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3 # Bacteria-mediated stabilization of a panel of Picornaviruses

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13 Short Title: Effects of bacteria on Picornaviruses

14

15 Keywords: Bacteria, Picornaviruses, Stability

16

17 Abstract word count: 206

18 Text word count: 4,390

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24 **ABSTRACT**

25 Several viruses encounter various bacterial species within the host and in the
26 environment. Despite these close encounters, the effects of bacteria on picornaviruses
27 specifically is not completely understood. Previous work determined that poliovirus (PV),
28 an enteric virus, has enhanced virion stability when exposed to bacteria or bacterial
29 surface polysaccharides such as lipopolysaccharide. Virion stabilization by bacteria may
30 be important for inter-host transmission since a mutant PV with reduced bacterial
31 binding had a fecal-oral transmission defect in mice. Therefore, we investigated whether
32 bacteria broadly enhance stability of picornaviruses from three different genera:
33 *Enterovirus* (PV and coxsackievirus B3 (CVB3)), *Kobuvirus* (Aichi virus) and *Cardiovirus*
34 (Mengo virus). Furthermore, to delineate strain-specific effects, we examined two
35 strains of CVB3 and a PV mutant with enhanced thermal stability. We determined that
36 specific bacterial strains enhance thermal stability of PV and CVB3, while Mengo virus
37 and Aichi virus are stable at high temperatures in the absence of bacteria. Additionally,
38 we determined that bacteria or lipopolysaccharide can stabilize PV, CVB3, Aichi virus,
39 and Mengo virus during exposure to bleach. These effects are likely mediated through
40 direct interactions with bacteria since viruses bound to bacteria in a pull-down assay.
41 Overall, this work reveals shared and distinct effects of bacteria on a panel of
42 picornaviruses.

43

44 **IMPORTANCE**

45 Recent studies have shown that bacteria promote infection and stabilization of
46 poliovirus particles, but the breadth of these effects on other members of the

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47 *Picornaviridae* family is unknown. Here, we compared the effect of bacteria on four
48 distinct members of the *Picornaviridae* family. We found that bacteria reduced
49 inactivation of all of the viruses during bleach treatment, but not all viral strains were
50 stabilized by bacteria during heat treatment. Overall, our data provide insight into how
51 bacteria play differential roles on picornavirus stability.

52

53 **INTRODUCTION**

54 The *Picornaviridae* family includes important human pathogens that can cause a
55 range of diseases such as the common cold, meningitis, hepatitis, and paralysis. The
56 *Picornaviridae* family is diverse and currently includes 80 species in 35 genera.
57 Members of this family are nonenveloped and contain a single-stranded, positive-sense
58 viral genome approximately 7,500 nucleotides in length (1).

59 Recent studies have shown that bacteria, in particular the gut microbiota, play
60 several important roles during viral infection. Enteric viruses encounter a milieu of
61 microorganisms, including bacteria, both within and outside of the host. It is estimated
62 that these viruses encounter approximately 10^{11} bacteria in the host and are expected
63 to encounter even more in the environment (2). Indeed, bacteria enhance infection of
64 several unrelated viruses, including poliovirus, reovirus, rotavirus, mouse mammary
65 tumor virus, and norovirus (3-9). These “pro-viral” effects are mediated by two known
66 mechanisms: 1) Direct interactions between bacteria and viruses that increased virion
67 stability and attachment to host cells, and 2) indirect interactions between bacteria and
68 the host immune system that modulated immune responses for productive viral infection
69 (3-8,10).

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70 Intriguingly, bacteria and bacterial molecules can inhibit infection with certain
71 viruses. For example, Ichinohe *et al*. demonstrated that certain bacteria promote host
72 immune responses during influenza infection of mice (11). More recently, Bandoro *et al*.
73 determined that exposure to the bacterial surface molecule lipopolysaccharide (LPS)
74 reduced stability of several strains of influenza virus by altering the morphology of the
75 virion envelope (12).

76 Based on the importance of bacterial-viral interactions on viral infection, we
77 sought to determine whether bacteria differentially affect different members of the same
78 viral family, the *Picornaviridae*. We used a panel of four picornaviruses that are spread
79 by the fecal-oral route and represent three separate genera: *Enterovirus* (coxsackievirus
80 B3 (CVB3) and PV), *Kobuvirus* (Aichi virus) and *Cardiovirus* (Mengo virus). We found
81 that a subset of the viral panel were stabilized by bacteria during heat treatment but that
82 all of the picornaviruses tested were stabilized by bacteria during bleach treatment. We
83 also determined that viruses bound to bacteria, indicating that direct interactions may be
84 facilitating viral stabilization of these viruses. This work expands on bacteria-mediated
85 enhancement effects previously observed with PV to other members of the
86 *Picornaviridae* family. Ultimately, this work defines the unique interactions between
87 specific viruses and bacteria which may provide insight into virion environmental
88 stability and transmission.

