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 lyses 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

48 microbial communities intact. EPEC and EAEC strains are associated with pediatric
49 acute gastroenteritis and chronic diarrhea, which can lead to stunted growth, life-
50 threatening dehydration, behavioral abnormalities, and a general failure to thrive. We
51 isolated and purified a strictly lytic phage and demonstrated selective, bacteriolytic
52 activity against EPEC and EAEC *E. coli* pathotypes, which supports the efficacy of
53 phage therapy in reducing pathogenic bacterial infections. Our data also suggest that
54 these pathogens can be eliminated by *PDX* with minimal microbiome dysbiosis.
55
56
57 **INTRODUCTION**
58 Enteropathogenic and enteroaggregative *E. coli* (EPEC and EAEC) cause diarrheal
59 diseases in infants and children and are endemic to Nigeria, other West African
60 countries, Asia, and South America (1). Current recommendations for the treatment for
61 EPEC and EAEC infections include the administration of fluoroquinolone antibiotics (2).
62 Consequently, increased antimicrobial resistances among bacterial pathogens over the
63 past decades have resulted in infections that fail to respond to frontline antibiotic
64 therapy, indicating that these particular pathovars have acquired a multidrug resistance
65 (MDR) phenotype (3). There has been a significant rise in EPEC and EAEC MDR
66 strains, and emergence of MDR in many other disease-causing bacteria, though few
67 novel antibiotic compounds have been developed during recent years (4).
68 One supplement, or alternative, to antibiotics that has been considered since
69 their discovery are bacteriophage (phage), viruses that infect and kill a bacterial host.
70 Recent estimates suggest that phage collectively comprise the most ubiquitous group of
71 microorganisms on Earth, with an estimated population exceeding 10^{31}, which out-

72 numbers the predicted population size of their bacterial hosts by a factor of ten (5). In
73 addition to the large population size, there is a great diversity of bacteriophage species;
74 recent publications estimate the existence of more than one million phage species (6, 7).
75 As a therapeutic agent, bacteriophage have been used successfully for treatment of
76 many bacterial pathogens, as exemplified in Poland (8). However, clinical research and
77 implementation of phage therapy in Western countries has been meager since the
78 development of antibiotics (6).

79 Few phage therapy clinical trials have been conducted in Western countries.
80 However, there have been two successful phage therapy applications as single dose
81 phage cocktails, which is a mixture of bacteriophage that target different pathogenic
82 bacteria. In one case, the phage cocktail was administered topically to burn victims in a
83 military hospital in Texas (9). The other successful Phase II clinical trial of phage
84 therapy was administered to patients suffering from chronic MDR *Pseudomonas*
85 *aeruginosa* ear infections (10). The study was terminated early as the treatment group
86 exhibited such impressive recoveries that the physicians wanted to be able to deliver
87 the phage preparation to the control group of patients to treat their infections. Three
88 patients enrolled in the study experienced full resolution of symptoms as determined by
89 both the study physicians and self-reporting. The results of these studies strongly
90 suggest that the efficacy of phage therapeutics is not the limiting factor in making such
91 applications available. Non-medicinal phage applications for agricultural products at risk
92 for contamination with known bacterial pathogens is also a booming area of phage
93 research. For example, a recent study demonstrated the efficacy of a phage cocktail to
94 reduce *Salmonella enteritidis* contamination in smoked and raw salmon tissue

95 preparations (11). Intralytix, a biotechnology company, has also received FDA approval
96 for administration of ListShield™, EcoShield™ and SalmoFresh™ for treating ready-to-
97 eat food to prevent foodborne illness (http://www.intralytix.com/index.php?page=faq).
98 Bacteriophage are ubiquitous in the environment, and sewage wastewater is a
99 rich resource for isolating potentially therapeutic phage. For phage isolation we used a
100 sequential scheme beginning with a laboratory strain of *E. coli* with sewage overlays
101 (see Figure 1). We show that the lytic *PDX* phage lyses pathogenic strains of EPEC and
102 a clinical isolate of EAEC. Our results indicate that the presence of *PDX* phage did not
103 correlate with reduction of the alpha diversity of cultured normal human feces by 16S
104 analysis, further demonstrating potential for this phage as a therapeutic against
105 diarrheal disease.
106
107 **RESULTS**
108 **Phage Isolation and Characterization.** Phages were isolated from clear plaques on
109 LB agar that formed against *E. coli* host strains when overlaid with raw sewage.
110 *Escherichia virus PDX* was initially isolated against *E. coli* MC4100 because the altered
111 LPS in laboratory strains allows for phage recognition of more conserved, core antigens
112 (12). The phage stock prepared from these plaques were further purified using the
113 sequential host isolation technique adapted from Yu *et al*., 2016 (13). Sequential host
114 isolation with EPEC and EAEC was used to enrich the stock in phages capable of
115 infecting all desired hosts (Fig. 1A). Five rounds of purification were carried out after the
116 sequential host isolation procedure until uniform 1 mm, circular plaques were observed
117 when overlaid on the EAEC host, to ensure that the stock was purified with the desired

118 phage (Fig. 1B). The high titer lysates for *Escherichia virus PDX* had a titer of 8.92×10^{12}
119 PFU/mL when propagated on EAEC. As a negative control, the phage was unable to
120 lyse a strain of *Bacillus subtilis* (ATCC #6051). To test how broadly *PDX* recognized
121 EPEC pathogens we found that the phage could lyse 9 and form plaques on 5 out of 20
122 clinical, diarrheal isolates from children living in the Seattle area (Table 1; (14)). Thus,
123 the *PDX* phage could be used to target some, but not a comprehensive set, of *E. coli*
124 pathogens.

125 In order to obtain a presumptive identification of *PDX*, we employed transmission
126 electron microscopy (TEM). Imagining revealed the phage belonged to the family
127 *Myoviridae,* identified by their canonical morphology: a small-isometric non-enveloped
128 head with a long contractile tail (Fig. 2; (15)). The head diameter of *PDX* was 76.4 ±
129 4.34 nm and the tail length was 114.0 ± 2.26 nm (n = 13) (Figs. 2ABC). Based on
130 morphological features and metrics obtained using TEM, we putatively identified *PDX* to
131 be a member of the *Myoviridae* family that infect *E. coli* (Fig. 2D).

132 To characterize the phage life cycle, we performed a one-step growth curve of
133 *Escherichia virus PDX,* infecting both EPEC and EAEC (Fig. 3). *PDX* infecting EAEC
134 had a shorter eclipse period, the time before the phage begin emerging from the
135 bacteria, than those infecting EPEC. At the 40-minute time point PFU/mL values were
136 2.40×10^6 and 1.70×10^5 for EAEC and EPEC, respectively ($P < 0.03$). *PDX* infecting
137 EAEC had a modestly greater relative virus count (PFU/mL) 100 minutes post infection
138 compared to EPEC, maximum concentrations reaching 3.60×10^6 PFU/mL and 2.57×10^6
139 PFU/mL for EAEC and EPEC, respectively. However, this difference did not reach
140 statistical significance ($P < 0.06$). Though able to infect both EPEC and EAEC, we

