exponential and stationary growth phases. For these, we observed that both P1 and P3 phages have an impaired replication in stationary phase bacteria, even more than in the colon and cecum samples, while P2 again replicates in both conditions, even if this rate decreased 1000 times when infecting cells in stationary phase. These results suggest that the phage replication rates do not depend solely on the microbiota composition, and that the conditions of the local gut environment can influence the efficiency of virulent phages.





Figure 2. *Ex vivo* phage replication. Amplification over 5h (n=5 biological replicates) of A) individual phages (P1, P2, P3, each at an MOI of approximately 10^{-2}) and B) 55989 cells in indicated homogenized gut sections (il-ileum (orange); cc-cecum (yellow); co-colon (red); fe-feces (red)) from 55989-colonized conventional or monocolonized mice and flasks of cultured 55989 cells during exponential (dark green) growth (OD600=0.5) or stationary (light green) (24h) phases. N-fold multiplication relative to the initial number of phages added is shown. Conventional mice data extracted from Maura et al 2012.

The previous results led us to further investigate the factors that modulate this differential phage efficiency, particularly by attempting to characterize the growth state of *E. coli* residing in the colon. This allows us to understand how the bacterial physiology in these conditions influence phage infection. Therefore, we performed a genome-wide RNA sequencing analysis of *E. coli* 55989 retrieved either from the ileum or the colon (fig3).

To find commonalities between the physiological state of bacteria experiencing these environments and either exponential or stationary phases, we also performed RNA-seq of *in vitro* grown bacteria, in either of these states.



Figure 3. Scheme of the RNAsequencing experimental design from *in vivo* and *in vitro* samples. Mice were colonized with *E. coli* and ileum and colon were collected after 3 days. These samples were snap-frozen in liquid nitrogen upon collection.

Gene expression pattern of bacteria in the colon is different from in vitro conditions, but better resembles exponential, rather than stationary phase

An initial analysis revealed that samples for three of the four analysed conditions (colon, stationary phase and exponential phase) have a total number of reads between 2.6 million and 1 million (fig20A). On the other hand, the number of reads obtained from the ileum samples (between 0.1 and 0.25 million) were not enough for its inclusion in the analysis, as we can observe by the high number of null counts of these samples (fig4C). This caused a high variation in at least one of the ileum samples, as observed by PCA (principal component analysis) (fig4B), which creates difficulties when attempting to compare them with the other samples (fig4A). On the other hand, the stationary phase culture sample shows a higher prevalence of null counts in comparison with the colon and exponential samples, consistent with the expected decrease of transcripts in such conditions (fig4D). Further work will be necessary to attempt to increase the analytical power of this approach, either by increasing the coverage or by further purifying the samples. Therefore, we will focus the remaining analysis on this chapter in the colon, exponential and stationary samples.


Figure 4. Statistical analysis. A) Number of mapped reads per sample. B) Variability within the experiment - first principal components of the PCA. Colours refer to the biological condition of the sample, stationary (blue), colon (red), exponential (pink) and ileum (green). C,D) Proportion of features with null read counts in each sample. Colours refer to the biological condition of the sample, stationary (blue), colon (red), exponential (green).

PCA analysis (statistical transformation of a set of complex variables for exploratory data visualization) on these three sets of samples revealed that all the replicates were well separated by biological condition, suggesting that the expression of bacterial genes changes between the different environments and according to their growth physiology (Fig5A). Moreover, this analysis also shows that despite a clear biological segregation, the colon samples show a gene expression pattern that is more closely related with bacteria in exponential phase, compared to those in stationary phase culture as we can see on the cluster dendrogram above the heatmap on fig 5B (clustering is performed after a Variance Stabilizing Transformation (VST) of the counts data and an euclidean distance is computed between samples, and the dendrogram is built upon the Ward criterion, see methods for more information).



Figure 5. Biological variability is the main source of variance on the experiment A) Variability within all the samples of the experiment - first principal components of the PCA, B) Heatmap and dendrogram obtained from VST-transformed data (see methods).

Characterization of the major physiological differences of E. coli strain 55989 between in vitro and in the colon environment of the mammalian gut

An initial comparison was performed between *in vivo* and *in vitro* gene expression, in an attempt to identify genes and pathways specifically involved in the colonization of the colon by 55989 *E. coli*. This comparison was performed by searching for the over or under expressed genes in colon when in comparison with exponential or stationary phase (fig 6). Genes equally over or under-expressed when comparing exponential and stationary were discarded, in order to focus the analysis on the conditions specific to the colon environment, and that might ultimately contribute to the physiological conditions that influence phage infection. Below we discuss some genes that were highlighted by our analysis.



Figure 6. Venn diagrams of over and under-expressed genes in the colon in comparison with the two in vitro conditions. CvsEXP – comparison colon with exponential and CvsSTA – comparison colon with stationary. 156 and 53 are the number of genes being over or under-expressed just in the colon samples.

Siderophores uptake and export down-regulation

The genes *entS*, responsible for export of enterobactin (Furrer et al., 2002) (siderophores that would allow *E. coli* to scavenge iron from the gut) to the exterior of the cell and *entC* involved in its biosynthesis (Fleming et al., 1983), show a decrease in expression when comparing colon data with both exponential (CvsExp) and stationary (CvsSta) phase. At the same time, genes *fepD* and *fhuD* involved in the import of enterobactin and iron-hydroxamate transporters (eg ferrichrome, aerobactin) respectively, are also down-regulated. Similarly, gene *fecl*, coding for the sigma-factor responsible for regulation of the genes responsible for the iron dicitrate transport (*fec*) is also under-expressed, as well as genes *yncE* and *yncD*, predicted to be associated with iron transport. This strongly suggests a global down-regulation of siderophores and iron-uptake systems by *E. coli* strain 55989 residing in the colon environment (fig7, table1 in orange). Siderophores are considered as virulence factors or virulence modulators (Kirienko et al., 2013) and mammals have evolved several mechanisms of protection against them, such as Lipocalin-2 for example. Lipocalin-2 is a molecule produced by the mammalian immune system that sequesters enterobactin siderophores (Singh et al., 2015). We can therefore hypothesise that 55989 *E. coli* reduces the expression of such genes in order to escape detection by, or activation of, the immune system.



Figure 7. Network of under-expressed genes in the colon. A network analysis was performed according to translated protein–protein interactions using STRING database on the under-expressed genes in the colon in comparison with stationary and exponential samples. Original transcriptomic data with annotations are given in table 1.

55989 E. coli experiences metabolism modulation in the mammalian gut

The genes with the highest increase in expression in bacteria retrieved from the colon were membrane transporter proteins, ompG and ycjV (table2). YcjV has been predicted has a component of an ABCtransporter, however no further characterization has been performed. OmpG protein, is a porin that has been shown to have a nonspecific function and to produce larger channels than OmpF and OmpC, which allows not only the efficient transport of monosaccharides, but also facilitates the diffusion of more complex molecules like dissacharides (sucrose) and trisaccharides (raffinose) (Fajardo et al., 1998). Moreover, ompG has been previously shown not to be expressed in laboratory conditions in E. coli (Fajardo et al., 1998). Interestingly, within the set of the most expressed genes in the colon, we observed that several belong to the pathway responsible for the molecular control of sucrose utilization. This pathway is composed by four genes cscB, which codes for the sucrose permease, cscA, which is the sucrose hydrolase or invertase, cscK, functions as fructokinase and the transcriptional regulator, cscR (fig8, table 2 in yellow) (Sabri et al., 2013). Our data supports the hypothesis that sucrose is an important sugar for bacteria (in particular, pathogenic bacteria) in the gut (Conway and Cohen, 2015). However, sucrose is not the only sugar with an increase in its metabolism, our analysis also shows also an up-regulation of several genes responsible for the metabolism of gluconate (*idnO*), glucurate (gudP, gudX, garL), galactarate (garD) and also galactonate (dgoD, dgoT), consistent with the hypothesis that pathogenic E. coli can metabolize different sugar acids whilst in the mammalian gut (fig43, table2 in coral) (Fabich et al., 2008; Suvorova et al., 2011). Furthermore, our results also revealed an over-expression of genes involved in the utilization of ethanolamine (eutN, eutG, eutJ and eutP), suggesting that this molecule can be used as carbon and/or nitrogen source in the gut. Ethanolamine is a compound that is derived from the membrane of cells and it has been suggested as an important metabolic source that serves as an advantage to pathogenic E. coli facilitating it to outcompete commensal *E. coli* (Bertin et al., 2011).

Overall, our results are consistent with the nutrient-niche hypothesis, suggested by Freter et al (Freter et al., 1983), which postulates that each individual species has a preference for one, or a few nutrients available in their environment, which creates a nutrient-defined niche that is occupied by a specific species. Given the reported low abundance of *E. coli* in the human gut, the nutrients available for *E. coli* must be low and therefore these bacteria must be ready to quickly adjust between a variety of different sources of nutrients (generalist), which is a clearly an advantageous strategy in these fluctuating environment (Chang et al., 2004; Peekhaus and Conway, 1998).

Oxygen fluctuations lead to overexpression of anaerobic pathways

E. coli has been shown to use the cytochome bo oxidase pathway in the presence of high oxygen levels, while cytochrome bd-I oxidase genes are expressed under low levels of oxygen (Jones et al., 2007). Our transcriptional comparison analysis between *in vivo* and *in vitro* samples revealed a down-regulation of the cytochome bo oxidase pathway (*cyoABCD*) in the colon (fig7, table1 in green). This is consistent with the oxygen limitation experienced by *E. coli* in the mammalian gut, and with the previous studies that report that this pathway is not to be essential for *E. coli* colonization of the mammalian gut (Jones et al., 2007). On the other hand, the expression of the fumarate metabolism (operon *frdBCD*) increased between 2 and 3 log2 fold changes in the colon samples, and fumarate hydratase (*fumB*) expression was also increased (fig8, table2 in green). Since *fumB* is regulated by anaerobic conditions and iron availability this suggests that despite the down-regulation of siderophores pathways, iron uptake is still being efficiently completed by other pathways allowing *E. coli* to scavenge this essential metal.

Table1. Log2-fold change of the genes found under-expressed in the *E. coli* 55989 colonizing the colon in comparison with the exponential and stationary 55989 growth cultures (colours represent the different pathways addressed on the text).

Under-expressed						
log2fol		dchange	function			
gene	Cvsexp	Cvssta				
ymgB	-5.412	-5.225	cellular response to acid chemical			
суоС	-5.309	-4.042	Cytochrome bo(3) ubiquinol oxidase subunit 3			
суоА	-5.137	-5.428	Cytochrome bo(3) ubiquinol oxidase subunit 2			
суоВ	-5.042	-4.094	Cytochrome bo(3) ubiquinol oxidase subunit 1			
fliE	-4.807	-2.92	Flagellar hook-basal body complex protein			
суоD	-4.806	-3.805	Cytochrome bo(3) ubiquinol oxidase subunit 4			
ymgA	-4.678	-4.09	Probable two-component-system connector protein YmgA			
lldP	-4.481	-4.123	L-lactate permease			
ybaQ	-4.366	-3.095	Uncharacterized HTH-type transcriptional regulator YbaQ			
EC55989_2605	-4.316	-4.07	DNA transfer protein			
ymgF	-4.073	-2.54	Inner membrane protein YmgF			
ycgZ	-3.973	-5.581	two-component-system connector protein			
putP	-3.899	-2.2	N/A			
EC55989_3383	-3.897	-3.597	Uncharacterized protein			
fepD	-3.876	-4.446	Ferric enterobactin transport system permease protein			
fecl	-3.681	-5.27	Probable RNA polymerase sigma factor			
lldR	-3.58	-2.736	Putative L-lactate dehydrogenase operon regulatory protein			
glpD	-3.57	-3.936	Aerobic glycerol-3-phosphate dehydrogenase			
суоЕ	-3.497	-2.582	Protoheme IX farnesyltransferase			
betA	-3.364	-2.393	Oxygen-dependent choline dehydrogenase			
EC55989_3834	-3.27	-2.233	N/A			
ynaJ	-3.225	-2.702	Uncharacterized protein			
crl	-3.223	-2.922	Sigma factor-binding protein			
ybjG	-3.026	-2.993	Putative undecaprenyl-diphosphatase			
entC	-3.023	-4.502	Isochorismate synthase			
yncE	-2.952	-3.965	Uncharacterized protein			
proV	-2.935	-2.299	Glycine betaine/proline betaine transport system ATP-binding protein			
fadL	-2.873	-4.251	Long-chain fatty acid transport protein			
proP	-2.866	-4.33	Proline/betaine transporter			
betB	-2.836	-3.751	NAD/NADP-dependent betaine aldehyde dehydrogenase			
yncD	-2.817	-4.107	TonB-dependent receptor			
ybcS	-2.679	-2.226	Lysozyme RrrD			
fhuD	-2.657	-1.948	Iron(3+)-hydroxamate-binding protein			
EC55989_0557	-2.618	-2.645	Uncharacterized protein			
EC55989_2615	-2.614	-2.375	Putative terminase small subunit			
EC55989_3897	-2.611	-3.026	N/A			
ylaC	-2.576	-2.114	Inner membrane protein			
gadW	-2.523	-4.024	HTH-type transcriptional regulator			
ompX	-2.458	-4.226	Outer membrane protein			
cysC	-2.348	-3.737	Adenylyl-sulfate kinase			
EC55989_2613	-2.249	-2.514	Portal protein p19			
ybjX	-2.243	-2.454	Uncharacterized protein			
kefG	-2.225	-4.068	Glutathione-regulated potassium-efflux system ancillary protein			
fabB	-2.224	-2.078	3-oxoacyl-[acyl-carrier-protein] synthase 1			
msrB	-2.207	-3.27	Peptide methionine sulfoxide reductase MsrB			
EC55989_tRNA26	-2.201	-2.668	N/A			
EC55989_2614	-2.19	-3.542	N/A			
ybdA	-2.166	-2.997	Enterobactin exporter EntS			
glpE	-2.146	-2.57	Thiosulfate sulfurtransferase			
EC55989_0285	-2.112	-2.174	Uncharacterized protein			
glnL	-2.106	-2.985	Sensory histidine kinase/phosphatase			
gltA	-2.087	-2.154	Citrate synthase			



Figure 8. Network of over-expressed genes in the colon. A network analysis was performed according to translated protein–protein interactions using STRING database on the over-expressed genes in the colon in comparison with stationary and exponential samples. Original transcriptomic data with annotations are given in table 2.

Table2. Log2-fold change of the genes found over-expressed in the *E. coli* 55989 colonizing the colon in comparison with the exponential and stationary 55989 growth cultures (colours represent the different pathways addressed on the text).

Over-expressed					
log2foldchange		change	function		
gene	Cvsexp	Cvssta			
ompG	9.805	10.086	Outer membrane protein		
ycjV	9.134	9.394	Putative uncharacterized ABC transporter ATP-binding protein		
cscK	7.93	8.282	Fructokinase (ATP + D-fructose = ADP + D-fructose 6-phosphate)		
lacZ	6.839	6.911	Beta-galactosidase		
cscB	6.51	7.645	N/A		
ygeW	6.338	7.786	Putative carbamoyltransferase		
cscA	5.807	6.557	Sucrose-6-phosphate hydrolase		
cchB	5.674	3.924	Ethanolamine utilization protein		
ygfK	5.612	5.316	Putative oxidoreductase		
ydhV	5.585	5.849	Uncharacterized oxidoreductase		
glxR	5.537	3.83	2-hydroxy-3-oxopropionate reductase		
melB	5.27	6.022	Melibiose carrier protein		
fumB	5.203	5.726	Fumarate hydratase class I, anaerobic		
ssnA	5.144	5.96	Putative aminohydrolase		
ydjH	5.104	4.612	Uncharacterized sugar kinase		
yqeB	5.066	4.474	Uncharacterized protein		
ygfU	5.052	4.196	Uric acid transporter		
ygfM	4.998	4.993	Uncharacterized protein		
EC55989_3335	4.988	3.077	N/A		
ydhW	4.867	5.537	Uncharacterized protein		
yjiM	4.723	3.752	Putative dehydratase subunit		
hgdC	4.719	6.069	HgdC protein (possible:ATPase, activator of (R)-hydroxyglutaryl-CoA dehydratase		
ygeX	4.654	4.301	Diaminopropionate ammonia-lyase		
yjiH	4.628	5.355	Uncharacterized protein		
EC55989 1671	4.623	4.449	Permease IIC component		
ybiY	4.599	3.233	Putative pyruvate formate-lyase 3-activating enzyme		
srlB	4.505	4.866	PTS system glucitol/sorbitol-specific EIIA component		
xdhC	4.5	4.977	Putative xanthine dehydrogenase iron-sulfur-binding subunit		
dgoD	4.433	2.457	D-galactonate dehydratase		
ygeY	4.43	3.553	Uncharacterized protein		
fixA	4.38	3.176	Protein FixA		
ynfG	4.319	2.746	Probable anaerobic dimethyl sulfoxide reductase chain		
eutP	4.314	4.223	Ethanolamine utilization protein		
ynfF	4.294	3.209	Probable dimethyl sulfoxide reductase chain		
fucA	4.269	3.337	L-fuculose phosphate aldolase		
xdhD	4.164	3.415	Probable hypoxanthine oxidase		
ydhT	4.163	2.743	Uncharacterized protein		
idnD	4.128	4.134	L-idonate 5-dehydrogenase (NAD(P)(+))		
araF	4.114	2.82	L-arabinose-binding periplasmic protein		
ygfT	4.028	3.323	Uncharacterized protein		
gudP	3.996	4.323	Probable glucarate transporter		
xdhA	3.978	3.885	Putative xanthine dehydrogenase molybdenum-binding subunit		
EC55989 1672	3.975	3.172	Putative cellobiose-specific phosphotransferase enzyme IIB component		
 xdhB	3.945	5.163	Putative xanthine dehydrogenase FAD-binding subunit		
ybbW	3.944	2.929	Putative allantoin permease		
fixC	3.886	2.797	Protein FixC		
yeiT	3.763	5.586	NAD-dependent dihydropyrimidine dehydrogenase subunit		
zraP	3.687	4.193	Zinc resistance-associated protein		
vdcA	3.656	2.946	Uncharacterized protein		
yghD	3.637	4.97	Alcohol dehvdrogenase		
allD	3.608	3.162	Ureidoglycolate dehydrogenase (NAD(+))		
ydeN	3.605	4.255	Uncharacterized sulfatase		

Over-expressed					
	log2foldchange		formation a		
gene	Cvsexp	Cvssta	tunction		
ybiW	3.582	3.739	Putative formate acetyltransferase 3		
eutG	3.573	2.358	Ethanolamine utilization protein EutG		
ydhU	3.52	3.433	Putative cytochrome		
ygcW	3.503	3.866	Uncharacterized oxidoreductase		
caiT	3.492	2.499	L-carnitine/gamma-butyrobetaine antiporter		
ydhX	3.47	5.166	Uncharacterized ferredoxin-like protein		
yjiG	3.451	4.492	Inner membrane protein YjiG;yjiG;ortholog		
pyrB	3.408	4.606	Aspartate carbamoyltransferase catalytic subunit		
fixB	3.359	2.64	Protein FixB		
hyuA	3.331	2.916	D-phenylhydantoinase		
xylF	3.33	4.583	D-xylose-binding periplasmic protein		
uspF	3.306	3.886	Universal stress protein F		
pspE	3.289	5.144	Thiosulfate sulfurtransferase		
yeel	3.271	3.873	Protein MtfA		
fucO	3.237	2.565	Lactaldehyde reductase		
hypC	3.197	3.472	Hydrogenase maturation factor		
ynfE	3.157	3.17	Putative dimethyl sulfoxide reductase chain		
carB	3.154	2.149	Carbamoyl-phosphate synthase large chain		
ibrA	3.135	2.838	catalytic activity (possible Immunoglobulin-binding regulator)		
EC55989 3295	3.11	3.004	Uncharacterized protein		
gldA	3.108	2.872	Glycerol dehydrogenase;gldA		
kdgT	3.1	2.251	2-keto-3-deoxygluconate permease		
hybF	3.095	4.449	Hydrogenase maturation factor		
hybE	3.053	4.153	Hydrogenase-2 operon protein		
ydjK	3.046	4.42	Putative metabolite transport protein		
C0664	3.031	2.236	N/A		
sgbU	3.022	4.146	Putative L-ribulose-5-phosphate 3-epimerase		
yiaK	3.014	2.868	2,3-diketo-L-gulonate reductase		
ydjl	3.011	4.49	Uncharacterized protein		
ydiL	2.966	3.356	Uncharacterized protein		
EC55989_3297	2.941	2.435	Uncharacterized protein		
ycjS	2.919	2.738	Uncharacterized oxidoreductase		
garR	2.916	3.243	2-hydroxy-3-oxopropionate reductase		
ulaA	2.902	3.067	Ascorbate-specific PTS system EIIC component		
ytfR	2.897	2.102	Uncharacterized ABC transporter ATP-binding protein		
rhaB	2.881	3.514	L-Rhamnulokinase		
tdcF	2.878	2.615	Putative reactive intermediate deaminase		
EC55989_3298	2.848	3.727	N/A		
ydjL	2.819	2.417	Uncharacterized zinc-type alcohol dehydrogenase-like protein		
EC55989_2250	2.816	2.65	Uncharacterized protein		
garL	2.792	3.876	5-keto-4-deoxy-D-glucarate aldolase		
EC55989_1408	2.776	2.606	Tail assembly protein I		
yiaO	2.775	3.051	2,3-diketo-L-gulonate-binding periplasmic protein		
idnO	2.773	2.031	5-keto-D-gluconate 5-reductase		
gudX	2.77	2.086	Glucarate dehydratase-related protein		
frdD	2.761	3.303	Fumarate reductase subunit D		
dmsC	2.751	2.251	Anaerobic dimethyl sulfoxide reductase chain C		
hybD	2.747	3.799	Hydrogenase 2 maturation protease		
exuT	2.726	2.851	Hexuronate transporter		
hybC	2.712	3.507	BU Hydrogenase-2 large chain		
purD	2.697	2.739	Phosphoribosylamineglycine ligase		
EC55989_3929	2.679	2.767	Tn7-like transposase TnsA		

Over-expressed				
	log2foldchange		function	
gene	Cvsexp	Cvssta	Tunction	
livK	2.676	3.517	Leucine-specific-binding protein	
eutJ	2.653	2.924	Ethanolamine utilization protein	
dgoT	2.653	4.223	D-galactonate transporter	
ulaE	2.646	3.536	L-ribulose-5-phosphate 3-epimerase	
yeaU	2.623	1.983	D-malate dehydrogenase [decarboxylating]	
EC55989_3306	2.563	2.276	Uncharacterized protein	
EC55989_3288	2.561	4.212	N/A	
EC55989_1435	2.557	3.433	Uncharacterized protein	
 J	2.54	2.549	Uncharacterized zinc-type alcohol dehydrogenase-like protein	
sgbH	2.54	3.143	3-keto-L-gulonate-6-phosphate decarboxylase	
EC55989_4883	2.526	4.274	N/A	
gntU	2.487	3.888	Low-affinity gluconate transporter	
tdcC	2.478	3.984	Threonine/serine transporter	
frdC	2.47	3.372	Fumarate reductase subunit C	
idnK	2.464	2.754	Thermosensitive gluconokinase	
yjiD	2.452	3.651	N/A	
livG	2.414	2.372	High-affinity branched-chain amino acid transport ATP-binding protein	
EC55989 3294	2.399	2.637	Uncharacterized protein	
 vbiA	2.385	3.864	N-glycosidase YbiA	
ompW	2.372	2.817	Outer membrane protein	
EC55989 3922	2.317	2.664	Putative transcriptional regulator	
fdhF	2.313	3.284	Formate dehydrogenase H	
vdiX	2.279	2.392	TVP38/TMEM64 family membrane protein	
hisH	2.271	2.522	Imidazole glycerol phosphate synthase subunit	
FC55989_3286	2.268	2.619	Uncharacterized protein	
iadA	2.26	2.59	Isoaspartyl dipeptidase	
vfbO	2.24	3.572	Glutamate-pyruvate aminotransferase	
FC55989_3415	2.239	2.658	Uncharacterized protein	
purM	2.23	1.97	Phosphoribosylformylglycinamidine cyclo-ligase	
FC55989 0235	2.223	1.963	Uncharacterized protein	
metR	2.221	2.281	HTH-type transcriptional regulator	
garD	2.188	3.083	Galactarate dehydratase (L-threo-forming)	
citF	2,168	3.242	Citrate Ivase alpha chain	
vigF	2.165	3.632	2-iminobutanoate/2-iminonronanoate deaminase	
	2.154	2.366	Uncharacterized sugar kinase	
uxaC	2.154	2.959	Uronate isomerase	
rhaS	2.151	2.682	HTH-type transcriptional activator	
vidF	2.134	3.869	Inner membrane protein	
FC55989 4980	2 133	2 924	Uncharacterized protein	
FC55989_3289	2 13	2.28		
	2,129	3.752	Aspartate carbamovitransferase regulatory chain	
nurF	2 126	2 131	N5-carboxyaminoimidazole ribonucleotide mutase	
citC	2 113	3 115	[Citrate [nro-35]-lyace] ligace	
viiM	2.086	2,157	Protein YiiM	
laci	2 083	2 068		
vøfO	2.071	2.126	Guanine/hypoxanthine permease	
FC55989 1864	2.071	2 289	Uncharacterized protein	
vkøG	2.007	3 854		
hcr	2.002	3 505		
FC55080 2781	2.045	2 444		
frdR	2.003	3.067	Fumarate reductase iron-sulfur subunit	
viiF	2.007	2 107	HTH-type transcriptional regulator	
ינע א	2.000	2.10/		

Comparative expression analysis of the 55989 natural plasmid

As an opportunistic pathogen, enteroaggegative *E. coli* 55989 possess several virulence factors. Some of these factors have been found to be encoded in its natural plasmid, therefore, and in order to perceive its expression in *in vivo* conditions, we included it within our transcriptomic analysis. An initial analysis of the data revealed that the samples of the three conditions have a total number of reads matching the natural plasmid sequence of strain 55989 with a number of reads between 0.035 million and 0.01 million, which corresponds to 70 and 100 times less than genomic reads, in agreement with the difference between the genome and the plasmid sizes (71x). We could observe a differential pattern in plasmid genes expression between the different conditions (fig9).



Figure 9. Biological variability is the main source of variance on the plasmid comparative analysis. A) Variability within the experiment - first principal components of the PCA; B) Heatmap and dendrogram obtained from VST-transformed data (see methods).

Adhesins and aggreagative fimbriae virulence factors overexpression is promoted in the mammalian gut

Enteroaggregative *E. coli* pathogenicity has been shown to be associated with the adherence to the mucosal layer of the intestine, from where they then release toxins (enterotoxins or cytotoxins) that damage the mucosal layer and subsequently the epithelial cells, inducing inflammation that ultimately leads to diarrhoea (Boll et al., 2017; Weintraub, 2007).

This adhesion phenotype is promoted by the production of aggregative adherence fimbriae (AAFs), that are related to Dr adhesins (Korotkova et al., 2006) and were reported to mediate the adherence to the intestinal mucosal layer. The biosynthesis of these structures is encoded by genes that can be found in the natural 55989 plasmid, belonging to the pAA plasmid family (Croxen and Finlay, 2010).

We found an overexpression of genes in the colonic environment (compared to exponential and stationary cells), both in the aforementioned plasmid and in chromosomal regions, that are relevant to adhesion mechanisms. These include adhesion biosynthesis (agg3C, agg3D) genes and also aggregative adherence fimbriae production gene ((pEC55989_0069) (table3), the three encoded in the plasmid, or genes described to promote adhesion at the expense of motility encoded in the chromosome (uspF) (table2). On the other hand, we also observed a decrease in the expression of the outer-membrane protein X (ompX), in agreement with previous studies that associate this repression to an increase of *E. coli* adhesion by type-1 fimbriae (Otto et al., 2001). These results show that the gut environment causes an up-regulation of some of 55989 *E. coli* virulence factors, of which some are carried by its natural plasmid and others are present in the bacterial chromosome.

Table3. Log2-fold change of the genes found over-expressed on the plasmid of the *E. coli* 55989colonizing the colon in comparison with the exponential and stationary 55989 growth cultures.

Over-expressed					
	log2fold	dchange	f		
gene	Cvsexp	Cvssta	Tunction		
pEC55989_0069	5.778	7.632	aggregative adherence fimbriae I protein (Fragment) (modular protein)		
pEC55989_0070	4.792	6.23	putative transposase ORF B (fragment) IS3		
pEC55989_0083	3.459	4.822	extrachromosomal origin - putative insertion sequence		
Agg3C	3.388	3.171	Outer membrane usher protein Agg3C		
pEC55989_0064	3.271	3.428	putative transposase (fragment)%2C IS630 family		
agg3D	2.857	2.255	Chaperone protein Agg3D		
agg3B	2.033	2.149	Protein Agg3B%2C putative invasin		

Colonic environment leads to an overexpression of transposases present in the natural plasmid of E coli 55989

We also detected an *in vivo* overexpression of two transposases that are found in the natural plasmid of strain 55989. One is an insertion sequence from the family of IS630, the other is a putative transposase. In work performed before my thesis we have shown the insertion sequences play an important role on the short term *E. coli* adaptation including in the mammalian gut, with several *in vivo* adaptation studies showing that the disruption of gene expression (by IS interruption of regulators or the genes themselves) might be involved in *E. coli*'s gut colonization (Barroso-Batista et al., 2014; Lourenco et al., 2016).

Transcriptomic analysis of factors that may influence phage infection

Next, we focused the transcriptomics analysis on the potential similarities between the expression profile of *E. coli* 55989 colonising the mouse colon and in planktonic liquid cultures reaching stationary growth phase (two conditions where phage infection is less efficient), but distinct of the exponential samples (where phage infection occurs normally). This analysis was performed in order to determine a possible cause for the differential phage replication rates *ex vivo* observed in the colon and stationary conditions (fig 2).

We performed a comparative analysis using a custom script that performs all the pairwise comparisons between the gene lists from the different samples (see methods). Our analysis revealed 238 candidate genes with similar expression between the colon and stationary phase. From these, 143 genes were under-expressed (fig10) while 95 were over-expressed (fig11). From this set of genes, several are consistent with the influence of physiological state of bacteria on the phage infection and/or replication: we found a high proportion of down-regulated genes related to cell wall structures (to which phages generally rely for adsorption), such as the whole flagellum biosynthesis genes, several genes involved in chemotaxis, some transporters and also LPS-biosynthesis related genes.



Figure 10. Network of over-expressed genes shared by colon and stationary samples. A network analysis was performed according to translated protein–protein interactions using STRING database on the under-expressed genes shared by colon and stationary samples in comparison with exponential samples. Original transcriptomics data with annotations are given in table 4.

Table4. Log2-fold change of the genes found under-expressed in the *E. coli* 55989 colonizing the colonand in stationary phase growth culture in comparison with the exponential growth cultures.

