

1 Two Lytic Bacteriophages That Depend on the *Escherichia coli* Multi-Drug Efflux Gene *tolC*  
2 and Differentially Affect Bacterial Growth and Selection

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12 Running Head: Phage Dependence on *tolC*

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15

## 16 **Abstract**

17 Bacterial pathogens are increasingly evolving drug resistance under natural selection from  
18 antibiotics in medicine, agriculture, and nature. Meanwhile, bacteria ubiquitously encounter  
19 bacteriophages and can rapidly evolve phage resistance. However, the role of phages in  
20 interacting with drug-resistant and drug-sensitive bacteria remains unclear. To gain insight into  
21 such relationships, we screened for and characterized phages that rely on the multi-drug efflux  
22 pump gene *tolC*. First, we screened a collection of 33 environmental and commercial  
23 *Escherichia coli* phages for their ability to infect cells that lacked *tolC*. Our screen revealed two  
24 phages that had reduced efficiency of plating (EOP) on the *tolC* knockout compared to wild type.  
25 We further characterized these phages with bacterial growth curves, transmission electron  
26 microscopy, and analysis of phage-resistant mutants. Phage U136B is a curly-tailed virus in  
27 family *Siphoviridae* with no ability to infect a *tolC* knockout, suggesting TolC is the U136B  
28 receptor. Phage 132 is a contractile-tailed virus in family *Myoviridae* with reduced EOP on cells  
29 lacking *ompF* and its positive regulators *tolC* and *ompR*. U136B and 132 differentially effect  
30 bacterial growth and lysis, and U136B-resistant mutants contain mutations of the *tolC* gene.  
31 Together, these results show that the *tolC* gene involved in drug resistance can modify bacteria-  
32 phage interactions in multiple ways, altering bacterial lysis and selection. These new phages  
33 offer utility for studying evolution, tradeoffs, and infection mechanisms.

34

## 35 **Importance**

36 Bacteria face strong selection by antibiotics in medicine and agriculture, resulting in increasing  
37 levels of drug resistance among bacterial pathogens. Slowing this process will require an  
38 understanding of the environmental contexts in which drug resistance evolutionarily increases or

39 decreases. In this study, we investigate two newly-isolated bacteriophages that rely on a bacterial  
40 antibiotic resistance gene. These bacteriophages vary in their interactions with drug-resistant  
41 bacteria, with one of the phages selecting for phage-resistant mutants that have mutations in the  
42 antibiotic resistance gene. Further study of these new phages will be useful to understanding  
43 evolutionary tradeoffs and how phages might be applied in natural settings to reverse the  
44 problem of drug resistance.

45

46

## 47 **Introduction**

48 Widespread use of antibiotics in medicine and agriculture has selected for the evolution of multi-  
49 drug resistant (MDR) bacterial pathogens (1). Meanwhile, bacteria frequently encounter phages,  
50 which are prevalent in the human microbiota, in hospital and farm settings, and in natural  
51 environments (2), and which exert selection pressure for bacteria to resist phage exploitation (3-  
52 7). However, the interaction between selection from antibiotics and phages, along with its role in  
53 driving bacterial evolution, remain unclear, in part because these interactions depend on both the  
54 environment and specific phage species.

55

56 Potential evolutionary interactions between drug resistance and phage resistance mechanisms in  
57 bacteria have been previously identified, including both positive and negative interactions that  
58 are highly genotype-dependent (8, 9). For example, *Pseudomonas aeruginosa* bacteria that  
59 evolve resistance to phage 14/1 simultaneously become *more* resistant to antibiotics (10),  
60 whereas *P. aeruginosa* that evolve resistance to phage OMKO1 become *less* resistant to  
61 antibiotics (5). In *Escherichia coli*, bacteria that evolve resistance to phage TLS also lose  
62 antibiotic resistance (11). Such interactions demonstrate that multiple selection pressures  
63 sometimes cause bacteria to evolve mutations with *trade-up* potential, whereby phages  
64 contribute to the problems of increased antibiotic resistance and virulence; in other cases, the  
65 mutations have *trade-off* potential, whereby phages reduce the problem of antibiotic resistance.

66

67 Bacteria-phage interactions can be highly dependent on cell membrane proteins. Such proteins  
68 are often exploited by phages for cell attachment and entry. In particular, multi-drug efflux  
69 pumps are protein complexes spanning the inner and outer membranes of some bacteria, such as

70 the homologous TolC-AcrAB system in *Escherichia coli* and OprM-MexAB system in *P.*  
71 *aeruginosa* (12). These efflux systems confer resistance to multiple antibiotics, acting as  
72 generalized transporters for multiple antibiotic classes as well as detergents, dyes, and bile acids  
73 (13). The outer membrane protein (OMP) components (TolC or OprM) are membrane-spanning  
74 beta barrels, with peptide loops that extend outside of the cell. The extracellular loops of OMPs  
75 are frequently exploited by phages as the specific binding sites for initiating phage infection (11,  
76 14-16). When phages use these OMPs as receptors, bacteria face selection for reduced or  
77 modified OMPs, catalyzing ecological restructuring or coevolutionary arms races that in turn  
78 alter selection on the phages (6, 15, 17, 18). Additionally, loss or modification of OMP genes has  
79 been shown to alter expression of other OMP genes. For example, *tolC* mutants have reduced  
80 expression of outer membrane proteins OmpF, NmpC, and protein 2 (19). Therefore, loss of an  
81 OMP gene might impact a phage either directly – by loss of the phage receptor – or indirectly,  
82 through changes to the expression of the phage receptor.

83  
84 In this study, we sought out phages that rely on the antibiotic resistance gene *tolC*, which  
85 encodes the outer membrane protein of the TolC-AcrAB efflux pump. Such phages, like the  
86 previously-characterized TolC-targeting phage TLS (11), might impose selection on bacterial  
87 communities to evolve phage resistance while losing antibiotic resistance. Such phages will be  
88 useful to the laboratory study of evolutionary tradeoffs, or more practically, to restore drug  
89 sensitivity in clinical settings. To search for such phages, we conducted a screen of our *E. coli*  
90 phage library on bacteria that lacked the *tolC* gene. Out of 33 phages, we found two with reduced  
91 plaquing efficiency on the *tolC* knockout. We found that these phages differentially affect both  
92 bacterial population dynamics and the potential for evolution of antibiotic resistance.