141 concluded that the *PDX* life cycle varied slightly when replicating in these two *E. coli*
142 pathotypes.
143
144 ***PDX* Kill *E. coli* in a Dose Dependent Manner Without Disrupting Normal**
145 **Microbiota.** The bacteriolytic activity of *PDX* against EPEC and EAEC was measured in
146 a time-kill assay revealing a dose-dependent inhibition of bacterial growth. At a
147 multiplicity of infection (MOI) of 100, *PDX* severely suppressed growth of EPEC and
148 EAEC (Fig. 4). Bacteria that were not treated with phage showed normal sigmoidal
149 growth curves that match the expected growth rates for their respective strains. A dose
150 dependent relationship between MOI and point of infection was observed: at greater
151 MOI, infection occurred earlier than for lower MOI (Fig. 4). In many phage treatments,
152 an increase in bacterial growth was observed around 8-10 hours of incubation. This is
153 likely due to the emergence of mutant bacteria that are resistant to phage infection and
154 has been observed in other, similar studies (16, 17).
155 The *in vitro* efficiency of *PDX* infection in the presence of commensal fecal
156 bacteria was tested by anaerobically culturing streptomycin-resistant derivatives of
157 EPEC and EAEC with feces and treating with *PDX*. EPEC or EAEC bacteria were
158 enumerated via colony counts of the culture after ten hours incubation and revealed that
159 *PDX* suppressed growth of pathogens when co-cultured with commensal fecal bacteria.
160 *PDX* significantly reduced the EPEC population, from 6.0 X 10^3 to 17 CFU/mL, and only
161 slightly reduced the population EAEC compared to cultures not treated with phage, from
162 5.2 X 10^3 to 3.2 X 10^3 CFU/mL. This is consistent with the results of the time-kill assays
163 (Fig. 4), which show that high doses of phage at MOI of 100 results in inhibition of

164 bacterial growth. It is unclear why the population of EAEC was not more reduced, as
165 was the case for EPEC, though a mutant could have arisen in the streptomycin-resistant
166 EAEC population that was no longer sensitive to phage infection (Fig. 4).
167 The impact of phage treatment on bacterial diversity of cultured human feces
168 was investigated using 16S metagenomic sequencing analysis of cultures containing
169 fecal bacteria treated with phage (Fig. 5A). Alpha diversity analysis, a metric to measure
170 the diversity to compare samples' relative diversity, was used to quantify the differences
171 between samples. Samples with *PDX* added to human feces were among the most
172 diverse and showed increased diversity compared to feces alone (Fig. 5B). Samples
173 treated with *PDX* and EPEC or EAEC had higher alpha diversities than samples treated
174 with only EPEC and EAEC. The relatively lower alpha diversity of fecal samples treated
175 with pathogens alone is consistent with the findings of the pathogen enumeration assay,
176 which confirmed the presence of populations of streptomycin-resistant EPEC and EAEC
177 in their respective cultures. While it would be expected that the culture containing only
178 feces might have the highest alpha diversity, our results do not show this, perhaps due
179 to sample variation. Nonetheless, we concluded that the presence of the *PDX* phage in
180 the fecal cultures did not reduce alpha diversity when compared to feces alone.
181
182 **Genomic and Phylogenetic Characterization.** Multiple genome analyses and
183 annotation software programs were used to characterize the *PDX* genome (Fig. 6). We
184 annotated the genome looking for closely related phage species and their relevance to
185 other phage therapy applications, as well as for markers that might indicate lysogenic
186 activity (Table 2). The genome of *PDX* is 138,828 bp dsDNA a midsized genome for the

187 *Myoviridae* family of viruses. The GC content is 43%, which is comparable to other lytic
188 phages (FastQC Galaxy). We identified 206 putative coding domain sequences (CDS)
189 (PROKKA, ORFfinder), but no toxin-encoding or lysogenic genes, which are
190 characterized as any genes which contained the terms “integrase”, “excisionase”,
191 “recombinase”, and “repressor” were identified in the genome when compared to the
192 NCBI BLAST database. Additionally, in a 20-standard amino acid (aa) search, a total of
193 6 putative tRNAs were predicted with open reading frames (ORFs) ranging from 72-88
194 bp in length (tRNAscan-SE). Virfam software uses the translated amino acid (aa) of
195 CDS from a phage genome as input and searches for homology to other structural
196 phage protein sequences deposited in the Aclame database. Based on the results of
197 the Virfam analysis, the head-neck-tail structure genome organization in *PDX* belongs
198 to the Neck Type One – Cluster 7 category (Fig. 7A). Neck Type One phage genomes
199 contain the following proteins: portal protein, Adaptor of type 1 (Ad1), Head-closure of
200 type 1(Hc1), Neck protein of type 1(Ne1), and tail-completion of type 1 (Tc1) (Fig. 7B).
201 Cluster 7 phage genomes are reported in Virfam to be strictly myophages with small
202 genome sizes and gene content (61-150 genes). However, it is noteworthy that the
203 genome of PDX contains 206 CDS, which is more than the other phage in this cluster in
204 the current Aclame database (Table 2). Cluster 7 myophages all infect Proteobacteria.
205 Cluster 7 phage genomes are organized such that there is a different gene order
206 compared to the canonical order that is observed in most clusters of the type 1 family
207 (Fig. 7B).
208 By BLAST analysis, the three genomes with the highest whole-genome
209 nucleotide identity match were *Escherichia coli* O157 typing phage 4 (98%), *Escherichia*

210 Phage JES-2013 (98%), and *Escherichia* Phage Murica. Murica is a *Myoviridae* phage
211 that shares a 97% nucleotide identity with *PDX*, is strictly lytic against enterotoxigenic
212 (ETEC), and is being researched as a potential treatment of contaminated food in lieu of
213 antibiotics (18). *E. coli* O157 typing phage 4 is lytic against *E. coli* O157, which also had
214 a 99% amino acid identity with the *PDX* tail fiber proteins (19). Phage JES-2013 is lytic
215 against *Lactococcus lactis* (20). The putative tail fiber sequences from *PDX* had a 99%
216 amino acid identity with *Myoviridae Escherichia* phage V18 (Table S1). V18 was in a
217 successful prophylactic phage cocktail used in the prevention of traveler's diarrhea in
218 mice infected with 6 bacterial strains: *E. coli, Shigella flexneri, Shigella sonnei,*
219 *Salmonella enetica, Listeria monocytogenes* or *Staphylococcus aureus,* and Lac (-) *E.*
220 *coli* K-12 C600 (21). Additionally, V18 phage was cited as having a broad host range
221 against ETEC, EAEC, and enterohaemorrhagic *E. coli* (EHEC). Host range data was
222 unavailable for the Slur16 *Myoviridae* phage, which shared 99% identity with the
223 putative PDX tail fiber proteins. However, Slur16 was isolated from dairy cow feces
224 using *E. coli* K-12 MG1655 as a host, suggesting that this phage is lytic against
225 pathogenic *E. coli* strains (22). Overall, the phage that had the highest percent identity
226 with *PDX* tail fiber amino acid sequences were all strictly lytic *Myoviridae* against
227 various *E. coli* strains.
228

229 **DISCUSSION**

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

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

250 In order for phage to be used as an efficacious therapeutic agent they must be
251 strictly lytic. Lysogenic phage, such as phage lambda, transfer bacterial genetic material

252 from one population to another upon infection and integration into the host genome.
253 This can lead to the transfer of antibiotic resistance genes and toxin encoding genes
254 between bacterial hosts (24). Several lines of evidence indicate that *PDX* is a lytic
255 phage. *PDX* was classified as a member of the strictly lytic (25) *Myoviridae*
256 bacteriophage family based on morphological characteristics from TEM imaging (Fig. 2).
257 Bioinformatic analysis of the head-neck structure CDS in the Virfam suite identified *PDX*
258 as belonging to the Neck Type One – Cluster 7 comprised only of members of the
259 myophage family (Fig. 7). Neither CI nor CI-associated genes that are responsible for
260 lysogeny in phage lambda were identified in the genome of *PDX.* Lastly, phage
261 induction, by treatment with Mitomycin C, did not induce *PDX* plaques in mutant
262 derivatives of EPEC and EAEC resistant to *PDX* infection (data not shown). Thus, we
263 concluded that the *PDX* phage was a member of the *Myoviridae* family having a strictly
264 lytic lifestyle.
265 The *PDX* genome is a midsized with 138,828 bp, compared to the 11,000 to
266 271,000 bp range found within the 547 complete *Myoviridae* genomes currently in the
267 NCBI database. Additionally, it is noteworthy that *PDX* has a higher putative CDS count
268 than any of the other members of Cluster 7 category in the Virfam and Aclame
269 databases.
270 One of the dangerous side effects of antibiotics is the drastic disruption of the
271 microbiome, or dysbiosis, due to the conserved nature of the drug targets. The
272 metagenomic analysis of fecal cultures treated with phage suggested that *PDX*
273 treatment did not significantly alter the community structure of the commensal
274 microbiome in feces cultured with *PDX*, in contrast to what occurs with antibiotic

