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Investigating the use of bacteriophages as a new decolonization strategy for intestinal carriage of CTX-M-15-producing ST131 *Escherichia coli*: An in vitro continuous culture system model

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ABSTRACT

Objectives: We investigated the use of bacteriophages as a strategy to decolonize intestinal carriers of multidrug-resistant *Escherichia coli*.

Methods: A fermentor was used as a continuous culture system for 48 h. Two different pools of faeces (studies I and II) obtained from volunteers were spiked with a CTX-M-15-producing ST131 *E. coli* (strain 4901.28) susceptible to bacteriophages and challenged with three doses of INTESTI Bacteriophage cocktail administered at 2, 6 and 10 h after the inoculum. Bacterial typing was performed by implementing microdilution panels, spot test, rep-PCR and whole-genome sequencing (including cgMLST and single-nucleotide variant analysis) obtained using Nanopore and Illumina platforms.

Results: In study I, bacteriophages decreased the numbers of 4901.28 dramatically ($\leq 10^1$ CFU/mL after 6 h). In contrast, during study II, a phage-resistant mutant of 4901.28 persisted in the continuous culture (10^4 CFU/mL at 48 h). Whole-genome sequencing revealed the presence of two additional plasmids in the mutant as well as 11 single-nucleotide variants, including one chromosomal in a glycosyltransferase family 2 protein that is responsible for the transfer of sugars to polysaccharides and lipids. In both studies, the commensal *E. coli* population remained unchanged by the phage treatment maintaining itself at 10^8 CFU/mL.

Conclusions: Our data indicates that bacteriophage cocktails may be implemented to decolonize some intestinal carriers. However, the individual microbiota composition may have an impact on the development of phage resistance. Mechanisms underlying this phenomenon are likely to be various and complex. Further in vivo studies and protein expression experiments are needed to confirm our observations and hypotheses.

1. Introduction

Multidrug-resistant (MDR) *Escherichia coli* are spreading worldwide due to hyperepidemic high-risk clones; among them, those of sequence type (ST) 131 are of particular concern. This lineage is a major driver of antibiotic resistance and is recognized as a highly prevalent, uropathogenic and pandemic clone harbouring numerous virulence factors. Clinical isolates of ST131 usually display an MDR phenotype, in which the extended-spectrum β-lactamases (ESBLs) are the main resistance mechanism (especially the CTX-M-15). The reasons behind the success of ESBL-producing ST131 *E. coli* expansion and dissemination on large scale are still to be elucidated. However, the main reasons are likely to be colonization at an intestinal level as well as prolonged persistence [1,2].

Notably, intestinal colonization with MDR organisms (MDROs) has four main consequences: (1) risk to spread these pathogens in the environment [1,3]; (2) cross-transmission among people and/or animals [4,5]; (3) risk to sporadically developing untreatable infections (e.g. bloodstream and urinary tract infections) [6,7]; and (4) risk of a lifelong carriage of MDROs with consequent potential horizontal transfer of resistance genes (e.g. via plasmids) to indigenous bacterial species in the gut [8,9].

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Several strategies aimed to decrease the density and relative abundance of MDR Gram-negatives at intestinal level have been suggested [10,11]. For example, it has been proposed that selective digestive decontamination using broad-spectrum antibiotic(s) administered for short periods be used. However, for Gram-negatives, only a few studies have examined its efficacy, especially to decolonize healthy carriers from ESBL-producing Enterobacterales [12,13]. This strategy seems not to completely eradicate the targeted strain, but rather decrease its number, which could lead to gut recolonization [13]. Moreover, these antibiotic-based approaches have the major disadvantage of reducing species diversity in the intestinal microbiota. This can lead to disrupted colonization resistance, increasing the risk for developing infections and resistance against last-line antibiotics [14,15].

More recently, the faecal microbiota transplantation, other than for preventing recurrent *Clostridium difficile* infections, has been implemented to lower the density of MDROs (alone or preceded by short courses of antibiotics). Although promising preliminary results have been recorded, a major drawback is patient compliance due to the difficult to accept nature of treatment [16]. Therefore, standardized, easy to use and effective strategies to decolonize intestinal carriers of MDROs are still not available.

In this overall context, bacteriophages could represent a new and alternative approach. Some of these bacterial viruses are highly species-specific - namely, with the potential to spare commensal populations selectively, unlike an antimicrobial treatment. Moreover, thanks to their self-propagating nature, they display a self-limiting action in the presence of the targeted bacterial species. However, although they have been part of the standard therapy regimens in Russia, Georgia and Poland for 100 years, they have yet received very little attention in the West [17,18]. As a consequence, we are facing a lack of rigorous scientific studies analyzing their efficacy for treating and preventing human infections [19].

To the best of our knowledge, bacteriophages have never been studied in the context of human intestinal decolonization of MDR *E. coli*. Therefore, we investigated the use of a commercial preparation of bacteriophages as a gut decolonization strategy against an ESBL-producing *E. coli* belonging to the pandemic ST131 lineage in a simplified in vitro model of intestinal colonization.

2. Materials and methods

2.1. Bacterial typing

E. coli strain 4901.28 was used as the wild-type (WT) targeted strain. It was isolated from a urine sample of a 69-year-old woman [7]. The isolate was previously characterized by phenotypic methods (MICs determined using the Sensititre GNX2F and ESB1F plates; Thermo Fisher Diagnostics, Waltham, MA) and genotypic methods (characterization of *bla* genes, multilocus sequence typing and plasmid replicon typing) [7]. In the present work, *E. coli* 4901.28 underwent whole-genome sequencing (WGS) analysis along with one representative bacteriophage-resistant mutant (see later).

2.2. Continuous culture system

A 2-L glass fermentation vessel, operated under the control of a New Brunswick™ BioFlo®/CelliGen® 115 Unit (Eppendorf, Hamburg, Germany) was chosen as the in vitro system (chemostat). The starting volume of the vessel was 1 L and the growth medium implemented was Brain Hearth Infusion (BHI) broth (Becton Dickinson, Franklin Lakes, NJ). Fresh sterilized medium was added via a peristaltic pump at a constant rate of 18 mL/h and waste culture liquid was removed at the same rate. The system was operated in aerobic conditions and the temperature was maintained at 37 °C using circulating water in the double wall. Moderate agitation at 70 rpm was applied.

