

Bacteriophage LM33_P1, a fast-acting weapon against the pandemic ST131-O25b:H4 Escherichia coli clonal complex.

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Bacteriophage LM33_P1, a fast weapon against the pandemic ST131-O25b:H4 *Escherichia coli* clonal complex

Running title: LM33_P1, a specific bacteriophage targeting the ST131-O25b:H4 clonal complex.

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Abstract (253 words)

Background and objectives. Amongst the highly diverse *Escherichia coli* population, the ST131-O25b:H4 clonal complex is particularly worrisome as it is associated with a high level of antibiotic resistance. The lack of new antibiotics, the worldwide continuous increase of infections caused by multidrug resistant bacteria and the need for narrow-spectrum antimicrobial agents have revived attention to phage therapy. In this article, we describe a virulent bacteriophage, LM33_P1, which specifically infects O25b strains and provide data related to its therapeutic potential.

Methods. A large panel of *E. coli* strains (n=283) were used to assess both the specificity and the activity of bacteriophage LM33_P1. Immunology, biochemistry and genetic-based methods confirmed this specificity. Virology methods and sequencing were used to characterize this bacteriophage *in vitro* while three relevant mice models were employed to show its *in vivo* efficacy.

Results. Bacteriophage LM33_P1 exclusively infects O25b *E. coli* strains with a 70% coverage on ST131 and ST69. This specificity is due to an interaction with the lipopolysaccharide mediated by an original tail fiber. LM33_P1 also has exceptional intrinsic properties with a high adsorption constant and produces over 300 virions per cell in less than 10 minutes. Using animal pneumonia, septicemia and urinary tract infection models, we showed the *in vivo* efficacy of LM33_P1 to reduce the bacterial load in several organs.

Conclusions. Bacteriophage LM33_P1 represents the first weapon that specifically and quickly kills O25b *E. coli* strains. Therapeutic approaches derived from this bacteriophage could be developed to stop or slow down the spread of the ST131-O25b:H4 drug-resistant clonal complex in humans.

Introduction

Amongst the highly diverse *Escherichia coli* population[1], the ST131-O25b:H4 clonal complex is of particular concern. Since its first description in 2008 in a limited number of countries, this clone has spread worldwide and shown an uncommon ability to propagate in humans.[2, 3] Moreover, ST131-O25b:H4 *E. coli* strains have a high pathogenic potential,[4] they belong to the B2 phylogroup where most extraintestinal-pathogenic *E. coli* are classified,[5] they express a large number of virulence factors[6] and are lethal in a mouse model of sepsis.[7] Involved in community as well as hospital-acquired infections, ST131-O25b:H4 isolates are responsible for a wide range of pathologies, from common cystitis to life threatening meningitis.[2] Finally, these clones are also particularly worrisome as they are associated with a high level of resistance to betalactams (mainly via the production of CTX-M-15 ESBL but also carbapenemase[8]) and fluoroquinolones.[9, 10] More recently described, the worrying plasmid-mediated colistin resistance gene, *mcr-1*, has been found in an ST131 strain, highlighting the propensity of this ST to carry antibiotic resistance genes.[11] To a lesser extent, O25b strains may belong to another spreading antibiotic resistant clonal complex with a high extraintestinal pathogenic potential, the ST69 ("clonal group A").[12]

The lack of new antibiotics and the worldwide continuous increase of the infections caused by multidrug resistant bacterial pathogens have revived attention to phage therapy,[13] boosting the search for novel bacteriophages. Numerous experimental data have been published demonstrating the proof of concept of this approach and clinical trials have been reported or are ongoing (http://www.clinicaltrials.gov).

In this work, we characterized a novel virulent bacteriophage, LM33_P1, which only infects *E. coli* O25b strains. The *in vitro* and *in vivo* efficacies of bacteriophage LM33_P1 are reported, showing its value for therapeutic applications.

Material and methods

Bacterial strains and bacteriophages, susceptibility testing

The bacterial strains used in this work belong to previously published collections: human commensal and extraintestinal *E. coli* gathered in France during the 2010s (n=83),[14-16] Spanish extraintestinal *E. coli* (n=16),[6] North American clinical *E. coli* (n=14),[17], commensal

and pathogenic *E. coli* from various parts of the world (Africa, America, Australia, Europa (n=18),[18, 19] the ECOR collection (n=8)[20] as well as the unpublished ColoColi collection (n=144, an ongoing French multicenter study collecting *E. coli* strains in the lower respiratory tract of mechanically ventilated patients). The phylogroup and the ST belonging were determined as described in.[21, 22] The O-type and the *fimH* allele were determined by PCR-based assays as previously described[18, 23] and as reported in **Supplementary Data - Procedures**, respectively. When needed, we confirmed the O25b phenotype using a monospecific O25 serum designed for *E. coli* serotyping purpose (*E. coli* mono O25, Statens Serum Institut, Denmark).

All the strains were grown in lysogeny broth (LB) (Difco™ Bacto-Tryptone 10 g/L, Difco™ Yeast extract Difco 5 g/L, NaCl 5 g/L). Their antibiotic susceptibility was determined using the disk diffusion method following the EUCAST guidelines.

Some *E. coli* strains, used for lipopolysaccharide (LPS) assays or bacteriophage susceptibility testing, are detailed below:

- LM33, LM36, AVC02 (ST131-O25b:H4) and AVC03 (O25b, non-ST131) are clinical strains responsible for ventilator-associated pneumonia,
- 536 (ST127-O6), LM02 (ST69-O17) and ECOR51 (ST73-O25a) have been used as a source of their corresponding LPS,
- 81009 WT (ST131-O25b:H4) and its isogenic rough derivative (a mutant strain obtained by deleting the gene encoding for the O-antigen ligase)[24] were used to prove the LPS-dependent interaction of LM33_P1.

The bacteriophages were isolated from sewage, using specific host.[25] By convention, bacteriophages are named as follows: “host bacteria_Px” (for example LM33_P1 represents the first bacteriophage isolated using strain LM33). In all experiments, bacteriophage solutions were obtained after purification by using ultracentrifugation on cesium chloride gradient as previously described.[26]

For bacteriophage susceptibility testing, we used the double spot test technique[27] as a screening method and then we calculated the efficiency of plaquing (EOP) for all susceptible strains. EOP was calculated as the ratio of the number of plaques formed by the bacteriophage on the non-host strain to the number of plaques formed on its host, using the same bacteriophage solution. More details are provided in **Supplementary Data – Procedures**.

LPS extraction.

LPS extracts were obtained and purified from the same amount of bacteria using a phenol-water-diethyl ether extraction[28] followed by extensive dialysis against sterile pyrolyzed water. High purity LPS was confirmed by performing an agarose gel electrophoresis with ethidium bromide staining (nucleic acids detection), an SDS-PAGE 12% followed by Coomassie blue staining (proteins detection) and a silver staining to visualize the LPS O-antigen pattern (SilverSNAP Stain Kit II, Pierce).

Plaque inhibition assays with LPS extracts

From a purified stock solution of bacteriophages in TN buffer (Tris-HCl 10 mM, NaCl 150 mM, pH 7.5), 3 solutions of 10^6, 10^5 and 10^4 pfu/mL in TN buffer were prepared. Each of these working solutions was used to prepare final tubes with bacteriophages alone (100 µL of working solution + 100 µL of pyrolyzed water) and tubes with bacteriophages + LPS (100 µL + 100 µL of undiluted LPS extract). Additional tubes containing bacteriophages and decreasing amounts of LPS were also prepared (pyrolyzed water was used to reach an identical final volume). Then, 10 µL of each final bacteriophage tubes, with and without LPS, were spotted in triplicate on an agar plate, previously overlaid by the bacteria to test. The plates were incubated for 4 hours at 37°C before the plaque-forming units were counted in each condition.

Characterization of bacteriophage LM33_P1

The adsorption assay and the one-step growth experiment were performed in triplicate as described by Hyman and Abedon[29] and as detailed in **Supplementary Data - Procedures**.

Lysis kinetics (with and without LPS extracts) and aggregation assays with O25 antibody

The lysis kinetic was performed as detailed in **Fig. S4**. Briefly, the growth of LM33 with and without LM33_P1 was followed overtime by recording optical density at 600 nm every 15 minutes. The aggregation assays were performed using an O25 *E. coli* anti-serum (see above) and observed under light microscope as detailed in **Fig. S7**.

Sequencing of strain LM33 and bacteriophage LM33_P1

The sequencing of bacteriophage LM33_P1 and strain LM33 was performed using Illumina sequencing technology (Illumina Inc., San Diego, CA). Genomes annotation was performed by the MicroScope plateform for strain LM33[30] and using the RAST server for bacteriophage LM33_P1.[31] See **Supplementary Data – Procedures** for more details.

Experimental murine infections models (additional details provided in **Supplementary Data - Procedures**)

The primary experimental outcome was the decrease in bacterial load in relevant organs.

As LM33 was originally responsible for a ventilator-associated pneumonia in an ICU patient, pneumonia was initiated by intranasal administration of $1x10^8$ cfu of strain LM33 on anesthetized mice (n=20) as previously described.[32] The septicemia model, essentially used to study intrinsic extraintestinal virulence of *E. coli* isolates,[7] was carried out with $1x10^9$ cfu of the strain H1659 (ST131-O25b:H4), injected subcutaneously into the nape of the neck (n=12 mice). We used this strain because of its virulence, previously investigated.[6] The non-lethal urinary tract infection model consists in a retrograde kidneys infection occurring after an intra-urethral injection of $5x10^7$ cfu of strain LM33 into the bladder (n=23 mice), as previously described.[33] In every case, the organs were aseptically removed and mechanically homogenized in cold PBS before counting bacterial and bacteriophage content.

Ethics

The animals were housed in animal facilities in accordance with French and European regulations on the care and protection of laboratory animals. The protocols were approved of by the veterinary staff of the Institut Pasteur and INSERM animal facilities together with the National Ethics Committee regulating animal experimentation (authorization CETEA #2012-0018). Food and drink were provided *ad libitum*.

