Isolation and Characterization of a Myoviridae phage that lyses two Escherichia coli pathotypes

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ABSTRACT

The emergence of pathogenic bacteria with multiple drug resistances (MDR) and the detrimental side effects to the microbiome from the overuse of antibiotics pose significant risks to human health. Bacteriophage (phage) can be an alternative or supplemental therapy to combat MDR infections. Phage can be isolated from environmental reservoirs, such as wastewater, and used to target desired hosts, including MDR bacterial pathogens. In this study, we isolate a Myoviridae phage from Portland, Oregon, wastewater named *Escherichia virus PDX* that lys es enteropathogenic (EPEC) and enteroaggregative (EAEC) *E. coli* strains. Host susceptibility assays, transmission electron microscopy, and genomic analysis confirmed the bacteriolytic activity of *PDX* against these *E. coli* pathogens, while time-kill assays demonstrate that *PDX* inhibited growth of both pathotypes in a dose-dependent manner. Metagenomic sequencing of cultured fecal bacteria treated with *PDX* revealed that alpha diversity of propagated fecal samples was not reduced in the presence of the *PDX* phage.

IMPORTANCE

MDR bacterial infections are exceedingly difficult and expensive to treat, which can lead to prolonged illness, disability, and even death. The need for antibiotic alternatives, or supplements, warrants the consideration of using phage therapy in Western medicine. Phage therapy offers the added benefit of lysing its specific hosts, while leaving healthy
microbial communities intact. EPEC and EAEC strains are associated with pediatric acute gastroenteritis and chronic diarrhea, which can lead to stunted growth, life-threatening dehydration, behavioral abnormalities, and a general failure to thrive. We isolated and purified a strictly lytic phage and demonstrated selective, bacteriolytic activity against EPEC and EAEC E. coli pathotypes, which supports the efficacy of phage therapy in reducing pathogenic bacterial infections. Our data also suggest that these pathogens can be eliminated by PDX with minimal microbiome dysbiosis.

INTRODUCTION

Enteropathogenic and enteroaggregative E. coli (EPEC and EAEC) cause diarrheal diseases in infants and children and are endemic to Nigeria, other West African countries, Asia, and South America (1). Current recommendations for the treatment for EPEC and EAEC infections include the administration of fluoroquinolone antibiotics (2). Consequently, increased antimicrobial resistances among bacterial pathogens over the past decades have resulted in infections that fail to respond to frontline antibiotic therapy, indicating that these particular pathovars have acquired a multidrug resistance (MDR) phenotype (3). There has been a significant rise in EPEC and EAEC MDR strains, and emergence of MDR in many other disease-causing bacteria, though few novel antibiotic compounds have been developed during recent years (4).

One supplement, or alternative, to antibiotics that has been considered since their discovery are bacteriophage (phage), viruses that infect and kill a bacterial host. Recent estimates suggest that phage collectively comprise the most ubiquitous group of microorganisms on Earth, with an estimated population exceeding $10^{31}$, which out-
numbers the predicted population size of their bacterial hosts by a factor of ten (5). In addition to the large population size, there is a great diversity of bacteriophage species; recent publications estimate the existence of more than one million phage species (6, 7). As a therapeutic agent, bacteriophage have been used successfully for treatment of many bacterial pathogens, as exemplified in Poland (8). However, clinical research and implementation of phage therapy in Western countries has been meager since the development of antibiotics (6).

Few phage therapy clinical trials have been conducted in Western countries. However, there have been two successful phage therapy applications as single dose phage cocktails, which is a mixture of bacteriophage that target different pathogenic bacteria. In one case, the phage cocktail was administered topically to burn victims in a military hospital in Texas (9). The other successful Phase II clinical trial of phage therapy was administered to patients suffering from chronic MDR *Pseudomonas aeruginosa* ear infections (10). The study was terminated early as the treatment group exhibited such impressive recoveries that the physicians wanted to be able to deliver the phage preparation to the control group of patients to treat their infections. Three patients enrolled in the study experienced full resolution of symptoms as determined by both the study physicians and self-reporting. The results of these studies strongly suggest that the efficacy of phage therapeutics is not the limiting factor in making such applications available. Non-medicinal phage applications for agricultural products at risk for contamination with known bacterial pathogens is also a booming area of phage research. For example, a recent study demonstrated the efficacy of a phage cocktail to reduce *Salmonella enteritidis* contamination in smoked and raw salmon tissue.
preparations (11). Intralytix, a biotechnology company, has also received FDA approval for administration of ListShield™, EcoShield™ and SalmoFresh™ for treating ready-to-eat food to prevent foodborne illness (http://www.intralytix.com/index.php?page=faq).

