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Virulent Bacteriophages Can Target O104:H4 Enteroaggregative *Escherichia coli* in the Mouse Intestine

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In vivo bacteriophage targeting of enteroaggregative *Escherichia coli* (EAEC) was assessed using a mouse intestinal model of colonization with the O104:H4 55989Str strain and a cocktail of three virulent bacteriophages. The colonization model was shown to mimic asymptomatic intestinal carriage found in humans. The addition of the cocktail to drinking water for 24 h strongly decreased ileal and weakly decreased fecal 55989Str concentrations in a dose-dependent manner. These decreases in ileal and fecal bacterial concentrations were only transient, since 55989Str concentrations returned to their original levels 3 days later. These transient decreases were independent of the mouse microbiota, as similar results were obtained with axenic mice. We studied the infectivity of each bacteriophage in the ileal and fecal environments and found that 55989Str bacteria in the mouse ileum were permissive to all three bacteriophages, whereas those in the feces were permissive to only one bacteriophage. Our results provide the first demonstration that bacterial permissivity to infection with virulent bacteriophages is not uniform throughout the gut; this highlights the need for a detailed characterization of the interactions between bacteria and bacteriophages *in vivo* for the further development of phage therapy targeting intestinal pathogens found in the gut of asymptomatic human carriers.

Bacteriophages—viruses that infect only bacteria — have been used for decades to treat human bacterial infections, mainly in Eastern Europe (2, 19, 24). The worldwide spread of panresistant bacteria and the shortage of new antibiotics are now leading to a worldwide revival of interest in phage therapy (43). Many studies of animal models have shown this therapeutic approach to be promising (8, 14, 28, 40). However, markedly different results have been obtained depending on the animal model, the nature of the bacteriophages, and the application method used (36). Nevertheless, the narrow host spectrum of bacteriophages, which are often restricted to a particular bacterial subspecies, may make it possible to target pathogens without affecting commensal strains from the same bacterial species. Several studies have reported the use of bacteriophages to target enterotoxigenic, enteropathogenic, and commensal *Escherichia coli* strains (7). They reported a decrease in diarrheal symptoms (21, 40, 41), a decrease in the concentration of the bacterium in the intestine (10, 33–35), an increase in the rate of natural bacterial clearance (9, 39, 42), or even no decrease in the bacterial concentration at all (16, 47). These data indicate that, within the scope of the intestinal microbiota, detailed investigations are required of the interactions between individual bacteriophages and their bacterial hosts, even within a single given bacterial species.

Enteroaggregative *E. coli* (EAEC) is now recognized as an emerging intestinal pathogen (30). It is frequently found in the gut of asymptomatic human carriers, who serve as reservoirs for interhuman EAEC transmission (3, 38). EAEC is the second leading cause of travelers' diarrhea worldwide (13) and the leading cause of diarrhea both in children in developing countries and in HIV-infected patients (31, 37, 46). We recently reported the isolation of three virulent bacteriophages able to infect the EAEC O104:H4 55989Str strain *in vitro*, whether growing under planktonic or biofilm conditions or as aggregates formed at the surface of enterocytes (26). These bacteriophages were also able to infect O104:H4 strains isolated from patients during an outbreak of diarrhea in western Europe in June 2011 that affected more than

4,000 people (15, 26, 32). In mice colonized with the 55989Str strain (25), we have shown that a 24-hour application of these three bacteriophages leads to their continuous replication in the gut for over 2 weeks (25, 26). We report here a detailed analysis of the impact of these three bacteriophages on their host in the intestine, demonstrating the ability of these bacteriophages to decrease ileal concentrations of 55989Str strongly but fecal concentrations only weakly. Thus, contrary to expectations, the replications of these bacteriophages along the length of the intestine were not uniform.

MATERIALS AND METHODS

Bacterial and viral strains and growth conditions. The 55989Str strain was routinely cultured at 37°C in Luria-Bertani (LB) broth, on LB agar, or on Drigalski agar plates. Streptomycin (100 mg/liter) was added to the culture media when required (25, 29, 44). Bacteriophages CLB_P1, CLB_P2, and CLB_P3 infecting the *E. coli* 55989Str strain were isolated from wastewater and prepared as described previously (26). When required, regular LB medium was adjusted to pH 2, 4, 5, or 7 by the addition of HCl.

Murine model of intestinal colonization. The colonization of 7-week-old female mice (BALB/c YJ from Charles River Laboratories or C3H from Institut Pasteur) and the collection of fecal samples were carried out as described previously (26). The mice were housed on grids to prevent coprophagy. When necessary, the animals were euthanized, and their intestines were removed and cut into four sections corresponding to the duodenum-jejunum, ileum, cecum, and colon (when required, the

adherent and luminal floras in the colonic samples were distinguished). Each of these sections was weighed (0.4 to 0.8 g/sample), homogenized in 5 ml of phosphate-buffered saline (PBS) (Ultra Turrax T25 with dispersing tool S25N-8G; IKA), and subjected to serial dilution. The detection limit was 5×10^2 CFU/g or PFU/g of tissue or feces. For 24 h, the mice received a cocktail of the three bacteriophages (equal amounts of each bacteriophage) in drinking water supplemented with 5 g/liter streptomycin. In the experiments with C3H axenic mice, no streptomycin was added to the drinking water. Tests of resistance to the individual bacteriophages were performed on 20 isolated colonies.

