PHAGES in Roscoff

Sixth symposium of the French PHAGE Network

11-12 October 2021, Marine station, Roscoff

Sessions

Ecology and evolution

Cellular and molecular interactions

Therapy and biotechnology

Invited Speakers

Joshua Weitz
Georgia Tech, USA

Aude Bernheim
INSERM, Paris

Adeline Goulet
CNRS, Marseille

Yves Briers
Ghent University, Belgium

Scientific committee

Frédérique Le Roux
Cécile Breyton
Aurélia Battesti
Baptiste Gaborieau
Claire le Hénaff

Organisation committee

Equipe génomique des vibrios, SBR, Roscoff

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Station Biologique de Roscoff

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MONDAY 11 OCTOBER

07:30	*08:15*	*Control of the sanitary passes, badges (Conference room)*	
08:15	08:30	**INTRODUCTION**	**Frédérique Le Roux**

Session cellular and molecular interaction

Chairs: Séraphine Degroux and Damien Piel

08:30	09:10	**Dissecting siphophage modus operandi: from host binding to hijacking.**	**Adeline Goulet**
09:10	09:30	Incidence and impact of lysogeny on the physiology of malolactic starters.	Amel Chaib
09:30	09:50	Exploring the mechanisms of host takeover by bacteriophage T5: role of the DNA-binding protein A2.	Muge Senarisoy
09:50	10:10	Construction and screening of an exhaustive prophage library of E. coli K12 to identify RpoS regulatory elements of prophage origin.	Paramita Sarkar
10:10	*10:40*	*Break*	
10:40	11:20	**Systematic and quantitative view of the antiviral arsenal of prokaryotes.**	**Aude Bernheim**
11:20	11:40	PHROG: families of prokaryotic virus proteins clustered using remote homology.	Paul TERZIAN
11:40	12:00	Phage-Antibiotic Synergy: The role of filamentation.	Julián Agustín Bulssico
12:00	12:20	Molecular multitasking: inhibition of phage infection by aminoglycoside antibiotics.	Aël Hardy
12:20	14:00	Lunch at the Hôtel d'Angleterre	
14:00	14:40	**Tailor-made phage lysins: as simple as Lego?**	**Yves Briers**
14:40	15:00	Molecular mechanisms of selective viral DNA recognition and packaging of the bacteriophage SPP1.	Mehdi El Sadek Fadel
15:00	15:20	Cyclic nucleotide in bacterial immune response: activate to better kill.	Gaelle Hogrel
15:20	*15:50*	*Break*	

Session therapy and biocontrol			
Chairs: Camille Kolenda and Ruben Barcia Cruz			
15:50	16:10	Evaluation of a phage cocktail to prevent and control avian colibacillosis.	Marianne Nicolas
16:10	16:30	Bacteriophage resistant clones emerging during pulmonary phage therapy are similar to those isolated *in vitro*.	Baptiste Gaboriau
16:30	16:50	Using bacteriophage T5's capsid as a therapeutic nanoparticle.	Nicolas Ducrot
16:50	17:10	PHAG-ONE: an ANR project for French academic production of therapeutic phages to fight multidrug resistant bacteria.	Frédéric Laurent
17:30	19:00	**Poster all sessions** (Hotel de France, 1st Floor, room 3)	
20:00		Dinner at the Hotel d'Angleterre	

TUESDAY 12 OCTOBER

Session ecology and evolution			
Chairs: Thomas Paillet and Wakinyan Benhamou			
08:30	09:10	**Ecological Dynamics and Therapeutic Impacts of a Not-So-Perfect Predator.**	**Joshua Weitz**
09:10	09:30	The gut environment shapes bacteriophages infection by modulating bacterial gene expression.	Lorenzo Chaffringeon
09:30	09:50	Coevolution and competition drive the diversification of CRISPR immunity.	Martin Guillemet
09:50	10:10	Regulation of prophage induction and lysogenization by phage communication systems.	Sylvain Gandon
10:10	*10:40*	*Break*	
10:40	11:00	Quantitative analysis of the intestinal and blood virome in inflammatory bowel disease.	Ilias Theodorou
11:00	11:20	Life and death after log: post-exponential phase infection of *Bacillus subtilis* by lytic bacteriophage SPP1 reveals new infection dynamics.	Jack Dorling
11:20	11:40	The paradoxical relationship between CRISPR-Cas and phage susceptibility in *Pseudomonas aeruginosa*.	Lood Cédric
11:40	12:00	Impact of anti-CRISPR on the ecology and evolution of phages and bacteria.	Anne Chevallereau
12:00	12:15	**CONCLUSION**	**Comité scientifique**
14:00	15:00	**GENERAL ASSEMBLY**	

ORAL SESSION

CELLULAR AND MOLECULAR INTERACTION

KEYNOTE SPEAKER

Adeline Goulet

Dissecting siphophage modus operandi: from host binding to hijacking

Incidence and impact of lysogeny on the physiology of malolactic starters

Amel CHAÏB[1], Yasma BARCHI[1], Magali DELERIS-BOU[2], Sibylle KRIEGER-WEBER[2] and Claire LE MARREC[1]

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The interactions between *Oenococcus oeni*, the lactic acid bacterium mainly responsible for malolactic fermentation (MLF) of wines, and its related phages are poorly documented. Yet, lysogeny is widespread in the species [1]. In this study, the prophages content of a panel of selected strains was assessed and lysogeny was found to be prevalent. The resistance spectra of strains to diverse lytic and temperate oenophages were evaluated and resistance mechanisms were highlighted in several strains, which is indicative of frequent phage-bacteria interactions. The impact of the prophages harbored by the panel strains on bacterial fitness was assessed by comparing the capacities of lysogenic and non-lysogenic isogenic derivatives to carry out MLF in the presence of different oenological constraints. Altogether, our results suggest that lysogeny is an integral part of *O. oeni* species and participates greatly in strain physiology and tolerance to oenological challenges.

[1] A. Lonvaud Funel. FEMS Microbiol. Lett. 126, 209 214 (1995)
[2] O. Claisse, A. Chaïb, F. Jaomanjaka, C. Philippe, Y. Barchi, P. M. Lucas, and C. Le Marrec. Microorganisms 9: 856. (2021)

Exploring the mechanisms of host takeover by bacteriophage T5: role of the DNA-binding protein A2

Muge SENARISOY[1], Philippe CUNIASSE[1], Sophie ZINN-JUSTIN[1], Pascale BOULANGER[1]

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At the onset of infection, bacteriophages defeat bacterial defences and hijack cellular functions to propagate inside their hosts. A century after phages were discovered and despite many scientific breakthroughs in phage research, the early mechanisms of host takeover during infection are still poorly understood. T5 is a virulent bacteriophage of *Escherichia coli*. It uses a unique two-step DNA injection mechanism, which gives an excellent opportunity to investigate host takeover uncoupled from the downstream phage multiplication steps [1]. The pre-early genes injected during the first step transfer (FST) are quickly expressed. They trigger massive host genome degradation and inactivate host defence systems. Then, during the second step transfer (SST), the remaining genome, including mid and late genes, are injected into the host, thus resuming the phage replication cycle. Two conserved pre-early proteins, A1 and A2, were found to be essential for these early events and to control the SST [2], but their functions are still unknown. We used a multidisciplinary approach combining biochemistry, bacterial genetics and structural biology to investigate the function and structure of T5 A2 protein. We found that A2 is a dimeric two-domain protein that interacts with DNA. We investigated the structure and DNA-binding properties of A2. NMR studies revealed the helix-turn-helix motif of the N-terminal DNA-binding domain and the alpha-helical structure of the C-terminal dimerization domain. A2 binds to double-stranded DNA with a high affinity without sequence specificity, as shown by EMSA. To explore the role of A2 during infection, we performed complementation assays with mutants of A2 impaired in DNA binding, and we showed that the DNA-binding feature of A2 is essential for T5 infection. We are currently investigating the protein partners of A2 to find out its function during T5 infection.

[1] Y.T. Lanni. Bacteriol. Rev. 32, 227–42 (1968).
[2] Y.T. Lanni. J. Mol. Biol. 44, 173–183 (1969).

Construction and screening of an exhaustive prophage library of *E. coli K12* to identify RpoS regulatory elements of prophage origin

Paramita Sarkar, Mireille Ansaldi and Aurélia Battesti

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Prophage refers to the latent form of the temperate bacteriophages, where the phage genome is integrated into the host bacterial chromosome [1]. In the prophage state, majority of its genes remain silent; however, the presence of prophages has been correlated with phenotypic variation in the traits of the lysogen [2]. This phenotypic effect posits an ongoing molecular dialogue between the virus and bacteria that can particularly be envisaged when the host bacterial cell experiences unfavourable environments as it also poses a threat to the virus.

Gamma proteobacteria mostly cope with stress by altering their gene expression controlled by the master regulator RpoS, an alternative sigma factor [3]. RpoS mediated regulation stimulates a state of general stress adaptation in the cell, triggered by various different signals [3]. RpoS itself is under complex regulation throughout bacterial life cycle [3]. The paradigm of RpoS regulation in the cell vary even amongst closely related species [4]. This plasticity in RpoS regulation can be correlated with the differential prophage content of the different species [5]. In addition, several RpoS regulators have been shown to have exogenous origin [6].

Following these leads, we hypothesised the resident prophages can contribute to the cellular stress adaptation of the host by influencing key regulators. This study aims to identify prophage encoded elements affecting the regulation of the master regulator of stress RpoS. To achieve this, we have constructed an exhaustive library of all 9 resident prophages from *E. coli K12*. The library is composed of ~ 4-7 Kb fragments cloned in a low copy number vector, where individual prophage genes are under the control of their native promoters. The library was then subjected to genetic screening using RpoS reporter strains that will inform alteration of RpoS regulation at the level of transcription, post-transcription and protein stability. Preliminary screening has identified two fragments from prophage Qin (10 genes) and CP4-6 (8 genes), that induce RpoS expression at late exponential phase. For prophage Qin, we have been able to narrow down to a shorter determinant composed of two genes: *gnsB*, encoding a putative effector of fatty acid abundance in cell membrane, and *ynfN,* encoding a protein of unknown function. Further genetic analysis is ongoing to establish the identity and mechanism of RpoS regulator from amongst the two candidates.

This study so far highlights a new paradigm of molecular interplay between prophage and host stress response. Furthermore, it demonstrates the utility of the prophage library which can be exploited under varied conditions to investigate the symbiotic association of bacteria and virus.

[1] C. Canchaya et al. Microbiology and Molecular Biology Reviews 67(2), 238–276 (2003)
[2] M. Touchon et al. Current Opinion in Microbiology 38, 66–73 (2017)
[3] A. Battesti et al. Annual Review of Microbiology 65, 189-213 (2011)
[4] S. Gottesman. Journal of Biological Chemistry 294(31), 11685-11700 (2019)
[5] H.E. Schellhorn. Future Microbiology 9, 497–507 (2014)
[6] A. Battesti et al. Journal of Bacteriology 194(10), 2470-2478 (2012

KEYNOTE SPEAKER

Aude Bernheim

Systematic and quantitative view of the antiviral arsenal of prokaryotes

PHROG: families of prokaryotic virus proteins clustered using remote homology

Paul Terzian[1], Eric Olo Ndela[1], Clovis Galiez[2], Julien Lossouarn[3], RE Pérez Bucio[1], Robin Mom[1], Ariane Toussaint[4], Marie-Agnès Petit[3] and François Enault[1]

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Phage diversity is high, both in terms of the number of different protein families encountered and in the sequence heterogeneity within each protein family. The constant increase in sequenced viral genomes constitutes a great opportunity to gain new insights into this diversity and consequently urges the development of annotation resources to help functional and comparative analysis. Here, we introduce PHROG (Prokaryotic Virus Remote Homologous Groups), a library of viral protein families generated using a new clustering approach based on remote homology detection by HMM profile-profile comparisons. A total of 17 473 reference (pro)viruses of prokaryotes were taken as input, among which 4975 were complete genomes from the NCBI and 12 498 were prophages extracted from genomes of cultivable bacterial isolates [1]. This dataset generated 938 864 non-redundant proteins, 868 340 of which were grouped into 38880 clusters. Compared to a classical strategy based on BLAST-like similarity searches, these clusters proved to be 2-fold deeper and yet to remain homogeneous.

Manual inspection of similarities to various reference sequence databases led to the annotation of 5108 clusters (containing 50.6 % of the total protein dataset) with 705 different annotation terms, included in 9 functional categories, specifically designed for viruses. Hopefully, PHROG will be a useful tool to better annotate future prokaryotic viral sequences thus helping the scientific community to better understand the evolution and ecology of these entities.

This work was recently published [2] and its associated website (https://phrogs.lmge.uca.fr) permits to browse protein families and phage genomes, as well as to download all profiles. A guide (in the *documentation* page) indicates how any user can compare its favorite phage proteins to this ordered catalogue of prokaryotic viral proteins. A constant annotation improvement of the families will take place, thanks to expert advices, collected through the *suggestion* page of this website.

[1] Roux,S., Hallam,S.J., Woyke,T. and Sullivan,M.B. (2015) Viral dark matter and virus–host interactions resolved from publicly available microbial genomes. Elife, 4, e08490.

[2] Terzian P, Olo Ndela E, Galiez C, Lossouarn J, Pérez Bucio RE, Mom R, Toussaint A, Petit MA, Enault F. PHROG: families of prokaryotic virus proteins clustered using remote homology. NAR Genom Bioinform. 2021 Aug 5;3(3):lqab067. doi: 10.1093/nargab/lqab067.

Phage-antibiotic synergy: the role of filamentation

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Phage-antibiotic synergy (PAS) is the phenomenon by which the presence of antibiotics increases phage efficacy to destroy bacteria [1]. In sensitive bacteria, antibiotics induce alterations in the physiological state of the cell allowing phage propagation to proceed faster. A morphological change that usually linked to synergy is cell filamentation [1,2]. We studied the contribution of filamentation to PAS using tailed phages on *E. coli*, with emphasis on the effect on bacterial population dynamics, phage reproductive cycle, and phage-bacterium interactions.

