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

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Running Head: Phage Dependence on *tolC*

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Abstract

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

Importance

Bacteria face strong selection by antibiotics in medicine and agriculture, resulting in increasing levels of drug resistance among bacterial pathogens. Slowing this process will require an 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
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Introduction

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

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

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

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

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

Results

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

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

Phage morphology. We used transmission electron microscopy to determine the general 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*$^{-}$ efficiency of plating data suggested that these
125 phages should also differentially affect the survival of wild-type and *tolC*$^{-}$ 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

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

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

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

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

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

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

Phage dependence on lipopolysaccharide synthesis genes. To test whether phage U136B and 132 may also rely on lipopolysaccharide (LPS) as primary receptors, we screened for efficiency of plating on knockouts of *rfa* genes, which are involved in LPS synthesis and modification. As expected, we found that knocking out single *rfa* genes greatly impacted efficiency of plating (Fig. 5). Neither phage formed any plaques on four of the knockouts (*rfaC*, *rfaD*, *rfaE*, *rfaP*). Phage 132 uniquely did not form plaques on three additional knockouts (*rfaF*, *rfaG*, and *rfgH*). For the eight remaining *rfa* knockouts for which EOP could be quantified for both phages (*rfaB*, *rfaI*, *rfaJ*, *rfaL*, *rfaQ*, *rfaS*, *rfaY*, *rfaZ*), the efficiency of plaquing was significantly effected by 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, Table S4). Together, these results suggest that phages U136B and 132 use LPS receptors, and that the two phages have different LPS specificity.

Discussion

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

Connecticut swine farm (Table 1). In spatially-structured solid media on *tolC*$^{+}$ bacteria, phage U136B forms easily-visible, medium-sized plaques while phage 132 forms smaller, more difficult-to-enumerate plaques. When *tolC* is absent, phage U136B completely fails to form plaques, while phage 132 forms plaques at reduced efficiency (see Results, '*Phage interactions with* tolC^{-} *bacteria'*). The two phages are also morphologically distinct from one another, belonging to different phage families (Fig. 1).

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

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

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

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

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

some other mechanism. It is also possible that phage 132 can use either TolC or OmpF as outer membrane protein receptors.

Two other *E. coli* phages (TuIa and T2) use OmpF as a receptor, and several phages can use multiple receptors. Phage T2 can use either OmpF or FadL as a receptor (14). To compare phage 132 to a known multi-receptor, OmpF phage, we tested the EOP of phage T2 on knockouts of *fadL*, *ompF*, and *ompR* and found only slight reductions in its EOP on either of its receptor knockouts (Fig. S4), while the *ompR* knockout had the greatest reduction in EOP. We hypothesize that this is because ompR affects the regulation of both *fadL* as well as *ompF* (24), thereby reducing the total number of T2 receptors on the cell surface. In contrast, phage 132 appears to be more dependent on *ompF* (Fig. 4B), and thereby more dependent on *tolC*.

In addtion to protein receptors, phages U136B and 132 may also require a primary receptor, such as lipopolysaccharaide (LPS) (11, 25, 26). Given that U136B and 132 appear to share some properties with lipopolysaccharide (LPS)-dependent phages TLS and T2, we reasoned that might may rely on LPS synthesis and modifcation genes, such as those in the *rfa* locus. While many other genes are also involved in LPS production, previous work with phage TLS found that half of phage-resistant mutants had mutations in the *rfa* locus (11), and so we used these genes here. Our screen on 13 *rfa* knockouts revealed that removal of many of these genes severely reduced plating efficiency for phage U136B, 132, or both (Fig. 4), with the two phages responding differently to the deletion of various genes. These results suggest that both U136B and 132 have LPS-dependent infection, and that these phages may have unique O-antigen requirements. More importantly, LPS-dependence may provide an easy way for bacteria to evolve resistance to phages U136B and 132, as mutations to *rfa* (or other LPS-related) genes could reduce or

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

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

Conclusion and Future Directions

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

Methods

Bacterial growth conditions and media. We grew bacteria in LB broth with 10 g tryptone, 5 g 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 µl 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-µL spots of phage on LB agar before and after each treatment in triplicate.
318