Histological and immunohistological analyses. Ileal and colonic samples were removed from the intestines of the euthanized animals. They were fixed by incubation in 4% formaldehyde for 24 h at 4°C and embedded in paraffin. Serial 4-mm sections were stained with hematoxylin and eosin (HE) and incubated with affinity-purified rabbit primary antibodies raised against *E. coli* lipopolysaccharide (LPS) (Biodesign Institute) diluted 1:1,000. Primary antibody binding was detected by incubation with a rabbit IgG coupled to horseradish peroxidase (EnVision+ system; Dako, Denmark).

Assays of the inhibition of bacteriophage infection. Several molecules have been shown to inhibit bacteriophage infection (4, 6). We tested EDTA (0 to 100 mM), $MgCl_2$ (0 to 2.5 M), sodium citrate (0 to 600 mM), and a combination of ascorbic acid (17 to 1,700 mg/liter) and cupric chloride (0.17 to 17 mg/liter) *in vitro*. Inhibitory molecules were added to strain 55989Str bacteria (1 ml of exponentially growing culture in LB). Subsequently, each bacteriophage was tested independently (multiplicity of infection [MOI], 500). After addition of the bacteriophages, followed by mixing and 10 min of incubation, the samples were serially diluted and plated to count viable bacteria.

Bacterial quantification by quantitative PCR. Bacterial DNA was extracted with the Qiagen stool DNA minikit 50 (Qiagen), including a 95°C incubation step after an initial 30 min of incubation with a mixture of DNase and RNase (0.2 mg/ml and 0.6 mg/ml final concentrations, respectively; Qiagen Lambda minikit) at 37°C to eliminate DNA from the lysed 55989Str cells. Amplification and detection were performed in 96-well plates (Abgene) with the iQ SYBR green supermix (Bio-Rad). Each amplification reaction was performed in duplicate, in a final volume of 25 μl, with a final concentration of 0.25 μM for each primer and 5 μl of extracted DNA (1 cycle at 95°C for 10 min; 45 cycles of 15 s at 95°C, 1 min at 60°C, and 1.5 min at 72°C; 80 cycles at 55°C with a 0.5°C increase in each cycle). *E. coli* 16S rRNA gene-specific primers and bacterial 16S rRNA gene universal primers were used in parallel in each PCR (17). The data are expressed as described previously (17).

Bacteriophage and 55989Str *ex vivo* multiplication in mouse intestinal samples. We added each individual bacteriophage (MOI, 2×10^{-2} or 100) to 55989Str liquid cultures in the exponential (optical density at 600 nm [OD_{600}], 0.6) and stationary (OD_{600}, 1.4) growth phases, which were then incubated for 5 h at 37°C. At 0 and 5 h, the bacteriophage and 55989Str concentrations were quantified by serial dilutions and plating on LB agar plates. Mouse ileal, cecal, and fecal samples were collected from both noncolonized mice and 55989Str-colonized mice (3 days) and homogenized in 1× PBS. 55989Str cells from an exponentially growing LB culture (OD_{600}, 0.3) were added to noncolonized samples (1×10^6 CFU/ml) and mixed vigorously for 1 min. Half of each sample was used as a control for the second half, to which each individual bacteriophage was added to a final MOI of 1×10^{-2}. Equal amounts of each bacteriophage were added to 55989Str-colonized samples. As a positive control, exponentially growing 55989Str cells (OD_{600}, 0.3) were added to LB medium, and each bacteriophage was then added under the same conditions as for the intestinal samples. Before and after 5 h of incubation at 37°C, the bacteriophage and 55989Str concentrations were determined.

Ethics statement. The animals were housed in an animal facility in accordance with Institut Pasteur guidelines and European recommendations. Food and drinking water were provided *ad libitum*. The protocols
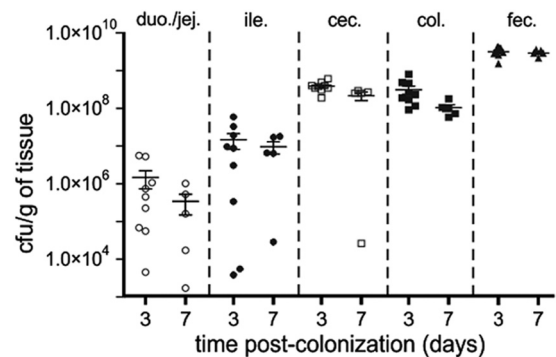

FIG 1 *E. coli* 55989Str colonizes both the large and small intestines. Quantification over time of the 55989Str strain in mouse intestinal sections: 3 days ($n = 9$) and 7 days ($n = 5$) after bacterial administration. ○, duodenum-jejunum (duo./jej.); ●, ileum (ile.); □, cecum (cec.); ■, colon (col.); ▲, feces (fec.).

were approved by the veterinary staff of the Institut Pasteur animal facility (approval identification 10.565).

Statistical analysis. Mann-Whitney tests were carried out with GraphPad InStat software (GraphPad Software, Inc.).