The presence of sub-inhibitory concentrations of ciprofloxacin (fluoroquinolone) and ceftazidime (□-lactam) impairs cell division, resulting in the production of multinucleated cells with enlarged cytoplasm. Treated populations display a similar OD_{600} and overall surface increase than the untreated ones but they consist in a smaller number of non-segmented, longer cells. A similar effect can be obtained by inhibiting division (FtsZ ring inhibition) through the production of the Kil peptide [3].

With filamentation we observed a delay of the latent period (the time between infection and lysis) of phage T7. This delay resulted in an increase in the number of phages produced per cell, an important parameter of phage virulence. It has been proposed that a larger membrane surface required higher amounts of holins to form a pore, delaying lysis [2]. To test this, time-lapse microscopy of infection by phage T7 allowed us to determine latent period among sub-populations of antibiotic-treated cells. No difference was observed between short and long sub-populations, suggesting latent period is not influenced by cell surface during T7 infection.
Phage can be tracked with fluorescence microscopy by labelling their capsids with GFP. Thank to this technique, we were able to track phage adsorption on the bacterial surface. We observed filamentation did not modify the adsorption per unit of surface. However, long bacteria adsorbed more phages due to their increased surface area. After phage infection the entire filament lysed as a single unit. The increased probability of phage adsorption to filamenting cells provokes an increased susceptibility to phage attack. This observation was supported by flow cytometry data indicating that filamenting cell population was the most affected after phage addition.

Our results suggest synergy happens due to a combination of effects on cell division and phage reproduction, reshaping dynamics of phage-bacteria interactions in a detrimental way for the bacterial population.

[1] Comeau, A. M., Tétart, F., Trojet, S. N., Prère, M.-F. & Krisch, H. M. Phage-Antibiotic Synergy (PAS): β-Lactam and Quinolone Antibiotics Stimulate Virulent Phage Growth. *PLoS ONE* **2**, e799 (2007).

[2] Kim, M. *et al.* Phage-Antibiotic Synergy via Delayed Lysis. *Appl Environ Microbiol* **84**, e02085-18, /aem/84/22/e02085-18.atom (2018)

[3] Haeusser, D. P. *et al.* The Kil Peptide of Bacteriophage λ Blocks Escherichia coli Cytokinesis via ZipA-Dependent Inhibition of FtsZ Assembly. *PLoS Genet* **10**, e1004217 (2014).

Molecular multitasking: inhibition of phage infection by aminoglycoside antibiotics

Aël Hardy[1], Larissa Kever[1], Tom Luthe[1], Max Hünnefeld[1], Cornelia Gätgens[1], Lars Milke[1], Johanna Wiechert[1], Johannes Wittmann[2], Cristina Moraru[3], Jan Marienhagen[1,4] and Julia Frunzke[1]*

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In response to viral predation, bacteria have evolved a wide range of defense mechanisms, which rely mostly on proteins acting at the cellular level. Here, we show that aminoglycosides, a well-known class of antibiotics produced by *Streptomyces*, are potent inhibitors of phage infection in widely divergent bacterial hosts. We demonstrate that aminoglycosides block an early step of the viral life cycle, prior to genome replication. Importantly, phage inhibition was also achieved using supernatants from natural aminoglycoside producers, hinting at a broad physiological significance of the antiviral properties of aminoglycosides. Strikingly, we show that acetylation of the aminoglycoside antibiotic apramycin abolishes its antibacterial effect, but retains its antiviral properties. Altogether, this study expands the known functions of aminoglycosides in bacterial communities. It further suggests that aminoglycosides are not only used by their producers as toxic molecules against their bacterial competitors, but could also provide community-wide protection against phage predation.

KEYNOTE SPEAKER

Yves Briers

Tailor-made phage lysins: as simple as Lego?

Molecular mechanisms of selective viral DNA recognition and packaging of the bacteriophage SPP1

Mehdi El Sadek Fadel [1, @] , Karima Djacem [1, 2] , Delphine Naquin [1] , Kévin Gorrichon [1], Yves D'aubenton-Carafa [1] , Paulo Tavares [1]

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Encapsidation of a double-stranded DNA genome into a preformed icosahedral procapsid is a crucial step of the viral cycle of tailed bacteriophages and herpesviruses. In these viruses, a protein complex, the terminase, mediates the recognition and encapsidation of viral DNA. This molecular complex recognizes specifically, cleaves, and translocates the viral DNA into the procapsid through a pore formed by a portal protein. In some cases, errors occur leading to bacterial DNA encapsidation. These events lead to generalized transduction of host genes rendering the virus a vector for horizontal transfer of genetic elements. SPP1 is a model system to study the molecular mechanisms of viral DNA packaging in tailed bacteriophages. In this virus, the terminase complex formed by gp1 and gp2 recognizes the viral DNA by binding to the sequence *pac*. In order to understand how the terminase discriminates between viral and bacterial DNA, we are studying the specificity of interaction between the terminase and *pac*. Sequential deletions and substitution mutagenesis of *pac* yielded infective phages demonstrating that a large part of *pac* is dispensable. However, extensive degeneration of the *pac*R sub-region was deleterious and suppressor mutations arose. In these revertants, we observed mutations in the oligomerisation domain of gene 1 that may affect its structure. We also identified some mutations in *pac*R itself showing that a poly-A sequence is important for phage DNA recognition. The effect of deletions in *pac*L, localized upstream of the gene encoding gp1, was compensated by mutations that led to overproduction of the terminase complex. The data show that increasing terminase concentration ensures correct *pac* recognition and cleavage in spite of elimination of the *pac*L site for gp1 binding region while degeneration of the other binding site, *pac*R, can be compensated by changes in the gp1 protein.

Cyclic nucleotide in bacterial immune response : activate to better kill

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Ubiquitous in the three domain of life, cycle nucleotides (cNT) act as key messengers to regulate cellular processes in response to both intra- and extra-cellular signals. In human innate immunity the cyclic GMP-AMP is well described for its role in the cGAS/STING signalling pathway triggered by intruder sequence. The interest in cNT has grown up again these last few years for their role in anti-viral signalling system in both Bacteria and Archaea with the description of completely new mechanisms relying on these small molecules [1], [2].
Recently gathered under the name of CBASS (cyclic oligonucleotide-based antiphage signalling system), genes involved in such processes are organised in defence island with a combination of a sensor protein to generate the cNT and effector proteins activated by this cNT [3]. While the sensor protein is commonly related to cGAS (cyclic GMP-AMP synthase) /DncV-like nucleotidyltransferase (CD-NTase) enzymes [4], the nature of the effector proteins cover diverse enzymatic functions [5] usually promoting cell-suicide to abort the infection.
We analysed the predicted CBASS operon of the gram-positive bacteria, *Microbacterium ketosireducens* (Mke) and identified a putative CD-NTase as the sensor surrounded by at least two putative effectors, TIR-SAVED and NucC, along with an ubiquitin-like modification system. Here we purified the *M. ketosireducens* CD-NTase and TIR-SAVED and demonstrated that the cyclase produced a cyclic tri-AMP messenger promoting the formation of TIR-SAVED proteo-filament detectable in microscopy. Once activated, TIR-SAVED hydrolyses NAD+, a vital co-factor for the cell. The precise mechanism of this enzyme activation will be discussed as well as how its activation can lead to abortive infection.

[1] C. Rouillon, J. S. Athukoralage, S. Graham, S. Grüschow, et M. F. White, *Elife*, vol. 7, 02 2018, doi: 10.7554/eLife.36734.
[2] D. Cohen *et al.*, *Nature*, vol. 574, n° 7780, Art. n° 7780, oct. 2019, doi: 10.1038/s41586-019-1605-5.
[3] A. Millman, S. Melamed, G. Amitai, et R. Sorek, *Nature Microbiology*, p. 1‑ 8, août 2020, doi: 10.1038/s41564-020-0777-y.
[4] P. J. Kranzusch, *Current Opinion in Structural Biology*, vol. 59, p. 178‑ 187, déc. 2019, doi: 10.1016/j.sbi.2019.08.003.
[5] A. T. Whiteley *et al.*, *Nature*, vol. 567, n° 7747, Art. n° 7747, mars 2019, doi: 10.1038/s41586-019-0953-5.

ORAL SESSION

THERAPY AND BIOCONTROL

Evaluation of a phage cocktail to prevent and control avian colibacillosis

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Avian pathogenic *Escherichia coli* (APEC) strains are responsible for the main bacterial disease in poultry, namely colibacillosis [1]. The control of this disease relies mainly on the use of antibiotics, which eventually led to the risk of emergence of antibiotic resistance. Therapeutic alternatives are urgently needed, and phage therapy, a century-old therapy, represents a promising alternative [2]. It consists of using bacteriophages, which are natural viruses of bacteria. In the team, we have isolated and sequenced 19 different phages (belonging 9 genus) active against *E. coli*, and for some of them, also active against *Salmonella*.

One of my objectives is to determine the prophylactic and therapeutic potential of a phage cocktail, constituted of 8 phages of different genus, to prevent and control colibacillosis induced by the APEC strain BEN4358 in chicks. This strain was chosen because it was virulent in an embryo lethality model, and because 8 phages of our collection were able to lyse the strain. Four of these phages (Rec, Esco3, Esco47 and Esco58) are able to replicate on BEN4358, leading to the death of the bacterial cells. At the opposite, 4 phages (Esco9, Esco10, Esco30 and Esco41) are not able to replicate on the strain, but are able to lyse bacterial cells by the action of virion-associated enzymes.

The ability of the phage cocktail to control embryo mortality induced by BEN4358 has been demonstrated since we observed that the combination of the 8 phages allowed 90% of chicken embryos to survive an infection by BEN4358, in contrast to the control (BEN4358 only), which gave a survival rate of 0%.

Thus, we tested the efficacy of the phage cocktail to prevent and control colibacillosis induced in chicks. The APEC strain was inoculated subcutaneously at one-day-old of chicks in isolator. The phage cocktail was either inoculated *in ovo* in the allantoic fluid at 16 or 17 days of incubation, or added to drinking water that was renewed each day. Mortality was monitored during 7 days. Bacterial and phage charge were determined in blood, liver and caeca of the surviving animals after euthanasia. When phages were detected, there were identified by PCR. Phage susceptibility of the recovered bacterial clones was studied.

Whatever the route of administration of the phage cocktail, no statistically difference of reduction of mortality was observed compared to the challenged group. However, the antibiotic-treated group (METHOXASOL® antibiotic added to drinking water) allowed 85% of chicks to survive, in contrast to the challenged group which gave a survival rate of 25%. So, at the opposite of antibiotic, phages didn't have a curative effect to control colibacillosis induced by BEN4358 strain.

In the phage-treated groups (*in ovo* and in drinking water), the intestinal carriage of APEC strain was statistically decreased, allowing a level lower excretion rate in the environment. In surviving chicks, Esco3 (100%) and Rec (75 to 92%) have been found in the caeca, highlighting their persistence *in vivo*. The majority of clones tested from caeca were resistant to Esco3 (92-100%) and Esco10 (100%), but all were still susceptible to Rec. Persistence of Rec and absence of emergence of resistance for this phage is of interest. Indeed, Rec belongs the Phapecoctavirus genus, some of which are already used in human medicine [3], reinforcing his potential for therapeutic used.

[1] Ghunaim H, Abu-Madi MA, Kariyawasam S. Veterinary Microbiology. août 2014;172(1-2):13-22.

[2] Ducrot C, Fric D, Lalmanach A-C, Monnet V, Sanders P, Schouler C. INRA Prod Anim. 14 juin 2018;30(1):77-88.

[3] McCallin S, Alam Sarker S, Barretto C, Sultana S, Berger B, Huq S, et al. Virology. sept 2013;443(2):187-96.

Bacteriophage resistant clones emerging during pulmonary phage therapy are similar to those isolated *in vitro*.

Authors : **Gaborieau Baptiste**[1,2], Delattre Raphaëlle[1,2], Adiba Sandrine[3], Debarbieux Laurent[2], Ricard Jean-Damien[1]
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Context: Antibiotic resistance is a major threat to public health worldwide. Phage therapy is a promising and effective solution but is also challenged by bacteria developing resistance to bacteriophages. Resistance mechanisms have mostly been identified and studied *in vitro*, while resistance emerging during phage therapy treatments remain poorly characterized.
Objective: This study aims to identify the mutations arising in the genome of *E. coli* strain 536 following a challenge by the virulent Myoviridae 536_P1, from either a liquid culture or the lungs of mice infected and treated, and their consequences on bacterial fitness.
Methods: We first determined the bacterial resistance rate to bacteriophage 536_P1 in the different conditions tested. Next, we collected several resistant clones from each condition and purified them. Then, whole genome sequencing was performed. The fitness of these clones was evaluated in three conditions: growth in nutrient-rich liquid medium; challenge of the amoeba *D. discoideum* (mimicking macrophage phagocytosis); virulence in a murine model of acute pneumonia.
Results: The mean rate of resistance reached 44% after 4 hours of incubation in nutrient-rich liquid medium, while it took 48 hours in infected and treated mice. The resistant clones from both *in vitro* (n=18) vs. *in vivo* (n=20 after 8 hours; n=26 after 48 hours) samples exhibited mutational convergence as identical pathways, genes and sometimes mutations were found. A large proportion (75%) of clones possess at least one mutation in a gene involved in the LPS biosynthesis. Besides genes related to LPS biosynthesis (77% of all mutations), we found that 18% of mutations were located in the K15 capsule coding region. The final 5% of mutations were located in genes coding membrane proteins. Subtle to mild variations of the fitness in liquid medium was observed for of all sequenced clones with no identical mutation (n=47) compared to the WT strain. The challenge with amoeba was not discriminative enough to distinguish LPS to capsule mutant while the virulence assay in mice was as only LPS mutants lost their virulence.
Conclusions: To resist to bacteriophage 536_P1 *in vitro* or *in vivo*, the *E. coli* strain 536 modifies first cell wall components. The role of the K15 capsule in resistance remains to be determined as mutations could not determine whether mutants were lacking capsule or overproducing it. Experiments with prolonged treatments are necessary to determine if other resistance mechanisms could take place and whether their emergence would be affected by environmental conditions including biodistribution in the organ, biofilms or the immune response.