2.3. Characterization of donor stools and preparation of faecal inoculum

Fresh faeces from healthy volunteers negative for extended-spectrum, cephalosporin-resistant Enterobacterales (ESC-R-Ent) were chosen for the experiments. Screening to confirm negativity was performed to detect ESC-R- and carbapenem-resistant Enterobacterales as previously done [5,8,9,20]. Briefly, ~20 μg of fresh stools was enriched overnight in 10 mL of Luria-Bertani (LB) broth containing a 10-μg disk of cefuroxime. Then, 100 μL was plated on ChromID ESBL (bioMérieux, Marcy-l'Étoile, France) and home-made SuperCarba (bioMérieux) selective plates. After overnight incubation, selected colonies were identified using matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS; Bruker, Billerica, MA).

Two different pools of faeces were tested (pool A for study I and pool B for study II), with each coming from three noncolonized volunteers and corresponding to a combined total of 1 g. Stools were uniformly suspended in 10 mL BHI and vigorously vortexed for 2 to 3 min. Homogenized faeces were equilibrated in a 37 °C incubator for approximately 15 min before starting the experiment. The chemostat vessel was then inoculated through a port in the top with the faecal suspension (1 g in 10 mL); after 15 min, the first time point sample (T_0) was taken.

2.4. Bacteriophages

INTESTI Bacteriophage (lot no. M2-801; Eliava Bio Preparations, Tbilisi, Georgia) was used as an antimicrobial agent to target *E. coli* 4901.28 selectively. This preparation represents a sterile filtrate phage lysate (total, $1 \times 10^{5-6}$ PFU/mL) of several pathogenic *E. coli*, *Shigella* spp., *Salmonella* spp., *Proteus vulgaris*/*mirabilis*, *Pseudomonas aeruginosa*, *Staphylococcus* spp. and *Enterococcus* spp. This biopreparation has been fully characterized with a metagenomic approach [21].

Susceptibility to the INTESTI Bacteriophage cocktail was determined by implementing the spot test with the double-agar method (opaque lysis/++ is part of the sensible phenotype scale; R stands for phage-resistant) after two passages on BHI plates [22]. Notably, *E. coli* 4901.28 was fully susceptible to the INTESTI cocktail [23].

2.5. Study design

In a first blank experiment (with pool A of faeces), 4901.28 was added (see later) in the chemostat system 30 min after the faecal inoculum (i.e. T_0 plus 15 min) to evaluate the growth trend of the pathogen compared with the total *E. coli* microbial population in the chemostat system (Fig. 1).

A second experiment consisted of investigating whether 4901.28 was able to maintain itself despite the introduction of INTESTI Bacteriophage cocktail aliquots. Specifically, three doses of 1-mL undiluted cocktail were added to the chemostat at T_2, T_6 and T_{10}. This experiment was performed in duplicate (experiments a and b) and also with two different pools of faeces (study I with pool A and study II with pool B). All experiments were conducted for 48 h, during which 20 time points were taken (15 time points for the first day and 5 for the second day). Graphs were generated with GraphPad Prism 7.0 (GraphPad Software, La Jolla, CA).

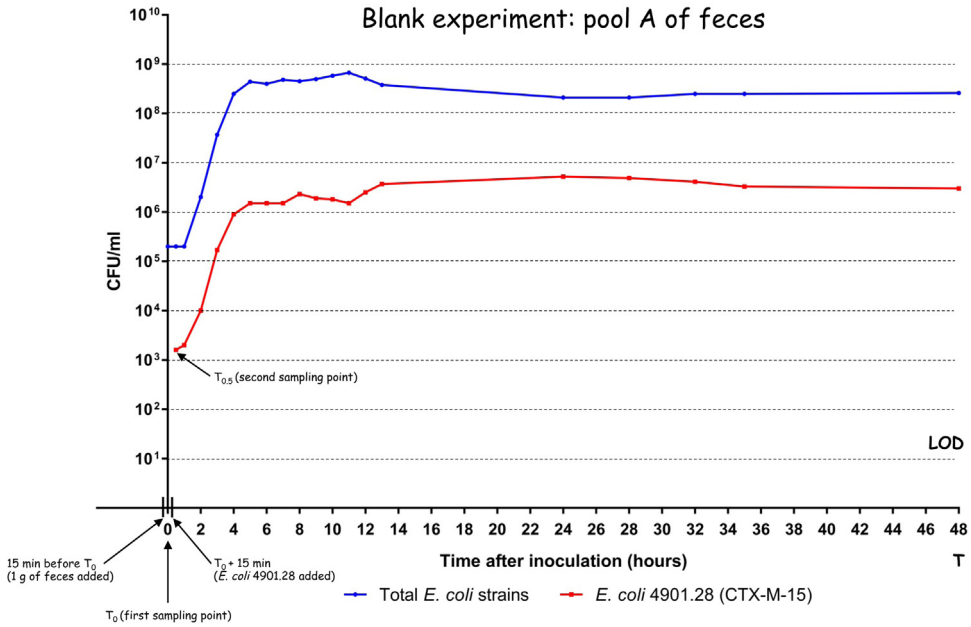

Fig. 1. Blank experiment of *E. coli* dynamics without bacteriophage treatment. Shown are the dynamics of the faecal *E. coli* community and *E. coli* 4901.28 alone in the chemostat system in the absence of bacteriophages (pool A of faeces, as for Study I). Faeces were inoculated into the chemostat 15 min before T_0 (first sampling point). Blue line, total *E. coli* population; red line, CTX-M-15-producing *E. coli* ST131 4901.28. LOD, Limit of detection (10^1 CFU/mL). The graph was generated with GraphPad Prism 7 on data from one experiment. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

2.6. Bacterial inoculum and population dynamics

E. coli 4901.28 was grown overnight on a MacConkey agar plate (Becton Dickinson, East Rutherford, NJ). Colonies were suspended in sterile NaCl, 0.9%, to reach a concentration of 1.2×10^8 CFU/mL (corresponding to 0.4 on the McFarland scale); then 80 μL of this suspension was added in 10 mL BHI to reach a total final concentration of 10^7 CFU. The 10 mL were finally poured into the 1-L BHI contained in the chemostat vessel 15 min after T_0. After an additional 15 min ($T_{0.5}$), the second sample was taken to measure the starting number (CFU/mL) of the targeted strain.