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

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

Bacterial growth curves. Cultures of wild type and Δ*tolC* were grown to exponential phase as measured by optical density at 600nm wavelength (OD_{600}) of 0.25-0.38. The exponential-phase cultures were diluted 1:5 into wells with LB to a total volume of 200 µl with phage to reach the 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_{600} 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
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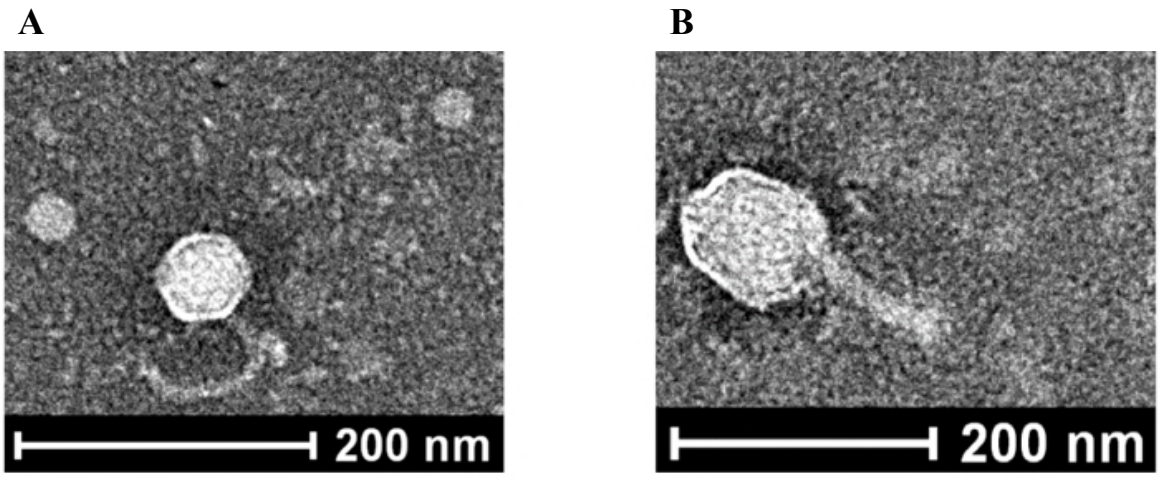
453 **Figures**



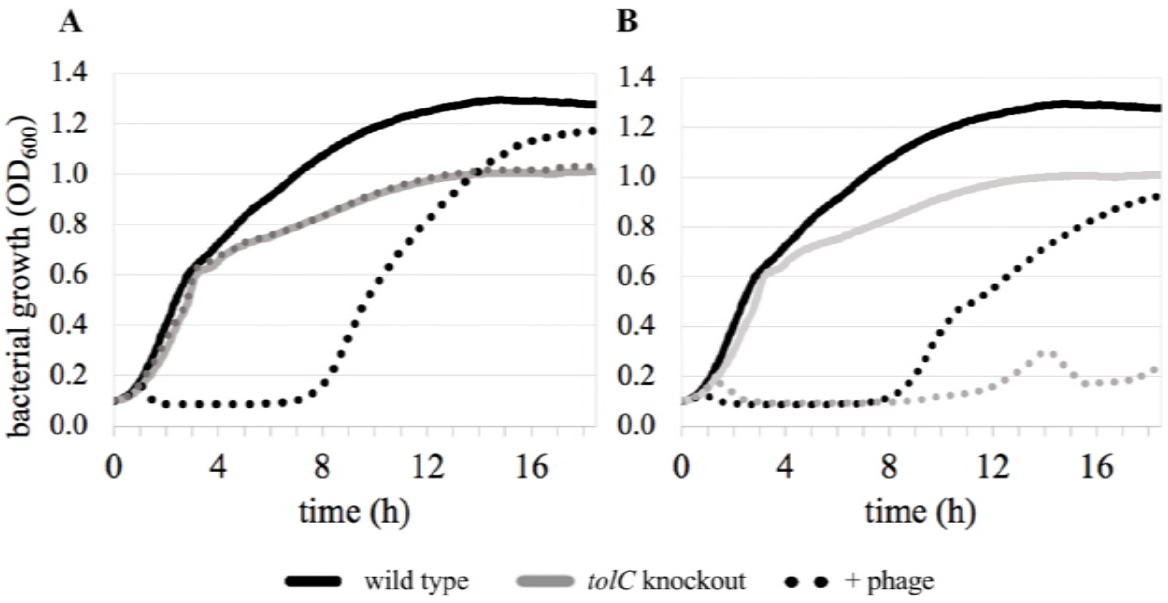
454

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

456 Phage U136B. B) Phage 132.

457

458



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.