89

90 **RESULTS**

91 **Panel of viruses from the *Picornaviridae* family and bacterial strains.** Previous
92 studies have indicated that bacteria can reduce the inactivation of poliovirus particles

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93 after heat or bleach treatment (3,7). In order to investigate whether bacteria stabilize
94 other members of the *Picornaviridae* family from these inactivating conditions, we
95 selected viruses from separate genera and viruses with differences in capsid sequence
96 similarity (**Fig. 1 A, B and Table 1**). These viruses differ in their capsid structure
97 sequence and topology, which may confer different interactions with bacteria (**Fig. 1A**).
98 The panel is composed of one virus from the *Kobuvirus* genus (Aichi virus), one virus
99 from the *Cardiovirus* genus (Mengo virus) and three viruses from the Enterovirus genus
100 (PV, CVB3-H3 and CVB3-Nancy). CVB3-Nancy and CVB3-H3 have 98.4% capsid
101 sequence similarity at the amino acid level and were compared to determine whether
102 there are strain-specific differences in bacteria-mediated stabilization (**Fig. 1B and**
103 **Table 1**). Additionally, our panel included a PV mutant with a single amino acid change
104 in the VP1 capsid coding region (PV-M132V), that confers enhanced thermal stability in
105 the absence of bacteria (13).

106 We also selected a representative panel of enteric bacteria, and bacterial and
107 non-bacterial molecules (**Table 2**). We included LPS, which is glycan found on the
108 surface of Gram negative bacteria. Additionally, we examined two representative Gram
109 negative bacterial strains (*Escherichia coli* 1470 and *Prevotella ruminicola)*, and two
110 representative Gram positive bacterial strains (*Bacillus badius* and *Lactobacillus*
111 *johnsonii).* We previously showed that *E. coli* 1470, *P. ruminicola*, *B. badius* and *L.*
112 *johnsonii* bind to PV (14), but whether these strains stabilize PV and other
113 picornaviruses was unknown. We also previously showed that non-bacterial
114 compounds, such as bovine albumin serum (BSA) and cellulose, had minimal effects on
115 PV stability and were included in this study as controls (**Table 2**) (3,7).

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116

117 **Specific bacteria enhance stability of a subset of picornaviruses during heat**
118 **treatment.** To determine whether bacteria enhance stability of picornaviruses, we first
119 examined viral inactivation at elevated temperatures. Picornavirus particles can be
120 inactivated by undergoing premature genome release at a range of temperatures, with
121 faster inactivation at higher temperatures (7,15,16). To increase tractability of our
122 assays, we used relatively high temperatures for our thermal inactivation experiments
123 because inactivation occurs relatively quickly. We first tested viral stability at 44°C for
124 4.5 h, a condition that we determined inactivates approximately 99% of PV infectivity
125 during incubation in PBS (**Fig. 2A**). Viruses were mixed with PBS, compounds (BSA,
126 Cellulose or LPS), or bacteria, incubated at 44°C for 4.5 h, and plaque assays were
127 performed to quantify the amount of viable virus remaining. When we incubated PV with
128 any of the bacterial strains or LPS, we observed >50-fold increase in viral stability
129 compared to PBS (**Fig. 2A**). A similar stabilization was observed for CVB3-H3 and
130 CVB3-Nancy when compared to PBS. Non-bacterial compounds (BSA and Cellulose)
131 did not stabilize PV or either CVB3 strain. Interestingly, Aichi virus and Mengo virus
132 were very stable in PBS under these conditions. We also tested a recently identified
133 heat-resistant PV mutant, PV-M132V (13). Like Aichi virus and Mengo virus, PV-M132V
134 was resistant to heat treatment, and thus incubation with any of the compounds or
135 bacterial strains did not increase stability (**Fig. 2A**).

136 We next wanted to determine whether bacteria could increase stability of the
137 heat-stable viruses at temperatures where they become heat labile. First, we increased
138 the temperature in the thermal stability assay to 46°C, and found that similar to the 44°C

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139 assay, PV, CVB3-H3, and CVB3-Nancy were stabilized by bacteria (**Fig. 2B**). Aichi
140 virus, Mengo virus, and PV-M132V were still stable in the 46°C assay and incubation
141 with any of the bacteria compounds or strains did not increase stability. To determine
142 the temperature necessary to inactivate Aichi virus and Mengo virus, we tested viability
143 at temperatures from 46-58°C for 4.5 h. These additional experiments at different
144 temperatures revealed that Aichi virus and Mengo virus were ~99% inactivated when
145 incubated at 50°C or 57°C for 4.5 h, respectively (**Fig. 2C-2F**). Despite viral inactivation
146 during these conditions, none of the bacterial strains or bacterial polysaccharides could
147 stabilize either of these viruses (**Fig. 2D and 2F**). Intriguingly, BSA stabilized Aichi virus
148 during incubation at 50°C, indicating this virus may have different requirements for
149 stabilization during heat treatment (**Fig. 2D**). Overall, these data indicate that bacteria
150 do not stabilize Aichi and Mengo virus during incubation at high temperatures, but that
151 bacterial stabilization of these viruses may be less important given their inherent high
152 stability.