At each time point (from T_0 to T_{48}), the cultivable microbiota was monitored by removing 5 mL of sample from the vessel; 1 mL was serially diluted in phosphate-buffered saline (PBS) and plated on CHROMagar™ Orientation (Paris) plus vancomycin (8 μg/mL; for the total *E. coli* count) and on CHROMagar™ Orientation plus vancomycin (8 μg/mL) and cefotaxime (2 μg/mL) (for selective ESBL-*E. coli* ST131 count). Plates were incubated overnight at 37 °C, and the next day only violet colonies (corresponding to *E. coli* sp.) were counted. Finally, sample aliquots were prepared; 1 mL/each sample was stored at −80 °C in 20% glycerol, and the remaining 3 mL was used for the viral titration (see later).

2.7. Viral population dynamics

The bacteriophage population was monitored by titration using the double-agar method on the host strain (*E. coli* 4901.28). At day 1, titration was performed at T_3, T_5, T_7, T_9, T_{11} and T_{13} (for ExIa, T_{10} was taken instead of T_9); on day 2, it was performed at each time point (T_{24}, T_{28}, T_{32}, T_{35} and T_{48}). Briefly, 1 mL of the undiluted chemostat sample was filtrated through a 0.22-μm syringe filter (Carl Roth GmbH, Karlsruhe, Germany) and further serially diluted up to 10^{-7} times. Then, 100 μl of 4901.28 (concentration of 1.5×10^8 CFU/mL) were supplemented with 1 mL of the dilutions 10^{-1}, 10^{-3}, 10^{-5} and 10^{-7} and with 5 mL of BHI soft agar (0.7%). The solutions were then poured on BHI agar plates and incubated for 24 h at 37 °C. Plaques were counted the next day to calculate the viral titre.

2.8. Repetitive extragenic palindromic polymerase chain reaction (rep-PCR)

The clonal relatedness of *E. coli* strains recovered from samples was studied using rep-PCR. Briefly, violet colonies were picked from CHROMagar™ Orientation plates supplemented with cefotaxime, followed by DNA extraction with Chelex® 100 sodium form (Merck KGaA, Darmstadt, Germany). Extracts were subjected to rep-PCR, and the resulting PCR products were run on a DNA chip (Agilent Technologies, Bethlehem, PA) using the Agilent 2100 Bioanalyzer (Agilent) [7,24,25].

2.9. Genotyping

WGS was carried out using both MinION (Oxford Nanopore, Oxford Science Park, UK) and HiSeq (Illumina, San Diego, CA), as previously described [25–28]. In brief, total DNA was extracted with the QIAamp Mini Kit (Qiagen, Hilden, Germany). For MinION, the SQK-LSK108 2D ligation sequencing kit, a R9.5 SpotON flow cell and MinION Mk1B device (Oxford Nanopore) were used for the 24-h run. Data acquisition, as well as base calling, was carried out with the MinKNOW software (Oxford Nanopore). Raw reads were converted to fastq with Poretools and assembled de novo with the Canu pipeline. For Illumina sequencing, reads were first trimmed with Trimmomatic software and then aligned to MinION contigs using Burrows-Wheeler Alignment (BAM) and Sequence Alignment/Map (SAM) for file conversion. FASTA sequences of each corrected contig were extracted from Geneious software and interpreted with Res-, Plasmid, Virulence-Finder (https://cge.cbs.dtu.dk/services), CRISPRCasFinder (https://crisprcas.i2bc.paris-saclay.fr/CrisprCasFinder/Index) and CRISPRone (http://omics.informatics.indiana.edu/CRISPRone).

In addition, assemblies of the raw Illumina reads with SPAdes Software were used for core genome MLST (cgMLST) analysis by implementing the cgMLSTFinder (https://cge.cbs.dtu.dk/services). Single-nucleotide variant (SNV) analysis was implemented to compare the chromosomes of 4901.28 and the phage-resistant mutant (ExIIa_T32_C2). Briefly, the core genome alignment was

performed with Parsnp v1.2 (https://github.com/marbl/parsnp). All strains were treated as curated genomes (–c parameter), and the chromosomal hybrid assembly of the mutant was used as a reference genome to fine-tune the core genome alignment, including only chromosomal sequences and excluding the plasmid ones. To maximize genome coverage across all genomes, the –c parameter was optimized to 6. Other parameters were set as default. Variant Call Format (VCF) data from Parsnp core genome alignment were extracted from the Gingr formatted binary archive output with Harvest-Tools v1.2 (https://github.com/marbl/harvest-tools). Core genome alignment coverage was determined with Gingr v1.2 (https://github.com/marbl/gingr). Variants with no flags (PASS) were determined as reliable [29] and used for downstream SNV analysis with a custom R v3.6.2 script (https://www.r-project.org). The translate tool ExPASy (http://www.web.expasy.org/translate) and followed by Protein BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) were finally used to identify and compare amino acid (AA) substitutions. Annotations of both hybrid and Illumina assemblies were conducted by the NCBI Prokaryotic Genome Annotation Pipeline.

2.10. GenBank accession numbers

These accession numbers are as follow: hybrid assembly (BioProject: PRJNA551948) for 4901.28: VMRI00000000 (chromosome, VMRI01000001, plasmid A, VMRI01000002); for ExIIa_T32_C2: VMRH00000000 (chromosome, VMRH01000003-VMRH01000006 - plasmid A, VMRH01000001 plasmid B, VMRH01000007, plasmid C, VMRH01000002); Sole Illumina (BioProject: PRJNA605932) for 4901.28: JAAHTE000000000; for ExIIa_T32_C2: JAAHTF000000000.

3. Results

3.1. E. coli *dynamics without bacteriophage treatment*

In the blank experiment, both monitored populations (*E. coli* 4901.28 and the total *E. coli*) exponentially increased for the first 5 h and then reached a plateau from T_5 to T_{48}. In particular, *E. coli* 4901.28 reached a stationary phase at a population size of 10^6 CFU/mL, whereas the total *E. coli* microbial population stabilized itself at 10^8 CFU/mL (Fig. 1).

3.2. E. coli *dynamics with three doses of bacteriophages and pool A of faeces (study I)*

For the first pool of faeces, phage treatment resulted in an immediate decrease of the population size of 4901.28 (from 10^5 to 10^1 CFU/mL) 2 h after inoculation of the first dose of phages. Moreover, after stopping phage treatment, the population of the target MDR pathogen never restored itself. On the other hand, the total *E. coli* microbial community was maintained constant despite the phage treatment (i.e. increasing for the first 7 h and then maintaining itself at 10^8 CFU/mL). Similarly, the bacteriophage population increased during the first 3 to 6 h to 10^7 PFU/mL and then, after 12 h, stabilized itself at 10^6 PFU/mL (Fig. 2; Supplementary Table S1).