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

284 Future studies should investigate the efficacy of this phage, both in isolation and
285 in combination with other phage (a phage cocktail), in attenuating bacterial infections.
286 Though certainly not exhaustive, we did observe that *PDX* could form plaques,
287 indicating replication, in 5 out of 20 clinical isolates of EPEC from Northwest children
288 (Table 1). The use of phage cocktails would then allow for the broadening of serotypes
289 and pathotypes that can be targeted by phage therapy. Further, an *in vivo* approach
290 could utilize mice whose microbiomes have been “humanized”, inoculating subjects with
291 phage susceptible bacteria and then treating subjects with varying titers and at different
292 times points post bacterial infection. Results would include monitoring virulence and
293 bacterial burdens. Notably, a recent study by Hodyra-Stefaniak and colleagues revealed
294 that the most important factors that determine the efficacy of a phage therapeutic agent
295 are: stage of bacterial pathogenesis, the bacterial population size at the time of
296 administration, the titer used, and the administration method (26). Just recently it was
297 announced that UC San Diego will launch The Center for Innovative Phage Applications

298 and Therapeutics (IPATH) to refine treatments and to bring them to market
299 (http://www.sciencemag.org/news/2018/06/can-bacteria-slaying-viruses-defeat-
300 antibiotic-resistant-infections-new-us-clinical). They hope to generate libraries of phages
301 and use cocktails for individual patients to treat MDR infections in order to meet FDA
302 approval challenges. Thus, our efforts and those of other researchers will be essential
303 for isolating therapeutic phages to be used against the increasing number of untreatable
304 bacterial infections.
305
306

307 **MATERIALS AND METHODS**

308 **Bacterial Strains and Growth Conditions.** Bacterial strains are listed in Table 1. All
309 strains were grown in Lysogeny Broth (LB) at 37°C with shaking at 225 RPM or on LB
310 agar at 37°C. All phage lysates were stored in SM gel buffer (50 mM Tris-HCl [pH 7.5],
311 0.1 M NaCl, 8 mM $MgSO_4$, 0.01% gelatin) at 4 ^{o}C, and warmed to room temperature for
312 all experiments unless otherwise stated.

313 **Phage Isolation and Propagation.** Bacteriophage *PDX* was initially isolated from
314 untreated (influent) sewage collected at the Portland Wastewater Treatment Plant on
315 October 1, 2015. The raw sewage was filtered through a 0.2 µm filter (Corning, Corning
316 NY) to remove bacteria. The filtered sewage was then added to host bacteria in liquid
317 culture (Fig. 1A). Phage-host solutions were combined with 0.5% LB soft agar and
318 overlaid on LB agar plates. Plates were then observed for the presence of plaques after
319 overnight growth at 37°C. Clear, defined plaques of interest were isolated and
320 transferred using a sterilized inoculating needle and agitation in 1 mL solution of lambda
321 diluent (10mM Tris, pH 7.5; 10 mM $MgSO_4$). To obtain a high titer phage preparation, a
322 crude lysate was generated. A clear *PDX* plaque was isolated and suspended in 1 mL
323 of sterile lambda diluent. The phage suspension was combined with overnight culture of
324 the bacterial host and incubated at 37°C with shaking at 225 RPM overnight. Next,
325 chloroform was added to kill any remaining bacteria. This solution was centrifuged at
326 4°C at 4000 x g for 10 minutes to remove bacterial cells. Finally, the supernatant was
327 recovered and treated with chloroform again to kill any remaining bacterial cells.

328 Several rounds of overlay purification were used to ensure lysate purity after
329 sequential host isolation. From the final overlay of phage sequentially isolated from

330 MC4100, EPEC, and EAEC, five rounds of purification were carried out (Fig. 1B).
331 Several clearly defined plaques from the final overlay plate were picked using a sterile
332 inoculating needle and suspended in microcentrifuge tubes containing SM buffer. The
333 phage suspension was incubated at room temperature for 15 minutes to allow the
334 phage to diffuse through the medium, then filtered through a sterile 0.2-µm filter to
335 remove any bacteria that may have been transferred from the overlay plate. A host
336 susceptibility assay was performed as described below to test the ability of each
337 selected plaque to lyse MC4100, EPEC, and EAEC. This process was performed four
338 more times until uniform plaques were observed indicating pure phage suspension.
339 The final phage suspension was then propagated to make a purified high titer
340 lysate. Exponential EAEC with undiluted phage suspension was incubated in LB
341 overnight at 37°C. Bacteria were removed from the culture by centrifugation at 6,055 x
342 g, then the supernatant was sterile filtered with a 0.2 µm filter. The titer of the lysate was
343 determined by plating dilutions (10^{-2}-10^{-16}) using the EAEC overlay procedure described
344 previously. After overnight incubation, the plates were observed and the number of
345 plaque forming units per mL (PFU/mL) was calculated. The purified high titer lysate in
346 SM buffer was stored at 4°C.
347 **Electron Microscopy**. *PDX* was analyzed using transmission electron microscopy
348 (TEM). Purified high titer lysate was precipitated in 25% PEG 6000-8000 in 2.5M NaCl
349 and stored overnight at 4°C. Precipitated phage solutions were centrifuged at 4°C, 4000
350 x g for 10 minutes. The supernatant was removed and the phage pellet was suspended
351 in 40µL of 50 mM Tris, pH 7.4, 10 mM $MgSO_4$. Phage suspension was placed on
352 copper coated formvar grids and negatively stained with 1% uranyl acetate. Samples

353 were examined under a FEI Tecnai™ Spirit TEM system at an operating voltage of 120
354 kV (FEI, Hillsboro, OR). All transmission electron microscopy was performed at the
355 Multi-Scale Microscopy Core (MMC) with technical support from the Oregon Health &
356 Science University (OHSU)-FEI Living Lab and the OHSU Center for Spatial Systems
357 Biomedicine (OCSSB).
358 **Host Susceptibility Assay.** A host susceptibility assay, or spot test was utilized to
359 determine the host range of *PDX* (Table 1). Each LB agar plate was streaked with 3-4
360 spatially separated bacterial strains using a sterilized inoculating loop. A small volume
361 of purified lysate was pipetted on top of the center of each linear bacterial streak and
362 incubated overnight at 37°C. Host susceptibility was indicated by a clearing within the
363 streak of a given bacterial strain.
364 **Growth Curves.** To determine the growth kinetics of *PDX*, one-step growth curve
365 experiments were performed. LB liquid cultures of *E. coli* were grown to an OD600 of
366 0.6-0.7, when the cultures had reached mid to late exponential phase. Next, the
367 indicated purified phage lysate was added to the liquid culture tubes to infect the
368 bacteria with a multiplicity of infection (MOI) of 0.01. The liquid bacterial culture and
369 phage lysate were incubated at room temperature for 10 minutes. Following the
370 incubation period, the solution was centrifuged at 8,000 x g for 10 minutes. The
371 supernatant was discarded and the pellet was re-suspended in LB to make the
372 absorption mixture. Immediately after re-suspension the absorption mixture was serially
373 diluted and plated with the bacterial host. The phage was incubated within a standard
374 plaque-overlay. This procedure was repeated at 20-minute time intervals for a total of
375 120 minutes. After 24 hours of incubation, the plaques on all the plates were recorded