Statistical analysis

All the statistical analyses were performed by using GraphPad Prism version 5.00 (Graph-Pad Software, La Jolla, CA). The normal distribution of all the variables was checked using the Kolmogorov-Smirnov test, and the results are then expressed as mean ±SD. In case of a non-

Gaussian distribution, the results are expressed as median [25th, 75th percentile]. The statistical tests (Student t test or Mann-Whitney test) were chosen accordingly.

Results

Bacteriophage LM33_P1 targets antibiotic resistant O25b *E. coli* strains. *E. coli* strain LM33 was used to isolate bacteriophage LM33_P1. The characteristics of strain LM33 are as follows: an O25b:H4 serotype, a B2 phylogroup (subgroup I), a ST131 (Warwick scheme)/ST43 (Pasteur Institute scheme)[22] sequence-type, a *fimH* allele *H22* as well as a multi-drug resistance phenotype with an extended spectrum beta-lactamase, a resistance to aminoglycosides (kanamycin, tobramycin, gentamicin, netilmicin except for amikacin where an intermediate phenotype is found), sulphonamides, chloramphenicol and an intermediate susceptibility to nalidixic acid. The beta-lactam resistance is supported by both a plasmid (pLM33) and the bacterial chromosome (**Table 1**).

We determined the host range of bacteriophage LM33_P1 on a panel of 283 *E. coli* strains belonging to various O-types (**Fig. 1** and **Data sheet 1** provided separately for O25 and O16 strains). One hundred and eighty-three (64%) of these strains were not O25b and none of them was infected by LM33_P1, including twelve O25a strains and six ST131-O16 strains. Among the remaining one hundred O25b strains (encompassing 83 ST131, 4 ST69, 10 ST95 and 3 other STs), 64 (64%) were infected by LM33_P1 with a median efficiency of plaquing of 0.46 [0.09-1.27] (**Fig. S1**). Interestingly, LM33_P1 was found to be more efficient on STs associated with high antibiotic resistance (ST131 and ST69, n=87) where 61 of these strains (70%) were lysed whereas its efficacy was weak on STs associated with low antibiotic resistance (ST95 and others, n=13) where only 3 of these strains (23%) were susceptible (**Fig. 1**). Finally, we did not find a correlation between the susceptibility to bacteriophage LM33_P1 and the *fimH* allele *H*30, which is strongly associated with fluoroquinolone resistance among ST131 strains.[34] Indeed, considering the two more frequent *fimH* alleles in our O25b strains (*H*22 and *H*30, n=83), the proportion of susceptible strains to LM33_P1 was 72% (21 out of 29) and 66% (36 out of 54) in the strains displaying respectively the *H*22 or the *H*30 allele (p=0.6, Fisher's exact test).

Bacteriophage LM33_P1 is a lytic *Podoviridae* distantly related to bacteriophage T7. The genome of bacteriophage LM33_P1 (38 979 bp; GC content of 50.8%; 49 ORFs predicted, accession number PRJEB12445) lacks putative ORFs with homologies to integrase or recombinase.

A BLAST analysis of the genomic sequence revealed that the four closest related bacteriophages were enterobacteria bacteriophages (**Table S1**): three coliphages called PE3-1, K1F,[35] EcoDS1 (with 94% identity on ≥ 88% of its length for all of them) and bacteriophage Dev2 infecting *Cronobacter turicensis* (with 83% identity on 85% of its length).[36] The alignment of these related bacteriophages with LM33_P1 revealed a similar spatial genome organization and confirmed the high homology between them (**Fig. 2**). Strikingly, the 5' extremity (the first 650 nucleotides) of the tail fiber gene is highly conserved in each bacteriophage genome, while the remaining part is highly divergent. The corresponding N-terminal region (IPR005604 / PF03906, InterPro / Pfam database) of this tail fiber protein is involved in its connection to the tail-tube[37] while the C-terminal part, involved in host recognition, often carries hydrolase activities such as the endosialidase of bacteriophage K1F used for exopolysaccharide degradation.[35, 38] BLAST searches on the C-terminal part of the tail fiber of bacteriophage LM33_P1 revealed a homology to a domain belonging to the pectin lyase superfamily (IPR011050). Tridimensional structure prediction using Phyre² database[39] confirmed its close proximity to the endopolygalacturonase of *Erwinia carotovora* that belongs to the pectin lyase superfamily (100% amino-acid predicted with a confidence >90% for the tertiary structure, index of confidence for homologous protein 94.1%, Protein Data Bank entry: 1BHE, **Fig. S2**).

Bacteriophage LM33_P1 is highly efficient and rapid *in vitro*. The adsorption of LM33_P1 bacteriophage on its host is fast with ≥ 90% of the viral population attached to cells after 3.5 minutes with an adsorption constant of 1.2×10^{-8} mL/min (**Fig. S3-A**). Newly produced virions are detected within the bacteria by 7 minutes post-infection (eclipse period) while host lysis occurs in 9 minutes (latent period) with a burst size of 317 (95% confidence interval: 289-345) (**Fig. S3-B**).

In liquid medium, when LM33_P1 was mixed with its host, the absorbance value of LM33 cells started to decline (a sign of lysis) within 15 minutes at a multiplicity of infection (MOI) of 1. With much fewer bacteriophages (MOI of 10^{-6}) lysis still occurred within 60 minutes

(**Fig. S4**). On solid medium, LM33_P1 forms clear and large plaques, whose diameter increases rapidly overtime with a visible halo around clear areas. This halo suggests the presence of a diffusible enzyme that most likely carries a depolymerase activity[40] (**Fig. S5**).

Bacteriophage LM33_P1 specifically binds to O25b LPS O-antigen. The host range of bacteriophage LM33_P1 strongly suggested that the O-chain of LPS could be involved in its specificity. Using LPS competition assays we observed that purified LPS from strain LM33 was able to inhibit the interaction between bacteriophage LM33_P1 and strain LM33 as well as other O25b strains.

First, we demonstrated that purified LPS reduced the number of plaque-forming units when mixed with bacteriophages before application on a bacterial layer (mean reduction of 1.0 ±0.23 Log_{10} from 15 assays with five different O25b strains, **Fig. S6-A**). Together with the reduction of the number of plaques, we observed a reduction of the plaque diameters, suggesting that the LPS molecules prevented newly released bacteriophages from infecting the surrounding hosts (**Fig. 3**). These observations are specific of the interaction of bacteriophage LM33_P1 with O25b strains since: i) an O25b LPS extract from strain LM33 was not able to affect the interaction of other bacteriophages targeting non O25b strains and ii) an LPS extract from non O25b strains (O25a, O6 and O17) was unable to alter the interaction between bacteriophage LM33_P1 and strain LM33 (**Table S2**).

Second, an LPS extract from O25b strain (LM33) also reduced the infectivity of bacteriophage LM33_P1 in liquid medium in a dose dependent manner (**Fig. S6-B**), whereas LPS extracts from O6 and O25a strains had no effect.

Third, using an O-type specific antibody to aggregate O25 strains for serotyping, we found that bacteriophage LM33_P1 prevented the aggregation of strain LM33 (**Fig. S7**).

Fourth, using *E. coli* O25b 81009 and its isogenic rough derivative (an LPS deficient strain obtained by deleting the gene encoding for the O-antigen ligase)[24] we observed that bacteriophage LM33_P1 infects the wild type strain 81009 while the LPS deficient strain is resistant. Conversely, we confirmed that bacteriophage LM33_P1 could not adsorb on the LPS defective strain.

The adsorption of bacteriophage LM33_P1 is most likely hindered by capsule production. The production of exopolysaccharides is a well-known bacteriophage resistance mechanism

and might be involved in the non-adsorption of bacteriophage LM33_P1 observed in five randomly chosen LM33_P1 resistant strains (81009 WT, JJ1886, S242, B-1, C-1). Since, in some cases (type II capsule), the synthesis of exopolysaccharides is temperature dependent, we investigated the susceptibility of LM33_P1 on all the O25b resistant strains (n=36) at 20°C. We observed that nine of them (25%) became susceptible at this temperature (**see Data sheet 1**), supporting this hypothesis.

Bacteriophage LM33_P1 efficiently infects its host *in vivo*. As bacteriophage LM33_P1 exhibited impressive *in vitro* characteristics, we investigated its *in vivo* activity in three different animal infection models relevant to ST131 clinical epidemiology: pneumonia, septicemia and urinary tract infection (**Fig. 4-6**). Since strain LM33 was isolated from a patient with pneumonia, we first attempted to trigger pneumonia in mice. Using an inoculum 50 times higher than previously reported in such model[32] and despite clear macroscopic lung lesions, strain LM33 was not lethal, preventing us from using survival as an indicator of the efficacy of the bacteriophage. We therefore evaluated LM33_P1 efficacy by counting the bacteria from lung homogenates collected 17 hours following infection. Three groups of mice were treated 4 hours post-infection either by control solution (PBS), or intranasal (MOI 50) or intraperitoneal (MOI 500) bacteriophages. Independently of the administration route, we observed a 3 Log_{10} reduction in bacterial load when the mice received bacteriophage treatments compared to control group (PBS-treated animal: $5.4x10^7$ cfu/g, intranasally LM33_P1-treated: $2.7x10^4$ cfu/g, intraperitoneally LM33_P1-treated: $3.3x10^4$ cfu/g, $p < 0.01$, **Fig. 4**). Interestingly, the number of bacteriophages in the lung tissue was similar between the intranasally and intraperitoneally-treated mice despite the fact that the latter had received a 10 times higher dose (**Fig. 4**).