93

## 94 **Results**

95 **Phage interactions with *tolC*<sup>-</sup> bacteria.** We conducted a screen of newly-collected phages with  
96 unknown receptors by plating for plaques on both wild-type *E. coli* and its isogenic *tolC*  
97 knockout from the Keio collection (20) (Table 1). Of 33 phages, we found two (phage U136B  
98 and phage 132) with dramatically reduced efficiency of plating (EOP, the number of plaques  
99 formed on a mutant strain of bacteria relative to a wild type) (Fig. S1, Table 1). Phage U136B,  
100 isolated from a swine farm in Connecticut, appears to obligately require bacterial *tolC* for  
101 infection, with EOP below our detection limit of  $10^{-10}$  (data not shown); we have never observed  
102 a plaque of U136B in the absence of the *tolC* gene. Phage 132, also isolated from a swine farm in  
103 Connecticut, has dramatically reduced plating efficiency on *tolC*<sup>-</sup> (EOP =  $9.5 \times 10^{-6}$ ) when plated  
104 with the standard Luria Bertani (LB) top agar formula (7.5 g/L agar). The phage 132 EOP on  
105 *tolC*<sup>-</sup> bacteria increases – but does not fully recover – when the top agar contains only 3.8 g/L  
106 agar, which we discuss in the Results section.

107

108 **Phage interactions with other drug efflux pump genes.** Finding that U136B and 132 rely fully  
109 or partially on presence of the *tolC* gene, we speculated that they might also require *acrA* and  
110 *acrB*, which encode the other components of the TolC multi-drug efflux pump. We performed  
111 spot tests for plaquing on the *acrA* and *acrB* knockouts of the Keio collection and found no  
112 change in plaquing ability (data not shown).

113

114 **Phage morphology.** We used transmission electron microscopy to determine the general  
115 structure and morphological families of U136B and 132. Phage U136B has a curly, non-

116 contractile tail of the *Siphoviridae* family type. It has a capsid width of 59 nm, capsid length of  
117 61 nm, and tail length of 115 nm (Fig. 1A). Phage 132 has a contractile tail of the *Myoviridae*  
118 family type. It has a capsid width of 72 nm, capsid length of 99 nm, extended tail length of 111  
119 nm and contracted tail length of 58 nm (Fig. 1B). Consistent with non-enveloped particle  
120 morphology, we also found that both phages were insensitive to chloroform treatment (Fig. S2).  
121 While both phages did have tails, they also appeared to be structurally robust with minimal loss  
122 due to mechanical agitation *via* vortexing (Fig. S2).

123

124 **Phage impact on bacterial growth.** The *tolC*<sup>-</sup> efficiency of plating data suggested that these  
125 phages should also differentially affect the survival of wild-type and *tolC*<sup>-</sup> bacteria in liquid  
126 cultures. To test this, we generated growth curves of wild-type and *tolC* knockout bacteria with  
127 and without addition of each phage. Indeed, the wild-type bacteria were killed by both phages  
128 (Fig. 2, black dashed lines), while the *tolC* knockout was unaffected by phage U136B (Fig. 2A,  
129 gray lines). Phage 132, which plaques with reduced efficiency on the *tolC* knockout, was able to  
130 kill both the *tolC* knockout and the wild-type bacteria (Fig. 2B, dashed lines).

131

132 **Effect of multiplicity of infection on bacterial lysis.** The ability for both phage U136B and  
133 phage 132 to rapidly lyse wild-type *E. coli* increases with higher multiplicity of infection (MOI,  
134 the ratio of phage particles to bacterial cells). Cell lysis still is rapid and efficient for both  
135 phages, beginning within two hours even at a low MOI of 0.01 (Fig. 3A-B). Increasing MOI  
136 significantly decreases the bacterial density at the onset of observed lysis (Fig. 3C), decreases the  
137 time to onset of lysis (Fig. 3D), and decreases the time to complete lysis (Fig. 3E) ( $p < 0.01$  in all  
138 cases, Table S2 ‘MOI’). The phage type significantly affects the bacterial density at the onset of

139 lysis and the time to complete lysis ( $p < 0.01$  in both cases, Table S2 ‘Phage’) but not the time of  
140 lysis onset ( $p = 0.15$ ). At lower MOIs, phage 132 has longer times to complete lysis than phage  
141 U136B (Fig. 3E).

142

143 **Phage-resistant mutants.** Our EOP and bacterial lysis data suggested that phage resistance in  
144 *tolC*<sup>+</sup> bacterial populations might come about by deletion or modification of the bacterial *tolC*  
145 gene. To test whether bacteria can obtain phage resistance mutations in *tolC*, we conducted a  
146 selection experiment using phage and bacteria on the surface of agar plates (7). Ten independent  
147 bacterial cultures (each seeded from a unique colony of the wild-type bacteria, BW25113) were  
148 exposed on plates to phage U136B or 132, and surviving bacterial colonies were readily obtained  
149 (Table 2). We randomly picked one colony from each independent culture for further  
150 characterization. We also picked additional, non-random colonies that appeared to have unique  
151 morphologies, such as the mucoidy phenotype (commonly caused by excess exopolysaccharide  
152 production that limits phage infection) (21). We isolated each mutant using the double-isolation  
153 technique, then checked for resistance using the cross-streak method. In some cases, the isolated  
154 cultures were not resistant to the phage that they had been selected on (Table 3). In those cases,  
155 we non-randomly chose another colony from the same independent bacterial culture to complete  
156 a set of 10 independent, phage-resistant mutants selected on each phage. We used those sets of  
157 10 independent mutants for further characterization including cross-resistance estimates and  
158 sequencing.

159



160 **Cross-resistance to phages.** We found that selection by phage U136B yielded cross-resistance  
161 to phage 132 (10/10 cases, Table 3). Conversely, selection by phage 132 yielded partial cross-  
162 resistance to phage U136B (8/10 cases, Table 3).

163

164 **Mutant *tolC* sequences.** We found that three of our ten U136B-resistant mutants had mutations  
165 in *tolC* (Table 4). None of the phage 132-selected mutants had mutations in *tolC*.

166

167 **Analysis of phage growth on *omp* gene knockouts.** Given that phage 132 could grow on the  
168 *tolC* knockout in solid media (albeit, with low efficiency) and lyse bacteria in liquid culture  
169 (Figs. 2-3), we hypothesized that it might indirectly interact with *tolC*. Alternatively, it might  
170 directly interact with TolC as well as another OMP as an alternative receptor. To test this, we  
171 determined efficiency of plating for phage 132 on a variety of OMP gene knockouts. We  
172 specifically chose to screen nine knockouts of OMPs known to serve as other phage receptors  
173 (14). To increase the resolution of this assay, we used top layer with 50% the normal amount of  
174 agar, which we find yields more and larger plaques of phage 132. We found an overall effect of  
175 gene knockout on the EOP for phage 132 ( $F_{9,20} = 58.0$ ,  $p < 0.0001$ , Table S3, Fig. 4B). The test  
176 also qualitatively replicated the initial result that phage 132 has reduced EOP on the *tolC*  
177 knockout, noting that EOP is greater in this modified agar environment (see Methods).  
178 Strikingly, the EOP was even further reduced by a single gene knockout of *ompF*, and to a lesser  
179 extent, the *ompF* positive regulatory gene *ompR* (Fig. 4B), suggesting that phage 132's receptor  
180 is OmpF.