Using bacteriophage T5's capsid as a therapeutic nanoparticle

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Bacteriophage T5 capsid is an icosahedral structure 90nm in diameter. This capsid auto-assembles from a major protein which makes the faces and vertices of the icosahedron, a portal protein that forms the gate at one of the vertices for the genome transport, and a maturation protease. Following this assembly and the DNA encapsidation, the decoration protein pb10 binds onto 120 sites on the outer surface of the icosahedron. In order to decipher the mechanisms which regulate T5 capsid assembly we developed capsid assembly systems independent of the phage infection by expressing the genes encoding for the capsid proteins in *E. coli* cells. These systems allow to obtain Capsid Like Particles (CLP), which show similar characteristics as the capsids assembled during phage infection. Those CLPs can be functionalized thanks to the decoration protein properties, which can be fused to peptides or proteins of interest, thus allowing their exposition onto the capsid surface [1]. The quasi-irreversible binding of these fusion proteins is mediated by high affinity protein/protein interactions and does not require any covalent crosslinking. To assess the potential of functionalized T5 capsids as a vaccination platform, we exposed the model antigen ovalbumin fused to the decoration protein onto their surface and immunized mice [2]. This immunisation, done without any adjuvant, induced a strong immune response, equivalent to the one observed in mice immunized with the same ovalbumin dose complexed to Freund adjuvant, and far superior to the response induced with the same dose of free ovalbumin. These results suggest a natural auto-adjuvant effect of the capsids and shows the strong immunogenicity of ovalbumin multimerised onto the capsid.
By their ability to expose a "modular" decoration protein, CLPs derived from T5 phage make a powerful polyvalent platform for the development of therapeutic applications, notably for vaccination.

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PHAG-ONE: an ANR project for French academic production of therapeutic phages to fight multidrug resistant bacteria

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The **rapid and inexorable spread of antibiotic resistance** is one of the critical challenges in health care for the coming decade. Patients increasingly encounter therapeutic dead-ends, with no effective molecule. The quest for **alternatives to antibiotic therapy** is a major public health issue and should, according to the WHO, be given priority status.

Phage therapy is one of these therapeutic options. They have been used for decades in some countries in Eastern Europe, but preparations from these countries cannot be imported in Western European countries as they fail to meet the standard European health authorities. With the agreement of the ANSM health products agency, HCL was authorized to use few phage preparations (Pherecydes Pharma) in **26 compassionate treatments of BJI or endocarditis, resulting in significant clinical improvements**.

In this context, the **PHAG-ONE project** brings together microbiologic, technological, pharmaceutical, pharmacologic and clinical expertise from public/academic teams experienced in phage biology, drug production and phage therapy. The aim is to produce and apply phage batches for therapeutic use in humans and lay the bases for cohort studies and clinical trials. Beyond the scientific answers to which the project aims to provide answers, this innovative pioneering project seeks **to set up the first academic European platform able to isolate, select, characterize, purify, produce therapeutic phages and provide phage therapies at affordable cost for the French medical community as a whole**. This responds to the expectations of the ANSM expressed in its April 2019 “Temporary Specialized Scientific Committee meeting on phage therapy”. Three target species (*S. aureus*, *S. epidermidis* and *E. coli*) were chosen at this first step.

This ambitious project comprises 9 axes:

1. **Constitution of a collection of lytic bacteriophages** and investigation of the in vitro and/or in vivo, phage/bacterium co-evolution (phage training /emergence of phage resistance) to shed light on molecular mechanisms and design predictive models.
2. **Optimization and validation of phage production protocols**, including selection of bacterial strains free of prophages and main virulence factors, or (ii) genetically-modified bacteria to eliminate prophages/virulence genes or avoid their expression/production.
3. **Validation and implementation of phage purification processes and QCs** in line with Good Preparation Practices via the **FRIPHARM-HCL pharmaceutic production platform**.
4. **Modeling phage PK/PD** from in vitro and in vivo data in planktonic bacteria and biofilm.
5. **Evaluation of therapeutic phages** in *S. aureus* infection models (Tissue-cage model, PJI mode) in rabbit.
6. **Establishment of guidelines for extemporaneous magistral phage preparation**
7. **Setting up and following up compassionate care cohorts** using PHAG-ONE phages to collect pharmacovigilance data and refine indications and administration ways.
8. **Development of innovative phage imaging tools** for phage **titration**, for rapid automated standardized **phagograms**.
10. **Socio-anthropological analysis of the emergence of this biomedical innovation** including socio-cultural, economic, legal, regulatory and political aspects.

ORAL SESSION

ECOLOGY AND EVOLUTION

KEYNOTE SPEAKER

Joshua Weitz

Ecological Dynamics and Therapeutic Impacts of a Not-So-Perfect Predator

The gut environment shapes bacteriophages infection by modulating bacterial gene expression

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Bacterial and bacteriophage (phage) populations, although antagonist, coexist in the gut of mammals and remain stable over time. We hypothesised that the mechanisms underlying this coexistence may involve the regulation of bacterial gene expression required for their adaptation to the intestinal environment.
We aimed to identify a set of genes specifically over- or under-expressed in the mammalian gut that may affect phage susceptibility. To this end, we performed genome-wide RNA sequencing on a human enteroaggregative Escherichia coli isolate, to identify genes differentially expressed between in vitro (flasks) and in vivo (mice colon) conditions.
In vivo, the global gene expression pattern was found to be closer to in vitro exponentially growing E. coli cells, compared to E. coli cells that reached stationary phase growth. Virulence determinants (adhesins and aggregative fimbriae) encoded by a plasmid, and chromosomal genes involved in adaptation to the gut environment (iron acquisition, anaerobic respiration and sugar metabolism) were over-expressed in vivo, whereas genes involved in aerobic respiration were under-expressed. We also identified a set of genes potentially involved in the differential replication of phages in vivo acting on the flagellum, the LPS biosynthesis, the biofilm formation and the quorum sensing. We experimentally demonstrated that four of these differentially expressed genes (rfaL, fliA, lsrC and bssR) affected the relationships between E. coli and three virulent phages.
This work demonstrates that the gut environment, by modifying microbial gene expression, modulates phage-bacteria interactions and highlights the role of tripartite relationships between bacteriophages, bacteria and host environment in intestinal homeostasis. These results emphasize the benefit of in vivo experiments to elucidate the mechanisms affecting phages efficacy in the gut.

Coevolution and competition drive the diversification of CRISPR immunity

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Host resistance diversity reduces the emergence and the evolution of pathogens [1,2,3]. But it is unclear what drives the dynamics of this diversity. Here we studied a coevolving phage population on the diversification of bacterial/host CRISPR immunity across space and time. We find that the negative-frequency-dependent selection generated by coevolution is a powerful force that maintains host resistance diversity and selects for new resistance mutations. But we also find that host evolution is driven by asymmetries in competitive abilities among different host genotypes. The fittest host genotypes are targeted by the evolving phages but they are also the ones that acquire faster new CRISPR immunities. Our study reveals the interplay between intraspecific and interspecific interactions on the evolutionary dynamics of host resistance diversity. The recognition of the joint impact of competition and coevolution on host resistance diversification challenges coevolutionary theory and has long-term implications on the evolution of interacting species.

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Regulation of prophage induction and lysogenization by phage communication systems

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Many viruses can cause both overt (or lytic) infections, where they release viral particles, and dormant infections, where they wait for a future opportunity to reactivate [1]. The benefits of each transmission mode depend strongly on the density of susceptible hosts in the environment [2, 3, 4]. It was recently discovered that some viruses that infect bacteria (phage) use molecular signalling to respond plastically to changes in host availability [5]. Specifically, these viruses produce a signal during lytic infection, and regulate, based on the accumulating signal concentration in the environment, the probability with which they switch to causing dormant infections [5,6]. Here we present an analytical framework to examine the adaptive significance of plasticity in viral life-history traits in fluctuating environments. Our model generalizes and extends previous theory [7], predicting that host fluctuations should select not only for plasticity in entering into lysogeny, but also for plasticity in the reactivation of the virus once signal concentrations decline. We experimentally test this prediction using *Bacillus subtilis* and its phage phi3T, and demonstrate that phage indeed use the signal to make informed decisions over prophage induction. We also demonstrate that lysogens produce signalling molecules and that signal is degraded by hosts in a density-dependent manner. A decline in signal concentration therefore potentially indicates the presence of uninfected cells and triggers prophage induction. Finally, we find that conflict over the responses of lysogenization and reactivation traits to signal is resolved through the evolution of different response thresholds for each trait. Collectively, these findings deepen our understanding of the ways in which viruses use molecular communication to regulate their infection strategies, which can be leveraged to manipulate host and phage population dynamics in natural environments using synthetic communication molecules.

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« Quantitative analysis of the intestinal and blood virome in inflammatory bowel disease »

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Inflammatory bowel disease (IBD) is a complex multifactorial condition that includes ulcerative colitis (UC) and Crohn's disease (CD). A major contributing factor to the pathogenesis of IBD is the gut microbiome, which in most patients exists in a state of dysbiosis. Recently, it has been suggested that the viral component of the human gut microbiome (virome) of people suffering from IBD can also diverge significantly from that of healthy individuals and as such contribute to the state of dysbiosis. IBD is also characterized by an increased permeability of the intestinal cell lining which could lead to increased "leakage" of luminal components into the circulatory system. With this in mind, we set out to perform a whole-virome analysis of the stool and blood samples derived from a cohort of five healthy volunteers and five IBD patients (participating in the Suivitech study undertaken in Hôpital St. Antoine, Paris). The protocol for the viral DNA extraction and amplification was developed in such a way as to acquire a quantitative representation of the viromes. Interestingly, we see a blood plasma virome exhibiting a viral load of approximately 10^6-10^7 pfu/mL. Although we did not observe quantitative differences in the viral load of IBD patients and healthy individuals, neither in stool nor in blood samples, we saw a consistent increase in the overlap of blood and stool viral DNA of IBD patients compared to that of healthy volunteers, which is in line with the observed rise in gut permeability in IBD. Additionally, in both types of samples, blood and stools, an increase in the prevalence of temperate phages was also observed. In conclusion, these findings provide for the first time strong evidence for the existence of a human blood virome and for its dysregulated state in IBD patients. Such an observation opens the possibility to consider the blood virome as a driver and/or a modulator of IBD patients' inflammatory response to the microbiota.

Life and death after log: post-exponential phase infection of Bacillus subtilis by lytic bacteriophage SPP1 reveals new infection dynamics

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The growth and proliferation of most bacteria, from the open ocean to the mammalian gut, is limited by nutrient availability. It is also well established that slow-growing or nutrient deprived bacterial populations support only poor phage replication. Thus, commonplace nutrient deprivation of bacterial hosts in the environment poses a problem for phage persistence. Some phages have evolved to circumvent this problem by integrating into the host chromosome and lysogenising their host to 'weather the hard times', waiting for better environmental conditions before completing their infection cycles and lysing their hosts. However, 'strictly lytic' (non-lysogenic) phages are not capable of host-genome integration and cannot deal with host nutrient deprivation in the same manner. This poses the question of how strictly lytic phages deal with the challenge of the slow or non-growing hosts that they are likely to most often encounter. In order to start to address this rather broad question, we investigated the growth phase-dependent infection of the Gram-positive soil bacterium *Bacillus subtilis* by it's strictly lytic phage SPP1 and focussing on the interaction of SPP1 with nutrient deprived, non-growing *B. subtilis*. We report that contrary to previous understanding SPP1 is indeed capable of infecting nutrient limited non-growing *B. subtilis* cells and that the dynamics that characterise this infection are qualitatively and quantitatively different to those observed in rapidly growing cultures. We additionally explore the role of SPP1 adsorption to *B. subtilis* in governing this interaction, how the outcome is affected by nutrient addition, and finally present data supporting a previously unobserved infection strategy of SPP1. Our results add to the currently very limited understanding of phage infection under nutrient limitation and have implications for both for understanding basic phage and bacterial biology in nature and for the use of phage therapy in treating persistent, slow- or non-growing bacterial infections.

The paradoxical relationship between CRISPR-Cas and phage susceptibility in *Pseudomonas aeruginosa*

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Pseudomonas aeruginosa is an important opportunistic pathogen and our labs focus on its interactions with bacteriophages [1]. From a genomics perspective, *P. aeruginosa* presents a tremendous amount of diversity, with pairs of strains sometimes differing by thousands of genes and harboring abundant accessory genetic components. As part of our research, we use machine learning approaches to explore omics datasets to understand interactions between given pairs of strains and phages.

We present here a story that takes us from an observation gathered using such computational analysis to a concrete biological insight into the evolutionary dynamics of the bacteria, its phages, and the CRISPR-Cas defense systems.

CRISPR-Cas systems are part of the pan-immune system of *Pseudomonas aeruginosa* and have recently been shown to limit horizontal gene transfers in that species [2]. Isolates equipped with these systems tend to have smaller genomes and CRISPR spacers targeting integrative conjugative elements, phages, and plasmids.

Based on features extracted from computational models of phage infectivity in *P. aeruginosa*, we investigated the genomic effects and phenotypic consequences of CRISPR-Cas systems in that species. First, we found multiple correlations between the presence of these systems, their subtypes (I-C, I-E, and I-F), and the population structure. Second, we highlight a paradoxical, positive correlation between the presence of CRISPR-Cas systems and the chances of the host being infected by a set of distinct, strictly virulent *Pseudomonas* phages.