Under-expressed					
	log2foldchange		for a strengt		
gene	Cvsexp	stavsexp	TUNCTION		
5055000 2444	7 526	7 207	FliC, or flagellin, is the basic subunit that polymerizes to form the rigid flagellar filament of		
EC55989_2144	-7.526	-7.207	Escherichia coli.		
treB	-6.992	-8.029	PTS system trehalose-specific EIIBC component		
EC55989_1639	-6.826	-8.296	N/A		
fliA	-6.736	-7.499	RNA polymerase sigma factor		
narK	-6.69	-6.304	Nitrate/nitrite transporter		
shf	-6.628	-5.237	N/A		
fliN	-6.188	-5.724	Flagellar motor switch protein		
tap	-5.949	-5.236	Methyl-accepting chemotaxis protein IV		
fliF	-5.81	-6.753	Flagellar M-ring protein		
flhB	-5.746	-6.336	Flagellar biosynthetic protein		
motA	-5.717	-6.13	Motility protein A		
fliH	-5.699	-4.684	Flagellar assembly protein		
fliJ	-5.681	-6.607	Flagellar Flij protein		
FC55989 4951	-5.638	-5.743	Glycosyl transferase:protein		
fliO	-5.523	-5.112	Flagellar biosynthetic protein		
nirC	-5.48	-4 566	Nitrite transnorter		
treC	-5 421	-6 409	Trebalose-6-phosphate hydrolase		
tsr	-5 409	-5 982	Methyl-accenting chemotaxis protein l		
elhA	-5 345	-3 51			
flgC	-5 33	-7 16	Elagellar basal-body rod protein		
cheB	-5 318	-5 871	Chemotaxis response regulator protein-dutamate methylecterase		
ubnT	-5.310	-3.071	Herose-&-nhosnhate-nhosnhate-antiporter		
vic7	-5.134	-5.064			
yjcz vbiH	-5.072	-1.024			
FC55989 21/15	-5.034	-4.934	flagellar canning protein flip		
flal	-5.048	-5.491			
tar	-1.045	-0.37	Methyl-accepting chemotavis protein II		
flaD	-4.933	-4.917	Recal body rod modification protein		
flgK	4.924	2 011	Elagellar book associated protein 1		
vidA	-4.863	-3.911			
flaN	4.004	-3.301 E 43			
narG	-4.032	-5.45	Fidgelid sylltilesis protein		
riard cho A	-4.704	-3.790	Chamatavic protoin		
flind	-4.779	-3.900	Elagellar motor switch protein		
motD	-4.077	-4.649	Flagellar motor switch protein		
fiit	-4.000	-5.194			
1111 vicC	-4.028	-4.555			
flaM	-4.010	-5.45	Negative regulator of flagollin synthesic		
fili	-4.595	-4.634			
1111 fl:r/	-4.527	-5.302	Flagellum-specific A IP synthase		
fink	-4.431	-4.209	Flagellar hook-length control protein		
fige	-4.396	-4.427	Flagellar hook protein		
	-4.358	-3.991	Peptidoglycan hydrolase		
cne2	-4.336	-2.83	Protein phosphatase		
CNEK	-4.306	-3.891	Chemotaxis protein methyltransferase		
figA	-4.285	-5.229	Fiagelia basal body P-ring formation protein		
yodA	-4.237	-3.452	N/A		
yaiE	-4.214	-4.114	UPFU345 protein YaiE; yaiE; ortholog		
flgG	-4.052	-3.285	Flagellar basal-body rod protein		
yjdK	-3.982	-4.935	N/A		
tliP	-3.972	-4.143	Flagellar biosynthetic protein		
cheY	-3.952	-4.364	Chemotaxis protein		
fecC	-3.856	-4.191	Fe(3+) dicitrate transport system permease protein		

gene log2foldchange function	
gene Cvsexp stavsexp	
flis -3.837 -5.348 Flagellar secretion chaperone	
ycgR -3.776 -4.097 Flagellar brake protein	
EC55989_4872 -3.718 -2.202 N/A	
flhA -3.63 -3.953 Flagellar biosynthesis protein	
flgF -3.521 -3.572 Flagellar basal-body rod protein	
vpeC -3.52 -4.929 N/A	
EC55989 tRNA91 -3.5 -4.836 N/A	
flgL -3.462 -3.197 Flagellar hook-associated protein 3	
EC55989 4866 -3.397 -2.484 hypothetical protein, putative exported protein -	
flhE -3.39 -4.63 Flagellar protein	
cheW -3.389 -3.804 Chemotaxis protein	
ykgB -3.379 -3.147 Inner membrane protein	
fruA -3.375 -2.8 PTS system fructose-specific EIIB'BC component	
EC55989 5013 -3.362 -2.862 N/A	
vobG -3.354 -2.908 conserved hypothetical protein.	
FC55989 4870 -3 349 -2 565 N/A	
EC55989 tRNA71 -3.319 -4.778 N/A	
vdiB -3 317 -3 769 Protein Ves	
FC55989 4860 -3 283 -3 016 putative operan control protein -	
EC55090 +000 -5.205 -5.010 putative operation control protein-	
voil 2 22 4 470	
vdbC 2 102 2 0E1	
EC55989_4858 -3.11/ -4.43 IV/A	
ginp -3.106 -2.725 Giutamine transport system permease protein	
EC55989_TKNA69 -3.096 -4.205 N/A	
dnak -3.029 -4.396 PEP-dependent dinydroxyacetone kinase, dinydroxyacetone-bindin	gsubunit
yeb0 -3.01/ -3.1/4 Uncharacterized protein	
EC55989_TRNA70 -3.014 -4.979 N/A	
EC55989_4598 -2.993 -2.905 alkylphosphonate uptake protein in phosphonate metabolis	n
EC55989_4869 -2.98 -4.011 N/A	
yncl -2.975 -2.137 N/A	
sdaC -2.969 -2.32 Serine transporter	
EC55989_tRNA89 -2.939 -4.779 N/A	
yehC -2.927 -3.153 Probable fimbrial chaperone	
rbsC -2.925 -3.798 Ribose import permease protein	
EC55989_2594 -2.902 -3.475 N/A	
EC55989_1577 -2.885 -2.06 N/A	
EC55989_4859 -2.85 -2.625 putative transcriptional regulator -	
yghG -2.84 -4.634 Uncharacterized lipoprotein	
ykgH -2.815 -3.425 Uncharacterized protein	
yafT -2.802 -3.607 Uncharacterized lipoprotein	
EC55989_4873 -2.795 -3.975 N/A	
gltL -2.786 -2.083 Glutamate/aspartate import ATP-binding protein	
fliZ -2.75 -3.018 Regulator of sigma S factor	
EC55989_4882 -2.728 -3.781 N/A	
EC55989_tRNA92 -2.683 -4.327 tRNA-Met -	
modB -2.683 -3.878 Molybdenum transport system permease protein	
EC55989_2618 -2.666 -2.286 hypothetical protein - unknown space extracromossomal original extraction by the second	in
yqeH -2.659 -3.283 Uncharacterized protein	
EC55989 tRNA68 -2.642 -4.168 N/A	
EC55989 4857 -2.64 -2.216 tetracvcline repressor protein TetR	
modA -2.638 -3.062 Molybdate-binding protein	

Under-expressed				
	log2fold	dchange	function	
gene	Cvsexp	stavsexp	Tuliction	
sdaB	-2.622	-3.747	L-serine dehydratase 2	
EC55989_4125	-2.62	-3.163	hypothetical protein - unknown space	
ybcY	-2.608	-3.974	Putative uncharacterized protein	
rfaL	-2.58	-3.072	O-antigen ligase	
yggX	-2.511	-4.218	Probable Fe(2+)-trafficking protein	
rbsA	-2.508	-3.942	Ribose import ATP-binding protein	
udp	-2.507	-3.774	Uridine phosphorylase	
ryfB	-2.491	-2.949	misc_RNA -	
yiiU	-2.443	-3.289	N/A	
gInQ	-2.423	-2.751	Glutamine transport ATP-binding protein	
EC55989_5015	-2.416	-3.929	N/A	
htrL	-2.405	-2.683	Protein HtrL	
yhcN	-2.401	-2.929	Uncharacterized protein	
yjfZ	-2.323	-2.252	Uncharacterized protein	
yfcL	-2.319	-3.821	Uncharacterized protein	
virK	-2.318	-2.484	regulator of virG protein -	
nupC	-2.302	-3.997	Nucleoside permease	
EC55989 2600	-2.286	-3.249	N/A	
yliF	-2.286	-3.142	Probable diguanylate cyclase	
EC55989_4856	-2.26	-3.759	ArsR family transcriptional regulator	
yliE	-2.23	-3.419	Probable cyclic di-GMP phosphodiesterase	
EC55989_tRNA77	-2.151	-2.805	tRNA-Ser -	
EC55989 4529	-2.128	-3.297	N/A	
ilvC	-2.116	-4.078	Ketol-acid reductoisomerase (NADP(+))	
ycgX	-2.115	-3.911	Uncharacterized protein	
ygfA	-2.115	-2.654	5-formyltetrahydrofolate cyclo-ligase	
gltP	-2.082	-4.035	Proton/glutamate-aspartate symporter	
EC55989_1378	-2.068	-2.443	N/A	
yeeX	-2.065	-3.389	UPF0265 protein YeeX	
yobF	-2.054	-2.917	Protein YobF	
yncM	-2.049	-2.399	N/A	
yedV	-2.038	-3.157	Probable sensor-like histidine kinase	
yifE	-2.008	-3.509	UPF0438 protein YifE	
epaO	-2.951	-3.304	pseudogene, fragment of Type III secretion apparatus protein (part 2),	
emrE	-1.665	-0.761	Multidrug transporter	
EC55989_4689	-2.145	-2.264	hypothetical protein - unknown space	
EC55989_tRNA17	-2.254	-2.668	tRNA-Arg -	
yccE	-2.217	-2.413	Uncharacterized protein YccE;yccE;ortholog	
efeO	-2.505	-2.57	ferrous iron transport binding protein -	

On the other hand, we found an over-expression of genes related to biofilm and communication (AI-2 signal), as well as genes that find homologues in related with prophage genomes (mostly structural proteins like tail proteins). We also found an over-expression of genes involved in sugar degradation and transport pathways, some of which include the assembly of proteins that can act as receptors for some phages.



Figure 11. Network of over-expressed genes shared by colon and stationary samples. A network analysis was performed according to translated protein–protein interactions using STRING database on the over-expressed genes shared by colon and stationary samples in comparison with exponential samples. Original transcriptomic data with annotations are given in table 5.

Table 5. Log2-fold change of the genes found over-expressed in the *E. coli* 55989 colonizing the colonand in stationary phase growth culture in comparison with the exponential growth cultures.

Over-expressed				
	log2foldcha		function	
gene	Cvsexp	stavsexp	Iditation	
bssR	6.391	6.063	Biofilm regulator	
yebV	5.068	6.945	Uncharacterized protein	
pps	4.798	3.2	Phosphoenolpyruvate synthase	
ytfQ	4.519	3.752	ABC transporter periplasmic-binding protein	
yjfN	4.122	3.989	Uncharacterized protein	
Т	4.099	4.313	Minor tail protein T	
hyaF	4.096	2.835	Hydrogenase-1 operon protein	
yniA	4.053	2.12	Putative kinase	
eutB	4.01	3.337	Ethanolamine ammonia-lyase heavy chain	
yicO	3.87	2.05	Adenine permease	
cchA	3.671	2.455	Ethanolamine utilization protein eutM	
hyaB	3.652	3.481	Hydrogenase-1 large chain	
EC55989_1395	3.644	5.288	N/A	
allB	3.642	4.082	Allantoinase	
ibrB	3.615	2.764	transcriptional regulator	
EC55989 3339	3.604	4.269	N/A	
EC55989 3338	3.543	3.813	conserved hypothetical protein	
 mokB	3.518	2.364	Regulatory protein	
vdiH	3.517	3.479	Uncharacterized protein	
EC55989 1402	3.461	4.308	prophage functions, minor tail protein T	
leuA	3.456	2.687	2-isopropylmalate synthase	
eutC	3,391	2.65	Ethanolamine ammonia-lyase light chain	
FC55989 3337	3.36	2,486	N/A	
eles	3,338	3.504	Surface composition regulator	
vlil	3,303	3.66	Aldose sugar dehvdrogenase	
araH	3.271	2,493	I-arabinose transport system permease protein	
FC55989 3296	3.179	2.027	N/A	
EC55989 1692	3.171	3.748	prophage functions. Putative Lom-like outer membrane protein	
hvaD	3 14	2 538	Hydrogenase 1 maturation protease	
vaeA	3 123	2 361	Carbamate kinase-like protein	
eutl	3.115	2,699	Ethanolamine utilization protein	
hvaC	3.075	3,492	Probable Ni/Fe-hydrogenase 1 B-type cytochrome subunit	
sokB	3 027	3 645	EC55989 misc RNA 34	
lsrC	3 009	3 537	Autoinducer 2 import system permesse protein	
FC55989 3334	3,006	2 602	nutative chaperone clnB	
vdbC	2 997	3 197	Putative oxidoreductase	
FC55989 3340	2 996	3 696	N/A	
csnD	2.550	3 038	Cold shock-like protein	
rihB	2.545	3 421	Pyrimidine-specific ribonucleoside hydrolase	
vhel	2.005	4 389	Lincharacterized protein	
gel	2.075	3 268	Glyoxylate carboligase	
glcG	2.005	2 75	Protein GlcG	
narY	2.032	3 421	Respiratory nitrate reductase 2 beta chain	
vdd⊔	2.025	4 751	kespiratory nitrate reductase 2 beta chain	
vahli	2.013	2 1/17		
FC55080 2272	2.70	2.147	hypothetical protein, putative membrane protein	
EC55980 2177	2.774	2.307	conserved hypothetical protein	
2005_21//	2.733	2.093	Glucose-1-phosphatasa	
αgμ vtfT	2.700	2.009		
	2.051	2.402	Ethonolomine utilization protoin	
eutA	2.040	2.93		
nype vibp	2.538	3.232		
	2.510	2.385		
FC22888_3311	2.46	2.580	N/A	

Over-expressed						
	log2foldchange		function			
gene	Cvsexp	stavsexp	Tunction			
ego	2.443	3.091	Autoinducer 2 import ATP-binding protein LsrA			
mak	2.418	2.348	Fructokinase			
yceF	2.414	3.762	Maf-like protein			
yeeO	2.387	3.195	Probable FMN/FAD exporter			
Ν	2.367	2.926	N/A			
pfIB	2.298	2.231	Formate acetyltransferase 1			
ychM	2.28	2.757	C4-dicarboxylic acid transporter DauA			
galS	2.237	3.681	HTH-type transcriptional regulator			
gst	2.208	3.747	Glutathione S-transferase GstA			
ychH	2.177	3.529	Uncharacterized protein			
EC55989_3424	2.176	2.388	conserved hypothetical protein			
lrhA	2.173	3.516	Probable HTH-type transcriptional regulator			
cbpM	2.146	2.796	Chaperone modulatory protein			
ycbK	2.129	2.301	Uncharacterized protein			
yodC	2.117	3.775	Uncharacterized protein			
rpoS	2.066	2.72	RNA polymerase sigma factor			
EC55989_1410	2.062	3.843	N/A			
ybiC	2.059	2.524	Uncharacterized oxidoreductase			
yhdL	2.047	2.078	Alternative ribosome-rescue factor A			
yfiQ	2.031	2.898	Protein lysine acetyltransferase Pka			

A particular concern of our data analysis was the validation of the approach itself considering the complexity of *in vivo* environments. Particularly, we wondered whether we could find factors associated with changes in gene expression that are actually related with phage-bacteria interactions. In this regard, we found in our dataset several examples of differentially regulated genes that had previously been described to be part of the interactions between bacteria and phage. For instance, one of the genes under-expressed in both colon and stationary phase is the *rfaL* gene, described as the O-antigen ligase. O-antigen has been shown, both in *E. coli* and Salmonella, to be a receptor for phages (Bertozzi Silva et al., 2016; Nobrega et al., 2018).

Moreover, the biosynthesis of the flagellum, another factor that was already shown to be a receptor for several phages in *E. coli* and salmonella, was observed to be completely down-regulated in the colon. This suggests that *in vivo* cells might be lacking a flagellum, and thus be less efficiently predated by phage in the colon (Bertozzi Silva et al., 2016). Overall, the identification of these genes demonstrates the validity of our approach in characterizing the *in vivo* transcriptomic profile of *E. coli*, which might ultimately be key factors to modulate the interaction between bacteria and phage as well. Future work will focus on investigation other genes highlighted by our analysis that were not previously reported to be a part of phage-bacteria interactions, to test their role in this ecological interactions.

The lack of *rfaL* gene (but not *fliA*) decrease the susceptibility to phages CLB_P1

After identification of possible factors that may be involved on phage differential replication, we decided to test knock-out mutants for the *rfaL* gene, encoding the O-antigen ligase and *fliA*, regulating genes involved in motility and flagellar synthesis, in the strain 55989. Phages CLB_P1, P2 and P3 were tested for their efficiency of plating (EOP) in 55989 *E. coli* clones with a knock-out mutation (gene replaced by a kanamycin resistance cassette gene by homologous recombination) of the rfaL and fliA genes. We could observe that deletion of the *fliA* gene did not alter the EOP of the three phages (fig12, table 6). On the other hand, we observed a 3-log decrease in susceptibility of the *rfaL* mutant to phage CLB_P1 (fig12, table 6) and a 0.5 to 1-log increase in susceptibility to phage CLB_P3 (fig12, table 6). Results confirm that genes for which the expression is altered in the gut environment can influence phage infection efficiency. Further studies, as lysis kinetics and adsorption assays, are required in order to better understand this influence.



Figure 12. Deletion of *rfaL* but not *fliA* gene decreases susceptibility of 55989 strain to phage CLB_P1. Several 10-fold dilutions of phage CLB_P1, P2 and P3 were spotted on an overlay of exponentially growing bacteria and incubated at 37° overnight.

Table 6. Efficiency of plating (EOP) calculated for *E. coli* strains $55989\Delta rfaL$ and $55989\Delta fliA$ relative to wt strain 55989.

	CLB_P1	CLB_P2	CLB_P3
55989∆rfaL	2.10x10-3 (+/- 3.11x10-4)	0.96 (+/- 0.16)	2.07 (+/- 0.6)
55989∆ <i>fliA</i>	1.19 (+/- 0.28)	1.23 (+/- 0.33)	0.99 (+/- 0.31)

Identification of non-essential host genes required for infection in a Myoviridade bacteriophage (CLB_P2)

CLB_P2 has been previously described as a virulent phage, from the Myoviridae family, with a genome of 172kb, belonging to the family of T4 phages and, more precisely, closely related to phage JS98 (fig13).



Figure 13. Genomic comparison of phages T4, CLB_P2 and JS98

Screening of the *E. coli* KEIO collection for genes required for permissivity to both CLB_P2 and T4 bacteriophages

After having established a link between differential gene expression of strain 55989 colonising the gut and susceptibility to phage infection, we focused on phage CLB_P2, which was found to be equally efficient in infecting its host in the different intestinal sections (*ex vivo* data – fig2). These observations led us to hypothesise that either CLB_P2 possesses all the enzymes that can compensate for those that the host could down-regulate, and/or that infection from phage CLB_P2 is not relying on any accessory host gene. To test the latter hypothesis we took advantage of the *E. coli* Keio collection (Baba et al., 2006; Yamamoto et al., 2009). The Keio collection is a library of *E. coli* clones that include single Knockout of all the genes non-essential for survival. CLB_P2 was shown to infect the KEIO collection ancestral *E. coli* strain with the same efficiency as the model phage T4. While phage T4 has been studied for years, no systematic screening of host genes required for T4 infection has been reported to date. Therefore, we performed the susceptibility test of the Keio collection towards phages T4 and CLB_P2. In order to detect possible weak alterations of efficiency, we tested for susceptibility of the mutants to both phages at a low MOI (10⁻⁵) (fig14).

Resistant mutant



Figure 14. Example of a double spot test where a phage resistant clone was observed.

Results revealed that only two knocked out clones, *rfaC* (*waaC*) and *lpcA* (*gmhA*) (gene names were changed in order to improve comparative genomics between species (Reeves et al., 1996)), showed a partial resistance to both phages, although EOP's were not possible to calculate, due to lack of detection of isolated plaques (fig 15). These genes are involved in Lipopolysaccharide (LPS) biosynthesis.



Figure 15. Mutations on *lpcA* and *rfaC* genes decreases the susceptibility to phages CLB_P2 and T4. Phage dilutions were spotted on bacteria (mutant strains, 55989 (host CLB_P2), BW25113 (Keio collection background) and BE (host T4) overlayed plate and incubated overnight.

The *rfaC* gene encodes for the ADP-heptose:LPS heptosyltransferase I (HepI) which is involved in the transfer of the first heptose sugar on the LPS inner core during biosynthesis (Kadrmas and Raetz, 1998). Deletion of this gene has been shown to cause a defect in the LPS core heptose region, causing a deeprough phenotype. At the same time, *lpcA* encodes for the sedoheptulose 7-phosphate isomerase which is also involved in the first step of the core LPS biosynthesis performing the isomerization of D-sedoheptulose 7-phosphate to D-glycero-D-manno-heptose 7-phosphate (fig16) (Brooke and Valvano, 1996). Mutation of this gene leads to the synthesis of LPS lacking heptose (Havekes et al., 1976). These data suggest that resistance to CLB_P2 is partially linked to LPS structure. Moreover, the lack of resistance on other LPS combined with the two mutations described here, suggest that CLB_P2 requires the transfer of the isomerised heptose on the LPS in order to successfully infect its host. Results show that phage CLB_P2 may rely in any *E. coli* essential gene or a combination of several non-essential genes, which may in part explain the fact that resistance is not found *in vivo*.

Surprisingly, ompC mutant did not displayed resistance to phage T4 (showing similar infection to KEIO collection background strain), as has been previously described in K12 MG1655 (Yu and Mizushima, 1982), this shows that phage infectivity can change even between similar *E. coli* strains supporting an highly flexible host-range and phage strain specificity.



Figure 16. A schematic diagram of the biosynthetic pathway of the nucleotide precursor ADP-L-glycero-D-mannoheptose, extracted from (Brooke and Valvano, 1996)

The lack of *lpcA* gene in 55989 decreased its susceptibility to phages CLB_P1 and CLB_P2

After identification of the two possible mutations conferring resistance to CLB_P2, we decided to test knock-out clones for these two genes (*lpcA* and *rfaC*) on the original host strain 55989. Phages CLB_P1, P2 and P3 were tested for their efficiency of plating (EOP). We could first observe that mutation on gene *lpcA* conferred complete resistance to phage CLB_P1. Second we observed a 0.5-log reduction on the mutant susceptibility to phage CLB_P2 (fig17, table 7). These results suggest that although important, gene *lpcA* is not completely required for success of the phage infection.



Figure 17. Deletion of *lpcA* decreases susceptibility of 55989 strain to phage CLB_P2 and confers total resistance to CLB_P1. Phages dilutions were spotted on an overlay of exponential growing bacteria and incubated at 37° overnight.

Table 7. Efficiency of plating (EOP) calculated for *E. coli* strains 55989 Δ /*pcA* relative to wt strain 55989.

	CLB_P1	CLB_P2	CLB_P3
55989∆ <i>lpcA</i>	0	0.4 (+/- 0.14)	1.89 (+/- 0.52)

Material and Methods

Ethics statement

C3H mice axenic mice (seven to nine-week-old) bred at Institut Pasteur (Paris, France) were housed in an animal facility in accordance with Institut Pasteur guidelines and European recommendations. Food and drinking water were provided ad libitum. Protocols were approved by the veterinary staff of the Institut Pasteur animal facility (Ref.#18.271) and the National Ethics Committee (Ref. #2015-0040).

Phages and Bacterial strains

Strains were routinely cultured in lysogeny broth (LB lennox - BD), or on LB lennox agar (BD) or Drigalski agar (Lactose agar with bromothymol blue and crystal violet- CONDA) plates, at 37°C. When required, streptomycin (100 mg/mL) or kanamycin (50 mg/mL) (Sigma) was added.

Phages used were previously described in Maura et al 2012.

55989 mutants were obtained by 3-step PCR in which the gene (*rfaL, fliA, and lpcA*) was disrupted by insertion of a Kanamycin resistance marker gene by lambda Red mediated homologous recombination. Genome comparison was performed with EasyFig (Easyfig_2.2.3) software.

Ex vivo assay

C3H mice received by oral gavage 200 μ L of strain 55989 (10⁷ cfu prepared from an overnight culture in LB at 37°C) in sterile sucrose sodium bicarbonate solution (20% sucrose and 2.6% sodium bicarbonate, pH 8) and three days after were sacrificed to collect and weight intestinal sections (ileum, cecum and colon). PBS was added to each sample (1.75mL for ileum and colon and 5mL for the cecum) before homogenization (Oligo-Macs, Mylteny). A volume of 150 μ L of each homogenized sample was distributed in the wells of a 96-well plate and 10 μ L of each individual phage was added to reach an MOI of 1 x 10⁻², and the plate was incubated at 37°C. A fraction of the homogenized samples was also serially diluted in PBS and plated on Drigalski medium to count Mt1B1 colonies at t=0. Following five hours of incubation, samples were serially diluted in PBS and plated on Drigalski medium as well as on LB agar plates overlayed with strain Mt1B1. Both set of plates were incubated at 37°C overnight. The same procedure was followed for *in vitro* assays with bacteria taken during exponential (OD 0.5) or stationary (24 hr) growth phase at 37°C with shaking.

Transcriptomics

Strain 55989 is grown overnight at 37°C with shaking in 4 different replicates. Cultures are refreshed until OD of approximately 0.5, when half culture is collected for RNA extraction and samples are kept at 37C with shaking until OD of approximately 5, to collect the second half corresponding to stationary phase cultures (see fig3 55989 *E. coli* strain growth curves). The RNA extraction process started with the centrifugation of the cells and the subsequent addition of TRIzol (sigma T-9424) for lysis.

For intestinal samples, axenic mice received by oral gavage 200µL of strain 55989 (10⁷ cfu prepared from an overnight culture in LB at 37°C) in sterile sucrose sodium bicarbonate solution (20% sucrose and 2.6% sodium bicarbonate, pH 8). Three days after gavage mice are sacrificed and the intestinal sections are collected and immediately frozen in liquid nitrogen (ileum, cecum and colon). TRIzol (sigma T-9424) is subsequently added to the frozen samples which were homogenized (Oligo-Macs, Mylteny). The total RNA of both in vitro and in vivo samples was purified through a standard organic extraction (phenol/chloroform) followed by ethanol precipitation. Afterwards RNA was submitted to RNeasy mini kit (Qiagen) for better purification and remaining genomic DNA was removed using oncolumn RNAse-free DNAse set protocol (Qiagen). RNA integrity was assessed with bioanalyser system (Agilent) and RNA Integrity Number (RIN) and the ribosomal ratio (23S/16S) was considered. (See platform protocol for the libraries and sequencing). All the next steps were performed by the Transcriptomics platform at Institut Pasteur. The rRNA-depleted RNA were obtained by using the RiboZero rRNA depletion kit, for eucaryote and procaryote called 'epidemiology' (Illumina). Libraries preparation were performed with TruSeq Stranded RNA LT prep kit (Illumina) with final validation on a DNA Agilent Chip (bioanalyser system) and final DNA quantifications were performed with sensitive fluorescent-based quantitation assays ("Quant-It" assays kit and QuBit fluorometer, Invitrogen). Samples were then normalized to 2nM concentrations and multiplexed. Afterwards samples were denatured at a concentration of 1nM using 0.1N NaOH, at room temperature and was finally diluted at 9,5 pM. Each sample was loaded on the sequencing flowcell at 9,5 pM. Single-read sequencing was performed using HiSeq 2500 Illumina sequencing machine (65 cycles, with 7 index base read).

Data analysis

After sequencing, the samples were analysed using Institut Pasteur cluster. We performed a first quality check using fastQC, after we proceeded with a cleanup of the reads using cutadapt. Read alignment was performed with bowtie and files were transformed in BAM and SAM with the samtools software. Finally, we performed read counts with featureCounts and the statistical analysis was performed using the R software (Core Team. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria, 2013.),(Gentleman et al., 2004)) packages including DESeq2 (Anders and Huber, 2010; Love et al., 2014) and the SARTools package developed at PF2 - Institut Pasteur (Varet et al., 2016). Normalization and differential analysis are carried out according to the DESeq2 model and package. This report comes with additional tab-delimited text files that contain lists of differentially expressed features. A gene ontology analysis was performed to the under and over-expressed gene lists (http://geneontology.org/). Gene functions were verified using EcoCyc database (Keseler et al., 2011). Network analysis was performed according to translated protein-protein interactions STRING database (https://stringusing db.org/cgi/input.pl?sessionId=6SFVgBOt3Ja6&input_page_show_search=on)

Lysis Kinetics

To record phage growth and bacteria lysis, an overnight culture of strain Mt1B1 was diluted in LB broth and grown to an OD_{600nm} of 0.2 from which 150 µL were distributed into each of the wells of a 96-well plate (Microtest 96 plates, Falcon). 10 µL of sterile phage lysates diluted in PBS to obtain a multiplicity of infection (MOI) of 1 x 10⁻² in each well. Plates were incubated in a microplate reader at 37°C, with a shaking step of 30 sec before the automatic recording of OD_{600nm} every 15 min over 20 hr (Glomax MultiDetection System, Promega, USA).

Phage efficiency of plating (EOP) test

MG1655 clones described above were challenged with serial dilutions of 65 phages from the lab collection. The efficiency of plating (EOP) was calculated (ratio of the number of plaques formed by the bacteriophage on the non-host strain to the number of plaques formed on its host) for each phage on all the clones individually. For the initial test three independent replicates were performed (except for the phages showing no activity against any of the clones during the first test). For these tests bacterial cultures were grown to an OD of approximately 0.2 and spread on LB before the phage dilutions were spotted. Plates were all incubated at 37°C overnight.

Keio collection resistance screening

We tested the keio collection strains for possible resistance to phages CLB_P2 and T4 by performing a double spot assay. For this assay overnight culture of all strains was diluted in LB broth in an 96-well plate and grown to an OD_{600nm} of approximately 0.2, after 10 μ L of each strain grown in broth were spotted on an LB square plate and dried, subsequently a 5 μ L drop of each phage suspension (between 1×10^5 and 1×10^4 pfu/mL) was spotted on top of the dried bacterial drops in order to reach an approximate MOI of 10^{-5} . The absence of plaques was considered full resistance and further testing was performed for confirmation.

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Chapter 3

Pathogenicity island confers increased susceptibility to phages in *E. coli*
Chapter 3

Pathogenicity island confers increased susceptibility to phages in E. coli

Introduction

Pathogenic bacteria are by definition, encoding for several virulence factors in their genomes. In *E.coli*, these virulence factors can often be found in pathogenic islands (PAI), which can be transferred between strains via horizontal gene transfer (Hacker et al., 1997; Messerer et al., 2017). Several virulence factors belonging to PAI, have already been described, including adhesins, toxins like α -Hemolysin and colibactin (Oelschlaeger et al., 2002; Putze et al., 2009). The activity of colibactin was first described in 2006 when its genotoxicity was revealed in eukaryotic cells, in which it induces DNA double-strand breaks that block the cell cycle both *in vitro* (Nougayrede et al., 2006) and *in vivo* (Cuevas-Ramos et al., 2010). Colibactin activity has also been linked to intestinal inflammation (Arthur et al., 2012). Its biosynthesis and secretion pathways are encoded on a genomic island named *pks* (polyketide synthase), that comprises the colibactin (*clbI*) polycistronic gene cluster, showed to be horizontally transmitted (Messerer et al., 2017; Putze et al., 2009) (fig 1). Although mostly associated with ExPEC (extraintestinal pathogenic) *E. coli* strains from the phylogenetic group B2, it has also been reported in *E. coli* strains from goup B1 and in species from the genus *Klebsiella* and *Citrobacter* (Putze et al., 2009).



Figure 1. A schematic diagram of the pks pathogenicity island extracted from (Putze et al., 2009)

Studies have suggested that *pks* island- positive *E. coli* strains are present in the intestinal microbiota of approximately 20% of healthy humans (Dubois et al., 2010; Johnson et al., 2002; Putze et al., 2009). This proportion increases for patients with inflammatory bowel diseases (IBD) and colorectal cancer (CRC), with an average carriage of *E. coli* pks strains of 40% and 60%, respectively. This suggests a possible influence of *pks* strains on IBD and CRC (Arthur et al., 2012; Dalmasso et al., 2014), either through a role of pathogenicity islands in the modulation of the intestinal microbiota or a competitive advantage of *pks*-positive *E. coli* against other non-carrier *E. coli* strains.

Due to its prevalence and possible impact on the gastrointestinal health (Johnson et al., 2008), the *pks* genomic island represents a potential antibacterial target.

The biosynthetic pathway of colibactin involves genes related to cytoplasmic metabolism, gene regulation and transport across the cell envelope. Therefore, we hypothesise that this pathogenicity island could modulate the surface of the bacterial cell with possible consequences on phage infection. It has also been shown that phages have complex interaction with virulence factors, including for example production of capsules, to which certain phages are adapted (Roach et al., 2013). In the following work, we tested whether *E. coli* strains carrying the *pks* island could be differentially infected by phages. Using an *E. coli* MG1655 strain carrying a pBAC vector, with or without the *pks* island. We screened a set of 65 *E. coli* phages from our laboratory collection. While we could not identify phages that are specific for the presence of the *pks* island, we observed that the presence of the pks island caused an increase by more than 3-logs on phage efficiency. We then explored the possible mechanisms involved in this differential activity.

Results and discussion

The presence of the *pks* genomic island increases the susceptibility to phages 412_P1, P4 and P5

MG1655 *E. coli* clones carrying an empty pBAC vector (pBAC-empty) or including the *pks* genomic island (pBAC-pks) were screened for their susceptibility to 65 *E. coli* phages from the laboratory collection. The phages were tested for their efficiency of plating (EOP) in comparison with their host of isolation and the MG1655 strain. From the 65 phages tested, only 5 were unable to infect any of the clones. Amongst the other 60 phages, we observed that the presence of the pBAC-*pks* in the host bacterium influenced the infection pattern for three of them. Curiously, these three phages were originally isolated against the same strain, 412. The three phages infected poorly the MG1655 ancestral strain, as well as the MG1655 clone with the pBAC-empty vector, with an EOP of approximately 10^{-6} compared to their efficiency on strain 412 (fig 2, table 1). In sharp contrast, the MG1655 clone with the pBAC-pks vector is infected with a significantly higher efficiency, showing an EOP of approximately 0.01 (replicated over four independent assays) ie. an 10^4 difference from the clones not carrying the *pks* island (fig 2, table 1).

These results support the hypothesis that the colibactin pathogenicity island increases the bacterial susceptibility to these three phages, suggesting that the *pks* genomic fragment allows a more successful phage infection. This phenomenon could be caused by i) an increase in expression of the phage receptor at the bacterial surface, ii) the presentation of a more suitable phage receptor or iii) the modification of a later step in the phage infection process.



Figure 2. The presence of the *pks* genomic island increases the susceptibility to phages 412_P1, P4 and P5. Phage dilutions were spotted on a bacteria overlay plate and incubated overnight.

Table 1. Efficiency of plating (EOP) of each of the 3 phages, calculated (from 4 independent replicates)for strain MG1655, MG1655 pBAC-empty and MG1655 pBAC-pks relative to strain 412.

	P1	Р4	Р5
MG1655	4.6x10 ⁻⁶ (+/- 1.6x10 ⁻⁶)	6.22x10 ⁻⁷ (+/- 1.96x10 ⁻⁷)	5.96x10 ⁻⁷ (+/- 1.39x10 ⁻⁷)
pBAC-empty	9.01x10 ⁻⁶ (+/- 2.8x10 ⁻⁶)	3.25x10 ⁻⁶ (+/- 1.1x10 ⁻⁶)	2.81x10 ⁻⁶ (+/- 1.27x10 ⁻⁶)
pBAC-pks	0.029 (+/- 0.014)	0.032 (+/- 0.0073)	0.015 (+/- 0.0073)

Phage Characterization

Lysis Kinetics

We then tested whether the differential infection observed in solid medium, was reproducible in homogenous liquid culture conditions. We recorded the growth of the bacteria overtime in the presence of the three phages, independently. We observed that at a multiplicity of infection (MOI) of 0.01, no lysis could be detected 20 hours after infection of the MG1655 strains (with or without the *pks* island), while the three phages show a rapid lysis (45 min) on their original host, strain 412 (fig3A). Following the lysis of the 412 bacterial cells, phage insensitive clones emerged after just 200 minutes (3.3hours) of co-culture (fig 3A).

When we increased the MOI to 1, both MG1655 clones, independently of carrying or not the *pks* island, are lysed within 3.5 to 4 hours, while the strain 412 is lysed in less than 45 minutes (fig 3B). The differences observed between the two MOI shows that infection of MG1655 requires a high number of phage particles to interact with the cells for the infection to be successful. In turn, this suggests a less efficient phage adsorption on the MG1655 strains independently of the presence of both pBAc-empty or pBAC-pks. The lack of high efficiency of infection on MG1655 pBAC-empty in solid environment may be related to the number of available receptors being modulated by the environmental conditions (homogenous liquid culture or structured solid media).

Even though these results point to a difference on the interaction with the receptor of MG1655, the lysis kinetics on both strains carrying or not the *pks* island suggest that this step is not the major factor influencing the differential phenotype observed in the solid medium.



Figure 3. Lysis Kinetics of phage P1, P4 and P5. Growth curves for 412, MG1655 pBAC-empty and pBAC-pks (n=3 for each condition) in liquid broth in the absence (412 – blue, MG1655 pBAC-empty – dark grey, MG1655 pBAC-pks – light blue) or presence of phage P1 (412 – orange, MG1655 pBAC-empty – brown, MG1655 pBAC-pks – purple), P4 (412 – light grey, MG1655 pBAC-empty – dark blue, MG1655 pBAC-pks – fluorescent pink) or P5 (412 – yellow, MG1655 pBAC-empty – green, MG1655 pBAC-pks – pink), added at t=0, at an MOI of 10⁻² and 1.

Genomic analysis

Phages P1, P4 and P5 display different plaques morphologies when tested against strain 412 (original host), with P1 forming large plaques surrounded by an halo, medium size plaques in P4 and P5 forms the smaller plaques (fig4). However, their lytic properties, as reported in figure 2, were indistinguishable, suggesting that they may represent genomic variants of the same phage strain.