Bacteriophage are ubiquitous in the environment, and sewage wastewater is a rich resource for isolating potentially therapeutic phage. For phage isolation we used a sequential scheme beginning with a laboratory strain of *E. coli* with sewage overlays (see Figure 1). We show that the lytic PDX phage lysed pathogenic strains of EPEC and a clinical isolate of EAEC. Our results indicate that the presence of PDX phage did not correlate with reduction of the alpha diversity of cultured normal human feces by 16S analysis, further demonstrating potential for this phage as a therapeutic against diarrheal disease.

**RESULTS**

**Phage Isolation and Characterization.** Phages were isolated from clear plaques on LB agar that formed against *E. coli* host strains when overlaid with raw sewage. *Escherichia virus PDX* was initially isolated against *E. coli* MC4100 because the altered LPS in laboratory strains allows for phage recognition of more conserved, core antigens (12). The phage stock prepared from these plaques were further purified using the sequential host isolation technique adapted from Yu *et al.*, 2016 (13). Sequential host isolation with EPEC and EAEC was used to enrich the stock in phages capable of infecting all desired hosts (Fig. 1A). Five rounds of purification were carried out after the sequential host isolation procedure until uniform 1 mm, circular plaques were observed when overlaid on the EAEC host, to ensure that the stock was purified with the desired
phage (Fig. 1B). The high titer lysates for *Escherichia virus PDX* had a titer of $8.92 \times 10^{12}$ PFU/mL when propagated on EAEC. As a negative control, the phage was unable to lyse a strain of *Bacillus subtilis* (ATCC #6051). To test how broadly *PDX* recognized EPEC pathogens we found that the phage could lyse 9 and form plaques on 5 out of 20 clinical, diarrheal isolates from children living in the Seattle area (Table 1; (14)). Thus, the *PDX* phage could be used to target some, but not a comprehensive set, of *E. coli* pathogens.

In order to obtain a presumptive identification of *PDX*, we employed transmission electron microscopy (TEM). Imaging revealed the phage belonged to the family *Myoviridae*, identified by their canonical morphology: a small-isometric non-enveloped head with a long contractile tail (Fig. 2; (15)). The head diameter of *PDX* was $76.4 \pm 4.34$ nm and the tail length was $114.0 \pm 2.26$ nm ($n = 13$) (Figs. 2ABC). Based on morphological features and metrics obtained using TEM, we putatively identified *PDX* to be a member of the *Myoviridae* family that infect *E. coli* (Fig. 2D).

To characterize the phage life cycle, we performed a one-step growth curve of *Escherichia virus PDX*, infecting both EPEC and EAEC (Fig. 3). *PDX* infecting EAEC had a shorter eclipse period, the time before the phage begin emerging from the bacteria, than those infecting EPEC. At the 40-minute time point PFU/mL values were $2.40 \times 10^6$ and $1.70 \times 10^5$ for EAEC and EPEC, respectively ($P < 0.03$). *PDX* infecting EAEC had a modestly greater relative virus count (PFU/mL) 100 minutes post infection compared to EPEC, maximum concentrations reaching $3.60 \times 10^6$ PFU/mL and $2.57 \times 10^6$ PFU/mL for EAEC and EPEC, respectively. However, this difference did not reach statistical significance ($P < 0.06$). Though able to infect both EPEC and EAEC, we
concluded that the *PDX* life cycle varied slightly when replicating in these two *E. coli* pathotypes.

**PDX Kill *E. coli* in a Dose Dependent Manner Without Disrupting Normal Microbiota.** The bacteriolytic activity of *PDX* against EPEC and EAEC was measured in a time-kill assay revealing a dose-dependent inhibition of bacterial growth. At a multiplicity of infection (MOI) of 100, *PDX* severely suppressed growth of EPEC and EAEC (Fig. 4). Bacteria that were not treated with phage showed normal sigmoidal growth curves that match the expected growth rates for their respective strains. A dose-dependent relationship between MOI and point of infection was observed: at greater MOI, infection occurred earlier than for lower MOI (Fig. 4). In many phage treatments, an increase in bacterial growth was observed around 8-10 hours of incubation. This is likely due to the emergence of mutant bacteria that are resistant to phage infection and has been observed in other, similar studies (16, 17).

The *in vitro* efficiency of *PDX* infection in the presence of commensal fecal bacteria was tested by anaerobically culturing streptomycin-resistant derivatives of EPEC and EAEC with feces and treating with *PDX*. EPEC or EAEC bacteria were enumerated via colony counts of the culture after ten hours incubation and revealed that *PDX* suppressed growth of pathogens when co-cultured with commensal fecal bacteria. *PDX* significantly reduced the EPEC population, from $6.0 \times 10^3$ to 17 CFU/mL, and only slightly reduced the population EAEC compared to cultures not treated with phage, from $5.2 \times 10^3$ to $3.2 \times 10^3$ CFU/mL. This is consistent with the results of the time-kill assays (Fig. 4), which show that high doses of phage at MOI of 100 results in inhibition of
bacterial growth. It is unclear why the population of EAEC was not more reduced, as was the case for EPEC, though a mutant could have arisen in the streptomycin-resistant EAEC population that was no longer sensitive to phage infection (Fig. 4).