181

182 Observing that phage 132 was affected by more than one OMP gene knockout, we then tested  
183 U136's plaquing efficiency on the same set of genes. U136B was unaffected by knocking out  
184 any of the outer membrane protein genes except for *tolC* (Fig. 4A).

185

186 **Phage dependence on lipopolysaccharide synthesis genes.** To test whether phage U136B and  
187 132 may also rely on lipopolysaccharide (LPS) as primary receptors, we screened for efficiency  
188 of plating on knockouts of *rfa* genes, which are involved in LPS synthesis and modification. As  
189 expected, we found that knocking out single *rfa* genes greatly impacted efficiency of plating  
190 (Fig. 5). Neither phage formed any plaques on four of the knockouts (*rfaC*, *rfaD*, *rfaE*, *rfaP*).  
191 Phage 132 uniquely did not form plaques on three additional knockouts (*rfaF*, *rfaG*, and *rfgH*).  
192 For the eight remaining *rfa* knockouts for which EOP could be quantified for both phages (*rfaB*,  
193 *rfaI*, *rfaJ*, *rfaL*, *rfaQ*, *rfaS*, *rfaY*, *rfaZ*), the efficiency of plaquing was significantly effected by  
194 both the phage type ( $F_{1,44} = 21.1$ ,  $p < 0.0001$ ) and *rfa* knockout ( $F_{8,44} = 4.7$ ,  $p < 0.001$ ) (Fig. 5,  
195 Table S4). Together, these results suggest that phages U136B and 132 use LPS receptors, and  
196 that the two phages have different LPS specificity.

197

## 198 **Discussion**

199 The interactions between bacteriophages, antibiotic-resistant bacteria, and antibiotic-sensitive  
200 bacteria remain unclear. In part, this lack of ecological and evolutionary knowledge is because  
201 few phages have been tested for interactions with antibiotic resistance genes. To gain insight into  
202 such relationships, we screened a collection of 33 environmental and commercial *E. coli* phages  
203 for their inability to infect cells that lacked the antibiotic efflux pump gene *tolC*. The screen  
204 identified two environmental phages, U136B and 132, which were recently isolated from a

205 Connecticut swine farm (Table 1). In spatially-structured solid media on *tolC*<sup>+</sup> bacteria, phage  
206 U136B forms easily-visible, medium-sized plaques while phage 132 forms smaller, more  
207 difficult-to-enumerate plaques. When *tolC* is absent, phage U136B completely fails to form  
208 plaques, while phage 132 forms plaques at reduced efficiency (see Results, ‘*Phage interactions*  
209 *with tolC<sup>-</sup> bacteria*’). The two phages are also morphologically distinct from one another,  
210 belonging to different phage families (Fig. 1).

211  
212 Other than the previously-described phage TLS, to the best of our knowledge these are the only  
213 two other *E. coli* phages shown to rely fully or partially on the multidrug efflux gene *tolC* (11,  
214 14). U136B has similar morphology to TLS, a curly-tailed *Siphoviridae* (11). Among other  
215 bacterial species, we know of only one phage, OMKO1, that relies on a *tolC* homolog, infecting  
216 *oprM*<sup>+</sup> strains of *Pseudomonas aeruginosa* (5). Despite this apparent rarity of *tolC*-dependent  
217 phages, we readily found two out of a modest-sized phage collection of 33 phages. We expect  
218 that other environmental phages also rely on *tolC*, and that their absence from the literature  
219 reflects under-characterization, rather than low natural abundances. In particular, we expect that  
220 future screens of phages from sources where antibiotics are used will yield especially high rates  
221 of *tolC*-dependent phages.

222  
223 While both U136B and 132 both had at least partial reliance on *tolC*, they differently affect lysis  
224 and selection in bacterial populations. In liquid culture, U136B rapidly lyses *tolC*<sup>+</sup> bacteria and  
225 has no effect on *tolC*<sup>-</sup> bacteria (Fig. 2A). Phage 132 efficiently lyses both *tolC*<sup>+</sup> and *tolC*<sup>-</sup>  
226 bacteria in liquid culture (Fig. 2B). Increasing the multiplicity of infection increases the rate of  
227 bacterial lysis for both phages, although the rate of lysis is greater for U136B than phage 132

228 (Fig. 3). These patterns suggest that phages U136B and 132 might affect bacterial communities  
229 in different ways, either by changing their ecological structure (e.g., population sizes, growth  
230 rates, death rates, etc.) or by impacting selection on bacteria (e.g., differential survival of phage-  
231 sensitive and phage-resistant cells).

232

233 To test how each phage might impact selection of phage-resistance mutations, we characterized  
234 mutants resistant to each phage. We expected that the phages would select for mutations in the  
235 *tolC* gene, as observed previously for *tolC*-dependent phage TLS (11). We observed *tolC*  
236 mutations in 3/10 U136B-selected mutants (Table 4). This result is consistent with phage TLS  
237 selection experiments, which found that half of TLS<sup>R</sup> mutants contained *tolC* mutations (11). No  
238 *tolC* mutations were observed in phage 132-selected mutants, although we did observe cross  
239 resistance between the two phages (Table 3).

240

241 Together, the lysis and selection experiments suggest that U136B and 132 interact with the *tolC*  
242 gene *via* different mechanisms. Phage U136B's completely restricted growth on *tolC*<sup>-</sup> bacteria  
243 and *tolC* resistance mutations suggest that it uses TolC as its outer membrane protein receptor.  
244 However, we have not yet verified this idea using adsorption assays, complementation tests, or  
245 other direct methods. In contrast to U136B, phage 132 seems to interact with *tolC* indirectly.  
246 While looking for additional genes required for phage 132 replication, we found that it has  
247 severely reduced EOP on *ompF*<sup>-</sup> bacteria, and to a lesser extent, *tolC*<sup>-</sup> and *ompR*<sup>-</sup> bacteria (Fig.  
248 4). As both TolC and OmpR increase *ompF* expression (19, 22, 23), one possibility is that phage  
249 132 uses OmpF as an outer membrane receptor. However, at this time it remains unclear whether  
250 phage 132 directly interacts with the TolC protein, or indirectly interacts with TolC *via* OmpF or