Figure 4. Phage P1, P4 and P5 plaque morphology differences. Phage dilutions were spotted on a bacteria overlay plate and incubated overnight.

The analysis of the genomes of these 3 phages revealed that they are indeed highly related, possibly being variants of a similar ancestor (Fig 5). The genome length is 44890 bp for phage P1, 45062 bp for phage P4 and 45312 bp for phage P5, encoding of 84 ORFs and 1 tRNA –Arg gene for P1 and P4, and 85 ORFs and 1 tRNA –Arg gene for P5, which includes one more hypothetical gene.



Figure 5. Genomic comparison of phages 412_P1, P4 and P5.

Blast analysis revealed that the closest sequenced phage sharing 81% similarity with these 3 phages and is *Escherichia* phage vB_Ecos_CEB_EC3a, belonging to the siphoviridae family (Oliveira et al., 2017)(table 2). We also sequenced and analysed the genome of the *E. coli* original host of these 3 phages, strain 412, in which we could not detect the presence of the *pks* genomic island.

Table 2. List of the closest phage homologs to phages P1, P4 and P5. Megablast tool from NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to search for the closest homologs ranked ranked in decreasing values of query cover.

Homologs to Phage P1, P4, P5 (44.8, 45 and 45.3 kb)							
Description	Max score	Total score	Query cover	E value	Identity	Accession	
Escherichia phage vB_Ecos_CEB_EC3a, complete genome	20367	43680	81%	0	87%	KY398841.1	
Enterobacteria phage vB_EcoS_ACG-M12, complete genome	12266	39513	82%	0	85%	JN986845.1	
Escherichia phage DTL, complete genome	11269	40754	80%	0	89%	MG050172.1	
Escherichia phage vB_EcoS-IME253, complete genome	9797	40737	78%	0	88%	KX130960.1	
Escherichia phage IMM-001, complete genome	7668	17499	60%	0	73%	MF630922.1	
Bacteriophage RTP, complete genome	6184	38499	79%	0	89%	AM156909.1	

Production, activation or restriction of colibactin (clbA,S,P and N) does not modulate susceptibility to phage infection

In order to test if the production of the colibactin toxin was involved in the increased susceptibility to the three phages, we performed EOP tests in MG1655 strains carrying pBAC-pks with deletions in individual genes (*clbA*, *clbP*, *clbS* and *clbN*) involved in the colibactin biosynthesis pathway (Taieb et al., 2016) (fig6).



Figure 6. Schematic diagram of the colibactin pathway adapted from (Taieb et al., 2016). We tested knock-out (yellow star) clones of clbA, clbS, clbP and clbN.

The *clbA* gene codes for a protein essential for the activation of the biosynthesis of the colibactin. Gene *clbP* codes for an amino-peptidase that modifies the active state of colibactin. *ClbS* codes for a protein that protects the bacteria from exposure to colibactin by restraining or modifying its presence in the bacterial cytoplasm. Finally, we tested also a mutant in the *clbN* gene which codes for a colibactin non-ribosomal peptide synthase essential for the initiation of the biosynthesis pathway. Results showed no significant difference in terms of phage susceptibility between the four strain carrying the different deletions and the strain carrying the complete *pks* island (EOP between 0.01 and 0.05, fig7, table 3). Therefore, these results suggest that the biosynthesis of the colibactin toxin does not interfere with the infectious cycle of these three phages.



Figure 7. Colibactin production does not affect susceptibility to phages 412_P1, P4 and P5. Phage dilutions were spotted on a bacteria overlayed plate and incubated overnight.

Table 3. Efficiency of plating (EOP) of each of the 3 phages, calculated (from 4 independent replicates) for strain MG1655 pBAC-empty, MG1655 pBAC-pks, MG1655 pBAC-pks $\Delta clbA$, MG1655 pBAC-pks $\Delta clbP$, MG1655 pBAC-pks $\Delta clbS$ and MG1655 pBAC-pks $\Delta clbN$ relative to strain 412.

	P1	P4	Р5
pBAC-empty	9.01x10 ⁻⁶ (+/- 2.8x10 ⁻⁶)	3.25x10 ⁻⁶ (+/- 1.1x10 ⁻⁶)	2.81x10 ⁻⁶ (+/- 1.27x10 ⁻⁶)
pBAC-pks	0.029 (+/- 0.014)	0.032 (+/- 0.0073)	0.015 (+/- 0.0073)
pBAC-pks∆clbA	0.032 (+/- 0.014)	0.055 (+/- 0.014)	0.055 (+/- 0.014)
pBAC-pks∆clbP	0.05 (+/- 0.033)	0.049 (+/- 0.011)	0.049 (+/- 0.011)
pBAC-pks∆clbS	0.048 (+/- 0.029)	0.046 (+/- 0.014)	0.046 (+/- 0.014)
pBAC-pks∆clbN	0.011 (+/- 0.0051)	0.056 (+/- 0.012)	0.056 (+/- 0.012)

pks genomic island is associated with overexpression of asparagine and aspartate tRNAs and underexpression of a type-1 restriction enzyme

In order to identify the possible factors involved in the increased susceptibility to the three phages of MG1655 carrying the *pks* pathogenicity island, we performed a gene expression profile analysis of strains pBAC –empty or the pBAC-pks. This analysis was performed by RNA sequencing from cells collected from solid medium. Although we observed a high transcriptomic similarity between clones carrying the *pks* island or the empty vector (suggesting that the production of colibactin does not have a large influence on the global gene expression), a small subset of genes were differentially expressed between the two backgrounds (Fig8, 3 independent replicates were used for each genomic background). We were also able to observe the expression of the colibactin synthesis pathway in the strains pBAC-pks, confirming that this toxin is produced in structured environments.



Figure 8. Variability within the experiment - first principal components of the PCA

Five genes were found to be down-regulated in strain pBAC-pks in comparison to the pBAC-empty strain (table 4).

Table 4. Log2-fold change of the genes found under-expressed in the pBAC-pks strain in comparisonwith the pBAC-empty carrier strain.

Under-expressed				
gene	log2(fold-change)	pvalue	function	
hsdS	-2.671	5.00E-15	Type-1 restriction enzyme EcoKI specificity protein	
lacZ	-2.389	2.53E-11	Beta-galactosidase	
mdaB	-1.613	7.84E-05	Modulator of drug activity B	
srlD	-1.439	8.40E-05	Sorbitol-6-phosphate 2-dehydrogenase	
ridA	-1.719	0.000138	2-iminobutanoate/2-iminopropanoate deaminase	

On the other hand, 10 genes were found to be over-expressed (table 5), from which two are pseudogenes described as CP4-44 putative defective prophage (*yoeH, yoeG*).

Table 5. Log2-fold change of the genes found over-expressed in the pBAC-pks strain in comparisonwith the pBAC-empty carrier strain.

Over-expressed				
gene	Log2(fold-change)	pvalue	function	
уоеН	2.012	1.60E-08	pseudogene	
aspT	2.177	4.23E-07	tRNA (<i>aspT</i>) is one of three aspartate tRNAs.	
asnW	1.493	8.62E-07	tRNA (<i>asnW</i>) is one of four asparagine tRNAs.	
asnU	1.707	1.56E-06	tRNA (<i>asnU</i>) is one of four asparagine tRNAs	
ldtA	1.311	1.47E-06	L,D-transpeptidase ErfK	
yoeG	1.981	4.85E-06	pseudogene	
asnT	1.194	2.96E-05	tRNA (<i>asnT</i>) is one of four asparagine tRNAs	
sibC	1.181	2.82E-05	small RNA SibC	
cobU	1.25	3.47E-05	cobinamide-P guanylyltransferase / cobinamide kinase	
aspU	1.196	0.00014	tRNA (<i>aspU</i>) is one of three aspartate tRNAs	

In the list of the over-expressed tRNA genes, only tRNA *asnW* is also encoded on the *pks* island, which could explain the overall over-expression.

Phages are parasites, and as such they depend on the cell physiology to have a successful and efficient replication. The fact that bacteria carrying the *pks* pathogenicity island overexpress tRNAs could be a possible explanation for the higher efficiency of phage infection and/or replication in these cells, compared to the cells carrying an empty vector. Some phages have been showed to encode tRNAs genes, but as parasites, they rely mostly on the host tRNA pool for a successful infection. Therefore, it is possible that cells with the pks island harbour a more favourable environment for the phage replication, as they can provide a more efficient translation of phage genomes, since the three phages studied only possess one arginine tRNA.

On the other hand, one of the underexpressed genes associated with the presence of the pks island is the *hsdS* gene. This gene codes for the specificity protein of type-1 restriction enzyme EcoKI, which suggests that it can be involved in lowering the expression of this defense mechanism, therefore, could increase the susceptibility to phages. Hypothesis supported by the presence of 3 restriction sites for this enzyme on this island. In addition, the genomes of the three phages possess 4 restriction sites for this enzyme.

Differential codon usage between the phages and the host

The translation of genomic information into proteins relies on the genetic code. Different nucleotide triplets, or codons, code for the amino acids required for protein synthesis. Some of these codons are synonymous, which means that different codons can code for the same amino acid. The frequency at which each codon is used differs between different organisms, phenomenon called codon usage bias (Grantham et al., 1980) that has been shown to affect the speed and accuracy of translation (Akashi, 1994; Tuller et al., 2010). Therefore, selection for an optimal codon usage plays an important role in bacterial genome evolution (Andersson and Kurland, 1990).

Both in bacteria and yeast, highly expressed genes preferentially use codons that are translated faster or more accurately (Gouy and Gautier, 1982). In prokaryotes, that the degree of codon usage bias can be associated with their environment. For instance, mesophiles and pathogenic bacteria have a less biased codon usage, allowing for a higher efficiency in adapting to fluctuations of the environmental conditions (Botzman and Margalit, 2011). Another factor that influences the codon usage bias in prokaryotes is the composition and abundance of tRNAs in their genomes (Ikemura, 1985; Percudani et al., 1997).

Phages, with their strictly parasitic lifestyle, are strongly dependent of the host machineries, including transcription and translation, in order to generate progeny. However, several phages were shown to possess a phage-specific codon usage that differs from the one in their host (Kunisawa et al., 1998; Sau et al., 2005) with a possible decreased phage efficiency.

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In order to lessen this effect, several phages encode for tRNAs corresponding to the codons highly used by themselves, and used less frequently to their host's codons (Bailly-Bechet et al., 2007). It has also been shown that there is an association between tRNA distribution on the genome and the codon usage (Bailly-Bechet et al., 2007). Since our transcriptomics data highlighted an overexpression of asparagine and aspartate tRNAs in bacteria carrying the *pks* island, we hypothesized that the differential susceptibility to the phage could be caused by differences on the codon usage bias of the phage in comparison with the strain MG1655. In order to test this hypothesis we performed a codon usage analysis from the coding sequences of the genomes of both *E. coli* strains 412 and MG1655 and also one of the phages (P4) (fig 9). Results showed that the codon usage varies between the three genomes, with usage biases in, for instance, Alanine-GGT in phage P4 and Leucine-CTG in strain 412.





Figure 9. Specific codon usage for the coding regions of phage P4 and bacterial strains 412 and MG1655. Bars represent the codon frequency in percentage (%) of each codon. P4 – orange, *E. coli* 412 – blue and *E. coli* MG1655 – green.

When comparing the codon usage frequency of the asparagine and aspartate codons, we observed that P4 displays a higher frequency of use of both amino acids relative to strains 412 and MG1655 (fig 10). About asparagine, we hypothesise that due to the fact that one of its tRNA is encoded on the *pks* island and also it is more used by the host strain 412 than by the strain MG1655 it could have an effect on the phage replication efficiency. On the other hand, although the aspartate tRNAs are not encoded by the *pks* genomic fragment it was also showed to be overexpressed in its presence, displays codon usage frequencies much lower in strain MG1655 than strain 412 (fig10). These results lead to other hypothesis in which the extra production of aspartate tRNAs by the MG1655 clones carrying the *pks* fragment may be sufficient to increase the efficiency of infection.

		differences(%) in codon usage				
		P4vs412 P4vsMG1655 412vsMG1655				
	GAT	0.44	2.02	0.4		
Asparagine	GAC	0.34	1.22	0.25		
	AAT	0.44	0.84	1.57		
Aspartate	AAC	0.67	0.92	0.89		



Figure 10. Differences in codon usage frequency for asparagine and asparate tRNAs. Bars represent the codon frequency in percentage (%) of each codon. P4 – orange, *E. coli* 412 – blue and *E. coli* MG1655 – green

Over-expression of tRNAs does not lead to an increase of susceptibility to phages

In order to test the effect of the aspartate or aparagine tRNAs on the efficiency of the phage infection, we inserted each tRNA individually into a pUC18 plasmid (constitutive expression) and introduced it into the MG1655 ancestral strain, and then tested their susceptibility to the three phages. We observed that neither the presence of the extra *asnW* (asparagine) nor *aspU* (asparatate) tRNAs could restore the phenotype observed for the clones carrying the *pks* island (fig11, table 6). This result demonstrates that the overexpression of one tRNA is not responsible for the different susceptibility of bacteria to phage infection. In the future the combination of both tRNAs should be tested.



Figure 11. Individual aspartate and asparagine tRNA over-expression does not affect susceptibility to phages 412_P1, P4 and P5. Phage dilutions were spotted on a bacteria overlayed plate and incubated overnight.

Table 6. Efficiency of plating (EOP) of each of the 3 phages, calculated for strain MG1655, MG1655 pBAC-empty, MG1655 pUC18 – aspU and MG1655 pUC18 – asnW relative to strain 412.

	P1	Р4	Р5
MG1655	4.6x10 ⁻⁶ (+/- 1.6x10 ⁻⁶)	6.22x10 ⁻⁷ (+/- 1.96x10 ⁻⁷)	5.96x10 ⁻⁷ (+/- 1.39x10 ⁻⁷)
pBAC-empty	9.01x10 ⁻⁶ (+/- 2.8x10 ⁻⁶)	3.25x10⁻⁶ (+/- 1.1x10⁻⁶)	2.81x10 ⁻⁶ (+/- 1.27x10 ⁻⁶)
MG1655 - puc18empty	4.39x10 ⁻⁶ (+/- 2.37x10 ⁻⁶)	5.57x10 ⁻⁶ (+/- 4.21x10 ⁻⁶)	5.57x10 ⁻⁶ (+/- 4.21x10 ⁻⁶)
MG1655 -puc18AspU	4.78x10 ⁻⁶ (+/- 3.9x10 ⁻⁶)	2.12x10 ⁻⁶ (+/- 1.04x10 ⁻⁶)	2.12x10 ⁻⁶ (+/- 1.04x10 ⁻⁶)
MG1655 -puc18AsnW	2.57x10 ⁻⁵ (+/- 2.48x10 ⁻⁵)	1.75x10 ⁻⁶ (+/- 6.12x10 ⁻⁷)	1.75x10 ⁻⁶ (+/- 6.12x10 ⁻⁷)

The lack of type-1 restriction enzyme *Eco*KI specificity protein leads to the increase of susceptibility to phages

We then tested the role of the type-1 restriction enzyme EcoKI biosynthesis pathway in phage infection efficiency, since one of its genes (hsdS) was found to be underexpressed in bacteria carrying the pks genomic island. The EcoKI pathway is composed of three genes, hsdM responsible for methylation, hsdR which is the restriction component and hsdS which codes for the sequence specificity protein. In order for DNA to be restricted by EcoKI, the protein complex of all three gene products is required (R2M2S). One of the roles of restriction-modification systems is to protect cells from foreign DNA (Ershova et al., 2015). For instance, it has been shown that the inactivation of the EcoKI restriction enzyme in strain *E. coli* MG1655 leads to an increase of the conjugational transfer of plasmid pOLA52, which carriers two EcoKI recognition sites (Roer et al., 2015). The role of the EcoKI pathway in phage infection was also previously shown, for instance, for phage T7, which produces a protein that binds to the EcoKI enzymatic complex, inhibiting DNA restriction (Atanasiu et al., 2002). Interestingly, neither of the three genes involved in the EcoKI biosynthesis was found on the phage's host strain 412 genomic analysis. Because MG1655 with pBAC-pks shows under-expression of hsdS relative to the pBAC-empty, it suggests that the pks island encodes for a gene responsible for the down-regulation of this restriction-modification system. This is in agreement with a strategy that a foreign DNA element could develop to ensure successful transfer to another strain. We observed that it may increase the susceptibility to phage infections, in agreement with previously shown involvement in increase of horizontal gene transfer events (Messerer et al., 2017). In order to test if the under-expression of gene hsdS in MG1655 with the pks genomic island could contribute to the differential efficiency of infection observed in structured environments, we tested phage P4 against a set of mutants, from the KEIO collection, each lacking one of the three genes of the EcoKI pathway. Unfortunately, the background strain of the KEIO is as susceptible as strain 412 to phage P4 leading to inconclusive results when testing these three mutants (EOP's relative to 412 between 0.1 and 0.7). In order to circumvent this problem, we will next i) knockout the hsdS gene on the MG1655 strain and ii) insert a plasmid carrying the hsdS gene into the pBAC-pks strain.

Materials and Methods

Phages and Bacterial strains

Strains MG1655, MG1655 pBAC-empty, MG1655 pBAC-*pks*, MG1655 pBAC-*pks* Δ *clbA*, MG1655 pBAC-*pks* Δ *clbP*, MG1655 pBAC-*pks* Δ *clbS* and MG1655 pBAC-*pks* Δ *clbN* were obtained from the laboratory of Professor Eric Oswald in Toulouse (MG1655+pBAC or MG1655pBAC-*pks* or MG1655BAC-*pks* Δ *clbA* have been already described in Martin et al 2013 (Martin et al., 2013).

Strain 412 is part of the collection of clinical strains of *E. coli* isolated from mechanically ventilated pneumonia patients (La Combe et al., 2018).

Phage isolation from sewage water was performed according to standard protocols (see methods chapter 4).

Strains were routinely cultured in lysogeny broth (LB lennox - BD), or on LB lennox agar (BD), at 37°C. When required, ampicilin (100 mg/mL) or chloramphenicol (25/30 mg/mL) (Sigma) was added.

Phage efficiency of plating (EOP) test

MG1655 clones described above were challenged with serial dilutions of 65 phages from the laboratory collection. The efficiency of plating (EOP) was calculated for each phage on all the clones individually. For the initial test, three independent replicates were performed (except for the phages showing no activity against any of the clones during the first test). For these tests bacterial cultures were grown to an optical density OD600 of approximately 0.2 and spread on LB or LB+chloramphenicol square plates before the phage dilutions were spotted. When testing strains with the pUC18 plasmid we used LB+ampicillin plates. Plates were all incubated at 37°C overnight.

Lysis Kinetics curves

An overnight culture in LB broth at 37°C of each strain was diluted in LB broth and grown to an OD_{600nm} of 0.2 from which 150 µL were distributed into each of the wells of a 96-well plate (Microtest 96 plates, Falcon). Ten µL of sterile phage lysates diluted in PBS to obtain a multiplicity of infection (MOI) of 1 x 10^{-2} were added in each well. Plates were incubated in a microplate reader (Glomax MultiDetection System, Promega, USA) at 37°C, with a shaking step of 30 sec before the automatic recording of OD_{600nm} every 15 min over 20 hr.

Trascriptomics

Strains 412, MG1655 pBAC-empty and MG1655 pBAC-*pks* were grown in triplicate overnight at 37°C with shaking. Cultures were refreshed until OD600 of approximately 0.3 and 1mL of these cultures were applied to cover the entire surface of LB plates. The excess of liquid was removed and plates

were let dry during 30 min. Three sets of 2 plates were prepared. On the first set phages were added and the second was used as control. Following 3.5 hours of incubation at 37°C, when phage plaques started to be visible, the control plates were used to collect bacteria by adding 2 mL of PBS. After centrifugation, the cells were lyzed in TRIzol (sigma T-9424) and total RNA was extracted through a standard organic extraction (phenol/chloroform) followed by ethanol precipitation. Afterwards RNA was submitted to RNeasy mini kit (Qiagen) for purification and remaining genomic DNA was removed using on-colunm RNAse-free DNAse set protocol (Qiagen). RNA integrity was assessed with bioanalyser system (Agilent) and RNA Integrity Number (RIN) and the ribosomal ratio (23S/16S) was considered. (See platform protocol for the libraries and sequencing). All the next steps were performed by the Transcriptomics platform at Institut Pasteur. The rRNA-depleted RNA were obtained by using the RiboZero rRNA depletion kit, for eucaryote and procaryote called 'epidemiology' (Illumina). Libraries preparation were performed with TruSeq Stranded RNA LT prep kit (Illumina) with final validation on a DNA Agilent Chip (bioanalyser system) and final DNA quantifications were performed with sensitive fluorescent-based quantitation assays ("Quant-It" assays kit and QuBit fluorometer, Invitrogen). Samples were then normalized to 2nM concentrations and multiplexed. Afterwards samples were denatured at a concentration of 1nM using 0.1N NaOH, at room temperature and it was finally diluted at 9,5 pM. Each sample was loaded on the sequencing flowcell at 9,5 pM. Single-read sequencing was performed using HiSeq 2500 Illumina sequencing machine (65 cycles, with 7 index base read).

Data analysis

After sequencing, the samples were analysed using Institut Pasteur cluster. We performed a first quality check using fastQC, and then proceeded with a cleanup of the reads using cutadapt. Read alignment was performed with bowtie and files were transformed in BAM and SAM with the samtools software. Finally we performed read counts with featureCounts and the statistical analysis was performed using the R software (Core Team. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria, 2013.),(Gentleman et al., 2004)) packages including DESeq2 (Anders and Huber, 2010; Love et al., 2014) and the SARTools package developed at PF2 - Institut Pasteur (Varet et al., 2016). Normalization and differential analysis are carried out according to the DESeq2 model and package. This report comes with additional tab-delimited text files that contain lists of differentially expressed features. A gene ontology analysis was performed to the under and over-expressed gene lists (http://geneontology.org/). Gene functions were verified using EcoCyc database (Keseler et al., 2011).

Bacteria and Phage genomes sequencing and analysis

Sterile phage lysates were treated by DNase (120 U) and RNase (240 µg/mL) and incubated for 30 minutes at 37°C. The RNase/DNase reaction was stopped by adding EDTA (20 mM). Lysates were then treated with proteinase K (100 µg/mL) and SDS (0.5%) and incubate at 55°C for 30 minutes. Afterwards DNA was extracted by a phenol-chloroform protocol modified from Pickard (Pickard, 2009). Sequencing was performed using Illumina technology (Illumina Inc., San Diego, CA) MiSeq Nano with paired-end reads of 250bp. Quality of reads was visualised by FastQC v0.10.1 Brabraham Bioinformatics (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Phage assembly was performed using a workflow implemented in Galaxy-Institut Pasteur using clc_assembler v4.4.2 and clc_mapper v4.4.2 (CLC Bio, Qiagen). Phage termini was determined by PhageTerm (Garneau et al., 2017). Phages annotation was performed by the RAST v2.0 server (Aziz et al., 2008). Genome comparison was performed with EasyFig (EasyFig_2.2.3) software.

Codon usage analysis

For the codon usage analysis we first sorted the Coding sequences (CDS) from all the genomes used using the webtool from https://rocaplab.ocean.washington.edu/tools/genbank to fasta/. Next we the CDS files used generated and tested for codon usage bias using http://www.bioinformatics.org/sms2/codon usage.html. Finally we calculated the each specific codon abundance relative to the total amount of codons.

tRNA cloning

Primers were designed to amplify the *asnW* gene carried by the *pks* island and clone it into plasmid pUC18 at the EcoRI and HindIII restriction sites. The pBAC *pks* plasmid was extracted from the MG1655 cells using the Nucleo-Spin Plasmid kit (Machery-Nagel). PCR reactions were performed in a total volume of 50 µL containing 1 µL of plasmid DNA, 10 µM of each primer (forward and reverse), 200 µM dNTPs, 1 U Taq polymerase and 1X Taq polymerase buffer. The PCR reaction conditions were as follows: 95°C for 3 min followed by 34 cycles of 95°C for 30 s, °C for 30 s, 72°C for 2 min, followed by 72°C for 5 min. Afterwards the PCR product was purified and digested with EcoRI and HindIII restriction enzymes. The pUC18 plasmid was also digested and then a ligation (T4 ligase) was performed between the PCR fragment and linearized pUC18. Ligation product was then transformed into a sig10 MAX electrocompetent cell (sigma-CMC0004) and plated on selective media supplemented with X-gal (concentration). White clones were then isolated and confirmed to carry the tRNA gene by PCR. All the strains carrying the pUC18 plasmid were grown in LB medium supplemented with ampicillin (100µg/mL).

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Chapter 4

The spatial heterogeneity of the gut drives the coexistence of antagonist bacteria and bacteriophages populations

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The spatial heterogeneity of the gut drives the coexistence of antagonist bacteria and bacteriophages populations

Virulent phages have been shown to coexist with their targeted bacteria for long periods of time within the mammalian gut. Several factors may underlie the coexistence of bacteria and phages, such as arms race dynamics, involving resistance to infection and viral counter-resistance, differential gene expression of the bacterial cells, the ability (inherent or evolved) of the phage to infect multiple hosts, and the spatial distribution of these two populations (Brockhurst et al., 2006; De Sordi et al., 2017; Heilmann et al., 2012; Stern and Sorek, 2011; Weitz et al., 2005).

The goal of the work presented in this chapter was to investigate the dynamic interactions of both populations in the mammalian gut identifying possible arms-race dynamics or ecological spatial constrains that can protect bacteria from phage infection and lead to coexistence between virulent phages and bacteria.

To achieve this aim we followed the populations' dynamics of both phages and bacteria when colonizing a new gnotobiotic murine model (OMM-12) (Brugiroux et al., 2016), in which the microbiota is composed of 12 murine strains. Coexistence between both populations was observed throughout the intestine, with the exception of the mucosal section of the ileum, in which a significant lower ratio of phages was observed, suggesting barriers for the phages to access this section of the gut. Moreover, we could not detect the emergence of phage resistant mutants. Altogether, results suggest a source-sink ecological scenario driving the coexistence of virulent phage and bacterial populations in the gut without major influence of arms-race dynamics.

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The author of this thesis performed the bacteria-phage *in vivo* dynamics experiments, qPCR assays, the *in vitro* characterization of the phages as well as the analysis of the whole genome sequencing analysis and drafted the manuscript. Luisa De Sordi helped with animal experiments and participated in the preparation of the manuscript. Quentin Lamy-Besnier provided technical help with the adsorption assays. Claudia Eberl performed the FISH experiment. The author and Pascal Campagne performed the statistical analysis.

Title

The spatial heterogeneity of the gut drives the coexistence of antagonist populations of bacteria and bacteriophages

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Abstract

Diverse populations of bacteriophages (phages) and bacteria are the most abundant components of the mammalian gut microbiota. The conditions underlying the coexistence of these antagonistic populations in this environment are unknown. We challenged a murine synthetic microbiota model with a set of three virulent phages, to investigate this coexistence in the gut. We found that coexistence was not principally dependent on fluctuations in the populations of phage-resistant bacteria and counter-resistant phages, or on the possibility of phages infecting other bacterial species. Instead, our data suggest that phage-inaccessible spatial refugees in the mucosa of the ileum serve as a source of bacteria, which can migrate into the gut lumen. The phage population is amplified locally through the infection of these bacteria in the gut lumen, leading to persistence of populations of virulent phages with phage-susceptible bacteria, providing an explanation for the sustained persistence of intestinal phages, such as the crAssphage, worldwide.

Introduction

The mammalian gut is a highly complex, diverse and heterogeneous organ composed of different eukaryotic cells that establish a symbiotic relationship with many different enteric microbes, including viruses. Bacteriophages (phages) are the most abundant viruses residing in the gut, but their precise role in the microbiome remains unclear (Manrique et al., 2017). However, changes in the viral and microbial communities of the gut are increasingly reported to be associated with pathological conditions, such as obesity, diabetes, *Clostridium difficile* infections, inflammatory bowel diseases, AIDS and autism(Lynch and Pedersen, 2016; Manrique et al., 2017). Furthermore, although there is a core phage community common to most humans, almost 50% of the phage community of any human individual is specific to that individual (Manrique et al., 2016).

For decades, the coexistence dynamics of predators (or parasites) and preys (or hosts) has been the subject of theoretical and experimental studies, mostly performed *in vitro* and *in silico*, with phages and bacteria as models (Betts et al., 2014; Brockhurst et al., 2006; Lenski and Levin, 1985; Weitz et al., 2013). Some studies have been performed in soil or the aquatic environment, but the coexistence of phages and bacteria in the mammalian gut has been explored only from metagenomics studies (Benler et al., 2018; Enav et al., 2018; Gomez and Buckling, 2011; Hannigan et al., 2018).

Genomic analyses of thousands of bacterial strains have confirmed the pervasive influence of temperate phages on bacteria behaviour and evolution (Keen and Dantas, 2018; Touchon et al., 2017). However, much less is known about the impact of virulent phages on bacterial communities in natural settings, as such phages can rapidly kill the bacteria they infect, in some cases within minutes, without leaving a traceable genomic signal (Maura and Debarbieux, 2012).

In the gut the role of temperate phages has recently been investigated experimentally. These studies revealed that phages can excise from bacterial chromosome, modulate the microbiome, acquire genetic information and even transfer between bacteria in response to inflammation (Cornuault et al., 2018; De Paepe et al., 2014; De Paepe et al., 2016; Diard et al., 2017). However, virulent phages have a limited effect in terms of their impact on the bacterial populations they target within the gut, as demonstrated by several phage therapy studies (Galtier et al., 2017; Maura et al., 2012a; Maura et al., 2012b; Weiss et al., 2009). Unexpected, these studies showed that phages could persist in the gut of animals for several weeks, and that they must, therefore, coexist with phage-susceptible bacteria. A similar situation has been described in the human gut coexisting with a high abundant population of Bacteroidetes, their proposed hosts (Guerin et al., 2018; Shkoporov et al., 2018; Yutin et al., 2018). The mechanisms driving the long-term coexistence of these antagonistic populations in the gut remain unknown.

Several factors may underlie the coexistence of bacteria and phages, such as arms race dynamics, involving resistance to infection and viral counter-resistance, the ability (inherent or evolved) of the phage to infect multiple hosts, and the spatial distribution of these two populations (Brockhurst et al., 2006; Doron et al., 2018; Galtier et al., 2017; Heilmann et al., 2012; Hilborn, 1975; Labrie et al., 2010). We investigated the contribution of these factors within the gut environment using the synthetic Oligo-Mouse-Microbiota (OMM¹²) model (Brugiroux et al., 2016). The stable colonization of an *Escherichia coli* strain was established and the population dynamics of this strain in the presence of three virulent phages, which were isolated and characterized, were studied. Our data ruled out the possibility of host-jumps (possibility of infecting other strains) and arms race fluctuations being the main drivers of phage-bacteria coexistence. Instead, an analysis of the phage and bacterial contents of different gut sections revealed that phages were less abundant in the ileal sections of the biogeography of the gut to protect themselves from phages. The phages, which reside in the lumen, persist by infecting the bacteria that leave the mucosal refugees.

Results and Discussion

Mice harbouring the OMM¹² consortium were colonized with *E. coli* strain Mt1B1, which became established within two to three days, forming a population that remained stable over a period of two weeks, with no sign of discomfort or change in feces consistency (Fig. 1A). Twelve days after the addition of strain Mt1B1, intestinal sections (ileum, cecum, colon) were examined and the location of strain Mt1B1 was determined by *in situ* fluorescence hybridization (Fig. 1B and Fig. S1). Strain Mt1B1 was found in all sections of the gut, including the ileum, consistent with the primary site from which it was isolated (mucosa from the ileum of conventional laboratory mice) (Garzetti et al., 2018; Lagkouvardos et al., 2016).



Figure 1. *E. coli* strain Mt1B1 stably colonizes the gut of the OMM¹² mice.

A. Fecal levels of *E. coli* strain Mt1B1 at the indicated time points for each OMM¹² mouse (n=11) receiving a single dose of 10⁸ cfu by oral gavage at day 0. **B.** Localization by FISH of the strain Mt1B1 in the ileal section of Mt1B1-colonized OMM¹² mice. Intestinal cells (nuclei) were stained with DAPI, and Mt1B1 (red+green=yellow) and Eubacteria (red) were stained with specific FISH probes. A representative image from a group of four mice is presented (images from colon and cecum are presented in Figure S1). Scale bar, 50μm.

We isolated and purified 28 phages infecting strain Mt1B1 from four sources of sewage water. The host-range of these 28 candidates were then characterized, with a panel of 98 different strains of *E. coli*. Three phages (P3, P10, P17) were chosen for these studies on the basis of their unique infection spectrums (Table S1). All three phages were able to infect strain Mt1B1 in liquid broth. Phages P3 and P10 displayed similar infection patterns, with rapid lysis followed by a moderate bacterial regrowth at 1.5 hours, whereas phage P17 halted the growth of strain Mt1B1 for several hours before the resumption of slow bacterial regrowth only after more than 10 hours. When used in combination, these three phages caused rapid lysis followed by very slow regrowth (Fig. 2A).

Phages P3 and P10 had similar affinity constants (5.26x10⁻¹⁰±2.8x10⁻¹⁰mL.min⁻¹ and 4.60x10⁻¹⁰±2.15x10⁻¹⁰mL.min⁻¹, respectively) and adsorption kinetics (90% of phages adsorbed in 2.8 ±0.14 min and 2.03 ±0.19 min, respectively), whereas phage P17 had a stronger affinity for the bacterium (1.83x10⁻¹⁰±5.60x10⁻¹¹mL.min⁻¹) but slower adsorption kinetics (5.53 ±1.28 min) (Fig. S2). The genomes of these three phages were sequenced and analysed, revealing that phage P3 (40.1 kb; 47 ORFs) and phage P10 (45.1 kb; 56 ORFs) belonged to the SP6virus and T7virus genera, both members of the *Autographivirinae* subfamily of *Podoviridae*, whereas phage P17 (150.9 kb; 295 ORFs) was closely related to phage ESCO13 and belonged to an unclassified subfamily of *Myoviridae* (Table S2). The annotation of predicted ORFs revealed an absence of integrases and recombinases homologs, with 57.44%, 75% and 91.53% of ORFs having unknown functions in phages P3, P10 and P17 respectively (Table S3).