376 and the PFU/mL was calculated. The titer over time (120 minutes) was plotted to obtain
377 a one-step growth curve. This experiment was repeated using EPEC and EAEC strains.
378 **Time-Kill Assays.** Bacterial density was measured over time to determine the course of
379 phage infection. Liquid cultures of EPEC and EAEC were incubated with *PDX*. The *in*
380 *vitro* lytic efficiency of the phage was examined at several MOIs. Wells of a flat-
381 bottomed 96-well microtiter plate were filled with 100 μl of inoculated double-strength LB
382 and 100 μL of purified lysate dilutions in SM buffer. Bacterial cultures were grown to late
383 exponential phase and diluted to 10^6 CFU/mL. *PDX* lysate was diluted to 10^5, 10^6, 10^7
384 or 10^8 PFU/mL, corresponding to MOIs of 0.1, 1, 10, and 100, respectively. Each
385 phage-host combination at specific MOIs was performed in triplicate wells, and each
386 experiment was performed three times. Controls for plate sterility, phage suspension
387 sterility, and bacterial growth without phage addition were also included. To prevent
388 between-well contamination by aerosolizing of bacteria, an adhesive transparent plate
389 cover was placed over the top of the plate. The plates were incubated at 37°C with
390 orbital shaking for 12 hours and OD at 600 nm was measured using a Microtiter Plate
391 Reader (Sunrise, Tecan Group Ltd., Austria) at 30-minute intervals.
392 **Anaerobic Culture.** Feces were collected from a 23-year-old male in Portland, Oregon
393 who had not taken antibiotics in the last 5 years. One gram of fresh feces was
394 suspended in sterile saline solution by vortexing and vigorous shaking. The fecal
395 suspension was then filtered through a sterile 1-mm mesh sieve twice to remove large
396 pieces of organic matter. The filtered fecal suspension was then concentrated by
397 centrifugation at 1500 x g for five minutes. The resulting fecal pellet was resuspended in
398 saline solution with 10% glycerol for a final concentration of 0.5g fresh feces/mL. Fecal

399 slurry was stored at -80°C.

400 Streptomycin-resistant (str^R) EPEC and EAEC were grown anaerobically under
401 100% CO_2 with fecal slurry and *PDX* using the Hungate method in M10 medium,
402 designed to mimic the nutrient, mineral, and pH content of the intestine (27). Cultures
403 were inoculated to final concentrations of 0.7 mg/mL feces, 1.4 x 10^6 CFU/mL *E. coli,*
404 and 1.4x10^8 PFU/mL phage treatments. Cultures were incubated for 16 hours in a
405 stationary incubator at 37°C. DNA from these cultures was isolated for 16s rDNA
406 sequencing analysis after 16 hours of incubation.

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

416 For DNA isolation, bacteria were pelleted by centrifugation at 5000 x g for 10
417 minutes. The pellets were washed with phosphate buffer solution to separate whole
418 cells from lysed cell debris, then spun down at 5000 x g for 5 minutes. DNA from
419 Hungate cultures was extracted using a DNeasy Blood and Tissue kit (Qiagen,
420 Germantown, MD), with the protocol adapted according to manufacturer instructions for
421 bacteria.

422 **16S Metagenomic Sequencing and Analysis.** Genomic DNA was sent to the Oregon
423 State University Center for Genome Research and Biocomputing for multiplex
424 sequencing analysis using the Illumina MiSeq sequencing platform. The V3-V4 region of
425 the 16S rRNA gene was amplified and used to prepare a dual index library using the
426 Nextera XT library preparation kit. The analysis produced forward and reverse paired
427 end reads of 150 bp at a sequencing depth of 1 million reads. Sequence quality was
428 quantified using FastQC and demultiplexed.
429 **Phage Genome Sequencing and Bioinformatics.** To initiate genomic sequencing, a
430 high titer lysate was purified using CsCl gradients. Genomic phage dsDNA was
431 extracted using an Epicentre DNA Isolation kit according to manufacturer's instructions
432 (Madison, WI). The quantity, quality, and concentration of the extracted DNA was
433 analyzed utilizing a high-sensitivity assay of dsDNA with a Qubit 2.0 (Invitrogen, Grand
434 Island, NY). All library preparation and Illumina sequencing performed at MGH Genome
435 Core (Cambridge, MA). A diagram illustrating work flow for genomic analysis is
436 presented in Figure 6. Upon receiving the genomic sequences in FASTQ format, we
437 used the Galaxy platform to perform quality control and filtering processes on the raw
438 reads (28–30). The Trimmomatic Galaxy (v. 0.32.3) software was used to recognize the
439 distinct adaptors to distinguish and gather all the reads for one phage separately from
440 the other (31). The quality of the reads was analyzed with FastQC (v. 0.11.4) suite with
441 default settings. The quality of the assembly was assessed using Quality Assessment
442 Tool for genome assemblies (QUAST, v. 2.3), as it reports relevant assembly quality
443 metrics (32). Following assembly, we used NCBI BLAST, tRNAscan-SE, FastQC
444 Galaxy, and Virfam to annotate the functional regions of the genome assembly. The

445 putative coding sequences (CDS) were filtered for only those with known protein
446 functions produced by NCBI smart BLAST with identity above 95%.
447 Genes of interest in the genome annotations were compared to other similar
448 sequences in the National Center for Biotechnology Information (NCBI) database using
449 the Basic Local Alignment Search Tool (BLAST; v. 2.3.1) suite, specifically using the
450 blastp and tblastn tools with default settings (21, 33). The genome assemblies were
451 deposited in the GenBank database. All phylogenetic analyses were performed using
452 Virfam webserver, which performs homology searches against phage sequences
453 available in the Aclame database (34, 35).
454 **Accession number.** The genomic sequence of the *Escherichia virus PDX* was
455 deposited in GenBank under accession number MG963916.
456
457

458 **Figure Legends**

459

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

466

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

475

476 **Figure 3.** One-step growth curve analysis of EAEC (A) and EPEC (B) infected by *PDX*
477 with an MOI of 0.01 for 120 minutes. Over the 120-minute infection period, bacterial
478 cultures were sampled, serially diluted and plated with a plaque-overlay procedure (see
479 Materials and Methods). After 24-hour incubation, the plaques were counted and the
480 titer (PFU/mL) was determined. Error bars represent the standard error of the mean. By

481 comparing the PFU/mL values at 40 min, the eclipse period was shorter for EAEC than
482 for EPEC when infected with *PDX* ($P < 0.04$).
483
484 **Figure 4.** Time-kill assay of EAEC (A) and EPEC (B) treated with *PDX*. In a 96-well
485 plate, exponential culture was inoculated with dilutions of *PDX* high titer lysate for MOI
486 (multiplicity of infection) ranging from 0.1, 1, 10 and 100. The plates were incubated at
487 37°C with orbital shaking for 12 hours and OD of 600 nm was measured using a
488 Microtiter Plate Reader (Tecan). Assays were performed in triplicate. Representative
489 data are presented.
490
491 **Figure 5.** Effect of phage treatment on bacterial community in human feces. (A)
492 Summary of taxa classified at the family level for fecal cultures with EPEC, EAEC and
493 *PDX*. The plot was generated based on OTU frequencies for each sample. OTUs were
494 assigned using a closed reference algorithm based on alignment of high throughput 16S
495 rDNA sequencing reads to reference genes in the Greengenes database. (B) Alpha
496 diversity of bacterial populations found in samples treated with feces, *PDX* and EPEC or
497 EAEC. Alpha diversity was calculated using the Choa1 algorithm in Qiita, which uses
498 the abundance of OTUs and number of different OTUs to determine a number
499 representing the samples relative diversity.
500
501 **Figure 6.** Bioinformatics workflow for the assembly, annotation and phylogeny of the
502 *PDX* genome. (A) Steps of the genome raw read processing. Names of programs used
503 appear in parenthesis; (B) Steps of the genome assembly; (C) Annotation of the

504 genome; (D) Genome assembly and annotation output; and (E) Phylogeny construction
505 using the Virfam program based on Aclame database.
506 *Only sequences with putative identity match of 95% were maintained for further
507 analysis.
508
509 **Figure 7.** Structural Protein Classification Analysis of *PDX*. (A) Tree representation of
510 *PDX* (black text in white box, lined red) classification among other *Caudovirales* phage,
511 constructed from a hierarchical agglomerative clustering procedure applied to a matrix
512 of similarity scores between pairs of phages (by combining HHsearch probabilities with
513 the percentage identity) and present in the Aclame database. The different branches of
514 the tree were sorted into 10 Clusters and are highlighted by different background colors.
515 The bacterial host(s) of the phages are listed for each Cluster in the legend at the
516 bottom of the figure. (B) Gene organization of *PDX* and other phage genomes that
517 belong to the Neck One – Cluster 7. The legend at the bottom indicates to which family
518 a protein belongs. (-) indicates the antisense DNA strand.
519

520 **Table 1.** *Escherichia coli* strains used in this study.