153

154 **The effect of feces on picornaviruses.** We next wanted to determine whether the
155 viruses in our panel are stabilized in feces. As enteric viruses are transmitted by the
156 fecal-oral route, the potential effects of fecal components on their stability and infection
157 is highly relevant. We previously showed that PV is stabilized in feces from conventional
158 mice (7). Here, we compared viral stability when viruses were incubated in PBS or feces
159 from conventional mice over the course of several days at 37°C followed by
160 quantification of remaining viable virus by plaque assay. We found that Aichi virus was
161 not stabilized by feces compared to PBS at 37°C after Day 1 (**Fig. 3A**). Feces only

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162 moderately stabilized CVB3-H3 at early timepoints (**Fig. 3B**). However, CVB3-Nancy
163 and PV were stabilized by feces at later timepoints (see Day 8) (**Fig. 3C and 3E**). We
164 also demonstrated that PV-M132V was stable during 8 days of incubation at 37°C in
165 both PBS and feces (13). Interestingly, Mengo virus exhibited significant inactivation
166 after 4 days at 37°C, but incubation in feces limited this inactivation (**Fig. 3D**). This
167 result was surprising given the stability of Mengo virus at 46°C for 4.5 h and the lack of
168 bacterial stabilization of Mengo virus at 57°C (**Fig. 2B and 2F**). However, Mengo virus
169 may have enhanced thermal sensitivity over longer time courses and bacterial effects
170 may be apparent only under these conditions and/or non-bacterial components of feces
171 could affect Mengo virus. When we incubated Mengo virus with mixtures of *E. coli, P.*
172 *ruminicola*, and *L. johnsonii* at 37°C for several days, Mengo virus was stabilized
173 compared to PBS at 37°C (see dashed line compared to dotted line) (**Fig. 3F**). These
174 findings indicate that bacteria stabilize Mengo virus during longer exposures to body
175 temperature (37°C) (**Fig. 3F**). Overall, these data indicate that several picornaviruses,
176 but not all, are stabilized in feces, which could facilitate transmission.
177
178 **Bacteria enhance stability of picornaviruses during bleach exposure.** In addition to
179 heat, virions can be inactivated by chlorine bleach via capsid penetration and damage
180 and/or genome release (17-21). To determine whether bacteria affect bleach
181 inactivation of viruses, we pre-incubated viruses in PBS, compounds, or bacterial
182 strains for 1 h followed by exposure to dilute bleach (0.0001%) for 1 min, neutralization,
183 and plaque assay to determine the amount of viable virus present. We determined that
184 when pre-incubated in PBS, all viruses lost ~90% of their infectivity (**Fig. 4**). However,

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185 when pre-incubated with LPS or bacterial strains, all viruses were stabilized by at least
186 some of the treatments (**Fig. 4**). Importantly, pre-incubation of the viruses with BSA or
187 cellulose did not prevent viral inactivation by bleach treatment, indicating that the effects
188 were specific to bacteria and LPS and not just due the presence of additional molecules
189 (**Fig. 4**). Interestingly, the heat stable PV-M132V mutant virus was inactivated by bleach
190 to the same extent at PV-WT, and bacteria limited bleach inactivation of PV-M132V.
191 These results suggest that thermal inactivation and bleach inactivation occur through
192 separable mechanisms, and that bacteria stabilize virions for both. Overall, these results
193 indicate that bacteria enhance viral stability of fecal-orally transmitted picornaviruses
194 during bleach treatment.

195

196 **Bacteria bind to a select panel of picornaviruses.** Since bacteria enhanced stability
197 of specific picornaviruses during heat or bleach inactivation, we wanted to determine
198 whether viruses directly interact with bacteria. In particular, we were curious whether
199 bacterial binding efficiencies vary among closely related viruses, such as CVB3-Nancy
200 and CVB3-H3, or between the PV-M132V heat stable mutant and PV-WT. Previously,
201 we showed that PV can bind directly to the surface of bacteria (3,7,14). ^{35}S-labeled
202 CVB3-Nancy, CVB3-H3, PV-WT, or PV-M132V were incubated with beads, *B. badius*,
203 or *E. coli* for 1 h followed by centrifugation, washing, and scintillation counting the
204 bacterial pellets to quantify viral binding. We determined that PV-WT and PV-M132V
205 bound to both bacterial strains to approximately the same extent (**Fig. 5A**). This
206 indicates that while the PV-M132V mutant does not require the presence of bacteria for
207 stability during heat treatment, it still binds to bacteria, which could explain why bacteria

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208 limit bleach inactivation of PV-M132V (**Fig. 4**). Additionally, we determined that both
209 CVB3 strains bind to the two bacterial strains tested (**Fig. 5B**). Interestingly, binding of
210 CVB3-Nancy to *E. coli* was nearly 3-fold higher than CVB3-H3 (**Fig. 5B**). Overall, these
211 results indicate that multiple picornaviruses bind to bacteria, but with different
212 efficiencies.
213

214 **DISCUSSION**

215 The *Picornaviridae* family is diverse and includes a large number of medically
216 relevant human pathogens. While it has been shown that bacteria promote infection, co-
217 infection, and transmission of poliovirus, the impact of bacteria on other picornaviruses
218 is unclear (3,7,14). Here, we show that bacteria increase stability of several viruses from
219 the *Picornaviridae* family, likely through direct interactions.