We then assessed the capacity of each phage to replicate in gut sections collected from Mt1B1colonized OMM¹² mice, in an assay previously used to demonstrate the differential activities of phages along the gut (Galtier et al., 2017; Maura et al., 2012a). We collected samples of ileum, cecum and colon from the mice. We then separated out the mucosal and luminal parts of the ileum and colonic tissues. We compared the replication of the three phages in these samples and in Mt1B1 planktonic cultures, at exponential and stationary growth phases. Similar patterns were observed for all three phages, with efficient replication in all gut sections tested and in cultures from exponential phase, but an absence of amplification in cells at stationary phase (Fig. 2B). Thus, Mt1B1 cell growth, even at low rates, is necessary for completion of the infection cycle in these three phages.

We investigated the ability of these three phages to infect the 12 bacteria comprising the synthetic microbiota of OMM¹² mice in vivo, by administering a single dose (6x10⁷ PFU) of a mixture of the three phages (equal amounts of each phage) to OMM¹² mice not inoculated with strain Mt1B1. Phage levels in feces and in some gut sections (cecum and luminal part of the colon) remain low (about 1x10⁴ pfu/g) 24 and 48 hr after administration. In both the luminal and the mucosal sections of the ileum, and in the mucosal part of the colon, the number of phages was below the threshold of detection (Fig. S3). This finding indicates that none of the phages used was able to multiply in any of the 12 strains within this time interval. These observations are consistent with findings for the administration of phages to human volunteers or to conventional mice not colonized with the targeted bacteria (Bruttin and Brussow, 2005; Maura et al., 2012b; Weiss et al., 2009).



Figure 2. Phages P3, P10 and P17 infect strain Mt1B1 both in vitro and ex-vivo.

A. Growth curves for strain Mt1B1 (n=3 for each condition) in liquid broth in the absence (grey) or presence of phage P3 (orange), P10 (blue) or P17 (green), added at t=0, at an MOI of 10⁻², or of a cocktail of these three phages (purple; equal proportions of each) added at t=0, at MOI of 10⁻². The inset shows an enlargement for early time points. **B.** Amplification over 5h (n=3 biological replicates) of individual phages (P3, orange; P10, blue; P17, green; each at an MOI of 10⁻²) and Mt1B1 cells (grey) in indicated homogenized gut sections (lu., luminal; mu, mucosal) from Mt1B1-colonized OMM¹² mice and flasks of cultured Mt1B1 cells during exponential growth (OD600=0.5) or stationary (24h) phases. N-fold multiplication relative to the initial number of phages added is shown.

We then investigated whether Mt1B1-colonized OMM¹² mice receiving equal amounts of each of the three phages (a single combined dose of $6x10^7$ PFU) could reproduce the condition resulting in the long-term coexistence of phages and their target bacterial populations within the gut. OMM¹² mice were fed strain Mt1B1 once by gavage, and colonization was monitored over a period of nine days. A single dose of the three phages was then administered. Over the next 15 days the levels of bacteria and phages in the feces were monitored, showing that these two populations coexisted in similar proportions (Fig. S4). For confirmation of the presence of phage-susceptible bacteria, Mt1B1 clones (n=120, 24 from each of the 5 mice) were isolated from fecal samples on days 10, 16 and 23, and tested with each of the three phages. All these clones were susceptible, confirming that this new murine model reproduces conditions similar to those previously obtained with mice harbouring conventional microbiota, and that it is, therefore, suitable for use in studies of the coexistence of phages and bacteria in the mammalian gut (Galtier et al., 2017; Maura et al., 2012b).

The lack of phage-resistant bacteria in the previous long-term coexistence experiment prompted us to focus on shorter time points. We also increased the phage load, to maximize the chances of capturing phage-resistant clones. In two independent experiments, during which the dose (6x10⁷ PFU) of the three phages was administered once daily on three consecutive days to a group of Mt1B1-colonized OMM¹² mice, we observed a decrease in fecal levels of strain Mt1B1 of less than one magnitude relative to the control group of mice receiving no phage, on each day(Fig. 3A). However, a comparison of the two groups of mice over time revealed a significant overall decrease in the levels of strain Mt1B1 in the group that received the phages (Fig. 3A, p=0.007; TableS4). Over the same period, phage levels remained roughly stable (Fig. 3B). The similar levels of phages and of strain Mt1B1 observed in fecal samples demonstrate the coexistence of these two antagonistic populations (Fig. 3C). This observation was not linked to a significant change in the community structure of the 12-strains consortium, as shown by 16S rRNA qPCR analysis, with daily fluctuations identified as the main source of the observed variations regardless of the presence or absence of added phages (Fig. 3D, Table S5). This finding is also consistent with the lack of amplification of these phages in OMM¹² mice (Fig. S3).



Figure 3. The coexistence of virulent phages with their target strain does not affect the microbiota composition of the OMM¹² mice.

A. OMM¹² mice (n=25) were colonized for 14 days before receiving PBS (red, n=11) or the three phages P3, P10 and P17 together (blue, n=14; 6x10⁷ PFU per dose made of the same amount of each phage) by oral gavage once daily on three consecutive days. Levels of *E. coli* strain Mt1B1 in the feces were recorded over time. **B.** Phage titers from the fecal samples reported in panel A. **C.** Phage:bacteria ratio for fecal samples collected on days 15, 16 and 17 demonstrating the coexistence of the two populations. **D.** Between-group PCA (BCA, axes 1 and 2) for the 16S rRNA qPCR data for mice thareceiving PBS (n=5, filled circle) or the phage cocktail (n=6, filled triangle) by oral gavage at the indicated time points (see the colors indicated) for 10 bacteria from the microbiota of the OMM¹² mice (strains YL2 and KB18 were not detected).

We then investigated whether the stability on the levels of both populations observed above might be due to the growth of a phage-resistant population, decreasing the density of available susceptible bacteria. Mt1B1 clones (n=160, 20 from each of 8 different mice) were isolated from fecal samples on the last day of the experiment. All were susceptible to each of the three phages tested. We can therefore conclude that phage-resistant clones were not selected, ruling out a major role of coevolutionary dynamics (arms race) in the observed coexistence. The selection of phage-resistant mutants was not a dominant element in our experimental design, but it may become more relevant for studies of bacterial pathogens for which phage resistance can affect virulence and, possibly, persistence (Seed et al., 2014; Smith et al., 1987). In the absence of evidence for off-target phage amplification or a coevolutionary arms race between phages and their hosts, we hypothesized that the spatial heterogeneity of the mammalian gut might result in the presence of local refuges, protecting the bacteria against phage predation (Heilmann et al., 2012). We tested this hypothesis by examining several gut sections (luminal and mucosal parts from ileum and colon, together with cecum samples) from mice sacrificed 24 hr after the third phage administration. We first confirmed the lack of "arms race" coevolution in these organs: all the colonies of strain Mt1B1 isolated (n=800; 20 colonies from each organ section of 8 mice) remained susceptible to the three phages (tested individually). We found that strain Mt1B1 levels were significantly lower in all sections, except for the cecum and the colon luminal sections (Fig.4A and Table S6). Phage levels were similar in all sections except the mucosal part of the ileum, in which they were strongly reduced (Fig. 4B). The ratio of phages to bacteria confirmed that the mucosal sections of the ileum harboured significantly fewer phages (p=0.006; Table S6) than the corresponding luminal section (Fig. 4C). Thus, the mucosa seems to be less accessible to phages than the lumen and may constitute a refuge in which a reservoir of strain Mt1B1 is protected against the phage predation. Phage diffusion in the gut may be limited by mucins and other glycoproteins, lipids and DNA molecules (Johansson et al., 2011; Qi et al., 2017). A specific in silico search for immunoglobulin motifs, which have been shown to favour phage binding to mucus, revealed that only ORF 118 of phage P17 possess such a motif (homologous to the bacterial Ig-like domain (Big2)) (Barr et al., 2013; Fraser et al., 2006). Data are consistent with our in vivo observation that these phages are less abundant in mucosal sections.



Figure 4. Lower abundance of phages in the ileum mucosa, consistent with source-sink dynamics underlying the coexistence of phages and bacteria.

A. OMM¹² mice (n=25) were colonized during 14 days before receiving PBS (red, n=11) or the three phages P3, P10 and P17 together (blue, n=14; 6x10⁷ PFU per dose, composed of equal proportions of each phage) by oral gavage once daily on three consecutive days. The mice were thesacrificed and the abundance of *E. coli* strain Mt1B1 in indicated gut sections was determined. **B.** Phage titers for the samples reported in panel A. **C.** Phage:bacteria ratio for the indicated gut sections in panels A and B. **D.** Schematic diagram of the source-sink dynamics between bacteria and virulent phage populations in the gut, in which the phages cannot reach bacteria located close to the mucosal epithelial cell layer.

Our observations of the coexistence of phages and susceptible bacteria fit well with the classical ecological theory of source-sink dynamics (Holt, 1985). Our data show that bacterial refugees in the mucosal layer may serve as a source, with the lumen acting as a sink in which the phages can infect their target. Phages exert no direct selective pressure on the source, so the bacteria reaching the gut lumen remain susceptible, and can therefore be infected by phages, enabling the phages to maintain their density in the lumen.

Other independent or co-dependent processes may also affect the coexistence of bacteria and phage populations in the mammalian gut, and may disturb the healthy balance of the microbiota (Lourenco et al., 2018). Bacteria can modulate the expression of genes encoding phage receptors, due to environmental stochasticity (He et al., 1999; Koziolek et al., 2015; Wang et al., 2007), with oscillation between phage-susceptible and phage-resistant cells, in a phenomenon known as phenotypic resistance (Bull et al., 2014; Chapman-McQuiston and Wu, 2008). This phenomenon, together with high rate of genetic transitions from resistance to susceptibility, accounts for the recently described "leaky resistance" mechanism (Chaudhry et al., 2018). Phages may also adopt a pseudolysogeny state, in which they are less infectious (Siringan et al., 2014). In our experimental setup, we characterized the role of spatial heterogeneity as a driver of the coexistence of populations of virulent phages and bacteria, and ruled out an effect of arms-race dynamics or off-target infections. Our results suggest that source-sink dynamics are involved in the resilience of the gut microbiota, which composition remains globally stable over time despite the permanent predation exerted by the virulent phages. Our work is calling for more in depth studies of the coevolution of phages and bacteria within the mammalian host to decipher how these three entities interact and influence health and diseases (Debarbieux, 2014).
Material and Methods

Ethics statement

All animal experiments were approved by the committee on animal experimentation of the Institut Pasteur and by the French Ministry of Research. C57BI/6J mice (seven to nine-week-old) OMM¹² gnotobiotic mice were bred at Institut Pasteur (Paris, France). A total of 32 mice were used.

Phage isolation

First, sewage water from four locations was filtered at 0.45µm and mixed with an equal volume of 2X Luria- Bertani (LB) medium. Second, these four mixtures were inoculated with a fresh growing culture of Mt1B1 (OD of 0.4 at 600nm, final dilution 1/200) and incubated on a shaker at 37°C overnight. The next day chloroform (1/10 volume) was added to the flasks and incubated at room temperature for one hour before to be centrifuged at 8000g for 10 min. One mL of the supernatant was mixed with 1/10 vol. of chloroform and centrifuged at 8000g for 5 min. A 100 fold dilution in TN buffer (10mM Tris HCl pH7.5, NaCl 100mM) of the aqueous phase was prepared. 10µL of the undiluted and diluted solutions were spread with an inoculation loop on the top of two separate LB agar plates and allowed to dry for 30 min under a safety cabinet. Subsequently, 1 mL of an exponentially growing culture of Mt1B1 was applied to cover entire each plate; the excess liquid culture was removed and the plates were incubated at 37°C overnight. The next day, individual plaques were picked and resuspended in tubes containing 200µl TN buffer. 1/10 vol. chloroform was mixed and tubes were centrifuged at 8000g for 5 min. These steps of plaque purification were performed three times. Finally, 10µL of the last resuspended plaque were added to 1mL of a liquid culture of Mt1B1 (OD of 0.4 at 600nm) and incubated at 37°C in a shaker for 5 hours. 1/10 vol. of chloroform was mixed and after centrifugation at 8000g for 5 min this stock was stored at 4°C and served as starting solution for large scale lysates.

Bacterial strains, and host range tests

Bacterial strains including Mt1B1 (DSM-28618) are listed in Table S1.

Host range tests were performed as follows: 3μ L of PBS-diluted phage solutions (0,2 μ m filtered sterilized crude lysates adjusted to 10^7 pfu/mL) were deposited side by side on the lawn of each tested bacterium on agar LB plates. Plates were incubated at 37°C overnight. Phages were grouped according to their host range and three representative phages of the main groups were chosen (Table S1).

Adsorption assays and phage growth

Three independent adsorption assays were performed for each phage according to the protocol previously described (Chevallereau et al., 2016). Data could be approximated using an exponential function and adsorption times were defined as the time required to reach a threshold of 10% of non-adsorbed phage particles. To record phage growth and bacteria lysis, an overnight culture of strain Mt1B1 was diluted in LB broth and grown to an OD_{600nm} of 0.2 from which 150 µL were distributed into each of the wells of a 96-well plate (Microtest 96 plates, Falcon). 10 µL of sterile phage lysates diluted in PBS to obtain a multiplicity of infection (MOI) of 1 x 10⁻² in each well. Plates were incubated in a microplate reader at 37°C, with a shaking step of 30 sec before the automatic recording of OD_{600nm} every 15 min over 20 hr (Glomax MultiDetection System, Promega, USA).

Phage genomes sequencing and analysis

Sterile phage lysates were treated by DNase (120 U) and RNase (240 µg/mL) and incubated for 30 minutes at 37°C. RNase/DNase reaction was stopped by adding EDTA (20 mM). Lysates were treated with proteinase K (100 µg/mL) and SDS (0.5%) and incubated at 55°C for 30 minutes. DNA was extracted by a phenol-chloroform protocol modified from Pickard (Pickard, 2009). Sequencing was performed using Illumina technology (Illumina Inc., San Diego, CA) MiSeq Nano with paired-end reads of 250bp. Quality of reads was visualised by FastQC v0.10.1 Brabraham Bioinformatics (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Assembly was performed using a workflow implemented in Galaxy-Institut Pasteur using clc_assembler v4.4.2 and clc_mapper v4.4.2 (CLC Bio, Qiagen). Phage termini were determined by PhageTerm (Garneau et al., 2017) and annotations were performed by the RAST v2.0 server (Aziz et al., 2008).

Search for Ig-like domains on phage genomes

Protein sequences of the three phages were scanned for homologs on Pfam database using the HMMER website (Potter et al., 2018). The results were compared to a comprehensive HMM (Hidden Markov Models) database of Ig-like domains found on Pfam that was kindly provided by Dr. Sean Benler.

Ex vivo assay

Oligo-MM12 mice received by oral gavage 200μ L of strain Mt1B1 (10^7 CFU prepared from an overnight culture in LB at 37°C) in sterile sucrose sodium bicarbonate solution (20% sucrose and 2.6% sodium bicarbonate, pH 8) and three days after were sacrificed to collect and weight intestinal sections (ileum,

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cecum and colon). PBS was added to each sample (1.75mL for ileum and colon and 5mL for the cecum) before homogenization (Oligo-Macs, Mylteny).

A volume of 150µL of each homogenized sample was distributed in the wells of a 96-well plate and 10µL of each individual phage was added to reach an MOI of 1 x 10⁻², and the plate was incubated at 37°C. A fraction of the homogenized samples was also serially diluted in PBS and plated on Drigalski medium to count Mt1B1 colonies at t=0. Following five hours of incubation, samples were serially diluted in PBS and plated on Drigalski medium as well as on LB agar plates overlayed with strain Mt1B1. Both set of plates were incubated at 37°C overnight. The same procedure was followed for *in vitro* assays with bacteria taken during exponential (OD 0.5) or stationary (24 hr) growth phase at 37°C with shaking.

Murine model of colonization and quantification of phages and bacteria

The long-term coexistence experiment included 7 mice (5 that received the phages and 2 that did not) and lasted 23 days. At day 0 mice feces were collected prior to Mt1B1 administration by oral gavage (200 µL of bacteria resuspended in sodium bicarbonate buffer from an overnight liquid culture). Fecal pellets were transferred in pre-weighted, sterile, 2 ml tubes, weighted and resuspended in 1 ml of PBS. Serial dilutions in PBS were performed and plated onto Drigalsky plates. The three phages (2x10⁷ PFU of each phage in 200 µL of PBS) were administered altogether once by oral gavage at day 9. The level of phages was assessed from serial dilutions in PBS spotted on LB plates overlayed with strain Mt1B1. Two shorter independent experiments with stronger phage selective pressure were performed with 11 (6 with phages and 5 without) and 14 mice (8 with phages and 6 without) respectively. At day 0 mice feces were collected prior to Mt1B1 administration (as described above) by oral gavage. Fecal pellets were transferred in pre-weighted, sterile, 2 ml tubes, weighted and resuspended in 1 ml of PBS. Serial dilutions in PBS were performed and plated onto Drigalsky plates. The three phages (2x10⁷ PFU of each phage in 200 µL of PBS) were administered altogether once by oral gavage at day 14, 15 and 16. The level of phages was assessed from serial dilutions in PBS spotted on LB plates overlayed with strain Mt1B1. Each mouse was sacrificed at day 17 to collect feces and intestinal sections all homogenized in PBS using gentleMACS[™] OCtoDissociator (Miltenyi Biotec) and plated on both Drigalski plates and LB plates overlayed with strain Mt1B1. The luminal sections correspond to the gut contents that were recovered by pressing the surface of the tissues with the back of a scalpel. The remaining tissues correspond to the mucosal sections that were homogenized together with the organ epithelium.

Identification of resistant clones

20 clones from each gut section and fecal samples were streaked vertically in LB agar plates and subsequently each of the three phages was horizontally streaked across. Plates were incubated at 37°C and phenotype was checked after 5h and overnight.

FISH

Fluorescence in situ hybridization was performed as previously described on intestinal samples from *E. coli* Mt1B1 colonized OMM¹² mice bred at the LMU Munich where the fecal level of strain Mt1B1 reached 10^9 CFU/g (Brugiroux et al., 2016). Ileal, cecal and colonic tissue was fixed in 4% paraformaldehyde (4°C overnight), washed in 20% sucrose (4°C overnight), embedded in O.C.T (Sakura), and flash frozen in liquid nitrogen. FISH was performed on 7µm sections, using double 3'and 5'-labelled 16S rRNA targeted probes specific for Enterobacteriaceae (Ent186-2xCy3 (CCC CCW CTT TGG TCT TGC)) and Eubacteria (1:1 mix of Eub338I-2xCy5 (GCT GCC TCC CGT AGG AGT) and Eub338III-2xCy5 (GCT GCC ACC CGT AGG TGT)). 1 µg/mL–1 4',6-diamidino-2-phenylindole (Roth) was used for DNA staining. Images were recorded with a Leica TCS SP5 confocal microscope (Leica, Wetzlar).

qPCR for 16S quantification

From homogenized fecal samples, 500 µL were centrifuged at 8.000g for 10 min and the supernatant removed. Pellets were diluted in 500 µL of lysis buffer (500 mM NaCl, 50 mM Tris-HCl, pH 8.0, 50 mM EDTA, 4% sodium dodecyl sulfate (SDS)) and incubated for 15 min at 50°C (Yu and Morrison, 2004). Then, 100 µL of lysozyme (25mg/ml) was added and samples were incubated at 37°C for 2 hr. DNA extraction was performed using the Maxwell Cell tissue kit (Promega). The primers, probes and qPCR protocol were used in conformity with previously described methods (Brugiroux et al., 2016) with the exception of the SsoAdvancedTM Universal Probes Supermix (BioRad). The qPCR reactions were performed in duplicate and in two independent runs using MasterCycler realplex4 from Eppendorf. Statistical analysis is described in the section below.

Statistical analysis

Statistical analysis on the number of bacteria and phages were carried out using the Ime4 and ImerTest packages of R (Bates et al., 2015; Kuznetsova et al., 2017). Both CFU and PFU were log10-transformed prior to analysis. In each experiment, two groups of mice were considered, a group exposed to phages and a control (unexposed) group. Beyond these groups, the effects of phages could be assessed based on the abundance of phages (log-PFU).

Given the non-linearity of responses, the day at which a measure was performed was considered as a categorical variable. Linear mixed-models were used to account for random experimental effects (i.e., both individual and cage effects).

Overall effects were assessed with Analysis of Variance (ANOVA) and post-hoc Tukey's comparisons and were performed using the *Ismeans* R package (Lenth, 2016).

16S-quantification data were analysed using multivariate analysis after standard normalization. A principal component analysis (PCA) was performed with the R package ade4 on the matrix of Δ Ct values of 10 bacterial strains (strains YL2 and KB18 were not detected). In addition, a between-group PCA was done in order to assess experimental effects, based on 12 groups of observations: 3 days (0, 14, and 17) and 4 cages (2 exposed, 2 unexposed).

Author contribution

ML, LDS and LD conceived the study. ML, LDS, QLB and CE performed experiments. ML and PC performed statistical analysis. MB, BS and LD secure funding. ML, LDS and LD drafted the paper and all the authors contributed and approved the submitted version.

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Extended Data (Figure S1, S2, S3, S4 and Table S1 to S6)





Figure S1. Localization by FISH of strain Mt1B1 in gut sections from OMM¹² mice.

Intestinal cells (nuclei) were stained with DAPI, and Mt1B1 (red+green=yellow) and Eubacteria (red) were stained with specific FISH probes. Representative images from a group of five mice are presented. Scale bar, 50µm.



Figure S2. Phages P3, P10 and P17 have different adsorption rates.

Adsorption assays for the three phages P3, P10 and P17. Three independent experiments were combined for each phage. A 2×10^5 pfu phage solution was added to exponentially growing culture (OD600=0.3) of strain Mt1B1 in LB medium. Samples were collected every 30 s overa period of 10 minutes for phages P3 and P10, and every 30 s until 5 minutes, and then every minute until 15 minutes had elapsed for phage P17, for counting of non-adsorbed phages. The percentage of phages remaining non-adsorbed (mean of three independent experiments ± s.e) is plotted against time.



Figure S3. Phages do not replicate in the gut in the absence of strain Mt1B1.

Phage titers were assessed from fecal samples and samples from the different sections of the gut collected at 24H (dark green) and 48H (light green) from OMM¹² mice that had received a single dose of the three phages (blue bar; 3x10⁷ pfu; phages mixed in equal proportions).



Figure S4. Mt1B1-colonized OMM¹² mice are suitable for use in studies of the long-term coexistence of phage and bacteria in the mammalian gut.

OMM¹² mice (n=7) were colonized during 14 days before a single administration of PBS (red, n=2) or the three phages P3, P10 and P17 together (blue, n=5; $6x10^7$ PFU per dose, consisting of equal proportions of each phage) by oral gavage on day 9. The levels of *E. coli* strain Mt1B1 were recorded at the indicated time points.

Supplementary Tables

Table S1. Host range of the 28 candidate phages isolated for strain Mt1B1.

(see Excel file)

Table S2. List of the closest phage homologs to phages P3, P10 and P17.

(see below)

Table S3. Table of genome annotations for phages P3, P10 and P17.

(see Excel file)

Table S4. Statistical analysis, with a mixed-effects model, of the abundance of strain Mt1B1 in feces.

(see below)

Table S5. p-values associated with the mixed models applied to the abundance of the 12 strains assessed from qPCR data.

(see below)

Table S6. Statistical analysis of the abundance of strain Mt1B1 and PFU/CFU ratios in intestinal samples.

(see below)

Strains / Phagos	D1	02	D		4 0	Ta	ble DG	\$1.	Hos	t rar	nge (of the	28 c	andid	late p	D15	s isol	ated u	using	strai	n Mt	1B1	022	022	D24	0.25	026	D 2 7	D29 Pafaranca
Strains / Phages	14	P2	P:		4 P	0	0	P7	P8 0	P9 0	019	P11 0	P12	P13	P14	P15	P10	P1/	018	019	P20	P21	P22	P23	P24	P25	P26	P27 0	0 laboratory collection gift from E. Donamur
PDP110				0	0	0	0	0	0	0	0						0	1	0	0				0			0	0	Olaboratory collection, gift from E. Denamur
T145				0	0	0	0	0	0	0	0							1	0	0								0	Olaboratory collection, gift from E. Denamur
PDP351				n	0	0	0	0	0	0	0						0	0	0	0				0			0	0	Olaboratory collection, gift from E. Denamur
FDF331				0	0	0	0	0	0	0	0						0	0	0	0								0	0 Przyczkiowicz E ot al. 2006 (PMID:16012116)
7074	1	1		1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1		1	1	1 AIEC collection from University of Lille France via N. Barnich
55089		-		1	1	1	-	1	1	1	-							0	-	0							0	0	0 Mossoro et al. 2002 (PMID:121/9388)
AL505	1	(0	0	0	0	0	0	0	1	0	1	1	0		0	0	0	0	0	0		0	0		0	0	0 Galtier et al., 2016 (PMID:26971586)
BE	0	0		0	0	0	0	0	0	0	0	0	0	0 0	0		0	0	0	0	0	0	0 0	0	0		0	0	0 Laboratory collection, gift from H. Krisch
BW25113	C	(0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0 0	0	0	0 Datsenko and Wanner, 2000 (PMID:10829079)
CR63	0	0		0	0	0	0	0	0	0	0	0	0	0	0		0	0	0	0	0	0	0 0	0	0	0 0	0	0	0 Laboratory collection, gift from H. Krisch
F22a	0	(0	0	0	0	0	0	0	0	0	0	0	0		0	0	0	0	0	0	0	0	0		0	0	0 Camguilhem and Milon, 1989 (PMID:2656746)
FCOR 1	0	(0	0	0	0	0	0	0	0	0	0	0	0		0	1	0	0	0	0	0	0	0		0	0	0 Ochman, H., and Selander, R.K. (1984) (PMID: 6363394)
ECOR 2	0	0		0	0	0	0	0	0	0	0	0	0	0	0		0	1	0	0	0	0		0	0		0	0	0 Ochman, H. and Selander, R.K. (1984) (PMID: 6363394)
FCOR 3	0	(0	0	0	0	0	0	0	0	0	0	0	0		0	0	0	0	0	0	0 0	0	0		0	0	0 Ochman, H., and Selander, R.K. (1984) (PMID: 6363394)
FCOR 4	0	(0	0	0	0	0	0	0	0	0	0	0	0		0	0	0	0	0	0	0	0	0		0	0	0 Ochman, H., and Selander, R.K. (1984) (PMID: 6363394)
FCOR 5	0	(0	0	0	0	0	0	0	0	0	0	0	0		0	0	0	0	0	0	0	0	0		0	0	0 Ochman, H., and Selander, R.K. (1984) (PMID: 6363394)
FCOR 6	0	(0	0	0	0	0	0	0	0	0	0	0	0		0	0	0	0	0	0	0	0	0		0	0	0 Ochman, H., and Selander, R.K. (1984) (PMID: 6363394)
FCOR 7	0	(0	0	0	0	0	0	0	0	0	0	0	0		0	0	0	0	0	0		0	0		0	0	0 Ochman, H., and Selander, R.K. (1984) (PMID: 6363394)
FCOR 8	0	(0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0 Ochman, H., and Selander, R.K. (1984) (PMID: 6363394)
ECOR 9	C	0		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	C	0 0	0	0	0 Ochman, H., and Selander, R.K. (1984) (PMID: 6363394)
ECOR 10	C	(0	0	0	0	0	0	0	0	0	0	0 0	0	0 0	0	0	0	0	0	0	0	0	C	0 0	0	0	0 Ochman, H., and Selander, R.K. (1984) (PMID: 6363394)
ECOR 11	C	(0	0	0	0	0	0	0	0	0	0	0 0	0	0 0	0	0	0	0	0	0	0	0	C	0 0	0	0	0 Ochman, H., and Selander, R.K. (1984) (PMID: 6363394)
ECOR 12	C	(0	0	0	0	0	0	0	0	0	0	0 0	0	0 0	0	0	0	0	0	0	0	0	C	0 0	0	0	0 Ochman, H., and Selander, R.K. (1984) (PMID: 6363394)
ECOR 13	C	(0	0	0	0	0	0	0	0	0	0	0 (0	0 0	0	0	0	0	0	0	0	0	C	0 0	0	0	0 Ochman, H., and Selander, R.K. (1984) (PMID: 6363394)
ECOR 14	C	(0	0	0	0	0	0	0	0	0	0	0 0	0	0 0	0	0	0	0	0	0	0	0	C	0 0	0	0	0 Ochman, H., and Selander, R.K. (1984) (PMID: 6363394)
ECOR 15	C	(0	0	0	0	0	0	0	0	0	0	0 (0	0 0	0	0	0	0	0	0	0	0	C	0 0	0	0	0 Ochman, H., and Selander, R.K. (1984) (PMID: 6363394)
ECOR 16	0	0		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	C	0 0	0	0	0 Ochman, H., and Selander, R.K. (1984) (PMID: 6363394)
ECOR 17	0	(0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0 Ochman, H., and Selander, R.K. (1984) (PMID: 6363394)
ECOR 18	0	0		0	0	0	0	0	0	0	0	0	0	0 0	0	0 0	0	0	0	0	0	0	0	0	0	0 0	0	0	0 Ochman, H., and Selander, R.K. (1984) (PMID: 6363394)
ECOR 19	0	0		0	0	0	0	0	0	0	0	0	0	0 0	0	0 0	0	0	0	0	0	0	0	0	0	0 0	0	0	0 Ochman, H., and Selander, R.K. (1984) (PMID: 6363394)
ECOR 20	0	(0	0	0	0	0	0	0	0	0	0	0 0	0	0 0	0	0	0	0	0	0	0	0	C	0 0	0	0	0 Ochman, H., and Selander, R.K. (1984) (PMID: 6363394)
ECOR 21	0	0		0	0	0	0	0	0	0	0	0	0	0 0	0	0 0	0	0	0	0	0	0	0	0	C	0 0	0	0	0 Ochman, H., and Selander, R.K. (1984) (PMID: 6363394)
ECOR 22	0	0		0	0	0	0	0	0	0	0	0	0	0 0	0	0 0	0	0	0	0	0	0	0	0	C	0 0	0	0	0 Ochman, H., and Selander, R.K. (1984) (PMID: 6363394)
ECOR 23	C	(0	0	0	0	0	0	0	0	0	0	0	0	0 0	0	1	0	0	0	0	0	0	C	0 0	0	0	0 Ochman, H., and Selander, R.K. (1984) (PMID: 6363394)
ECOR 24	C	(0	0	0	0	0	0	0	0	0	0	0 0	0	0 0	0	1	0	0	0	0	0	0	C	0 (0	0	0 Ochman, H., and Selander, R.K. (1984) (PMID: 6363394)
ECOR 25	0	(0	0	0	0	0	0	0	0	0	0	0 0	0	0 0	0	1	0	0	0	0	0 0	0	C	0 0	0	0	0 Ochman, H., and Selander, R.K. (1984) (PMID: 6363394)
ECOR 26	0	(0	0	0	0	0	0	0	0	0	0	0 0	0	0 0	0	0	0	0	0	0	0	0	0	0 0	0	0	0 Ochman, H., and Selander, R.K. (1984) (PMID: 6363394)
ECOR 27	0	(0	0	0	0	0	0	0	0	0	0	0 0	0	0 0	0	0	0	0	0	0	0 0	0	C	0 0	0	0	0 Ochman, H., and Selander, R.K. (1984) (PMID: 6363394)
ECOR 28	0	0		0	0	0	0	0	0	0	0	0	C	0 0	0	0 0	0	0	0	0	0	0	0	0	C	0 0	0	0	0 Ochman, H., and Selander, R.K. (1984) (PMID: 6363394)
ECOR 29	0	(0	0	0	0	0	0	0	0	0	0	0 0	0	0 0	0	0	0	0	0	0	0 0	0	C	0 0	0	0	0 Ochman, H., and Selander, R.K. (1984) (PMID: 6363394)
ECOR 30	0	(0	0	0	0	0	0	0	0	0	0	0 0	0	0 0	0	1	0	0	0	0	0	0	0	0 0	0	0	0 Ochman, H., and Selander, R.K. (1984) (PMID: 6363394)
ECOR 31	0	(0	0	0	0	0	0	0	0	0	0	0 0	0	0 0	0	0	0	0	0	0	0 0	0	C	0 0	0	0	0 Ochman, H., and Selander, R.K. (1984) (PMID: 6363394)
ECOR 32	0	(0	0	0	0	0	0	0	0	0	C	0 0	0	0 0	0	1	0	0	0	0	0 0	0	C	0 0	0	0	0 Ochman, H., and Selander, R.K. (1984) (PMID: 6363394)
ECOR 33	C	(0	0	0	0	0	0	0	0	0	C	0 0	0	0 0	0	1	0	0	0	0	0 0	0	C	0 0	0	0	0 Ochman, H., and Selander, R.K. (1984) (PMID: 6363394)
ECOR 34	0	(0	0	0	0	0	0	0	0	0	0	0 0	0	0 0	0	0	0	0	0	0	0 0	0	0	0 0	0	0	0 Ochman, H., and Selander, R.K. (1984) (PMID: 6363394)
ECOR 35	0	(0	0	0	0	0	0	0	0	0	0	0 0	0	0 0	0	1	0	0	0	0	0 0	0	0	0 0	0	0	0 Ochman, H., and Selander, R.K. (1984) (PMID: 6363394)
ECOR 36				1	1	1	1	1	1	1	1	1		. 1			1	0	1	1	1		1	1			1	1	1 Ochman, H., and Selander, R.K. (1984) (PMID: 6363394)
ECOR 37	0		-	1	4	0	0	0	0	0	0						0	0	0	0		0		0			0	0	U Ochman, H., and Selander, R.K. (1984) (PMID: 6363394)
ECOR 30	1			1	1	1	1	1	1	1	1						1	0	1	0				1	1		1	1	0 Ochman, H., and Selander, R.K. (1984) (PMID: 6363394)
ECOR 39	1	1	H	1	1	1	1	1	1	1	1	1	1	1	1		1	0	1	1	1	1	1	1	1		1	1	1 Ochman, H., and Selander, R.K. (1984) (PMID: 6363394)
ECOR 40	1	1	-	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1		1	1	1 Ochman, H. and Selander, R.K. (1984) (PMID: 6363394)
ECOR 42		-		1	1	1	0	0	1	1	-							1	0	0					0		0	0	0 Ochman, H. and Selander, R.K. (1984) (PMID: 6363394)
ECOR 43	0	0		0	0	0	0	0	0	0	0						0	0	0	0	0			0	0		0	0	0 Ochman, H. and Selander, R.K. (1984) (PMID: 6363394)
ECOR 43				0	0	0	0	0	0	0	0						0	0	0	0				0			0	0	0 Ochman, H. and Selander, R.K. (1984) (PMID: 6363394)
ECOR 45	0	0		0	0	0	0	0	0	0	0				0		0	0	0	0	0			0	0		0	0	0 Ochman, H. and Selander, R.K. (1984) (PMID: 6363394)
FCOR 46	0	(0	0	0	0	0	0	0	0	0	0	0	0		0	0	0	0	0	0	0	0	0		0	0	0 Ochman, H., and Selander, R.K. (1984) (PMID: 6363394)
ECOR 47	C	(0	0	0	0	0	0	0	0	0	0	0 0	0	0 0	0	0	0	0	0	0	0	0	C	0 0	0	0	0 Ochman, H., and Selander, R.K. (1984) (PMID: 6363394)
ECOR 48	C	(0	0	0	0	0	0	0	0	0	0	0 0	0	0 0	0	0	0	0	0	0	0	0	C	0 0	0	0	0 Ochman, H., and Selander, R.K. (1984) (PMID: 6363394)
ECOR 49	1	(0	0	0	0	0	0	0	1	0	1	. 1	0	0 0	0	0	0	0	0	0	0	0	C	0 (0	0	0 Ochman, H., and Selander, R.K. (1984) (PMID: 6363394)
ECOR 50	1	(0	0	0	0	0	0	0	1	. 0	1	. 1	0	0 0	0	0	0	0	0	0	0	0	0	0 (0	0	0 Ochman, H., and Selander, R.K. (1984) (PMID: 6363394)
ECOR 51	0	(0	0	0	0	0	0	0	0	0	0	0 0	0	0 0	0	0	0	0	0	0	0 0	0	C	0 0	0	0	0 Ochman, H., and Selander, R.K. (1984) (PMID: 6363394)
ECOR 52	0	0		0	0	0	0	0	0	0	0	0	0	0 0	0	0 0	0	1	0	0	0	0	0	0	C	0 0	0	0	0 Ochman, H., and Selander, R.K. (1984) (PMID: 6363394)
ECOR 53	0	0		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0		0	C	0 0	0	0	0 Ochman, H., and Selander, R.K. (1984) (PMID: 6363394)
ECOR 54	0	0		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	C	0 0	0	0	0 Ochman, H., and Selander, R.K. (1984) (PMID: 6363394)
ECOR 55	0	0		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	C	0 0	0	0	0 Ochman, H., and Selander, R.K. (1984) (PMID: 6363394)
ECOR 56	0	0	1	U	0	0	0	0	0	0	0	0	0	0 0	0	0	0	0	0	0	0	0		0	C	0	0	0	0 Ochman, H., and Selander, R.K. (1984) (PMID: 6363394)
ECOR 57	0	0		U	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	U Ochman, H., and Selander, R.K. (1984) (PMID: 6363394)
ECOR 58	0		1	U	U	0	0	0	0	0	0	0		0	0	0	0	0	0	0	0	0		0	0	0	0	0	UUchman, H., and Selander, R.K. (1984) (PMID: 6363394)
ECOR 59	0	0		U	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0		0	C	1 0	0	0	0 Ochman, H., and Selander, R.K. (1984) (PMID: 6363394)
ECOR 60	0		"	U	0	0	0	0	0	0	0	0		0	0	0	0	0	0	0	0	0		0	0	10	0	0	UCchman, H., and Selander, R.K. (1984) (PMID: 6363394)
ECOR 61	1	1		1	1	1	1	1	1	1	1	1	1	. 1		1	1	1	1	1	1	1	1	1	1	1	1	1	1 Uchman, H., and Selander, R.K. (1984) (PMID: 6363394)
ECOR 62	1	1		1	1	1	1	1	1	1	1	1	1	1		1	1	0	1	1	1	1	1	1		<u> 1</u>	1	1	1 Ucnman, H., and Selander, R.K. (1984) (PMID: 6363394)
ECOR 63			-	U	0	0	0	0	0	0	0	0					0	0	0	0	0	0		0	0	1 0	0	0	UCCIMman, H., and Selander, R.K. (1984) (PMID: 6363394)
ECON 64			1		0	0	U	U	0	0	0							0	0	0						1 0		0	Operating P. and Selender, R.K. (1984) (PMID: 6363394)
ECOR 65		F,			0	0	0	0	0	0	0						0	0	0	0	0			0		+ 0		0	Ochman H, and Selander, R.K. (1984) (PMID: 6363394)
ECON 60					0	0	0	U		0	0		-					0	0	0						1		0	0 Ochman, H., and Solandor, P.K. (1984) (PMID: 5353394)
ECOR 6/		F,			0	0	0	0	0	0	0						0	0	0	0	0			0		+ 0		0	Ochman H, and Selander, R.K. (1984) (PMID: 6363394)
FCOP 60		F			0	0	0	0	0	0	0						0	0	U	0								0	Ochman H and Selander P.K. (1994) (PMID: 6363394)
ECOR 70	0				0	0	0	0	0	0	0						0	0	0	0				0				0	Ochman H and Selander R K (1984) (PMID: 6363394)
LECOR /U	0				0	0	0	0	0	0	0						0	0	0	0				0		1		0	0 Darfauille-Michaud et al. 2004 (PMID: 15200573)
1F31	0				0	0	0	0	0	0	0) n	1		0	1	0	0	0	0		0			0	0	0 Darfeuille-Michaud et al 2004 (FWID:15200573)
1550	0	1		0	0	0	0	0	0	0	0				1		0	1	0	0	0	0		0			0	0	0 Darfeuille-Michaud et al. 2004 (PMID:15300573)
LF73	0			0	ō	0	0	0	0	0	0				0		0	0	0	0	0	0		0	0		0	0	0 Darfeuille-Michaud et al., 2004 (PMID:15300573)
LF82	0	1		0	0	0	0	0	0	0	0			0 0	0		0	0	0	0	0	0		0	0		0	0	0 Miguel et al ., 2010 (PMID:20862302)
LM33	0	1		0	0	0	0	0	n	0	0	0	6	0 0	n) (0	0	0	0	0	0		0	1		0	0	0 Dufour et al., 2016 (PMID:27387322)
M1/5	0	1		0	0	0	0	0	0	0	0	0	0	0 0	0	0 0	0	0	0	0	0	0		0	0	0	0	0	0 Laboratory collection, gift from E. Oswald
MG1655	0	6		0	0	0	0	0	0	0	0	0	0	0 0	0	0 0	0	0	0	0	0	0		0	0	0 0	0	0	0 Laboratory collection
Mt1B1	1	1		1	1	1	1	1	1	1	1	. 1	1	. 1	1	1	1	1	1	1	1	1	1	1	1	1 1	1	1	1 Garzetti et al 2018 (DSM-28618)
Nissle 1917	0	6		0	0	0	0	0	0	0	0	0	0	0 0	0	0 0	0	1	0	0	0	0		0	0	0	0	0	0 Laboratory collection, gift from E. Oswald
NRG857c	0	0		0	0	0	0	0	0	0	0	0	0	0 0	0	0 0	0	0	0	0	0	0		0	0	0 0	0	0	0 Small et al ., 2013 (PMID:23748852)
042	0			0	0	0	0	0	0	0	0	0	0	0 0	0	00	0	0	0	0	0	0	0	0	0	0 0	0	0	0 Laboratory collection, gift from C. Le Bouguénec
OP50	0	0		0	0	0	0	0	0	0	0	0	0	0 0	0	0 0	0	0	0	0	0	0	0	0	0	0 0	0	0	0 Laboratory collection, gift from J. Ewbank
SE15	0	1		0	0	0	0	0	0	0	0	0	0	0_0	0	0 0	0	0	0	0	0	0	0	0	C	0_0	0	0	0 Laboratory collection
Sp15	0	0		0	0	0	0	0	0	0	0	0	0	0 0	0	0 0	0	0	0	0	0	0	0	0	0	0 0	0	0	0 Laboratory collection, gift from E. Oswald
ST24	0			0	0	0	0	0	0	0	0	0		0	L C	0	0	0	0	0	0	0		0	C	0	0	0	0 Laboratory collection
total score	11	9		8	8	8	8	8	8	8	11	. 8	11	. 11	8	8 8	8	16	8	8	8	8	8	8	8	3 8	8	8	8
_																		-			-6.01	000			1.0.0	7.05	1		
scoring	0	wa	sg	ive	n to	ph	lage	es th	nat v	vere	e not	able	to cl	ear th	ie lav	vn of	pacte	ria wi	th a s	of	ot 30	.000 F	PFU (:	of 10	10^	7 PFU	/mL)		
	1	Wa	sg	ve	n to	pr	iage	es th	iat v	vere	apl	e to c	ear	ine la	wn o	ir pact	eria	with a	spot	ot 3	0.000	J PFU	(JHL	or 10	/ PF	-u/ml	-)		