RESULTS

The 55989Str strain can colonize both the large and small intestines. We characterized in detail the colonization of the mouse intestine with the EAEC 55989Str O104:H4 strain. We quantified the 55989Str bacteria in various sections of the gut, from the duodenum to the colon, and in the feces, 3 and 7 days after oral gavage. We found 55989Str cells in the large intestine and feces at a mean concentration of 10^8 to 10^9 CFU/g and in the small intestine at a mean concentration of 10^3 to 10^8 CFU/g of duodenum-jejunum and ileum tissues (Fig. 1). No major differences (exceeding 1 order of magnitude) in the bacterial concentrations were observed between days 3 and 7. We also carried out histological and immunohistochemical analyses on ileal and colonic tissues. Interestingly, 55989Str bacteria were present not only in the lumen but also between ileal villi and in crypts, where they formed aggregates on the surfaces of intestinal epithelial cells (Fig. 2). Aggregates on intestinal epithelial cells of the colon were also observed (Fig. 3). However, histological examinations of the ileal and colonic epithelia of colonized and noncolonized mice revealed no differences between these two groups of animals. Together with previous observations (animal weight and stool consistency) (C. Le Bouguénec, unpublished data), our data confirmed that this mouse model could be considered an appropriate model of asymptomatic intestinal 55989Str carriage.

The bacteriophage cocktail reduces the concentration of 55989Str in the ileum. We recently reported the isolation and characterization of three virulent bacteriophages infecting the 55989Str strain that were able to replicate for a long period in a mouse intestinal colonization model (26). In two groups of mice colonized by 55989Str, we first quantified both the viral and the bacterial concentrations in the ileal section of the intestines 4 and 7 days after colonization. One group received bacteriophage-free drinking water, whereas the other received drinking water containing 3×10^8 PFU/ml of the bacteriophage cocktail for 24 h (days 3 to 4), and bacteriophage-free drinking water thereafter. The bacteriophage cocktail yielded 55989Str concentrations in the ileum only one-eighth of those of the control after 24 h of treat-
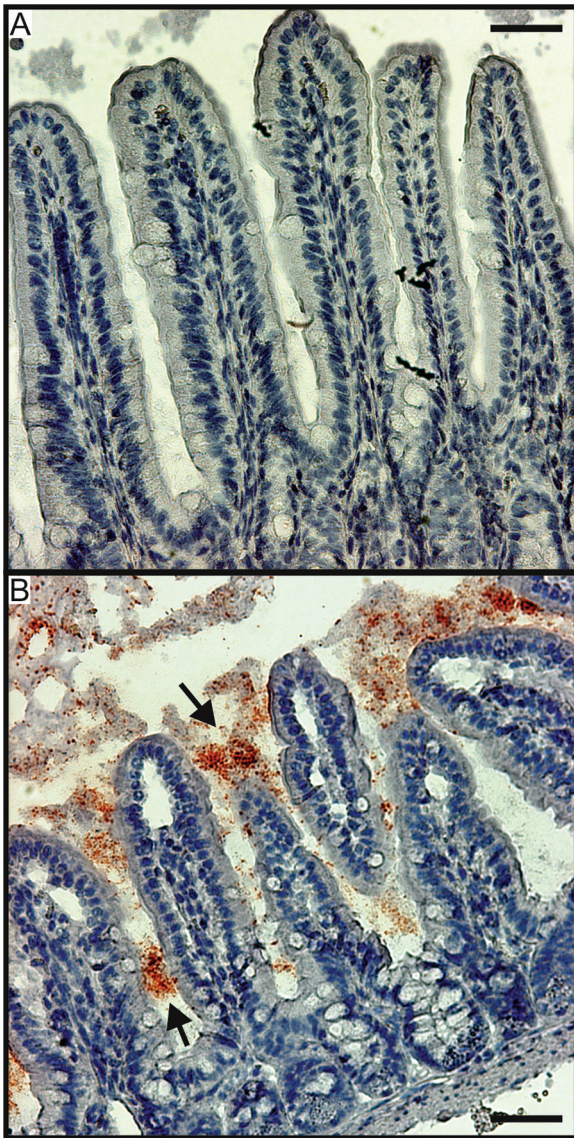
FIG 2 *E. coli* 55989Str bacteria aggregate at the surface of the mouse ileal epithelium. Histological sections of mouse ileum not colonized (A) or colonized (B) with *E. coli* 55989Str were immunolabeled for the detection of *E. coli* cells (red). Black arrows indicate bacterial aggregates (scale bars, 50 μm).

FIG 3 *E. coli* 55989Str bacteria adhere to the surface of colonic epithelium. Histological sections of mouse colon not colonized (A) or colonized (B) with *E. coli* 55989Str were immunolabeled for the detection of *E. coli* cells (red). Black arrows indicate bacterial aggregates (scale bars, 50 μm).

ment ($P < 0.01$) (Fig. 4A). Moreover, no viable 55989Str cells could be detected in the ileum of 40% of these mice. However, on day 7, ileal 55989Str concentrations were found to have increased to levels similar to those in the control group. The ileal bacteriophage concentrations, which were relatively high (10^8 PFU/g), did not change significantly between days 4 and 7 (Fig. 4B).