We propose a link between this increased phage susceptibility in the presence of CRISPR-Cas and the other accessory defense system genes. Indeed, we show that the defense systems are often found on genomic islands of *P. aeruginosa* which are depleted in the presence of CRISPR-Cas. We also propose that there could be a co-selection of antibiotic resistance and resistance to phages when thinking of potential therapeutic applications, and project how this analysis expands to other bacterial species.

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Impact of anti-CRISPR genes on the ecology and evolution of phages and bacteria

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Bacteria are under constant threat from their viruses (known as phages) which led to the evolution of a large repertoire of resistance mechanisms, amongst which 'CRISPR-Cas' is one of the most widespread. This immune system captures elements from the invader's genome (spacers) and insert them into CRISPR loci to provide sequence-specific immunity against (re)infecting phages. The modalities of spacer acquisition allow the generation of high levels of spacer-diversity within bacterial populations, which is key to limit phage epidemics and evolution of escape mutations. This CRISPR-mediated selection has likely driven the evolution of 'anti-CRISPR' (Acr) mechanisms in phage populations. Acr-phages have remarkable ecological dynamics: a first 'sacrificial' phage takes CRISPR-Cas defences down but fails to replicate, which allows a second phage to successfully multiply on this immunosuppressed host. The benefits of Acr proteins may also be shared more largely in the phage community. Indeed, the immunosuppressed bacteria generated by Acr-phage infections can be exploited by other phages that lack *acr* genes. These shared benefits depend on the 'strength' of the Acr and eventually impact the ecological dynamics of mixed phage populations. In addition to their ecological effects, Acr prevent the host from acquiring new spacers and hence inhibit the evolution of phage-resistance. Overall, the fitness benefits that CRISPR-Cas provide to bacteria, when they are exposed to predatory lytic phages, are annulled when phages encode Acr proteins. However, in the presence of parasitic (or mutualistic) lysogenic phages, CRISPR-Cas may become maladaptive owing to autoimmunity effects that result from imperfect interactions between the spacers that pre-exist in the host CRISPR array and the prophage integrated in the host genome. These fitness costs may drive the evolutionary loss of CRISPR-Cas locus from bacterial populations, unless phages carry *acr* genes. Altogether, these findings highlight that Acr may provide wide fitness benefits, not only to the phages (whether they carry *acr* genes or not) but also to the host under certain circumstances, and therefore, Acr may have important impacts on the ecology and evolution of phages and bacteria.

POSTERS

CELLULAR AND MOLECULAR INTERACTION

Functional and structural study of T5 bacteriophage immunity protein Llp

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Phage infection is triggered by host recognition thanks to the Receptor Binding Protein binding to its receptor at the surface of the cell: this interaction allows viral DNA to be delivered into the host cytoplasm. This first step of infection is followed by viral replication and eventually liberation of the new virions. During this vulnerable time, phages protect the new viral factory from over-infection. In coliphage T5 protection is mediated by a periplasmic lipoprotein, Llp[1], targeted to the inner leaflet of the outer-membrane, which binds the phage receptor FhuA [2], [3]. Llp biological function is probably also to prevent the inactivation of progeny phage by active receptors present in outer-membrane debris of lysed cells[1], thereby increasing their chances of infecting a new host. We aim to decipher the mechanisms of T5 host inhibition by Llp at the molecular level.

During my thesis I over-expressed Llp in an acylated (Ac-Llp) and soluble (Sol-Llp) form in quantities compatible with biochemical and structural studies, and solved Sol-Llp (7.5 kDa) structure by NMR: it aligns with the N-ter glycoside hydrolase domain of bacillus-phage PBC5 lysin (DALI Z-score 2.8) and a putative Salmonella lipoprotein (Z-score 2.2). I could show that Ac-Llp protects the overexpressing strain from T5 infection and I am characterizing the FhuA:Ac-Llp complex by to several biochemical and biophysical methods (phenotypic assay, nano-DSF, SPR, MST, AUC, SEC-MALS, NMR, mutagenesis): the My perspectives for the coming year is to solved the FhuA:Ac-Llp structure to understand at the molecular level the protection mechanism against phage infection (by crystallography and/or Cryo-EM).

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Deciphering the host response and transcriptome reprogramming during infection of *Streptococcus pyogenes* by a lytic phage

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Antimicrobial resistance is increasing worldwide and threatens humanity. The recent resurgence of phages research could be the key to face the rise of superbugs. The evolutionary history of phages with their hosts has led to intricate hijacking mechanisms and their dissection may lead to the identification of new antibiotics targets [1,2]. Additionally, phage therapy implementation is impeded by the weak knowledge we have about their lifecycles and their impact on the pathophysiology of their bacterial host [3]. In the laboratory, we are mainly focused on deciphering the virulence of *Streptococcus pyogenes* and on vaccine development. This pathogen is responsible for diseases ranging from mild pharyngitis to life-threatening infections like necrotizing fasciitis. The worldwide burden of GAS infection is estimated to more than 700 million cases and 50 000 deaths a year [4]. The complexity of GAS epidemiology (emm-types diversity and their worlwide distribution) is slowing down the development of vaccines [5]. Therefore, the treatment of GAS infection exclusively relies on antibiotics use like penicillin. Although no resistant strains have emerged, allergy to β-lactams and therapy failure (up to 40% in some regions) force to choose other antibiotics like MLS for which resistant clones have emerged [6]. In an effort to find alternatives, we decided to revisit the old model system *Streptococcus pyogenes* - bacteriophage A25 [7] with omics technologies. Recently, the sequencing of the phage A25 genome revealed a temperate phage which became strictly lytic by escaping lysogeny, *i.e.* integration as a prophage in the host genome, through the losses of the lytic cycle repressor *cI* and the integrase *int* genes [8]. We sequenced the genome of the susceptible M25 strain and set up infection with phage A25. Using RNAseq, we found that phage A25 transcriptional program is a classical 3 steps process with the transcription of (i) early genes involved in phage genome replication and host hijacking, (ii) middle genes involved in capsid formation and genome packaging and (iii) late genes involved in tail formation and bacterial lysis. Considering its bacterial host, we found a reprogramming of up to 27% of the transcriptome, turning *S. pyogenes* into a virocell to sustain phages production. To discriminate the host response to initial infection steps from the hijacking of its transcriptional program, we expressed the repressor *cI in trans* and determined transcriptome changes. We found that the expression of genes involved in ribosome assembly, carbohydrate and lipid metabolisms are differentially expressed due to phage activity. We are currently looking for phage products and host targets involved in these changes to seek for new antibiotics targets and to better understand the different mechanisms by which lytic phages wipe out their host.

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What's up about phages at Langevin institute?

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At the Institut Langevin, ESPCI Paris, we have developed a new, sensitive, interferometric, and non-destructive optical approach to detect label-free single nanoparticles (NPs) that allows us to count and sort different types of virus (Boccara et al, Biomed. Opt. Exp. 2016). In short, we measure the interference between the illuminating light and the light scattered by a nanoparticle; this measurement is complemented with single particle tracking analysis of their Brownian motion in order to determine the NPs size and nature, allowing us to discriminate objects of the same size like for example virus and membrane vesicles. We will present new developments and applications of this technique.

Coupling interferometric scattering microscopy with fluorescence imaging
We developed a multimodal microscopy system that combines the advantages of both, common-path interferometry and fluorescence microscopy. We labeled phages with sybergold and were able to identify, quantify, and characterize phages and viruses from 30nm to 150nm. We were able to identify viruses and vesicles in mixtures of phages and vesicles (phages 1%). We also challenged our set up with more complex samples from aquatic environments or stools.

Label-free interferometric detection and characterization of single virus by single-particle tracking analysis: application to antibodies recognition
We have developed an assay based on antibody recognition of targeted virus in which we associate changes in diffusion to antibody recognition and possibly aggregation. We have applied this approach to different bacteriophages (T5 and SPP1), with antibodies targeting different surface proteins, using purified IgGs or serum. In all cases, we have observed a significant change in diffusion, an increase in signal at the time mark of around one minute, and a decrease of number of detected particles at longer time scales indication aggregation (tens of minutes), validating thus our assay as a tool for rapid virus identification or to highlight antibody-antigen interactions.

Dynamic cell imaging (DCI) a method to analyze phage infected bacteria
Our aim is to try to detect "viral factories" ("organelles") within bacteria without using fluorescence. DCI principles rely on the light scattering by subcellular structures and the fact that the scattered light interferes with the illumination beam either constructively or destructively depending on whether the scatterer is placed after or before the focus respectively (known as the Gouy's phase shift). Our aim is to do time-lapse movies of phage infection and to monitor the dynamics of "organelles" with imageJ.

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Bacteriophage M13 for phage display: selection of highly specific interaction modules and their applications

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Phage display is a laboratory technique that enables the selection of peptides or proteins with a high afinity for a target. We use a library of phages derived from bacteriophage M13. Although often called a "phage library", constructs are phagemids that are unable to reproduce without the addition of a helper phage. A random genetic sequence is inserted into a phage coat protein gene, causing the phage to “display” the corresponding random peptide or protein on its envelope. We thus establish a connection between genotype (the gene) and phenotype (the protein). The peptide or antibody, fused with the coat protein pIII of the phage, is exposed at the surface of the phage. After three to five rounds of selection / amplification, an enriched library of phages with a high affinity for the target is obtained. Most of the libraries are composed of scFv recombinant antibodies. ScFv consist of only the light chain (VL) and heavy chain (VH) variable regions of immunoglobulins connected by a peptide linker. It is thus possible to screen large recombinant antibody libraries of human origin, mimicking human immune repertoires, in order to generate antibodies to target molecules for fundamental, diagnostic and therapeutic applications. For example, we have generated antibodies that specifically recognize a particular topology of the DNA G-quadruplex found in the telomers of eucaryotic chromosomes (collaboration with Pr Eric Defrancq, Département Chimie Moléculaire at Grenoble). This structure is thought to be important in the development of cancers. Moreover, phage display has been successfully applied to the generation of peptides as molecular tools. In an other collaboration (with Yanxia HOU, Systèmes Moléculaires et nanoMatériaux pour l'Énergie et la Santé at Grenoble), we are screening peptides as biological sensors of Volatile Organic Compounds to be used as key components of a new generation of electronic noses.

AppY, a transcriptional regulator from phage origin, provides multiple benefits to *Escherichia coli*

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Bacterial genome diversity is largely due to prophages, which are viral genomes integrated into the ones of bacteria [1]. Most prophage genes are silent, but those that are expressed can provide new properties to their host [2]. In *E. coli* K-12, that carry 9 defectives prophages in its genome, the impact of many of these viral genes on their host remains to be determined. Some of these genes code for transcriptional regulators. These regulators are poorly studied but *appY*, encoded on the DLP12 prophage, has been shown to regulate the expression of bacterial genes [3]. Indeed, *appY* encodes a transcriptional regulator belonging to the AraC/XylS family with two known bacterial targets: the *hya* operon coding for the hydrogenase 1 and the the *cbd* operon coding for the cytochrome bd-II oxidase [4]. Moreover, another study suggested that AppY affects the level of more than 30 proteins without identifying them [5]. **The purpose of our study is to identify bacterial processes under AppY's control.**

By performing RNA-Seq experiments, we have shown that AppY production modulates the expression of more than 200 genes involved in stress adaptation. Using a ChIP-Seq approach, we identified 13 of them as direct AppY targets. AppY regulates directly and positively *nhaR* and *gadY*, 2 genes involved in biofilm formation, but also several genes involved in acid stress response including the master regulator gene *gadE*. Moreover, AppY indirectly and negatively impacts mobility by favouring the degradation of FlhDC, the master regulator of the flagella biosynthesis. As a consequence of these regulatory effects, overproduction of AppY in the cell increased **biofilm formation**, **acid stress resistance** and led to a strong defect in **motility**.

Across our research, AppY has emerged as a **central regulator** in the cell that controls the expression of bacterial master regulators in order to provide benefits to its host under stress conditions. Many genes from prophages are not characterized yet and their studies will allow to understand the mechanisms by which bacterial fitness is increased as well as the co-evolution between bacterial viruses and their host.

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Discovery of novel antiviral compounds produced by prokaryotic viperins

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Bacteria live under the constant threat of viruses. To fight off phage infections, they have developed a variety of defence systems. Recently, a new type of anti-phage defence system was discovered: chemical defence systems, which produce small antiviral compounds. These naturally produced antiviral compounds could be a pharmaceutical gold mine for future drug development.

Prokaryotic Viperins (pVips) are a group of bacterial and archaeal enzymes with anti-phage properties. Through their catalytic activity, they produce modified nucleotides that inhibit phage replication. However, five pVips protect bacteria against phage infection, although they do not seem to produce modified nucleotides. These pVips could produce different types of anti-phage compounds.

We analysed the cell lysate content of pVips-expressing *E. coli* using LCMS (Liquid Chromatography combined with Mass Spectrometry). We found out that three of the five pVips for which no antiviral product was identified do in fact produce modified nucleotides. Moreover, we found 11 compounds potentially produced by pVips during phage infection. Further study is needed to determine the antiviral activity of these compounds. Our study could be a step toward understanding new antiviral mechanisms behind pVips anti-phage activity.

Key words: Antivirals, Phages, Viperins, Defence systems

Characterization of phagic recombination proteins and their impact on *E. coli* LF82 pathogenicity

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Escherichia coli LF82 is an adherent-invasive pathogen (AIEC) frequently found in the gut microbiota from Crohn's disease patients. This bacterium is able to survive and multiply itself in macrophages, where it sustains the gut inflammation.