464

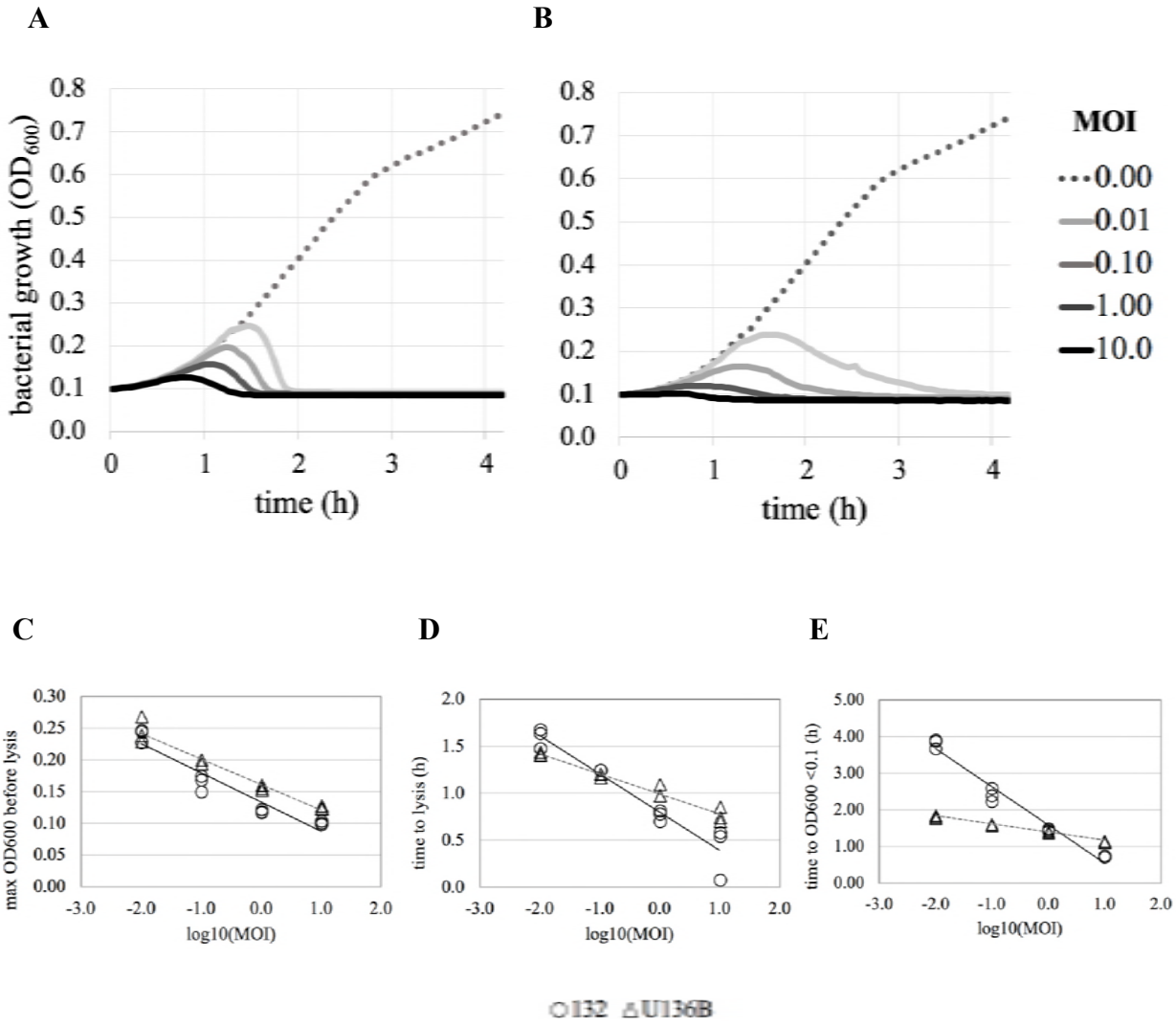