3.3. E. coli *dynamics with three doses of bacteriophages and pool B of faeces (study II)*

For the second pool of faeces, as in study I, 4901.28 increased for the first 4 h and dropped below the limit of detection (LOD) 2 h after inoculation of the first treatment dose (T_5; Fig. 3; Supplementary Table S1). However, in contrast to study I, a phage-resistant population started to emerge after T_5. It then continued to grow with some oscillations during the second (T_6) and third (T_{10}) doses of cocktail treatment, eventually stabilizing itself at 10^{3-4} CFU/mL. We also noted that the total *E. coli* population showed similar dynamics as study I and blank experiments (i.e. increasing for the first 8 h and reaching a plateau of 10^8 CFU/mL). In contrast, the bacteriophage population showed a more rapid and higher titre than observed in study I (i.e., at 5 h, 10^9 PFU/mL, which then stabilized at 10^8 PFU/mL after about 12 h).

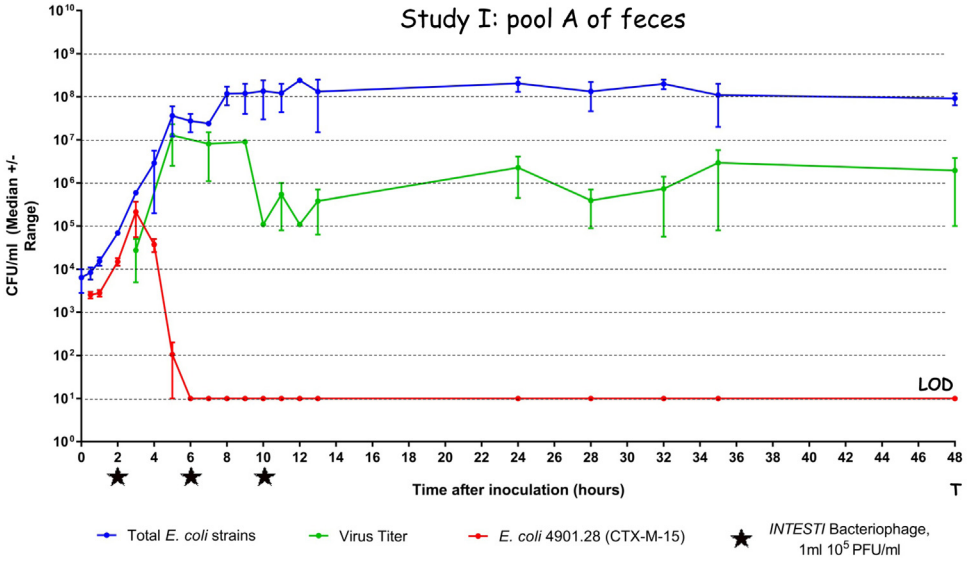

Fig. 2. Study I: *E. coli* dynamics with three doses of INTESTI Bacteriophage cocktail and pool A of faeces. Shown is the influence of bacteriophage treatment on the faecal *E. coli* community and on *E. coli* 4901.28 performed in the chemostat system with the first pool (A) of faeces. Faeces were inoculated into the chemostat 15 min before T_0 (that was the first sampling point). Blue line, total *E. coli* population; red line, CTX-M-15-producing *E. coli* ST131 4901.28; black stars, administered bacteriophage doses. LOD: Limit of detection (10^1 CFU/mL). The graph was generated with GraphPad Prism 7 on data from two experiments. Appearance, median and error. Plot, range. Error bars are not drowned by the software when shorter than the height of the symbol. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

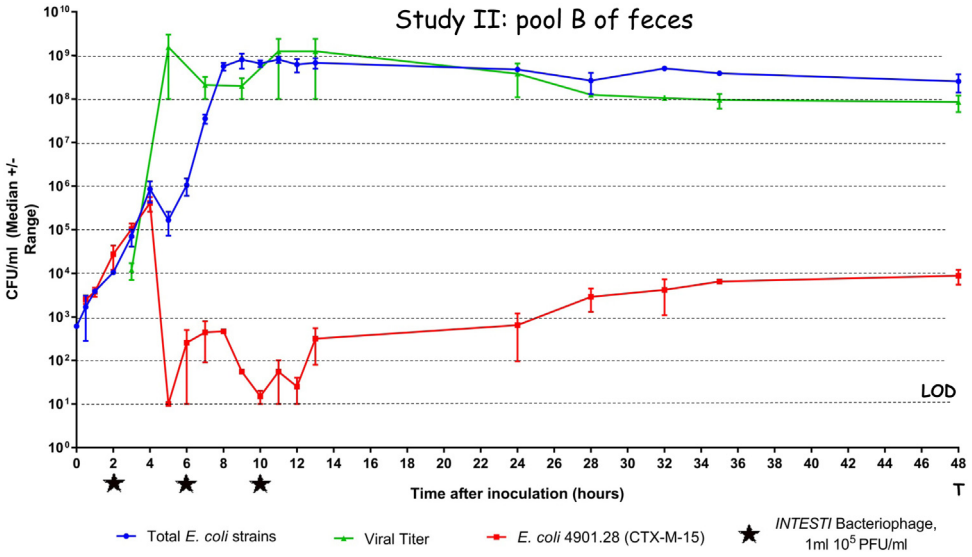

Fig. 3. Study II: *E. coli* dynamics with three doses of INTESTI Bacteriophage cocktail and pool B of faeces. Shown is the influence of bacteriophage treatment on the faecal *E. coli* community and on *E. coli* 4901.28 performed in the chemostat system with the second pool (B) of faeces. Faeces were inoculated into the chemostat 15 min before T_0 (that was the first sampling point). Blue line, total *E. coli* population; red line, CTX-M-15-producing *E. coli* ST131 4901.28; black stars, administered bacteriophage doses. LOD, limit of detection (10^1 CFU/mL). The graph was generated with GraphPad Prism 7 on data from one duplicate experiment. Appearance, median and error. Plot, range. Error bars are not drowned by the software when shorter than the height of the symbol. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.4. Characterization of phage-resistant mutants

For study II, six regrowing cefotaxime-resistant *E. coli* colonies taken from time points T_{28} and T_{32} of experiment IIa (ExIIa) and two from T_{35} and T_{48} from experiment IIb (ExIIb) were isolated and analyzed. In particular, their rep-PCR profiles were identical to each other, but slightly different compared with 4901.28 (i.e. with three less intense or absent bands; Supplementary Fig. S1). One of these cefotaxime-resistant isolates (strain ExIIa_T32_C2), recovered during study IIa at T_{32} and phenotypically resistant to the phage cocktail using the spot test, was randomly chosen as a representative strain for further analyses.