Strain	Genotype/ Serotype	Syndrome[1]	Spot Test Result[2]	Plaque Formation[3]	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)
TB209E	O15:HN	CD	-	-	(14)
TB216A	O118:H8	D	-	-	(14)
TB220B	O153:HN	CD	+	+	(14)
TB227A	O8:H41	CD	-	-	(14)
TB269C	O145:HN	D, F	+	-	(14)
TB280A	O66:HN	CD	-	-	(14)

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)

521 [1]***Key:*** BD, bloody diarrhea; CD, chronic diarrhea; D, acute diarrhea; F, fever; U, details
522 unknown; V, vomiting.
523 [2]A plus sign indicates strain is killed by *Escherichia virus PDX.*
524 [3]A plus sign indicates *PDX* replicates (can form plaques on) the strain.

525

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

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

527

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

	% Identity with PDX Genome[b]	E-Value[c]	Total Score[d] (max score 255600)	Accession Number
Escherichia Phage Murica	97%	0.0	40234	AKU44119.1
Escherichia coli O157 typing phage 4	98%	0.0	52649	AKE45376.1

Escherichia Phage JES-2013	98%	0.0	52649	YP_008530272.1

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

536

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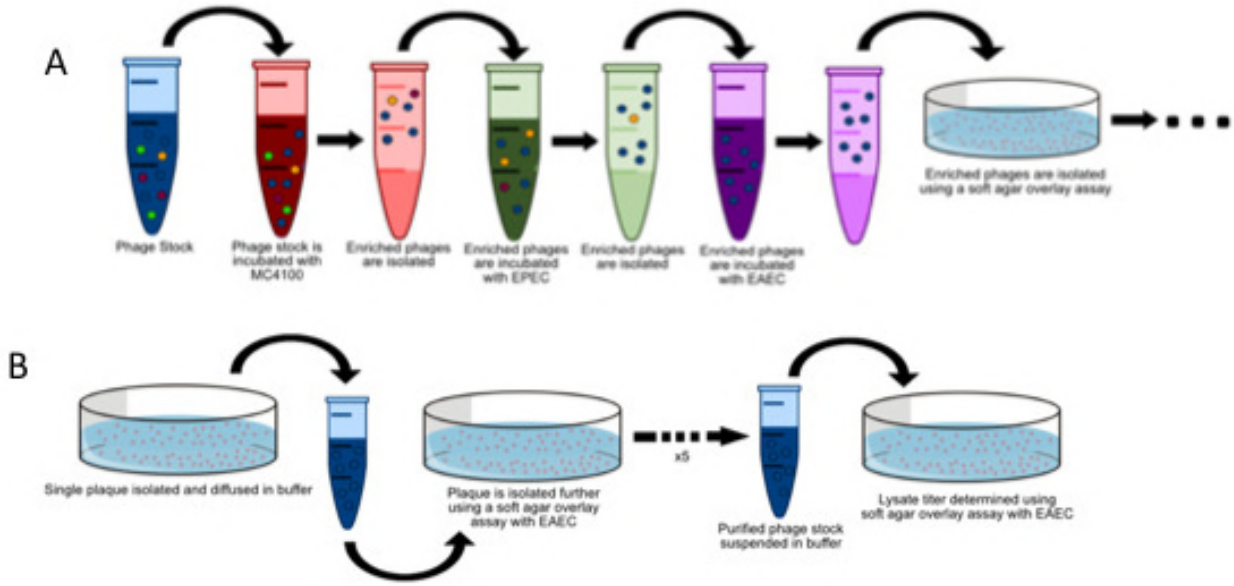
666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Enriched phages are incubated with EAEC
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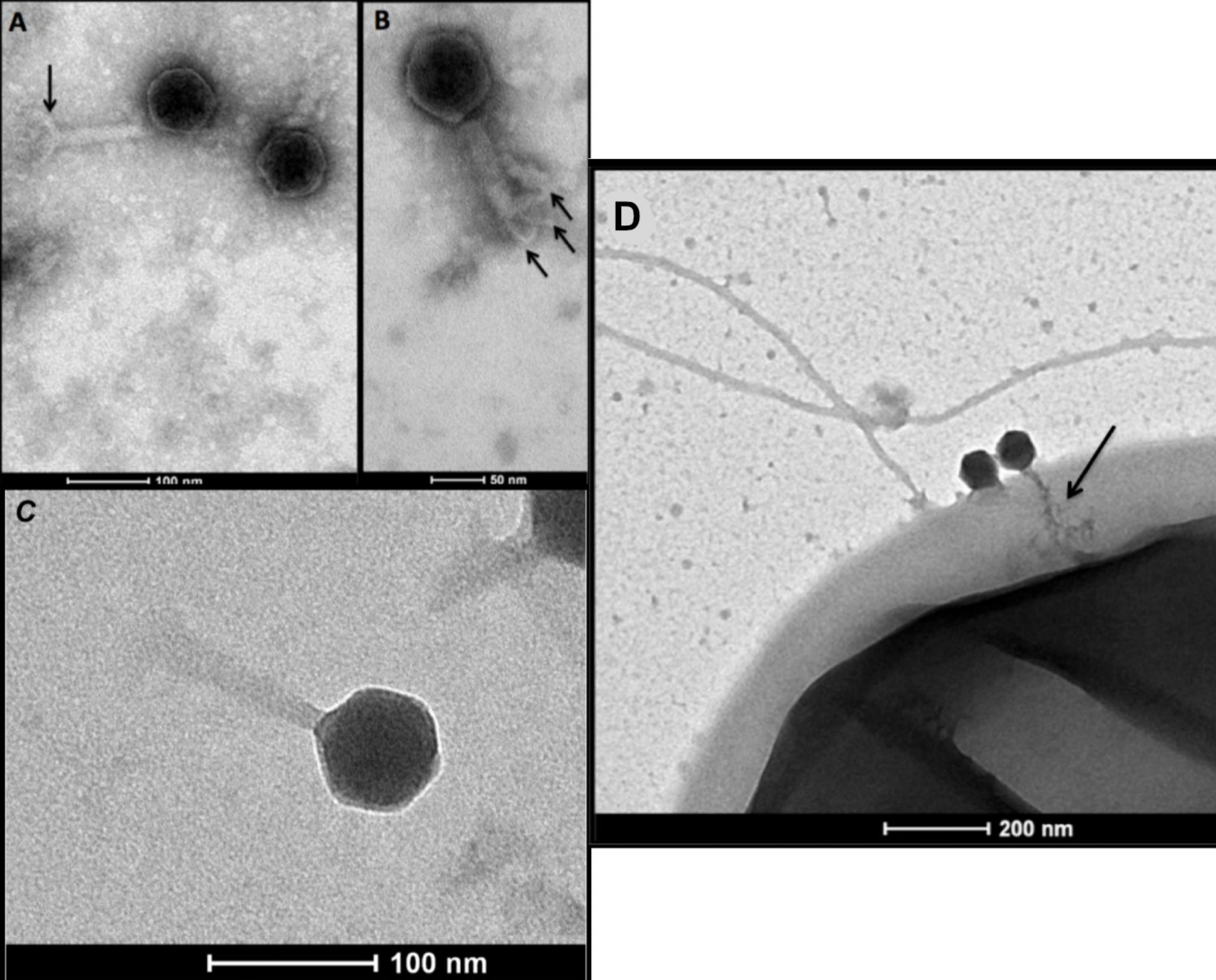
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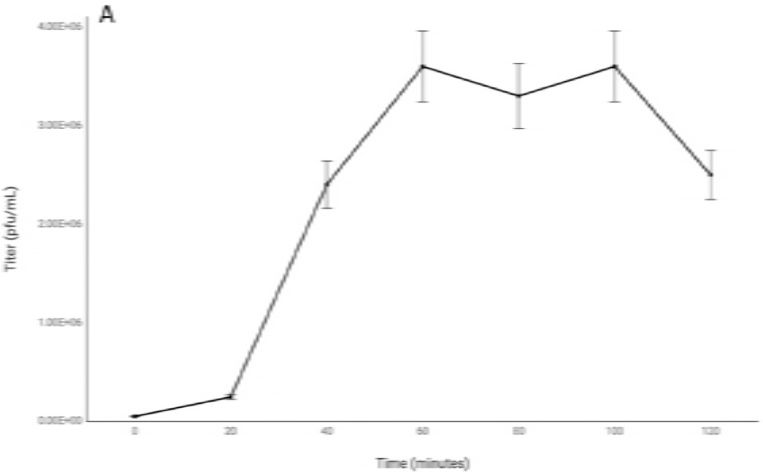