In order to see if prophages could be involved in the physiology of *E. coli* LF82, we started the characterization of the five prophages identified in this strain [1]. We shew that one of them, a P22 homologue called Gally that is strongly associated with *E. coli* strains from Crohn's disease patients [2], is produced in high quantity in *E. coli* LF82 virome ($7{,}3.10^8$ phages/mL) and is able to package virulence genes from *E. coli* LF82 thanks to lateral transduction. We also noticed that Gally and two other LF82 phages, Perceval and Cyrano, are overproduced in presence of ciprofloxacin, drug that is known to induce SOS response. *E. coli* LF82 bacteria are subjected to the same kind of stress in macrophages [3], but interestingly these phages are not detected in macrophage lysates after *E. coli* LF82 infection.

Focusing on the Gally prophage, we observed that some of its genes seem to be important for the survival of its host in macrophage [4]. One of them was annotated *gam* thanks to the homology of its related protein with the NHEJ phage protein (Ku vir) Gam from Mu [5]. However, after PHROG analysis [6] we suspect that there are several Ku vir categories, distinguished thanks to their genetic neighborhood : one that includes Gam-Gally, surrounded by genes expressing SSA proteins, and another with Gam-Mu, for which the genetic environment does not include this kind of proteins. *In vivo* experiments seem to confirm that Gam-Gally is not implied in NHEJ repair. Moreover, identification of Gam-Gally partners with Tap-Tag allowed us to isolate RecT-Gally protein that is expressed from the neighbor gene of *gam*. As *gam*, *recT* is overexpressed when *E. coli* LF82 infect macrophages [4]. We shew that RecT-Gally is able to do single-strand annealing (SSA) *in vivo* on single-stranded DNA. However, with a double-stranded DNA (dsDNA) substrate, the presence of Gam-Gally is necessary for the SSA activity mediated by RecT. Gam-Mu is not able to do SSA with RecT-Gally on dsDNA, confirming that Gam-Gally and Gam-Mu are implied in different cellular processes.

The study of *recT-gam* genes deletion indicates that they are implied in the production of Gally virions (÷3 in *E. coli* LF82 deleted). But this phagic mechanism of DNA repair could also be implied in the pathogenicity of *E. coli* LF82 by providing a gain-of-function to its bacterial host during macrophage infection.

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Treatment of intestinal infections with bacteriophages: setting up a model with gnotobiotic OMM[12] mice as host and *Citrobacter rodentium* as pathogen

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Gnotobiotic OMM[12] mice are colonized by a synthetic community of 12 commensal murine strains. This model is increasingly being used to decipher the role of individual components of the intestinal microbiota including bacteria and bacteriophages. Here we aimed i) to characterize the intestinal infection initiated by the murine pathogen *Citrobacter rodentium*, which displays similarities with enterohaemorrhagic and enteropathogenic *Escherichia coli*, and ii) to assess the efficacy of virulent phages to control this intestinal infection.

We found that as low as 10 cells of *C. rodentium* were sufficient to initiate an intestinal colonization reaching 10^8 CFU/g of feces within 4 days. Ten days after the introduction of *C. rodentium*, the level of Lipocalin-2, a marker of inflammation, strongly increased. Histological examination of gut sections confirmed the colonic crypt hyperplasia and the increase of KI-67 positive cells 10 days post-infection. 20 days post-infection these markers return to levels similar to those of uninfected mice.

Future experiments will include the administration of virulent phages, single phages and cocktail, which are currently being characterized.

Visualizing phage - bacteria Interactions on a population and single cell level

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In an era of unprecedented emergence of antibiotic resistant bacteria, alternative therapeutics are being urgently sought. There is a renewed interest in phage - viruses that replicate within and lyse bacteria cells - therapy to combat pathogenic infections. Phage therapy has been demonstrated to be effective against bacterial infection. However, a better understanding of phage-host interactions and how they are affected by the environment is still needed in order to improve the prospects of future treatments.
We investigate how spatial barriers effect the rate of phage attachment to host cells using the *Pseudomonas aeruginosa* pathogen. We have developed a system to track infection events both on population and single cells levels using fluorescent and bioluminescent markers. Bacteria used for these experiments express the fluorescent protein GFP. We can also detect infection events through a nano luciferase reporter gene system, in which the nanoLUC gene has been introduced into the genome of a phage. When the genome of the phage enters the host cell, the luciferase gene is expressed, and the enzyme is produced, it breaks down its substrate to create a bioluminescent signal that can be detected. This allowed us to record two indications of infection : the disappearance of the fluorescent signal when cells die upon phage infection, and the appearance of the bioluminescent signal indicating that the cells are being infected by the phage. We have shown that we can detect luminescence from different bacteria infected by the luciferase producing phage in liquid culture using a microplate reader. We also detected infection events on a single cell level using a high throughput confocal microscope.

POSTERS

THERAPY AND BIOCONTROL

Towards a phage therapy against multi-drug resistant *Klebsiella pneumoniae*

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The misuse of antibiotics has led to the emergence of multi-drug resistant (MDR) bacteria, being a major global threat [1]. The World Health Organization has drawn up a list of multidrug-resistant pathogenic bacteria, among which are critical nosocomial pathogens that can cause serious and often lethal infections, such as *Klebsiella pneumoniae*. *K. pneumoniae* MDR strains are an outstanding pathogen in need of alternative solutions. Phages, viruses of bacteria, have been proposed as promising biomedical tools [2], and this project has focused on the isolation of novel lytic phages from the environment against all reference capsular types of *K. pneumoniae*, including high-risk clinical clones [3-4]. We have isolated and characterized (phenotypically and genomically) more than one hundred *Klebsiella* phages. Our results are encouraging and provide a battery of phages against pathogenic *K. pneumoniae* MDR that could be used in biomedicine.

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Isolation and characterisation of virulent phages against European *Xylella fastidiosa* subspecies

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Xylella fastidiosa (*Xf)* is ranked among the top 10 most important bacterial plant pathogens in the world [1]. This bacterium colonizes the xylem vessels of plants and forms biofilms that block water flow, resulting in plant wilting and eventually death [2]. *Xf* is transmitted by biting-sucking insects such as leafhoppers or meadow spittlebugs in Europe but also, on a larger scale, by the trading of contaminated plants. To date, the host range of *X. fastidiosa* is estimated to be over 638 plant species belonging to 87 botanical families [3], and new host plants are discovered regularly.

Native to Americas, *Xf* has invaded many regions including Asia, the Middle East and Europe [4]. The most spread and damaging subspecies (*Xf fastidiosa*, *Xf pauca*, and *Xf multiplex*) are present in many countries and infect a large variety of plant species including vine, olive, fruit trees (*Prunus spp*.), citrus, coffee, oak, and ornamentals, therefore provoking a huge economic impact on agriculture worldwide. *Xf* is listed as a quarantine pest in Europe [3] and its potential economic, environmental and social impact are considered the most serious in the Union [5]. Since 2013, *Xfp* has been formally identified for the first time in Europe on olive trees in the Apulia region of Italy [6]. To date, 18 900 hectares are affected by the disease, which represents 2.1 million potentially infected olive trees, most of them centenarians. In Spain, the situation quickly deteriorated with infection clusters on olive and almond trees, and in France the first cases of heavy infection have been reported on olive trees in the PACA region. Since 2017, Corsica is entirely considered an infected area with regard to *Xfm* strain.

No curative or prophylactic technique exists against this bacterium and the current control measures are destructive leading to the destruction of all trees surrounding an infected tree. It is therefore of global importance to find new and sustainable means of biocontrol against this disease. Phage biocontrol is one of the most promising method to fight this pathogen and our objective is to develop a biocontrol product targeting Mediterranean strains of *Xf*. For this, *Xf*-associated (plant and soil samples in contaminated areas) and non-*Xf*-associated (water sample from Marseille) environments were tested for the presence of bacteriophages efficient on *Xf* strains.
To bypass the difficulties of *in vitro* experimentation on *Xf*, a surrogate host strategy on *Xanthomonas albilineans* was implemented to isolate bacteriophages whose efficacy is then tested on a range of hosts. After three years of research, three phages were selected as potential candidates [7]. These results led to further research, isolation and characterization of new phages. To date, we have about ten characterized phages having at least efficiency on *Xfm* of which, some being also efficient on *Xff*.

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PHAGE*in*LYON: caractérisation et développement des process de production de dix-sept bactériophages anti-*Staphylococcus*

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Introduction. Les bactériophages, virus spécifiques des bactéries, constituent une stratégie thérapeutique prometteuse pour lutter contre les bactéries résistantes aux antibiotiques et/ou formant du biofilm, comme les staphylocoques. *Staphylococcus aureus* est en effet un pathogène majeur responsable de multiples formes d'infections sévères et fait partie des bactéries ESKAPE définies par l'OMS comme cibles prioritaires pour la lutte contre l'antibiorésistance. Les staphylocoques à coagulase négative sont quant à eux des pathogènes fréquemment multirésistants aux antibiotiques et très prévalents dans les infections chroniques comme les infections ostéo-articulaires (IOA). Dans ce contexte, le programme PHAGE*in*LYON a pour objectif de développer une production de phages thérapeutiques répondant à la volonté de l'ANSM d'encourager le développement d'une plateforme académique nationale de phagothérapie. Nous rapportons ici l'isolement et la caractérisation de 17 nouveaux phages anti-*Staphylococcus* ainsi que l'évaluation de leur activité sur une collection de souches cliniques de *S. aureus* (SA) et de staphylocoques à coagulase négative (SCN) pour évaluer leur potentiel thérapeutique.

Méthodes. Dix-sept phages ont été isolés à partir d'échantillons d'eaux usées. Leur identification a été obtenue par séquençage complet de leur génome par la technique Illumina. Pour évaluer leur spectre d'activité, 30 souches cliniques de SA génétiquement caractérisées et représentatives des fonds génétiques les plus prévalents ainsi que 33 souches appartenant à 7 espèces de SCN responsables d'IOA ont été testées. La technique du spot test, basée sur la détermination du ratio Efficiency Of Plating, a été utilisée (EOP, avec un seuil de 10^{-3} pour retenir une efficacité du phage). Nous avons par ailleurs optimisé les conditions expérimentales des protocoles permettant la production des phages d'intérêt thérapeutique avec i) des rendements importants compatibles avec une utilisation en clinique (>10^{10} PFU/mL) et ii) grâce à des souches bactériennes ne produisant pas de facteurs de virulence majeurs et dont le génôme ne contient pas de prophages, sources potentielles de contamination des phages thérapeutiques lytiques.

Résultats. Tous les phages isolés appartiennent à la famille des *Myoviridae* dont respectivement 14/17 et 3/17 aux genres *Kayvirus* et *Silviavirus*. Les phages *Silviavirus* étaient plus actifs sur les souches de SA (EOP>0,001 pour 73 à 90% des souches) que les phages *Kayvirus* (EOP>0,001 pour 13 à 70% des souches). Une action concomitante sur 80% des souches testées a été mise en évidence en combinant chacun des phages au spectre le plus large au sein de chaque genre. Il est à noter que les phages *Kayvirus* avaient une activité étendue à plusieurs espèces de SCN (47% des souches de SCN testées pour le phage avec le spectre le plus large), notamment *S. lugdunensis*, *S. capitis* et *S. caprae*, alors que les phages *Silviavirus* étaient seulement actifs sur 6 à 12% des souches testées. Deux souches de production candidates permettant d'obtenir les pré-requis et les rendements attendus ont été identifiées.

Conclusion. Nous rapportons la caractérisation d'une large collection de nouveaux phages appartenant à deux genres différents présentant des spectres complémentaires contre une collection

de souches de SA et SCN. Les travaux ultérieurs se concentrent actuellement i) sur l'isolement de phages anti-*S. epidermidis*, espèce peu sensible aux phages décrits alors qu'elle constitue un pathogène majeur dans le contexte des IOA, ii) au développement des protocoles de purification et de contrôles de ces phages afin de répondre aux exigences de l'ANSM pour une future administration chez l'homme.

ABSTRACT

Towards customized bacteriophage cocktails: study of phage-bacteria interactions of the genus *Escherichia*

The development of alternatives to antibiotics has become a crucial issue for the World Health Organization (WHO). Phage therapy is an interesting alternative, but its effectiveness remains controversial.

The aim of this work is to study phage-bacteria interactions at the species level on the *Escherichia coli* model, causal agent of multiple infections and particularly involved in bacterial resistance to antibiotics.

Our hypothesis is that the anti-phage systems plays a role in bacterial resistance to phages and would be an obstacle to phage therapy.

To test this hypothesis, we set out to study phage-bacteria interactions of collections representative of the species *Escherichia coli*. We established a collection of 187 phages. We then used it to generate a phenotypic interaction matrix against a collection of 370 strains representative of the genus *Escherichia*. In parallel, a program to detect antiviral defense systems within bacterial genomes is under developmentwas used to characterize the collection. These genomic results could provide answers insights on how to our understand the diverse phage-bacteria interactionsphenotypic observations.

Preliminary results, on 98 bacteria, suggest on the one hand that the high specificity of phages does not seem to be related to phylogenetic distance at the species level. On the other hand, despite its ability to adsorb to a host, phage infection leading to cell lysis and release of new virions can be prevented. The role of defense systems, in view of our first statistical analyses and recent work on the subject, is very strongly suspected.

Overall, our first results show that several defense systems are significantly associated with a bacterial resistance profile.

These results require extensive validation work and could pave the way for the development of targeted antibacterial therapies.

Phage genome annotation: is this phage actually safe for therapeutic use?

