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In Vitro and In Vivo Bacteriolytic Activities of *Escherichia coli* Phages: Implications for Phage Therapy

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Four T4-like coliphages with broad host ranges for diarrhea-associated *Escherichia coli* serotypes were isolated from stool specimens from pediatric diarrhea patients and from environmental water samples. All four phages showed a highly efficient gastrointestinal passage in adult mice when added to drinking water. Viable phages were recovered from the feces in a dose-dependent way. The minimal oral dose for consistent fecal recovery was as low as 10^3 PFU of phage per ml of drinking water. In conventional mice, the orally applied phage remained restricted to the gut lumen, and as expected for a noninvasive phage, no histopathological changes of the gut mucosa were detected in the phage-exposed animals. *E. coli* strains recently introduced into the intestines of conventional mice and traced as ampicillin-resistant colonies were efficiently lysed in vivo by phage added to the drinking water. Likewise, an in vitro phage-susceptible *E. coli* strain freshly inoculated into axenic mice was lysed in vivo by an orally applied phage, while an in vitro-resistant *E. coli* strain was not lysed. In contrast, the normal *E. coli* gut flora of conventional mice was only minimally affected by oral phage application despite the fact that in vitro the majority of the murine intestinal *E. coli* colonies were susceptible to the given phage cocktail. Apparently, the resident *E. coli* gut flora is physically or physiologically protected against phage infection.

Diarrhea is the second most common cause of morbidity and mortality among infants and children in developing countries, exceeded only by respiratory diseases (47). Diarrhea has a complex etiology (22). However, *Escherichia coli* and rotavirus account for up to 50% of childhood diarrhea cases in developing countries (2, 3, 16, 23, 26). Enterotoxigenic *E. coli* (ETEC) is also a leading cause of traveler's diarrhea (9). Although the use of oral rehydration solutions has substantially reduced mortality from dehydration, it has little or no effect on the diarrhea itself and no effect on the transmission of the disease (7). Vaccines against diarrhea-causing *E. coli* are still in an early developmental stage (42, 43). A potentially low-cost treatment option for bacterial diarrhea was proposed 80 years ago by Félix d'Hérelle in the form of bacterial viruses (bacteriophages). Indeed, in the 1930s American physicians used pharmaceutical phage preparations for the treatment of both diarrheal diseases and staphylococcal infections (reviewed in references 15 and 50). The development of antibiotics in the 1940s replaced phages as therapeutic agents in the West, although enteric diseases, nosocomial infections, burns and wound infections continued to be treated with phage preparations in the Soviet Union on a very large scale (49). Western scientists were either unaware of this work or remained dubious about the reported high success rate despite the positive results obtained with the phage treatment of *E. coli* infections in a number of farm animals (calves, piglets, lambs, and chickens) (6, 45, 46).

An ideal candidate for phage therapy of *E. coli* infections is the coliphage T4 family. T4 is arguably the best-characterized biological system (25). This phage family is a natural component of the mammalian gut and can be easily isolated from the environment (stool and sewage) (1, 20, 21, 29). The richest sources of T4-like phages are apparently stools of diarrhea patients (20, 21). T4-like phages can be grown to high titer on laboratory *E. coli* strains. Early during the infection cycle T4 degrades the host DNA to the nucleotide level, preventing any integration of phage DNA into the bacterial chromosome (lysogeny) (34). T4 and a number of related phages have been completely sequenced (http://phage-.bioc.tulane.edu/) (33), and no phage-associated bacterial virulence factors have been detected in these phages.

Despite these assets of the T4 phage system, the potential of bacteriophage therapy in human infections has not yet been carefully documented in scientific publications. Part of the academic community is therefore still "phage skeptic." They point to the experience that the tremendous in vitro lytic activities of coliphages was rarely, if ever, demonstrated in carefully documented in vivo situations. In fact, not much is known about the factors governing the phage-bacterium interaction in the context of the complex microbial environment of the mammalian gut. To help fill this gap, we report here on the gastrointestinal passage of a set of orally applied T4-like phages in mice. In addition, we studied their in vivo bacteriolytic activities on the resident gut *E. coli* flora and towards *E. coli* strains introduced into the gut.

MATERIALS AND METHODS

Phage isolation. Phage JS4 and JS94.1 were isolated from stool samples of pediatric patients with undifferentiated diarrhea hospitalized at the International Center for Diarrheal Disease Research in Dhaka, Bangladesh. The stool sample (~10g) was resuspended in TS (NaCl [8.5 g/liter], tryptone [1 g/liter]) to a final volume of 30 ml and centrifuged for 15 min at 14,500 × *g* in 50-ml Falcon tubes.

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One milliliter of each stool preparation was filtered through a Millex AP20 prefilter followed by a 0.45-μm-pore-size Minisart filter. Subsequently, the samples were stored at 4°C. Phage JSD.1 was isolated from environmental water in Dhaka, Bangladesh, and phage JSL.6 was isolated from a sewage station in Vidy (Lausanne), Switzerland. Fifty milliliters of water samples was centrifuged at 10,000 rpm for 15 min and filtered through a 0.45-μm-pore-size Minisart filter.

The presence of phages was screened on the laboratory strain K803 (a K-12 derivative lacking prophage lambda, described in reference 5). The strain lacks restriction-modification systems and prophage lambda. K-12 is one of the major strains that have been widely used for phage studies as well as for recombinant DNA work. It is susceptible, for example, to nearly all of the over 100 T4-like phages in the Evergreen collection, most of which were isolated on *E. coli* B or on some pathogenic strain. The K803 strain was propagated in Hershey broth (prepared according to the recipe in reference 26) at 37°C with agitation (240 rpm). After overnight growth, the strains were streaked on a Hershey agar petri dish. Each time needed, a new culture was grown from a single colony. The stock cultures were kept as stab cultures at 4°C.

Spot testing was done on Hershey plates (15 g/liter agar) overlaid with 3.5 ml of Hershey top agar (7.5 g/liter). Ten microliters of filtered samples was put as eight spots in clockwise distribution around the plate after the top agar with plating bacteria solidified. For phage plaque assays, top agar (7.5 g/liter) was inoculated with 200 μl of a fresh overnight culture and 100 μl of positive sample and incubated overnight at 37°C

One well-separated phage plaque was chosen for amplification from each positive stool sample, picked with a sterile toothpick, and inoculated into 5 ml of Hershey broth together with 1% of an overnight culture of the *E. coli* strain K803. Incubation was performed with agitation at 240 rpm at 37°C. When lysis occurred, 3 drops of chloroform was added. The lysate was left overnight at room temperature followed by centrifugation at 14,500 × *g* for 10 min. The supernatant was transferred into a screw-cap glass tube. Three drops of fresh chloroform was added, and the phage stock was stored at 4°C. The phage lysate was at least diluted 1,000-fold into mineral water for the mouse feeding experiments.

Lysis in tube. The lysis test was done as follows. Five milliliters of Hershey broth (23) was inoculated with 1% of a freshly grown culture (10^9 CFU/ml) and 1% phage lysate (10^8 PFU/ml). Incubation at 37°C was continued under aerobic conditions in a shaking incubator (240 rpm) for 3 to 5 h until the uninfected control cells reached the stationary growth phase. The optical density (OD) in the phage-inoculated cell was compared to that of mock-infected control cells. For anaerobic conditions, tubes were held in an anaerobic jar at 37°C for 5 to 10 h. The media were not prereduced; there was thus significant oxygen present during the early hours of the experiment.

Broth culture of phages. *E. coli* was inoculated 1:100 in 200 ml of Hershey medium and incubated at 37°C in a shaking incubator (240 rpm). When an OD at 600 nm of 0.1 was reached, stock phages were inoculated with 10^7 PFU/ml. Each 15 min, samples were taken and OD readings of infected and uninfected cells were done at 600 nm. Samples were then centrifuged (10,000 × *g*, 5 min, 20°C), and chloroform-treated phage was titrated in the supernatant.