Then, we tested bacteriophage LM33_P1 in a murine model of septicemia previously reported[6, 7] using the H1659 ST131-O25b:H4 strain[6] (strain LM33 was not lethal in this model), on which LM33_P1 is as efficient as on strain LM33 (EOP = 1). Following a subcutaneous inoculation of $1x10^9$ cfu, septic metastases were rapidly observed in several organs (the first deaths occurred in less than 24 hours). Intraperitoneal administrations of bacteriophage LM33_P1 (MOI 60, a single dose 2 hours post-infection or two doses 2 hours and 12 hours post-infection) were not sufficient to prevent the death of the animals. However, in the subset of the animals that died within the same time interval (between 24

and 30 hours), the bacteria and bacteriophage content was analyzed in the liver, the spleen and the lung-heart homogenates (**Fig. 5**). In these organs, the number of bacteria was reduced compared to the control group (untreated infected animals). A two doses regimen appeared to be more efficient than a single one, enabling to reach a significant reduction of approximately 1.4 Log_{10} (the median bacterial count decreases from $8.5x10^6$ to $2.9x10^5$ in the heart-lungs, from $7.7x10^5$ to $3.2x10^4$ in the liver and from $3.5x10^5$ to $1.4x10^4$ cfu/g in the spleen). The bacteriophage counts were in the same order of magnitude in all the organs, but were significantly higher when two doses had been administered ($2.0x10^{10}$ versus $4.0x10^9$ pfu/g, $p < 0.01$). In addition, the amount of bacteriophages was 3 to 6 Log_{10} higher than the amount of bacteria in each mouse for all the organs. All these observations revealed that bacteriophage LM33_P1 was able to infect and multiply *in vivo* in strain H1659. Finally, as *E. coli* is a major pathogen in UTIs, we assessed the efficacy of bacteriophage LM33_P1 in a murine UTI model (**Fig. 6**). Twenty-four hours following intra-urethral injection of 5.10^7 cfu of strain LM33, the mice received a single bacteriophage treatment intraperitoneally (MOI of 200). Forty-eight hours post-infection, a 2 Log_{10} reduction of bacterial load was observed in the kidneys in the treated group compared to control group ($1.5x10^5$ versus $8.8x10^2$ cfu/g, $p < 0.001$).

Altogether these data firmly show the ability of bacteriophage LM33_P1 to infect O25b strains *in vivo*.

Discussion

One of the main advantages of bacteriophages which has often been reported is their specificity to infect a few strains only within a species, having then a limited impact on the patient's microbiota. Along with monoclonal antibodies (anti-O25b antibodies have been proven to exert a protective effect in mouse septicemia model),[41] bacteriophages are the only anti-infectious tools that could reach such specificity. Using an ST131-O25b:H4 clinical isolate of *E. coli* (strain LM33), we isolated a bacteriophage, LM33_P1, which was found to exclusively infect O25b strains. Interestingly, O25b O-antigen is present in the archetypal ST131 clonal complex but also in the ST69, another antibiotic resistant spreading clone of *E. coli*, the "clonal group A".[12, 42] In a therapeutic projection and taking into account the pandemic lineages of extraintestinal pathogenic *E. coli*,[43] we observed a greater

susceptibility among both of these STs (70%) compared to less antibiotic-resistant O25b STs such as ST95 and minor ones (23%).

Additionally, a majority of the strains belonging to the ST131 clonal complex display an O25b O-antigen whereas a minor part, less resistant to antibiotics, display an O16 serogroup.[17] The specificity of bacteriophage LM33_P1 is linked to the O25b O-antigen and not to the sequence type (*i.e.* none of the non-O25b ST131 strains were susceptible to bacteriophage LM33_P1 while all the O25b-ST69 strains tested were susceptible). Furthermore, the susceptibility of ST131-O25b:H4 strains to bacteriophage LM33_P1 is independent of the *fimH* allele, a marker of the epidemiologic evolution of this clone.[34] Besides, bacteriophage LM33_P1 was unable to infect the O25a strains, despite a highly similar O-antigen structure where polysaccharides repeated units only differ by one monosaccharide (fucose *versus* rhamnose), a fine discrimination that is not possible with the classical antibodies used for serotyping until the recent description of O25b monoclonal antibodies.[24]
Our investigations led us to estimate that the global host coverage of bacteriophage LM33_P1 on O25b strains is 64%. We consider that this coverage is reliable as we first avoided sampling bias by screening a large collection obtained from different sources with many serotypes. Second, we assessed strain susceptibility in a rigorous way using EOP determination that excludes atypical results and false positives such as those obtained with lysis from without.[44, 45] On the other hand, one limitation of our study is the origin of the strains we tested: as most of them originate from European countries (76%), further studies will be required to assess whether coverage of LM33_P1 is higher, equal or lower if submitted to strains originating from Asian, Indian, African or American countries.

Finally, compared to the data available in literature, we found that LM33_P1 is the quickest T7-like bacteriophage ever reported, lysing its host within 10 minutes while T7 takes 13 to 16 minutes.[46, 47] Part of this success is due to its absorption constant (1.2×10^{-8} mL/min) which was found to be 10 times higher than that of most bacteriophages[48-51] and its burst size which is also in the top half of the values usually observed.[52]

To prevent phage adsorption, bacteria can mask phage receptors by the production of extracellular exopolysaccharides (capsules), which can also help bacteria to escape recognition by immune cells.[53, 54] We found that 25% of the strains reversed their phenotype towards bacteriophage LM33_P1 from resistant to susceptible, when grown and tested at

20°C, a temperature known to turn off type II capsule production.[55] Therefore, the bacteriophage LM33_P1 coverage increased to 80% among all the ST131-O25b:H4 strains and to 73% among all the O25b strains tested. It was also previously shown that bacteriophages can defeat such an exopolysaccharide shield by using tail fibers that possess depolymerase activities.[56] We can reasonably assume that the discovery of new bacteriophages and/or the isolation of LM33_P1 variants could provide viruses equipped with such a tail fiber-associated enzyme and thus enable to improve (by restoring the O25b antigen accessibility) the coverage rate of O25b strains.[57-59]

With the goal of using bacteriophages to treat human bacterial infections, the translation from *in vitro* activity (forming plaques) to *in vivo* efficacy (curing a disease) is not guaranteed, despite a high success rate.[60] Our investigation into the *in vivo* curative potential of bacteriophage LM33_P1 revealed indeed that, in the three models tested, this bacteriophage was able to infect targeted bacteria in several body compartments and via different administration routes. With all the limits inherent to animal experiments, our data should not be over-translated to the clinical setting. However, these results clearly show that bacteriophages, including LM33_P1 as shown in this study, can quickly reduce the load of their host within a complex environment including the gut of mammals.[61] In a therapeutic approach, such bacteriophages could be used as a selective antimicrobial agent to control the passive carriage of ST131-O25b:H4 strains in human gut in order to reduce its dissemination, particularly in healthcare-associated environments. Indeed, *E. coli* strains residing in the digestive tract constitute a well-known reservoir for urinary tract infections but probably also for ventilator-associated pneumonia.[15]

Beside the classical phage therapy approach, bacteriophage LM33_P1 or its proteins offer opportunities to develop several tools. The tail fiber could be used to specifically kill O25b *E. coli* strains using bacteriocins, as previously shown for the O104 *E. coli* strains involved in enterohemorragic colitis.[62] Other approaches could be considered where bacteriophages are reprogrammed and could suppress antibiotic resistance genes using CRISPR-Cas system[63] or express well-chosen beneficial enzymes to fight biofilm.[64] Deeper investigations on the infectious cycle of this bacteriophage are now required to determine what molecular mechanisms are responsible for its fast-killing component. Bacteriophage LM33_P1 could

403 also be used from now as a starting platform to develop highly virulent synthetic
404 bacteriophages with various host specificity.[65]

405

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Transparency declarations

None to declare.

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579

580 **Table 1. Main genotypic characteristics of strain LM33 and its plasmid pLM33**

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Strain LM33 chromosome (accession number: PRJEB9970)

General information		
Genome size: 5 450 287 bp	GC content: 51.5 %	Number of genes: 5 276
Sequence type : ST131 (according to the Achtman scheme)	Serotype: O25b:H4	Phylogroup: B2 *fimH* allele: 22

Genes coding for antibiotic resistance*
Aminoglycoside resistance: *strB*, *aacA4*, *strA*, *aac(6')-IIc*
Beta-lactam resistance: *blaDHA-7*, *blaSHV-12*, *blaTEM-1C*
Quinolone resistance: *aac(6')Ib-cr*, *qnrB4*
MLS resistance: *ere(A)*
Sulphonamide: *sul1* ; thrimethoprim: *dfrA18*

Plasmid pLM33 (accession number: PRJEB9970)

General information		
Plasmid size: 296 909 bp	GC content: 47.2%	Number of genes: 382
Incompatibility group: H		

Genes coding for antibiotic resistance*
Aminoglycoside resistance: *strA, strB*, *aacA4*, *aac(6')-IIc*
Beta-lactam resistance: *blaSHV-12*, *blaTEM-1C*
Quinolone resistance: *aac(6')Ib-cr*
MLS resistance: *ere(A)*

*data obtained using the center for genetic epidemiology server[66]

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Figure 1: O-type distribution of the 283 *E. coli* strains tested for their susceptibility to bacteriophage LM33_P1. The numbers in brackets represent the number of strains tested, for each O-type. None of the non-O25b strains was infected by bacteriophage LM33_P1. The proportion of O25b strains regarding bacteriophage LM33_P1 susceptibility (S, susceptible or R, resistant) and sequence type is represented on the right part.

O25b+INS stands for the strains possessing an O25b genotype based on the *rfb* locus sequence but with an insertion sequence within the *wbbL* gene located at the end of the operon, just upstream the *gnd* gene, and responsible for a non-O25b phenotype.