nago D3 (
lage i J (40.1 kb)										
/lax score	Total score	Query cover	E value	Identity	Accession						
26378	58105	92%	0	96%	AM084414.1						
26378	58054	92%	0	96%	DQ111067.1						
17570	52746	87%	0	95%	LT594300.1						
17538	51871	84%	0	95%	KJ748011.1						
17533	50929	83%	0	95%	KX689784.1						
12052	51523	83%	0	95%	KX669227.1						
11795	51840	86%	0	95%	EU734172.1						
Homologs to Phage P10 (45.1 kb)											
Aax score	Total score	Query cover	E value	Ident	Accession						
31115	60634	89%	0	93%	KX279892.1						
23555	63566	90%	0	94%	AM084415.1						
20670	57278	89%	0	92%	JN986844.1						
17897	58201	89%	0	91%	AY370674.1						
3954	13337	43%	0	77%	AY288927.2						
age P17 (150.9 kb)										
/lax score	Total score	Query cover	Evalue	Ident	Accession						
63247	2,41E+05	93%	0	98%	KX552041.2						
45930	2,35E+05	91%	0	98%	JX561091.1						
43853	2,33E+05	91%	0	98%	KX664695.2						
4379	29518	28%	0	76%	FR775895.2						
	age P3 (ax score 26378 26378 26378 17570 17538 17533 12052 11795 ge P10 ax score 31115 23555 20670 17897 3954 ax score 532477 43930 4379	age P3 (40.1 kb) ax score Total score 26378 58105 26378 58054 17570 52746 17533 50929 12052 51523 1795 51840 ge P10 (45.1 kb) ax score Total score 31115 60634 23555 63566 20670 57278 17897 58201 3954 13337 3954 13337 3247 2,41E+05 43853 2,33E+05 4379 29518	age P3 (40.1 kb) ax score Total score Query cover 26378 58105 92% 26378 58054 92% 26378 58054 92% 26378 58054 92% 26378 58054 92% 26378 58054 92% 26378 58054 92% 26378 58054 92% 26378 58054 92% 17570 52746 87% 17533 50929 83% 12052 51523 83% 12052 51840 86% ge P10 (45.1 kb) 86% 89% 23555 63566 90% 23555 63566 90% 20670 57278 89% 2057 58201 89% 3954 13337 43% 3954 13337 43% 3247 2,41E+05 93% 4379 2,9518 28% <td>age P3 (40.1 kb) ax score Total score Query cover E value 26378 58105 92% 0 26378 58054 92% 0 26378 58054 92% 0 26378 58054 92% 0 17570 52746 87% 0 17538 51871 84% 0 17533 50929 83% 0 12052 51523 83% 0 12052 51840 86% 0 ge P10 (45.1 kb) 86% 0 ax score Total score Query cover E value 31115 60634 89% 0 23555 63566 90% 0 20670 57278 89% 0 2053 58201 89% 0 20670 57278 89% 0 3954 13337 43% 0 3247 2,41E+05</td> <td>age P3 (40.1 kb)Identityax scoreTotal scoreQuery coverE valueIdentity263785810592%096%263785805492%096%263785805492%096%263785805492%095%175705274687%095%175335092983%095%17955184086%095%120525152383%095%120525184086%095%120525184086%095%120525184086%095%120525184086%095%120525184086%095%120525184086%095%120525184086%095%120525184086%093%120525184089%093%235556356690%094%235556356690%092%178975820189%091%39541333743%077%32472,41E+0593%098%438532,33E+0591%098%43792951828%076%</td>	age P3 (40.1 kb) ax score Total score Query cover E value 26378 58105 92% 0 26378 58054 92% 0 26378 58054 92% 0 26378 58054 92% 0 17570 52746 87% 0 17538 51871 84% 0 17533 50929 83% 0 12052 51523 83% 0 12052 51840 86% 0 ge P10 (45.1 kb) 86% 0 ax score Total score Query cover E value 31115 60634 89% 0 23555 63566 90% 0 20670 57278 89% 0 2053 58201 89% 0 20670 57278 89% 0 3954 13337 43% 0 3247 2,41E+05	age P3 (40.1 kb)Identityax scoreTotal scoreQuery coverE valueIdentity263785810592%096%263785805492%096%263785805492%096%263785805492%095%175705274687%095%175335092983%095%17955184086%095%120525152383%095%120525184086%095%120525184086%095%120525184086%095%120525184086%095%120525184086%095%120525184086%095%120525184086%095%120525184086%093%120525184089%093%235556356690%094%235556356690%092%178975820189%091%39541333743%077%32472,41E+0593%098%438532,33E+0591%098%43792951828%076%						

Table S2. Closest phage homologs to phage P3, P10 and P17.

Magablast tool from NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to search for the closest homologs ranked ranked in decreasing values of query cover.

Table S3. Table of genome annotations for phages P3, P10 and P17.										
				P3						
contig_id	start	stop	strand	predicted function						
P3_gp1	600	442	-	Phage protein						
P3_gp2	2661	898	-	Phage DNA packaging						
P3_gp3	3107	2658	-	Phage endopeptidase (EC 3.4) Rz						
P3_gp4	3472	3209	-	DNA packaging protein A, T7-like gp18						
P3_gp5	3666	3469	-	Phage holin, class II						
P3_gp6	6972	3778	-	hypothetical protein						
P3_gp7	10924	7037	-	hypothetical protein						
P3_gp8	13211	10929	-	hypothetical protein						
P3_gp9	13810	13223	-	Phage internal (core) protein						
P3_gp10	14265	13795	-	Phage internal (core) protein						
P3_gp11	16699	14342	-	hypothetical protein						
P3_gp12	16936	16709	-	Phage protein						
P3_gp13	17502	16936	-	Phage tail fiber protein / T7-like tail tubular protein A						
P3_gp14	18835	17792	-	hypothetical protein						
P3_gp15	19882	18962	-	Phage capsid and scaffold						
P3_gp16	21523	19955	-	Phage collar / T7-like phage head-to-tail joining protein						
P3_gp17	21786	21535	-	hypothetical protein						
P3_gp18	22201	21779	-	Phage protein						
P3_gp19	22430	22206	-	Phage protein (ACLAME 1292)						
P3_gp20	22717	22445	-	Phage protein						
P3_gp21	23794	22928	-	phage exonuclease, putative						
P3_gp22	24012	23794	-	Phage protein						
P3_gp23	24218	24009	-	Phage HNS binding protein (ACLAME 440)						
P3_gp24	24487	24215	-	hypothetical protein						
P3_gp25	26658	24487	-	DNA polymerase						
P3_gp26	26886	26722	-	Phage protein						
P3_gp27	28598	26898	-	T7-like phage primase/helicase protein						
P3_gp28	28882	28667	-	Phage protein						
P3_gp29	29365	28907	-	Phage lysin, N-acetylmuramoyl-L-alanine amidase (EC 3.5.1.28)						
P3_gp30	29825	29355	-	Phage protein						
P3_gp31	30244	29825	-	T7-like phage endonuclease (EC 3.1.21.2)						
P3_gp32	30975	30277	-	hypothetical protein						
P3_gp33	31191	31033	-	Host RNA polymerase inhibitor, T7-like gp2						
P3_gp34	31506	31279	-	Phage protein						
P3_gp35	31936	31496	-	Phage protein						
P3_gp36	32189	31929	-	Phage protein (ACLAME 1535)						
P3_gp37	33398	32322	-	DNA ligase, phage-associated						
P3_gp38	33661	33398	-	dGTP triphosphohydrolase inhibitor						
P3_gp39	33845	33666	-	Phage protein						
P3_gp40	34232	34017	-	Phage protein						
P3_gp41	34475	34245	-	Phage protein						
P3_gp42	37172	34491	-	DNA-directed RNA polymerase (EC 2.7.7.6)						
P3_gp43	37530	37270	-	Phage protein						
P3_gp44	38250	37903	-	hypothetical protein						
P3_gp45	38520	38353	-	Phage protein 160						
P3_gp46	39050	38520	-	Phage receptor binding protein kinase (ACLAME 1534)						
P3_gp47	39378	39124	-	Phage protein						

				P10
contig_id	start	stop	strand	predicted function
P10_repeat	1	267	+	repeat region
P10_gp1	3053	618	-	hypothetical protein
P10_gp2	4578	3139	-	hypothetical protein
P10_gp3	4986	4783	-	Phage protein
P10_gp4	5146	4997	-	Phage protein
P10_gp5	5261	5130	-	hypothetical protein
P10_gp6	5599	5255	-	Phage protein (ACLAME 488)
P10_gp7	5908	5621	-	Phage protein (ACLAME 971)
P10_gp8	6199	5921	-	Phage protein (ACLAME 971)
P10_gp9	8252	6354	-	Phage DNA packaging
P10_gp10	8682	8239	-	HNH homing endonuclease
P10_gp11	8983	8684	-	Phage terminase, small subunit
P10_gp12	9161	8967	-	Phage holin #or could be lysin as there is no membrane signal or lipoP
P10_gp13	10133	9171	-	hypothetical protein
P10_gp14	13441	10133	-	hypothetical protein
P10_gp15	16454	13506	-	hypothetical protein
P10_gp16	17200	16454	-	Phage internal virion protein
P10_gp17	19602	17200	-	Phage tail fibers
P10_gp18	20339	19602	-	Phage tail fibers
P10_gp19	21600	20395	-	Phage capsid and scaffold
P10_gp20	22597	21677	-	hypothetical protein
P10_gp21	24147	22597	-	Phage collar
P10_gp22	24358	24149	-	Phage protein
P10_gp23	24829	24368	-	hypothetical protein
P10_gp24	24948	24826	-	Phage protein
P10_gp25	25174	24959	-	Phage protein
P10_gp26	26093	25146	-	DNA ligase, phage-associated
P10_gp27	26263	26090	-	hypothetical protein
P10_gp28	26735	26265	-	hypothetical protein
P10_gp29	27811	26804	-	hypothetical protein
P10_gp30	28217	27804	-	Phage endonuclease
P10_gp31	29227	28202	-	Phage exonuclease
P10_gp32	29934	29563	-	Phage protein
P10_gp33	30258	30040	-	Phage protein
P10_gp34	31074	30268	-	hypothetical protein
P10_gp35	31451	31152	-	Phage protein
P10_gp36	31835	31458	-	Phage protein
P10_gp37	32089	31991	-	Phage protein
P10_gp38	32205	32101	-	Phage protein
P10_gp39	34751	32205	-	hypothetical protein
P10_gp40	34956	34738	-	Phage protein
P10_gp41	35050	34943	-	Phage protein
P10_gp42	35281	35108	-	Phage protein
P10_gp43	35528	35274	-	hypothetical protein
P10_gp44	35743	35528	-	Phage protein
P10_gp45	36476	35754	-	hypothetical protein
P10_gp46	38538	36553	-	hypothetical protein

contig_id	start	stop	strand	predicted function	
P10_gp47	38896	38540	-	HNH homing endonuclease	
P10_gp48	39028	38900	-	Phage protein	
P10_gp49	41982	39355	-	-like phage DNA-directed RNA polymerase (EC 2.7.7.6) (ACLAME 323)	
P10_gp50	42930	42046	-	hypothetical protein	
P10_gp51	43179	42994	-	Phage protein	
P10_gp52	43369	43256	-	Phage protein	
P10_gp53	43703	43371	-	Phage receptor binding protein kinase (ACLAME 1534)	
P10_gp54	44017	43853	-	Phage protein	
P10_gp55	44368	44144	-	Phage protein	
P10_gp56	44547	44368	-	Phage protein	
P10_repeat	45093	45359	+	repeat region	
P10_gp57	45359	45156	-	hypothetical protein	

	P17								
contig_id	start	stop	strand	predicted function					
P17_repeat	1	328	+	repeat region					
P17_gp1	1664	1245	-	Phage protein					
P17_gp2	2172	1804	-	hypothetical protein					
P17_gp3	2484	2251	-	Phage protein					
P17_gp4	2871	2737	-	Phage protein					
P17_gp5	3158	2868	-	Phage protein					
P17_gp6	3450	3214	-	hypothetical protein					
P17_gp7	3794	3495	-	hypothetical protein					
P17_gp8	4262	3876	-	hypothetical protein					
P17_gp9	4474	4259	-	Phage protein					
P17_gp10	4810	4673	-	hypothetical protein					
P17_gp11	5025	4855	-	hypothetical protein					
P17_gp12	5395	5111	-	hypothetical protein					
P17_gp13	5584	5474	-	hypothetical protein					
P17_gp14	5873	5595	-	hypothetical protein					
P17_gp15	6115	5954	-	hypothetical protein					
P17_gp16	6245	6126	-	hypothetical protein					
P17_gp17	6658	6344	-	hypothetical protein					
P17_gp18	6906	6700	-	hypothetical protein					
P17_gp19	7177	6992	-	hypothetical protein					
P17_gp20	7379	7233	-	hypothetical protein					
P17_gp21	7812	7459	-	hypothetical protein					
P17_gp22	8112	7891	-	hypothetical protein					
P17_gp23	8404	8186	-	Phage protein					
P17_gp24	8650	8483	-	hypothetical protein					
P17_gp25	8954	8742	-	hypothetical protein					
P17_gp26	9272	9045	-	hypothetical protein					
P17_gp27	9603	9364	-	hypothetical protein					
P17_gp28	10058	9693	-	hypothetical protein					
P17_gp29	10335	10156	-	Phage protein					
P17_gp30	10781	10419	-	Phage protein					

contig_id	start	stop	strand	predicted function
P17_gp31	10967	10872	-	hypothetical protein
P17_gp32	11607	11050	-	hypothetical protein
P17_gp33	11866	11609	-	hypothetical protein
P17_gp34	12215	11943	-	hypothetical protein
P17_gp35	12502	12305	-	hypothetical protein
P17_gp36	12776	13042	+	hypothetical protein
P17_gp37	14391	13858	-	Uncharacterized domain COG3236 / GTP cyclohydrolase II (EC 3.5.4.25)
P17_gp38	14567	14388	-	hypothetical protein
P17_gp39	14946	14581	-	hypothetical protein
P17_gp40	15947	14940	-	hypothetical protein
P17_gp41	17156	16017	-	hypothetical protein
P17_gp42	17512	17156	-	hypothetical protein
P17_gp43	17688	17524	-	hypothetical protein
P17 gp44	17862	17698	-	hypothetical protein
P17 gp45	18127	17876	-	hypothetical protein
P17 gp46	18465	18127	-	hypothetical protein
P17 gp47	18652	18476	İ_	hypothetical protein
P17 gp48	19011	18655	-	hypothetical protein
P17 gp49	19295	19008	-	hypothetical protein
P17 gp50	19824	19333	-	hypothetical protein
P17 gp51	20127	19837	-	hypothetical protein
P17 gp52	20245	20670	+	hypothetical protein
P17 gp53	20722	21708	+	hypothetical protein
P17_gp54	21711	21860	+	hypothetical protein
P17_gp54	21861	22184	+	hypothetical protein
P17_gp56	22181	22438	+	hypothetical protein
P17_gp57	22435	22647	_	hypothetical protein
P17_gp57	22400	22047		hypothetical protein
P17_gp50	22040	22330	+	hypothetical protein
P17_gp55	23327	23596	+	hypothetical protein
P17_gp60	23679	23876	+	hypothetical protein
P17 gp62	23920	24093	+	hypothetical protein
P17_gp62	24093	24401	+	hypothetical protein
P17 gp64	24401	24706	+	hypothetical protein
P17_gp65	24750	25070	+	hypothetical protein
P17_gp65	25067	25/20	- -	
P17_gp00	25/31	25700		
P17_gp07	25796	26155	т _	
P17_gp00	26170	26679	т _	
P17 an 70	26604	20019	<u>'</u>	
P17 an71	20034	20200	<u>'</u>	
P17_gp71	20207	20000	т _	
P17 ap72	20000	20130 20103	т _	
P17_gp73	29000	20432	т —	
P17_gp74	20669	30430	-	Rippotnetical protein
P17_gp75	20000	22047	+	Ribonucleotide reductase of class III (anacrobic), faige subunit (EC 1.17.4.2)
P17_gp70	32344	32014	т _	hypothetical protein
P17 gp77	32020	33640	<u>'</u>	
P17 ap70	33640	3/200	' _	
P17 an90	24204	25110	+	
P17 ap91	34291	32110	+	
P17 ap92	25640	30000	+	
Г 17_урод D17_со02	35019	26004	+	
P17 cold	30920	30094	+	
P17_gp84	30937	31281	+	hypothetical protein
P17_gp85	3/4/1	37908	+	Inypotnetical protein 163

contig_id	start	stop	strand	predicted function
P17_gp86	37923	38516	+	hypothetical protein
P17_gp87	38562	39110	+	hypothetical protein
P17_gp88	39107	40219	+	hypothetical protein
P17_gp89	40216	40416	+	hypothetical protein
P17_gp90	40416	40589	+	hypothetical protein
P17_gp91	40582	40980	+	hypothetical protein
P17_gp92	40980	41543	+	hypothetical protein
P17_gp93	41536	41814	+	hypothetical protein
P17_gp94	41816	42610	+	hypothetical protein
P17_gp95	42607	42855	+	hypothetical protein
P17_gp96	42836	43105	+	hypothetical protein
P17_gp97	43121	43723	+	hypothetical protein
P17_gp98	43823	44344	+	hypothetical protein
P17_gp99	44346	44630	+	hypothetical protein
P17_gp100	44630	44860	+	hypothetical protein
P17_gp101	44838	45029	+	hypothetical protein
P17 gp102	45046	45609	+	hypothetical protein
P17 gp103	45618	46301	+	hypothetical protein
P17 gp104	46291	47238	+	hypothetical protein
P17 gp105	47246	48190	+	hypothetical protein
P17 gp106	48190	48468	+	hypothetical protein
P17 gp107	48481	50259	+	hypothetical protein
P17 gp108	50352	50783	+	hypothetical protein
P17 gp109	50793	51587	+	hypothetical protein
P17 gp100	51587	54184	+	hypothetical protein
P17_gp110	54234	54425	+	hypothetical protein
P17 gp112	54425	54592	+	hypothetical protein
P17 gp112	54603	54821	+	hypothetical protein
P17 gp110	54814	55407	+	hypothetical protein
P17 gp115	55453	55845	+	hypothetical protein
P17_gp116	55862	56794	+	hypothetical protein
P17_gp118	56853	57260	+	hypothetical protein
P17 gp118	59960	57288	-	hypothetical protein
P17 gp119	62026	60002	-	hypothetical protein
P17_gp110	65142	62089	-	hypothetical protein
P17 gp120	65481	65146	_	hypothetical protein
P17_gp121	66036	65491	_	hypothetical protein
P17 gp122	67098	66037	-	hypothetical protein
P17 gp128	67737	67108	-	hypothetical protein
P17 gp125	69227	67740	_	hypothetical protein
P17_gp126	69487	69227	_	hypothetical protein
P17_gp120	71543	69480	_	hypothetical protein
P17 gp128	74428	71552	_	nhage tail protein l
P17_gp120	74901	74428	_	hypothetical protein
P17_gp120	75527	7/001	_	
P17_gp130	76263	74901	-	
P17_gp131	77273	76263	_	hypothetical protein
P17 gp132	77606	77292	-	hypothetical protein
P17_gp133	79400	77600	-	hypothetical protein
P17 ap125	10400 20515	70507	-	
P17 ap126	8075A	10021 80526	- _	
P17 ap127	00/04	00000	-	
P17 co100	01204	00002	-	
P17_cc120	01009	01330	-	
P17_gp139	83229	00001	-	hypothetical protein
r17_gp140	o3902	o3264	1-	nypothetical protein 164

contig_id	start	stop	strand	predicted function
P17_gp141	84311	83895	-	hypothetical protein
P17_gp142	84845	84357	-	hypothetical protein
P17_gp143	85393	84845	-	hypothetical protein
P17_gp144	85798	85403	-	hypothetical protein
P17 gp145	86833	85832	-	hypothetical protein
P17 gp146	87253	86855	-	hypothetical protein
P17 gp147	88388	87273	-	hypothetical protein
P17 gp148	88870	88391	-	hypothetical protein
P17 gp149	90529	88964	_	hypothetical protein
P17_gp140	02706	00631	_	
P17_gp150	92700	02051	-	
P17_gp151	02564	92901	-	
P17_gp152	93304	93394	-	
P17_gp153	94001	93705	-	
P17_tRNA1	94287	94212	-	
P17_tRNA2	94439	94369	-	tRNA-lie-GA I
P17_tRNA3	94522	94449	-	tRNA-Pro-IGG
P17_tRNA4	94603	94531	-	tRNA-GIn-TTG
P17_tRNA5	94775	94705	-	tRNA-Gly-TCC
P17_tRNA6	95164	95093	-	tRNA-Thr-TGT
P17_tRNA7	95340	95258	-	tRNA-Asn-GTT
P17_tRNA8	95434	95350	-	tRNA-Pseudo-GTA
P17_tRNA9	95650	95568	-	tRNA-Ser-GCT
P17_tRNA10	96054	95983	-	tRNA-Arg-TCT
P17_tRNA11	96131	96060	-	tRNA-Met-CAT
P17_gp154	96626	96378	-	hypothetical protein
P17_gp155	96805	96635	-	hypothetical protein
P17_gp156	97109	96924	-	hypothetical protein
P17_gp157	97591	97914	+	hypothetical protein
P17_gp158	97898	98014	+	hypothetical protein
P17_gp159	98011	98205	+	hypothetical protein
P17 gp160	98395	98748	+	hypothetical protein
P17 gp161	98745	99026	+	hypothetical protein
P17 gp162	99023	99205	+	hypothetical protein
P17 gp163	99202	99408	+	hypothetical protein
P17 gp164	99405	99875	+	hypothetical protein
P17 gp165	99886	100242	+	hypothetical protein
P17_gp166	100252	100242	+	hypothetical protein
P17_gp166	100202	100758	+	Phage anti-restriction nuclease (ACI AME 1193)
P17 ap168	100752	101200	+	hypothetical protein
P17 an160	101211	102242		hypothetical protein
P17 ap170	102244	102243	т _	
D17 ap174	102244	102040	т 1	
P17_gp171	102038	102904	+	
F1/_gp1/2	102994	103185	+	
P17_gp173	103182	103403	+	
P17_gp174	103403	104518	+	DNA ligase, phage-associated
P17_gp175	104528	104722	+	hypothetical protein
P1/_gp176	104719	104901	+	nypotnetical protein
P17_gp177	104911	105636	+	hypothetical protein
P17_gp178	105693	106571	+	hypothetical protein
P17_gp179	106644	106853	+	hypothetical protein
P17_gp180	107295	107984	+	hypothetical protein
P17_gp181	107981	108940	+	hypothetical protein
P17_gp182	109028	109255	+	hypothetical protein
P17_gp183	109248	109649	+	hypothetical protein
P17_gp184	109633	109827	+	hypothetical protein165

contig_id	start	stop	strand	predicted function
P17_gp185	109838	110392	+	hypothetical protein
P17_gp186	110429	111037	+	hypothetical protein
P17_gp187	111047	112045	+	hypothetical protein
P17_gp188	112144	112554	+	hypothetical protein
P17_gp189	112551	112805	+	hypothetical protein
P17_gp190	112822	115062	+	Ribonucleotide reductase of class la (aerobic), alpha subunit (EC 1.17.4.1)
P17_gp191	115107	116192	+	Ribonucleotide reductase of class la (aerobic), beta subunit (EC 1.17.4.1)
P17_gp192	116192	116401	+	hypothetical protein
P17_gp193	116401	116553	+	hypothetical protein
P17_gp194	116543	116707	+	hypothetical protein
P17_gp195	116697	117023	+	hypothetical protein
P17_gp196	117033	117599	+	dTDP-4-dehydrorhamnose 3,5-epimerase (EC 5.1.3.13)
P17_gp197	117593	118435	+	dTDP-4-dehydrorhamnose reductase (EC 1.1.1.133)
P17_gp198	118435	118695	+	hypothetical protein
P17_gp199	118701	118850	+	hypothetical protein
P17_gp200	118858	119358	+	T4-like phage baseplate hub + tail lysozyme
P17_gp201	119396	120154	+	hypothetical protein
P17_gp202	120190	120690	+	hypothetical protein
P17_gp203	120690	121382	+	hypothetical protein
P17_gp204	121428	121862	+	hypothetical protein
P17_gp205	121846	122355	+	hypothetical protein
P17_gp206	122368	122604	+	hypothetical protein
P17_gp207	122604	122807	+	hypothetical protein
P17_gp208	122853	123047	+	hypothetical protein
P17_gp209	123062	123634	+	hypothetical protein
P17_gp210	123645	123974	+	hypothetical protein
P17_gp211	123971	124714	+	hypothetical protein
P17_gp212	124714	125022	+	hypothetical protein
P17_gp213	125030	125611	+	hypothetical protein
P17_gp214	125601	126113	+	hypothetical protein
P17_gp215	126092	126382	+	hypothetical protein
P17_gp216	126366	126563	+	hypothetical protein
P17_gp217	126575	126850	+	hypothetical protein
P17_gp218	126850	127068	+	hypothetical protein
P17_gp219	127078	127350	+	hypothetical protein
P17_gp220	127441	128382	+	Phage protein (ACLAME 1471)
P17_gp221	128384	128779	+	hypothetical protein
P17_gp222	128772	129455	+	Phage protein (ACLAME 536)
P17_gp223	129455	130108	+	hypothetical protein
P17_gp224	130167	130652	+	hypothetical protein
P17_gp225	130637	131452	+	NAD-dependent protein deacetylase of SIR2 family
P17_gp226	131496	131768	+	hypothetical protein
P17_gp227	132068	131838	-	hypothetical protein
P17_gp228	132440	132159	-	hypothetical protein
P17_gp229	132626	132453	-	hypothetical protein
P17_gp230	132780	132679	-	hypothetical protein
P17_gp231	133398	132790	-	hypothetical protein
P17_gp232	133670	133407	-	hypothetical protein
P17_gp233	133834	133667	-	hypothetical protein
P17_gp234	134280	133831	-	hypothetical protein
P17_gp235	134461	134264	-	hypothetical protein
P17_gp236	134682	134464	-	hypothetical protein
P17_gp237	134911	134696	-	hypothetical protein
P17_gp238	135481	135272	-	hypothetical protein
P17_gp239	135894	135475	-	hypothetical protein166
P17_gp240	136048	135914	-	hypothetical protein

contig_id	start	stop	strand	predicted function
P17_gp241	136244	136041	-	hypothetical protein
P17_gp242	136569	136231	-	hypothetical protein
P17_gp243	137232	136621	-	hypothetical protein
P17_gp244	137509	137237	-	hypothetical protein
P17_gp245	137874	137509	-	hypothetical protein
P17_gp246	138274	137852	-	hypothetical protein
P17_gp247	138954	138283	-	Methionine ABC transporter ATP-binding protein
P17_gp248	139237	139025	-	Phage protein
P17_gp249	139561	139238	-	hypothetical protein
P17_gp250	139871	139572	-	hypothetical protein
P17_gp251	140338	139925	-	hypothetical protein
P17_gp252	140713	140393	-	hypothetical protein
P17_gp253	140922	140710	-	hypothetical protein
P17_gp254	141152	140925	-	hypothetical protein
P17_gp255	141478	141152	-	hypothetical protein
P17_gp256	141976	141779	-	hypothetical protein
P17_gp257	142338	141976	-	hypothetical protein
P17_gp258	142603	142340	-	hypothetical protein
P17_gp259	143054	142608	-	hypothetical protein
P17_gp260	143463	143065	-	hypothetical protein
P17_gp261	143765	143559	-	hypothetical protein
P17_gp262	143989	143768	-	hypothetical protein
P17_gp263	144179	143991	-	hypothetical protein
P17_gp264	144394	144194	-	hypothetical protein
P17_gp265	144699	144385	-	hypothetical protein
P17_gp266	144876	144700	-	hypothetical protein
P17_gp267	145251	144892	-	hypothetical protein
P17_gp268	145452	145261	-	hypothetical protein
P17_gp269	145651	145433	-	hypothetical protein
P17_gp270	146121	145648	-	hypothetical protein
P17_gp271	146368	146135	-	hypothetical protein
P17_gp272	146588	146379	-	hypothetical protein
P17_gp273	146812	146588	-	hypothetical protein
P17_gp274	147111	146812	-	hypothetical protein
P17_gp275	147576	147139	-	hypothetical protein
P17_gp276	148044	147589	-	hypothetical protein
P17_gp277	148505	148050	-	hypothetical protein
P17_gp278	148692	148498	-	hypothetical protein
P17 gp279	149146	148685	-	hypothetical protein
P17_gp280	149382	149143	-	hypothetical protein
P17_gp281	149625	149395	-	hypothetical protein
P17_gp282	149827	149618	-	hypothetical protein
P17_gp283	150255	149887	-	hypothetical protein
P17_gp284	150589	150245	-	hypothetical protein
P17_repeat	150875	151202	+	repeat region

Table S4. Statis	tical analysis of the co	lonization	level of strai	n Mt1B1 i	in fecal san	nples
ANOVA						
day: testing dif	ference between the	different	days			
phage: effect o	of the phage exposure					
Response:	<pre>scale(log10(CFU.g))</pre>					
	Chisq	Df	Pr(>Chisq)			
day	15,4517	3	0,0015			
phage	7,0961	1	0,0077			
day:phage	12,8145	3	0,0051			
Post-hoc tests	Comparison betweer	"nhage" a	and "no phage	" at the o	lifferent ti	me points

df contrast estimate SE t.ratio p.value Day=14 no phage vs. phage -0,20947 0,3581942 72,33 -0,585 0,5605 Day=15 no phage vs. phage 1,217633 0,3581942 72,33 3,399 0,0011 2,641 Day=16 no phage vs. phage 0,946077 0,3581942 72,33 0,0101 Day=17 no phage vs. phage 0,678505 0,3581942 72,33 1,894 0,0622

Bacterial abundance (CFU) as a function of time (day) and exposure to phages. Random effects include individual IDs as well as the cage in which they were reared. Overall analysis of variance (ANOVA) reveals significant effects of day (p=0.001469), phage (p=0.007725) and their interaction (p=0.005056). The post-hoc Tukey comparisons displayed below were performed between mice exposed to phage and not exposed within each day.