Pathogenic *E. coli* strains. The tested collection of pathogenic *E. coli* strains included 12 enteropathogenic *E. coli* (EPEC) strains, representing the major serotypes isolated worldwide from pediatric diarrhea patients (41). This set of strains covered 10 different somatic O antigens and 10 different capsular K antigens (Table 1). In addition, the collection contained 12 major ETEC serotypes isolated from either pediatric gastroenteritis patients or adults suffering from traveler's diarrhea (Table 1). The ETEC strains represented 11 further O antigens, 10 distinct H antigens, and various combinations of heat-stable (ST) and heat-labile (LT) toxin producers (Table 1). These 22 pathogenic *E. coli* strains were obtained from B. Rowe (Central Public Health Laboratory, London, United Kingdom). Twelve further distinct ETEC and six EPEC strains were obtained from the microbiology laboratory of the International Center for Diarrheal Disease Research. They represent predominant *E. coli* isolates from their hospitalized pediatric diarrhea patients. The pathogenic *E. coli* strains from Dhaka were typed by DNA probes for the presence of ST and LT enterotoxin, ST, colonization factor antigen (CFA), *E. coli* surface antigens (CS), and the attaching-effacing genes (A/E) (Table 1) according to published methods (5, 17, 24).

Phage purification. One liter of Hershey medium was inoculated with a bacterial colony, grown to an OD of 0.1, and then infected at a multiplicity of infection of 5. NaCl was added to the lysate to a final concentration of 0.5 M and incubated 1 h at 4°C. After centrifugation at 10,000 rpm (16 min at 4°C) in a Sorvall RC5B centrifuge, polyethylene glycol 6000 was added to the supernatant to a final concentration of 10%. The lysate was incubated overnight at 4°C with gentle stirring. Polyethylene glycol-precipitated phages were collected by centrifugation at 14,500 × *g* for 16 min. The resulting pellets were resuspended in 3 ml of phage buffer (20 mM Tris-HCl [pH 7.4], 100 mM NaCl, 10 mM $MgSO_4$), loaded on a discontinuous CsCl gradient (CsCl at 1.35, 1.53, and 1.65 g/ml), and centrifuged at 4°C in an SW55 rotor at 40,000 rpm for 3 h in a Beckman L8-60 M ultracentrifuge. Purified phage were recovered with a syringe and dialyzed against phage buffer.

Electron microscopy. A drop of the phage suspension was applied to a Formvar carbon-coated copper grid for 5 min; the suspension was removed with a pipette and immediately replaced by a mixture of solutions A and B (solution A, 2% ammonium molybdate at pH 7.0 or 2% PTA; solution B, 11% bacitracin in distilled water) or a solution of 3% uranyl acetate. After 1 min the liquid was removed with a filter paper. The grids were examined in a Philips CM12 transmission electron microscope at 80 kV (magnification, ×176,000 or ×224,000). The dimensions of the phage were calibrated with T4 phage particles (25).

DNA purification. Purified phages were treated with proteinase K at a final concentration of 1 mg/ml for 2 h at 37°C, and 3 M sodium acetate (pH 4.3) was added. DNA was extracted twice by phenol-chloroform and precipitated with 2 volumes of ethanol. After centrifugation, pellets were washed with 70% ethanol and resuspended in 50 μl of Tris-EDTA. DNA was digested with restriction enzymes according to the instructions provided by the manufacturer.

Experimental animals. Eight-week-old C3H male mice (Charles River, St. Germain sur l'Arbre, France) were held under standard animal house conditions and fed irradiated 03-40 chow from Usine d'Alimentation Rationelle (Villemoissin-Orge, France). The drinking water, which did or did not contain phage at the specified titer, consisted of Vittel mineral water. We initially used this water since it contained bicarbonate at 258 mg/liter, reasoning that bicarbonate would buffer the stomach acidity and allow more efficient stomach passage of the phage. However, in later experiments we observed that mineral waters containing less bicarbonate (65 mg/liter) allowed an equally efficient gut passage of the phage (data not shown). The mineral water was changed every two days. Feces were sampled once a day directly from the hand-held animal into a sterile tube by gently pressing the abdomen of the animals to avoid contamination of the stool with bedding material or dripping from the water bottles. For each experimental series, five mice were used. Each mouse was held in a Makrolon type 3 cage with a filtered lid (Indulab, Gams, Switzerland), preventing cross-contamination between the cages. Stool samples were resuspended in 1 ml of phosphate-buffered saline. Tenfold dilutions of the resuspended feces were plated on Drigalski agar (Bio-Rad). This is a medium recommended for coproculture (Diagnostics Pasteur). The medium is not specific for *E. coli* but allows the differentiation of lactose-fermenting colonies (*E. coli*, *Klebsiella*, *Enterobacter*) yielding yellow colonies from lactose-nonfermenters (*Salmonella*, *Shigella*, *Proteus*, *Providencia*, *Hafnia*, *Serratia*, *Levinea*, *Edwardsiella*, *Alcaligenes*, *Pseudomonas*) yielding blue-green colonies (Diagnostics Pasteur). Practically all colonies from mouse fecal pellets were lactose positive. Since *Klebsiella* and *Enterobacter* species do not belong to the normal mouse fecal flora (52), the yellow colony count is practically an *E. coli* count. This diagnosis was confirmed by phage susceptibility: practically all colonies were lysed by one of the T4-like *E. coli* phages from our collection (see Results). We confirmed that the T4-like phages did not lyse a distinct genus of *Enterobacteriaceae*, for example, the food pathogen *Enterobacter sakazaki* (P. Breeuwer, unpublished results). The various T4-like phages were added to the drinking water in dilutions as specified in the text. In the experiments four mice received mineral water with phage while one negative mouse control in each experiment received only mineral water. Phages were titrated by the plaque assay in filtered fecal samples on the *E. coli* indicator cell K803. At the end of the experiment, the mice were sacrificed and standard gross anatomical and histopathological examinations were conducted. Different parts of the gut were rinsed with physiological salt before cells and phages were counted by colony and plaque assay.

Mouse experiments with ampicillin-resistant cells. *E. coli* K803 was grown in Hershey broth to an OD (600 nm) of 0.7. Cells were centrifuged for 20 min at 4,500 rpm. The pellet was carefully resuspended in cold H_2O (4°C). The cells were then washed twice with cold 10% glycerol. Finally the pellet was resuspended in 1 ml of cold glycerol and kept at −80°C as competent cells.

One hundred microliters of cells was electroporated with 0.5 ng of pUC18 using the following settings: 25 μF, 2.5 kV, and 200 Ω. The cells were incubated for 1 h in SOC (2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCL_2$, 20 mM Mg SO_4, 20 mM glucose) medium at 37°C and plated on Hershey agar containing 20 μg of ampicillin.