As shown in Table 1, the phenotype, ST, plasmid replicons and resistance genes of ExIIa_T32_C2 were identical to those of the WT strain *E. coli* 4901.28. WGS data of ExIIa_T32_C2 revealed the presence of two additional plasmids of 4 kb and 7 kb (plasmids B and C, respectively), as compared with *E. coli* 4901.28, which originally only carried a 170-kb $bla_{CTX\text{-}M\text{-}15}$-positive plasmid (plasmid A). Plasmid A carried several resistance genes, the virulence factor for increased serum survival and the three replicon types FII, FIB, and FIA as well as the colicinogenic marker Col156. Plasmid B carried five genes encoding two replication proteins and three that were functionally uncharacterized. Plasmid C carried eight genes encoding proteins for mobilization, replication, conjugal transfer and unknown function ($n = 2$ each). Resistance genes or virulence factors were not found in plasmids B and C (Table 1).

Large chromosomal deletions or insertions were not detected in the mutant. However, core genome analysis revealed that ExIIa_T32_C2 possessed 11 chromosomal SNVs compared with the WT strain (Table 2). Three were located in the IS*3* family transposase gene, and the remaining were in the AAA family transposase, glycosyltransferase family 2 protein (transfer of nucleotide-diphosphate sugars to polysaccharides and lipids), IS*66* family transposase, hypothetical protein, DUF945 domain-containing protein (domain of unknown function), RadC family protein (DNA repair and recombination protein) and polB (DNA polymerase β) genes and one in a noncoding region (Table 2). Finally, CRISPR-*cas* analysis showed only the presence of questionable CRISPR spacers and the complete absence of *cas* genes (data not shown).

4. Discussion

E. coli belonging to ST131 is responsible for the increasing prevalence and spread of cephalosporin resistance worldwide. Particularly worrisome is the silent carriage at the intestinal level, which may translate into future difficult to treat infections [1]. Efforts to try decolonizing the gut using antibiotic treatment can cause disturbance of the normal bacterial flora, leading to overgrowth of pathogenic strains (exogenous or already present in the gut) [30]. As an alternative, bacteriophages could help maintain colonization resistance (i.e. protection by the endogenous flora against pathogenic bacteria) at the physiological level.

4.1. The in vitro model

Operated with 1-L volume and spiked stool, our system can host both the pathogenic strain and commensal *E. coli* populations. Moreover, compared to more simplistic in vitro systems, this continuous culture approach allows us to come a step closer in mimicking the in vivo conditions of the gut (e.g. through introduction of fresh nutrients and elimination of leftovers in the chemostat). However, the aerobic conditions used are not able to comprehensively reflect the complex diversity of bacterial populations comprehensively that are present in the bowel (i.e. for a total of 10^{11} CFU/g of faeces) [31]. Anaerobic species could play a role in colonization resistance and could modulate the population size of the targeted ST131 *E. coli* strain, with consequent influence on the success or failure of phage treatment.

Nevertheless, among the enriched facultative anaerobe Enterobacterales, we could observe a total count of *E. coli* of about 10^8 CFU/mL, in line with concentrations recovered in vivo (i.e. reaching 10^{8-9} CFU/g of faeces in the gut) [32]. Concerning the dosage protocol, we chose to administer multiple doses to simulate a continuous treatment because the effectiveness of phage therapy is known to be correlated to the dosage and treatment time point. Several studies have shown that early administration of multiple

Table 1
Molecular and phenotypic features of phage-sensitive WT strain 4901.28 and phage-resistant mutant ExIIa_T32_C2.

Characteristics	*E. coli* 4901.28	*E. coli* ExIIa_T32_C2 (mutant)
ASTs (MICs, μg/mL)[a]	P/T4 (≤8/4), FOT (>32), TAZ (16), FEP (16), AZT (>16), ETP (≤0.25), GEN (8), AMI (16), CIP (>2), SXT (>4/76), DOX (16), TGC (1), COL (≤0.25), FOX (≤4), AMP (>16), T/C (≤0.12/4), F/C (≤0.12/4)	P/T4 (≤4/4), FOT (>32), TAZ (16), FEP (8), AZT (>16), ETP (≤0.25), GEN (≤4), AMI (>32), CIP (>2), SXT (>4/76), DOX (16), TGC (0.5), COL (≤0.25), FOX (≤4), AMP (>16), T/C (≤0.12/4), F/C (≤0.12/4)
Spot test results[b]	++	R
ST	131	131
Plasmid Finder (replicon)		
Plasmid A (170 kb)	FII, FIB, FIA, Col156	FII, FIB, FIA, Col156
Plasmid B (4 kb)	NA	Col (BS512)
Plasmid C (7 kb)	NA	–
ResFinder (resistance genes)[c]		
Chromosome	*mdf(A)*	*mdf(A)*
Plasmid A (170 kb)	*bla*$_{CTX-M-15}$, *bla*$_{OXA-1}$, *aadA5, aacA4, aac(6′)-Ib-cr, mph(A), catB3, sul1, dfrA17, tet(A)*	*bla*$_{CTX-M-15}$, *bla*$_{OXA-1}$, *aadA5, aacA4, aac(6′)-Ib-cr, mph(A), catB3, sul1, dfrA17, tet(A)*
Plasmid B (4 kb)	NA	–
Plasmid C (7 kb)	NA	–
VirulenceFinder (virulence genes)[d]		
Chromosome	*gad, iha, sat, nfaE, iss*	*gad, iha, sat, nfaE, iss*
Plasmid A (170 kb)	*senB*	*senB*
Plasmid B (4 kb)	NA	–
Plasmid C (7 kb)	NA	–