251 some other mechanism. It is also possible that phage 132 can use either TolC or OmpF as outer  
252 membrane protein receptors.  
253  
254 Two other *E. coli* phages (Tula and T2) use OmpF as a receptor, and several phages can use  
255 multiple receptors. Phage T2 can use either OmpF or FadL as a receptor (14). To compare phage  
256 132 to a known multi-receptor, OmpF phage, we tested the EOP of phage T2 on knockouts of  
257 *fadL*, *ompF*, and *ompR* and found only slight reductions in its EOP on either of its receptor  
258 knockouts (Fig. S4), while the *ompR* knockout had the greatest reduction in EOP. We  
259 hypothesize that this is because *ompR* affects the regulation of both *fadL* as well as *ompF* (24),  
260 thereby reducing the total number of T2 receptors on the cell surface. In contrast, phage 132  
261 appears to be more dependent on *ompF* (Fig. 4B), and thereby more dependent on *tolC*.  
262 In addition to protein receptors, phages U136B and 132 may also require a primary receptor, such  
263 as lipopolysaccharide (LPS) (11, 25, 26). Given that U136B and 132 appear to share some  
264 properties with lipopolysaccharide (LPS)-dependent phages TLS and T2, we reasoned that might  
265 may rely on LPS synthesis and modification genes, such as those in the *rfa* locus. While many  
266 other genes are also involved in LPS production, previous work with phage TLS found that half  
267 of phage-resistant mutants had mutations in the *rfa* locus (11), and so we used these genes here.  
268 Our screen on 13 *rfa* knockouts revealed that removal of many of these genes severely reduced  
269 plating efficiency for phage U136B, 132, or both (Fig. 4), with the two phages responding  
270 differently to the deletion of various genes. These results suggest that both U136B and 132 have  
271 LPS-dependent infection, and that these phages may have unique O-antigen requirements. More  
272 importantly, LPS-dependence may provide an easy way for bacteria to evolve resistance to  
273 phages U136B and 132, as mutations to *rfa* (or other LPS-related) genes could reduce or

274 eliminate phage infection. This might explain why few of our phage-resistant mutants contained  
275 *tolC* mutations (Table 4).

276

277 Together, our results show that the antibiotic resistance gene *tolC* can modify bacteria-phage  
278 interactions in multiple ways, potentially through a direct interaction as the phage receptor (as  
279 we hypothesize for phage U136B) or by modifying expression of the outer membrane protein  
280 phage receptor (as we hypothesize for phage 132).

281

## 282 **Conclusion and Future Directions**

283 Our goal here was to identify bacteriophages that rely on the *E. coli* outer membrane protein  
284 TolC, which confers multi-drug resistance as part of the AcrAB-TolC efflux pump. Our two  
285 newly-isolated phages, U136B and 132, have unique morphologies and differentially impact  
286 bacterial populations, including lysis dynamics and selection for phage resistance. Together,  
287 these phages will be useful for future studies of evolutionary trade-offs between phage resistance  
288 and antibiotic resistance. These phages will also be useful for studying the specific biochemical  
289 interactions between phages and their hosts, including whether phage U136B uses TolC and  
290 phage 132 uses OmpF for outer membrane attachment. A potential further possibility is the  
291 development of these phages for practical application, where they might help to restore drug  
292 sensitivity.

293

## 294 **Methods**

295 **Bacterial growth conditions and media.** We grew bacteria in LB broth with 10 g tryptone, 5 g  
296 yeast extract, and 10 g/L NaCl. LB agar included 15 g/L agar and LB top agar included 7.5 g/L

297 agar unless otherwise noted. Overnight culture incubation was performed at 200 RPM shaking at  
298 37°C.

299

300 **Phage library screen.** We screened 33 phage isolates collected previously from various sources.  
301 We screened each isolate using the plaque spot test on host lawns of wild-type and knockout *E.*  
302 *coli* from the Keio collection obtained from the Yale Coli Genetic Stock Center (Table 1).

303

304 **Efficiency of plating.** Those phages for which a difference in plaquing was observed in the  
305 library screen were then screened for differences in efficiency of plating (EOP, the number of  
306 plaques formed on a mutant relative to wild-type bacteria). We determined the initial EOP for  
307 phages U136B and 132 on the *tolC* knockout using a full plate dilution series in standard top  
308 agar (7.5 g/L agar). For all other EOP assays, we used we used top agar that contained 3.8 g/L,  
309 which is 50% the typical amount of agar, and serial dilution spot tests of 2  $\mu$ l to obtain phage  
310 titers on each bacterium. (We found that phage 132 generally formed small, difficult-to-count  
311 plaques, were more easily enumerated in the 3.8 g/L formula.)

312

313 **Chloroform/vortex sensitivity assay.** We tested phage sensitivity to vortexing and to  
314 chloroform exposure under the typical conditions we use during phage preparation. We subjected  
315 test phages to three treatments: LB only, LB plus vortexing at moderate speed (setting 4/10 on a  
316 Scientific Industries Vortex Genie 2) for 3 seconds, and LB with 1% v/v chloroform. We plated a  
317 dilution series of 1.5- $\mu$ L spots of phage on LB agar before and after each treatment in triplicate.

318

319 **Mutant selection procedure.** To select for phage-resistant bacteria, we mixed wild-type bacteria  
320 and phages, then spread plated them onto LB agar and incubated overnight at 37°C. To ensure  
321 the isolation of independent mutations, each replicate was grown from a single isolated colony.  
322 To confirm the multiplicity of infection (MOI, the ratio of phage particles to bacterial cells) on  
323 the agar plate, we plated bacteria and phage in triplicate and counted CFU/ml and PFU/ml,  
324 respectively. From each mutant-selection plate, we picked a random colony, plus any colonies of  
325 notable morphology, and streaked each onto LB agar plate and incubated at 37°C overnight. We  
326 re-streaked the colonies to obtain isolates and grew each in 10 mL LB at 37°C with shaking  
327 overnight. We archived freezer stocks of each mutant in 20% glycerol, stored at -80°C.

328

329 **Mutants' phage resistance and cross-resistance.** We checked for resistance and cross-  
330 resistance using the cross-streak method (27), modified as follows. A line of 10 µl of high-titer  
331 phage stock was placed along an LB agar plate. When the phage streak had dried, 1 µl of  
332 bacterial culture was streaked across the phage. Cultures with no growth past the phage line were  
333 scored as phage-susceptible. Cultures with equal growth past the phage line were scored as  
334 phage-resistant. Cultures with decreased growth past the phage line were scored as intermediate-  
335 resistant.

336

337 **Bacterial growth curves.** Cultures of wild type and  $\Delta tolC$  were grown to exponential phase as  
338 measured by optical density at 600nm wavelength ( $OD_{600}$ ) of 0.25-0.38. The exponential-phase  
339 cultures were diluted 1:5 into wells with LB to a total volume of 200 µl with phage to reach the  
340 target multiplicities of infection (MOI, the ratio of phage to cells). Cultures were incubated at



341 37°C with shaking at 288 RPM for 18 h and OD<sub>600</sub> read every 2 minutes using an automated  
342 spectrophotometer (TECAN microplate reader).

343

344 **TEM.** Transmission electron micrographs were collected on high-titer phage lysates at the Yale  
345 Electron Microscopy facility in the Center for Cellular and Molecular Imaging. Samples were  
346 negatively stained with 2% uranyl acetate and imaged with FEG 200kV transmission EM.