The experiments with the Amp^r cells were conducted with a total of 21 animals, i.e., three groups of seven mice per experiment. In each experiment, three mice received the Amp^r cells without phage, three received Amp^r cells and phage in the drinking water, while one mouse received phage orally but no Amp^r cells. Six-week-old C3H male mice were taken for the experiment. The three experiments differed with respect to addition of oral ampicillin (experiment 1, no

TABLE 1. Susceptibilities of *E. coli* strains to infection with T4-like phages

Strain	Lysis[b] caused by:				Strain	Lysis[b] caused by:			
	JS4	JSD.1	JSL.6	JS94.1		JS4	JSD.1	JSL.6	JS94.1
EPEC O18:K77	X				ECOR				
EPEC O20:K84	X			X	1				
EPEC O26:K60	X				2				
EPEC O55:K59	X		X		4			X	X
EPEC O86:K61					5				
EPEC O111:K58				X	6				
EPEC O112:K66	X				8				X
EPEC O119:K69				X	9				
EPEC O124:K72	X				11				
EPEC O125:K70					12				
EPEC A/E+[a]	X			(X)	13				X
EPEC A/E+[a]	X				14				X
EPEC A/E+[a]	X		X		15	X		X	
EPEC A/E+[a]					24		X	X	
EPEC[a]		(X)		X	26	X			
EPEC[a]					28				
EPEC[a]		(X)		X	35	X			
EPEC[a]					36	X			
					38	X			
ETEC O6:H16					39	X			
ETEC O8:H9			X		40	X			
ETEC O15:H11					41				
ETEC O25:H42	X				42				
ETEC O78:H12					43				
ETEC O115:H51					48	X			
ETEC O20:H11					49	(X)			
ETEC O27:H7					50				
ETEC O128:H18					51				
ETEC O63:H−					53				
ETEC O148:H28	X				54				
ETEC O153:H12					55				
ETEC LT+/ST+; CFA 1[a]					56		X		
ETEC LT−/ST+; CS6[a]					59				
ETEC LT−/ST+; PCF O166[a]					60	(X)			X
ETEC LT−/ST+; CFA1[a]		X			61	(X)			
ETEC LT−/ST+; CS4; CS6[a]					62				
ETEC LT+/ST+; CS1; CS3[a]		X	(X)		63		X		
ETEC LT+/ST+; CS5; CS6[a]					64				
ETEC LT+/ST+[a]					71				
ETEC LT+/ST+[a]					72				
ETEC LT+/ST−[a]	X								
ETEC LT+/ST−[a]									
ETEC LT+/ST−[a]	X								

[a] Isolated from the Dhaka hospital laboratory.
[b] X, lysis; (X), partial lysis.

ampicillin; experiment 2, ampicillin was given together with Amp^r cells; experiment 3, ampicillin was given first, followed by Amp^r cells). Their drinking water consisted of Vittel supplemented with the four-phage cocktail (10^6 PFU/ml) and ampicillin (20 mg/ml) as specified in the text. The animals were force fed with ampicillin-resistant K803 (5×10^7 CFU) supplemented with 6 mg of ampicillin as specified in the text. Feces were sampled twice a day for the first 4 days and once a day for the rest of the study. Tenfold dilutions of resuspended feces were plated on Drigalski agar containing 20 μg per ml of ampicillin.

Axenic mice. A total of six 8-week-old C3H axenic male mice from our own animal house breeding colony were allotted to three experiments. Each group consisted of two animals held under sterile conditions in a Makrolon type 2 cage maintained in the same cage without a filtered lid within a positive pressure isolator of the animal house. In experiment 1 mice were force-fed with *E. coli* K803 (0.5 ml at a concentration of 10^8 CFU/ml) using intubation. Daily fecal samples were investigated for *E. coli* cell counts on Drigalski plates. One week after colonization, sterile-filtered phage JS94.1 was given continuously at a concentration of 10^5 PFU/ml in the drinking water followed by daily fecal cell and phage counts. In the next week a second force-feeding was performed with *E. coli* ECOR5 followed two weeks later by force-feeding with ECOR56. In experiment 2 mice received cells and phage at the same time, while in experiment 3 the mice received first the phage and then the bacteria.

RESULTS

Isolation and characterization of the phages. When tested on the *E. coli* strain K803, stool samples from pediatric diarrhea patients and environmental water samples in Dhaka, Bangladesh, and sewage from Switzerland yielded nearly exclusively *Myoviridae* (phages with a contractile tail). The elongated heads measured 110 nm by 75 nm. A collar separated the head from the tail sheath, which measured about 95 nm in length and 18 nm in width, with an annular substructure (Fig. 1A). The tail is terminated by a base plate structure to which both short tail spikes and 150-nm-long tail fibers with a central knee joint are attached (Fig. 1A, subpanel b). Phages

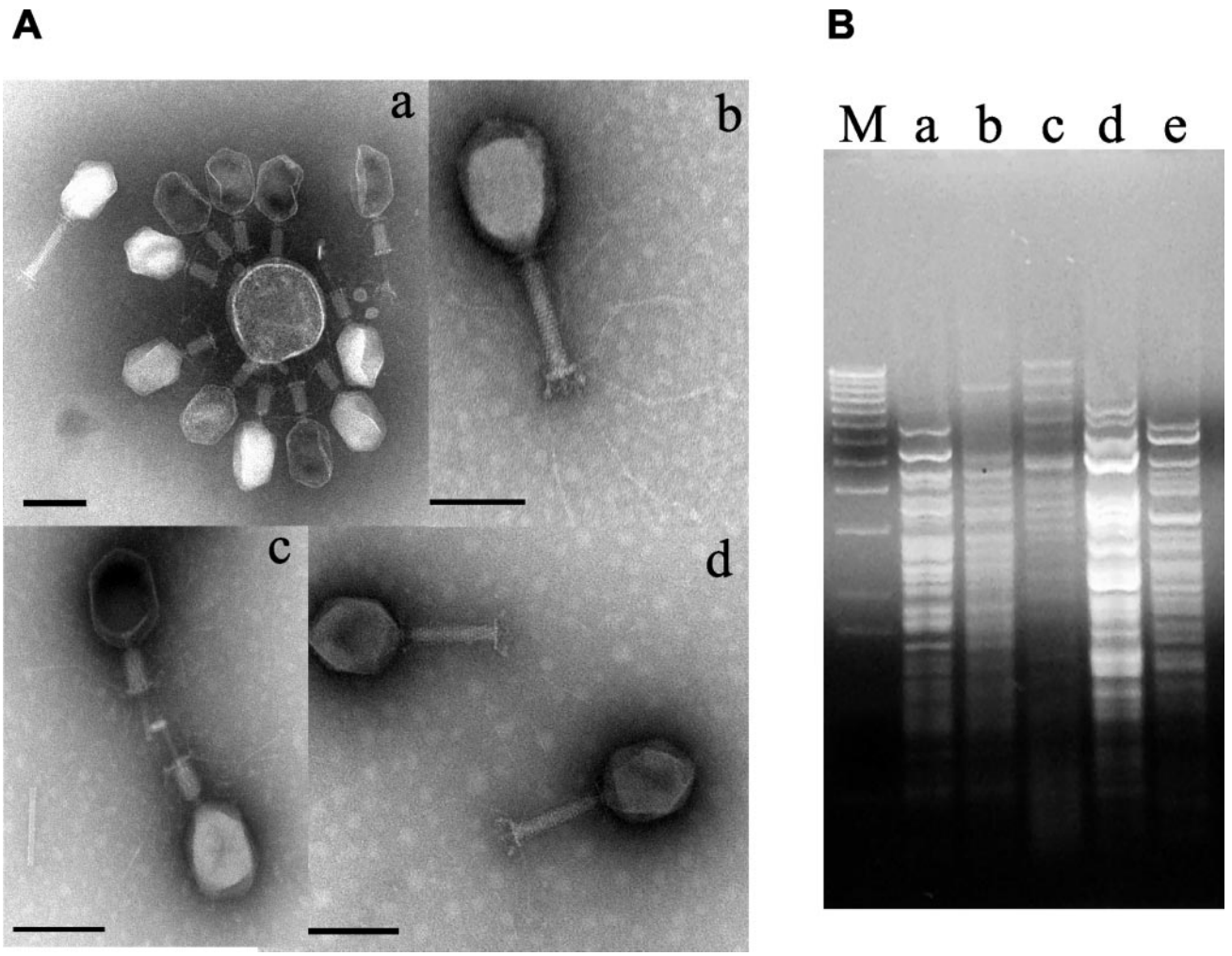

FIG. 1. Four T4-like phages used in the mouse experiments. (A) Transmission electron microscopy picture of CsCl density gradient-purified bacteriophage JS4 (a), JSD.1 (b), JSL.6 (c), and JS94.1 (d). Negative staining was performed with uranyl acetate (c), ammonium molybdate (a and d), or phosphotungstic acid (c). The size bar corresponds to 100 nm. (B) Restriction analysis of phages (for lanes a to d, see corresponding subpanel in panel A; lane e, phage T4) with enzyme DraI. Lane M, DNA size marker (1-kb lambda DNA ladder; Invitrogen).