We then investigated whether an increase in the concentration of the cocktail could increase both the amplitude and duration of the decrease in the 55989Str concentration in the ileum. The use of a 100-times-greater concentration of the bacteriophage cocktail resulted in a decrease in ileal 55989Str concentrations 70 times greater than those achieved with the lower cocktail concentrations and levels of bacteria lower than those in the untreated controls by a factor of 470 ($P < 0.001$). Moreover, in 14 of 15 mice (93%), no 55989Str cells were detectable in the ileum sections after treatment
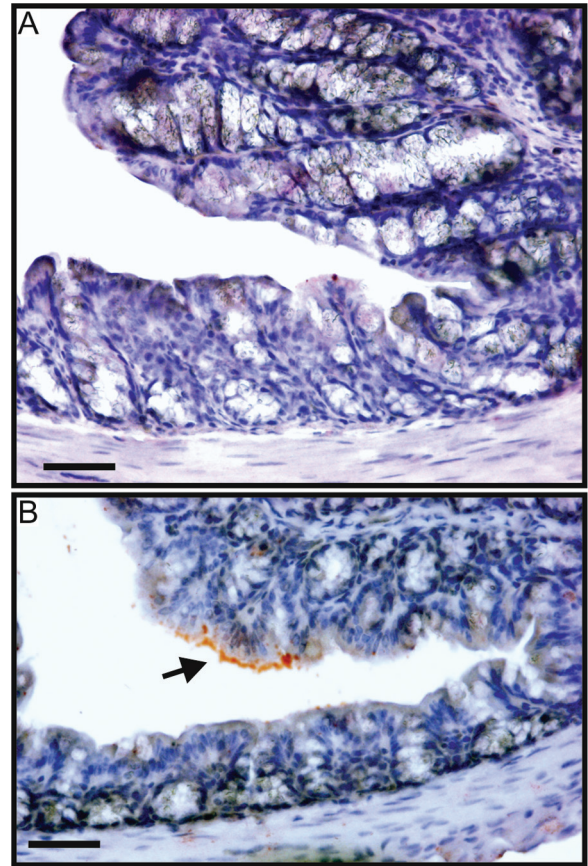
for 24 h. However, on day 7, the ileal 55989Str concentrations had returned to the levels in the untreated control animals, as observed previously with the lower cocktail concentration (Fig. 4A). In both experiments, 100% of the 55989Str cells isolated from day 7 ileal samples were found to be susceptible to the phage cocktail.

Bacterial counts in intestinal samples containing an excess of bacteriophages. The apparent recolonization of the ileum with 55989Str that was observed between days 4 and 7 suggested that the bacteria either returned to the gut through coprophagy (mice eating their own feces) or were not completely eliminated from the ileum. Animals were housed on grids to prevent coprophagy, so we considered that the 55989Str bacteria were not cleared from the ileum on day 4. Assuming that this was indeed the case, the total clearance observed in up to 93% of mice may have been due to an underestimation of the number of viable 55989Str cells in the presence of excess bacteriophages, leading to an increase of the frequency of contact between bacteriophages and their hosts during the homogenization step (see bacteriophage counts in Fig. 4B). We confirmed this hypothesis by showing *in vitro* that the presence of bacteriophages at a multiplicity of infection (MOI) of 500 or more in the samples prevented the direct counting of bacteria on plates by the serial dilution method (data not shown).

We then carried out several *in vitro* assays in an attempt to inhibit the interactions between bacteriophages and bacteria in
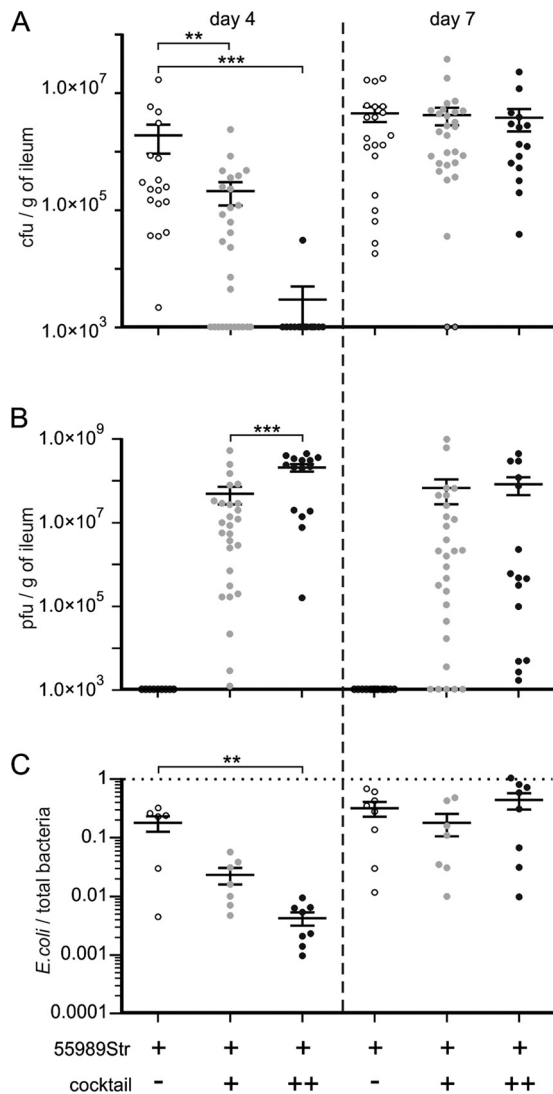
FIG 4 The bacteriophage cocktail reduces 55989Str concentrations in the mouse ileum. Ileal concentrations of bacteria (A) and bacteriophages (B) were determined on days 4 and 7 for three groups of mice colonized with *E. coli* 55989Str on day 0 and receiving, on day 3, bacteriophage-free drinking water (white circles) or drinking water containing a cocktail of bacteriophages at 3×10^8 PFU/ml (gray circles) or 3×10^{10} PFU/ml (black circles) for 24 h only. Experiments were performed in triplicate ($n = 15$ to 29 per condition). Two asterisks indicate P values of <0.01, and three asterisks indicate P values of <0.001. (C) Ileal concentrations of *E. coli* 55989Str were determined by qPCR. The ratios of CFU equivalents of *E. coli* normalized with respect to the total numbers of bacteria are shown. Experiments were performed in duplicate ($n = 6$ to 8 per condition).