The impact of phage treatment on bacterial diversity of cultured human feces was investigated using 16S metagenomic sequencing analysis of cultures containing fecal bacteria treated with phage (Fig. 5A). Alpha diversity analysis, a metric to measure the diversity to compare samples’ relative diversity, was used to quantify the differences between samples. Samples with PDX added to human feces were among the most diverse and showed increased diversity compared to feces alone (Fig. 5B). Samples treated with PDX and EPEC or EAEC had higher alpha diversities than samples treated with only EPEC and EAEC. The relatively lower alpha diversity of fecal samples treated with pathogens alone is consistent with the findings of the pathogen enumeration assay, which confirmed the presence of populations of streptomycin-resistant EPEC and EAEC in their respective cultures. While it would be expected that the culture containing only feces might have the highest alpha diversity, our results do not show this, perhaps due to sample variation. Nonetheless, we concluded that the presence of the PDX phage in the fecal cultures did not reduce alpha diversity when compared to feces alone.

Genomic and Phylogenetic Characterization. Multiple genome analyses and annotation software programs were used to characterize the PDX genome (Fig. 6). We annotated the genome looking for closely related phage species and their relevance to other phage therapy applications, as well as for markers that might indicate lysogenic activity (Table 2). The genome of PDX is 138,828 bp dsDNA a midsized genome for the
Myoviridae family of viruses. The GC content is 43%, which is comparable to other lytic phages (FastQC Galaxy). We identified 206 putative coding domain sequences (CDS) (PROKKA, ORFfinder), but no toxin-encoding or lysogenic genes, which are characterized as any genes which contained the terms “integrase”, “excisionase”, “recombinase”, and “repressor” were identified in the genome when compared to the NCBI BLAST database. Additionally, in a 20-standard amino acid (aa) search, a total of 6 putative tRNAs were predicted with open reading frames (ORFs) ranging from 72-88 bp in length (tRNA scan-SE). Virfam software uses the translated amino acid (aa) of CDS from a phage genome as input and searches for homology to other structural phage protein sequences deposited in the Aclame database. Based on the results of the Virfam analysis, the head-neck-tail structure genome organization in PDX belongs to the Neck Type One – Cluster 7 category (Fig. 7A). Neck Type One phage genomes contain the following proteins: portal protein, Adaptor of type 1 (Ad1), Head-closure of type 1 (Hc1), Neck protein of type 1 (Ne1), and tail-completion of type 1 (Tc1) (Fig. 7B). Cluster 7 phage genomes are reported in Virfam to be strictly myophages with small genome sizes and gene content (61-150 genes). However, it is noteworthy that the genome of PDX contains 206 CDS, which is more than the other phage in this cluster in the current Aclame database (Table 2). Cluster 7 myophages all infect Proteobacteria. Cluster 7 phage genomes are organized such that there is a different gene order compared to the canonical order that is observed in most clusters of the type 1 family (Fig. 7B).

By BLAST analysis, the three genomes with the highest whole-genome nucleotide identity match were Escherichia coli O157 typing phage 4 (98%), Escherichia
Phage JES-2013 (98%), and *Escherichia* Phage Murica. Murica is a *Myoviridae* phage that shares a 97% nucleotide identity with *PDX*, is strictly lytic against enterotoxigenic (ETEC), and is being researched as a potential treatment of contaminated food in lieu of antibiotics (18). *E. coli* O157 typing phage 4 is lytic against *E. coli* O157, which also had a 99% amino acid identity with the *PDX* tail fiber proteins (19). Phage JES-2013 is lytic against *Lactococcus lactis* (20). The putative tail fiber sequences from *PDX* had a 99% amino acid identity with *Myoviridae Escherichia* phage V18 (Table S1). V18 was in a successful prophylactic phage cocktail used in the prevention of traveler's diarrhea in mice infected with 6 bacterial strains: *E. coli*, *Shigella flexneri*, *Shigella sonnei*, *Salmonella enterica*, *Listeria monocytogenes* or *Staphylococcus aureus*, and Lac (-) *E. coli* K-12 C600 (21). Additionally, V18 phage was cited as having a broad host range against ETEC, EAEC, and enterohaemorrhagic *E. coli* (EHEC). Host range data was unavailable for the Slur16 *Myoviridae* phage, which shared 99% identity with the putative *PDX* tail fiber proteins. However, Slur16 was isolated from dairy cow feces using *E. coli* K-12 MG1655 as a host, suggesting that this phage is lytic against pathogenic *E. coli* strains (22). Overall, the phage that had the highest percent identity with *PDX* tail fiber amino acid sequences were all strictly lytic *Myoviridae* against various *E. coli* strains.
DISCUSSION