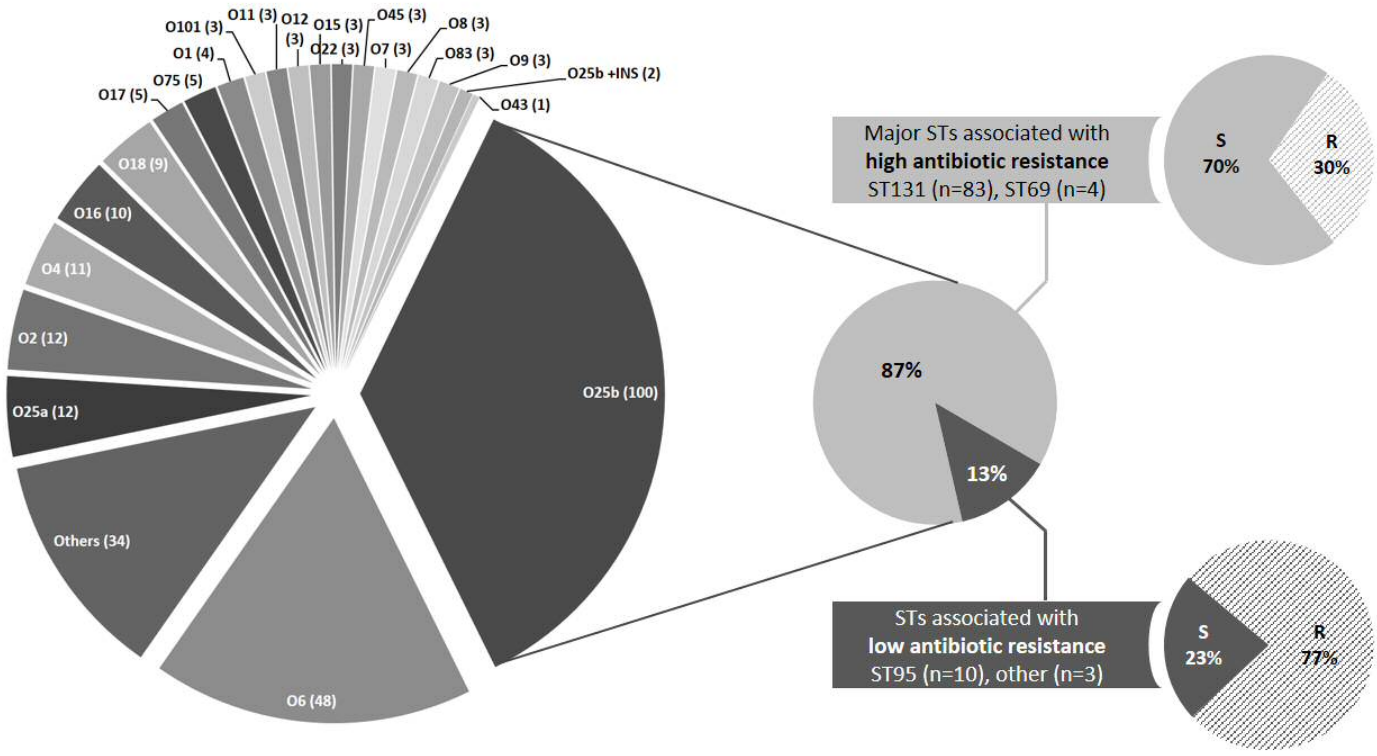


Figure 2. Genome alignment of bacteriophage LM33_P1 with its four closest related bacteriophages and model bacteriophage T7. The homology in nucleotide sequence is color-coded (see scale). The blue arrows correspond to the genes and indicate their transcription direction. Blue arrows correspond to the gene coding for the tail fiber protein. Only the homologies with an E-value ≤ 10^{-3} and a nucleotide length ≥ 230 are represented.

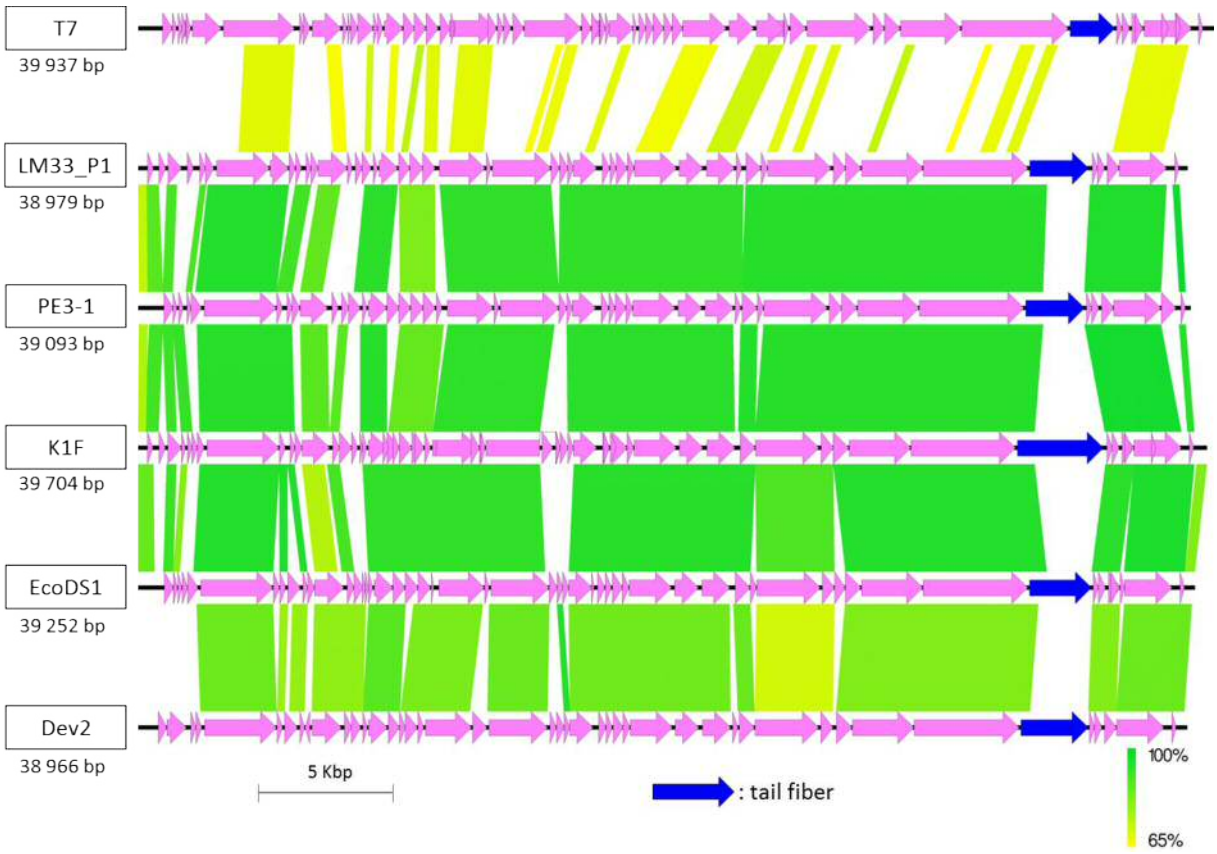


Figure 3. O25b LPS extract inhibits bacteriophage LM33_P1 infection: appearance on agar plates. An LPS extract from strain LM33 was mixed with bacteriophage LM33_P1 (left) or 536_P1 (right) at two different concentrations (10^5 and 10^4 pfu/mL) and assayed on two agar plates overlaid with an O25b strain (AVC02) or an O6 strain (536) as control. Enlargements of these two plates are shown to facilitate the observation.

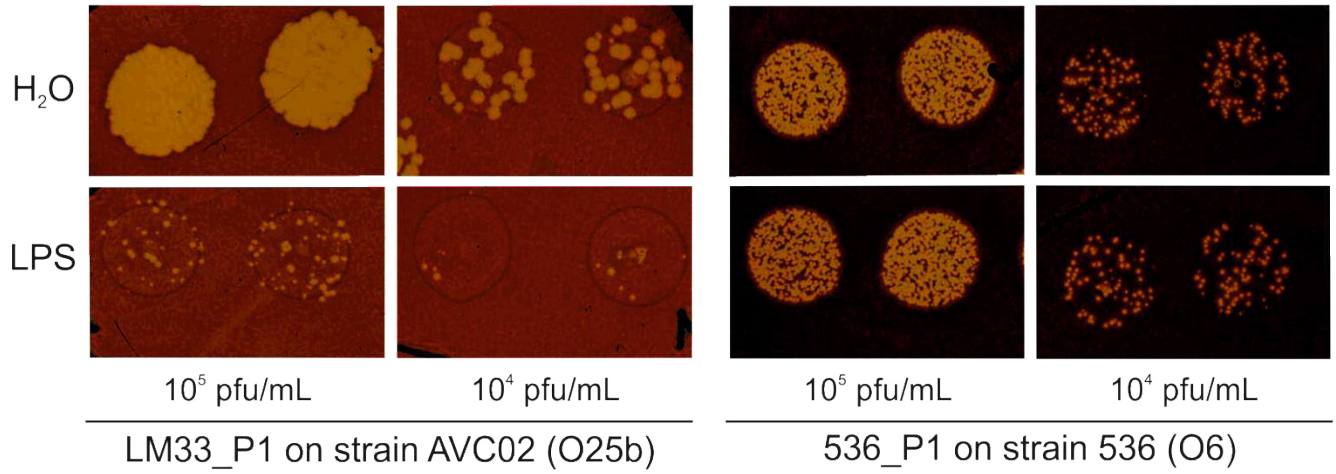


Figure 4. Bacteriophage LM33_P1 *in vivo* activity in a lung infection model. Bacterial (panel A) and viral (panel B) counts 17 hours post-infection in lungs homogenates of mice infected with $1x10^8$ cfu of strain LM33. Four hours post-infection, the mice received either PBS (Ctrl, n=8, one half intranasally and the other half intraperitoneally) or bacteriophage LM33_P1 by intranasal route (φ IN, MOI 50, n=6) or by intraperitoneal route (φ IP, MOI 500, n=6). The results are expressed as individual values with median and interquartile ranges (25^{th} and 75^{th} percentiles). *: p <0.001 compared to control group.