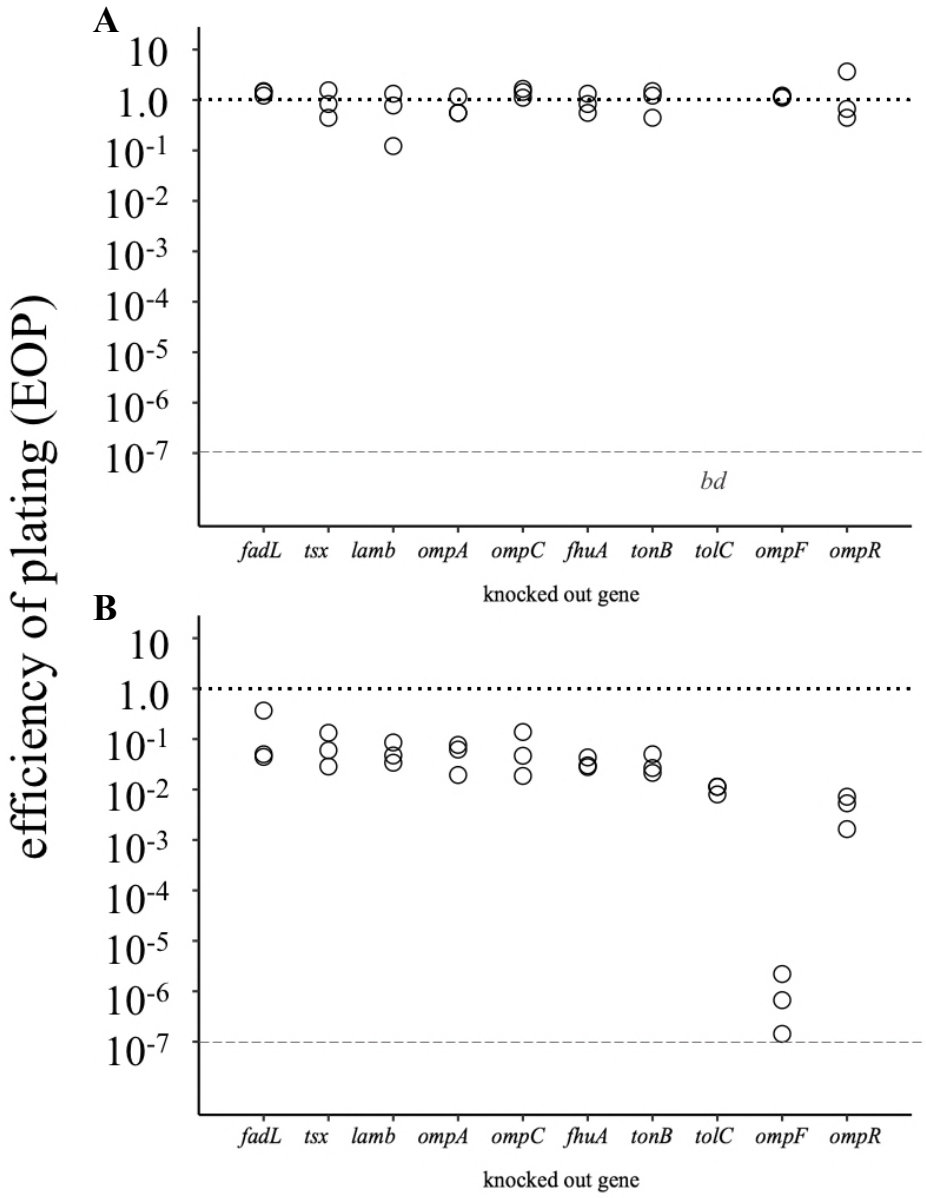