347

348 **PCR and Sanger sequencing.** We extracted DNA from overnight cultures grown in LB using  
349 the Qiagen DNeasy Blood and Tissue Kit (Cat. # 69506), following the product's protocol for  
350 Gram-negative bacteria and the optional RNase step. We amplified the *tolC* gene using Promega  
351 GoTaq DNA polymerase (Cat. # M829) with standard buffer and primers AJL-001 and AJL-002  
352 (Table S1), with an annealing temperature of 54°C. Sanger sequences were collected by the  
353 DNA Analysis Facility on Science Hill at Yale using all four primers (Table S1). We aligned  
354 sequences to the reference sequence BW25113 (Table 1) using SeqMan Pro (LaserGene,  
355 DNASTAR, Madison, WI), confirming mutations by assessing the sequence trace data. We  
356 determined the identity of the insertion in the case of RGB-071 using BLAST (28) via the NCBI  
357 Nucleotide online tool (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) against reference sequence  
358 CP009273.1 for *E. coli* strain BW25113 (Table 1).

359

360 **Statistical analysis.** We used linear regression to test the effect of log-transformed MOI and  
361 phage type on bacteria lysis time parameters. We used ANOVAs to test the effect of bacterial  
362 *omp* gene knockouts on log-transformed efficiency of plating (EOP) data. (We conducted these  
363 analyses separately for phage 132 and U136, as the two phages were tested in separate

364 experiments and were thereby potentially subjected to block effects.) To test the effects of  
365 bacterial *rfa* gene knockout and phage type on EOP, we used an ANOVA with both gene and  
366 phage type independent variables. All analyses were performed in R (29) using custom scripts.

367

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375

### 376 **References**

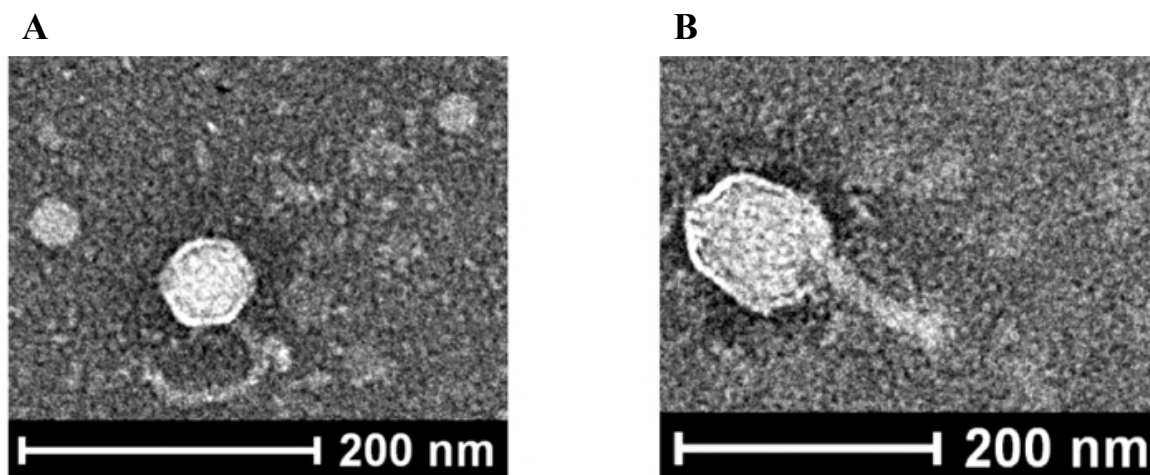
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453 **Figures**



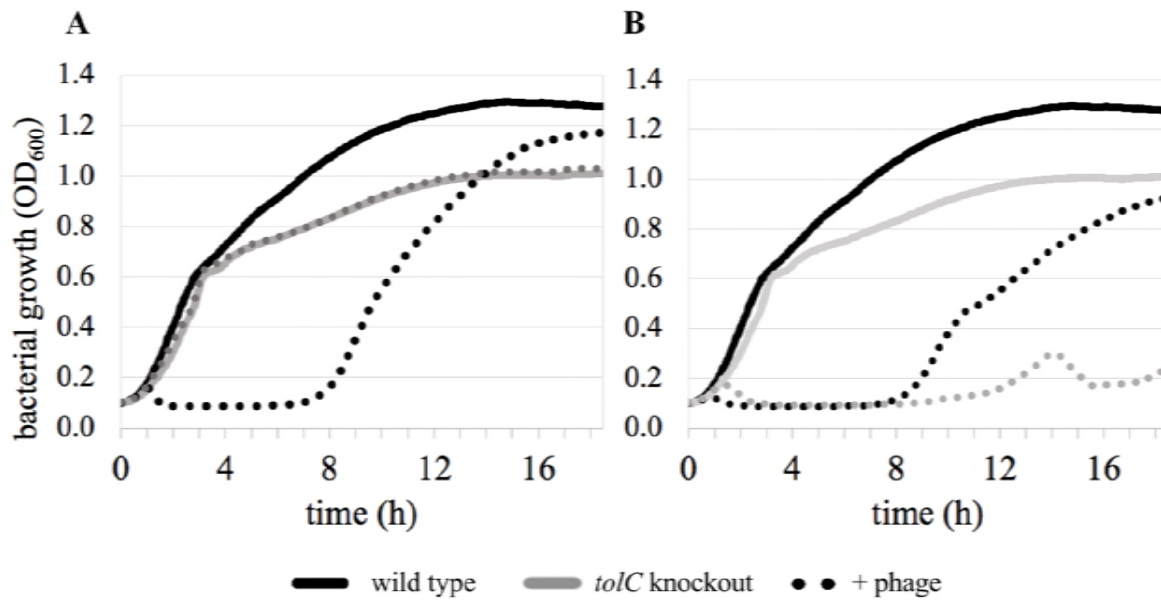
454

455 **Figure 1.** Transmission electron microscopy of the newly-isolated, *tolC*-dependent phages. A)

456 Phage U136B. B) Phage 132.

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459

460 **Figure 2.** Effects of *tolC*-dependent phages U136B and 132 on bacterial growth in liquid culture.