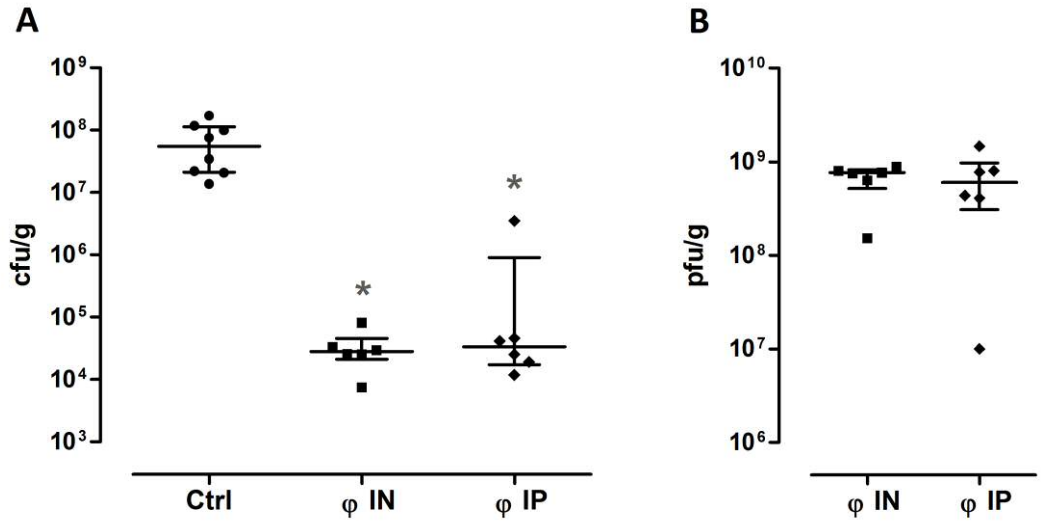


Figure 5. Bacteriophage LM33_P1 *in vivo* activity in a septicemia model. Bacterial (panel A) and viral (panel B) counts 20 hours post-infection in the indicated organs of mice infected with $1x10^9$ cfu of strain H1659 (ST131-O25b:H4). Two hours post-infection, the mice received intraperitoneally either PBS (Ctrl) or bacteriophage LM33_P1 at a MOI of 60 (φ X1: one dose 2 hours post-infection, φ X2: two doses 2 and 12 hours post-infection). The results are expressed as individual values (4 animals per condition) with median and interquartile ranges (25^{th} and 75^{th} percentiles). §, #: $p < 0.05$ (§) or $p = 0.057$ (#) compared to the control group (panel A) or the single-dose treatment (panel B).

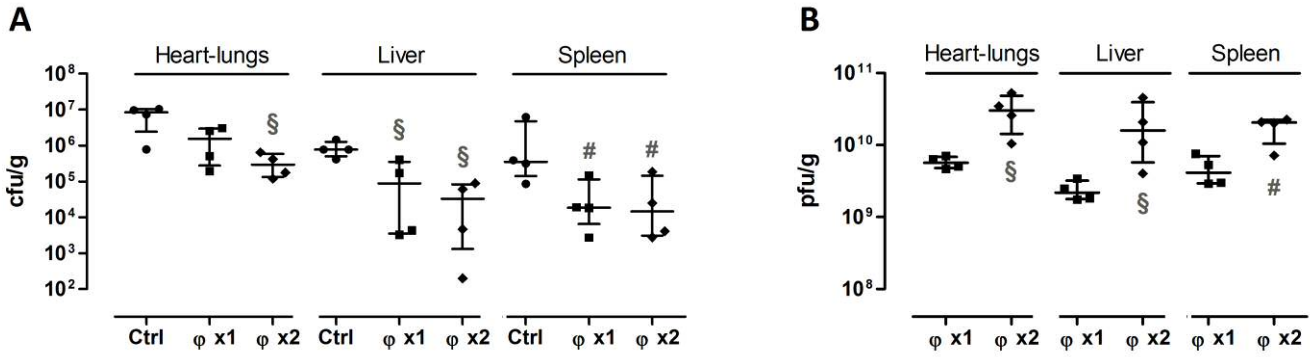


Figure 6. Bacteriophage LM33_P1 *in vivo* activity in a urinary tract infection model. Bacterial (panel A) and viral (panel B) counts 48 hours post-infection in kidneys homogenates of mice infected with $5x10^7$ cfu of strain LM33. Twenty four hours post-infection, the mice received intraperitoneally either PBS (Ctrl, n=13) or bacteriophage LM33_P1 (φ, MOI 200, n=10). The results are expressed as individual values with median and interquartile ranges (25^{th} and 75^{th} percentiles). *: p <0.001 compared to control group.

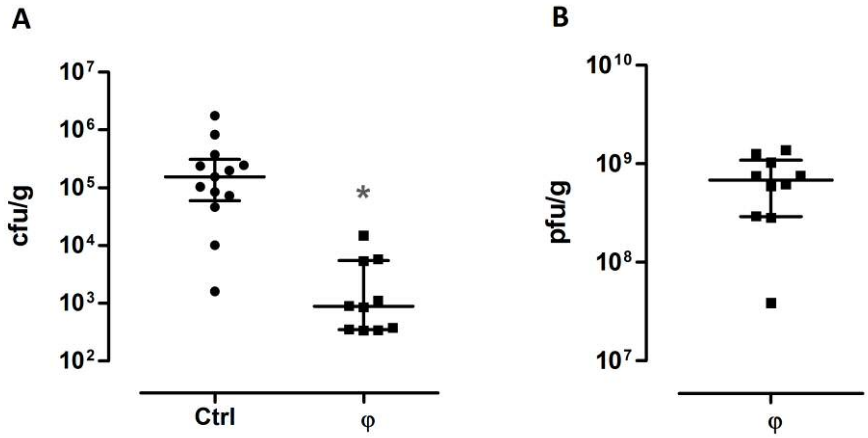


Table S1

Genomic characteristics of bacteriophage LM33_P1, its four closest homologs and the reference bacteriophage T7, all belonging to the *Autographivirinae* subfamily of viruses.

Bacteriophage	Host	Genome size (bp)	ORFs (n)	GC %	Accession number
LM33-P1	*E. coli*	38 979	49	50.8	PRJEB12445
T7	*E. coli*	39 937	60	49.0	NC_001604.1
PE3-1	*E. coli*	39 093	48	50.4	NC_024379.1
K1F	*E. coli*	39 704	43	49.8	NC_007456.1
EcoDS1	*E. coli*	39 252	53	49.9	NC_011042.1
Dev2	*C. turicensis*	38 966	45	52.6	NC_023558.1

Table S2

Data obtained during plaque test inhibition assays with different LPS extracts and randomly chosen couples of viruses-bacteria. (+)/(-): presence/absence of an inhibitory effect of LPS extract, – : not tested.

Interaction tested		**Inhibitory effect of various LPS extracts**			
Bacteriophage	Bacteria (serotype)	O25b (LM33)	O6 (536)	O17 (LM02)	O25a (ECOR51)
LM33_P1	LM33 (O25b)	(+)	(-)	(-)	(-)
" "	LM34 (O25b)	(+)	(-)	(-)	(-)
" "	LM36 (O25b)	(+)	(-)	(-)	(-)
" "	AVC02 (O25b)	(+)	(-)	(-)	(-)
" "	AVC03(O25b)	(+)	(-)	(-)	(-)
536_P1[a]	536 (O6)	(-)	(-)	–	–
423_P1[b]	H17 (O16)	(-)	–	–	–
416_P1[b]	LM49 (O2b)	(-)	–	–	–
LF82_P2[c]	LF82 (O83)	(-)	–	–	–
LF82_P2[c]	RY09 (O4)	(-)	–	–	–

[a] described in reference 31 in the manuscript, [b] bacteriophages isolated using ventilator-associated pneumonia (VAP) strains (423, 416) and active on others VAP strains (H17, LM49), [c] bacteriophage isolated using an adherent-invasive *E. coli* (LF82) and active on VAP strain RY09.

Figure S1

Distribution of the efficiency of plaquing values of bacteriophage LM33_P1. Dots represent individual values for each of the 64 strains tested whereas median (0.46) and 25th-75th percentiles [0.09-1.27] are indicated by lines. Y-axis is in Log scale.

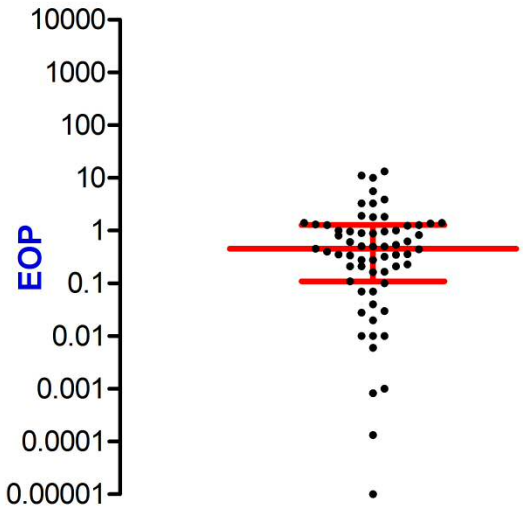


Figure S2

Predicted tertiary structure of the the C-terminal part of bacteriophage LM33_P1 tail fiber using Phyre², compared to its closest homolog (*Erwinia carotovora* endopolygalacturonase).