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In-depth study of phages genomic properties is one of the main steps to assess their safety for therapeutic use [1]. This process enables detection of genes that make phages potentially harmful for the subject of the therapy or the environment, such as antibiotic resistance, lysogeny, and pathogenicity genes. Given phages are bacterial viruses, genome annotation is traditionally performed using bioinformatic tools that are designed for bacterial genomes [2]. However, the different genome structure of phages and lack of phage gene entries in annotation databases yields poor gene calling performance and poor gene function annotation of phage genomes. In most of recent scientific literature describing promising phages for therapy, only 20-30% of the genes were successfully given a function, which implies that dangerous genes could be present in the 70-80% of the genes of unknown function. Taking the example of Vibrio vulnificus phage VVP001 [3], this poster describes how combining multiple manual and automatic approaches for structural and functional annotation improved the results of genome annotation. The number of detected genes was increased by 24% and 73% of the genes' function were predicted, yielding 5 times more information on the phage than the original analysis. This powerful analysis method therefore allows a finer study of phages, which is essential to prove their harmlessness as a medicine.

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Isolation and characterization of a novel JUMBO phage active against the emerging ST307 carbapenemase-producing *K. pneumoniae* clone

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5. CNR résistance aux antibiotiques

Introduction. The emergence of carbapenemase-producing Enterobacteriaceae, especially *Klebsiella pneumoniae* (KP), is a significant health concern across the world. This major burden has been related to the dissemination of few successful high-risk clones, such as the emerging KP ST307 multi-drug resistant clone. Bacteriophages, viruses specific of bacteria, are promising alternative antibacterial agents to fight against antimicrobial resistance. In this study, we report the first description of a lytic phage active against KP ST307 isolates.

Methods. Bacteriophage V1KP1 was isolated from a wastewater sample using a ST307 clinical strain. Whole genome Illumina sequencing was used for phage identification using miniKraken and genome annotation using PATRIC 3.6.9. To assess its activity spectrum, we tested 30 strains identified as ST307 based on multilocus sequence typing and 52 strains belonging to 27 other STs. The 82 strains produced various types of carbapenemases (OXA48-like,n=53; NDM,n=16; KPC,n=11; VIM,n=2). The spot test assay based on the determination of the Efficiency Of Plating ratio (EOP), ([phage titer on a test strain]/ [phage titer on a reference strain]) was used to determine the lytic activity. As capsular type has been reported to be a determinant of the high specificity of anti-KP phages, we determined the *wzi* allele (predictive of the capsular type) of all strains.

Results. Phage V1KP1 has a 346,057-bp genome and thus belongs to a unique group of phages, known as “JUMBO” phages. Sequence analysis showed that it belonged to the *Myoviridae* family and the *Alcyoneusvirus* genus. We identified 626 predicted protein-coding genes, covering 94.5% of the genome (Figure 1). The phage is active on 24/30 ST307 isolates, irrespectively of the type of carbapenemase harboured, with high EOP scores (>0.1) but on only 5/52 non-ST307 isolates (3/3 ST16, 1/1 ST20, 1/1 ST327). The results of molecular screening for the *wzi* allele highlighted that capsular type is not the only factor predictive of phage activity as all ST307 isolates carried the *wzi*173 allele but were not all susceptible to V1KP1 while non-ST307 isolates susceptible to V1KP1 presented highly variable alleles (Figure 2).

Conclusion. Phage V1KP1 is a JUMBO phage with promising activity on clinical KP isolates especially carbapenemase producers, including those belonging to the worrisome multi-drug resistant ST307 clone.

Figure 1. Circular genomic map of bacteriophage V1KP1. Proteins with predicted or unknown functions are marked in green or blue respectively. The map was generated using CG viewer.

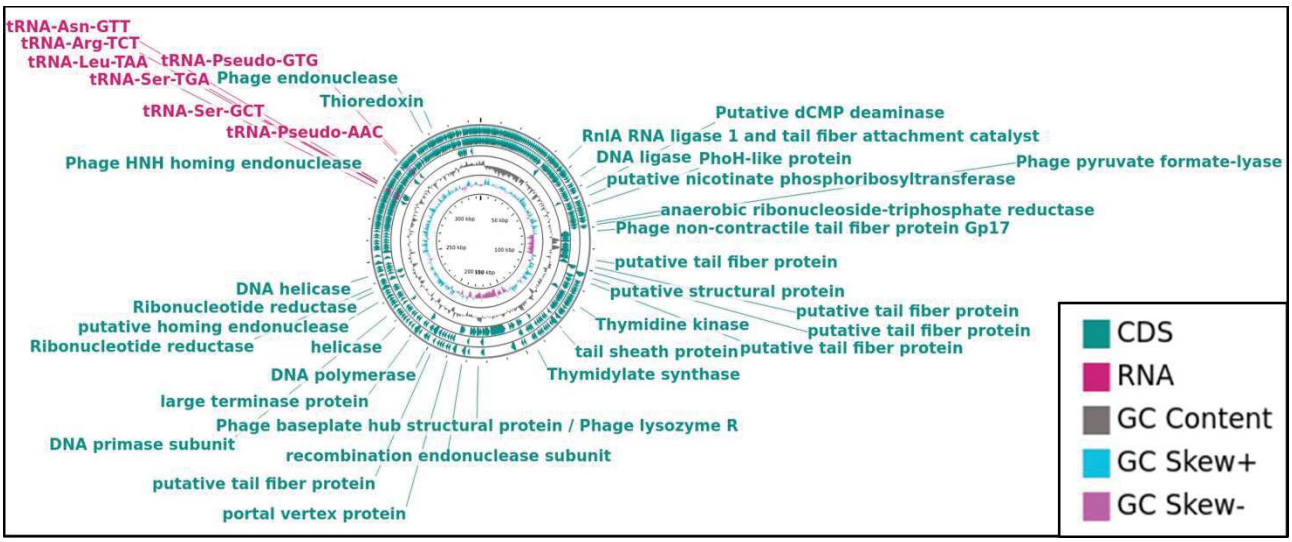
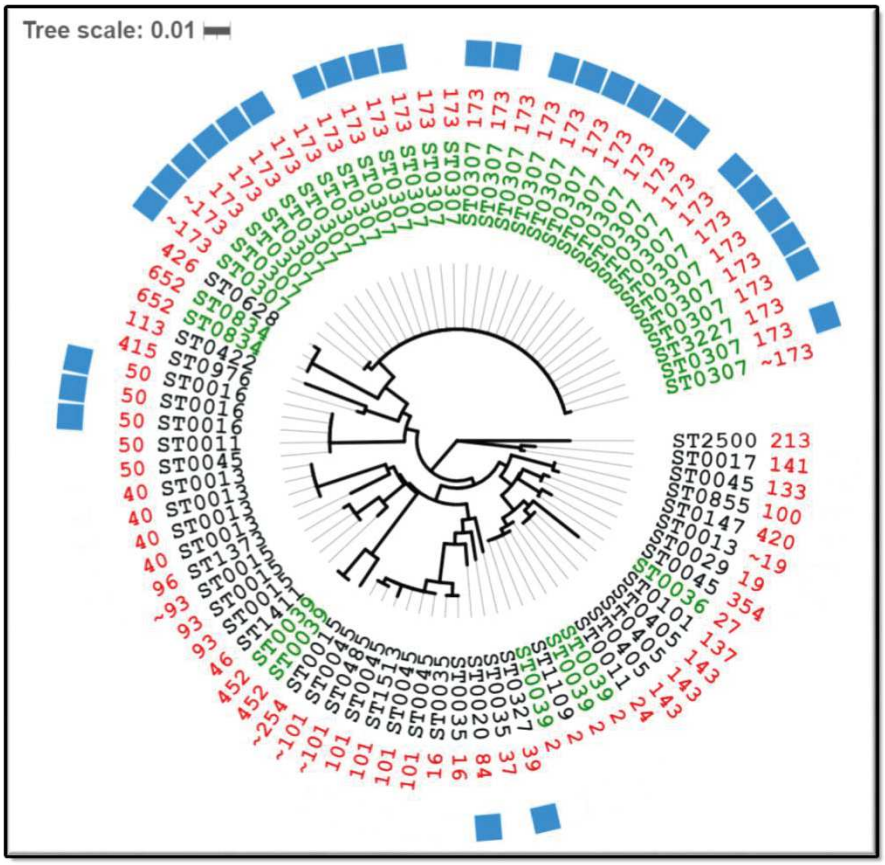


Figure 2. Phage V1KP1 host range according to ST and *wzi* allele sequence. Phylogenetic tree built based on *wzi* gene sequences.
First layer: green = ST307 strains; Black = others
Middle layer: WZI allele type; ~ = closest *wzi* allele
External layer: V1KP1 activity (blue square=EOP>0,1 ; empty=no or weak lysis)



Towards a phage therapy against multi-drug resistant *Klebsiella pneumoniae*

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The misuse of antibiotics has led to the emergence of multi-drug resistant (MDR) bacteria, being a major global threat [1]. The World Health Organization has drawn up a list of multidrug-resistant pathogenic bacteria, among which are critical nosocomial pathogens that can cause serious and often lethal infections, such as *Klebsiella pneumoniae*. *K. pneumoniae* MDR strains are an outstanding pathogen in need of alternative solutions. Phages, viruses of bacteria, have been proposed as promising biomedical tools [2], and this project has focused on the isolation of novel lytic phages from the environment against all reference capsular types of *K. pneumoniae*, including high-risk clinical clones [3-4]. We have isolated and characterized (phenotypically and genomically) more than one hundred *Klebsiella* phages. Our results are encouraging and provide a battery of phages against pathogenic *K. pneumoniae* MDR that could be used in biomedicine.

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Production de phages thérapeutiques en conditions « Low-Tech »

Étude de faisabilité sur *Staphylococcus epidermidis*

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Les maladies infectieuses provoquées par des bactéries résistantes aux antibiotiques pourraient tuer plus que le cancer dans le monde à l'horizon 2050. La thérapie phagique semble être une alternative ou un complément crédible aux antibiotiques et présente l'avantage d'être techniquement relativement peu coûteuse. Il est donc nécessaire et possible de démocratiser la recherche sur les bactériophages afin d'obtenir des phages provenant de la plus grande diversité de localité et d'environnements.

Notre étude consiste à référencer et tester expérimentalement les modes « Low-Tech » et « Opensource » d'isolement et de production de phages avec des moyens techniques simples et peu coûteux. Les populations à faibles revenus pourraient ainsi se saisir de cette méthode thérapeutique. Nous avons ainsi cherché à appliquer cette démarche sur les bactériophages de *Staphylococcus epidermidis*. Cette espèce bactérienne très commune a longtemps été considérée comme inoffensive, mais, depuis 1980 elle est classée comme un pathogène opportuniste. Elle est responsable d'infections cutanées, nasales ou urinaires et est redoutée dans le milieu hospitalier, car elle forme facilement des biofilms à la surface des cathéters ou des implants médicaux (Peerayeh et al., 2016) , (Otto, 2009). Elle ne produit pas de toxine mais possède des facteurs de virulence et peut servir de réservoir de gènes de résistance pour sa cousine S.aureus. Elle est souvent résistante à une grande variété d'antibiotiques, y compris la pénicilline et la méticilline.

Pour arriver à produire des bactériophages, il convient (i) d'isoler et identifier la souche bactérienne pathogène, (ii) d'isoler des phages infectant cette souche de manière efficace et (iii) de produire en forte concentration une solution de bactériophages dans des conditions sanitaires satisfaisantes. Nous présenterons dans notre poster les moyens « low-techs » qui nous ont semblé les mieux à même de répondre à ces trois étapes.

DefenseFinder : a systematic detection tool for anti-phages defense system of prokaryotes

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Phage therapy could be a solution against antibiotic resistance. However, the mechanisms governing the infection of bacteria by phages are still not well understood. The first anti-phage defense systems were discovered in bacteria in the 50s. For the following 70 years, only a few types of anti-phage mechanisms (Abi and CRISPR-Cas) were uncovered and thought to constitute the anti-viral arsenal of bacteria. However, in the last five years, dozens of systems have been discovered fundamentally changing our understanding of how bacteria protect themselves against viruses. However, little is known about the global distribution of anti-phage systems in prokaryotic genomes. We created DefenseFinder, a tool for the systematic detection of all known anti-phage defense systems. DefenseFinder is based on MacSyFinder and uses HMM detection to detect all the defense systems. This tool, DefenseFinder, allows the detection of 60 different defense systems. Our tool is already available online and as a command line interface. Here, we will present our tool and several of its applications.

Synergistic anti-biofilm activity of a recombinant phage lysin (Exebacase), alone or in association with rifampin, vancomycin or daptomycin, against *Staphylococcus epidermidis* strains responsible for bone and joint infections

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Background

Staphylococcus epidermidis (*S. epidermidis*) is one of the main pathogens responsible for bone and joint infections especially those involving prosthetic materials (PJI). Although less virulent than *S. aureus, S. epidermidis* is involved in chronic infections notably due to its ability to form biofilm. Moreover, *S. epidermidis* is frequently multiresistant to antibiotics. In this context, the development of additional or alternative antibacterial therapies targeting the biofilm is a priority.

Methods

The aim of this study was to evaluate *in vitro* the activity of lysin exebacase (CF-301) against biofilms formed by 19 *S. epidermidis* clinical strains responsible for PJI. We determined the remaining viable bacteria inside the biofilm and the biomass (bacteria and extracellular matrix) after 24h of exposition to exebacase at different concentrations, alone or in combination with antibiotics commonly used to treat multi-resistant *S. epidermidis* PJI (rifampin, vancomycin and daptomycin). In this study, synergy was defined as a significantly higher effect of the association in comparison to the sum of the effect of each molecule.

Results

Exebacase showed a dose-dependent reduction of biomass, ranging from 11 % at 0.5 mg/L to 66 % at 150 mg/L. Exebacase showed a significant bactericidal activity at 50 and 150 mg/l, with a mean decrease of the inoculum of 0.94 and 1.7 log, respectively. In addition, synergistic effects were observed in association with i) rifampin (1 mg/L) showing a mean decrease up to 84% of the biomass and 3.5 log CFU at 150 mg/L of exebacase, ii) vancomycin (10 mg/L) showing a mean decrease up to 81% of the biomass and 2.82 log CFU at 150 mg/L of exebacase, iii) and daptomycin (10 mg/L) showing a mean decrease up to 85% of the biomass and 3.1 log CFU at 150 mg/L of exebacase.