220 Our data show that bacteria-mediated thermal stability can vary among a family
221 of viruses. We determined that certain picornaviruses (*Enterovirus* genus members:
222 CVB3-H3, CVB3-Nancy and PV) are sensitive to heat treatment and that bacteria
223 increase stability of these viruses (**Fig. 2 and Fig. 3**). We also determined that another
224 picornavirus (*Cardiovirus* genus: Mengo virus) has mixed phenotypes depending on the
225 condition tested. While Mengo virus was very stable at high temperatures during
226 relatively short incubation times (4.5 h) and was not impacted by bacteria under these
227 conditions, it was inactivated after 4 days at 37°C and exposure to feces reduced this
228 inactivation (**Fig. 3D**). This suggests that Mengo virus may be stabilized by bacteria at
229 physiological temperatures in the host. Finally, we determined that a distantly related
230 picornavirus (*Kobuvirus* genus: Aichi virus) is relatively resistant to high temperature,

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231 but is not stabilized by bacteria or bacterial products (**Fig. 2 and Fig. 3A**). In fact,
232 exposure to feces slightly reduced Aichi virus infectivity (**Fig. 3A**). Although Aichi virus is
233 transmitted by the fecal-oral route, there are large sequence and structural differences
234 between Aichi and other picornaviruses that may contribute to the different phenotype
235 (**Fig. 1 A and B**) (22,23). Although a member of the *Caliciviridae*, human norovirus can
236 bind to and is stabilized by bacteria that express certain histo-blood group antigens
237 (24,25). Similarly, reovirus (*Reoviridae* family) can be stabilized by exposure to certain
238 bacteria or bacterial surface molecules, but stabilization efficiency and specificity varies
239 among different reovirus strains (8). Taken together, these results indicate that viruses
240 from separate viral families can be stabilized by bacteria, but that not all viruses within a
241 given family share phenotypes.

242 While picornaviruses vary in bacteria-mediated thermal stabilization, we found
243 that bacteria enhanced viability of all picornaviruses tested during bleach treatment
244 (**Fig. 4**). Although the PV-M132V mutant was not inactivated at high temperatures, it
245 was inactivated by bleach treatment and bacteria limited this inactivation. Indeed, the
246 PV-M132V virus was determined to bind to bacteria, which could explain stabilization
247 during bleach treatment (**Fig. 4**). Thus, heat inactivation and bleach inactivation are
248 independent and could have separate requirements for stabilization.

249 Overall, this study provides insight into the effects of bacteria on a panel of
250 viruses from the same family, the *Picornaviridae*. Understanding the role of bacteria
251 during stabilization and infection of viruses could provide insight into efficient infection
252 within specific hosts (i.e. harboring specific microbiota) as well as between hosts (i.e.
253 environmental bacteria).

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254

255 **MATERIALS AND METHODS**

256 **Cells and viruses.** HeLa cells were propagated in Dulbecco's modified Eagle's medium
257 (DMEM) supplemented with 10 % calf serum and 1 % penicillin/streptomycin. HeLa
258 cells were used for CVB3, Mengo virus, and PV propagation and quantification of viral
259 titer by plaque assay (26-28). Vero cells were propagated in DMEM supplemented with
260 10% fetal bovine serum (FBS) and 1 % penicillin/streptomycin. Vero cells were used for
261 Aichi virus propagation and quantification of viral titer by plaque assay. All infections
262 were performed using viruses derived from infectious cDNA clones (the Mengo virus
263 clone was a kind gift from Marco Vignuzzi) (29,30). All viruses were confirmed by
264 Sanger sequencing.

265 To quantify virus, plaque assay was performed as previously described
266 (26,27,30) Briefly, virus was diluted in phosphate-buffered saline supplemented with
267 100 µg/mL $CaCl_2$ and 100 µg/mL $MgCl_2$ (PBS+) and added to cells for 30 min at 37°C in
268 presence of 5 % CO_2 to allow for attachment. Agar overlay containing DMEM,
269 supplemented with 20 % calf serum, and 2 % agar was used for CVB3 and PV samples
270 and removed after 48 h. Agar overlay containing DMEM, supplemented with 20 % FBS,
271 and 2 % agar was used for Aichi virus samples and removed after 48 h. Agar overlay
272 containing P5 buffer and 2 % agar was used for Mengo virus samples and removed
273 after 48 h (27).

274 Radiolabeling of picornaviruses was performed as previously described (3,7,28) .
275 Briefly, viruses were propagated in the presence of ^{35}S-Cysteine/Methionine and were
276 purified using cesium chloride gradients. Purity of viruses were confirmed by

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277 phosphorimaging of radiolabeled capsid proteins on SDS-PAGE, and scintillation count
278 to determine CPM and viable fractions (3).
279
280 **Bacterial strains.** Strains of bacteria were from ATCC or from the cecum of mice, as
281 previously described (14). Cultures were inoculated from glycerol stocks in strain-
282 specific nutrient media as previously described (14). Briefly, cultures were grown
283 overnight, bacterial cell pellets were collected and washed in PBS+. After resuspension
284 in 1 mL PBS+, OD_{600} values were obtained by spectrophotometer (Eppendorf
285 BioPhotometer D30) to determine colony forming units (CFUs) needed specific for each
286 assay. Bacteria were UV inactivated prior to use in assays. The amount of bacteria was
287 confirmed by plating on nutrient-specific agar and conditions prior to UV inactivation
288 (14).
289
290 **Quantifying picornavirus binding to bacterial cells.** Bacterial binding assay was
291 performed as previously described for poliovirus (14). Briefly, approximately 3,000 CPM
292 (approximately 1 x 10^6 PFU) of ^{35}S-radiolabeled virus was mixed with PBS+ or 1 x 10^8
293 CFU of bacteria and incubated at 37°C in presence of CO_2 for 1 h. After incubation,
294 bacteria was pelleted and washed with PBS+ to remove unbound virus. The amount of
295 CPM (virus bound to bacterial cells) was determined by scintillation counting.
296
297 **Quantifying effects of bacteria on virion stability.** To determine the effect of bacteria
298 on thermal stability of picornaviruses, 1 x 10^5 PFU of each virus was mixed with PBS+,
299 1 mg/mL of bacterial surface polysaccharides, or 1 x 10^{10} CFU of bacteria and