with a 40-nm contracted tail sheath were also observed (Fig. 1A, subpanels a and c). In these phages, an internal tail tube extended beyond the contracted tail sheath. The morphology suggests T4-like phages. All phage isolates were tested individually in the spot test against a collection of pathogenic *E. coli* strains associated with diarrhea. From this screening a group of four phages was selected that offered the broadest combined theoretical host range. All four phages showed the typical morphology of T4-like phages (Fig. 1A); showed, like phage T4, a 170-kb genome upon pulsed-field gel electrophoresis; yielded with gp23- and gp32-specific primers the diagnostic PCR products for T4-like phages (36, 53) (data not shown); and showed distinct restriction patterns (Fig. 1B).

These four selected phages were rescreened for their lytic potential on pathogenic *E. coli* strains by a tube lysis test. This test is more labor-intensive than the spot test but offers a more rigorous assessment of the bacterial lysis activities of the test phages (Table 1). Also in the tube lysis test, the stool phages JS4 and JS94.1 and the environmental water phages JSD.1 and JSL.6 showed a complementary lytic potential on our pathogenic *E. coli* strain collection (Table 1). Most notably, the combined theoretical host range determined by adding up the host ranges of the four individual phages was 19 out of 40 (47%) of the pathogenic *E. coli* strains. When tested on the K803 strain, progeny phage was detected in broth culture infections at about 40 min postinfection and phage titer increased afterwards, sometimes in a biphasic way (Fig. 2B). The OD of phage-infected K803 cultures lagged from the beginning behind the OD development of the uninfected culture (Fig. 2A).

The fact that *E. coli* is also a normal constituent of the gut flora of humans could present a peculiar problem for phage therapy of *E. coli* diarrhea. The four phages constituting the cocktail were therefore also investigated individually for their lytic potential on nonpathogenic *E. coli* strains from the ECOR collection (30) in the tube lysis test (38). The ECOR collection is a widely used set of 72 reference *Escherichia coli* strains isolated between 1973 and 1983 from a variety of animal hosts and a variety of geographic locations. In broth culture, JS4, JS94.1, JSL.6, and JSD.1 lysed eight, three, three, and five

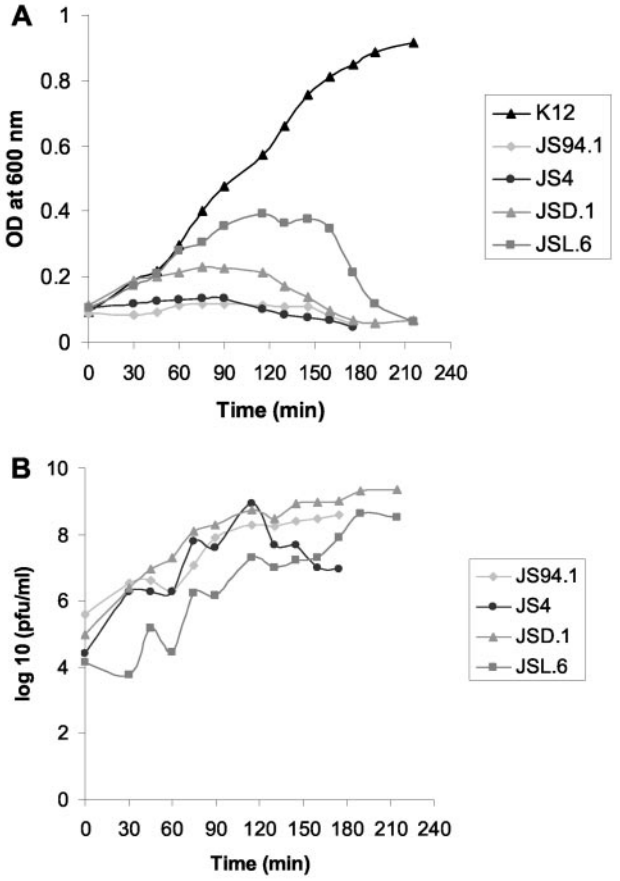

FIG. 2. Lysis of *E. coli* K803 strain by the four T4-like phages in broth culture. (A) OD development of an uninfected control culture (K-12) and parallel cultures infected with phages JS94.1, JS4, JSD.1, and JSL.6. (B) Progeny phage release from the four phage-infected cultures depicted in panel A. Phage infectivity was measured by plaque assay.

strains, respectively, from 39 ECOR strains included in the test (Table 1). The combined theoretical host range determined by adding up the host ranges of the four individual phages was 18 (46%) of the nonpathogenic *E. coli* strains.

In the next step, we explored the in vitro lytic activities of the four phages on the endogenous *E. coli* gut flora from a group of ten conventional adult mice. Over a 10-day observation period, feces were recovered every day for each mouse and lactose-positive yellow colonies were counted on Drigalski agar plates. The fecal cell counts showed an average of 10^6 CFU/g (data not shown), which is in agreement with similar results obtained by Poulsen et al. (39). Five random colonies were selected per day for each mouse, and the total of 500 colonies was tested against the four phages in the spot test to reduce the workload. Between 85 and 100% of the tested colonies were lysed by phages JS4 and JSD.1. JSL.6 lysed about 80% of the colonies. More variation was observed with phage JS94.1, which lysed less than 40% of the isolated murine *E. coli* gut strains in six animals. When the results were combined, practically all cells were lysed by one of the *E. coli* phages, confirming the attribution of the vast majority of the yellow colonies from the feces of conventional mice to *E. coli*. One fecal sample of each mouse was tested over 5 days for the presence of phage on the K803 indicator cell in the plaque assay. No phage plaques were detected.

Effect of oral phage on fecal *E. coli* count in mice. Next we wanted to determine the threshold for an in vivo lytic effect of orally applied phages on the intestinal *E. coli* population in laboratory mice. To this end the four phages were added as a cocktail to the drinking water of 10 mice in increasing doses separated by 3 days of phage-free drinking water. Substantial variation was seen for the lactose-positive cell count on the Drigalski plates in all animals, even before phages were added to the drinking water. This variation was also seen during the periods of phage feeding to the animals. Using a two-way analysis of variance with phage dose as a fixed factor and animals as a random factor, we derived the following means and standard errors of the means for fecal colony counts on Drigalski plates: for water only, $10^{6.2 \pm 0.04}$; for 10^3 phage/ml, $10^{5.9 \pm 0.10}$; for 10^5 phage/ml, $10^{6.1 \pm 0.06}$; for 10^7 phage/ml, $10^{5.7 \pm 0.06}$. The effect of the phage dose on the cell count was highly significant (P value < 0.0001) but was in absolute terms very small and thus biologically not significant.

In view of the in vitro phage susceptibility of the most prevalent lactose-positive fecal colonies on Drigalski agar, the lack of a bacteriolytic effect of the oral phage on the fecal cell count was surprising. We considered several hypotheses to explain this observation. First, under the selective pressure of the phages the prevalent phage-susceptible strains might have been replaced by phage-insensitive strains. Second, without protection (antacid or microencapsulation) phages might not survive the gastric passage and thus not be available in the intestine. Third, phages might be present in the gut, but for physiological reasons the endogenous intestinal *E. coli* cell population resists phage infection.