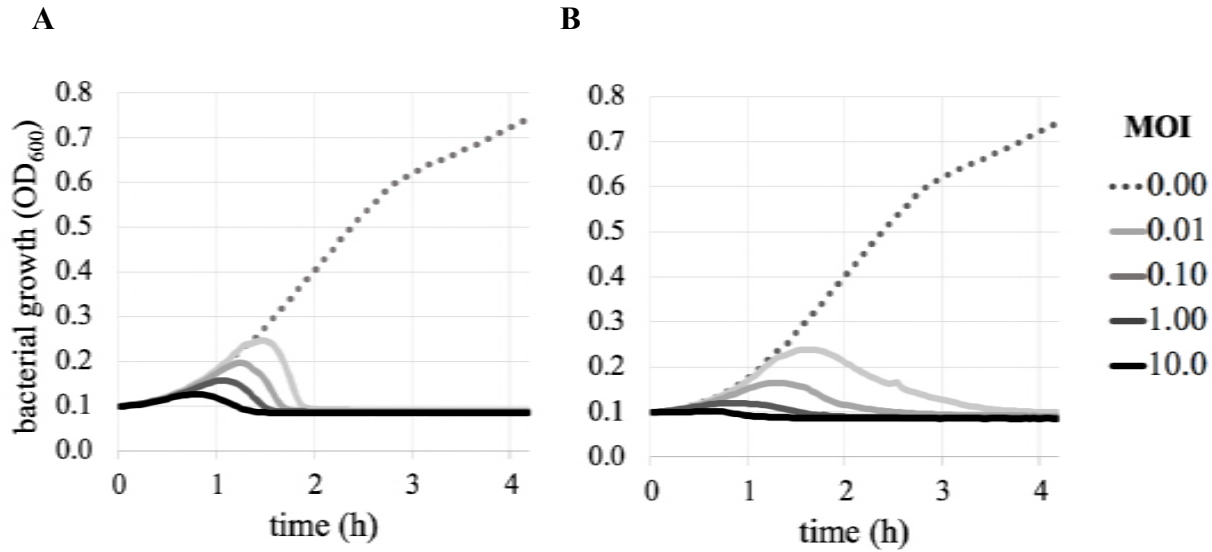
461 A) Knocking out *tolC* protects *E. coli* from infection by phage U136B, while the wild-type is

462 rapidly lysed. B) Both the *tolC* knockout and wild-type are lysed by phage 132. Each curve shows

463 the mean of three to six replicates. Individual replicates are shown in Fig. S3.

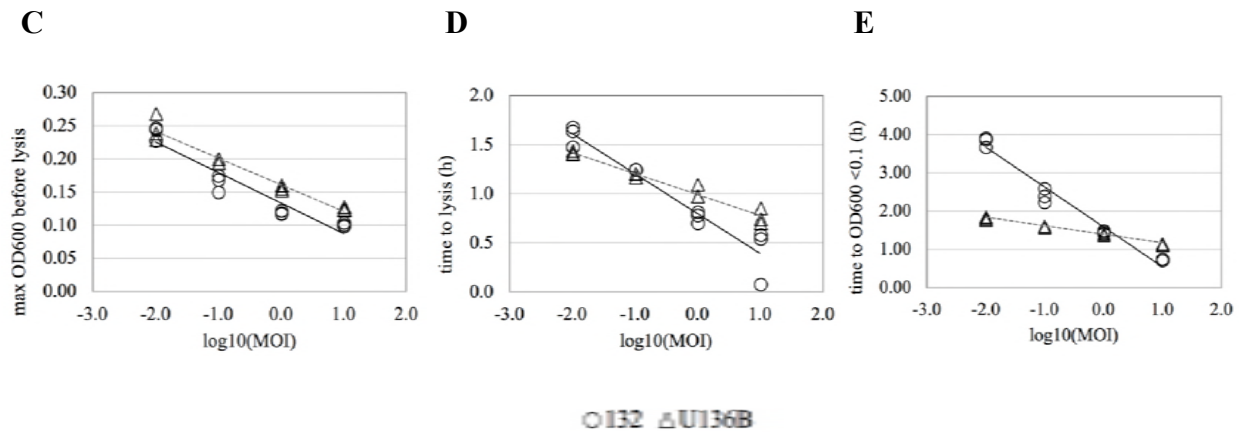
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470 **Figure 3.** Bacterial lysis at different multiplicities of infection (MOI, the ratio of phage to bacteria)

471 of the *tolC*-dependent phages U136B (Panel A) and 132 (Panel B). Phage addition to bacterial

472 cultures results in cell lysis, with increasing rates of lysis at higher multiplicities of infection

473 (MOI). Each curve shows the mean of three replicates. Panel C) The maximum OD reached before

474 the onset of bacterial lysis, Panel D) The time on lysis onset, and Panel E) The time to complete

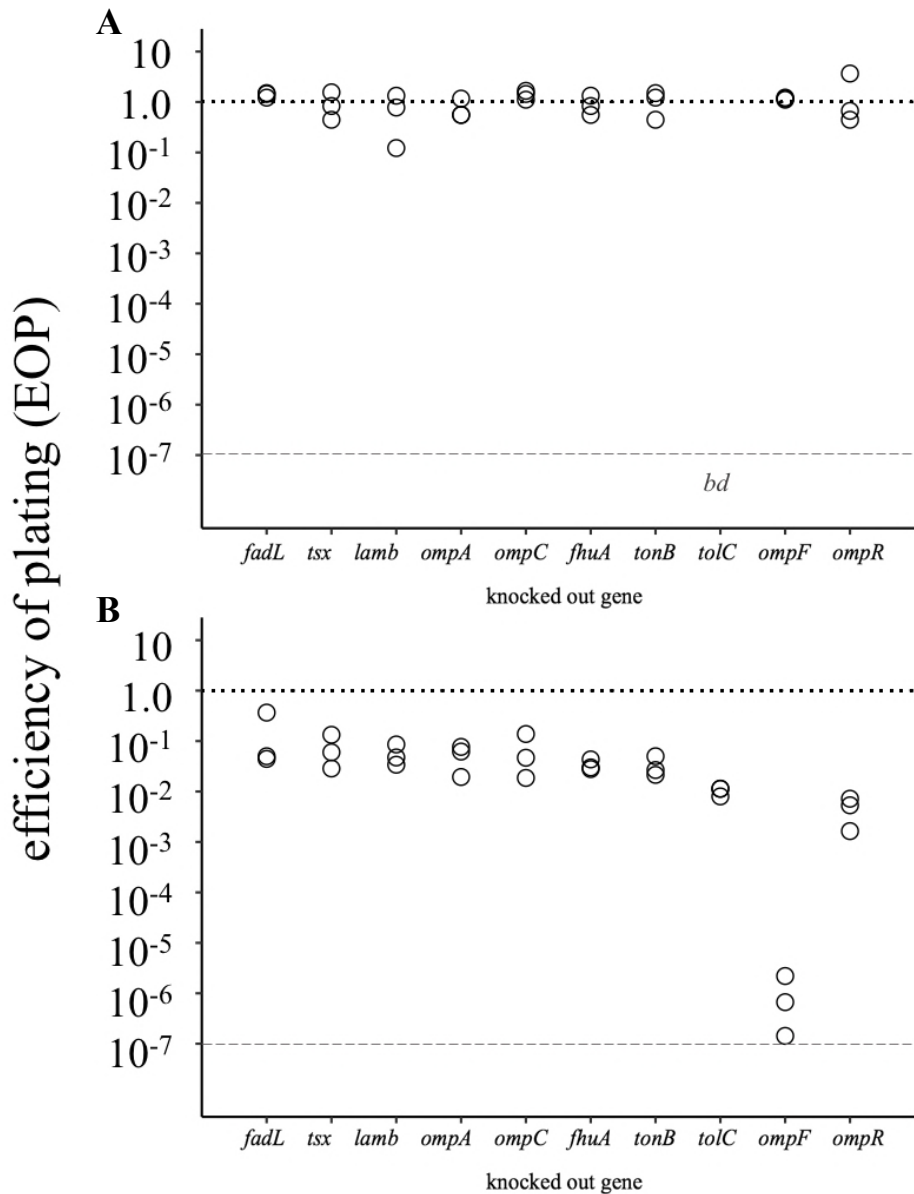
475 lysis. Increasing MOI significantly decreased the maximum OD, the time of lysis onset, and the

476 time to complete lysis (p < 0.00001 all cases, Table S2). Phage type significantly affected the



477 maximum OD and the complete time to lysis ( $p < 0.01$  both cases) but not the time of lysis onset ( $p$   
478  $= 0.15$ ) (Table S2).