[a] AMI, Amikacin; AMP, ampicillin; ASTs, Antimicrobial susceptibility tests (MICs interpreted according to EUCAST 2019, version 9.0, except for doxycycline for which CLSI 2019, M100-S29, was used); AZT, aztreonam; CIP, ciprofloxacin; COL, colistin; DOX, doxycycline; ETP, ertapenem; F/C, cefotaxime-clavulanic acid; FEP, cefepime; FOT, cefotaxime; FOX, cefoxitin; GEN, gentamicin; NA, not applicable; –, no output (genes not previously annotated); P/T4, piperacillin/tazobactam; ST, sequence type; SXT, trimethoprim-sulfamethoxazole; TAZ, ceftazidime; T/C, ceftazidime-clavulanic acid; TGC, tigecycline.
[b] Spot test performed with the double-agar method where 'opaque lysis/++' is part of the sensible phenotype scale, and 'R' stands for phage-resistant.
[c] *mdf(A)*, macrolide-associated resistance; *aadA5*, aminoglycoside resistance; *aadA4*, aminoglycoside resistance; *bla*$_{CTX-M-15}$, β-lactam resistance; *bla*$_{OXA-1}$, β-lactam resistance; *aac(6′)Ib-cr*, fluoroquinolone and aminoglycoside resistance; *mph(A)*, macrolide resistance; *catB4*, phenicols resistance; *sul1*, sulphonamides resistance; *dfrA7*, trimethoprim resistance.
[d] *gad*, glutamate decarboxylase; *Iha*, adherence protein; *sat*, secreted autotransporter toxin; *nfaE*, diffuse adherence fibrillary adhesion gene; *gad*, glutamate decarboxylase; *iss*, increased serum survival; *senB*, plasmid-encoded enterotoxin.

doses is more effective than a single dose in eradicating the targeted bacterial strain [33].

4.2. Occurrence of resistant mutants

An interesting finding in our study was the identification of phage-resistant mutants isolated only from one of the two tested faecal pools. Bacteriophage resistance is a known phenomenon in natural environments where phages outnumber bacteria by 10:1 and thus exert a strong predatory pressure on them. It therefore represents a predictable evolutionary response to viral attack [34]. In 1943–1945, Demerec and Fano, together with Luria and Delbrück, described multiple resistance mechanisms that simultaneously occur in *E. coli* against different bacteriophages [35,36].

Nowadays, various phage resistance mechanisms have been well characterized; these include preventing phage adsorption (e.g. by blocking phage receptors or producing extracellular matrix), preventing phage DNA entry (e.g. superinfection exclusion system, Sie), cutting phage nucleic acid (e.g. restriction-modification system, R-M; CRISPR-*Cas* system), and abortive infection (Abi) systems. Other resistance strategies have been observed, but their mechanisms are still to be elucidated; moreover, many other completely unknown phage resistance mechanisms are likely to exist [37]. In particular, the CRISPR-*Cas* system is comprised of CRISPR-motifs scattered in the genome, each one containing sets of conserved inverted direct repeats intercalated by a spacer sequence originating by exogenous DNA and accompanied by *cas* genes. This represents an antiphage and antiplasmid adaptive immunity harboured by ∼40% of all bacteria [38,39].

In the present study, we could not find any *cas* gene indicative of a functional CRISPR system [40]. Only questionable CRISPR systems were detected, likely corresponding to repeated regions in the genome (data not shown). This is not surprising because some groups of *E. coli* in the phylogenetic group B2 to which our strains belong were previously shown to lack this system completely [41].

We hypothesize that more than one resistance mechanism coexists in our phage-resistant mutant when in the presence of a complex cocktail containing multiple lytic phages against the ST131 *E. coli* strain. In this regard, the chromosomal amino acid substitution that we detected in the glycosyl transferase family 2 protein domain could potentially block one or more phage receptors by overtransferring sugars to outer membrane substrates. However, a functional study of the mutated enzyme should be done to confirm this hypothesis. Additionally, to understand a possible link with the resistant phenotype better, a protein expression level approach should be implemented by comparing the mRNA profiles of mutant and WT strains. This analysis would also be essential to explore the Abi and R-M systems, which exploit several heterogeneous proteins to provide resistance [37].

Finally, several genes present in the newly acquired plasmids could not be assigned to a known function. Their implication in the acquisition of resistance could not be further confirmed with conjugation experiments. In fact, due to their living and evolving nature, it is technically unfeasible to prepare stable plates selective for any phage or phage cocktail, enabling the further selection of transconjugants.

4.3. Host microbiota may affect activity of bacteriophages

Regarding the divergence of results between studies I and II, we hypothesize that the emergence of phage resistance in only one pool of faeces (pool B) could be dependent on the different profiles

Table 2
Results of SNV analyses comparing the chromosomes of WT strain 4901.28 and its phage-resistant mutant ExIIa_T32_C2.

SNV environment[a]	ExIIa_T32_C2 hybrid assembly[b]	ExIIa_T32_C2 sole Illumina[b]	4901.28 sole Illumina[b]	Target CDS	AA change[c]	AAs identity
GGCTTTCCAG CCCTTATTT	C	C	A	IS*3*-like element IS*1397* family transposase	Q33L E37A	99% (198/200)
ACAGGGAGCT CCGCTTTGA	G	G	T	IS*3* family transposase		
CGCTTTGAAC GTCGCTGAA	A	A	T	IS*3* family transposase		
AAATGTATAA TCATACTTT	T	T	G	Non-coding region	NA	NA
TAACCCCGGC TTTCGTTTC	T	T	C	AAA family ATPase	–	100% (170/170)
TACATCGGGG TAACAAAGA	G	G	T	Glycosyltransferase family 2 protein	N49T	99% (223/224)
CGATGGGCCG GAAGGCGCG	T	C	T	IS*66* family transposase	–	100% (512/512)
ACGTGCGCGC CCCGTGCCA	T	T	G	Hypothetical protein	A123S	99% (130/131)
CCCGGCGTCG GGCGTCAGA	C	T	C	DUF945 domain-containing protein	–	100% (158/158)
TGTATCTGAA AACCAGAAT	C	C	T	RadC family protein	–	100% (158/158)
AGATCTGCGT ACCAGCTCG	C	C	T	PolB	–	100% (649/649)

[a] Space between bases in each sequence represents the nucleotide position of the mutation.
[b] Letters represent the bases contained in the sequence spaces reported in the first column: A, adenine; C, cytosine; G, guanine; T, thymine.
[c] The first AA abbreviation belongs to 4901.28 (wild-type); the second belongs to the phage-resistant mutant ExIIa_T32_C2.A, Alanine; AA, amino acid.; E, glutamic acid; L, leucine; N, asparagine; NA, not applicable; Q, glutamine; S, serine; SNVs, single-nucleotide variants; T, threonine.

of their bacterial populations. In particular, some faecal bacteria may help each other by means of quorum sensing (QS) signalling to fight against viral predators. Notably, QS represents chemical signals exploited by some bacteria and eukaryotic cells to communicate within or between different bacterial populations (e.g. leading to expression of biofilm or of virulence factors). They have also been recognized as playing a role in the relationship between bacteria and phages – namely, to communicate the presence of viruses in the environment and further control and coordinate the expression of antiphage defences [34].