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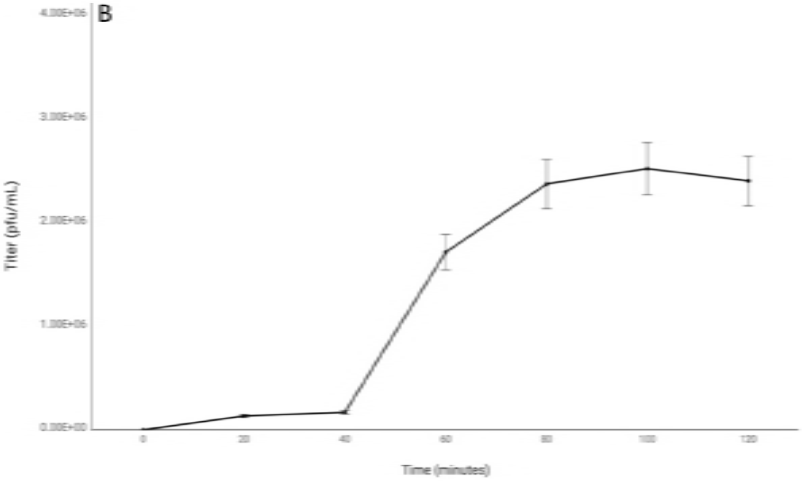
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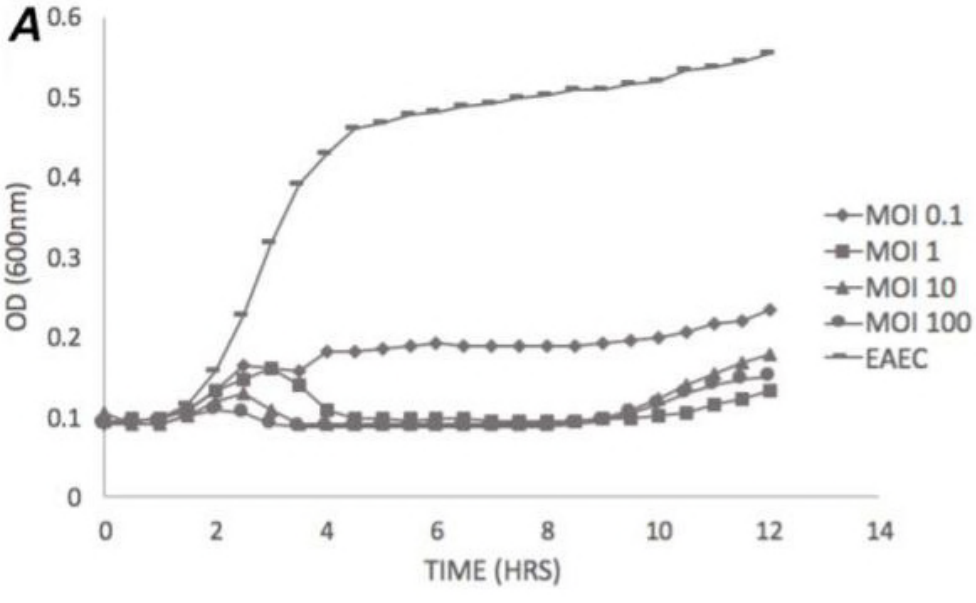
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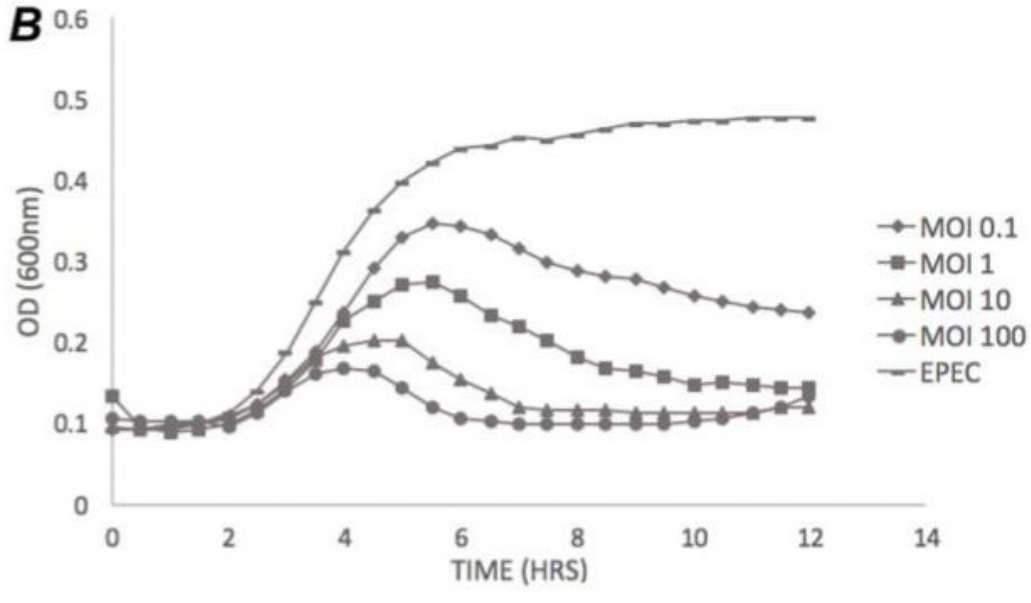
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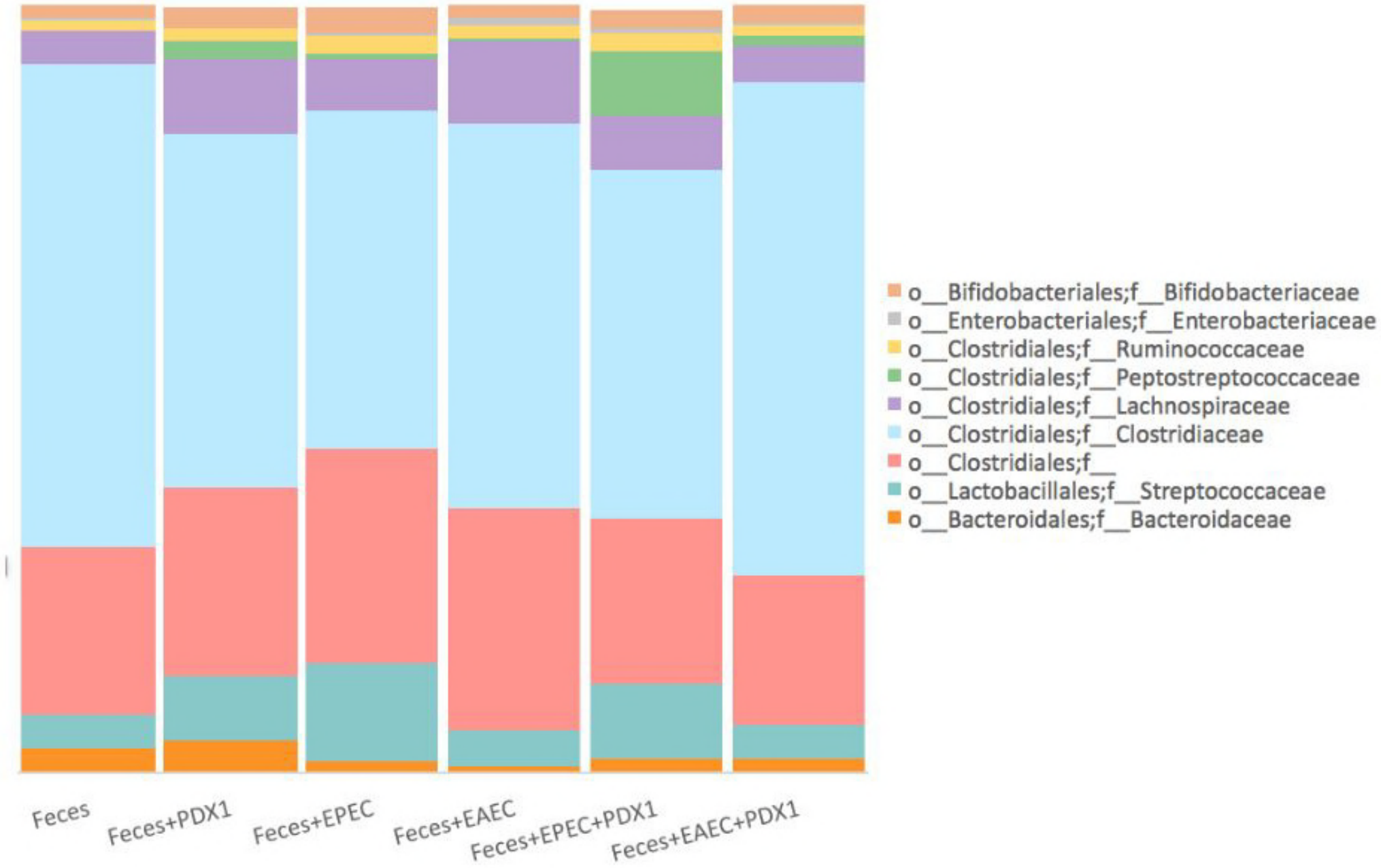
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o__Lactobacillales;f__Streptococcaceae
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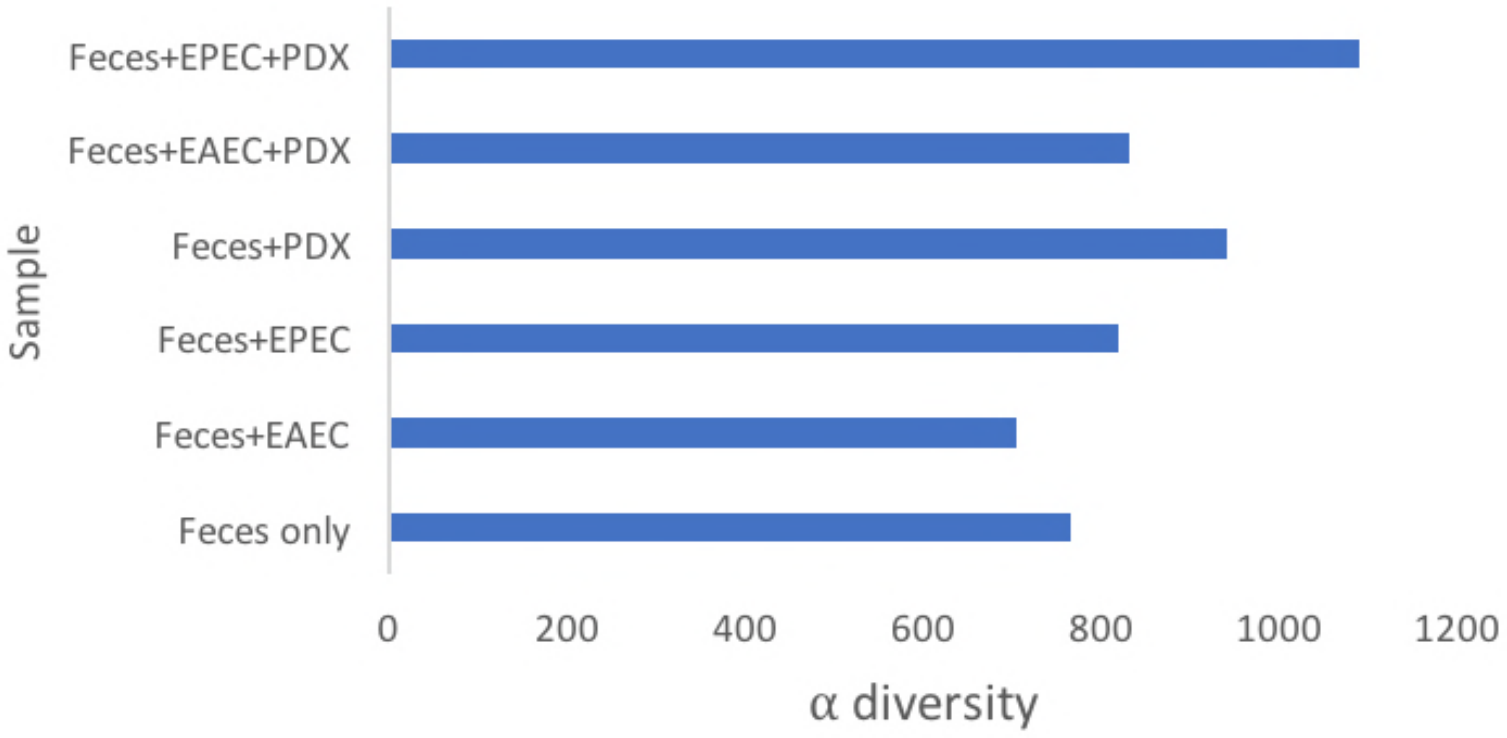
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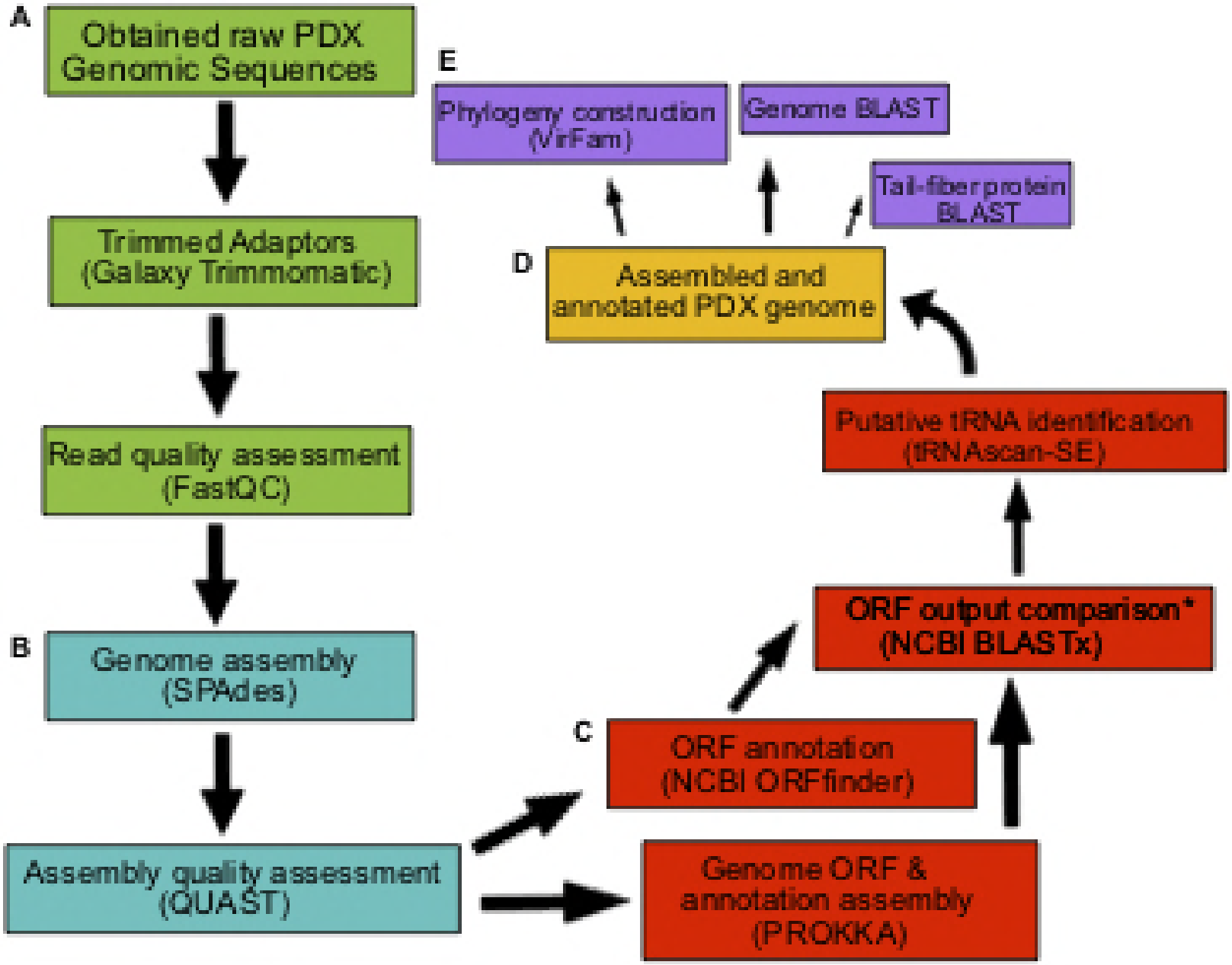
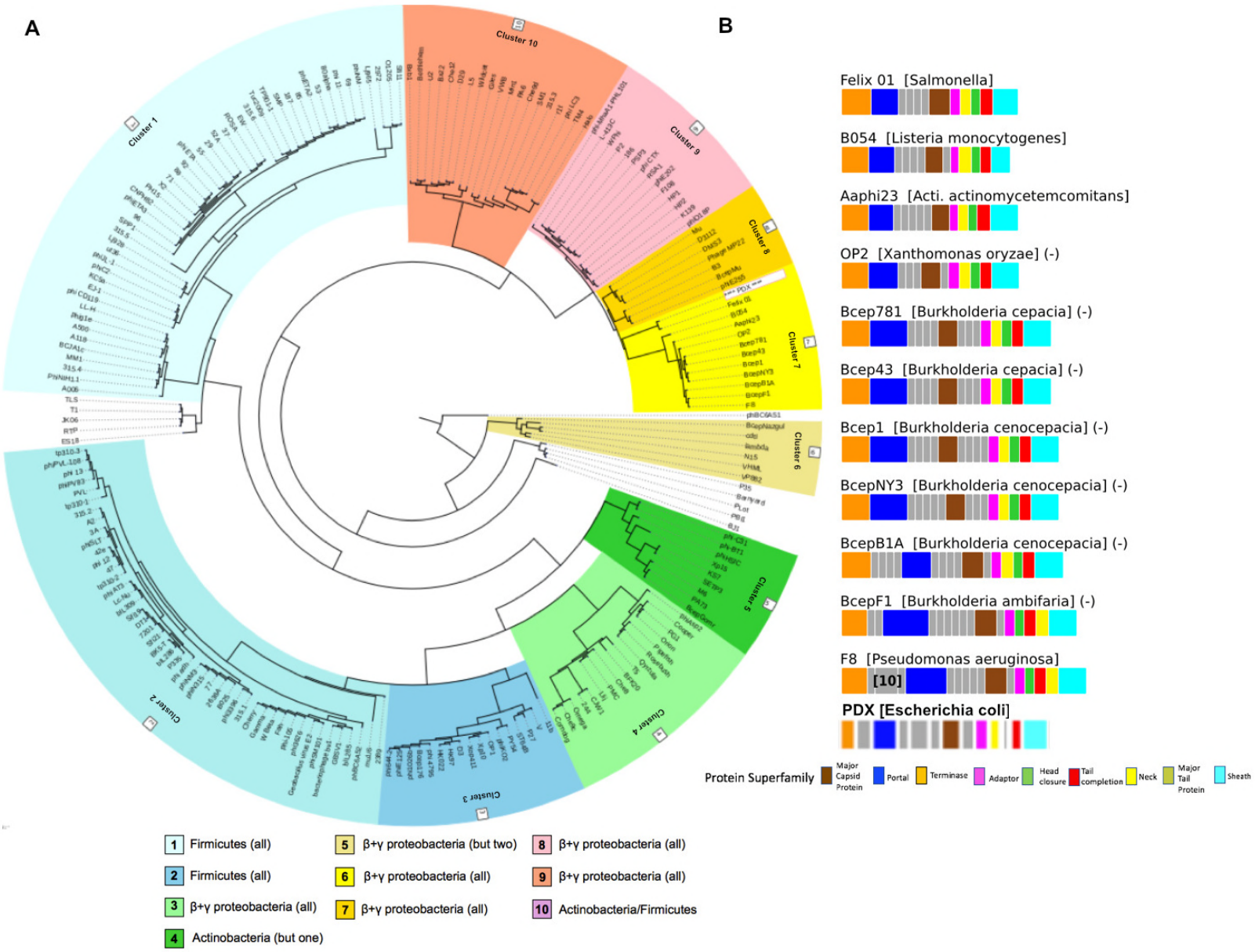
0
200
400
600
800
1000
1200
α diversity