•				-
day 14: variation	ns within samples day 14			
day 17: variation	ns within samples day 17			
experiment: vai	riations taking in account th	ne 3 days (0, 14 and 17)		
phage: variation	ns caused by phage expositi	ion		
ref#	148	YL44	YL27	YL32
Strain name	Bacteroides caecimuris	Akkermansia muciniphila	Muribaculum intestinale	Clostridium clostridioforme
day 14	0,238	0,354	0,403	0,182
day 17	0,45	0,01	0,838	0,01
experiment	0,078	0,862	0,442	0,179
phage	0,28	0,071	0,867	0,275
ref#	146	149	YL58	YL45
Strain name	Clostridium innocuum	Lactobacillus reuteri	Blautia coccoides	Turicimonas muris
day 14	0	0,279	0	0,55
day 17	0,782	0,256	0,022	0,035
experiment	0,509	0,556	0,338	0,295
phage	0,269	0,269	0,434	0,434
ref#	KB1	YL31	YL2	KB18
Strain name	Enterococcus faecalis	Flavonifractor plautii	Bifidobacterium longum s	Acutalibacter muris
day 14	0,022	0,078	NA	NA
day 17	0,269	0,011	NA	NA
experiment	0,112	0,684	NA	NA
phage	0,403	0,579	NA	NA
The model tested	variations within day 14. withi	in day 17 and also variations	on the samples taking into a	account days 0. 14 and 17.

Table S5. p-values associated with the mixed models applied to the abundance of the 12 strains assessed from qPCR data

The model tested variations within day 14, within day 17 and also variations on the samples taking into account days 0, 14 and 17 The model also tested variations on each strain taking into account the phage exposure.

Table S6. Statistical analysis of the abundance of strain Mt1B1 and PFU/CFU ratios in intestinal sections

CFU abundance i	in sections						
ANOVA/ANODE							
	<pre>scale(log10(CFU.g+1))</pre>						
	Chisq	Df	Pr(>Chisq)				
section	77,354	4	6,33E-16	***			
phage	25,713	1	3,96E-07	***			
section:phage	10,113	4	0,03857	*			
Post-hoc tests							
	Comparison between "phage" and "no phage" in the different sections						
	contrast	estimate	SE	df	t.ratio	p.value	
ileum_lumen	no phage vs. phage	1,0723044	0,2959727	110,16	3,623	0,0004	
ileum_mucosa	no phage vs. phage	0,9855932	0,2959727	110,16	3,33	0,0012	
cecum	no phage vs. phage	0,1633725	0,2959727	110,16	0,552	0,5821	
colon_lumen	no phage vs. phage	0,525542	0,2959727	110,16	1,776	0,0786	
colon_mucosa	no phage vs. phage	1,2514249	0,2959727	110,16	4,228	<.0001	

Bacterial abundance (CFU) as a function of organ and exposure to phages. Random effects include individual IDs as well as the cage in which they were reared. Overall analysis of variance (ANOVA) reveals significant effects of *organ* (p=6.33E-16), *phage* (p=3.96E-07) and their *interaction* (p=0.03857). The posthoc Tukey comparisons displayed below were performed between the mice exposed to phage and not exposed within each day.

PFU/CFU ratios	

ANOVA – ANODI							
Response:	scale(L.ratio)						
	Chisq	Df	Pr(>Chisq)				
organ	2,4925	2	0,287582				
group ^a	9,7241	1	0,001819				
organ:group	0,7109	1	0,399152				
^a group= luminal	or mucosal						
Post-hoc test							
organ	contrast	estimate	SE	df	t.ratio	p.value	
colon	lumen-mucosa	0,5535417	0,3440682	62,13	1,609	0,1127	
ileum	lumen-mucosa	0,9637997	0,3440682	62,13	2,801	0,0068	

The ratios of phages over bacteria abundance (PFU/CFU) as a function of organ and group (mucosa or lumen). Random effects include individual IDs as well as the cage in which they were reared. Overall analysis of variance (ANOVA) reveals no significant effects of *organ* (p=0.287582), but significant of *group* (p=0.001819). The post-hoc Tukey comparisons displayed below were performed between the lumen and mucosa data. Post-hoc tests revealed a significant difference between lumen and mucosa of the ileum (p=0.0068). 170

Chapter 5

"I will survive": A tale of bacteriophage-

bacteria coevolution in the gut

Chapter 5

"I will survive": A tale of bacteriophage-bacteria coevolution in the gut

Introduction

A previous phage-bacteria coevolution experiment, from our laboratory, using a tripartite network of one virulent phage (P10) and its host (LF82) and a strain (MG1655) insensitive to the phage, showed an adaptation of the phage to the insensitive strain in conventional mice but not in *in vitro* cultures or monoaxenic mice. This adaptation (host-jump) was shown to be mediated by an intermediate bacterial host present on the microbiota of conventional mice (De Sordi et al., 2017), demonstrating the role of the microbiota as a driver of phage diversification. From these experiments it was observed, in the mice in which the host-jump was detected, the emergence of MG1655 clones resistant to the adapted P10 as well as second-adapted phages to these new clones. The goal of the work presented in this chapter was to explore the phenotypic and genomic characteristics of this coevolution.

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The author of this thesis performed bacteria and phage genomes analysis and comparisons and participated in the preparation of the manuscript. Luisa De Sordi performed the remaining experimental work and drafted the manuscript.

Title:

"I will survive": a tale of bacteriophage-bacteria coevolution in the gut

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Abstract

Viruses that infect bacteria, or bacteriophages, are among the most abundant entities in the gut microbiome. However, their role and the mechanisms by which they infect bacteria in the intestinal tract remain poorly understood. We recently reported that intestinal bacteria are an evolutionary force, driving the expansion of the bacteriophage host range by boosting the genetic variability of these viruses. Here, we expand these observations by studying antagonistic bacteriophage-bacteria coevolution dynamics and revealing that bacterial genetic variability is also increased under the pressure of bacteriophage predation. We propose a model showing how the expansion of bacteriophage-bacteria infection networks is relative to the opportunities for coevolution encountered in the intestinal tract. Our data suggest that predator-prey dynamics are perpetuated and differentiated in parallel, to generate and maintain intestinal microbial diversity and equilibrium.

Introduction

The homeostasis of the intestinal microbiome is crucial to health, as shown by the ever-growing list of chronic conditions linked to microbiota dysbiosis, including obesity, diabetes, asthma, inflammatory bowel disease (IBD) and central nervous system disorders(Fujimura and Lynch, 2015; Gallo et al., 2016; Sampson et al., 2016; Tremaroli and Backhed, 2012). The antagonistic coevolution between the two most abundant components of the microbiome, bacteria and their viruses, bacteriophages, is a key candidate player in the maintenance of this microbial equilibrium (Scanlan, 2017).

The perpetuation of bacteriophages is intrinsically dependent on their ability to predate on the bacterial populations and experimental coevolution studies have characterised the dynamics of interactions between bacteria and bacteriophages (Buckling and Rainey, 2002; Gandon et al., 2008). The development of bacterial resistance, and the consequent bacteriophage adaptation towards such resistance, have been identified as major forces driving their antagonistic coevolution *in vitro* and in environmental samples. This arms race necessarily results in an increase in the genomic diversity of both partners to ensure population survival (Gomez et al., 2016; Koskella and Brockhurst, 2014), as seen in aquatic ecosystems (Enav et al., 2018; Middelboe et al., 2009). However, most studies of this type are limited to single pairs of bacteria and bacteriophages and are frequently performed in laboratory settings.

Metagenomic analyses of intestinal bacterial populations have revealed that these organisms are diverse and differently abundant in healthy humans and diseased patients. Fewer studies have focused on viral populations (virome), but those that have been performed have revealed an unprecedented complexity of relationships between bacteriophages, bacteria and the mammalian host (Mirzaei and Maurice, 2017). A recent comparative study showed that healthy humans share a pool of conserved intestinal bacteriophages that differs significantly from the viruses found in patients with inflammatory bowel disease (IBD) (Manrique et al., 2016). Also, in these patients, lower bacterial diversity is associated with a significantly larger number and diversity of bacteriophages (Norman et al., 2015). Similarly, a recent microbiome study conducted on malnourished pediatric patients hospitalized with acute diarrhea showed an increase in *Escherichia coli* bacteriophages compared to healthy individuals, that negatively correlated with the abundance of the bacterial host (Kieser et al.). Other studies suggest that bacteriophages play a key role in regulating intestinal bacterial populations by showing that filtered (bacteria-free) faecal microbiota transplantation (FMT) yields curative results comparable to those obtained with traditional FMT, and that viral transfer correlates with the resolution of gut infections caused by *Clostridium difficile* (Ott et al., 2016)[,] (Zuo et al., 2017). Nonetheless, exploitation of this genomic information at the molecular level remains limited, because most of the sequences obtained do not match to a known function. Another major hurdle is the lack of association between bacteriophage sequences and those of their specific bacterial hosts. There is, therefore, a considerable gap between studies of interactions between bacteriophages and bacteria in laboratory conditions and the complexity of these interactions in the gut (Manrique et al., 2016; Reyes et al., 2015).

In the environment, bacteria and bacteriophages coexist in intricate, structured interaction networks (Flores et al., 2011; Weitz et al., 2013). Bacterial species are represented by distinct genetic lineages (strains) and bacteriophages are mostly strain-specific: rare are bacteriophages that infect most strains within one given species and even fewer are those infecting distinct species. Thus, little is known about the role of bacteriophage-bacteria infection networks in driving the diversification of the gut microbial ecosystem in the context of health and disease.

Microbiota-driven bacteriophage adaptation

Reductionist approaches using E. coli and its bacteriophages have successfully deciphered major mechanisms of molecular biology (Brenner et al., 1961; Cairns et al., 2007; Hershey and Chase, 1952). By lifting the reductionist approach to the next level of complexity, namely the study of the intestinal microbiota, we recently described the coevolution of one bacteriophage with multiple host strains within the mouse gut (De Sordi et al., 2017). We studied P10, a virulent bacteriophage from the Myoviridae family, infecting the *E coli* strain LF82, and we assessed its ability to adapt to E. coli strain MG1655, to which it was initially unable to bind and therefore could not infect. Such host-range expansion was observed, but only occurred during coevolution in the gut of conventional mice hosting *E. coli* strains LF82 and MG1655 within their microbiota. In planktonic in vitro cultures or in the gut of dixenic mice colonized solely by the two E. coli strains, this event was never detected. Based on these findings, we hypothesized that the mouse microbiota played a crucial role in promoting adaptation. Indeed, we showed that this adaptation was initiated by the infection of an intermediate host, *E. coli* strain MEc1, which we isolated from the murine microbiota. Mixing bacteriophage P10 in vitro with the three E. coli strains also promoted viral host-range expansion. This adaptation was accompanied by genomic differentiation in the bacteriophage population: a single point mutation in a tail fibre-encoding gene was found to be sufficient to promote host adaptation, but additional mutations were required to optimise the infectious cycle.

The spatial and temporal dynamics of the acquisition of these mutations in the structured intestinal environment remain unclear. However, our data are consistent with the hypothesis that the genomic differentiation of bacteriophage subpopulations depends on the diversity of the bacteria encountered, making the microbiota an ideal site to generate viral diversity. In addition to bacterial diversity, the spatial distribution of bacterial populations along the gut may also influence the dynamics of bacteriophage evolution (Suzuki and Nachman, 2016; Wang et al., 2005). The colonisation of macro-environments, such as the small versus the large intestine and the their compartments (luminal and mucosal), and the occupation of specific niches within these contexts (nutrient-niche hypothesis (Freter et al., 1983)), give rise to structured networks of single or mixed bacterial populations (Pereira and Berry, 2017) likely to promote the diversification of bacteriophages into multiple subpopulations with diverging infectivity profiles.
Genetic bacterial resistance in the gut

Here, we analyse a second source of genomic diversity, the emergence of bacterial resistance, one of the drivers of antagonistic evolution (Hall et al., 2011; Koskella and Brockhurst, 2014; Scanlan, 2017). Faecal pellets of mice in which P10 adaptation had occurred, yielded five MG1655 clones displaying different degrees of resistance to adapted P10 bacteriophages (Fig. 1A). The genomes of these five strains presented different mutations in the *waaZ* gene, which encodes a protein involved in the biosynthetic pathway for the core lipopolysaccharide (LPS) (Fig. 1B; Table S1). We identified four convergent paths of adaptation, characterised by gene disruption by insertion sequences (ISs), IS5 and IS2, at different gene positions. We hypothesise that independent convergent events leading to modifications of the LPS core biosynthesis pathway had served as the first step towards adaptation of the newly targeted strain MG1655, under the selective pressure of bacteriophage predation.



Figure 1. Bacteriophages and bacteria coevolve in the mouse gut.

A) Adapted (ad) P10 bacteriophages show differential infectivity towards coevolved (ev) clones of *E. coli* strain MG1655 (MG) isolated at the same time point and that have developed bacteriophage resistance. Infectivity of five P10 bacteriophages (1-3,5-6) was tested against five MG1655 clones (a-e) by double spot technique (Saussereau et al., 2014) with two amounts of bacteriophages (10^6 and 10^4 pfu) in three replicates. Positive results of infection were determined by recording bacterial lysis and are shown as black dots. B) Bacterial genomic mutations under bacteriophage selective pressure in the mouse gut: ev MG clones a-to-e were sequenced by Illumina technology and mutations were called using the Breseq variant report software v0.26 (Barrick et al., 2009). Mutations (orange, red and blue triangle - IS1, IS2 and IS5 respectively, black triangle pointing down - 1-5bp insertion, black triangle pointing up - 1-5bp deletion, vertical black rectangle – SNP and black horizontal rectangle - >1kb deletion) are reported relative to their positions in the genome. For mutation hotspots, the relative targeted genes are reported as purple arrows. For a complete list of bacterial genomic mutations see Table S1. The corresponding sequences are deposited at ENA under project PRJEB24878. C) Bacteriophage genomic mutations accumulated during coevolution with strain MG1655 in the mouse gut. Sequences of five adapted P10 bacteriophages (ad P10 1-3,5-6) were analysed as described for bacterial clones. Mutations are relative to their positions in the bacteriophage genome (ORFs are shown as purple arrows) and mutation hotspots are indicated (same legend as for panel B). For a complete list of viral genomic mutations, see Table S2. The corresponding sequences are deposited at ENA under project PRJEB18073. D) Bacteriophages overcome genetic bacterial resistance. A time-shift experiment shows the percentage infectivity of fourty P10 bacteriophages from different time points tested towards fourty MG1655 clones isolate from past, present and future time-points during coevolution in the mouse gut. Bacterial lysis was tested by double-spot assay (Saussereau et al., 2014).

Another gene, *waaY*, flanking *waaZ*, was also targeted by IS elements in three of the coevolved MG1655 clones. The occurrence of these mutations, coupled to the high degree of sequence identity between bacteriophage P10 and the LPS-binding WV8 and Felix-O1 bacteriophages (Hudson et al., 1978; Villegas et al., 2009), suggests a bacterial resistance strategy based on the masking of the bacteriophage receptor. Interestingly, natural populations of *Vibro cholerae* isolated from patients with diarrhoea have also been shown to consist of heterogeneous mixtures of unique mutants resistant to bacteriophage predation (Seed et al., 2014). However, these mutants were subject to fitness and virulence costs that might arguably affect their infection potential. Similarly, experimental phage therapy studies revealed that bacterial pathogens can develop bacteriophage resistance at the expenses of their major virulence factors, as shown in bovine enteropathogenic *E. coli* (Smith and Huggins, 1983) or during experimental endocarditis due to *Pseudomonas aeruginosa (Oechslin et al., 2017)*.

Further genomic analysis of the MG1655 clones that had coevolved with P10 identified a second hotspot for mutations in the galactitol operon, which was previously shown to be pervasive in *E. coli* clones adapting to the gut environment (Barroso-Batista et al., 2014)⁻(Lourenco et al., 2016). In addition, two sugar metabolism pathways (maltose and galactonate) were targeted by IS insertions in genes encoding the DNA-binding transcriptional regulators (*malT*, *IgoR*), with probable positive or negative overall effects on pathway activation.

The contextual genomic variability of bacteriophages was also analysed by sequencing five adapted bacteriophages differing in their ability to infect the five MG1655 clones considered (Fig. 1C). The only bacteriophage able to infect all the bacterial clones had the largest number of mutations (12 mutations, versus 5 to 9 in the other bacteriophages isolated; Table S1), suggesting a possible faster pace of adaptation in response to bacterial resistance. The mutations were clustered into four genomic regions. The first corresponds to the *rIIA* (*gp37*) gene, the function of which is probably related to infection fitness, as this gene was also highlighted in our population genomics study in *in vitro* conditions (De Sordi et al., 2017). A second, larger region encompasses several structural genes, including the tail fibre genes. The *gp55* and *gp57* genes, which are predicted to encode two subunits of the class I ribonucleotide reductase, were also affected, together with *qp108*, the function of which is unknown.

However, the functions of the affected genes were not sufficient to associate genomic mutations with differences in bacteriophage infectivity, highlighting the versatility of bacteriophage infection. It remains to be determined which of these mutations accumulated before and after the development of bacterial resistance.

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We investigated these dynamics further, by performing a time-shift interaction study. We isolated P10 clones (*n*=40) from three time points during coevolution: one time-point before, and two after the adaptation of P10 to strain MG1655. We characterised the ability of these clones to infect MG1655 clones (*n*=40) isolated at past, present and future time points in the same experiment. As expected, bacteriophages isolated before the adaptation event were unable to infect any of the contemporary MG1655 clones (present) (Fig. 1D). While adapted bacteriophages were always able to infect bacterial clones from the past time points, those isolated at the first time point after the adaptation event (day 1) showed reduced infectivity towards MG1655 bacterial clones isolated at the present and, particularly, future time points. However, all bacteriophages isolated subsequently (day 21) were able to infect past, present and future bacterial clones, overcoming the bacterial resistance that had developed and demonstrating the occurrence of continuous adaptive evolution in the mouse gut (Fig. 1D). It could, therefore, be argued that bacteriophage adaptation in the gut led to a two-step coevolution pathway, in which the evolutionary arms race was initially characterised by the rapid development of bacterial resistance followed by a refining of bacteriophage adaptation.

The two populations subsequently continued to coexist, with no evidence of renewed bacterial resistance, suggesting that transient resistance occurred *in situ*, protecting the bacteria against bacteriophage predation, as discussed below.

Transient bacterial resistance in the gut

Bacterial resistance to bacteriophages has long been studied and characterised *in vitro*(*Luria and Delbruck, 1943*), and is known to involve several mechanisms. These include the prevention of adsorption, superinfection exclusion, restriction modifications, CRISPR-Cas systems, bacteriophage exclusion (BREX), and many new recently discovered systems revealing the extreme versatility of bacterial resources for defence (Doron et al., 2018; Goldfarb et al., 2015; Labrie et al., 2010). Nonetheless, little is known about the mechanisms activated *in vivo*, and their relevance and impact in natural communities. In our study, the resistance of strain MG1655 to the newly adapted bacteriophage P10 seemed to depend on preventing adsorption by modifying the bacteriophage receptor. However, this may simply reflect part of the process of bacteriophage adaptation to a new bacterial host, as the bacteriophage could rapidly fine-tune its mechanism of infection to overcome this resistance. This hypothesis is supported by the lack of emergence of resistant clones of the original bacterial host, strain LF82, in mouse faeces (data not shown), despite the presence of large numbers of both the bacteriophage and the bacterium during the course of the experiment.

We have already reported similar observations for a different *E. coli* strain, 55989, coevolving in mouse gut with either a cocktail of three virulent bacteriophages or with each bacteriophage separately. No resistance was ever detected when 20 bacterial isolates were tested against the individual bacteriophages (Maura and Debarbieux, 2012; Maura et al., 2012a). However, two to six hours of co-incubation with the same bacteriophages *in vitro* was sufficient to trigger the development of bacterial resistance (Maura et al., 2012b). We also previously tested the ability of each bacteriophage to replicate in the intestinal environment *ex vivo*, both in homogenates of the small and the large intestines and in the faeces of mice colonised with *E. coli* strain LF82 or strain 55989 (Galtier et al., 2017; Maura et al., 2012a). We found that all bacteriophages were infectious in the ileal sections, but that replication in colonic or faecal samples was significantly impaired for some of them (Galtier et al., 2017; Maura et al., 2017; Maura et al., 2012a).

These results support the hypothesis that the metabolic state of bacteria, which is not uniform throughout the gut (Denou et al., 2007), is the principal barrier to bacteriophage infection. Indeed, several factors, such as the availability of carbon sources, oxygen, and stress responses, can have a marked effect on cell surface structures, some of which are required for bacteriophage infection. This physiological and structural versatility provides bacteria with opportunities for transient resistance to bacteriophages without paying the cost or irreversible mutations, but remaining susceptible when the physiological conditions change, such as during growth in the laboratory environment. Conversely, bacteriophages can escape such resistance strategies by entering into a state of pseudolysogeny or hibernation, in which the infectious cycle is halted until better conditions for progeny production occur (Bryan et al., 2016; Los and Wegrzyn, 2012).

Model of bacteriophage – bacteria coevolution in the gut

This dynamic picture of the coevolution of bacteria and bacteriophages serves as the basis of a theoretical model describing how microbiome diversity is generated and expanded via these interactions (Fig. 2). Mutations in the bacteriophage genome accumulate when they confer a fitness advantage and favour perpetuation of the infection cycle. This corresponds to adaptation to new host strains, and/or host strains that have acquired resistance. However, it remains unclear whether bacteriophage evolution discriminates between these two bacterial situations, since each adaptation event would involve specific mechanisms to overcome the obstacles to predation.

The evolution of the microbiome results in a growing number of bacteriophage populations infecting new bacterial hosts with which perpetuating the process of antagonistic coevolution.

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This is likely to occur at the expense of the most abundant and available bacterial populations, providing a major contribution to microbiome homeostasis and to bacterial differentiation. Bacterial hosts also have opportunities to escape bacteriophage predation, resulting in genomic differentiation between microbial populations. In addition, some of these populations are likely to be protected against bacteriophage predation due to their physical inaccessibility in the environment, their limited density and/or the development of transient resistance due to their metabolic and phenotypic states. An example of such viral diversification in the human gut can be found with the expanding population of Crassphage (Yutin et al., 2018).



Figure 2. Model of bacteriophage-bacteriacoevolutionanddifferentiation in the gut.

From the bottom, three bacterial populations (blue, green and orange) are differentially susceptible to one bacteriophage (yellow). Under bacteriophage predation, sub-populations of resistant bacteria can emerge (lighter colours). These either can become dominant, leading to of extinction other subpopulations, or be maintained in equilibrium. Contextually, bacteriophage sub-populations diverge (represented by different colours) by adapting to changes in the coevolving bacteria or to new hosts (host-jump, black arrows). The consequence (top) is the progressive differentiation of both antagonistic populations.

Concluding remarks

The timing, frequency and conditions required for bacteriophage adaptation and bacterial resistance during coevolution in the intestinal microbiota remain largely unpredictable. However, we propose that, in healthy conditions, bacteriophage communities play a crucial role in controlling bacterial populations, both by promoting heterogeneous microbial differentiation and by adapting in a flexible manner to new patterns of abundance and diversity in susceptible bacteria. If this fails to occur, dysbiotic conditions may arise, leading to extinction or abnormal proliferation of the viral and bacterial partners, with consequences for human health.

Disclosure of potential conflicts of interest

The authors declare no potential conflicts of interest.

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Chapter 6

Discussion and perspectives

Chapter 6

Discussion

Even though phages have been studied for more than a century, their interactions with bacteria in human associated environments are still poorly understood. Further studies are urgently needed in order to decipher the factors and mechanisms that underlie these interactions. This is fundamental to improve our knowledge on the feasibility of using phages as tools for microbiota engineering and therapy. One of the potential targets for the clinical use of phages are pathogenic gastrointestinal bacteria. The mammalian gastrointestinal tract is a highly complex and heterogeneous environment, divided in several contiguous sections (Donaldson et al., 2016) that generate a range of different micro-environments. These environments are characterized by fluctuations in several ecological parameters (pH, nutrients, water, oxygen or density (from liquid to semi-solid or solid)) that strongly impact the physiology of bacteria (He et al., 1999; Koziolek et al., 2015; Wang et al., 2007). The aim of this thesis was to uncover how, and by which mechanisms, the physiological and ecological changes experienced by bacteria in the mammalian gut environment impact their antagonistic interactions with virulent phages.

Major physiological changes and differential spatial distribution imposed by the gut environment play an important role in the coexistence of virulent phages and their target bacteria populations

Studies on the interactions between virulent phages and bacteria in the gut environment consistently show a coexistence of both populations for long periods of time, without the emergence and dominance of bacterial resistant clones (Maura et al., 2012; Weiss et al., 2009). Thus, one major aim of this thesis was to study how the coexistence between these antagonistic populations can occur in the gastrointestinal tract. In **chapter 2** we started by confirming a previous observation concerning the differential replication efficacy of three phages in different gut sections of mice mono-colonised with the bacterial host of these phages. In addition to the previous results, which showed also that the presence of other microbial species did not change these observations. This suggests that physiological differences in the target bacteria itself could play an important role in modulating phage infection (Maura et al., 2012). However, we also observed that not all phages show the same behaviour towards gut-colonising bacteria, as described in **chapter 4.** When using a strain of *E. coli* isolated from mice (Mt1B1), no differential replication was observed for the different sections of the gut. This suggests that the efficacy of phages in the gastrointestinal environment is intrinsically dependent on specific phages and hosts pairs, and might not be a generalizable pattern.

Previous studies on *E. coli* 55989 could not detect the presence of phage resistant mutants in the gut of mice (Maura et al., 2012), a phenomenon that was also observed on chapter 4 within the coexistence of Mt1B1 and a phage cocktail. This was observed both during long-term coevolution with only one administration of the phage cocktail or during short-term (3days) coevolution with multiple phage inoculations suggesting that it occurs independently of the coevolution time and dose of phage administration. This suggests that arms-race dynamics, in which both populations accumulate adaptive mutations of resistance and counter-resistance, might have a less important role in the coexistence of both populations in the gut, in contrast to what has been shown in *in vitro* conditions or in soil and sea environments (Breitbart et al., 2018; Gomez and Buckling, 2011; Hall et al., 2011; Harrison et al., 2013; Weitz et al., 2005). On chapter 5, in another experiment that also takes place in the gastrointestinal environment, we also did not observe the emergence of phage resistant mutants on a bacterial strain (LF82), even after the phage had evolved to infect another host (MG1655). However, resistant mutants were detected to emerge from this latter host background, suggesting that the evolution of phage resistance may be contingent on the genetic background of bacteria, and particularly on how well adapted they are to the environment.

In sum, data show that resistant mutants were never detected on the *E. coli* strain for which the phage was originally isolated (55989, Mt1B1 or LF82) but were detected on newly infected strains (MG1655). From this, we propose two non-mutually exclusive hypotheses. The first postulates that the arms race coevolution between the original hosts and the phage already took place in the past and was then "saturated", reaching a state in which both populations already acquired most possible changes against one another without losing important physiological traits. Because strain MG1655 was naïve to the phage that had recently adapted to infect it, both populations quickly adapted to each other, leading first to the evolution of the phage's ability to infect the new host, and the former's adaptive response of generating resistance mutations against its new predator. If so, this could be the origin of new arms race dynamic.

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On the other hand, it has been suggested that fitness costs can play an important role in modelling resistance both in natural populations facing multiple selection pressures, exerted by multiple different phages or when the phage-bacteria interactions occur in the soil environment (Gomez and Buckling, 2011; Koskella et al., 2012). This led us to a second hypothesis, taking into account possible fitness costs on the bacterial host inherent to phage resistance in the gut environment due its multiple selective pressures imposed not only by the microbial community, but also the environmental fluctuations (pH, nutrients) and the immune system of the mammalian host.

Resistance to phages is often associated with changes in the bacterial cell outer membrane or its flagellum (Bertozzi Silva et al., 2016), which many cause impairment of the colonization of the gut by resistant clones. Therefore, it is possible that phage resistant clones emerge but because they could be very costly in the gastrointestinal environment, as previously shown for other natural environments (Gomez and Buckling, 2011), they could be rapidly outcompeted by the sensitive clones. At the same time, the inherent fitness costs may also lead to a reversion of resistance, as previously observed for antibiotic resistances (Andersson and Hughes, 2010; Moura de Sousa et al., 2017). Our data suggests that these costs may be contingent on the genetic background, which could explain the opposite results obtain for strain MG1655 compared to the other E. coli strains. Moreover, despite the fact that the strain MG1655 is derived from the K12 strain that was isolated from the human gut in 1922, it has been cultured in laboratory environments for decades, becoming one of the most used strains for in vitro studies (Bachmann, 1972). In a previous work, I contributed to the observation that strain MG1655 has a high rate of adaptation upon colonization of the gut of mice, with high levels of clonal interference (several beneficial mutations arise at the same time, leading to competition within the population) (Barroso-Batista et al., 2014; Lourenco et al., 2016; Sousa et al., 2017). On the other hand, E. coli strains 55989, Mt1B1 and LF82 were isolated more recently (Darfeuille-Michaud et al., 1998; Lagkouvardos et al., 2016; Mossoro et al., 2002) so we hypothesise that they may be less adapted to laboratory conditions. Their genetic configuration, better suited for in vivo conditions, could contribute to the lack of emergence phage resistant clones, which can be easily outcompeted by the sensitive ones. Fitness costs of resistance to phages in the gut environment are, to the best of our knowledge, unknown. In particular, how they differ from the costs measured in vitro, and how dependent they are on the genetic background (e.g., 55989 vs MG1655) are an important focus for future work.

Nevertheless, the lack of emergence of resistant clones suggests that arms-race coevolution is not a major cause of coexistence between the antagonistic population of virulent phages and its host bacteria in the gut environment, at least in our experimental setting. Thus, we postulated that this coexistence could be driven by two other, potentially linked, factors: the heterogeneous spatial structure of the gut, that might promote a compartmentalization of different populations of bacteria (susceptible or not) and phage; and the distinct bacterial gene expression that might result from residing in different sections of the gut (Denou et al., 2007; Li et al., 2015; Pereira and Berry, 2017; Tropini et al., 2017).