EPEC and EAEC infections in humans are endemic to underdeveloped regions of the world and are commonly isolated from stool samples of diarrheal patients living in these countries (1). The rise in MDR strains of EPEC and EAEC demand an alternative treatment (3), and in this study we identified and characterized a bacteriophage, \textit{PDX}, and investigated its potential as a therapeutic agent to treat EPEC and EAEC disease. Our findings revealed that \textit{PDX} is a good candidate for phage therapy based on its efficiency and specificity in killing pathogenic \textit{E. coli}. Furthermore, the presence of \textit{PDX} did not correlate with reduced alpha diversity of the microbiota when cultured with normal human feces (Fig. 5B). This is in stark contrast to the up to 40\% reduction in gut microbiome alpha diversity observed in patients given ciprofloxacin, a fluoroquinolone commonly prescribed for \textit{E. coli} infections (23).

Phage are generally thought to be present in the same environment as their bacterial hosts. However, we isolated the \textit{PDX} phage, able to infect two pathotypes, from the wastewater treatment plant located in Portland, Oregon even though infections by EPEC and EAEC bacteria are rare in the Pacific Northwest of the United States. The phage isolated might also replicate in related \textit{E. coli} host bacteria via the recognition of conserved receptors. However, to the initial point, these pathogens may be more common in the Pacific Northwest than initially thought because \textit{PDX} was able to form plaques on 5 of 20 EPEC strains, clinical samples from children living in the Seattle area (see Table 1; (14)).

In order for phage to be used as an efficacious therapeutic agent they must be strictly lytic. Lysogenic phage, such as phage lambda, transfer bacterial genetic material
from one population to another upon infection and integration into the host genome. This can lead to the transfer of antibiotic resistance genes and toxin encoding genes between bacterial hosts (24). Several lines of evidence indicate that PDX is a lytic phage. PDX was classified as a member of the strictly lytic (25) Myoviridae bacteriophage family based on morphological characteristics from TEM imaging (Fig. 2). Bioinformatic analysis of the head-neck structure CDS in the Virfam suite identified PDX as belonging to the Neck Type One – Cluster 7 comprised only of members of the myophage family (Fig. 7). Neither CI nor CI-associated genes that are responsible for lysogeny in phage lambda were identified in the genome of PDX. Lastly, phage induction, by treatment with Mitomycin C, did not induce PDX plaques in mutant derivatives of EPEC and EAEC resistant to PDX infection (data not shown). Thus, we concluded that the PDX phage was a member of the Myoviridae family having a strictly lytic lifestyle.

The PDX genome is a midsized with 138,828 bp, compared to the 11,000 to 271,000 bp range found within the 547 complete Myoviridae genomes currently in the NCBI database. Additionally, it is noteworthy that PDX has a higher putative CDS count than any of the other members of Cluster 7 category in the Virfam and Aclame databases.

One of the dangerous side effects of antibiotics is the drastic disruption of the microbiome, or dysbiosis, due to the conserved nature of the drug targets. The metagenomic analysis of fecal cultures treated with phage suggested that PDX treatment did not significantly alter the community structure of the commensal microbiome in feces cultured with PDX, in contrast to what occurs with antibiotic...
treatment. We did observe, however, that the relative abundance of bacteria in the

*Enterbacteriaceae* family was reduced when comparing the feces and feces + *PDX*
samples in Figure 5. This suggested that some commensal bacteria belonging to this
family were targeted by *PDX*. Even so, bacteria in this family make up a small fraction of
human gut microbiomes, in most cases less than 1% of the population, and the overall
impact on the microbiota from treatment with *PDX* is predicted to be minimal. Phage
with a limited host range avoid the consequences of non-specific microbiome disruption,
and thus offer an alternative to antibiotic treatment against MDR, or even antibiotic
sensitive, EPEC and EAEC infections.

Future studies should investigate the efficacy of this phage, both in isolation and
in combination with other phage (a phage cocktail), in attenuating bacterial infections.
Though certainly not exhaustive, we did observe that *PDX* could form plaques,
indicating replication, in 5 out of 20 clinical isolates of EPEC from Northwest children
(Table 1). The use of phage cocktails would then allow for the broadening of serotypes
and pathotypes that can be targeted by phage therapy. Further, an *in vivo* approach
could utilize mice whose microbiomes have been “humanized”, inoculating subjects with
phage susceptible bacteria and then treating subjects with varying titers and at different
times points post bacterial infection. Results would include monitoring virulence and
bacterial burdens. Notably, a recent study by Hodyra-Stefaniak and colleagues revealed
that the most important factors that determine the efficacy of a phage therapeutic agent
are: stage of bacterial pathogenesis, the bacterial population size at the time of
administration, the titer used, and the administration method (26). Just recently it was
announced that UC San Diego will launch The Center for Innovative Phage Applications
and Therapeutics (IPATH) to refine treatments and to bring them to market
(http://www.sciencemag.org/news/2018/06/can-bacteria-slaying-viruses-defeat-
antibiotic-resistant-infections-new-us-clinical). They hope to generate libraries of phages
and use cocktails for individual patients to treat MDR infections in order to meet FDA
approval challenges. Thus, our efforts and those of other researchers will be essential
for isolating therapeutic phages to be used against the increasing number of untreatable
bacterial infections.
MATERIALS AND METHODS