such complex samples. We tested EDTA, MgCl$_2$, sodium citrate, and a combination of ascorbic acid and cupric chloride *in vitro* (see Materials and Methods). Only the combination of ascorbic acid (170 mg/liter) and cupric chloride (1.7 mg/liter) led to the inhibition of 55989Str infection by each of the three bacteriophages (MOI, 500), with a loss of bacterial viability of less than 1 order of magnitude. However, this inhibition was not efficient when we used tissue samples from 55989Str-colonized mice to which bacteriophages at an MOI of 500 were added (data not shown).

We therefore developed a molecular approach based on quantitative PCR (qPCR) (see Materials and Methods). With this technique, we observed that 3×10^8 and 3×10^{10} PFU/ml of the cocktail of bacteriophages induced decreases in ileal 55989Str concentrations by factors of 8 ($P < 0.01$) and 42 ($P < 0.01$), respectively, as demonstrated by comparisons with data from the control animals. This is consistent with a significant decrease in 55989Str concentrations observed from direct plating. We also confirmed, for both groups, that the 55989Str concentrations were similar to those in the untreated controls on day 7 and that bacteriophage concentrations were similar to those in previous experiments (Fig. 4C).

Impact of the bacteriophage cocktail in the colon and feces. In the same experimental conditions described above, we then investigated the impact of the bacteriophages in the colon, on adherent and luminal floras, and in feces. Concentrations of 55989Str in the adherent and luminal colonic floras remained roughly stable regardless of the number of bacteriophages added to the drinking water (Fig. 5A). 55989Str concentrations in the feces of mice receiving the highest dose of the cocktail represented one-third ($P < 0.001$) of those of the untreated controls (Fig. 5A). By comparison, the highest dose of bacteriophages decreased the concentration of 55989Str in the ileum by a factor of 430 ($P < 0.001$), suggesting that one or several factors (physical and/or physiological) may affect bacteriophage infection differently at different locations in the intestine.

The infection of 55989Str by bacteriophages in the intestine is not limited by the mouse microbiota. In conventional mice, the composition of the microbiota differs between the small and large intestines, and such differences may have been responsible for the differences observed following the addition of bacteriophages here (12). Using two groups of gnotobiotic mice colonized exclusively with the 55989Str strain, we carried out experiments similar to those described above, with one group receiving the 3×10^8 PFU/ml cocktail and the control group receiving bacteriophage-free drinking water. In both ileal and fecal samples, we observed no significant changes in bacteriophage concentrations between day 4 and day 7 (Fig. 6B). As in mice with conventional floras the bacteriophage cocktail decreased the ileal 55989Str concentration by a factor of 8 on day 4, but it had no effect on the bacterial concentration in the feces, which remained similar to those for the untreated control (Fig. 6A). On day 7, bacteriophages had no significant effects on either the ileal or fecal samples. Thus, the intestinal microbiota has no major effect on the infection of 55989Str with bacteriophages in the intestine.

55989Str permissivity to bacteriophage infection is not uniform throughout the gut. We then investigated the role of the physiological state of 55989Str in the limitation of bacteriophage infection. We first compared the amplification *in vitro* of each bacteriophage on 55989Str cells in the exponential and stationary growth phases in LB medium. At an MOI of 0.2, the amplifications of CLB_P1 and CLB_P3 were weaker (by factors of 28 and 8, respectively) in stationary phase than in the exponential growth phase, whereas CLB_P2 was affected much less than that (factor of 2) (Fig. 7A). At an MOI of 100, similar results were obtained with reduced amplification factors (6, 1, and 6, respectively, for CLB_P1, CLB_P2, and CLB_P3; data not shown). Therefore, the physiological state of the 55989Str host strain can play a role in the bacteriophage infection cycle.