479

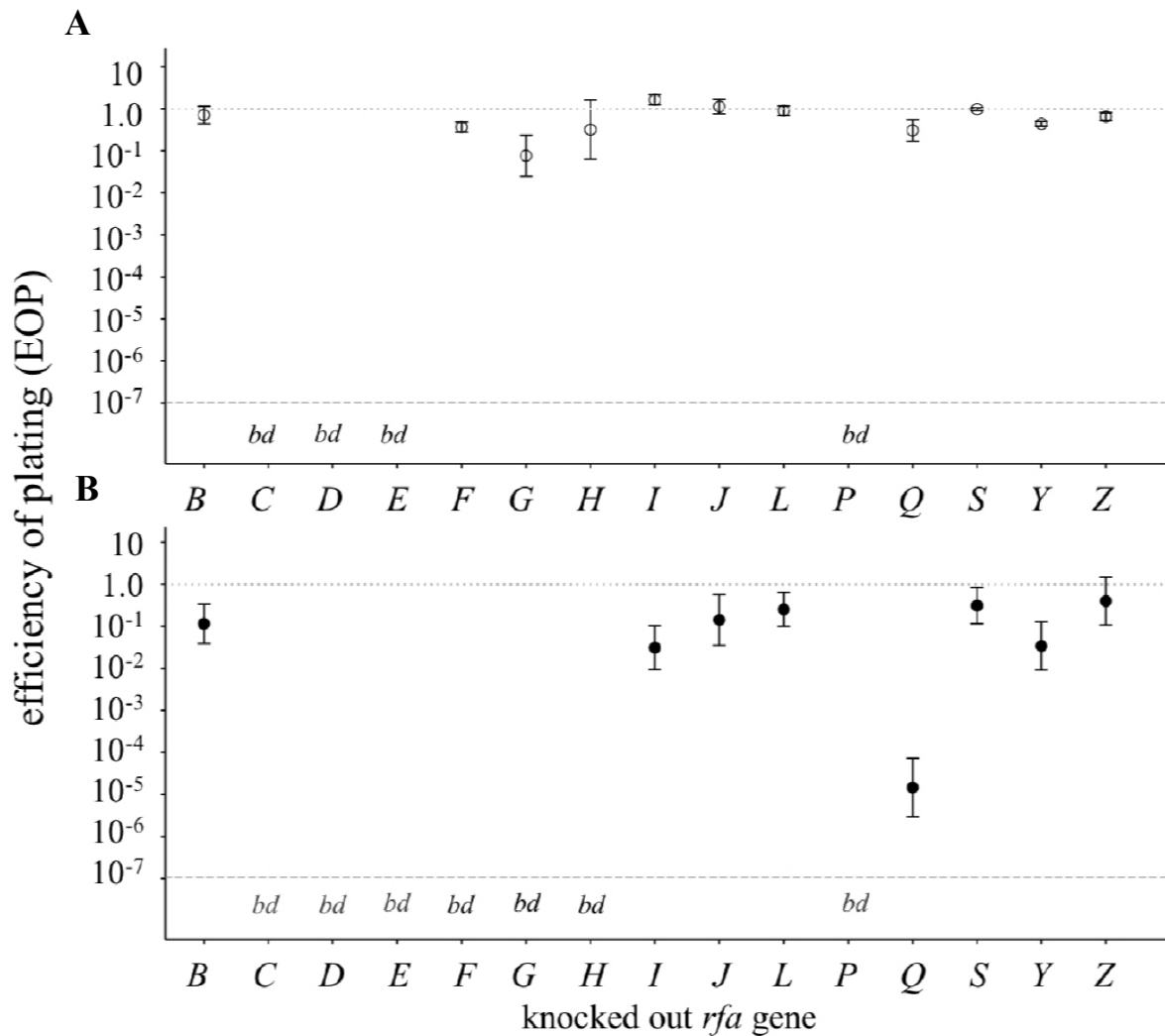


480

481 **Figure 4.** Efficiency of plating of phages U136B (Panel A) and 132 (Panel B) on outer  
482 membrane protein-coding gene knockouts. A phage that grows the same (produces an equal  
483 number of plaques) on a knockout as on wild-type bacteria has EOP of 1.0 (dotted line). Each  
484 assay was conducted in triplicate biological replicates (each replicate phage stock grown

485 independently from an isolated plaque of 132), with technical replicates performed in duplicate.  
486 Each point shows the mean of the two technical replicates. A “bd” below the lower, dashed line  
487 indicates that the efficiency of plating was below the limit of detection ( $\sim 10^{-7}$ ). The gene  
488 knockout significantly affected the EOP for phage 132 ( $F_{9,20} = 58.0$ ,  $p < 0.00001$ ) but not for  
489 phage U136B ( $F_{8,18}$ ,  $p = 0.68$ ) (Table S3).

490



491

492 **Figure 5.** Role of lipopolysaccharide synthesis genes on replication of phages U136B (upper  
493 panel) and 132 (lower panel). A phage that grows the same (produces an equal number of  
494 plaques) on a knockout as on wild-type bacteria has EOP of 1.0 (upper, dotted line in each

495 panel). Each assay was conducted in triplicate biological replicates (each replicate phage stock  
 496 grown independently from an isolated plaque). A “bd” below the lower, dashed line within each  
 497 panel indicates that the efficiency of plating was below the limit of detection ( $\sim 10^{-7}$ ). For phage-  
 498 bacteria combinations that were above the detection limit, efficiency of plating was influenced  
 499 by both the phage ( $F_{1,39} = 22.4$ ,  $p < 0.0001$ ) and *rfa* gene knockout ( $F_{7,39} = 4.5$ ,  $p < 0.001$ ) (Table  
 500 S4).

501

## 502 Tables

503 **Table 1.** *E. coli* and bacteriophage strains used in this study. All bacteria were obtained from the  
 504 Yale Coli Genetic Stock Center and were originally described by Baba *et al.* 2008 (20).