Conclusion

Exebacase showed *in vitro* synergistic activity with antibiotics against *S. epidermidis* biofilms. It is a promising adjuvant therapy to rifampin, vancomycin and daptomycin in the context of PJI. Further in vitro studies are needed to understand the mechanism of action on *S. epidermidis* biofilm and the heterogeneity of strain behavior and *in vivo* to confirm the present data.

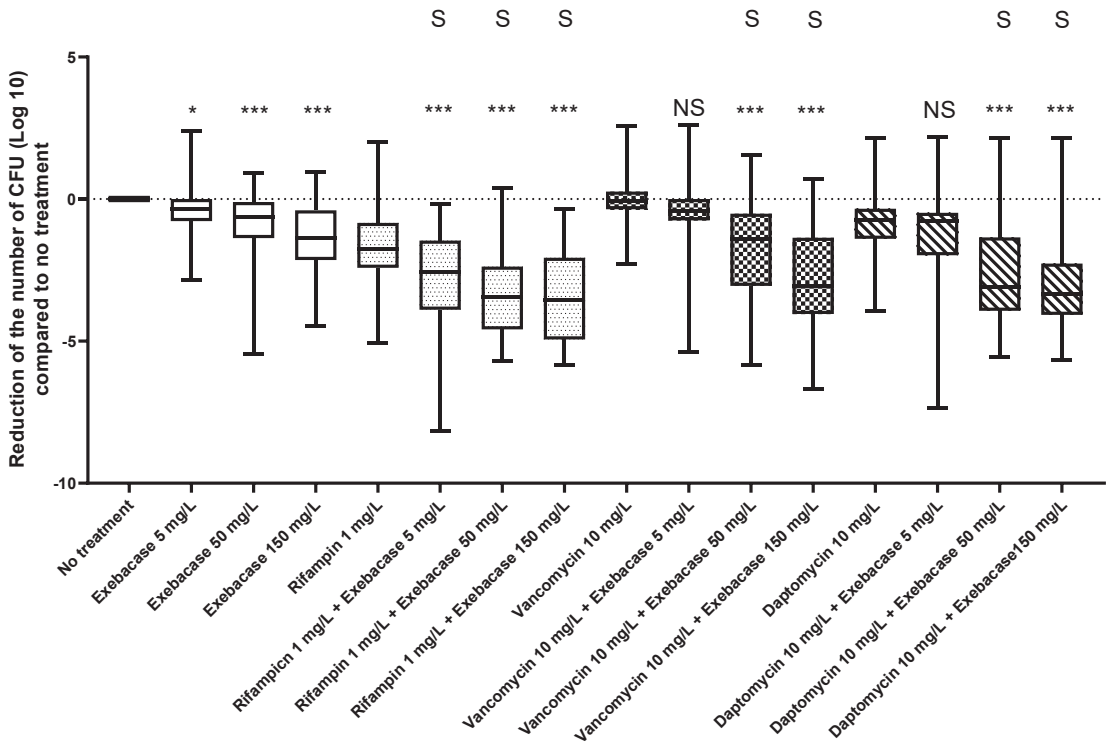

Figure 1 : Associations of Exebacase with antibiotics antibiofilm activity in comparison to Exebacase and antibiotics alone, on *S. epidermidis* preformed biofilm. Living bacteria inside biofilm after 24 hours Exebacase treatment at 5; 50 and 150 mg/L, and/or rifampin at 1 mg/L, vancomycin 10 mg/L and daptomycin at 10 mg/L. Evaluated by enumeration on COS media after serial dilutions, showed as mean ± min-max. * Significant effect of the treatment (* = $p<0.05$; ** $p<0.01$; *** $p<0.001$; NS not significant) ; S = synergistic effect of the association exebacase-antibiotic at the indicated concentrations.

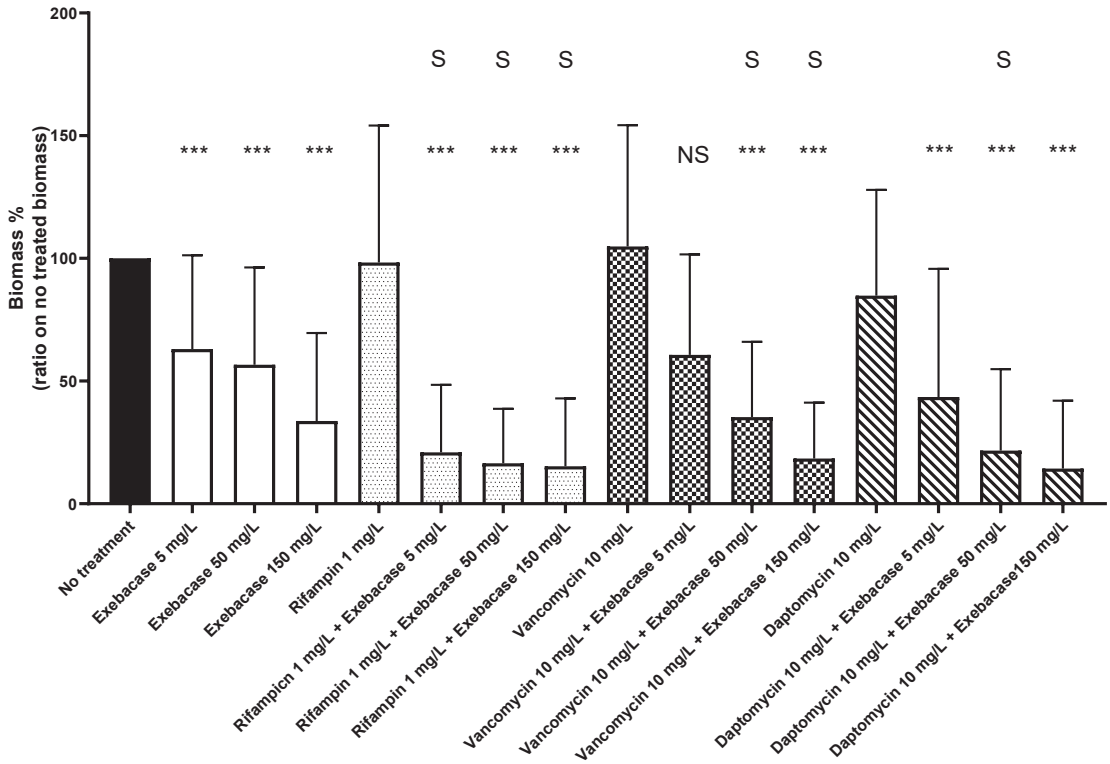

Figure 2: Associations of Exebacase with antibiotics anti-biomass activity in comparison to Exebacase and antibiotics alone, on *S. epidermidis* preformed biofilm. Remaining biomass after 24 hours Exebacase treatment at 5; 50 and 150 mg/L, and/or rifampin at 1 mg/L, vancomycin 10 mg/L and daptomycin 10 mg/L. Evaluated by Crystal Violet staining, showed as mean + sd. * Significant effect of the treatment (* = $p<0.05$; ** $p<0.01$; *** $p<0.001$; NS not significant); S = synergistic effect of the association exebacase-antibiotic at the indicated concentrations.

POSTERS

ECOLOGY AND EVOLUTION

A new mobile genetic element hitchhiking a virulent vibriophage

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Natural populations of vibrio are genetically extremely diverse and genomes comparison led to the identification of numerous strain-specific regions that could frequently not be assigned to a specific mechanism of recombination and spread (Le Roux 2009). These genomic islands often encode adaptive functions, notably for phage resistance (Hussein 2021, Piel 2021, questioning about a role of phages in spreading and/or selecting these elements. Sequencing the genome of a virulent phage infecting *Vibrio chagasii*, revealed two contigs, the phage genome of 47 kbp and an additional circularized element of 6kb. This element was found integrated in the genome of the host strain at the end of the highly conserved gene *fis*. The integrated element is flanked by two direct repeats of a 15 base motif. This motif is likely a recombination and circularization site of a new mobile genetic element. Among the six genes this element carries, one encodes a site-specific integrase and another a AlpA regulator previously identified in satellite phages. We further showed that this element could be excised, circularized and transferred to another host by hitchhiking a virulent phage. Finally this mobile genetic element is broadly distributed in vibrios with variable size and genes content. Notably the identification of restriction modification systems, retrons and abortive infection systems led us to speculate a role of this mobile genetic elements in phage defense. Since phage therapy favor the use of virulent phages, the study of their ability to transfer mobile genetic elements has great interest to ensure the sustainability of such treatment.

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From *Escherichia coli* to *Citrobacter rodentium*: three approaches for *in vitro* phage host adaptation

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Citrobacter rodentium is an enteric murine pathogen that shares infection strategy and virulence factors with human *Escherichia coli* diarrheagenic pathogens. Therefore, it is considered as a more relevant pathogen than human isolates of *E. coli* for studying in murine models of intestinal infection mechanisms and testing the efficacy of treatments. We previously characterized the Myoviridae CLB_P2, which is distantly related to phage T4. Phage CLB_P2 was isolated using the diarrheagenic *E. coli* strain 55989 and we found that this phage was able to actively replicate in the gut of mice colonized by strain 55989. Despite infecting *in vitro* numerous different *E. coli* strains, phage CLB_P2 does not form plaque neither lyses in liquid culture the strain ICC180, the prototypical strain of *C. rodentium* commonly used in the laboratory.

The aim of this work was to test three *in vitro* strategies to isolate one or several phage CLB_P2 variants that would infect strain ICC180. These include (i) a dynamic approach: CLB_P2 (MOI 1:100) was incubated with a mix of strain ICC180 and strain 55989 in a ratio of 20:1 under dynamic conditions during 5 h and refreshed every day during 5 days; (ii) a direct host jump approach: strain ICC180 was incubated with CLB_P2 at MOI 10:1 and incubated for 24 h and refreshed every day during 20 days; (iii) an intermediate host jump approach: CLB_P2 (MOI 1:100) was incubated with either a single *E. coli* strain or in a mix with strain ICC180 in static condition during one week and renewed thrice. This approach was attempted with three *E. coli* strains (MG1655, LF31 and LF110).

No plaques on a lawn of the strain ICC180 were detected from the co-incubation of strain ICC180 with strain 55989, while several plaques were isolated from the two other approaches. Two plaques with different morphologies were then isolated from each individual condition and subsequently purified by three passages on strain ICC180. Nearly half of the variant's lysates displayed a higher titer on strain ICC180 than on strain 55989 with one variant unable to infect this ancestral host, showing that the host jump was associated with a fitness cost on strain 55989. Interestingly, we noted an exclusive phenotype with some variants amplifying much more efficiently on lawns than in liquid and vice versa. An investigation of the host range of these variants on 14 putative hosts revealed that the variants display different patterns suggesting that they carry different mutations. Genomic analysis of these variants will be performed shortly and subsequent *in vivo* activity to target *C. rodentium* in the gut of mice will be engaged.

Analysis of the microbiome and virome of a gnotobiotic mouse model by Chromosome Conformation Capture

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The gut microbiota houses a complex and diverse microbial community that coexists in a balance which is crucial for human health. Indeed, the alteration of the composition of bacteria has been associated to various chronic diseases such as inflammatory bowel disease, asthma or obesity. More recently, variations of intestinal viruses, predominated by bacteriophages (phages), have also been associated with dysbiosis, calling for combined studies of both bacterial and viral populations [1, 2].
Chromosome Conformation Capture (3C) applied to microbial communities is an innovative method to obtain information on both bacterial and viral populations as well as their interactions through DNA collisions [3, 4]. Here, we applied this method and developed the tools to analyze longitudinal samples from OMM12 mice, which are gnotobiotic animals colonized by a synthetic microbiota comprising 12 bacteria [5]. In addition, we performed deep sequencing of the total viral fraction to identify free viruses.
Data from the 3C method led us to improve the assembly of the 12 bacterial genomes and revealed specific 3D structures of their chromosomes, providing novel information on both the diversity of architecture of non-model bacterial chromosomes and the metabolic activities of these bacteria in the gut environment. In particular, we detected the induction of prophages amongst which several formed free particles as confirmed by virome sequencing. The temporal stability of bacteria and phage populations was assessed over time as well as the reproducibility of the method.
These data demonstrate that the dynamic interactions between phages and bacteria can be determined by *in situ* by 3C associated to viromes, which provide a solid base for implementation of this gnotobiotic model to study microbial communities in a relevant gut environment.

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A family for lambda:

a possible genome-based taxonomy for lambdoid viruses?

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Discovered by Esther Lederberg in 1951, the temperate bacterial virus (or bacteriophage) lambda infecting the laboratory *Escherichia coli* strain K-12 has notably owed its fame to being at the center of the molecular genetic universe during three decades[1]. Over the years, many phages related to lambda, and susceptible to recombine with it, were isolated and named « lambdoids » (or « lambda-like »). Due to the sequencing development, the ill-defined term of lambdoid was expanded to mean a phage with the same functional gene order as lambda and carrying patches of nucleotide sequence similarity with it or another lambdoid phage [1]. Whole genomic comparison indeed revealed that lambdoid phage genomes were highly mosaic with each other and thus appeared to regularly share and exchange a wide pool of genes.