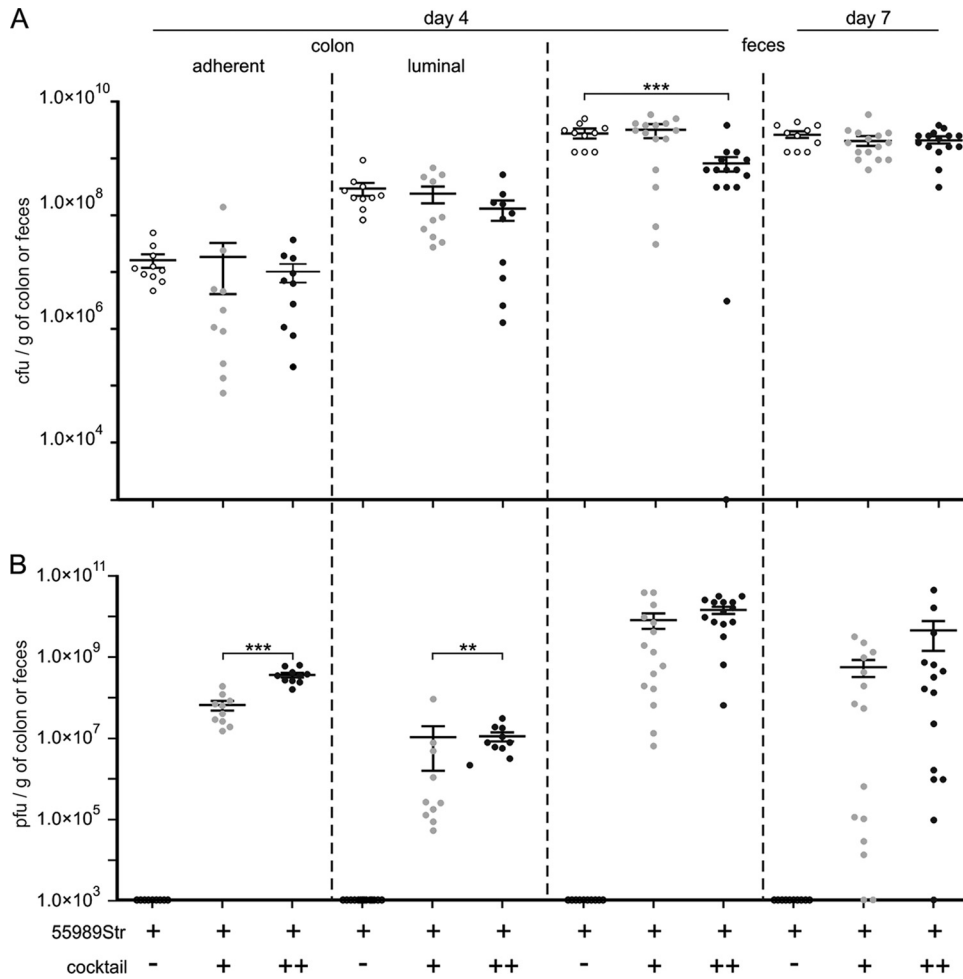We also examined the role of pH on the host permissivity to

FIG 5 The bacteriophage cocktail has a limited impact on 55989Str bacteria in the colon and feces of colonized mice. Three groups of mice colonized with *E. coli* 55989Str on day 0 received, on day 3, bacteriophage-free drinking water (white circles) or drinking water containing a cocktail of bacteriophages at 3×10^8 PFU/ml (gray circles) or 3×10^{10} PFU/ml (black circles) for 24 h only. The concentrations of bacteria (A) and bacteriophages (B) were determined on days 4 and 7. The experiments were performed in duplicate or triplicate ($n = 10$ to 15 per condition). Two asterisks indicate P values of <0.01, and three asterisks indicate P values of <0.001.

phage infection upon a 5-h exposure time. The cocktails of bacteriophages and their hosts were unable to survive at pH 2. At pH 4, the 55989Str bacteria could not multiply as well as bacteriophages (variation inferior to 1 log), while at pH 5 and 7, both bacteria and bacteriophages multiplied over 3 logs, showing that *in vitro* variations of pH could affect bacterial physiology and, consequently, the bacteriophage infection cycle (27).

We then evaluated bacteriophage amplification *ex vivo* in the intestinal context by adding the bacteriophages separately to ileal and fecal homogenates obtained from noncolonized mice or 55989Str-colonized mice (Fig. 7B). We added 55989Str to samples from noncolonized mice and mixed before adding each type of bacteriophage separately. Under these conditions, the three bacteriophages displayed levels of amplification in the ileal and fecal samples that were slightly higher than or similar to those in LB medium. The intestinal environment therefore contained no specific inhibitors of bacteriophage infection (data not shown). When individual bacteriophages were added separately to 55989Str-colonized samples, they were all amplified as efficiently in ileal samples as in control LB medium (Fig. 6B). However,

CLB_P1 and CLB_P3 were amplified less efficiently in fecal samples than in ileal samples (by factors of 8,900 and 200, respectively), whereas CLB_P2 amplification was affected only a little (decreasing by a factor of only 2) (Fig. 7B). Interestingly, 55989Str growth rates were identical in the ileal and fecal samples (Fig. 7B). Thus, under these conditions, bacteriophage amplification was not dependent on the growth rate of the host cells. The replication of CLB_P1 and CLB_P3 along the length of the gut *in vivo* is not uniform, due to the changes in 55989Str permissivity.