The ST131 *E. coli* strain 4901.28 may thus be able to sense the presence of phages thanks to signals produced by other species present in the faeces of specific individuals and consequently may be prepared against a possible attack [34]. This could enable bacterial populations to increase their defences only in presence of high viral titre, thereby sparing the energy required to maintain a constant high-level defence in case of a lower danger of infection. Notably, Hoyland-Kroghsbo et al. found a particular pathway of QS signalling in *E. coli* that causes a temporarily diminished number of phage receptors. It is activated only during high phage density and despite the consequent diminished fitness (e.g. lower absorption of specific nutrients) [34].

In our case, producers of QS signals could be individual faecal bacterial populations or, alternatively, eukaryotic cells (also known to exert QS towards bacterial cells in natural environments), specifically colonic epithelial cells that are part of the normal stool composition. The consequent reversible decreased expression of particular receptors may have spared *E. coli* 4901.28 from being infected by bacteriophages in the second pool of faeces (study II), but not in the first one (study I). This hypothesis is supported by the observation that in study II, the viral titre that resulted was much higher than in study I (Fig. 2 vs. Fig. 3, respectively). It can be speculated that in pool B of faeces, some of the bacteriophages included in the INTESTI cocktail found that specific bacterial host (s) were to replicate better and faster than in pool A. Then, the higher viral concentration induced QS signals able to protect bacteria at risk for infection.

Our work suggests that a deeper and detailed knowledge of the nature of bacterial populations favouring or hampering the emergence of phage resistance is necessary for the future application of phage therapy as a decolonization strategy.

5. Conclusion

We hypothesized that bacteriophages could represent a possible alternative strategy to decolonize intestinal carriers of MDR *E. coli*. Certainly, phage cocktails are lacking the major drawbacks presented by antibiotic regimens as well as by other strategies aimed to decolonize intestinal carriers from MDROs. Nevertheless, phage decolonization should be performed with caution because phage resistance may emerge in certain circumstances. Our data indicate that bacteriophages' efficacy may be influenced by individual microbiota composition. Moreover, the phenomenon of resistance against bacteriophages may involve different and simultaneous mechanisms, especially in the presence of complex phage cocktails. Evidently, an in vivo model of intestinal colonization should be developed along with protein expression level experiments to further confirm these findings.

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Competing interests

None.

Ethical approval

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.jgar.2020.05.018.