Bacterial Strains and Growth Conditions. Bacterial strains are listed in Table 1. All strains were grown in Lysogeny Broth (LB) at 37°C with shaking at 225 RPM or on LB agar at 37°C. All phage lysates were stored in SM gel buffer (50 mM Tris-HCl [pH 7.5], 0.1 M NaCl, 8 mM MgSO_4_, 0.01% gelatin) at 4°C, and warmed to room temperature for all experiments unless otherwise stated.

Phage Isolation and Propagation. Bacteriophage PDX was initially isolated from untreated (influent) sewage collected at the Portland Wastewater Treatment Plant on October 1, 2015. The raw sewage was filtered through a 0.2 µm filter (Corning, Corning NY) to remove bacteria. The filtered sewage was then added to host bacteria in liquid culture (Fig. 1A). Phage-host solutions were combined with 0.5% LB soft agar and overlaid on LB agar plates. Plates were then observed for the presence of plaques after overnight growth at 37°C. Clear, defined plaques of interest were isolated and transferred using a sterilized inoculating needle and agitation in 1 mL solution of lambda diluent (10mM Tris, pH 7.5; 10 mM MgSO_4_). To obtain a high titer phage preparation, a crude lysate was generated. A clear PDX plaque was isolated and suspended in 1 mL of sterile lambda diluent. The phage suspension was combined with overnight culture of the bacterial host and incubated at 37°C with shaking at 225 RPM overnight. Next, chloroform was added to kill any remaining bacteria. This solution was centrifuged at 4°C at 4000 x g for 10 minutes to remove bacterial cells. Finally, the supernatant was recovered and treated with chloroform again to kill any remaining bacterial cells.

Several rounds of overlay purification were used to ensure lysate purity after sequential host isolation. From the final overlay of phage sequentially isolated from
MC4100, EPEC, and EAEC, five rounds of purification were carried out (Fig. 1B).

Several clearly defined plaques from the final overlay plate were picked using a sterile inoculating needle and suspended in microcentrifuge tubes containing SM buffer. The phage suspension was incubated at room temperature for 15 minutes to allow the phage to diffuse through the medium, then filtered through a sterile 0.2-μm filter to remove any bacteria that may have been transferred from the overlay plate. A host susceptibility assay was performed as described below to test the ability of each selected plaque to lyse MC4100, EPEC, and EAEC. This process was performed four more times until uniform plaques were observed indicating pure phage suspension.

The final phage suspension was then propagated to make a purified high titer lysate. Exponential EAEC with undiluted phage suspension was incubated in LB overnight at 37°C. Bacteria were removed from the culture by centrifugation at 6,055 x g, then the supernatant was sterile filtered with a 0.2 μm filter. The titer of the lysate was determined by plating dilutions (10^{-2}-10^{-16}) using the EAEC overlay procedure described previously. After overnight incubation, the plates were observed and the number of plaque forming units per mL (PFU/mL) was calculated. The purified high titer lysate in SM buffer was stored at 4°C.

**Electron Microscopy.** PDX was analyzed using transmission electron microscopy (TEM). Purified high titer lysate was precipitated in 25% PEG 6000-8000 in 2.5M NaCl and stored overnight at 4°C. Precipitated phage solutions were centrifuged at 4°C, 4000 x g for 10 minutes. The supernatant was removed and the phage pellet was suspended in 40μL of 50 mM Tris, pH 7.4, 10 mM MgSO4. Phage suspension was placed on copper coated formvar grids and negatively stained with 1% uranyl acetate. Samples
were examined under a FEI Tecnai™ Spirit TEM system at an operating voltage of 120 kV (FEI, Hillsboro, OR). All transmission electron microscopy was performed at the Multi-Scale Microscopy Core (MMC) with technical support from the Oregon Health & Science University (OHSU)-FEI Living Lab and the OHSU Center for Spatial Systems Biomedicine (OCSSB).

Host Susceptibility Assay. A host susceptibility assay, or spot test was utilized to determine the host range of PDX (Table 1). Each LB agar plate was streaked with 3-4 spatially separated bacterial strains using a sterilized inoculating loop. A small volume of purified lysate was pipetted on top of the center of each linear bacterial streak and incubated overnight at 37°C. Host susceptibility was indicated by a clearing within the streak of a given bacterial strain.