DISCUSSION

The interest in phage therapy has grown steadily over the last 10 years, but our knowledge of the factors affecting the interactions between bacteriophages and bacteria *in vivo*, within the body of an animal, remains incomplete (36). In this study, we analyzed the targeting of a human pathogenic O104:H4 EAEC strain by bacteriophages in an animal model of stable and asymptomatic intestinal bacterial carriage. We also investigated the factors mediating the infection process of 55989Str bacteria with these bacteriophages within the intestine.
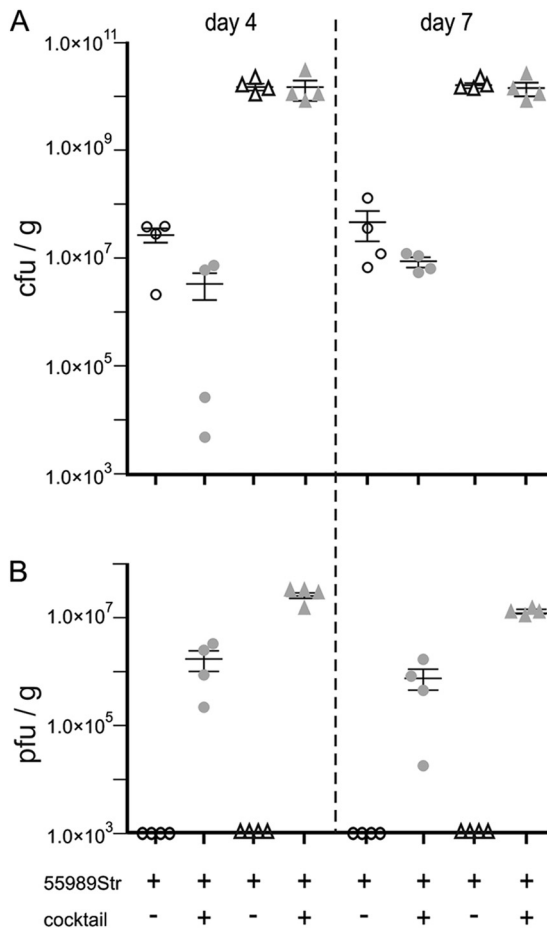
FIG 6 The infection of *E. coli* 55989Str bacteria with bacteriophages is not limited by the intestinal microbiota. Two groups of axenic mice (*n* = 4) colonized with *E. coli* 55989Str on day 0 received, on day 3, bacteriophage-free water (white symbols) or drinking water containing a cocktail of bacteriophages at 3×10^8 PFU/ml (gray symbols) for 24 h only. Ileal (circles) and fecal (triangles) concentrations of bacteria (A) and bacteriophages (B) were determined on days 4 and 7.

FIG 7 The location of 55989Str bacteria within the gut affects permissivity to bacteriophage infection. (A) Exponentially growing (white) or stationary-phase (black) *E. coli* 55989Str cells were mixed with each individual bacteriophage (CLB_P1, CLB_P2, or CLB_P3 at an MOI of 2×10^{-2}) and incubated for 5 h before quantification. The results are expressed as *n*-fold multiplication with respect to the initial number of bacteriophages added. (B) LB medium containing exponentially growing 55989Str (Ct) and ileal (i) and fecal (f) parts of the intestine collected from mice colonized with the 55989Str strain for 3 days were mixed with each individual bacteriophage at an MOI of 1×10^{-2} and incubated for 5 h. The results are expressed as *n*-fold multiplication with respect to the initial number of bacteriophages added.

riophages (CLB_P1, CLB_P2, and CLB_P3) infecting 55989Str in mice, with the aim of reducing its intestinal carriage.

Using two different concentrations of the bacteriophage cocktail, we showed that these viruses induced a significant dose-dependent decrease in the ileal concentration of 55989Str. Conventional bacterial counts for some ileal samples suggested that complete clearance had occurred, but qPCR quantifications revealed that this was not the case. The apparent clearance of bacteria was due to the presence of an excess of bacteriophages in the samples that prevented bacteria from growing on agar plates. None of the inhibitors of bacteriophage-bacterium interactions tested was efficient with biological samples, unlike buffer conditions. Indirect quantification techniques (qPCR, fluorescent or luminescent reporters) are therefore required for obtaining accurate bacterial counts at an MOI above 500.

Our results provided evidence of a significant decrease in the number of bacteria following the addition of bacteriophages on day 4, but incomplete 55989Str clearance allowed this bacterial strain to recolonize this section to the same extent as in untreated controls by day 7. These results, obtained under conditions in which coprophagy was prevented, are similar to those of a previous study in which coprophagy was not prevented, suggesting that coprophagy is not a major source of recolonization (47).