The first hypothesis was addressed by the isolation of 100 additional lactose-positive colonies from the feces of two animals during the phage treatment period. The colonies showed a comparable phage susceptibility pattern before and during the phage treatment period, leading to the rejection of the hypothesis of an intestinal outgrowth of phage-resistant *E. coli* or other *Enterobacteriaceae* under the selective pressure of the oral phages.

Gastrointestinal passage of orally applied phages. The following experiments demonstrated that unprotected T4-like phages could survive the gastric passage in conventional adult laboratory mice. These experiments refute the second hypothesis.

To begin, we determined the lowest phage concentration leading to stable fecal phage excretion. To this end four animals received in the drinking water successively the four individual phages added at 10-fold dilution steps. Fecal phage titers decreased with the titer of the phage in the drinking water in an approximate dose-response pattern (Fig. 3). With the lowest phage concentration of 10^3 PFU/ml in the drinking water, only low fecal phage titers over short time periods were observed, while exposure to 10^4 PFU/ml resulted in fecal phage detection nearly over the entire exposure period (Fig. 3).

In the next experiment, we asked whether the gastrointestinal passage differed between the individual phages or between individual mice. To answer this question, four mice received

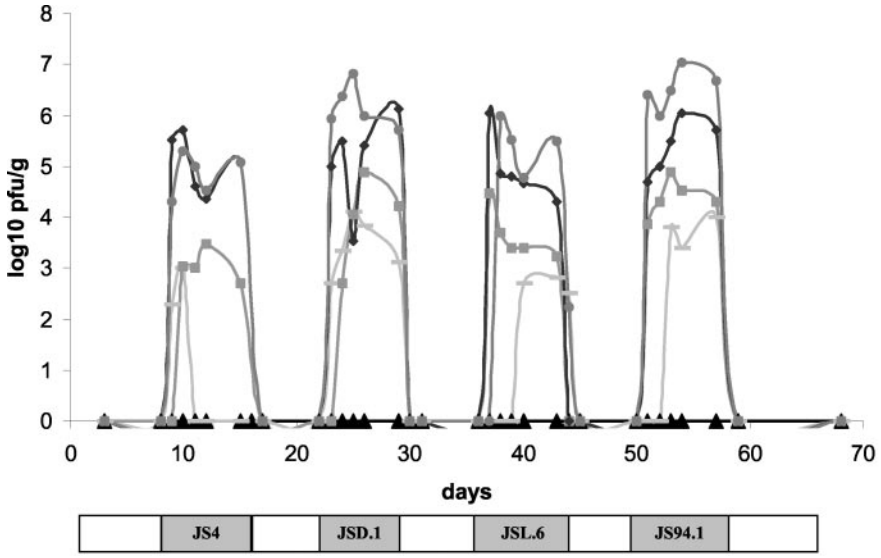

FIG. 3. Gastrointestinal passage of the orally added phages in conventional mice. Fecal phage titer after oral addition of the specified phage strain at 10^6 (circles), 10^5 (diamonds), 10^4 (squares), and 10^3 (bars) PFU/ml, fed to four mice at the times indicated by the shaded bars at the bottom of the figure. The triangles give the phage titers for the control mice. The periods of phage-free drinking water are indicated by white boxes.

successively each of the four individual phages at a fixed concentration of 10^7 PFU/ml in the drinking water. Each phage addition was followed by a phage-free drinking water period before the next phage isolate was added to the drinking water. The fecal phage counts were assessed on a daily basis (data not shown). Three observations were made in this experiment. First, no significant difference was detected between the individual animals with respect to gastrointestinal phage passage. Second, some difference in gastrointestinal passage was detected between the different phage isolates as documented by a 50-fold fecal phage titer difference between mice fed phage JS94.1 and JSL.6 which achieved the highest and the lowest fecal phage titers, respectively (data not shown). Third, in one mouse the identity of the fecally excreted phage with the phage in the drinking water was demonstrated for all four feeding periods by restriction analysis of the fecally reisolated phage.

Next we asked whether the stool phage isolates could not infect their host cells due to the anaerobic atmosphere of the gut environment. This was not the case: during in vitro growth in an anaerobic jar, 11 of 32 T4-like stool phage isolates and one of the four test phages (JSL.6) lysed its target cells under both anaerobic and aerobic conditions.

Finally, we asked whether phages could be given repetitively without interference by an intestinal immune response. Phage JS94.1 was given three times to two mice. Each intervention was followed by a 2-week rest period. In each case infectious phage was detected in the stools samples with titers approximately proportional to concentration of the phage in the drinking water (data not shown).

Phage treatment of axenic mice. To test the in vivo lytic activities of the isolated stool phages, we inoculated two axenic mice with a single *E. coli* strain, namely, the indicator cell K803, resulting in a cell concentration of 10^8 CFU/g of feces (Fig. 4A). One week later, the K803-colonized mice were exposed to phage JS94.1 at 10^5 PFU/ml in the drinking water. Within a day, the fecal phage titer in the JS94.1-exposed mice rose from undetectable titers to beyond 10^{10} PFU/ml (Fig. 4A). The 100,000-fold titer increase with respect to the phage concentration in the drinking water documented an active replication of phage JS94.1 in the guts of the experimental animals. Concomitantly, the fecal *E. coli* cell count dropped from 10^8 to 10^4 CFU/ml or even lower, documenting a substantial in vivo bacteriolytic activity of the orally applied phage (Fig. 4A). Despite this serious drop in host cell density, the very high fecal phage titers decreased only slowly over the next four days. Interestingly, over the same time period the fecal cell count increased from undetectable levels to about 10^5 CFU/ml, which is still 1,000-fold lower than the original fecal cell count. At days 9 and 11, several dozen colonies were picked. All were sensitive to phage JS94.1 suggesting that some of the cells are now in gut sites protected from phage.

Two weeks after the colonization with K803, the mice were force-fed the ECOR5 strain that is insensitive to phage JS94.1 in vitro. Within days, the fecal cell count rose to 10^{10} CFU/ml, suggesting successful colonization with the new *E. coli* strain (Fig. 4A). In parallel with the renewed fecal cell count increase, the fecal phage titer dropped by 4 logs or more, consistent with the expected replacement of a phage-sensitive by a phage-insensitive intestinal cell population. Alternatively, the phage-sensitive population did not change, but the ability of the phage to infect was diminished under the circumstances created. The mice were then force-fed with ECOR56 strain (sensitive to phage JS94.1) followed by phage JS94.1 in the drinking water. This operation did not rescue the fecal phage titer (Fig. 4A), suggesting colonization resistance or host immune defenses. At day 42, no phages were detected in four different rinsed gut segments (duodenum, jejunum, ileum, colon) or in the liver or in mesenteric lymph nodes.