Growth Curves. To determine the growth kinetics of PDX, one-step growth curve experiments were performed. LB liquid cultures of E. coli were grown to an OD600 of 0.6-0.7, when the cultures had reached mid to late exponential phase. Next, the indicated purified phage lysate was added to the liquid culture tubes to infect the bacteria with a multiplicity of infection (MOI) of 0.01. The liquid bacterial culture and phage lysate were incubated at room temperature for 10 minutes. Following the incubation period, the solution was centrifuged at 8,000 x g for 10 minutes. The supernatant was discarded and the pellet was re-suspended in LB to make the absorption mixture. Immediately after re-suspension the absorption mixture was serially diluted and plated with the bacterial host. The phage was incubated within a standard plaque-overlay. This procedure was repeated at 20-minute time intervals for a total of 120 minutes. After 24 hours of incubation, the plaques on all the plates were recorded.
and the PFU/mL was calculated. The titer over time (120 minutes) was plotted to obtain
a one-step growth curve. This experiment was repeated using EPEC and EAEC strains.

**Time-Kill Assays.** Bacterial density was measured over time to determine the course of
phage infection. Liquid cultures of EPEC and EAEC were incubated with *PDX*. The *in
vitro* lytic efficiency of the phage was examined at several MOIs. Wells of a flat-
bottomed 96-well microtiter plate were filled with 100 µl of inoculated double-strength LB
and 100 µL of purified lysate dilutions in SM buffer. Bacterial cultures were grown to late
exponential phase and diluted to 10^6 CFU/mL. *PDX* lysate was diluted to 10^5, 10^6, 10^7
or 10^8 PFU/mL, corresponding to MOIs of 0.1, 1, 10, and 100, respectively. Each
phage-host combination at specific MOIs was performed in triplicate wells, and each
experiment was performed three times. Controls for plate sterility, phage suspension
sterility, and bacterial growth without phage addition were also included. To prevent
between-well contamination by aerosolizing of bacteria, an adhesive transparent plate
cover was placed over the top of the plate. The plates were incubated at 37°C with
orbital shaking for 12 hours and OD at 600 nm was measured using a Microtiter Plate
Reader (Sunrise, Tecan Group Ltd., Austria) at 30-minute intervals.

**Anaerobic Culture.** Feces were collected from a 23-year-old male in Portland, Oregon
who had not taken antibiotics in the last 5 years. One gram of fresh feces was
suspended in sterile saline solution by vortexing and vigorous shaking. The fecal
suspension was then filtered through a sterile 1-mm mesh sieve twice to remove large
pieces of organic matter. The filtered fecal suspension was then concentrated by
centrifugation at 1500 x g for five minutes. The resulting fecal pellet was resuspended in
saline solution with 10% glycerol for a final concentration of 0.5g fresh feces/mL. Fecal
Streptomycin-resistant (strR) EPEC and EAEC were grown anaerobically under 100% CO2 with fecal slurry and PDX using the Hungate method in M10 medium, designed to mimic the nutrient, mineral, and pH content of the intestine (27). Cultures were inoculated to final concentrations of 0.7 mg/mL feces, 1.4 x 10^6 CFU/mL E. coli, and 1.4x10^8 PFU/mL phage treatments. Cultures were incubated for 16 hours in a stationary incubator at 37°C. DNA from these cultures was isolated for 16s rDNA sequencing analysis after 16 hours of incubation.

Immediately prior to DNA extraction, a sample from each culture was plated to enumerate the concentrations of strR EPEC and EAEC in the anaerobic cultures. EPEC and EAEC were distinguished from fecal bacteria by plating the culture on selective media containing streptomycin. As a control, fecal samples were plated on LB agar with streptomycin, and no growth was observed (data not shown). Dilutions from 10^-1-10^-5 were performed in a 96-well plate in sterile LB, then plated in triplicate 10-μL drops of each concentration on LB-agar containing 100-μg/mL streptomycin. Plates were incubated for 20 hours at room temperature and colonies were counted using a dissection microscope.

For DNA isolation, bacteria were pelleted by centrifugation at 5000 x g for 10 minutes. The pellets were washed with phosphate buffer solution to separate whole cells from lysed cell debris, then spun down at 5000 x g for 5 minutes. DNA from Hungate cultures was extracted using a DNeasy Blood and Tissue kit (Qiagen, Germantown, MD), with the protocol adapted according to manufacturer instructions for bacteria.
16S Metagenomic Sequencing and Analysis. Genomic DNA was sent to the Oregon State University Center for Genome Research and Biocomputing for multiplex sequencing analysis using the Illumina MiSeq sequencing platform. The V3-V4 region of the 16S rRNA gene was amplified and used to prepare a dual index library using the Nextera XT library preparation kit. The analysis produced forward and reverse paired end reads of 150 bp at a sequencing depth of 1 million reads. Sequence quality was quantified using FastQC and demultiplexed.