References

[1] Manges AR, Geum HM, Guo A, Edens TJ, Fibke CD, Pitout JDD. Global extraintestinal pathogenic *Escherichia coli* (ExPEC) lineages. Clin Microbiol Rev 2019;32:e00135–e138.
[2] Vila J, Saez-Lopez E, Johnson JR, Romling U, Dobrindt U, Canton R, et al. *Escherichia coli*: an old friend with new tidings. FEMS Microbiol Rev 2016;40:437–63.
[3] Doi Y, Iovleva A, Bonomo RA. The ecology of extended-spectrum beta-lactamases (ESBLs) in the developed world. J Travel Med 2017;24:S44–51.
[4] Hilty M, Betsch BY, Bogli-Stuber K, Heiniger N, Stadler M, Kuffer M, et al. Transmission dynamics of extended-spectrum beta-lactamase-producing *Enterobacteriaceae* in the tertiary care hospital and the household setting. Clin Infect Dis 2012;55:967–75.
[5] Pires J, Bernasconi OJ, Kasraian S, Hilty M, Perreten V, Endimiani A. Intestinal colonisation with extended-spectrum cephalosporin-resistant *Escherichia coli* in Swiss pets: molecular features, risk factors and transmission with owners. Int J Antimicrob Agents 2016;48:759–60.
[6] Kronenberg A, Hilty M, Endimiani A, Muhlemann K. Temporal trends of extended-spectrum cephalosporin-resistant *Escherichia coli* and *Klebsiella pneumoniae* isolates in in- and outpatients in Switzerland, 2004 to 2011. Euro Surveill 2013;18.
[7] Seiffert SN, Hilty M, Kronenberg A, Droz S, Perreten V, Endimiani A. Extended-spectrum cephalosporin-resistant *Escherichia coli* in community, specialized outpatient clinic and hospital settings in Switzerland. J Antimicrob Chemother 2013;68:2249–54.
[8] Pires J, Kuenzli E, Hauser C, Tinguely R, Kasraian S, Atkinson A, et al. Intestinal colonisation with extended-spectrum cephalosporin-resistant Enterobacteriaceae in different populations in Switzerland: prevalence, risk factors and molecular features. J Glob Antimicrob Resist 2018;12:17–9.
[9] Pires J, Kuenzli E, Kasraian S, Tinguely R, Furrer H, Hilty M, et al. Polyclonal intestinal colonization with extended-spectrum cephalosporin-resistant Enterobacteriaceae upon traveling to India. Front Microbiol 2016;7:1069.
[10] Tavoukjian V. Faecal microbiota transplantation for the decolonization of antibiotic-resistant bacteria in the gut: a systematic review and meta-analysis. J Hosp Infect 2019;102:174–88.
[11] Bar-Yoseph H, Hussein K, Braun E, Paul M. Natural history and decolonization strategies for ESBL/carbapenem-resistant Enterobacteriaceae carriage: systematic review and meta-analysis. J Antimicrob Chemother 2016;71:2729–39.
[12] Huttner B, Haustein T, Uckay I, Renzi G, Stewardson A, Schaerrer D, et al. Decolonization of intestinal carriage of extended-spectrum β-lactamase-producing Enterobacteriaceae with oral colistin and neomycin: a randomized, double-blind, placebo-controlled trial. J Antimicrob Chemother 2013;68:2375–82.
[13] Rieg S, Kupper MF, de With K, Serr A, Bohnert JA, Kern WV. Intestinal decolonization of Enterobacteriaceae producing extended-spectrum β-lactamases (ESBL): a retrospective observational study in patients at risk for infection and a brief review of the literature. BMC Infect Dis 2015;15:475.
[14] de Lastours V, Poirel L, Huttner B, Harbarth S, Denamur E, Nordmann P. Emergence of colistin-resistant Gram-negative Enterobacterales in the gut of patients receiving oral colistin and neomycin decontamination. J Infect 2020;80:578–606.
[15] Ducarmon QR, Zwittink RD, Hornung BVH, van Schaik W, Young VB, Kuijper EJ. Gut microbiota and colonization resistance against bacterial enteric infection. Microbiol Mol Biol Rev 2019;83:e00007–e19.
[16] Saha S, Tariq R, Tosh PK, Pardi DS, Khanna S. Faecal microbiota transplantation for eradicating carriage of multidrug-resistant organisms: a systematic review. Clin Microbiol Infect 2019;25:958–63.
[17] Kutateladze M, Adamia R. Phage therapy experience at the Eliava Institute. Med Mal Infect 2008;38:426–30.
[18] Sarker SA, Brussow H. From bench to bed and back again: phage therapy of childhood *Escherichia coli* diarrhea. Ann N Y Acad Sci 2016;1372:42–52.
[19] Domingo-Calap P, Georgel P, Bahram S. Back to the future: bacteriophages as promising therapeutic tools. HLA 2016;87:133–40.
[20] Pires J, Bernasconi OJ, Hauser C, Tinguely R, Atkinson A, Perreten V, et al. Intestinal colonisation with extended-spectrum cephalosporin- and colistin-resistant Enterobacteriaceae in HIV-positive individuals in Switzerland: molecular features and risk factors. Int J Antimicrob Agents 2017;49:519–21.
[21] Zschach H, Joensen KG, Lindhard B, Lund O, Goderdzishvili M, Chkonia I, et al. What can we learn from a metagenomic analysis of a Georgian bacteriophage cocktail? Viruses 2015;7:6570–89.
[22] Clockie MRJ, Kropinski AM, editors. Bacteriophages: methods and protocols, vol 1. Isolation, characterization, and interactions. New York: Humana Press; 2009.
[23] Bernasconi OJ, Dona V, Tinguely R, Endimiani A. In vitro activity of three commercial bacteriophage cocktails against multidrug-resistant *Escherichia coli* and *Proteus* spp. strains of human and non-human origin. J Glob Antimicrob Resist 2017;8:179–85.
[24] Hilty M, Betsch BY, Bogli-Stuber K, Heiniger N, Stadler M, Kuffer M, et al. Transmission dynamics of extended-spectrum β-lactamase-producing Enterobacteriaceae in the tertiary care hospital and the household setting. Clin Infect Dis 2012;55:967–75.
[25] Budel T, Kuenzli E, Clement M, Bernasconi OJ, Fehr J, Mohammed AH, et al. Polyclonal gut colonization with extended-spectrum cephalosporin- and/or colistin-resistant Enterobacteriaceae: a normal status for hotel employees on the island of Zanzibar, Tanzania. J Antimicrob Chemother 2019;74:2880–90.
[26] Clement M, Ramette A, Bernasconi OJ, Principe L, Luzzaro F, Endimiani A. Whole-genome sequence of the first extended-spectrum beta-lactamase-producing strain of *Salmonella enterica* subsp. enterica Serovar Napoli. Microbiol Resour Announc 2018;7:e00973–e1018.
[27] Dona V, Bernasconi OJ, Pires J, Collaud A, Overesch G, Ramette A, et al. Heterogeneous genetic location of mcr-1 in colistin-resistant *Escherichia coli* isolates from humans and retail chicken meat in Switzerland: emergence of *mcr-1*-carrying IncK2 plasmids. Antimicrob Agents Chemother 2017;61:e01245–e1317.
[28] Luzzaro F, Clement M, Principe L, Viaggi V, Bernasconi OJ, Endimiani A. Characterisation of the first extended-spectrum beta-lactamase (ESBL)-producing *Shigella sonnei* clinical isolate in Italy. J Glob Antimicrob Resist 2019;17:58–9.
[29] Treangen TJ, Ondov BD, Koren S, Phillippy AM. The Harvest suite for rapid core-genome alignment and visualization of thousands of intraspecific microbial genomes. Genome Biol 2014;15:524.
[30] Ducarmon QR, Zwittink RD, Hornung BVH, van Schaik W, Young VB, Kuijper EJ. Gut microbiota and colonization resistance against bacterial enteric infection. Microbiol Mol Biol Rev 2019;83:e00007–e19.
[31] Pompei A, Cordisco L, Raimondi S, Amaretti A, Pagnoni UM, Matteuzzi D, et al. In vitro comparison of the prebiotic effects of two inulin-type fructans. Anaerobe 2008;14:280–6.
[32] Ruppe E, Andremont A. Causes, consequences, and perspectives in the variations of intestinal density of colonization of multidrug-resistant enterobacteria. Front Microbiol 2013;4:129.
[33] Ly-Chatain MH. The factors affecting effectiveness of treatment in phages therapy. Front Microbiol 2014;5:51.
[34] Hoyland-Kroghsbo NM, Maerkedahl RB, Svenningsen SL. A quorum-sensing-induced bacteriophage defense mechanism. mBio 2013;4:e00362–e412.
[35] Luria SE, Delbruck M. Mutations of bacteria from virus sensitivity to virus resistance. Genetics 1943;28:491–511.
[36] Demerec M, Fano U. Bacteriophage-resistant mutants in *Escherichia coli*. Genetics 1945;30:119–36.
[37] Doron S, Melamed S, Ofir G, Leavitt A, Lopatina A, Keren M, et al. Systematic discovery of antiphage defense systems in the microbial pangenome. Science 2018;359:eaar4120.
[38] Gudbergsdottir S, Deng L, Chen Z, Jensen JV, Jensen LR, She Q, et al. Dynamic properties of the Sulfolobus CRISPR/Cas and CRISPR/Cmr systems when challenged with vector-borne viral and plasmid genes and protospacers. Mol Microbiol 2011;79:35–49.
[39] Westra ER, Brouns SJ. The rise and fall of CRISPRs – dynamics of spacer acquisition and loss. Mol Microbiol 2012;85:1021–5.
[40] Labrie SJ, Samson JE, Moineau S. Bacteriophage resistance mechanisms. Nat Rev Microbiol 2010;8:317–27.
[41] Touchon M, Charpentier S, Clermont O, Rocha EP, Denamur E, Branger C. CRISPR distribution within the *Escherichia coli* species is not suggestive of immunity-associated diversifying selection. J Bacteriol Int J Antimicrob Agents 2011;193:2460–7.