Since the 2000s and especially in recent years, the Bacterial and Archaeal Viruses Subcommittee of the International Committee on Taxonomy of Viruses has moved largely to a molecular taxonomy based upon overall DNA and protein similarity [2]. The question raised whether lambdoid genomes, despite their recombinational flexibility, could be placed into a classification scheme. For this, we started with a set of 70 complete lambdoid genomes, and submitted them to analysis with two recent tools: VIRIDIC (Virus Intergenomic Distance Calculator), computing pairwise ANI-like intergenomic distances/similarities amongst phage genomes [3] and ViPTree, which is a viral proteomic tree server based on genome-wide sequence similarities computed by tBLASTx [4]. The first one was used for species and genus delineations using respective thresholds of 95% and 70% nucleotide identity while the second helped us to distinguish a potential family demarcation. Only 43 of the 70 genomes, (representing 30 species and 12 genera), could be included in a modern, genome-based “lambdoid” family subdivided into three distinct subfamilies, while the remaining genomes did not share enough ancestry to be included into a family. The structural genes coding for virion components were delineating clearly these subfamilies, while four tail tip encoding-genes (*I, J, K, L* on lambda) formed the core genome of the lambdoid family level. This classification scheme seemed robust, as a challenge with 36 new *E.coli* prophages (size >42kb) did not perturb the ViPTree-based subfamily branchings. We propose that despite its mosaicism, the lambdoid family can be recognized as such, with a core genome restricted to its tail tip (>73% aa identity).

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Transposable prophages in pathogenic *Leptospira*: similar to and different from all other *Saltoviridae.*

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Weil's disease, also named leptospirosis, is a global zoonotic disease currently causing more than 1 million severe cases and 60,000 deaths per year. Its causative agent, the corkscrew shaped bacterium *Leptospira*, has been identified for a century and is thoroughly studied at the genomic level, more than 700 completely sequenced genomes being now available. Yet, little is known about the viruses associated to these bacteria, as only 3 phages have been isolated on the laboratory strains *L.biflexa*. Moreover, the number of genetic tools available for these bacteria remains very limited. We addressed these two issues by searching leptospirales genomes for putative transposable prophages and predicted as many as 236 of them, 156 being distinct. These prophages were found more often and more abundantly in *Leptospira* genomes of the most pathogenic clade, consistent with the hypothesis that they facilitate acquisition of virulent traits by the host through increased horizontal gene transfer and genome reorganization.

Based on DNA comparisons, these 156 prophages could be divided into 126 species, 25 genera and 6 large groups. These 6 groups could be retrieved on the phylogeny of the transposition proteins, characteristic of this type of phages and prophages. One of these groups only contained prophages that had a TA-TA direct repeat at their ends instead of the standard TG-CA inverted repeat found on already known transposable (pro)phages and were found in the five *Leptospir*a species analyzed. The characteristic scar of a transposition event, a short direct target sequence repeat, was identified flanking a significant fraction of both types of prophages, supporting their ability to transpose.

Genome length, gene functions and overall genomic organization in two arms transcribed in opposite direction, resembled that of *Casadabanvirus* B3, but with different relative positions of the transposition/replication, semi-essential region, lysis, head and tail modules in the 6 groups. At a large scale level, the structural and transposition modules displayed different evolutionary histories, consistent with recombination having occurred between ancestors of the 6 groups. The gene content was coherent with the baseplate phylogeny, with 2 large clades sharing a significant number of orthologous groups, most of which contained structural proteins. Despite a wide sequence divergence that prevented the identification of sequence similarities, even with HMM comparisons, functional similarities point to a largely conserved gene content.

A few prophage proteins involved in host recognition (spike, tail fibers) and lysis, were related to orthologs in the 3 phages infecting *L.biflexa*, suggesting gene transfer between these different Caudovirales, and more importantly, the possibility to use some of the transposable prophages in that model strain.

Evolutionary training of bacteriophage targeting *Salmonella enterica* serotype Tenessee : increase in host range and virulence

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The efficacy of phage therapy depends on the ability of therapeutic bacteriophages not only to infect pathogenic bacteria but also to limit the emergence of new resistant bacterial genotypes or to select rare resident resistant genotypes.

Using a collection of 31 genotypes of *Salmonella enterica* serotype Tenessee, we isolated and characterized several new bacteriophages (*Myo-* and *Siphoviridae*) that infected one or the other bacterial genotype. We realized *in vitro* evolutionary training of one of the bacteriophages (four independent lineages) by performing 6 to 7 serial passages against 8 "naive" (i.e. not co-evolving) bacterial genotypes.

While the isolated (ancestral) bacteriophage population was capable of infecting only 3/8 bacterial genotypes, the evolved populations of the 4 lineages expanded their host range (8/8 infected bacterial genotypes) and adaptation such that bacterial growth inhibition efficiency was maintained without appearance of resistant bacteria for more than 20 hours despite a 3-4 log dilution of the bacteriophages.

With the objective of maximizing the chances of therapeutic success, these results demonstrate the importance of evolutionary training across the diversity of bacteria isolated *in situ* prior to the use of bacteriophages.

Cider is a harsh ecosystem for bacteriophage communities.

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Although the occurrence of bacteriophages (phages) in fermented foods and beverages has been known for decades, data highlighting the diversity and roles of phage communities (phageomes) in such environments are scarce. This is especially true for cider, an alcoholic fermented apple beverage, presenting harsh physicochemical conditions. The current work aimed at describing the diversity of phageomes in cider throughout the fermentation process, which is mainly performed by yeast and lactic acid bacteria (LAB).

The lytic phageome diversity was investigated by attempting to isolate phages from 120 cider samples targeting 150 potential LAB host strains from genera found in cider (i.e. *Leuconostoc* sp., genera belonging to *Lactobacillaceae*, *Oenococcus* sp.), and to perform a metagenomic approach based on the extraction and sequencing of the total viral DNA from cider samples. Different methods for the concentration of lytic phages and DNA extraction were designed and tested, such as tangential flow filtration, flocculation, and the development of several extraction buffers. The diversity of the temperate phageome (prophages) was also explored by a shotgun metagenomic study in the bacterial communities of cider samples from two producers, at 4 time points during the fermentation process.

Despite the different tested methods, the lytic phageome diversity and occurrence in cider seemed very poor as only one lytic *Siphoviridae* phage UCMA 21115 was isolated from the 120 cider samples. It targeted *Liquorilactobacillus mali* UCMA 16447 strain and was thoroughly characterized (adsorption, one step growth curve, pH sensitivity, etc.). The different methods used for extracting the DNA of the lytic phageome failed, confirming the probable low occurrence of lytic phages in such fermented beverages. The temperate phageome was thus explored and showed that 93% of the contigs were assigned to prophages but only 2 were identified with a high quality level. These results highlighted also the low occurrence of temperate phages in cider even if incomplete traces of prophages were found in the different samples, suggesting that phages potentially played an earlier role in this harsh ecosystem. Getting knowledge about both lytic and temperate phageomes in fermented foods and beverages is nevertheless crucial in terms of microbial ecology and, to a larger extent, for a better understanding of fermentation processes.

Rapid *in vitro* cross-resistance evolution to different phages of the phytopathogenic bacterium *Ralstonia solanacearum*

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Ralstonia solanacearum species complex (RSSC) is one of the most devastating bacterial plant pathogens causing bacterial wilt in a wide range of hosts including crops and wild plants. These soil-borne Gram-negative bacteria have a worldwide geographic distribution and significantly reduce crop yields especially in tropical regions such as the southwestern Indian Ocean. Due to the lack of efficient curative measures and the high persistence of RSSC, phage therapy represents a sustainable plant disease management strategy to be applied in agricultural settings. However, using phages as a biocontrol tool raises concerns about the potential of phage resistance evolution in plant pathogenic bacterial populations. To explore this question, we first evaluated the *in vitro* virulence of a local phage collection against the most prevalent strain of RSSC in Reunion Island. Then, we examined the temporal adaptation of the strain to these phages, and its consequences for bacterial fitness. Analysis of the virulence of the 10 phages tested showed that they were all able to effectively reduce bacterial growth. The emergence of bacterial resistance was reported after two or three days. Absence of clear lysis plaques were observed in evolved bacteria not only against the phage encountered but also against the entire phage collection, which included several phage species and genera. These results show high cross-resistance emergence, which resulted in a fitness cost for the bacteria, as measured by a 8 to 22% reduction in growth rate and loss of swimming ability for 8 of 10 evolved bacteria exposed to the different phages. These results suggest that phylogenetically distant phages can be rapidly blocked in bacteria. Mechanistically different phages or complementary strategies may be needed to efficiently control phytopathogens. Conversely, fitness costs in bacteria point in the direction of a reduced virulence *in planta*. Further *in vivo* and genetic analysis will bring light to these questions.

Toward the prediction of the epidemiological dynamic of *E.Coli* during infection by bacteriophages

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Measure, describe and predict the co-evolution of bacteria and their phages is essential for the development of bacteriophages as clinical antibacterial treatments. When studying epidemiological dynamics of bacteria/phage systems, one of the main issues is to find a precise and high throughput, yet not too laborious measurement, as a proxy of the bacterial population size over time while phage infection occurs. In our study, the production of living cells, dead cells and biomass of Escherichia coli in the presence of bacteriophage T7 were measured in parallel on a real-time basis, using several fluoro-luminometric measurement (Fluorescence with CFP and orange SYTOX and bioluminescence), combined with optical density that is a rough proxy of the bacterial population kinetic. We evaluate the ability of a classic Sensitive/Infected/Phage (SIP) epidemiological model to jointly fit these distinct measured bacterial kinetics and their mutual consistency as a test of model validity. Using these four fluoroluminometric measurements combined with their theoretical modelisation allow to describe the dynamics of infections with accuracy. Such parameterization of the epidemiological dynamic opens the perspective of predicting with good confidence the kinetics of co-evolution in bacteria/phage systems.
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Genetic mining of newly isolated salmophages for phage therapy

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Salmonella enterica - a Gram negative zoonotic bacterium – is mainly a foodborne pathogen and the main cause of diarrhea in humans worldwide. Main reservoirs are found in poultry farms but also in wild birds. The development of antibiotic resistance in *S. enterica* species raises concerns about the future of efficient therapies against this pathogen. More generally speaking, the development of resistance to antibiotics revived the interest in bacteriophages as a useful therapy against bacterial infections. During the course of their work to develop an epigenetic biosensor for bacteriophage detection and phage receptor discrimination, Olivenza et *al.* [1] isolated in the Sevilla region (Spain) various bacteriophages infecting *S. enterica* species. In the present study, we aimed at analyzing 14 of these Salmonella phage genomes and characterizing them in the optic of *S. enterica* biocontrol. To that avail, we designed a bioinformatic pipeline using available building blocks in order to de novo assemble genomes from raw sequencing reads, perform their syntaxic and functional annotations and tentatively assign a position in the phage taxonomic landscape. We finally searched for useful functions for phage therapy such as systems encoded by the phage to circumvent cellular defenses including CRIPSR-Cas and Restriction-Modification (R-M) systems. We were also particularly interested in comparing "traditional" functional annotation relying solely on protein sequence similarities within available public protein databases with more recent annotation methods using protein orthologous groups as those defined in the phage-specific PHROG database [2] to infer protein functions.

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New virulent bacteriophages isolated from the surface of Epoisses cheese infect ripening bacteria

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Cheese surface hosts complex microbiota, comprising fungi, bacteria and bacteriophages (phages). The latter are thought to have a substantial impact on the dynamics of microbial ecosystems. However, to date, ecological roles of phages were mainly studied in natural aquatic and terrestrial ecosystems and in the human gut. The hypothesis of this work is that phages affect the microbial succession observed on the cheese surface during the ripening process. In order to explore this question, isolation and characterization of "indigenous" bacteria and phages targeting them represent the first objectives.

According to shotgun metagenomics data obtained from the surface of Epoisses, a French washed-rind cheese, members of the *Pseudoalteromonas*, *Psychrobacter* and *Glutamicibacter* genera represent the dominant bacteria present in this ecosystem. This result guided the phage isolation step which consisted in plating cheese rind viral fractions on strains from these 3 main genera (as well as on *Brevibacterium* and *Leuconostoc* strains, 5 to 20 strains per species were tested) isolated from the same cheese rind. Phages were isolated for all genera except *Pseudoalteromonas* and 4 of them (one infecting each of the 4 genera) were characterized from a morphological point of view: one was a myovirus, 2 siphoviruses and one podovirus. Their genome was sequenced and annotated. Genomic analysis suggested they are all virulent (absence of integrase, no partition system) and 3 of them represented new phage genera. Moreover, evaluation of their host range on 20 strain per species revealed that all were very specific, as they generally infect only the strain on which it was isolated.

In the near future, we plan to use this phage-bacteria collection for testing the hypothesis of an ecological role of phages on the cheese surface using a synthetic ecology approach. Dynamics of both phages and their hosts will be monitored in real time within a simplified cheese matrix.

Quantification of bacteriophages, extracellular vesicles and bacteria in the stools of IBD patients and healthy donors

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It is now largely accepted that the intestinal microbiota plays a key role in Intestinal Bowel Diseases (IBD). There are indications that phages, the viruses infecting bacteria, but also extracellular vesicles (EVs), might play a role in this family of diseases. Indeed, it was found that patients had higher loads of virus like particles (VLPs) in intestinal biopsies compared to healthy controls. In addition, a recent publication also shows an increase of the exosome proteins proportion in the proteome of EVs isolated from IBD patients. To investigate further a potential role of EVs and VLPs in IBD, our project is to quantify EVs, VLPs and bacteria in fecal filtrates from IBD patients, either in crisis or remission (from the cohort Suivithèque, at the hospital Saint-Antoine in Paris) and healthy volunteers.

From healthy donors, we first devised and validated protocols for VLPs and EVs quantification in fecal samples by using, respectively, epifluorescence microscopy and Interferometric Light Microscopy (Videodrop, by Myriade). Using flow cytometry, we found bacterial concentrations around 5x10^10 per gram of stool, as expected. EVs were generally slightly above this concentration (approximately 3x10^11 EVs per gram of stool). Concerning the VLPs, their concentrations were very dispersed, ranging from 10^9 to 2x10^11 viruses per gram of stool (median: 10^10). We conclude that EVs are a major component of fecal filtrates in healthy donors, while VLPs are usually less numerous than bacteria.

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