In chapter 4 we tested the first part of this hypothesis, and showed that the distribution of phages and bacterial hosts changes in different gut section. For that, we used a recently developed mice model (OMM-12), colonized with an E. coli strain, to also test the effects of phage cross-infection towards other microbiota strains. This E. coli strain was targeted with a cocktail of three different phages, which showed no cross-infection towards the other 12 strains. Once again, the emergence of phage resistant clones was not detected, even though phages were detected at constant numbers throughout the 15 days of the experiment after inoculation. Our results showed that this coexistence between both populations was observed throughout the intestine, with the exception of the mucosal section of the ileum, in which a significant lower ratio of phages was observed. This suggests that phages might have difficulties in either accessing or remaining in this section of the gut. Moreover, phage diffusion in the gut may be limited by mucins and other glycoproteins, lipids and DNA molecules (Johansson et al., 2011; Qi et al., 2017). Interestingly, our data showed that amongst the phages tested only one possessed an IG-like motif, shown previously to favour phage binding to the mucus (Barr et al., 2013), which is consistent with our in vivo observation that these phages are less abundant in mucosal sections. Thus, the reduced opportunities of contact between phages and bacteria might create a context where potentially costly resistance mutants have a low selective value. This could contribute to the lack of detection of phage resistance mutants, at least in this section of the gut.

Altogether these results led us to hypothesise a source-sink scenario in which the mucus is the source of (susceptible) bacteria, to which the phages have difficulties in reaching. However, when these bacteria leave the mucus (the source), they supply the lumen of the gut (the sink) with sensitive hosts that can be infected by lumen-residing phages, which can now replicate and keep their numbers high.

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Coupled with the concept of costly phage resistance mutations, this hypothesis and our results are in accordance with observations from *in silico* individual based stochastic spatial models, which showed that structured environments can create spatial refuges that lead to coexistence between bacteria and phages without the emergence of resistant clones (Heilmann et al., 2012; Sousa and Rocha, 2019). The model for source-sink ecological dynamics was first proposed in 1985, by Robert D.Holt to explain possible stabilization of prey-predator dynamics. Its use in microbiology is rare, potentially due to the fact that many studies assume homogeneous environments. However, it has recently been applied to explain how the transfer of plasmids can maintain gene mobility in soil bacterial communities (Hall et al., 2016).

An alternative explanation for the lower abundance of phages in the ileum mucosal section can be due to the reduced numbers of bacteria, compared to the other sections, underlying a possible density-dependent phenomenon that was observed in several *in vitro* structured environments (Eriksen et al., 2018). This density-dependent phenomenon has been linked to the "proliferation threshold" proposed by Wiggins and Alexander in 1985. These authors proposed that a threshold number of bacteria (10⁴ CFU/ml) is needed for the phage to start an effective infection and amplify (Payne et al., 2000; Wiggins and Alexander, 1985). In regards to our sourcesink hypothesis, this "proliferation threshold" could play an important role in allowing the creation of bacterial refuges towards phages.

In chapter 2, we focused on bacterial physiology by testing whether bacterial differential gene expression in the gut affects phage efficiency. For this, we performed a transcriptomic analysis of the opportunistic E. coli, strain 55989 colonising the mammalian colon in comparison with in vitro exponential and stationary phase cultures. We found major differences in gene expression *in vivo* relative to *in vitro* conditions, from iron uptake, to nutrition and oxygen consumption. This strongly support previous results that have identified these functions as essential for E. coli colonization of the mammalian gut (Conway and Cohen, 2015). When taking into account the phage differential efficiency observed in ex vivo experiments, we also found several genes and pathways linked to phage infection. We observed differential expression of genes related to phage receptors or an increase in biofilm formation, which have been shown to play important roles in bacterial defences against phage infection (Lourenco et al., 2018). These results demonstrated the validity of our approach in characterizing the transcriptomic profile of E. coli in the gut environment in order to study possible key factors that may modulate the coexistence between the antagonistic populations of virulent phages and bacteria. We further confirmed this premise by showing that a knockout mutation in one of the highlighted genes (*rfaL*), coding for the O-antigen ligase, affected the efficiencies of two of the three phages tested.

Since the O-antigen is a known receptor for several phages, these results show that the expression of phage receptors can be influenced by the gut environment henceforth altering the phage efficiencies. On the other hand, mutation on the fliA gene (flagellum regulator), did not show any effect on any of the three phages efficiency. Thus, it is possible that none of receptors in these phage relies on the bacterial flagellum for infection, although evidently that it will not be true for all phages (Bertozzi Silva et al., 2016). Further characterization of other parameters related to phage infection, such as phage adsorption rate, are needed in order to fully evaluate the influence of these host genes.

These results confirm that the differential gene expression of *E. coli* 55989 in the mammalian gut can affect the efficiency of phage predation, in accordance with previous *in vitro* studies showing that changes in bacterial physiology affect phage efficiency (Lourenco et al., 2018). Moreover, our gene expression analysis has highlighted several additional genes and pathways, some of which were completely undescribed in relation to bacteria-phage interactions. It will be very interesting, and it can be very enlightening from the point of view of mechanisms of phage infection, to test mutants in these genes for differences in phage infection efficiency. This will allow not only to further understand the impact of phage efficiency towards bacterial physiological shifts that occur in the gut, but also to learn about phage-bacteria interaction mechanisms in general.

Of course, there are several other processes, which were not directly tackled in this thesis that can also contribute to the coexistence of phage and bacteria populations in the mammalian gut. For example, phenotypic resistance is described as the ability of bacteria to oscillate between phage-susceptible and phage-resistant cells by modulating the expression of genes that encode for phage receptors, led by environmental or cellular stochasticity. This phenomenon could also lead to coexistence, creating bacterial populations of physiologically less susceptible clones (Bull et al., 2014; Chapman-McQuiston and Wu, 2008). On the other hand, the coexistence of both populations with no detection of phage resistant clones could also be explained by the recently described mechanism named "leaky resistance" (Chaudhry et al., 2018). This process is characterized by high rates of genetic transitions (mutation versus reversion) from resistance to susceptibility. It is currently unknown whether these transitions occur in vivo, but it is possible that, if they do, they might occur at too short time scales for being detected. However, they could still influence the persistence of the bacterial population at high numbers. An interesting experiment would be to sequence several bacterial and phage clones in short distance time points to verify this possibility.

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The occurrence of this process would not invalidate our observations, since the environmental driven physiological changes shown to influence the phage infection were observed in the absence of phage selection. Moreover, such mechanisms of genetic transition would also be influenced by spatial segregation, by lowering their selective value.

Yet another factor with possible influence on the coexistence between phages and bacteria in the gut is the mammalian immune system, which have been shown to play an important role in these interactions in the lungs (Roach et al., 2017). In the future, this influence could be tested for example by using bacterial strains pathogenic to the mice (eg *citrobacter rodentium*), towards which gut inflammation is generated.

Increase in bacterial virulence can be linked to increase susceptibility to phage infection

One of the final aims of this thesis is to expand our knowledge on the interaction of phage and bacteria in *in vivo* conditions in order to improve the use of phages as therapeutics against pathogenic bacteria. Opportunistic and strictly pathogenic *E. coli* strains encode many virulence factors, both in their chromosome or in plasmids, that are usually found in genomic islands called pathogenicity islands (Gal-Mor and Finlay, 2006).

The differential gene expression profiles studied in chapter 2 showed an overexpression of several virulence genes, including the ones encoded in the plasmid of *E. coli* 55989. These genes are responsible for the production of proteins linked to the aggregative adherence of the strain to the mucosal layer of the intestine (Blanton et al., 2018). These virulence factors have been shown to be crucial for some bacteria, as 55989, to colonize the mammalian gut (Harrington et al., 2009). The overexpression of such virulence factors, that are related, for example, to adherence proteins, but also to toxins or bacterial capsules (Wagner and Waldor, 2002), may also exert an influence on phage and bacteria interactions, as we showed in chapter 3. Here, we observed that the presence of the pks pathogenicity island (PAI), responsible for the production of colibactin, increased the bacterial susceptibility to phage. Moreover, we showed that the production of the toxin per se was not responsible for the phenotype. Transcriptomic analysis revealed an overexpression of two different tRNAs (asparagine and asparatate). Codon bias analysis on these tRNAs showed that they are preferentially required by the phage, and less by the MG1655 host strain, which, in our experiment, received the pks island. Previously, tRNAs have been shown to play an important role in phage infection, for example the deletion of tRNA genes in phage T4 decreases the burst sizes and the rate of protein synthesis (Wilson, 1973).

The distribution of tRNAs in phages have been shown to be associated with its codon usage and its presence in phage genome usually correspond to codons that are highly required by the phage and less required by the host (Bailly-Bechet et al., 2007). The phage used in our experiment has only one tRNA, coding for arginine, and therefore, it requires all other tRNAs from the bacterial cell. However, in our system the over-expression of each, of the highlighted tRNA, alone in the MG1655 strain could not restore the phenotype given by the pks island. Further study is needed to understand if the co-expression of both tRNAs could restore the increased susceptibility or if the deletion of this tRNA genes on the original host strain (412) would decrease phage efficiency.

Moreover, the acquisition of this specific PAI also led to a reduced expression of the specificity protein required for the EcoKI restriction-modification system (R-M system). Horizontal gene transfer (HGT) of elements like phages, plasmids or pathogenicity islands have been described as playing a major role in bacterial evolution, in special in bacterial pathogenicity (Mazodier and Davies, 1991; Pallen and Wren, 2007). On the other hand, R-M systems have been described to play a role as a barrier to HGT (Berndt et al., 2003; Waldron and Lindsay, 2006). For example, the deletion of the EcoKI restriction enzyme in MG1655 has been shown to increase conjugational transfer of a plasmid carrying 2 sites specific for this enzyme (Roer et al., 2015). At the same time, it has been shown that several of these transmissible elements, like phages (Samson et al., 2013) or plasmids (Read et al., 1992) evolved mechanisms to evade these systems. Regarding pathogenicity islands, although it has been shown that ssDNA methylases encoded by the bacterial cells can play a role in protection of these elements (Johnston et al., 2013) specific evasion systems are still poorly understood. Taking in account this information and the important role of R-M systems in bacterial defence against phages (Labrie et al., 2010), we hypothesise that the down-regulation of *hsdS* gene allows the phage DNA to enter the cell and complete its cycle without being destroyed by the bacterial defences. Further studies are need to test this hypothesis, both by deleting this gene on the MG1655 background and by overexpressing it on MG1655 carrying the pks island. Our results led us to hypothesise that the mechanisms employed, or influenced, by the pathogenicity island, as DNA foreign to the cell, against bacterial defences can confer an advantage to the phage. This will allow to understand the impact of pathogenicity islands and the associated evasion systems in phage – bacteria interactions and infection efficiency.

The two mechanisms with a potential influence on phage efficiency revealed by this transcriptomics analysis are internal to the bacterial cell (R-M systems and tRNAs), contrasting with the mechanisms revealed by the transcriptomics analysis performed in **chapter 2**, which are associated with the outer membrane of the cell (phage receptors). Together, these results highlight how bacterial susceptibility to phages can be linked to subtle changes in gene expression caused by factors that are not directly linked to the phages themselves. These processes can occur both within and outside the bacterial cell, and are not limited to a specific physiological response. Interestingly, phages themselves have been shown to play an important role in disseminating bacterial virulence (Wagner and Waldor, 2002). Our results uncovered possible new relationships between virulence and increased susceptibility to phages, which expands the concept that phages can interfere with bacterial virulence.

These results also reinforce that the antagonistic interactions between bacteria and phages are defined by adaptive trade-offs: for a bacterium to be a successful pathogen, it can become, as a consequence, more susceptible to phage. For example it has been demonstrated that selection for phage resistance can decrease virulence, in particular when virulence factors are membrane structures used by the phages as receptors (Leon and Bastias, 2015). Conversely, it could happen that because bacteria are more susceptible to phage, they can become more virulent as temperate phages have been shown to carry virulence factors that can be transferred between strains, effectively transforming nonvirulent strains in virulent ones (Boyd, 2012). These types of evolutionary and ecological interactions further underline how the lifestyle of bacteria and phages are intertwined.

Impact of phage receptors flexibility on phage efficiency

The study of phage receptors in **chapter 3** shows that the role of these structures depends on environmental conditions (well-mixed vs structured environments). This led us to investigate the concept of receptor specificity, hypothesising that some of these receptors may not be ideal for phage adsorption, leading to a higher rate of failure which would in turn require a higher rate of contacts to produce a successful infection. Variations on these interactions between the phage and the bacterial receptors can lead to a variable host-range, i.e., a variation on the number of strains that can be infected by the phage. For example, in 2017, De Sordi et al, showed that one single point mutation on the tail fiber of phage P10 allowed it to infect a new *E. coli* strain to which it was previously unable to infect. Moreover, the authors also observed that these phages, when coevolving in the gut with a microbial community, acquired a variable set of small mutations that led to expansion or narrowing of their host range.

On the other hand, these mutational variations on the phages, change their interactions with their bacterial receptors, which in turn can also affect how bacteria become resistant, as showed in **chapter 2**. When screening the *E. coli* KEIO collection for clones resistant to phage CLB_P2 and T4, we observed that the *ompC* mutant, a mutation previously shown to confer resistance to T4 in *E. coli* YA21 (Yu and Mizushima, 1982), did not result in similar resistance phenotype. This suggests that even between closely related bacterial strains, small variations in receptors that are used by the phage can play a role in an efficient infection.

Furthermore, in chapter 3, we showed that the increased susceptibility to phage in clones carrying the pks island, could only be recovered in solid and not in well-mixed media. One hypothesis is that this differential phenotype in well-mixed environments may be caused by a lower adsorption rate of the phage in MG1655 cells in comparison with its original host due to defective phage receptor. This defective receptor requires higher rates of contact between the bacteria and phage, which is less likely to occur with low phage doses in a well-mixed environment. Nonetheless, we showed that this does not represent the major factor for the differences in susceptibility, since lysis intervals were shared by both clones carrying or not the pks island. An interesting question is then to investigate where the differences in the described phenotypes originate from. Are there environmental effects, such as the one we observed in chapter 3, that affect the expression of the receptors? Or can small genetic mutations alter the structure of the protein modulating the susceptibility phenotype associated with the ompC mutation, as shown in the work of De Sordi et al. 2017. Further work will focus on comparing the sequences of these two bacteria ($\Delta ompC$ resistant or $\Delta ompC$ susceptible) to uncover possible reasons for these differences. Altogether, these hypotheses suggest that the variations on the interactions of phages towards their bacterial receptors, could be driven by environmentassociated changes in gene expression or the rapid accumulation of mutations, which would strongly determine the outcome of an infection and the fate of the interactions between bacteria and phages.

Virulent phage and bacteria interactions are highly influenced by the gut environment



Figure 1. Coexistence between virulent phages and bacteria in the gut can be influenced by the presence of spatial refuges due to structural heterogeneity of the gut, by differential bacterial gene expression which can also have an impact on the interactions of the phages with their receptors on the bacterial cells.

Overall, the research presented in this thesis shows how the complexity of the environment can interfere on phage – bacteria interactions and how these intricate interactions can influence phage infection efficiency. Most of the current knowledge about these interactions are based on more than a century of experiments mostly performed in conditions that differ from the natural environments where the relationships between bacteria and phages have evolved. Although crucial in characterizing many aspects of phage-bacteria interactions, from their cellular responses to ecological or evolutionary outcomes, *in vitro*, and particularly liquid and homogeneous environments might hide variability in those interactions. Accordingly, the use of mouse models has been shown to be a very useful system to study these interactions, providing a realistic and natural scenario. On the other hand, more work is required to understand the dynamics of this complex environment, as well as the physiological responses associated with

it. The spatial configuration of the gastrointestinal tract is very distant from a test tube, and the richness of the communities that might be present mean that bacteria are susceptible both to the spatial compartmentalization driven by other species and also to potential foreign DNA acquired from them (e.g. pathogenicity islands).

The data presented show that both physiology and spatial distributions in the mammalian gut play an important role in modulating the coexistence between virulent phages and their host bacterial strains (fig1), without a major contribution from an arms-race coevolution. Our transcriptomic analysis was shown to be a valid approach for the identification of possible factors influencing phage infection *in vivo*, particularly by identifying physiological differences in comparison to *in vitro* environments, or the ones caused by the interference of foreign DNA in the cell, in the shape of mobile pathogenicity islands.

We believe this work is a relevant contribution to lift the tip of the veil on the many factors that influence phage and bacteria interactions in an environment as complex as the gut. It is the type of research that is necessary if phages are to be considered as a much needed viable alternative to antibiotics in dealing with infections by pathogenic bacteria, which are often multi-drug resistant. It also emphasizes the importance of studying phage-bacteria interactions in their natural environments, in order to understand their roles, both individually and together as an ecological unit, in human health and disease.

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Annexes

Strain List

Strains	Reference
55989	Mossoro et al ., 2002 (PMID:12149388)
55989∆rfaL	this work
55989∆ <i>fliA</i>	this work
55989∆ <i>lpc</i> A	this work
55989∆rfaC	this work
MG1655	Laboratory collection, gift from E. Oswald
MG1655 pBAC	doi:10.1371/journal.ppat.1003437
MG1655 pBACpks	doi:10.1371/journal.ppat.1003437
MG1655 pBACpks∆clbA	doi:10.1371/journal.ppat.1003437
MG1655 pBACpks∆clbP	Laboratory collection, gift from E. Oswald
MG1655 pBACpks∆clbS	Laboratory collection, gift from E. Oswald
MG1655 pBACpks∆clbN	Laboratory collection, gift from E. Oswald
MG1655 puc18	this work
MG1655 puc18asnW	this work
MG1655 puc18aspU	this work
PDP110	laboratory collection, gift from E. Denamur
T145	laboratory collection, gift from E. Denamur
T147	laboratory collection, gift from E. Denamur
PDP351	laboratory collection, gift from E. Denamur
536	Brzuszkiewicz E et al ., 2006 (PMID:16912116)
7074	AIEC collection from University of Lille, France, via N. Barnich
AL505	Galtier et al ., 2016 (PMID:26971586)
BE	Laboratory collection, gift from H. Krisch
BW25113	Datsenko and Wanner, 2000 (PMID:10829079)
CR63	Laboratory collection, gift from H. Krisch
E22a	Camguilhem and Milon, 1989 (PMID:2656746)
ECOR collection	Ochman, H., and Selander, R.K. (1984) (PMID: 6363394)
LF110	Darfeuille-Michaud et al ., 2004 (PMID:15300573)
LF31	Darfeuille-Michaud et al ., 2004 (PMID:15300573)
LF50	Darfeuille-Michaud et al ., 2004 (PMID:15300573)
LF73	Darfeuille-Michaud et al ., 2004 (PMID:15300573)
LF82	Miquel et al ., 2010 (PMID:20862302)
LM33	Dufour et al., 2016 (PMID:27387322)
M1/5	Laboratory collection, gift from E. Oswald
MG1655	Laboratory collection
Mt1B1	Garzetti et al 2018 (DSM-28618)
Nissle 1917	Laboratory collection, gift from E. Oswald
NRG857c	Small et al ., 2013 (PMID:23748852)
042	Laboratory collection, gift from C. Le Bouguénec
OP50	Laboratory collection, gift from J. Ewbank
SE15	Laboratory collection
Sp15	Laboratory collection, gift from E. Oswald
ST24	Laboratory collection
Keio collection	Baba et al., 2006 (PMID: 16738554)
Primers List

name	description	primers
rfaC_bf_F	3-step PCR <i>rfaC</i> gene deletion and insertion of kanR - up	GCAGGTGAGCGAAGGTGAAA
rfaC_bf_R_kan	3-step PCR <i>rfaC</i> gene deletion and insertion of kanR - up	gtgagctatgagaaagcgccTCCGTCAGGCTTCCTCTTGTA
rfaC_af_F_kan	3-step PCR rfaC gene deletion and insertion of kanR - down	aaacaaataggggttccgcgACCAATAAGTTGACATCGGAGA
rfaC_af_R	3-step PCR rfaC gene deletion and insertion of kanR - down	TCGACATCTTCTCTTTTCTCGTCT
lpcA_bf_F	3-step PCR <i>lpcA</i> gene deletion and insertion of kanR - up	ACGAAAAGCCCCTTACTTGTAG
<pre>lpcA_bf_R_kan</pre>	3-step PCR IpcA gene deletion and insertion of kanR - up	gtgagctatgagaaagcgccAGTGTACCGGATACCGCCAA
lpcA_af_F_kan	3-step PCR IpcA gene deletion and insertion of kanR - down	aaacaaataggggttccgcgCACTTGTGCGCTGATGCCTG
lpcA_af_R	3-step PCR <i>lpcA</i> gene deletion and insertion of kanR - down	ACAGCGGGAGCGCATGG
rfaL_bf_F	3-step PCR rfaL gene deletion and insertion of kanR - up	ATCACGGTTTATGGACCAAC
rfaL_bf_R_kan	3-step PCR rfaL gene deletion and insertion of kanR - up	gtgagctatgagaaagcgccCTTATCTCCGATGTCAACTT
rfaL _af_F_kan	3-step PCR rfaL gene deletion and insertion of kanR - down	aaacaaataggggttccgcgAAAATAAAAAAGGCTGCATA
rfaL_af_R	3-step PCR rfaL gene deletion and insertion of kanR - down	GCAAAGCAAGGTCAGGACTT
fliA_bf_F	3-step PCR fliA gene deletion and insertion of kanR - up	TAAGAACTCCTGGTAGTCA
fliA_bf_kan_R2	3-step PCR <i>fliA</i> gene deletion and insertion of kanR - up	gtgagctatgagaaagcgccCGTCAGTAAATGCCGCACT
fliA_af_kan_F	3-step PCR <i>fliA</i> gene deletion and insertion of kanR - down	aaacaaataggggttccgcgGATAAACAGCCCTGCGTTAT
fliA_bf_R	3-step PCR <i>fliA</i> gene deletion and insertion of kanR - down	AACCTGCCTGACCCCGCTA
KanF	kanamycin resistance cassete	GGCGCTTTCTCATAGCTCAC
KanR	kanamycin resistance cassete	CGCGGAACCCCTATTTGTTT
aspUrv_F	Asparatate tRNA aspU gene (hindIII)	tttttttAAGCTTACAGACGAAAAAAAACCTCG
aspUrv_R	Asparatate tRNA aspU gene (ecoRI)	gggggGAATTCAATTCGGTGGAGCGGTA
asnW_F	Asparatate tRNA aspU gene (hindIII)	gggggAAGCTTTGAGGATATCAAGCGCCAGG
asnW_R	Asparatate tRNA aspU gene (ecoRI)	tttttgaattcTCGCCGTTAAGATGTGCCTC
M13_fw	cloning confirmation (pUC18)	GTAAAACGACGGCCAGT
M13 _rv	cloning confirmation (pUC18)	CAGGAAACAGCTATGAC

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Review Article

The Diversity of Bacterial Lifestyles

Hampers Bacteriophage Tenacity





Review

The Diversity of Bacterial Lifestyles Hampers Bacteriophage Tenacity

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Abstract: Phage therapy is based on a simple concept: the use of a virus (bacteriophage) that is capable of killing specific pathogenic bacteria to treat bacterial infections. Since the pioneering work of Félix d'Herelle, bacteriophages (phages) isolated in vitro have been shown to be of therapeutic value. Over decades of study, a large number of rather complex mechanisms that are used by phages to hijack bacterial resources and to produce their progeny have been deciphered. While these mechanisms have been identified and have been studied under optimal conditions in vitro, much less is known about the requirements for successful viral infections in relevant natural conditions. This is particularly true in the context of phage therapy. Here, we highlight the parameters affecting phage replication in both in vitro and in vivo environments, focusing, in particular, on the mammalian digestive tract. We propose avenues for increasing the knowledge-guided implementation of phages as therapeutic tools.

Keywords: virus-host interactions; bacteriophage efficacy; gastrointestinal tract; phage therapy

1. Introduction

With the alarming worldwide increase in the prevalence of multidrug-resistant bacteria, phage therapy—the use of phages to target pathogenic bacteria [1]—has recently returned to the spotlight in the USA and Europe, although it had never fallen out of favour in countries such as Georgia [2]. The three main characteristics of phages that make phage therapy an appealing strategy are (i) the self-replication of phages, leading to a local increase in their concentration; (ii) the lack of broad off-target effects due to the narrow host specificity of phages and (iii) genomic flexibility making it possible to rapidly develop optimised variants. The recent publication of a successful compassionate clinical case treatment with phages has highlighted the potential value of phage therapy in the context of human health [3,4]. However, in modern phase II clinical trials, the efficacy of phage therapy was highly variable in a small number of patients with chronic otitis, and phage therapy was ineffective in a larger trial with children with diarrhoea [5,6]. This lack of success may partly reflect the paucity of data relating to the translation from in vitro to clinical settings [7]. We must, therefore, address the challenge of identifying the parameters characterising effective phage treatments. For example, in studies of several experimental models investigating the use of phages to target bacteria residing in the digestive tract of animals, treatment efficacy has been reported to range from complete inefficacy to highly successful [8–12]. These findings contrast strongly with in vitro observations in which most, if not all, phages are highly efficient at infecting their host. These discrepancies may be explained by the influence of the bacterial lifestyle on phage infection, as discussed below.

2. Bacteria Provide Essential Support for the Parasitic Lifestyle of Phages

Bacteria are among the most ubiquitous organisms on the planet and their high levels diversity are regularly confirmed in metagenomics studies [13–15]. Bacteria colonise a multitude of environments, from oceans to deserts, demonstrating their great ability to thrive in different environments and to regulate major global processes, such as the biogeochemical cycles of essential elements (carbon, nitrogen, oxygen) [16].

From an anthropocentric point of view, most bacteria are harmless while a few are beneficial or pathogenic. Bacteria isolated from many body sites have been shown to survive in various conditions, such as the acidic medium of the stomach or the highly oxygenated respiratory tract. Even within a single species, bacteria may display considerable phenotypic flexibility. This is illustrated by the well-known model bacterium Escherichia coli, a facultative anaerobe able to survive in environmental conditions that are very different from its natural habitat, the digestive tract of warmblooded animals [17].

Bacterial physiological responses play a crucial role in shaping the interactions of bacteria with their environment. The recent development of several techniques (membrane, chip, RNASeq), which facilitate the capture of mRNAs, has made a fundamental contribution to the description of global physiological responses in bacteria. These techniques have made it possible for researchers to describe the transcriptomic profile of bacteria growing in several different types of conditions [18–23]. For example, Denou et al. compared Lactobacillus johnsonii gene expression between in vitro (in flasks) and in vivo (mouse gastrointestinal tract) conditions and in different sections of the gastrointestinal tract (stomach, caecum and colon) [18]. Their observations confirmed that the animal host, either directly or indirectly via other microbes, influences gene expression in the bacterial populations colonizing different body sites.

Phages are obligate parasites and, as such, their distribution matches that of the bacteria they infect. Bacteria may be susceptible to phages or resistant via many mechanisms developed by bacteria during the course of their coevolution with phages. Bacteria can prevent phage adsorption by deleting phage receptors, modifying their conformation, or releasing factors that occupy the binding site or even mask it. Other mechanisms of protection involve the prevention of phage DNA injection, the digestion of phage DNA by restriction-modification enzymes or by the CRISPR-Cas machinery. For a more comprehensive and detailed description of these phage resistance mechanisms, we refer the reader to the review by Labrie, S.J., et al. [24]. In 2015, a novel system called BREX (bacteriophage exclusion) was described and reported to specifically prevent phage DNA replication [25]. Doron et al. (2018) recently used comparative genomics to predict an impressive list of 26 new putative antiphage systems, nine of which were experimentally validated [26]. In addition, environmental fluctuations driving bacterial modifications can directly or indirectly influence phage infection, as discussed in the chapters below focused on virulent phages and schematically illustrated in Figure 1.



Figure 1. Schematic illustration summarising the obstacles that bacteriophages must overcome to be considered as antibacterial weapons

3. Bacterial Physiology Affects the Outcome of Phage Infection

In optimal in vitro conditions, bacterial growth is characterised by four different phases: (i) the lag phase (initial phase) during which the bacteria are still adapting and adjusting to the growth conditions; (ii) the exponential growth or log phase during which the bacteria replicate rapidly; (iii) the stationary phase during which nutrients are depleted from the medium, limiting replication rates (during this phase, growth rate and death rate are usually matched); and (iv) death, which occurs when the nutrients are exhausted. The physiological state of a bacterium is linked to its growth conditions, which are, in turn, highly dependent on abiotic factors, such as nutrient variety and density, in particular [19]. Changes in growth conditions can affect the antibacterial activity of phages by preventing infection, replication or lysis. In vitro studies of phage—host interactions are typically performed in exponential phase cultures in liquid broth and little is known about these interactions in other conditions resembling those found in natural environments. The initial isolation of phages itself introduces a selection bias in that it often occurs in growth conditions that are optimal for the host (rich medium with shaking), i.e., those in which the bacteria are constantly in a planktonic state.

Many in vitro studies on the model system consisting of the phage T4 and its host, *E. coli*, have characterised the effects of host physiology on the infection efficiency of the phage. At high growth rates, phage T4 is absorbed and released more rapidly, its burst size increases and its eclipse and latent periods decrease [27–30]. These observations led to the suggestion that phage synthesis and assembly rates depend on the protein synthesis machinery of the host, whereas lysis time is correlated with cellular dimensions [29]. Other studies have shown that phages T4 and ms2 can enter a dormant state during the infection of stationary-phase cells. This state has been referred to as "hibernation" and is reversible. Some phage proteins are synthesised during hibernation but particle assembly is placed on hold until additional nutrients become available in the environment, which allows the phage infection processes to resume [27,31,32].

Bacteria may display various physiological states due to environmental stochasticity, which can convert a phage-susceptible bacterial host into a phage-resistant host. Indeed, stochastic differential gene expression can generate a heterogeneous population of cells within which a subpopulation may express lower levels of phage receptors, with consequences for the rate of phage adsorption. Such stochastic expression renders cells effectively resistant to phages without the need to acquire resistance through mutation. Although this phenomenon, known as phenotypic resistance, remains underappreciated and understudied, it may potentially account for the difference in infection efficiency between in vitro and in vivo conditions [33–35].

Another example of differences in phage infection efficiency due to shifts of environmental conditions is provided by phage T5. The infection efficiency of this phage has been shown to be dependent on temperature, which alters the host cell's membrane rigidity [36]. By contrast, E. coli phage infection efficiency seems to be independent of oxygen concentration, at least in vitro, as shown by studies in both aerobic and anaerobic conditions [11,12]. Nevertheless, it was shown that different aeration conditions imposed on Bacillus thuringiensis could affect the duration of the infectious cycle of phage BAM35 [37]. In 2004, Sillankorva et al. performed an extensive study with the phage US1 and its host, Pseudomonas fluorescens [38]. These authors showed that temperatures lower (4 \degree C) or higher (37 \degree C) than the optimal temperature (26 \degree C) had a major effect on phage infection efficiency, leading to an absence of phage amplification (37 °C) or rare (4 °C) phage infections. Furthermore, this phage cannot infect its host in a glucose medium despite its high infection efficiency in nutrient-rich conditions. Studies of the outer membrane protein profiles of cells grown in these two environments identified two proteins-17.5 and 99.0 kDa-with differential abundance under these growth conditions. These proteins were not detected in bacteria growing at 37 °C or in a glucose medium and the smaller protein was not detected at 4 °C, suggesting a possible role for these proteins as phage receptors. Environmental shifts can also, in some cases, trigger the production of capsules, which may mask phage receptors or allow other phages to use these same receptors [39–41]. In other cases, these environmental fluctuations can promote the induction (resumption of lytic cycle) of prophages present in the genome of bacteria, causing the destruction of their host [42]. Interestingly, prophage induction is frequent in the digestive tract of mammals as suggested by metagenomics data, however, their precise role waits to be defined [43,44].

4. Bacterial Community Lifestyle Influences Phage Infection

In any environment, including body sites, bacterial populations do not generally adopt the planktonic state of growth that is frequently observed in laboratory experiments. Instead, they tend to live in multilayer aggregates of cells that adhere to each other and frequently to surfaces via the production of a matrix of extracellular polymeric substances (EPSs) [45]. These EPSs include exopolysaccharides and proteins but also lipids and DNA. The resulting biofilms limit the efficacy of antibiotics, principally by decreasing their diffusion. As a result, the bacteria are not completely eradicated by such treatments, favouring the development of chronic bacterial infections [46]. In such situations, phages may constitute a potential solution given their impact on microbial communities [47]. However, the efficacy of phages against biofilms in vitro is variable and certain biofilm components may act as barriers against phage infection. For example, the presence of an amyloid fibre network of CsgA (curli polymer) can physically prevent phages from penetrating biofilms [48]. Phages can also attach to these amyloid fibres, preventing the viral binding to receptors [48]. On the other hand, some phages are equipped with enzymes that can degrade the polysaccharides produced by bacteria, thereby facilitating the diffusion of viral particles in biofilms [49,50]. The efficacy with which phages infect bacteria in biofilms is also strongly influenced by nutrient availability and nutrient concentrations that are highly heterogeneous within the biofilm structure [51].

An additional layer of complexity in interactions between phages and biofilms has been reported in studies of biofilms formed by the gut pathogen *Campylobacter jejuni*. Following phage infection, some of the cells in *C. jejuni* biofilms enter a carrier state. This involves phenotypic modifications to the bacterial cells, conferring advantages that enable them to survive in extraintestinal environments but preventing them from colonising the gut of chickens. Nevertheless, such carrier bacteria can import the phage into chickens that are already colonized by *C. jejuni*, providing the phage with opportunities to infect new cells following its release from the carrier [52,53].

Biofilms can also provide bacteria with a spatial refuge, reducing the probability of contact between a phage and its host, driving coexistence dynamics between the two populations without extinction of either the bacteria or the phage. This has been studied in vitro and modelled in silico. Spatially explicit individualbased stochastic models have shown that these structured refuges may maintain coexistence between the two populations within their boundaries, without the emergence of resistant clones [54]. In vitro experiments on populations of *P. aeruginosa* and bacteriophage PP7 in a heterogeneous artificial environment (static bacterial growth) showed a decrease in viral transmission and the emergence of refuges for the bacterial cells, stabilising interactions between the two antagonistic entities [55]. Similar observations were made when biofilms were grown on the wall of chemostats [56]. Finally, Eriksen et al. showed in a much more structured environment (solid agar in a Petri dish) that populations of phages and bacteria can co-exist in the long term but that this phenomenon is dependent on bacterial density, requiring the presence of at least 50,000 cells [57]. This threshold for phage replication is close to the threshold of 10,000 cells previously determined for well-mixed populations in several systems (*Bacillus subtilis, Escherichia coli and Staphylococcus aureus*), a phenomenon known as the "threshold for phage replication" or "proliferation threshold" [58,59].

5. Human Health and the Gut Phageome

Many aspects of phage biology, from initial adsorption to final lysis, can be affected by host behaviour, making it harder to reliably predict the overall efficacy of a phage in a given situation. This challenge is even greater when the complexity of viral species inhabiting the human gut is taken into account, as the cellular hosts of most of these viruses have yet to be identified [60,61]. The human gastrointestinal tract is a highly diverse and heterogeneous environment [62] that is inhabited by many different microorganisms [63]. It is also characterised by changes in conditions between sections, exposing its inhabitants to fluctuations in pH, nutrient levels, water and oxygen concentrations and even structure (ranging from liquid to semi-solid) [64–68].