Phage Genome Sequencing and Bioinformatics. To initiate genomic sequencing, a high titer lysate was purified using CsCl gradients. Genomic phage dsDNA was extracted using an Epicentre DNA Isolation kit according to manufacturer’s instructions (Madison, WI). The quantity, quality, and concentration of the extracted DNA was analyzed utilizing a high-sensitivity assay of dsDNA with a Qubit 2.0 (Invitrogen, Grand Island, NY). All library preparation and Illumina sequencing performed at MGH Genome Core (Cambridge, MA). A diagram illustrating work flow for genomic analysis is presented in Figure 6. Upon receiving the genomic sequences in FASTQ format, we used the Galaxy platform to perform quality control and filtering processes on the raw reads (28–30). The Trimmomatic Galaxy (v. 0.32.3) software was used to recognize the distinct adaptors to distinguish and gather all the reads for one phage separately from the other (31). The quality of the reads was analyzed with FastQC (v. 0.11.4) suite with default settings. The quality of the assembly was assessed using Quality Assessment Tool for genome assemblies (QUAST, v. 2.3), as it reports relevant assembly quality metrics (32). Following assembly, we used NCBI BLAST, tRNAscan-SE, FastQC Galaxy, and Virfam to annotate the functional regions of the genome assembly. The
putative coding sequences (CDS) were filtered for only those with known protein functions produced by NCBI smart BLAST with identity above 95%.

Genes of interest in the genome annotations were compared to other similar sequences in the National Center for Biotechnology Information (NCBI) database using the Basic Local Alignment Search Tool (BLAST; v. 2.3.1) suite, specifically using the blastp and tblastn tools with default settings (21, 33). The genome assemblies were deposited in the GenBank database. All phylogenetic analyses were performed using Virfam webserver, which performs homology searches against phage sequences available in the Aclame database (34, 35).

**Accession number.** The genomic sequence of the *Escherichia virus PDX* was deposited in GenBank under accession number MG963916.
Figure Legends

Figure 1. Propagation and purification of polyvalent bacteriophages. (A) Phage stock was introduced to liquid culture of hosts, MC4100, EPEC LRT9, and lastly EAEC strain EN1E-0227, then enriched phages were collected. (B) For purification of polyvalent bacteriophages one well-differentiated plaque from the propagation step was diffused in SM buffer and isolated with multiple rounds of overlay plate assays using EAEC strain EN1E-0227 as host until all plaques were morphologically uniform.

Figure 2. Transmission electron micrographs of bacteriophage *Escherichia virus PDX*. Samples were negatively stained with 1% uranyl acetate solution, prepared on a carbon formvar grid, and were imaged at 120 kV on a FEI Tecan-i Spirit TEM system. The arrow in (A) indicates the base plate. The arrows in (B) identify three putative tail fibers. The image in (C) illustrates the small isometric, non-enveloped head with long contractile tail, specific to the family *Myoviridae*. (D) illustrates *PDX* infecting EAEC, with contractile tail, indicated by arrow, penetrating the outer membrane and periplasmic space. Scale bars are as indicated.

Figure 3. One-step growth curve analysis of EAEC (A) and EPEC (B) infected by *PDX* with an MOI of 0.01 for 120 minutes. Over the 120-minute infection period, bacterial cultures were sampled, serially diluted and plated with a plaque-overlay procedure (see Materials and Methods). After 24-hour incubation, the plaques were counted and the titer (PFU/mL) was determined. Error bars represent the standard error of the mean. By
comparing the PFU/mL values at 40 min, the eclipse period was shorter for EAEC than for EPEC when infected with \textit{PDX} \((P < 0.04)\).

**Figure 4.** Time-kill assay of EAEC (A) and EPEC (B) treated with \textit{PDX}. In a 96-well plate, exponential culture was inoculated with dilutions of \textit{PDX} high titer lysate for MOI (multiplicity of infection) ranging from 0.1, 1, 10 and 100. The plates were incubated at 37°C with orbital shaking for 12 hours and OD of 600 nm was measured using a Microtiter Plate Reader (Tecan). Assays were performed in triplicate. Representative data are presented.

**Figure 5.** Effect of phage treatment on bacterial community in human feces. (A) Summary of taxa classified at the family level for fecal cultures with EPEC, EAEC and \textit{PDX}. The plot was generated based on OTU frequencies for each sample. OTUs were assigned using a closed reference algorithm based on alignment of high throughput 16S rDNA sequencing reads to reference genes in the Greengenes database. (B) Alpha diversity of bacterial populations found in samples treated with feces, \textit{PDX} and EPEC or EAEC. Alpha diversity was calculated using the Choa1 algorithm in Qiita, which uses the abundance of OTUs and number of different OTUs to determine a number representing the samples relative diversity.