In the next experiment, two axenic mice were force-fed with 10^4 K803 cells and received at the same time the four-phage cocktail at 10^6 PFU/ml in the drinking water (data not shown). One mouse showed an initial high fecal cell count (10^9 CFU/g

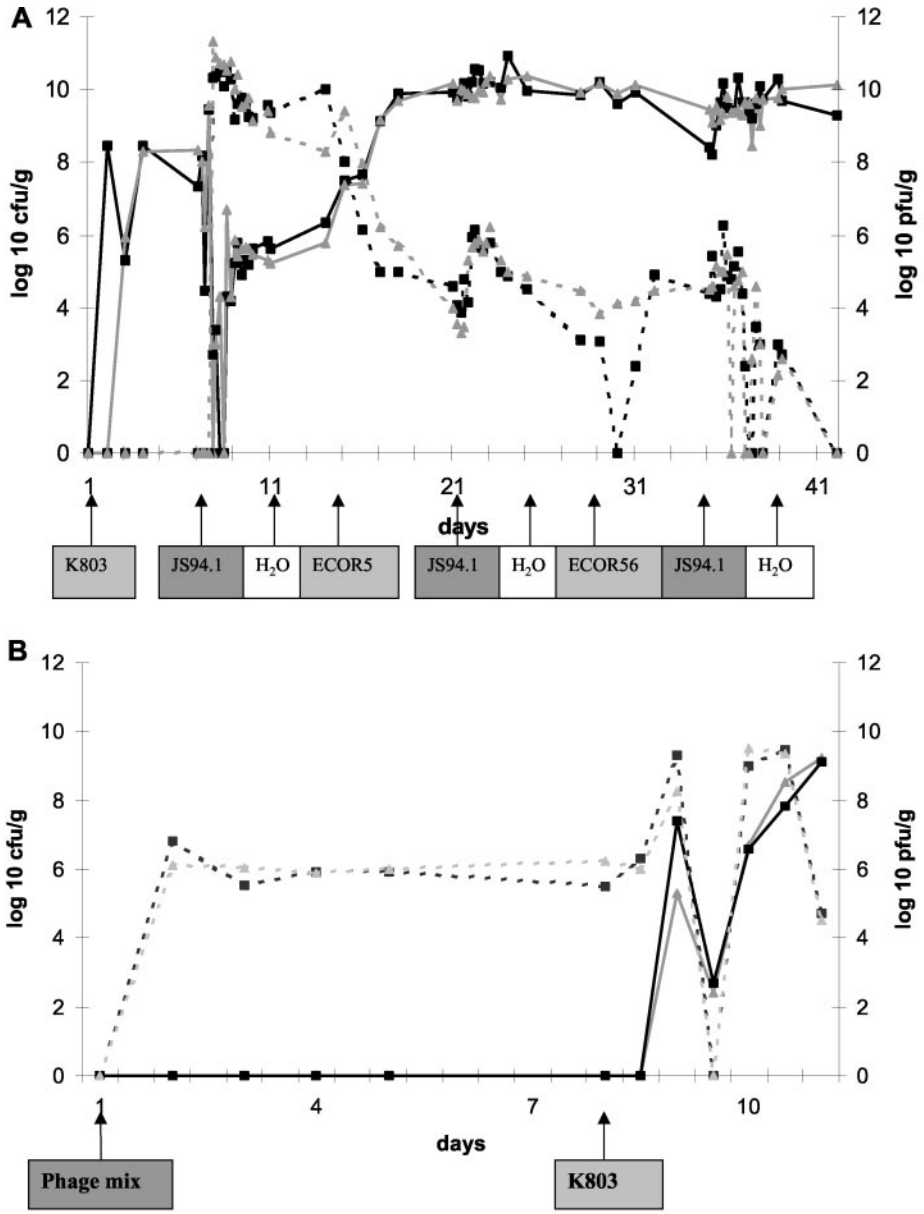

FIG. 4. Effect of oral phage on the inoculated *E. coli* strain in axenic mice. (A) Fecal *E. coli* counts (solid line in log CFU per milliliter) and fecal phage counts (dashed line in log PFU per milliliter) in two axenic mice exposed to the specified *E. coli* strains, phage JS94.1, or water at the specified time points; the start day is indicated with an arrow below the abscissa. A black line with squares or a gray line with triangles identifies values from an individual mouse. (B) 10^6 PFU/ml was given from day 1 in the drinking water to axenic mice lacking intestinal bacteria. The mice were force-fed with 10^4 CFU of K803 at day 8. Logarithmic fecal cell (solid line) and phage counts (dashed line) per gram of stool were plotted over 10 days. A black line with squares or a gray line with triangles identifies values from an individual mouse.

of feces), followed by a precipitous drop to 10^4/g and lower. Another mouse showed a fecal cell count decrease from 10^6 to 10^4 CFU/g (data not shown). Both mice showed a fecal titer 1,000-fold higher than the drinking water phage titer over the first days of the experiment, suggestive of active in vivo phage replication.

Finally, two axenic mice were first exposed to the four-phage cocktail at 10^6 PFU/ml in the drinking water before receiving cells. Notably, in the absence of intestinal bacteria, 10^6 PFU of phages were also detected per g of stool (Fig. 4B), demonstrating that the fecal phages are not the result of intestinal replication of phages after a reduction of phage titers in the stomach, but the consequence of a passive transit through the entire gastrointestinal tract including the stomach. One week later the mice were force-fed with 10^4 K803 cells. Introduction of *E. coli* into the gut resulted in a transient 1,000- to 10,000-fold fecal phage titer increase (Fig. 4B). During the initial phase of intestinal phage replication, a low and variable fecal cell number was observed. This phase was followed by a steady increase of bacteria to 10^9 CFU/g stool over the next days (Fig. 4B), and bacteria remained at this level until 2 weeks later (data not shown), while the phage titers dropped to low levels.

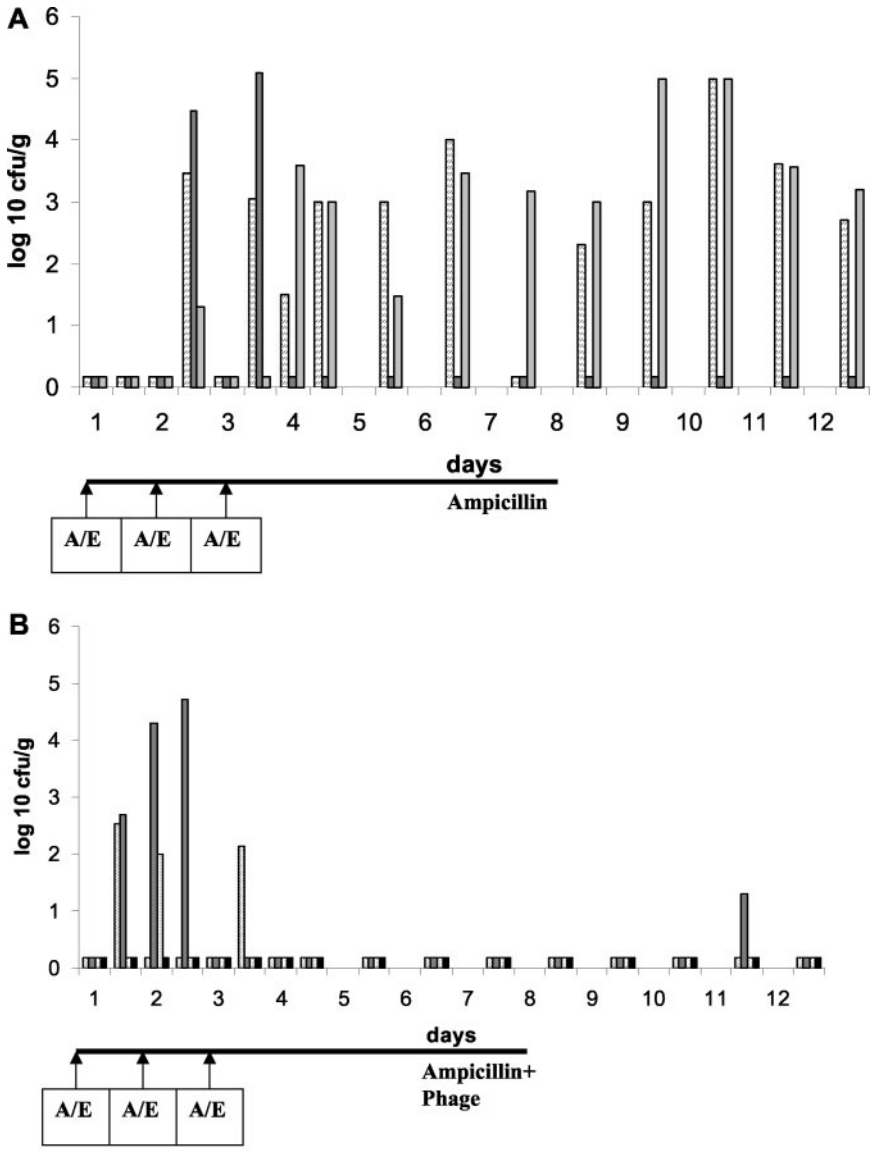

FIG. 5. Effect of oral phage on the introduction of ampicillin-resistant *E. coli* in mice. (A) Fecal cell counts in three mice force-fed with 5×10^7 CFU of ampicillin-resistant *E. coli* and ampicillin (A/E) at the time points marked with an arrow below the time axis. The ordinate shows the logarithm of CFU per gram of stool. Each vertical bar represents the fecal cell count for one animal at the specified time point. (B) The same experiment as depicted in panel A except that in addition to ampicillin the mice also received the phage cocktail at 10^6 PFU/ml in the drinking water. The rightmost black data points refer to a control mouse not receiving ampicillin-resistant *E. coli*.