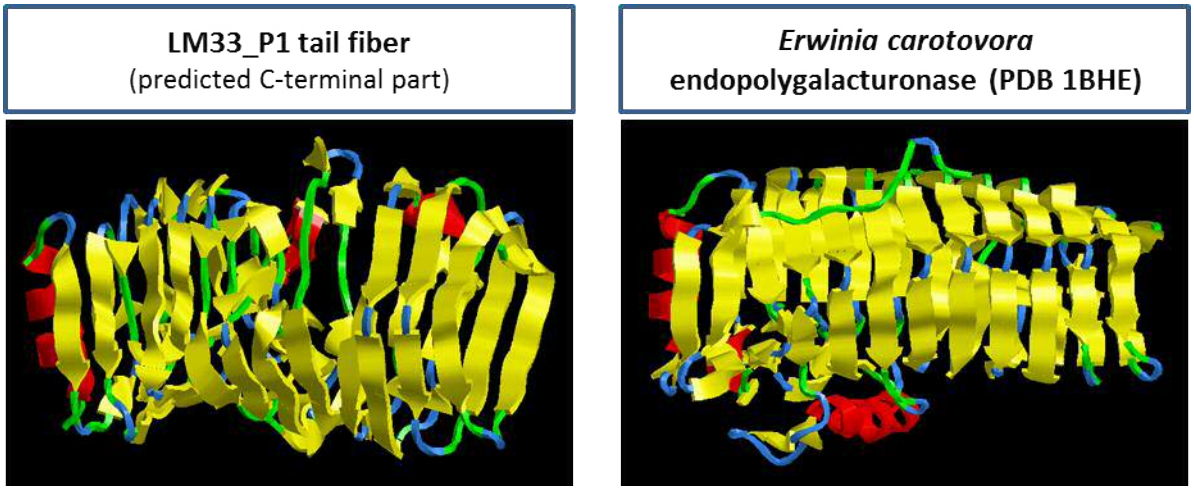


Figure S3

A. Adsorption of bacteriophage LM33_P1 on its host LM33. Strain LM33 grown in LB at 37 °C with shaking (100 rpm) was mixed with bacteriophage LM33_P1 at a MOI of 10^{-4} and aliquots were taken at the indicated time points. Dots represent the mean of 3 independent experiments with the standard deviation.

B. Bacteriophage LM33_P1 growth parameters. Bacteriophage LM33_P1 was mixed with strain LM33 (MOI 10^{-1}) at 37 °C with shaking. At the indicated time points, samples were analyzed in absence (grey lines) or in presence (black lines) of chloroform. The continuous lines represent the experimental measurements (mean of 3 independent experiments with standard error) while the dashed lines are the nonlinear regression obtained from these points.

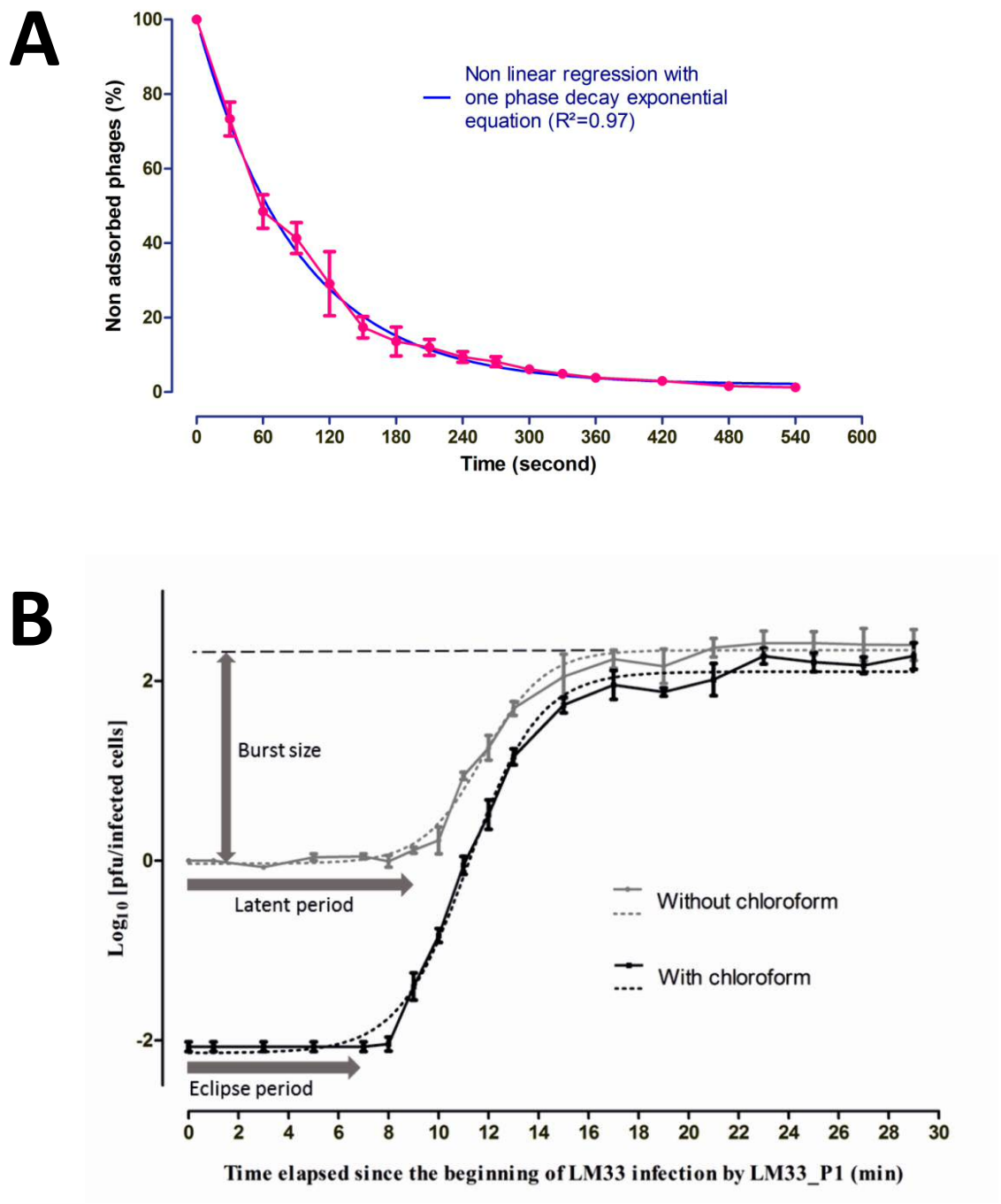


Figure S4

Lysis kinetics of strain LM33 by bacteriophage LM33_P1 at different multiplicity of infection. The **panel A** displays the first 22 hours of kinetic and the **panel B** displays a magnification of the first 2 hours. The lysis kinetics of the strain LM33 were performed as described previously (Maura et al., Environ Microbiol, 2012;14(8):1844-1854) in LB, at 37°C and with various multiplicity of infection (MOI). Optical density (600 nm) was followed over time and recorded each 15 minutes on a Glomax plate reader (Promega, Madison, USA). Strain LM33 without LM33_P1 was used as a control. All the conditions were performed in triplicates and the experiment was repeated three times independently (only one representative experiment is shown). For readability purpose, only mean of triplicates are displayed without the corresponding error bars.

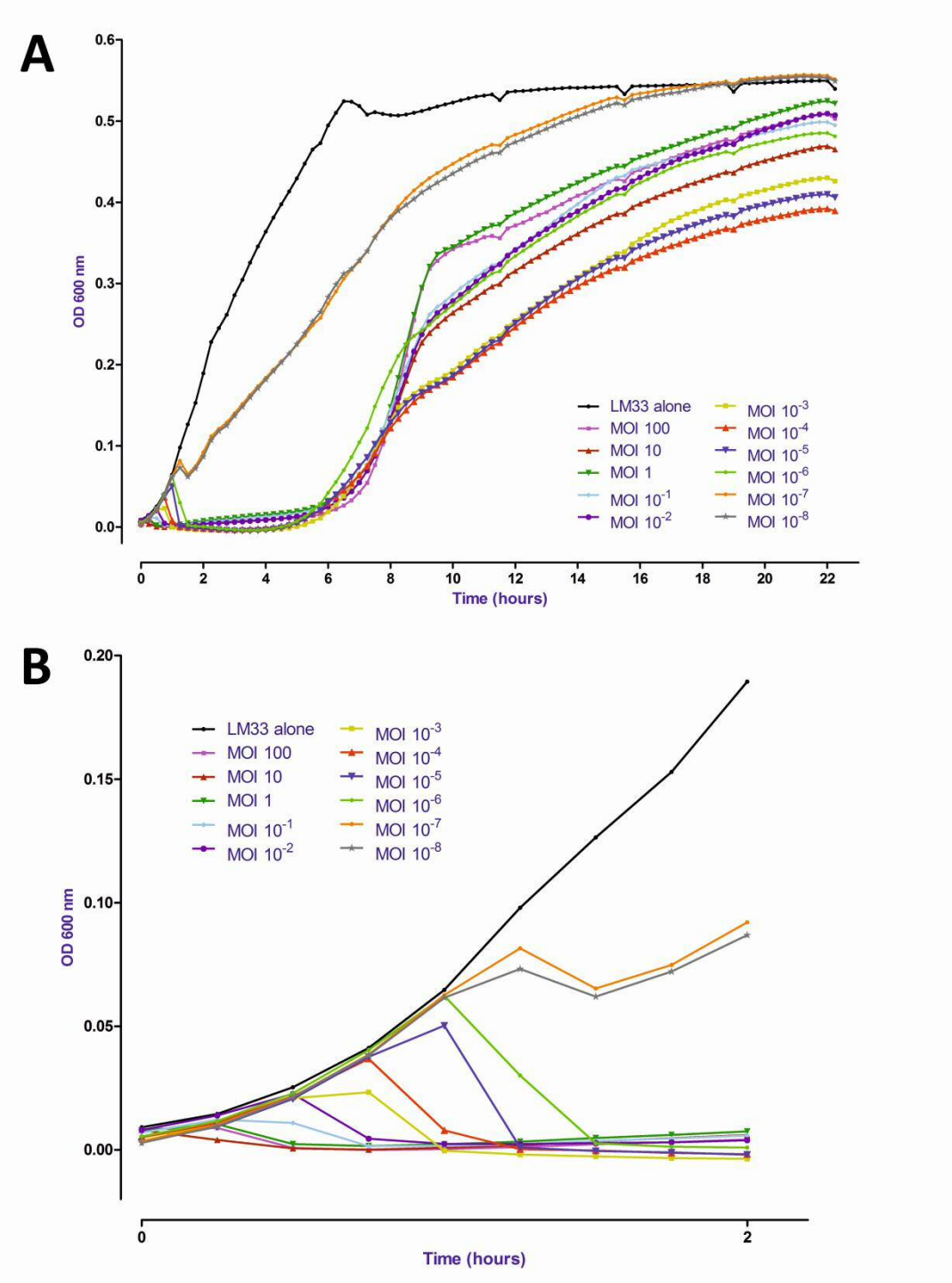
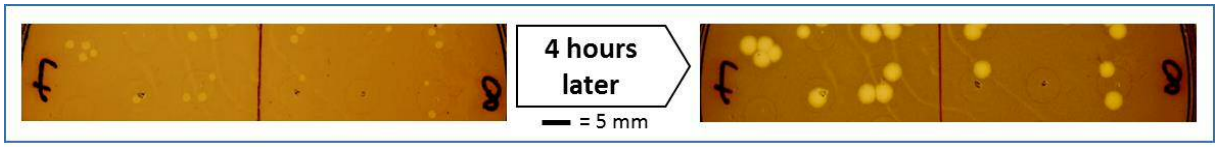