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300 incubated at 44°C for 4.5 h. The same procedure was followed for elevated temperature
301 assays. After incubation, plaque assays were performed using virus-specific conditions
302 to determine the amount of viable virus before and after heat treatment.

303 Bleach inactivation assay was performed as previously described for PV, except
304 that a lower concentration of bleach was used here (7). Briefly, 1 x 10^5 PFU of each
305 virus was mixed with PBS+, 1 mg/mL of bacterial surface polysaccharides, or 1 x 10^8
306 CFU of bacteria. Samples were incubated at 37°C for 1 h and then added to 0.0001 %
307 fresh bleach for 1 min. Bleach neutralization was done by adding 0.01 % sodium
308 thiosulfate (Sigma). Plaque assays using virus-specific conditions were performed to
309 determine amount of viable virus before and after bleach treatment.

310 To examine effects of feces on viral stability, feces from four- to ten-week-old
311 male C57BL/6 *PVR-IFNAR -/-* mice were collected and resuspended in PBS+ to a final
312 concentration of 0.0642 mg/μL. Briefly, 1 x 10^5 PFU of virus was mixed with 300 μL of
313 PBS+ or resuspended fecal samples and incubated at 37°C in the presence of 5 %
314 CO_2. Additional samples in PBS+ were placed at 4°C as a control. Samples were taken
315 at designated time points and processed by chloroform extraction as previously
316 described (3,7). Plaque assay was performed to determine amount of viable virus
317 before and at designated time points, as described earlier. In Figure 3F, 1 x 10^5 PFU of
318 Mengo virus was mixed with approximately 1 x 10^5 CFU each of *E. coli, P. ruminicola*,
319 and *L. johnsonii* in a total volume of 300 μL and samples were incubated at 37°C.
320 Samples were collected and titers were determined as described above.
321

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322 **Mouse Experiments.** Animals were handled according to the Guide for the Care and
323 Use of Laboratory Animals. C57BL/6 *PVR-IFNAR -/-* mice were obtained from S. Koike
324 (Tokyo, Japan) (31). Feces collection was performed at UT Southwestern Medical
325 Center.
326
327 **Data Analysis.** Figures of viral structures were generated using the UCSF Chimera
328 software (http://www.rbvi.ucsf.edu/chimera). The Electron Microscopy Data Bank
329 (EMDB) IDs used for each virus are as follows: Aichi virus (EM-9517), CVB3 (EM-6637)
330 and Saffold virus (EM-3097) to represent their respective genera. The phylogenetic tree
331 was generated using the MEGA7 software and following the Neighbor-Joining Method
332 (32). The optimal tree with the sum of branch length = 2.65090397 is shown. The tree is
333 drawn to scale, with branch lengths in the same units as those of the evolutionary
334 distances used to infer the phylogenetic tree. The evolutionary distances were
335 computed using the Poisson correction method and are in the units of the number of
336 amino acid substitutions per site. (32). The analysis involved 5 amino acid sequences.
337 All positions containing gaps and missing data were eliminated. There were a total of
338 766 positions in the final phylogeny tree dataset.
339 All statistical analyses were performed using the GraphPad Prism Software.
340 Outliers were identified and removed by the ROUT method, Q = 1%. All one way
341 ANOVA tests were performed with Dunnett's multiple comparisons post hoc test. All
342 two-way ANOVA tests were performed with Tukey's post hoc test.
343
344 **ACKNOWLEDGEMENTS**

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345 We thank Andrea Erickson, Broc McCune and Arielle Woznica for critical review of the
346 manuscript. We thank Marco Vignuzzi for the Mengo virus infectious clone. We also
347 thank Mikal Woods-Acevedo for providing ^{35}S-labeled CVB3-H3 and CVB3-Nancy
348 viruses. We also thank Nam Nguyen for assistance with structural modeling. Molecular
349 graphics and analyses were performed with the UCSF Chimera package. Chimera is
350 developed by the Resource for Biocomputing, Visualization, and Informatics at the
351 University of California, San Francisco (supported by NIGMS P41-GM103311).
352
353 **FUNDING INFORMATION**
354 Work in J.K.P.'s lab is funded through NIH NIAID grant R01 AI74668, a Burroughs
355 Wellcome Fund Investigators in the Pathogenesis of Infectious Diseases Award, and a
356 Faculty Scholar grant from the Howard Hughes Medical Institute. E.R.A. was supported
357 in part by the National Science Foundation Graduate Research Fellowship grant
358 2014176649.
359

360 **FIGURE LEGENDS**

361 **Figure 1. Panel of picornaviruses used in this study.**

362 **A**) Structural models of picornaviruses. Structural comparisons were performed using
363 EMDB ascension numbers for each viral genus and topological distances from the
364 center of the virion calculated from 135 Å (blue) to 155 Å (red), as indicated by the scale
365 bar (22). Representative viruses for each genus are Aichi virus (*Kobuvirus*), CVB3
366 (*Enterovirus*), and Saffold virus (*Cardiovirus*). The Aichi virus structure is at 3.7 Å
367 resolution, CVB3 structure is at 3.9 Å resolution and Saffold virus is at 10.6 Å resolution.