Follow-up of Amp^r *E. coli* cells in conventional mice. The preceding experiments suggested that orally applied phages lysed only *E. coli* cells that were recently introduced into the intestine. To differentiate newly introduced from resident *E. coli* strains, 10^8 CFU of K803 cells transformed with plasmid pUC18 containing an ampicillin resistance marker were force-fed to three conventional mice. Transient peaks of fecal Amp^r *E. coli* cells were detected half a day after the force-feeding, but they were lost from the intestine half a day later (data not shown). No spontaneous fecal phage excretion was seen in these mice or the corresponding control mice of the experiments reported below. Three further mice received in addition 10^6 PFU of phage per ml in the drinking water. As in the preceding experiment, fecal Amp^r *E. coli* cells were only transiently observed directly after the force-feeding (data not shown). Phage was detected only during the phage feeding period with a 1-day time lag for appearance and 2 days for disappearance.

To overcome the colonization resistance of the resident intestinal flora against the introduction of new cells, the Amp^r-labeled cells were given together with ampicillin during the force-feeding. During the first week ampicillin was also added to the drinking water (20 μg/ml). Under these conditions, 10^3 to 10^5 CFU of the Amp^r *E. coli* cells were detected per g of stool and for at least 5 days maintained after omission of ampicillin from the drinking water (Fig. 5A). When similarly treated mice received phage in addition in the drinking water, Amp^r *E. coli* cells were detected after day 2 only in two fecal

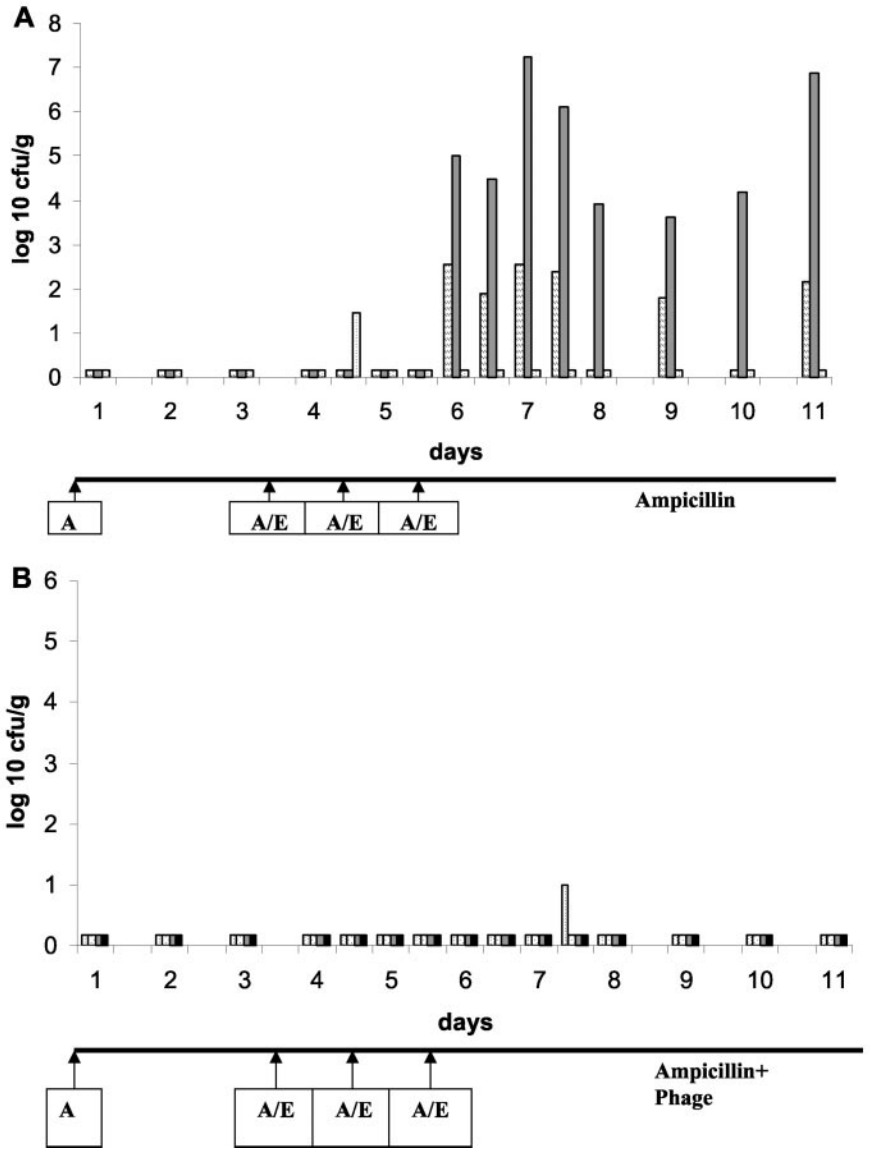

FIG. 6. Effect of oral phage on the introduction of ampicillin-resistant *E. coli* in mice pretreated with ampicillin in the drinking water. (A) Seven mice received ampicillin by force-feeding at day 1 and in the drinking water throughout the experiment. At the time points indicated with arrows marked with A/E, six mice were force-fed with ampicillin and ampicillin-resistant *E. coli* (a control mouse received only buffer instead of *E. coli*). Both groups of mice received ampicillin in the drinking water, but some mice were in addition exposed to 10^6 PFU/ml of the phage cocktail in the drinking water (B). Both panels show the fecal counts of ampicillin-resistant cells. The rightmost black data points in panel B refer to a control mouse not receiving ampicillin-resistant *E. coli*.

samples with low counts, suggesting elimination of Amp^r *E. coli* from the gut (Fig. 5B) by oral phage.

In the next experiment, six mice were pretreated with ampicillin both by force-feeding and in drinking water before being fed with Amp^r *E. coli* cells. In this experiment only one stool sample from three mice receiving phages in the drinking water showed a low fecal cell count of Amp^r cells (Fig. 6B) compared to 15 stool samples from the three mice receiving plain water (Fig. 6A).

Orally fed T4-like phages remain restricted to the gut. Four axenic mice were inoculated with K803 cells and then force-fed with 10^9 PFU of the phage cocktail and maintained on phage at 10^7 PFU/ml in the drinking water and finally sacrificed. Four different gut segments (once rinsed with buffer to remove the gut content), the liver, and the mesenteric lymph nodes were tested for the presence of K803 cell and phages. Neither cells (<10 CFU/ml) nor phages (<10 PFU/ml) were detected in the mesenteric lymph nodes or the liver. Four further axenic mice were sacrificed 4 days after a change to phage-free drinking water. None of the investigated tissues were associated with phages (data not shown). Tissue samples from eight phage-treated mice and two control mice were processed for standard histology analysis. For the intestinal samples, both longitudinal and cross-sectional histological cuts were analyzed. The tissue

morphology was normal in all animals. No behavioral or fur changes were observed in the animals during the treatment period.