It is now acknowledged that there are at least as many phages as bacterial cells in the mammalian gastrointestinal tract [69]. In healthy humans, only a small proportion of the phageome (phage community) is common to large numbers of individuals, with most of the phages present being subject specific [44]. Moreover, patients with inflammatory bowel disease (ulcerative colitis and Crohn's disease) or AIDS have been shown to have gut viral populations that are very different in size and diversity from those of healthy individuals [70,71]. Furthermore, changes in viral diversity have been shown to precede the appearance of type I diabetes in children [72]. Phageome variations are of course connected with bacteriome deviations, demonstrating the intimate but still poorly characterised link between these two antagonistic populations. These conditions of viral and cellular dysbiosis raise questions about whether certain diseases are caused by changes in the microbiome rather than a single pathogen, defining the new concept of a "pathobiome" [73]. This concept underlies a paradigm shift with a move away from targeting single pathogens to targeting whole communities. Within this framework, phages are potentially useful as modulators of the microbiome as a whole. A striking example of this approach is provided by the similar efficacies of treatments for recurrent Clostridium difficile infections based on faecal microbiota transfer or sterile faecal transfer with filtering to exclude bacteria (but not phages), highlighting the role of non-bacterial components of the microbiota in the clinical effect of treatment [74,75]. Interestingly, the virome composition of patients treated by sterile transfer was found to be similar to that in the donor [75].

Interesting features of these phages can be linked to their adaptation to this environment; for example, some phages carry specific motifs in their capsids that allow them to bind to the intestinal mucus, potentially creating an additional layer of protection against bacteria [76]. Moreover, a direct role of the microbiome in phage evolution has also been suggested by the results of a study reporting the evolution of an ability to infect new hosts through the use of a second strain as a stepping stone [9]. No such evolution was observed in vitro or in dixenic mice and it was, therefore, suggested that the gut microbiota can promote phage and bacterial population diversification [9,77].

In summary, each partner in this tripartite interaction (the phage, the bacterium and the mammalian host) plays an important role in phage—bacterium dynamics. It is therefore vital to consider these partners as an ecosystem rather than as two separate paired entities (phage/bacterium or bacterium/host) [78,79]. There are currently gaps in our knowledge that we need to overcome if we are to implement effective strategies based on phage treatments for intestinal pathogens or for the development of microbiota engineering strategies.

6. Overcoming the Limitations of Phage Infection Efficacy In Vivo

To optimise the output of applications based on phages, the gap between in vitro studies and in vivo conditions may be bridged in several ways. First, phages can be isolated and characterised in more realistic and ecologically relevant conditions than under the conditions for optimal bacterial growth that are typically used. For example, we can decide to start from in vitro biofilms consisting of single bacterial species or multi-species communities, and then proceed to ex-vivo conditions using organs [11,80] and, ultimately, in vivo environments [60]. Second, the precise identification of phage receptors and their expression profiles in ecologically relevant conditions will not only provide us with information about phage biology but will also guide the optimisation of conditions for in vivo efficacy. Adaptation of the phage to the targeted pathogen has also been shown to increase phage efficacy in some cases [81]. Moreover, the use of different doses and the localised release of microencapsulated phages may overcome some of the difficulties related to bacterial refuges and bacterial density thresholds [82].

Third, the use of phages together with other treatments (e.g., antibiotics) may improve overall treatment efficacy, an idea that has gained ground since the publication of the Phage Antibiotic Synergy system in 2007 [83]. Several studies have since confirmed the advantages of combining these two antibacterial weapons, although some of the mechanisms involved have yet to be identified (not all phage and antibiotic combinations display such synergy [84,85]). Such combinations may also be effective against biofilms, overcoming the limitations of each of these agents used separately [86–88]. The selection of resistant cells is a key concern in the use of both antibiotics and phages. However, there is no overall

association between antibiotic resistance and phage resistance profiles supporting further their use in combination [89]. Nevertheless, double resistance or persister cells could provide a means for bacteria to protect themselves from these threats, however, this requires further studies. Interestingly, it was observed that the growth of phage-resistant bacteria during phage therapy in experimental models can be controlled with two independent allies: antibiotics, as demonstrated in an endocarditis model, and the innate immune response, as shown in a model of pulmonary infection [84,90].

About a century after their first use as an antibacterial agent for treating infections, phages have not yet revealed all their secrets. Phage biology is presenting scientists with new challenges every day. Many of the mechanisms involved in phage infection of bacteria remain unknown, hindering the effective use of phages as an ecological and sustainable alternative or complement to overcome the antibiotic resistance crisis and to tackle diseases caused by microbiome dysbiosis.

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Review Article

The Battle Within:

Interactions of Bacteriophages

and Bacteria in the Gastrointestinal Tract

The Battle Within: Interactions of Bacteriophages and Bacteria in the Gastrointestinal Tract

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The intestinal microbiota is intimately linked to human health. Decoding the mechanisms underlying its stability in healthy subjects should uncover causes of microbiota-associated diseases and pave the way for treatment. Bacteria and bacteriophages (phages) are the most abundant biological entities in the gastrointestinal tract, where their coexistence is dynamic and affixed. Phages drive and maintain bacterial diversity by perpetuating the coevolutionary interactions with their microbial prey. This review brings together recent in silico, in vitro, and in vivo work dissecting the complexity of phage-bacteria interactions in the intestinal microbiota, including coevolution perspectives. We define the types of dynamics encountered in the gastrointestinal tract and the parameters that affect their outcome. The impact of intestinal physiology on phagebacterial coevolution is analyzed in the light of its potential contribution to the relationship between the microbiota and human health.

Introduction

Polymicrobial communities, hereafter termed microbiota, are present in most environments and shape the ecology of these environments through metabolic reactions vital to ecosystem function. Within such communities, microbes can establish interspecies networks that can coordinate energetic pathways with effects on the environment at the global scale, as illustrated by the biogeochemical cycling processes that occur in bodies of water and the soil (Singer et al., 2017). Several microbiota also establish intimate associations with multicellular organisms, such as plants or animals, sometimes causing disease but more often developing a symbiotic coexistence of mutual benefit. The human intestinal microbiota is an example of such mutualism that is currently extensively studied, being associated with vital functions, such as digestion, the immune response, and the nervous system (Belkaid and Hand, 2014; Sharon et al., 2016; Sonnenburg et al., 2005). Analyses of clinical samples have revealed that several diseases and disorders are associated with alterations in the composition of the intestinal microbiota compared to controls (Frank et al., 2007; Lev et al., 2006; Qin et al., 2012; Zhu et al., 2017). This active field of research is currently focusing on delineating the role of individual components of the microbiota, with the goal to re-establish health (Gentile and Weir, 2018).

The intestinal microbiota consists of bacteria, archaea, fungi, protists, and viruses. The advent of high-throughput sequencing has opened the door to revealing the very large microbial diversity associated to the intestinal ecosystem, which is further expanding as new cohorts are analyzed worldwide (Pasolli et al., 2019). However, not all the genomic information can be assigned to a defined organism, and this is particularly true for the identification of viruses, at least in part because of the lack of common genomic markers, such as the 16S or 18S ribosomal RNA genes (Paez-Espino et al., 2016). Parasitic interactions occur within the gastrointestinal tract (GIT), as exemplified by

the most abundant microbes in this environment: bacteria and the viruses that predate on them, bacteriophages (phages). The perpetuating antagonistic coevolution between the predator (phages) and the prey (bacteria) populations results in fluctuations of both these populations (Faruque et al., 2005; Koskella and Brockhurst, 2014).

The presence of viruses, including phages in particular, in the human GIT has been known for a century, but their role in the intestinal microbiota has been little studied (d'Herelle, 1917; Reyes et al., 2012). The number of active phage species in a healthy subject has been estimated between 35 and 2,800, with more than 50% being predicted to be unique to each individual (Manrique et al., 2016; Minot et al., 2011; Reves et al., 2010). The most abundant viral families include Myoviridae, Podoviridae, and Siphoviridae, all with double-stranded DNA genomes, as well as the Microviridae family, which possess single-stranded DNA genomes (Manrique et al., 2016; Reyes et al., 2015). As mentioned, the exploitation of virome data has proved more challenging than that of genomic data for other components of the microbiota. Besides viral identification, an even greater hurdle is the lack of a universal tool for matching the predating phages to their host bacteria. Progress in this direction has been made to refine predictions-for example, by looking for bacterial CRISPR spacer sequences with homology to known viral genome sequences. The technique was successful in identifying the match between phage and bacterial species-the next challenge being to define this match at the strain level (Paez-Espino et al., 2019). As a result, progress in the role of phages in microbiota has been based mostly on experimental models built from data obtained in silico, in vitro, and in vivo (Scanlan, 2017). In this Review, we will integrate recent data from phage biology into the broader context of the coevolution of phages and bacteria within the GIT and discuss the possible effects of this coevolution on the human host.

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Interactions between Phages and Bacteria: From Test Tube to the Intestinal Organ

Studies of phage-bacterium interactions (PBIs) began with the launch of phage therapy a century ago (d'Herelle, 1917). Many original molecular mechanisms affecting these interactions have since been identified, mostly from studies of individual phage-bacterium pairs cultured in optimal laboratory conditions. A broad summary of these mechanisms is presented in Figure 1, and several reviews have described the resistance systems developed by bacteria and the counterdefense strategies used by phages (de Jonge et al., 2019; Labrie et al., 2010). In addition to broad mechanisms, such as alterations to receptor and restriction-modification systems, more specific and novel systems have been recently uncovered by data mining and large-scale screening, and it is thought that many more remain to be discovered (Doron et al., 2018; Kronheim et al., 2018). The flexibility of genetic information forms the cornerstone of all these systems. Therefore, integration of the evolution of PBIs in the intestinal context requires the consideration of multiple levels of information, from small viral genomes to the behavior of large organs. Evidences for the Coevolution of Phages and Bacteria in

the GIT

The coevolution of phages and bacteria gives rise to structured nested and modular networks. Nested interaction networks are characterized by a hierarchy of bacteria and phages ranked according to the susceptibility or resistance of bacteria and to the specialist (infecting few strains) or generalist (infecting many strains) nature of the phages. By contrast, in modular networks, interactions occur within distinct groups of phages and bacteria different from those present in other modules, with very little overlap (Weitz et al., 2013). These two types of interactions may also coexist within the nested-modular networks of complex ecosystems, including the mouse GIT, in which generalist phages are prevalent (De Sordi et al., 2017; Kim and Bae, 2018). This level of interaction is subject to dynamic modulation by the evolution of defense and counter-defense systems of bacteria and phages. For example, resistance to phages has been

Figure 1. Bacterial Mechanisms of Defense against Phage Predation

No single bacterium has been found to possess all of these defense systems, but each bacterium can have several. A large pink star indicates the essential mechanisms of DNA replication, transcription, and translation underlying the genetic and phenotypic variations inherent to life. Red crosses correspond to an arrest of the infection process. Red triangles correspond to both bacterial proteins involved in the abortive infection (Abi) system leading to cell suicide and phage proteins involved in the super-infection exclusion (Sie) mechanism to prevent further infection by related phages. Blue and green DNA molecules correspond to bacterial and phage DNA, respectively. R-M, restrictionmodification.

observed in studies characterizing clinical samples from Vibrio cholerae-infected patients and phage-treated chickens infected with Campylobacter jejuni or calves

infected with Escherichia coli (Holst Sørensen et al., 2012; Seed et al., 2014; Smith and Huggins, 1983).

Moreover, intestinal metagenomic analyses have revealed the existence of considerable variability in bacterial surface epitopes, including phage receptors, within a given bacterial species isolated from different subjects (Zhu et al., 2015), supporting a hypothesis of active local coevolution between phages and bacteria. A similar conclusion was drawn from the metagenomic detection of highly variable and rapidly evolving CRISPR sequences, suggesting multiple attempts to escape phage predation (Stern et al., 2012). These genomic events do not necessarily give rise to a dominant population of bacteria with a phenotype of phage resistance. Indeed, experimental phage-bacteria coevolution studies in animal models have failed to recover phenotypically resistant bacteria from isolated colonies, and large viromic studies in humans have failed to detect metagenomic signs of coevolution (De Sordi et al., 2018: Minot et al., 2013). Nevertheless, coevolution has persisted over time, as demonstrated by the existence of mutations of bacterial loci relating to phage receptors. This suggests that either phages deploy rapid counter defense mechanisms or that alternative resistance mechanisms operate. This phenotypic resistance of bacteria is conceptually similar to the tolerance to antibiotics and persistence of bacteria in the presence of antibiotics and may emerge within the intestinal organ (Lourenco et al., 2018).

A complementary hypothesis is that bacteria may display a different physiological state locally, rendering them less permissive to phage infection (Denou et al., 2007). Indeed, bacteria may display differential susceptibility to phages between the niches occupied in the GIT, as shown by replication data obtained ex vivo. The CLB_P1 phage is capable of replicating in ileal sections but not in feces collected from the same mouse colonized by the E. coli strain targeted by this phage (Maura et al., 2012). Using the same assay, other phages were independently efficient in other gut sections, demonstrating that the observed phenotypic resistance is phage specific (Galtier et al., 2016b; Maura et al., 2012). Gradients of abjatic factors, such as pH and oxygen concentration, and of metabolites, such as bile salts



and short-chain fatty acids, along the digestive tract can alter the physiology of bacteria and, consequently, their susceptibility to phages. Furthermore, a recent study by Kronheim et al. show that bacteria can produce molecules that interfere with the phage infection cycle, revealing an additional source of phenotypic resistance (Kronheim et al., 2018). The GIT is a structured environment with transverse and longitudinal differences in microbial density and layers of mucus and villi. This spatially heterogeneous organ can provide niches in which coevolution does not occur. As an example, T4 phages have been shown to display differential ability to persist in a cell line model of mucosal layer, depending on the presence or absence of immunoglobulin (Ig)like domains on the viral capsid, therefore affecting the frequency of encountering their hosts (Barr et al., 2013). In addition, immune cells that are patrolling the human body can also interact with both bacteria and phages, the latter having attracted much less attention from researchers than the first (Van Belleghem et al., 2018). More importantly, each of the above parameters may affect the coevolution of individual pairs of phages and bacteria, highlighting the complexity of studying PBI in the GIT (Figure 2) (Lourenço et al., 2018).

Population Dynamics

The antagonistic coevolution of phages and bacteria has an impact on the dynamic fluctuations of both populations. Such dynamics have been described in different models, such as the arms-race dynamics (ARD) and the density-dependent fluctuating-selection dynamics (FSD) models (Gandon et al., 2008). In the ARD model, both phages and bacteria accumulate genomic mutations, which enable the bacteria to develop resistance and the phages to counteract that resistance, thereby generating predator-prey cycles. Instead, the FSD model is

Figure 2. Factors Influencing Phage-Bacteria Interactions in the Gastrointestinal Tract

In healthy subjects, abiotic and biotic factors can affect bacteria gene expression (rods in different shades of blue) or mammalian cells, with direct consequences for phage populations. Microbiota in Crohn's disease patients is characterized by an inverse correlation between phage and bacterial diversities (decrease of different colored rods and increase of phages with various colors). Intestinal villi can serve as spatial refuges for bacteria, enabling them to escape phage predation, represented by dashed lines and multiple phages. Epithelial cells and the mucus layer are colored in rose and yellow, respectively, with the rare goblet and Paneth cells noted. B, B cells; DC, dendritic cells; M, macrophages; N, neutrophils; T, T cells.

not based on the evolution of phages to overcome bacterial resistance, as it takes into account the pleiotropic costs associated with the mutations enabling bacteria to become resistant. Strong predation by phages selects for resistant bacterial populations, thereby decreasing the number of phages present locally. A subsequent absence of phage-selective pressure thus favors an expansion of the population of bacteria susceptible to phages, which is not subject to the possible fitness cost

associated with the mutations conferring resistance to phages (Hall et al., 2011; Lennon et al., 2007; Middelboe et al., 2009). Conversely, mutations overcoming bacterial resistance may also be a burden to the phage in situations in which such a counterresistance is not selected. This FSD model applies to both single phage-bacterium pairs and to the heterogeneous populations derived from their coevolution (Breitbart et al., 2018).

In any given microbiota in which phages interact with diverse populations of strains, antagonistic coevolution proceeds within a more intricate network of interactions. For example, the antagonistic coevolution of multiple wild-marine T7-like cyanophages with their targeted bacteria, Prochlorococcus, is characterized by genomic mutations, host-range expansion, and fitness costs (Enav et al., 2018). The authors confirmed the concomitant occurrence of ARD, shown by the detection of genomic mutations responsible for bacterial resistance and phage re-infectivity, and FSD, due to the genetic hypervariability of the two populations. However, many phage populations did not carry mutations that could overcome Prochlorococcus resistance, suggesting that these two coevolutionary models alone cannot account for the maintenance of these phage variants. The authors postulated that host jumps might constitute a third concomitant mechanism based on the selection of genomic mutations in phages that confer an ability to infect alternative bacteria, thereby avoiding the risk of phage extinction and termination of the predator-prev cycle. Host jumps were also reported in a mouse model of coevolution in the GIT (De Sordi et al., 2017). In this model, the E. coll phage P10 evolved to infect an initially inaccessible E. coll strain during multiple passages in two other E. coll strains. The selection of a combination of mutations resulted in greater fitness associated with infection of the

inaccessible E. coli strain. In these settings, the genomic heterogeneity of both the phage and bacterial populations may also result from simultaneous dynamic population fluctuations and the sustained antagonistic coevolution and generation of community variability (De Sordi et al., 2018).

The application of coevolution models to the intestinal microbiota would theoretically be able to identify a moment in space and time at which the most fit population of phages and bacteria would have the opportunity to replace the most abundant ones. However, from birth to adulthood, the bacterial diversity of the intestinal microbiota is dominated by the same phyla, Bacteroidetes and Firmicutes, to which the most abundant species belong. This observation gave rise to a theoretical royal family model, in which a bacterial population declining after an antagonistic fluctuation is replaced by a related bacterial population, which is already adapted for occupation of the same environmental niche, rather than any other bacteria (Breitbart et al., 2018). This model was first proposed based on analysis of aquatic ecosystems and is supported by the repeated isolation of the same bacterial and viral taxa in parallel antagonistic coevolutions.

A prime example of the dominance of a particular viral group in the GIT is provided by the crAssphage family, the members of which infect bacteria from one of the most widespread phyla, Bacteroiodetes. The crAssphage family was first identified metagenomically in 2014 and was rapidly characterized and found to be widespread, but the first isolated target strain of these phages (Bacteroiodes intestinalis) was not identified until 2018, after a laborious search (Dutilh et al., 2014; Shkoporov et al., 2018; Yutin et al., 2018). If that much effort was required to characterize the most abundant antagonistic populations in the human GIT, we can only imagine how difficult the identification of less abundant coevolving pairs of phages and bacteria is likely to be. However, further studies of the evolution of crAssphage and Bacteroides populations would provide useful insight into the keys to microbiota stability.

Phage Activities Related to Health and Disease Virulent and Temperate Phages

It is widely agreed that most phages have the capacity to lyse the bacteria they infect (M13 being a well-known exception relying on chronic infection), and some have, in addition, a dedicated set of genes (encoding integrase, excisionase, and repressors, for example) required for the integration of their genome into the chromosomes of the bacteria to postpone lysis. These

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genes encoding such functions. Due to the abundance of integrases on virome analysis, it has been suggested that the majority of phages in the gut would be temperate (Minot et al., 2011; Reyes et al., 2010). The phage genome integrated into the bacterial chromosome is named prophage. Many sequence-analysis tools have been developed for scanning bacterial genomes, and they have revealed that putative prophage sequences can account for up to 20% of the total length of the bacterial genome (Canchaya et al., 2003; Casjens, 2003). The integration of a phage genome is known as lysogenic conversion and has been studied in bacteria for more than 50 years, particularly for the infection of E. coli by phage lambda (Lwoff, 1953). This model phage has been studied in detail, and many reports have focused on the conditions governing the excision of the phage lambda genome from the chromosome to re-establish virulent infection. Indeed, several environmental stresses, such as UV light or chemicals (including antibiotics), as well as inflammation in the GIT, can induce prophage excision (Banks et al., 2003; Barnhart et al., 1976; Goerke et al., 2006). Oh et al. recently added dietary fructose and short-chain fatty acids to the list of inducers of prophage excision, suggesting a mechanism of phage-mediated alteration of the intestinal microbiota depending on the bacterial metabolism (Oh et al., 2018). The induction of excision is essential for the perpetuation of temperate phages, as it provides a means of infecting more bacteria and disseminating. The exit of the phage from a bacterial chromosome must, therefore, be precisely controlled by a defined molecular mechanism. This mechanism has been dissected in great detail for phage lambda and is based on a genetic switch governing the production of the CI (lysogenypromoting) and Cro (excision/lytic-promoting) proteins. Most of the inducers of phage excision provoke DNA damage, triggering an emergency response that is used by the phage to express the genes required for the excision. However, signaling molecules also impact the decision for excision. A V. cholerae phage encodes for a receptor able to activate the phage lytic pathway upon binding to a quorumsensing molecule produced by the bacterial host (Silpe and Bassler, 2019). An alternative system, called arbitrium, has recently been described (Erez et al., 2017). Upon bacterial lysis, a peptide produced by the prophage is released; when the concentration of this peptide in the environment exceeds a particular threshold, the surrounding bacteria perceive the signal (through a set of genes also originating from the phage), leading to the cessation of lysis and the promotion of lysogeny. This novel system is thought to be only one of many original systems awaiting discovery (Howard-Varona et al., 2017). In the confined intestinal environment, where accumulation of small signaling molecules is favored compared to open environments, such new mechanisms are likely to play a role in shaping the microbial communities. Recent studies on prophage dynamics in the GIT have shown that prophages can excise from bacterial chromosomes, modulate the microbiome, acquire genetic information, or even transfer between bacteria in response to inflammatory pro-cesses (Cornuault et al., 2018; De Paepe et al., 2014, 2016; Diard et al., 2017; Oh et al., 2018). Overall, in the GIT, the activity of phages-whether temperate or virulent-influences not only phage abundance, but also bacterial behavior.

Impact of Phages on Bacterial Behavior and Virulence The lysogenic conversion of bacteria is accompanied by wideranging effects on their behavior. For example, the toxin genes carried by temperate phages, encoding cholera, or Shiga toxins can affect bacterial virulence (Bille et al., 2017; Muniesa et al., 2012). Prophage induction in pathogenic bacteria in the GIT may then provide opportunities for the dissemination of such virulence factors. Genes carried by prophages can also influence bacterial physiology by supplying new functions, such as an expansion of metabolic capability conferring an enhanced fitness in this competitive niche (Bille et al., 2017; Br€ussow et al., 2004; Harrison and Brockhurst, 2017; Obeng et al., 2016). Importantly, prophages—and phages in general— do not usually carry antibotic resistance genes, suggesting possible counter-selection against such genes within phage genomes (Enault et al., 2017).

In addition to these direct consequences, prophage integration can also lead to indirect effects that are currently underappreciated. For example, prophage integration may change the conformation of the bacterial chromosome, with various effects on gene expression. When the prophage excision mechanism is altered or lost, the genomic information of the phage is locked in the bacterial chromosome, where it is subjected to purifying selection (deletion of deleterious functions) (Bobay et al., 2014). By contrast, lysogenic behavior is lost on prophage excision and lysis of the host bacterium. Lysogens may then be assimilated as a subpopulation of bacteria with a higher probability of death than non-lysogenic bacteria. On leaving the bacterium, phages may encapsidate some of the genomic information from the bacterium, which may then transduce another bacterium. This property was exploited extensively in the early days of bacterial genetics, with phage P1 the best-known example of a transducing phage. Horizontal gene transfers of this type are now recognized as a major driving force behind bacterial evolution and adaptation to environmental cues (Howard-Varona et al., 2017).

In the GIT, in which many, if not all, of these events can take place, evidence supporting dynamic prophage induction is emerging. For instance, the cost of carrying a prophage was assessed for E. coli in a model of axenic mice colonized by lysogens, and the results revealed a high rate of prophage induction (De Paepe et al., 2016). Induction was also recorded in vivo with Enterococcus faecalis, and this process was shown to be involved in killing competitors (Duerkop et al., 2012). Studies of this bacterium have also revealed the intricate behavior of several prophage elements within the same cell: some defective prophages were shown to hijack structural proteins from other intact prophages to form the virions required for their dissemination (Matos et al., 2013). The mammalian host has also recently been considered in studies of the inflammatory response promoting phage transfer from one Salmonella spp. strain to another (Diard et al., 2017). This work highlighted the need to take the mammalian host into account in studies of PBI dynamics and vice versa, as well as to study the processes that may lead to alteration in the microbiota associated to infections and inflammatory diseases (Debarbieux, 2014; Galtier et al., 2016a). In addition to the host and its response to the presence of microbes and their dynamics, other factors, such as changes in diet, may affect PBI dynamics by altering metabolic pathways (Oh et al., 2018). Metabolic changes may, in turn, influence the competition between bacteria for particular niches, thereby affecting the mammalian host response and potentially resulting in a shift in the overall stability and evolution of the microbial consortium. Phages in **Disease Cohorts**

Without the advent of metagenomic sequencing, the link between phages and health would probably never have been discovered, because traditional culture methods for viruses are based on the high specificity of PBI. Indeed, the quantification of phages by direct plaquing is restricted by the number of bacterial strains used to perform these tests. This laborious task managed to identify one crAssphage-susceptible strain in laboratory conditions only because it was guided by metagenomics information for a fecal sample highly enriched in this family of phages (Shkoporov et al., 2018; Yutin et al., 2018).

One of the earliest studies of the richness and diversity of the phage community associated with changes in intestinal microbiota was performed on fecal samples from patients with Crohn's disease and ulcerative colitis. Surprisingly, both the richness and diversity of phages were higher in these patients than in healthy subjects, but bacterial richness and diversity were lower (Norman et al., 2015). A similar observation was also reported for healthy twins during the first 24 months of life (Reyes et al., 2010). The drivers of these dynamics remain unknown. Do the disease conditions lead to an expansion of the population of prophages excised from low-abundance bacterial populations below the radar of metagenomics analysis? Or do changes in bacterial composition provide newcomers with an opportunity to invade the GIT with the necessary adaptation steps, including shifts in phage populations? The two hypotheses are not mutually exclusive, and methods for increased exploitation of the genomic information are still being developed (Roux et al., 2017). However, for the time being, the interpretation of these observations cannot yet extend beyond associations or trends based on the abundance of reads. Nevertheless, growing examples of changes in viral metagenomes are being associated with diseases, like AIDS or diabetes (Manrique et al., 2016; Monaco et al., 2016; Norman et al., 2015; Zhao et al., 2017).

Fecal microbiota transfer (FMT) to treat recurrent Clostridium difficile infections is a recent development providing support for an active role of phages in shaping the intestinal microbial community. First, viruses from the donor were found to be transferred to the recipient after 6 weeks of FMT treatment. All the transferred viruses were phages, providing an additional argument in favor of the safety of FMT and the putative role of phages in the success of this treatment (Zuo et al., 2018). A 12-month follow-up study recently showed that phages from the donors were still detectable in the recipients, demonstrating the longterm invasion of the initial microbiota by the phages and, thus, the ability of these phages to adapt to a different environment (Draper et al., 2018). Moreover, FMT treatment with a sterile filtrate was found to be as effective for reducing C. difficile infections as standard FMT (Ott et al., 2017). Overall, these data highlight a major role for phages in the manipulation of the intestinal microbial population. However, it remains undetermined whether phages exert these effects on their own and by which mechanisms they establish and evolve over time.

Perspectives

After 100 years of research mostly focused on single-phage/ singlebacterium pairs, the field of PBI research is now enjoying a new lease on life thanks to novel technologies facilitating the study of complex microbial communities and the need to support phage therapy as one possible solution to the problem of antibiotic resistance (Roach and Debarbieux, 2017). When trying to understand what maintains or disturbs the intestinal microbial balance in healthy subjects, efforts should focus on modeling and defining how different coevolutionary models can concurrently shape microbial diversity in spatially heterogeneous environments (Hannigan et al., 2018). The type and cost of resistance and counter-resistance should be considered, together with the physiological advantages of colonizing different niches. The aim is to describe the coexistence of many different phages and bacterial hosts in the microbiota mathematically and to predict the



Figure 3. Schematic Diagram of the Antagonistic Coevolution of Phages and Bacteria in a Mammalian Host

Heterogeneous populations of bacteria (differentially colored rods) with different phenotypes (distinct shading within clusters) coexist with various phages (different colors are consistent with bacterial diversity, and distinct shades of colors correspond to evolved phages including host jumps). The infection of bacteria by phages affects the fitness of both phage and bacterial populations within a defined spatial niche that is represented by an assemblage of bacteria either colored in blue (corresponding to phage-resistant/ inaccessible populations), orange (corresponding to bacteria lysed by virulent phages [dashed lines]), or green (corresponding to lysogens from which prophage [red circles] excise). The mammalian host (represented as a pink area) underlies these microbial interactions and can modulate and be modulated by the outcome of these interactions.

sweeps likely to alter or maintain the equilibrium in a steady state in the long term. Viral ecologists can provide a framework for achieving this goal, generally by focusing on global ecosystems (Bolduc et al., 2017). The next stage will be to develop models of these coevolution dynamics, taking into account the mammalian host in the context of both healthy and diseased subjects (Hochberg, 2018).

Experimental model systems will also be required to decipher individual mechanisms at the molecular level. One-to-one interaction studies can reveal novel ways in which phages and bacteria can manipulate each other's evolution, but access to the mammalian environment remains limited. However, gnotobiotic murine models-germ-free mice colonized by a defined set of bacterial strains-are emerging as a surrogate system in which the impact of the intestinal microbiota on health can be investigated. Germ-free mice colonized with human bacterial strains have been used to describe phages from human viral fecal material and could be further developed for the isolation of human-associated phages (Reyes et al., 2013). Another model was recently established with murine bacterial strains, providing a more natural intestinal ecosystem compared to introducing human bacterial strains into germ-free mice. This model was then used to demonstrate the basis of the microbial competition between S. typhimurium and E. coli strains for the same niche (Brugiroux et al., 2016). Such a model has the advantage of high reproducibility between breeding facilities, making it possible for multiple teams with specific objectives to work with the same tool. Murine models also provide biological samples that can be difficult to obtain from humans, such as intestinal biopsy specimens, which are required to assess the influence of spatial distribution on the interaction and evolution of microbial species. Last, but not least, beyond PBI, diverse microbial interactions, such as those between fungi and bacteria, are currently underappreciated together with interactions among other GIT inhabitants, such as enteric parasites.

Finally, an area still overshadowed by the work on PBI and evolution is the effect of the mammalian response to this dy

namic consortium of microbes (Figure 3). Disease states, in particular inflammation, have been shown to affect PBI, but many other processes remain to be studied in the GIT and in humanassociated microbiota in general (Diard et al., 2017; Norman et al., 2015). Following on from the unexpected discovery that relationships between microbiota and drugs can influence the efficacy of immunotherapy against tumors, we can reasonably assume that some PBI play a role on biological processes beyond microbiology (Routy et al., 2018). It remains unclear whether such effects are driven by the antagonistic coevolution of phages and bacteria or a subtler diversion of function exploited by the immune system, but these possibilities highlight how exciting phage biology has become as it enters a new era of full integration into studies of microbes and their interactions within ecosystems.

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Résumé :

L'intestin des mammifères est peuplé de nombreux et divers microbes comprenant des bactéries et leurs prédateurs viraux, des bactériophages (phages). Les interactions entre les phages et les bactéries intestinales sont encore mal comprises.

Des expériences indépendantes ont montré que les phages virulents n'avaient aucun effet majeur sur l'abondance des bactéries intestinales ciblées, en dépit de leur amplification durable. Cela suggère que des facteurs encore inconnus de l'environnement intestinal modulent ces interactions.

À l'aide d'une analyse transcriptomique comparative de la bactérie *Escherichia coli* cultivée *in vitro* et *in vivo* (dans l'intestin de mammifères), nous avons constaté que, dans l'intestin, les bactéries réduisent l'expression de gènes liés aux récepteurs du phage. Ceci permet d'expliquer l'absence de sélection des bactéries mutées devenues résistantes aux phages lors d'expériences *in vivo*. D'autre part, nous avons montré que l'acquisition d'un îlot de pathogénicité, souvent associé aux souches intestinales humaines d'*E. coli*, affecte la susceptibilité aux phages en régulant négativement un mécanisme de défense contre l'ADN étranger. Enfin, nous avons examiné la répartition des phages et des bactéries dans les parties mucosales et luminales de l'intestin et avons observé une distribution spatiale hétérogène de ces deux populations antagonistes, corroborant l'hypothèse d'une dynamique « source-sink ».

Globalement, nos données démontrent que de multiples facteurs incluant la distribution spatiale, la physiologie bactérienne et les défenses contre l'ADN étranger modulent les interactions entre bactéries et phages dans l'intestin des mammifères.

Mots clés : interactions bacteriophage-bactérie, microbiote, écologie bactérienne, physiologie bactérienne, transcriptomique, *Escherichia coli*, bacteriophage virulent

Abstract :

The mammalian gut is a heterogeneous environment inhabited by a large and diverse microbial community, including bacteria and their viral predators, bacteriophages (phages). Dynamic interactions between virulent phages and bacteria in the gut are still poorly understood, which is also an obstacle for the design of successful therapeutic interventions based on phages.

Independent experiments have shown that virulent phages were found to have no major effects on their targeted bacteria in the gut, in spite of sustainable phage amplification. This suggests that there are unknown factors in the gut environment that modulate these interactions.

Using comparative transcriptomics analysis of *E. coli* grown *in vitro* and *in vivo* (within the mammalian gut) we found that in the gut, bacteria downregulate the expression of genes related to phage receptors, which provides an explanation for the lack of selection of phage-resistant bacteria during *in vivo* experiments. We also found that the acquisition of a pathogenicity island commonly found in human *E. coli* isolates affects phage susceptibility possibility by downregulating a defense mechanism against invading DNA. Finally, we examined the repartition of phages and bacteria through mucosal and luminal gut sections and observed a heterogeneous spatial distribution of these two antagonist populations, supporting the hypothesis of source-sink dynamics.

Altogether our data demonstrates that multiple factors encompassing, spatial distribution, bacterial physiology and defenses against foreign DNA modulate the interactions between bacteria and phages within the gut.

Keywords : Phage-Bacteria interactions, Microbiota, Bacterial ecology, Bacterial physiology