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368 Models and distances were generated with UCSF Chimera software. **B**) Phylogenetic
369 tree of picornaviruses based on the amino acid sequence of the capsid-coding region.
370 The tree was generated using MEGA7 software. The evolutionary history was inferred
371 using the Neighbor-Joining method. The scale bar represents the number of
372 substitutions per site.
373

374 **Figure 2. Effects of bacteria and compounds on picornavirus stability at elevated**
375 **temperatures.** Thermal stability assays were performed by incubating 1 x 10^5 PFU
376 viruses in PBS, 1 mg/mL BSA, cellulose, LPS, or 1 x 10^{10} CFU of bacterial strains at
377 various temperatures for 4.5 h. The amount of viable virus following each assay was
378 determined by plaque assay and compared to PBS viral titer at 0 h to determine percent
379 of input PFU. **A**) 44°C assay. Data are representative of ten to eighteen independent
380 experiments, n= 4-47. **B**) 46°C assay. Data are representative of nine to fourteen
381 independent experiments, n= 4-25. **C**) Incubation of Aichi virus in PBS at various
382 temperatures. Data are representative of two to three independent experiments, n=3-5.
383 **D**) Incubation of Mengo virus in PBS at various temperatures. Data are representative
384 of one to three independent experiments, n=2-6. **E**) Aichi virus 50°C assay. Data are
385 representative of two independent experiments, n=4. **F**) Mengo virus 57°C assay. Data
386 are representative of two experiments, n=4. Bars are shown in SEM. Statistical
387 significance was determined by one-way ANOVA, * = $P<0.05$. n.s., not significant.
388

389 **Figure 3. Picornavirus stability in feces.** 1 x 10^5 PFU of A) Aichi virus, B) CVB3-H3,
390 C) CVB3-Nancy, D) Mengo virus, or E) PV was incubated with PBS or a slurry of feces

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391 from mice and incubated at 37°C (PBS, dashed lines; Feces, dotted lines) or 4°C (PBS,
392 solid lines). Data are representative of two to three experiments, n = 4-5. In F) 1 x 10^5
393 PFU of Mengo virus was incubated with PBS or a mixture of *E. coli, P. ruminicola*, and
394 *L. johnsonii* and incubated at 37°C (PBS, dashed lines; Bacteria, dotted lines) or 4°C
395 (PBS, solid lines). Data are representative of two independent experiments, n = 4.
396 Samples were taken at designated time points and processed prior to plaque assay for
397 quantification of viable virus. Bars are shown in SEM. Statistical significance between
398 PBS and feces or bacteria at 37°C was determined by two-way ANOVA, * = P<0.05.
399

400 **Figure 4. Effects of bacteria on picornavirus stability during bleach treatment.** 1 x
401 10^5 PFU viruses were incubated individually in PBS, 1 mg/mL BSA, cellulose, LPS, or 1
402 x 10^8 CFU of bacterial strains at 37°C for 1 h. After incubation, samples were treated
403 with 0.0001% bleach for 1 minute and neutralized with sodium thiolsulfate. Amount of
404 viable virus was determined by plaque assay and compared to PBS viral titer at 0 h to
405 determine % of input PFU. Data are representative of seven to twenty independent
406 experiments, n = 4-40. Bars are shown in SEM. Statistical significance was determined
407 by one-way ANOVA, * = P<0.05. n.s., not significant.

408 **Figure 5. Picornaviruses bind to bacteria.** ^{35}S-labeled viruses (3,000
409 CPM/approximately 1 x 10^6 PFU) were incubated with 1 x 10^8 CFU of bacteria for 1 h at
410 37°C. After incubation, samples were spun down and washed to remove unbound virus.
411 Bound virus was quantified by scintillation counting. Data are representative of two
412 independent experiments, n=3-4. Bars are shown in SEM. * = P<0.05, based on
413 student's T test.

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422 **Table 1. Percent sequence identity between panel of picornaviruses**

		Capsid Amino Acid Sequence Similarity (%)				
Family and genus	**Virus**	Aichi virus	Mengo virus	CVB3 H3	CVB3 Nancy	Poliovirus
Picornaviridae						
Kobuvirus	Aichi virus	100.0	28.6	23.3	23.3	24.3
Cardiovirus	Mengo virus		100.0	30.0	29.8	29.0
Enterovirus	CVB3 H3			100.0	98.6	54.4
	CVB3 Nancy				100.0	54.2
	Poliovirus					100.0