DISCUSSION

The scientific literature shows a renewed interest in phage therapy. Part of this interest stems from a series of spectacular experiments conducted with phage lysins (31, 37, 44), another part comes from a number of recent animal experiments with viable phage particles as antibacterial agents (8, 11, 14, 28, 30, 32, 49, 54), and a further part derives from historical reviews of the Soviet experience with phage therapy (4, 10, 12, 18, 48, 49, 50). It is currently difficult to critically assess the potential of the clinical trials conducted in the former Soviet Union. The trials were mainly published in Russian, most of the trials were uncontrolled, and the therapeutic phages were not described in published reports. With the present study, we wanted to address some of the basic preclinical problems of phage therapy in the context of *E. coli* diarrhea. We have chosen this example because a substantial body of knowledge has been accumulated on *E. coli* and its phages; the pathogenic target bacteria are located in the gut and are thus principally accessible to orally applied phages; the Soviet experience reported efficiency of phages against dysentery; and we have some experience in the treatment of *E. coli* diarrhea with biologicals (bovine antibodies [13, 51]) and probiotic lactobacilli (S. Sarker et al., unpublished data).

For the experiments reported here we selected two stool- and two environmental water-derived T4-like phage isolates with a broad host range for diarrhea-associated *E. coli* serotypes. The chosen T4-like phages survived the gastrointestinal transit in adult mice. More specifically, the fecal phage count corresponded roughly to the orally applied phage titer. This quantitative correlation should on its own not be overinterpreted since it could represent a combination of inactivation of some phage in the stomach and the reproduction of some phage in the endogenous intestinal *E. coli* population. However, no major inactivation of T4-like phages occurred in the stomachs of adult mice since in axenic mice (lacking any intestinal microbial flora and thus the possibility to amplify phage), a similar oral-fecal phage titer correlation was found. Unprotected T4-like phage thus has the capacity to transit the entire gastrointestinal tract without appreciable infectivity loss. When compared to the timing of a pulse of phage in the drinking water, the appearance and disappearance of the phage in the feces took approximately 1 to 2 days. If coprophagy (the eating of feces, which in contrast to rabbits, was only occasionally observed in mice from our animal house) or water dripping from the bottles into the cage and not the orally applied phage were the source for maintenance of fecal phage titers in mice, one would have expected longer fecal phage disappearance times due to phage recycling. Notably, fecal pellets were not collected from the bedding of the cages excluding passive phage contamination. The murine stomach differs substantially from the human stomach by allowing the thriving of an endogenous *Lactobacillus* flora in the esophagus-proximal part, showing a mean pH value of 3.8 (52). However, in the pylorus-proximal part of the murine stomach the mean pH value was only 2.2 (52). This acidity killed 95% of acid-sensitive bacteria like *Vibrio cholerae* and half of the fragile *E. coli* mutant χ1666, but had little bactericidal effect on *E. coli* K-12 (19).

In the case of *E. coli* diarrhea, a peculiar problem for phage therapy is presented by the fact that nonpathogenic *E. coli* strains are a normal constituent of the gut flora of humans and many animals (52). The in vivo experiments demonstrated that collateral damage on nonpathogenic gut *E. coli* strains is an unlikely complication of oral application of T4-like phages. In conventional mice we observed no detrimental effect of the phage transit on the physiological *E. coli* flora. The total count of lactose-positive fecal colonies on the Drigalski plates decreased only slightly during phage treatment. No change from phage-sensitive to phage-resistant *E. coli* was seen, as if the cells had not experienced a phage infection pressure. In addition, in conventional mice we did not observe evidence for intestinal phage replication. Two basic hypotheses might explain this result. First, the intestinal *E. coli* cells might be in an altered physiological state (stationary state, starvation, anaerobic growth) that does not permit phage infection. Alternatively, physical factors might prevent the infection of the resident intestinal *E. coli* flora (reduced phage diffusion in the thickened gut content, difficulty for the phage in finding its target cell in the presence of a large excess of nontarget bacterial cells, and seclusion of the resident *E. coli* in a nonaccessible niche). Anaerobic growth prevented the in vitro growth of some, but by far not all, T4-like phages. However, residual oxygen was not rigorously excluded in these experiments. Nevertheless, in vivo growth of phages was demonstrated in axenic mice excluding anaerobiosis as a major limiting factor for phage replication.

Data from the literature help to understand the lack of activities of the orally applied T4-like phages on the resident *E. coli* flora. Fluorescent oligonucleotide probes targeting rRNA were used to localize *E. coli* cells in the large intestines of mice by the in situ hybridization technique. *E. coli* cells were seen embedded in the mucosal material overlying the epithelial cells of the large intestine, and no direct attachment to the epithelium was observed (39). Extension of these studies revealed that *E. coli* consisted in the murine intestine of two populations, one in the mucus which has an apparent generation time of 40 to 80 min and one in the luminal contents which is static (40). Interestingly, a defined *E. coli* strain when introduced into the murine intestine differentiated into two distinct populations, one that has the characteristics of the laboratory-grown strain and one that appears as a coccoid cell. The authors observed natural selection for the coccus-type cell in the intestine and for the rod-shaped *E. coli* cell in laboratory medium growth (27).

On the basis of our observations and the literature data, the most conclusive interpretation is the following. Orally applied T4-like phages pass the stomach and intestine as efficiently as *E. coli* K-12. Transit times of less than a day without amplification or death were reported for radiolabeled K-12 (19) and confirmed by us with Amp^r-labeled K-12. The phage meets the viable but nongrowing *E. coli* in the gut lumen which we counted in the feces. The metabolic state (of starvation?) in this intestinal *E. coli* population does not permit phage replication. On laboratory media the cells resume growth and become fully susceptible to phage infection. We suspect that the

E. coli cells growing as microcolonies in the mucin layer (52) are physically protected against phage infection. However, this cell population might only show up to a limited extent in the fecal flora. It will be interesting to scrape the mucin layer from the large intestines of sacrificed mice and test their *E. coli* population for T4 phage susceptibility in media supplemented with cecal mucus (35).

Our experiments provide, however, clear evidence that *E. coli* recently introduced into the murine intestine is susceptible to phage infection. This was demonstrated in both conventional and axenic mice. In conventional mice, the colonization resistance of the resident intestinal flora had to be upset by the feeding of an antibiotic. This treatment permitted an at least temporary foothold to the externally added ampicillin-resistant K-12 *E. coli* cell even after the cessation of ampicillin feeding. Orally added phage prevented the fecal appearance of ampicillin-resistant K-12 *E. coli* cells over the observation period, suggesting the in vivo lysis of the added *E. coli* cells. In axenic mice inoculated with K-12 cells, the orally added phage had a lytic effect on the cells when given before, concomitant with, and after feeding of the cells. However, after a transient lytic phase, *E. coli* cells were again observed in the fecal samples. It is not clear what factor prevented the phage from permanently clearing the intestine of *E. coli*. Without the competition of other intestinal bacteria, K-12 might achieve in axenic mice an association with the mucus layer and thus escape phage infection. However, in axenic mice the outgrowth of the cells did not reach the original cell titers. Only when phage-resistant cells were fed in a second wave were the initial high fecal *E. coli* titers achieved, suggesting that the first *E. coli* strain remained under phage control.

The success of the phage therapy approach against *E. coli* diarrhea hinges on the in vivo phage susceptibility of the infecting pathogenic *E. coli* strains. As pathogenic *E. coli* targets the small intestine and does not colonize the intestine beyond the acute phase of diarrhea, it is unlikely that EPEC or ETEC strains occupy the phage-resistant niche of the resident *E. coli* strains in the large intestine. The effectiveness of phages in treating experimental *E. coli* diarrhea in mice, calves, piglets, and lambs (45, 46) suggests strongly that pathogenic *E. coli* is susceptible to orally applied phage.

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