Strain	Description	Relevant Characteristics
<b>Bacteria</b>		
BW25113	<i>E. coli</i> K12, parental strain of Keio collection	
JW5503-1	<i>tolC</i> knockout in Keio collection	$\Delta tolC732::kan$
JW0451-2	<i>acrB</i> knockout in Keio collection	$\Delta acrB747::kan$
JW0452-3	<i>acrA</i> knockout in Keio collection	$\Delta acrA748::kan$
JW2341-1	<i>fadL</i> knockout in Keio collection	$\Delta fadL752::kan$
JW0401-1	<i>tsx</i> knockout in Keio collection	$\Delta tsx773::kan$
JW3996-1	<i>lamB</i> knockout in Keio collection	$\Delta lamB732::kan$
JW0940-6	<i>ompA</i> knockout in Keio collection	$\Delta ompA772::kan$
JW2203-1	<i>ompC</i> knockout in Keio collection	$\Delta ompC768::kan$
JW0146-2	<i>fhuA</i> knockout in Keio collection	$\Delta fhuA766::kan$
JW5195-1	<i>tonB</i> knockout in Keio collection	$\Delta tonB760::kan$
JW0912-1	<i>ompF</i> knockout in Keio collection	$\Delta ompF746::kan$
JW3368-1	<i>ompR</i> knockout in Keio collection	$\Delta ompR739::kan$
JW3603-2	<i>rfaB</i> knockout in Keio collection	$\Delta rfaB739::kan$
JW3596-1	<i>rfaC</i> knockout in Keio collection	$\Delta rfaC733::kan$
JW3594-1	<i>rfaD</i> knockout in Keio collection	$\Delta rfaD731::kan$
JW3024-1	<i>rfaE</i> knockout in Keio collection	$\Delta rfaE745::kan$
JW3595-2	<i>rfaF</i> knockout in Keio collection	$\Delta rfaF732::kan$
JW3606-1	<i>rfaG</i> knockout in Keio collection	$\Delta rfaG742::kan$
JW3818-1	<i>rfaH</i> knockout in Keio collection	$\Delta rfaH783::kan$
JW3602-1	<i>rfaI</i> knockout in Keio collection	$\Delta rfaI738::kan$
JW3601-3	<i>rfaJ</i> knockout in Keio collection	$\Delta rfaJ737::kan$
JW3597-1	<i>rfaL</i> knockout in Keio collection	$\Delta rfaL734::kan$
JW3605-1	<i>rfaP</i> knockout in Keio collection	$\Delta rfaP741::kan$
JW3607-2	<i>rfaQ</i> knockout in Keio collection	$\Delta rfaQ743::kan$
JW3604-2	<i>rfaS</i> knockout in Keio collection	$\Delta rfaS740::kan$
JW3600-1	<i>rfaY</i> knockout in Keio collection	$\Delta rfaY736::kan$
JW3599-1	<i>rfaZ</i> knockout in Keio collection	$\Delta rfaZ735::kan$
<b>Phage</b>		
Phage 132	Environmental isolate from swine farm	
Phage U136B	Environmental isolate from swine farm	
Phage T2	Obtained from the Yale Coli Genetic Stock Center, CGSC #12141	

505

506 **Table 2.** Selection of phage-resistant mutants. Independent cultures of *E. coli* BW25113 were

507 plated at MOI of 1 (phage U136B) or 10 (phage 132).

Selecting Phage	Surviving colony frequency	Cross resistance to the other phage <sup>1</sup>
Phage 132	2.75 x 10 <sup>-7</sup>	8/10
Phage U136B	3.20 x 10 <sup>-6</sup>	10/10

508

509 **Table 3.** Phage-resistant *E. coli* mutants. ‘Culture’ indicates the independent culture the selection

510 was performed on. ‘Selected on’ indicates the phage used for selection. ‘Type’ indicates whether

511 the culture was chosen randomly or nonrandomly from the selection plate.

Strain	Culture	Selected on	Colony Morphology	Type	Phage 132 <sup>R</sup>	U136B <sup>R</sup>	<i>tolC</i> Sequenced
RGB-034	1	U136B	Mucoid	Nonrandom	R	R	
RGB-036	1	U136B	S translucent	Random	R	R	*
RGB-040	2	U136B	L opaque	Random	IS	R	*
RGB-045	3	U136B	L opaque	Random	IS	R	*
RGB-049	4	U136B	S opaque	Random	R	R	*
RGB-050	4	U136B	Mucoid	Nonrandom	R	R	
RGB-058	5	U136B	L opaque	Random	IS	R	*
RGB-060	6	U136B	S opaque	Random	IS	R	*
RGB-065	7	U136B	S opaque	Random	R	R	*
RGB-071	8	U136B	S translucent	Random	IS	R	*
RGB-074	9	U136B	S opaque	Random	R	IR	*
RGB-079	10	U136B	S opaque	Random	IS	R	*
RGB-082	1	132	L	Random	R	R	*
RGB-083	1	132	S	Nonrandom	S	S	
RGB-084	2	132	L	Random	R	R	*
RGB-085	2	132	S	Nonrandom	S	S	
RGB-086	3	132	S	Random	S	S	
RGB-087	3	132	L	Nonrandom	R	R	*
RGB-088	4	132	L	Random	R	R	*
RGB-089	4	132	S	Nonrandom	S	S	
RGB-090	5	132	L	Random	R	S	*
RGB-091	5	132	S	Nonrandom	IR	S	
RGB-092	6	132	L	Random	IR	R	*
RGB-093	6	132	S	Nonrandom	R	S	
RGB-094	7	132	S	Random	S	S	
RGB-095	7	132	L	Nonrandom	R	R	*
RGB-096	8	132	S	Random	S	S	
RGB-097	8	132	L	Nonrandom	R	R	*
RGB-098	9	132	L	Random	R	R	*
RGB-099	9	132	S	Nonrandom	IS	R	
RGB-100	10	132	S	Random	S	S	
RGB-101	10	132	L	Nonrandom	R	R	*

512

S: small colony size

- 513 L: large colony size  
514 S: susceptible (complete clearing of bacterial growth along line of phage, few resistant colonies may be visible)  
515 R: resistant (line of bacterial growth looks same before/after/on line of phage)  
516 IS: intermediate susceptible (growth is much less dense but still visible after line of phage, more than a few resistant colonies)  
517 IR: intermediate resistant (growth is a bit less dense after line of phage but does not look as confluent as totally resistant)  
518

519 **Table 4.** List of *tolC* mutations identified by targeted Sanger sequencing in mutants resistant to  
520 phage U136B. No *tolC* mutations were identified in mutants resistant to phage 132. Location  
521 indicates the mutation position from the start of the *tolC* gene.

U136B <sup>R</sup> Mutant	Mutation Type	Description
RGB-040	C472T	premature stop codon (coding 157/493 amino acids)
RGB-045	ΔA at position 1092	frame shift
RGB-071	~1.3 kb insertion	IS5 insertion

522