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423

424 **Table 2. List of reagents and bacterial strains used in this study.**

Name	Gram Status	Phylum	Source
Bovine Albumin Serum (BSA)			Fischer Scientific
Cellulose			Sigma
Lipopolysaccharide			Sigma (0127:B8)
Escherichia coli 1470	Gram negative	Proteobacteria	Mouse cecum
Prevotella runimicola	Gram negative	Bacteroidetes	ATCC 19189
Bacillus badius	Gram positive	Firmicutes	Mouse cecum
Lactobacillus johnsonii	Gram positive	Firmicutes	Mouse cecum

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Figure 1

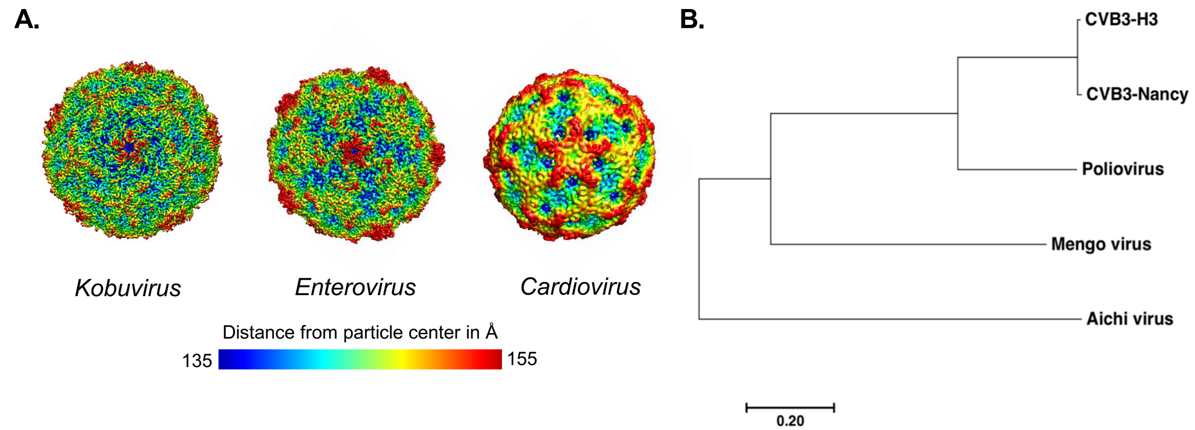

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Figure 2

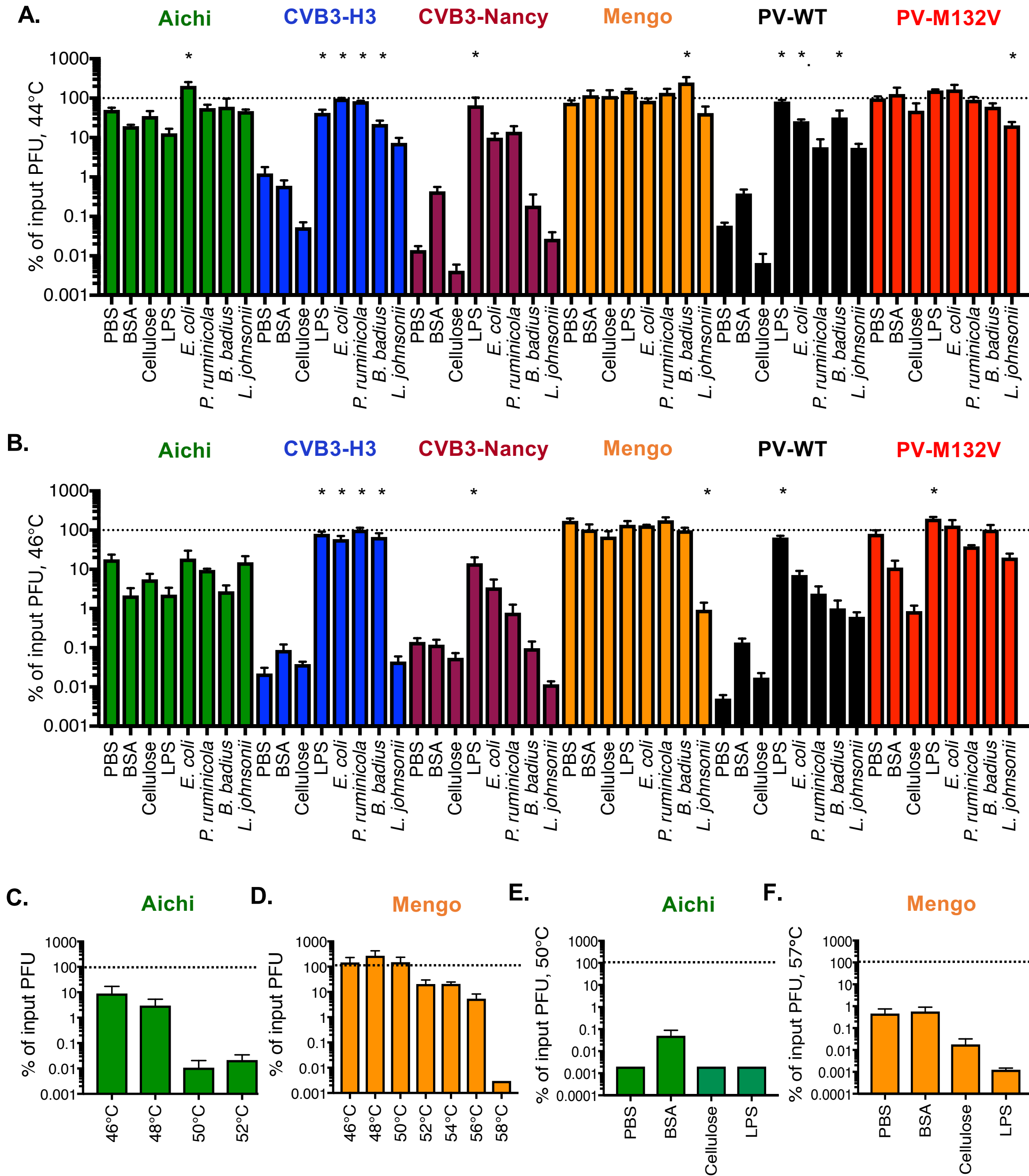

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Figure 3

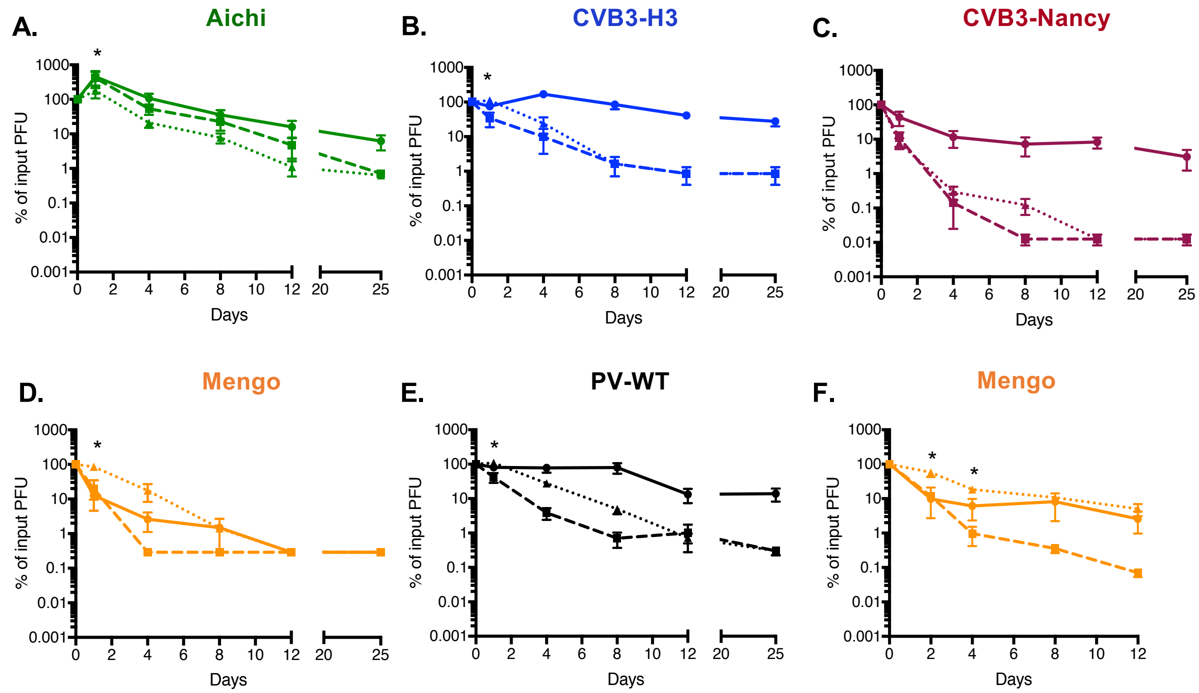

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Figure 4

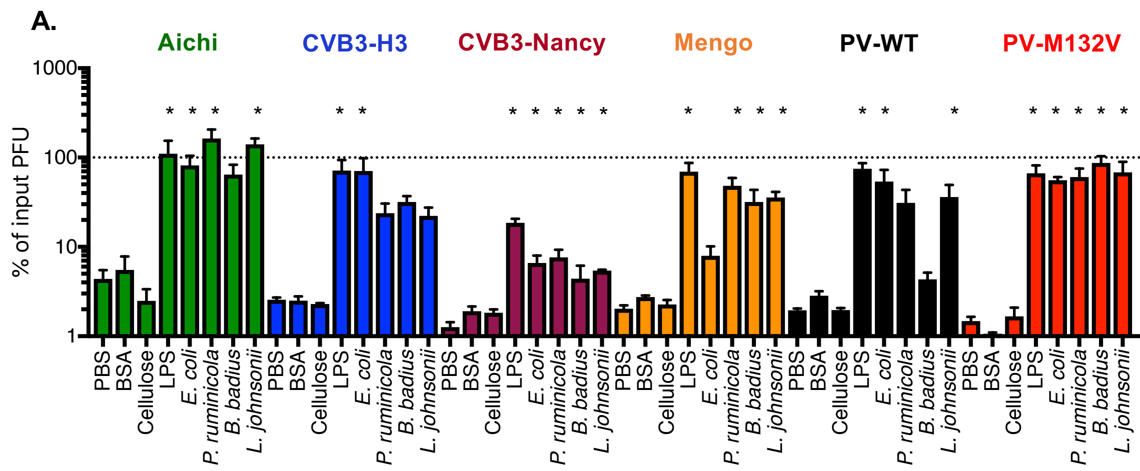

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Figure 5