Figure S5

A. The size of the plaques made by bacteriophage LM33_P1 increases rapidly over time. LM33_P1 plaque-forming units on strain LM33 as visualized on LB-agar plate after 2 hours of incubation at 37 °C (left picture) and after 6 hours of infection (right picture).

B. A large halo surrounds plaques and area of lysis made by bacteriophage LM33_P1. Three drops of 10 µL of LM33_P1 ($5x10^3$ pfu/mL) have been dropped off on a bacterial host layer and incubated at 37 °C for 21 (left) and 36 hours (right). A halo is clearly visible at 36 hours, surrounding the area of lysis.

A

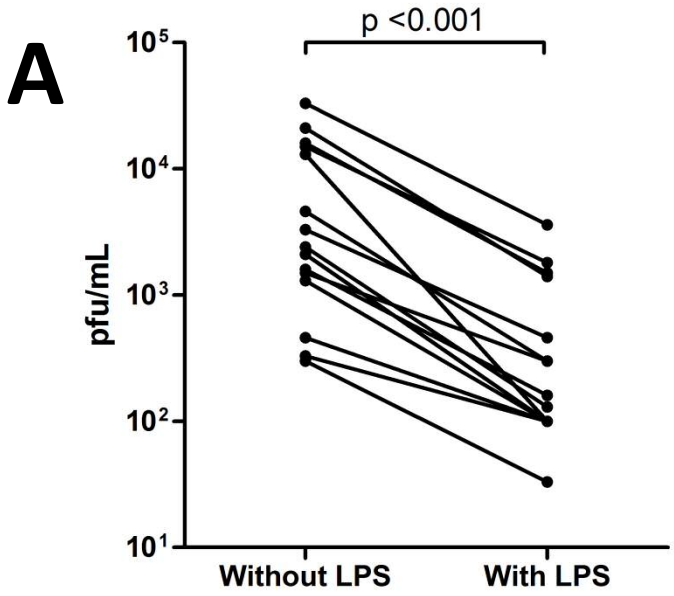


B



Figure S6

A: An O25b LPS extract inhibits interactions between bacteriophage LM33_P1 and O25b strains. Variations of bacteriophage titers from 15 individual plaque tests in presence or absence of O25b LPS extract are shown. For each individual assay, the same starting bacteriophage solution was titrated in triplicate, without and with LPS extract obtained from strain LM33. Each dot represents therefore the mean titer of the 3 replicates. Four O25b strains and different concentrations of LM33_P1 have been tested (see methods). **B: O25b LPS extract inhibits bacteriophage LM33_P1 activity in liquid medium.** The growth of strain LM33 in liquid broth was recorded every 15 minutes using optical density (OD600nm) in absence (red curve) or in presence of bacteriophage LM33_P1 (MOI of 10^{-6}) without (green) or with undiluted (black), 2-fold diluted (dark blue), 4-fold diluted (light blue) O25b LPS extract.



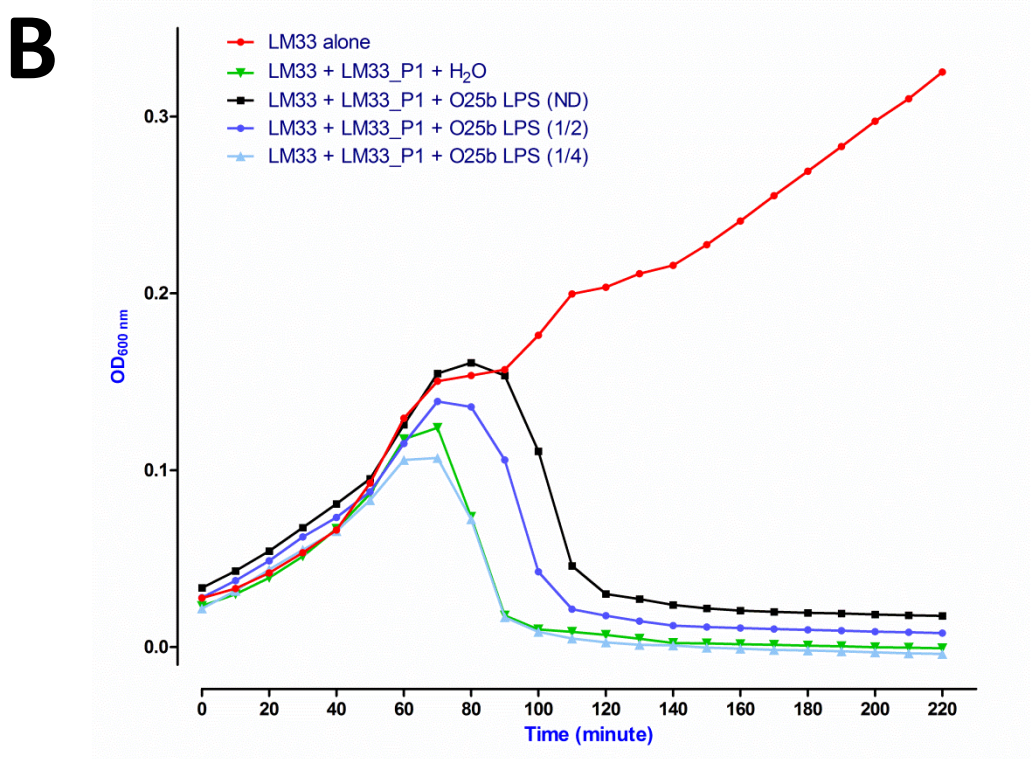


Figure S7

Bacteriophage LM33_P1 prevents O25 antibody-mediated aggregation of strain LM33. A commercially available specific O25 *E. coli* anti-serum, sold to perform O-antigen serotyping, was obtained from the Statens Serum Institut (Copenhagen, Denmark). The tests were performed using old liquid cultures of strain LM33 in stationary phase in LB (in order to slow down and delay bacterial lysis). Four conditions were tested, with the following mixes:

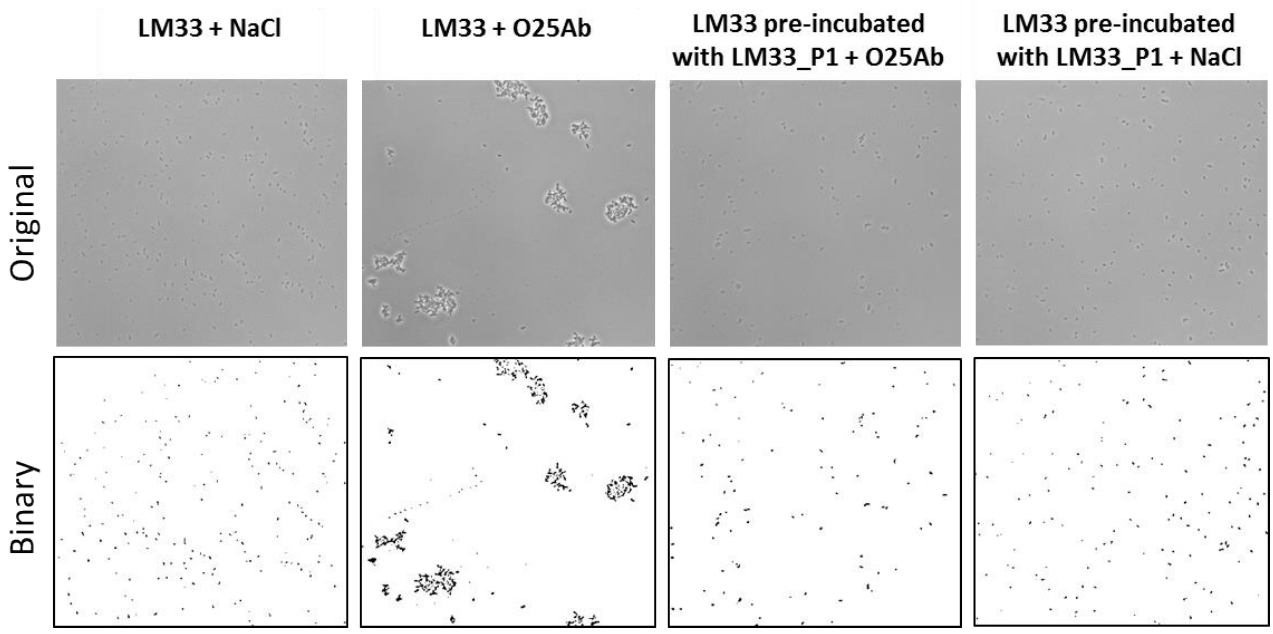
I- LM33 (5 µL) + Saline (NaCl 0.9%, 5 µL) + TN buffer (2 µL),

II- LM33 (5 µL) + O25Ab (5 µL, in NaCl 0.9%) + TN (2 µL),

III- LM33 (5 µL) pre-incubated 10 min at 20 °C with LM33_P1 (in TN, 2 µL, MOI of 10) + O25Ab (5 µL),

IV- LM33 (5 µL) pre-incubated 10 min at 20 °C with LM33_P1 (in TN, 2 µL, MOI of 10) + Saline (5 µL).