A
Obtained raw PDX Genomic Sequences
Trimmed Adaptors (Galaxy Trimmomatic)
Read quality assessment (FastQC)
B
Genome assembly (SPAdes)
Assembly quality assessment (QUAST)
C
ORF annotation (NCBI ORFfinder)
Genome ORF & annotation assembly (PROKKA)
ORF output comparison* (NCBI BLASTx)
Putative tRNA identification (tRNAscan-SE)
D
Assembled and annotated PDX genome
E
Phylogeny construction (VirFam)
Genome BLAST
Tail-fiber protein BLAST


A
B
Cluster 1
Cluster 2
Cluster 3
Cluster 4
Cluster 5
Cluster 6
Cluster 7
Cluster 8
Cluster 9
Cluster 10
Felix 01 [Salmonella]
B054 [Listeria monocytogenes]
Aaphi23 [Acti. actinomycetemcomitans]
OP2 [Xanthomonas oryzae] (-)
Bcep781 [Burkholderia cepacia] (-)
Bcep43 [Burkholderia cepacia] (-)
Bcep1 [Burkholderia cenocepacia] (-)
BcepNY3 [Burkholderia cenocepacia] (-)
BcepB1A [Burkholderia cenocepacia] (-)
BcepF1 [Burkholderia ambifaria] (-)
F8 [Pseudomonas aeruginosa]
[10]
PDX [Escherichia coli]
Protein Superfamily
Major Capsid Protein
Portal
Terminase
Adaptor
Head closure
Tail completion
Neck
Major Tail Protein
Sheath
1 Firmicutes (all)
2 Firmicutes (all)
3 β+γ proteobacteria (all)
4 Actinobacteria (but one)
5 β+γ proteobacteria (but two)
6 β+γ proteobacteria (all)
7 β+γ proteobacteria (all)
8 β+γ proteobacteria (all)
9 β+γ proteobacteria (all)
10 Actinobacteria/Firmicutes