We did not expect clearance on day 4 in the ileum to be incom-

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Localization experiments showed that the 55989Str strain could colonize not only the mouse large intestine but also, more interestingly, the small intestine for at least 7 days. These results are consistent with those of previous studies reporting the colonization of pig, rabbit, and mouse small intestine by other EAEC strains and the detection of these bacteria on human ileal biopsies (20, 22, 23, 45). In ileal sections, 55989Str cells were observed within the intestinal crypts, which are usually devoid of bacteria due to the presence of several antimicrobial molecules (5). In the large intestine, 55989Str bacteria were also present on the surface of epithelial cells. This distribution suggests that this strain may display particular colonization properties. Despite the lethality of 55989Str in the systemic infection model, this strain induced no apparent diarrhea or intestinal epithelial lesions (44). We therefore considered this intestinal colonization model to mimic the asymptomatic intestinal carriage of pathogenic bacteria, as is commonly observed in human populations. This provided us with an opportunity to study the behavior of the EAEC strain within the autochthonous intestinal microbiota (38). We focused, in particular, on the impact of a cocktail of three virulent bacte-
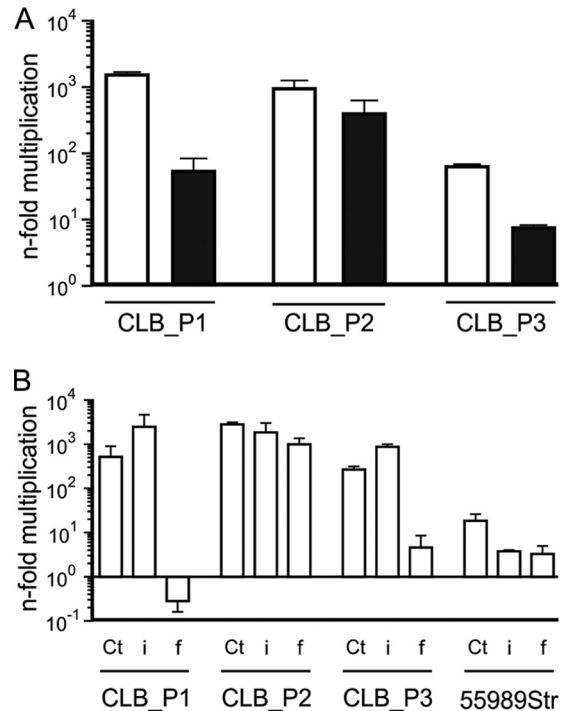
plete with the highest dose of the cocktail, as we calculated there to be more than a 4,000-fold excess of bacteriophage (10^8 PFU/g versus 2.5×10^4 CFU/g, the number of bacteria calculated from the 42-fold reduction of the 1×10^6 CFU/g observed for ileal samples from the control group on day 4). We also showed that bacteriophage infection was less efficient in the large intestine and feces than in the ileum. Both the adherent and luminal parts of the colonic flora were examined, and no significant differences either between them or with the feces were observed. We therefore investigated the factors that might prevent bacteriophages either from clearing the entire 55989Str population from the ileum or from reducing it in the large intestine and feces. Our data suggest that (i) 55989Str cells resistant to bacteriophages played no major role, as no such cells were detected in our experiments, (ii) the gut microbiota did not act as a major physical barrier (see the comparison of gnotobiotic and conventional mice), (iii) the accessibility of 55989Str bacteria to bacteriophages might not be strongly reduced in biofilm structures, as we showed previously that this cocktail of bacteriophages reduced bacterial levels strongly *in vitro* in biofilms and in aggregates at the surface of epithelial cells (26), (iv) no inhibitors of intestinal homogenates were detected in our *ex vivo* experiments, ruling out a role for bile salts and carbohydrates, which have been shown to interfere with bacteriophage lambda infection (18), and (v) 55989Str did not seem to be protected from bacteriophage infection by an intracellular stage, as no intracellular bacteria were detected on histological samples, but that (vi) permissivity to CLB_P1 and CLB_P3 infection was related to the location of 55989Str in the gut.

We plan to investigate the molecular mechanisms involved in this reduced permissivity in both the viral and bacterial partners (e.g., changes in receptor availability, modifications of host proteins involved in viral cycles, expression of specific viral genes). For example, CLB_P1 and CLB_P3 may not be able to perform a complete viral cycle in the large intestine, resulting in their persistence in a pseudolysogenic stage within infected—but not lysed—cells. Pseudolysogeny has been suggested to have ecological consequences for the intestinal microbiota (e.g., gene transfer, bacteriophage maintenance, clustered regularly interspaced short palindromic repeats [CRISPR] acquisition, limitation of predator-prey relationships) (1, 11). Local conditions (e.g., pH, nutrients, and electrolytes) in some spatial niches within gut parts may also affect bacteriophage permissivity. Special attention will also be paid to comparative genomics studies of these three bacteriophages, which could lead to the identification of specific functions that each has adapted for the gut environment.

The three bacteriophages studied here belong to three different viral families (*Podoviridae*, *Myoviridae*, and *Siphoviridae*). The *Myoviridae* bacteriophage CLB_P2 clearly had the most appropriate characteristics for use as a therapeutic agent, because it was effective in all sections of the intestine, whereas the *Podoviridae* bacteriophage CLB_P1 had the least appropriate characteristics for therapeutic use. These findings conflict with previous reports that members of the *Podoviridae* family replicate more efficiently than members of the *Myoviridae* family (47). This clearly indicates that generalization from a single given model is not possible and that the *in vivo* biological properties of each bacteriophage should be studied.

In conclusion, despite the many unknowns in virus-host interactions within the gut, a single application of bacteriophages decreased the level of carriage of an *E. coli* O104:H4 strain, dem-

onstrating the potential value of bacteriophages for targeting intestinal pathogens.

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