Following the incubation at 37 °C during 30 min, a drop (5 µL) of each condition was put on a glass slide and covered with a cover slip for direct examination under a phase contrast microscope. The upper line shows original pictures as obtained with a 40x magnification and the lower line represents a binary black and white transformation of the above pictures generated by ImageJ software (rsb.info.nih.gov/ij/).



Procedures

Allele-specific PCR amplification for the identification of the *fimH* alleles 22, 30 and 41

The primers for the *fimH* allele-specific PCR and the length of the PCR products were as follows:

fimH22.f (5'-TATTGGCGGTGGCAGCGCC-3'), *fimH22*.r (5'-GTTTCGCTGGTAGTAGGGAAA-3'), 234 bp;
fimH30.f (5'-CCGCCAATGGTACCGCTATT-3'), *fimH30*.r (5'-CAGCTTTAATCGCCACCCCA-3'), 354 bp;
fimH41.f (5'-TTTATGTAAACCTTGCGCCC-3'), *fimH41*.r (5'-AACATCACAGCCGCCAGTG-3'), 431 bp.

PCR reactions were carried out in a 20-µl volume containing 4 µl of 5X buffer (supplied with Taq polymerase), 10 pmol of each primer, 200 µM each dNTP, 2 U of Taq polymerase (Promega, Charbonnières-les-Bains, France), and 3 µl of bacterial lysate or 2 µl of DNA. PCR was performed with an Eppendorf Mastercycler with MicroAm tubes in the following conditions: denaturation 4 min at 94° C, 30 cycles of 5 sec at 94° C and 10 sec at 65°C, and a final extension step of 5 min at 72° C.

PCR products were loaded on 2% agarose gel with SYBR® Safe DNA gel stain (Invitrogen, Cergy-Pontoise, France). Following electrophoresis, gels were photographed under UV light.

The method was validated on a panel of *E. coli* strains from which the *fimH* sequence was available, i.e. 7, 19 and 47 strains exhibiting the *fimH* allele *H22*, *H30* and *H41*, respectively, as well as 67 strains exhibiting none of these alleles (Ochman H, Selander RK. J Bacteriol. 1984 Feb;157(2):690-3 ; Clermont O, Gordon D, Denamur E. Microbiology. 2015 May;161(Pt 5):980-8 ; Johnson JR, Clermont O, Johnston B, Clabots C, Tchesnokova V, Sokurenko E, Junka AF, Maczynska B, Denamur E. J Clin Microbiol. 2014 May;52(5):1358-65.). The sensitivity and the specificity of the method were of 100%.

Bacteriophage susceptibility testing

For bacteriophage susceptibility testing, we used the double spot test technique as screening method and EOP calculation for all susceptible strains. The double spot test consisted in dropping off 10 µL of a growing liquid culture of the bacterial strain (OD_{600nm} 0.5) on an agar plate. After drying, 1 µL of the bacteriophage solution (LM33_P1, 10^7 pfu/mL) was added on one half of the bacterial drop. The plate was then incubated at 37°C during 4-8 hours before reading. A susceptible strain was identified by the presence of a crescent-shaped lysis area on the bacterial drop or the visualization of individualized plaques. Efficiency of plaquing (EOP) was determined for all susceptible strains by titrating the solution of LM33_P1 on both its host (LM33) and the evaluated strain. EOP was calculated as the ratio of the number of plaques formed by the bacteriophage on the non-host strain to

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the number of plaques formed on its host, using the same bacteriophage solution. Only the strains for which individualized plaques were observed were considered as susceptible strains. For strain 81009 WT and its rough derivative mutant, tests were performed at 20 °C to turn-off type II capsule expression.

Characterization of bacteriophage LM33_P1

The adsorption assay and the one-step growth experiment were performed in triplicate, using Lysogeny Broth (Difco™ Bacto-Tryptone 10 g/L, Difco™ Yeast extract Difco 5 g/L, NaCl 5 g/L), under constant shaking (100 rpm) at 37°C, as described by Hyman and Abedon (Practical methods for determining phage growth parameters, Methods Mol Biol 501:175-202, 2009). A correlation curve was extrapolated from raw data using nonlinear regressions (GraphPad Prism 5.0, GraphPad software, California): a dose-response model was used for the one step growth experiment (Y=Bottom + (Top-Bottom)/(1+10^((LogEC50-X)*HillSlope)) with Y=log(pfu/infected cell) and X=time) and an exponential model with one phase decay for adsorption experiment (Y=(Y0 - Plateau)*exp(-K*X) + Plateau with Y=free phages(%), X=time). The growth parameters (eclipse and latent period, burst size) were then derived from these regressions. The adsorption constant was calculated as –p/N where p is the slope of the straight line obtained after a natural logarithm transform and N the concentration of bacteria at the beginning of the adsorption assay.

Sequencing of the strain LM33 and bacteriophage LM33_P1

The sequencing of bacteriophage LM33_P1 and strain LM33 was performed using Illumina sequencing technology (Illumina Inc., San Diego, CA). The LM33_P1 DNA was extracted from a purified bacteriophage solution, using DNase and RNase pretreatments followed by a phenol-chloroform extraction as described by Pickard (Pickard DJ, 2009, Preparation of bacteriophage lysates and pure DNA. Methods Mol Biol). The LM33 genomic DNA was extracted using a MaxWell Tissue DNA Purification kit (Promega, Madison, WI). Genomes annotation was performed by the MicroScope plateform for strain LM33 (Vallenet D, 2013, MicroScope: an integrated microbial resource for the curation and comparative analysis of genomic and metabolic data. Nucleic Acids Res) and with the RAST server for bacteriophage LM33_P1 (Aziz RK, 2008, The RAST Server: rapid annotations using subsystems technology. BMC Genomics), followed by manual curation.

Procedures

Murine experimental infections models

The animals were housed in authorized animal facilities in accordance with French and European regulations on the care and protection of laboratory animals. The protocols were approved of by the veterinary staff of the Institut Pasteur and INSERM animal facilities, as well as the National Ethics Committee regulating animal experimentation. The animals were housed in a SPF animal facility rated biosafety level 3 (Institut Pasteur) or 2 (INSERM). Food and drink were provided *ad libitum*. The mice were housed in separate ventilated cages corresponding to each group (control or phage-treated) and were visited at least twice daily to monitor health status. A daily weighing was performed and the mice were euthanized if weight loss greater than 20%, a limit that had never been reached here. The control group was always handled first (before the phage-treated group) to avoid phage contamination of the control group. The period of time between the infection and the treatment (phage or mock) was recorded and was identical between each group.

For each infection model, the results provided are the sum of two or three independent experiments.

The bacteriophage solutions administered in the animal experiments were obtained from purified stock (as described in Material and Methods) and were submitted to an additional purification procedure to reduce endotoxin level using an affinity chromatography-based endotoxin removal kit (EndoTrap blue, Hyglos, Germany).

Pneumonia was initiated by intranasal administration of $1x10^8$ cfu of strain LM33 on 20 anesthetized eight-week-old 25 g BALB/cJRj male mice (Janvier, Le Genest Saint Isle, France). The mice were treated using bacteriophage LM33_P1 four hours post-infection (n=12), either by using the intranasal route (multiplicity of infection of 50, *i.e.* a ratio of viruses to bacteria equal to 50) or the intraperitoneal route (MOI of 500). Control mice (n=8) received accordingly an intranasal or intraperitoneal identical volume of PBS (phosphate-buffered saline). The lungs were collected 17 hours post-infection on euthanized animals. An intraperitoneal administration of 1.25 µg (50 µg/kg) of buprenorphine (Buprecare; AST Farma, Oudewater, The Netherlands) was systematically performed on all the mice at 8 hours post infection to limit the pain and the dyspnea.

The septicemia model, as previously described, is essentially used to study the intrinsic extraintestinal virulence of *E. coli* isolates. Twelve four-week-old 17 g OF1 female mice (Janvier, Le Genest Saint Isle, France) were injected subcutaneously into the nape of the neck with $1x10^9$ cfu of strain H1659 (ST131-O25b:H4). Because of the high inoculum used, we tested both a single and a double dose of bacteriophages: the single dose (MOI 60) was administered by an intraperitoneal injection 2 hours post-infection (n=4) while the double dose consisted in an injection (MOI 60) administered 2 and 12 hours post-infection (n=4). Control mice (n=4) received an identical volume of PBS. Organs targeted by septic metastasis

Procedures

(heart-lung, spleen and liver) were collected on the animals that died between 24 to 30 hours post-infection.

The urinary tract infection model consists in a retrograde kidneys infection occurring after an intra-urethral injection of $5x10^7$ cfu of strain LM33 in the bladder (23 mice). Twenty-four hours after the infection, 8-week-old 17 g CBA/j female mice (Charles River, Chatillon-sur-Chalaronne, France) were treated intraperitoneally with LM33_P1 (MOI of 200, n=10) while control mice (n=13) received an identical volume of PBS. The kidneys were collected 48 hours post-infection.

The euthanasia of animals was performed by asphyxiation with rising concentration of CO_2. In every case, the organs were mechanically homogenized in cold PBS using a gentleMACS Octo Dissociator (Milteny Biotec, Bergisch Gladbach, Germany) before being serially diluted and spread on Drigalski agar plates containing appropriate antibiotic to numerate colony, in triplicate. The bacteriophage count was performed in triplicate on supernatant after centrifugation of the homogenates according to routine methods.