**Figure 6.** Bioinformatics workflow for the assembly, annotation and phylogeny of the \textit{PDX} genome. (A) Steps of the genome raw read processing. Names of programs used appear in parenthesis; (B) Steps of the genome assembly; (C) Annotation of the
genome; (D) Genome assembly and annotation output; and (E) Phylogeny construction using the Virfam program based on Aclame database.

*Only sequences with putative identity match of 95% were maintained for further analysis.

**Figure 7.** Structural Protein Classification Analysis of *PDX*. (A) Tree representation of *PDX* (black text in white box, lined red) classification among other *Caudovirales* phage, constructed from a hierarchical agglomerative clustering procedure applied to a matrix of similarity scores between pairs of phages (by combining HHsearch probabilities with the percentage identity) and present in the Aclame database. The different branches of the tree were sorted into 10 Clusters and are highlighted by different background colors. The bacterial host(s) of the phages are listed for each Cluster in the legend at the bottom of the figure. (B) Gene organization of *PDX* and other phage genomes that belong to the Neck One – Cluster 7. The legend at the bottom indicates to which family a protein belongs. (-) indicates the antisense DNA strand.
### Table 1. *Escherichia coli* strains used in this study.

| Strain | Genotype/ Serotype | Syndrome | Spot Test Result | Plaque Formation | Source |
|--------|--------------------|----------|------------------|------------------|--------|
| EN1E-0227 | EAEC O86:H27 | D      | +   | + | (36) |
| LRT9   | EPEC O11:abH2 | U      | +   | + | (37) |
| MC4100 | F- araD139 Δ(argF- lac) U169 rpsL 150 StrR relA1 flhD5301 deoC1 ptsF25 rbsR | U      | +   | + | (38) |
| TB85A  | 0131:HN            | CD     | +   | - | (14) |
| TB96A  | 075:HN            | BD     | +   | + | (14) |
| TB135A | 0128:H2        | CD     | -   | - | (14) |
| TB156A | O55:H7         | CD     | -   | - | (14) |
| TB171A | O125:H6       | D, V   | +   | + | (14) |
| TB182A | O55:H7       | CD     | -   | - | (14) |
| TB183A | O127:H40    | D      | -   | - | (14) |
| TB204A | O96:HN        | D, V, F | +   | + | (14) |
| TB206A | O26:HN      | BD     | -   | - | (14) |
| TB209A | O15:HN      | CD     | -   | - | (14) |
| TB216A | O118:H8   | D      | -   | - | (14) |
| TB220A | O153:HN    | CD     | +   | + | (14) |
| TB227A | O8:H41     | CD     | -   | - | (14) |
| TB269C | O145:HN    | D, F   | +   | - | (14) |
| TB280A | O66:HN   | CD     | -   | - | (14) |
| Strain | Serotype | Diarrhea Type | Virulence | Replication | Notes |
|--------|-----------|---------------|------------|-------------|-------|
| TB309A | O127:H2   | CD            | +          | -           | (14)  |
| TB320A | ON:HN     | BD, V         | -          | -           | (14)  |
| TB342C | O115:NM   | CD            | +          | -           | (14)  |
| TB353E | ON:HN     | CD            | +          | +           | (14)  |
| TB425B | ON:HN     | CD            | -          | -           | (14)  |

Key: BD, bloody diarrhea; CD, chronic diarrhea; D, acute diarrhea; F, fever; U, details unknown; V, vomiting.

* A plus sign indicates strain is killed by *Escherichia virus PDX*.

* A plus sign indicates *PDX* replicates (can form plaques on) the strain.

**Table 2.** Genomic analysis of *PDX*

| Feature                        | Quantity |
|--------------------------------|----------|
| Size (bp)                      | 138,828  |
| GC content (%)                 | 43       |
| Coding domain sequences (CDS)  | 206      |
| tRNAs                          | 6        |

**Table S1.** NCBI BLAST results of bacteriophage with the highest whole genome nucleotide percent identity to *PDX*.

| **% Identity with PDX** Genome | E-Value | Total Score (max score 255600) | Accession Number |
|-------------------------------|---------|--------------------------------|------------------|
| *Escherichia Phage Murica*    | 97%     | 0.0                            | AKU44119.1       |
| *Escherichia coli* O157 typing phage 4 | 98% | 0.0                            | AKE45376.1       |
| **Escherichia Phage JES-2013** | 98% | 0.0 | 52649 | YP_008530272.1 |
|--------------------------------|-----|-----|-------|----------------|

*a*: Default BLAST parameters used, with standard 20-amino acid search.  
*b*: With default parameters, % identity is calculated by subtracting the number of bases and spacers from a perfect identity match.  
*c*: Statistical test determining the probability that the significance of alignment was due purely to chance.  
*d*: Under default settings, the BLOSUM62 scoring matrix was used which subtracts 11 points for the existence of a gap with a 1-point deduction for each gap extension made.

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