Figure 3. Bacterial lysis at different multiplicities of infection (MOI, the ratio of phage to bacteria) of the *tolC*-dependent phages U136B (Panel A) and 132 (Panel B). Phage addition to bacterial cultures results in cell lysis, with increasing rates of lysis at higher multiplicities of infection (MOI). Each curve shows the mean of three replicates. Panel C) The maximum OD reached before the onset of bacterial lysis, Panel D) The time on lysis onset, and Panel E) The time to complete lysis. Increasing MOI significantly decreased the maximum OD, the time of lysis onset, and the 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

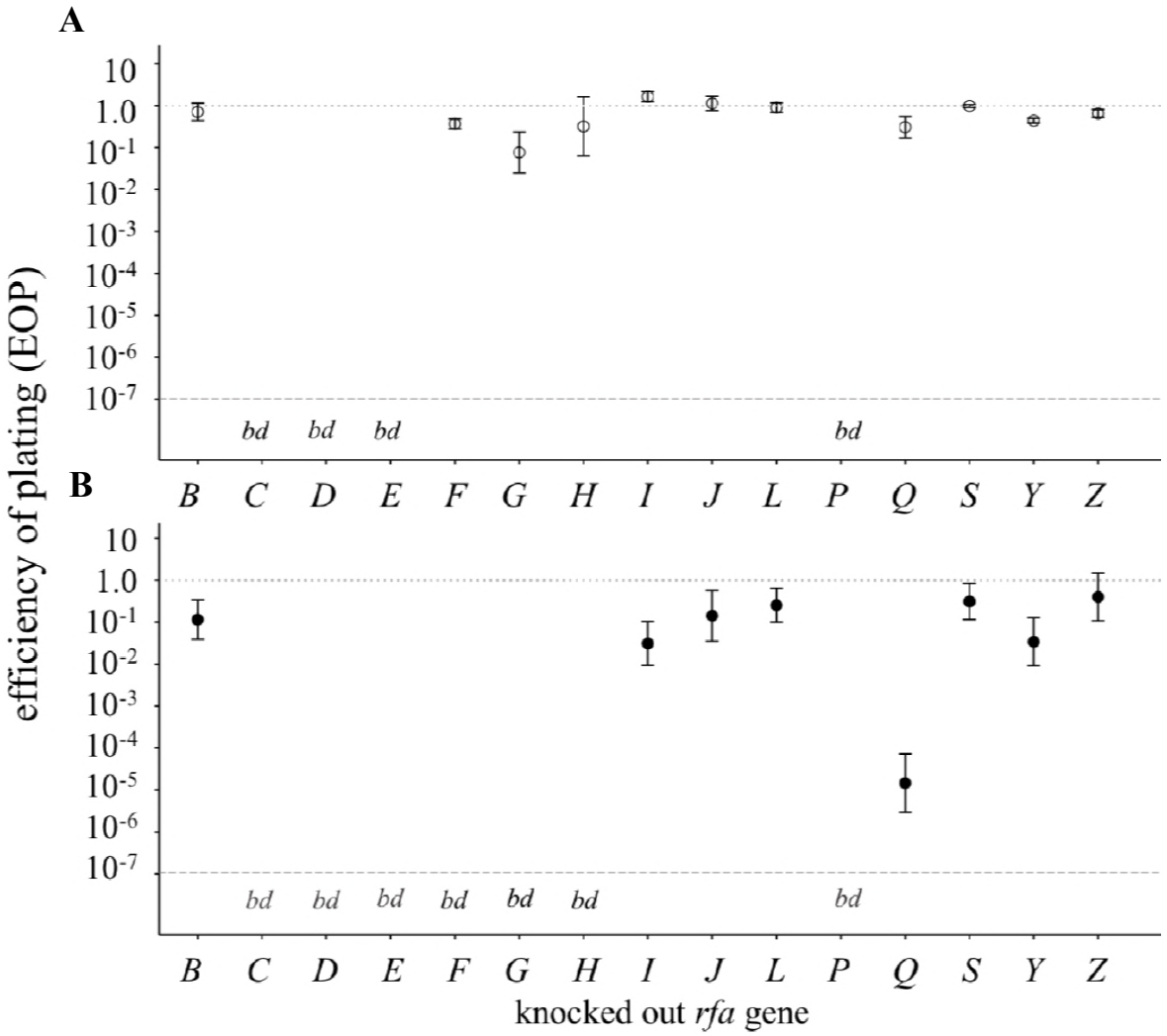
481 **Figure 4.** Efficiency of plating of phages U136B (Panel A) and 132 (Panel B) on outer

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 (~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

panel). Each assay was conducted in triplicate biological replicates (each replicate phage stock grown independently from an isolated plaque). A "bd" below the lower, dashed line within each panel indicates that the efficiency of plating was below the limit of detection (~10^{-7}). For phage-bacteria combinations that were above the detection limit, efficiency of plating was influenced 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 S4).

Tables

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

Strain	Description	Relevant Characteristics
Bacteria		
BW25113	*E. coli* K12, parental strain of Keio collection	
JW5503-1	*tolC* knockout in Keio collection	Δ*tolC732::kan*
JW0451-2	*acrB* knockout in Keio collection	Δ*acrB747::kan*
JW0452-3	*acrA* knockout in Keio collection	Δ*acrA748::kan*
JW2341-1	*fadL* knockout in Keio collection	Δ*fadL752::kan*
JW0401-1	*tsx* knockout in Keio collection	Δ*tsx773::kan*
JW3996-1	*lamB* knockout in Keio collection	Δ*lamB732::kan*
JW0940-6	*ompA* knockout in Keio collection	Δ*ompA772::kan*
JW2203-1	*ompC* knockout in Keio collection	Δ*ompC768::kan*
JW0146-2	*fhuA* knockout in Keio collection	Δ*fhuA766::kan*
JW5195-1	*tonB* knockout in Keio collection	Δ*tonB760::kan*
JW0912-1	*ompF* knockout in Keio collection	Δ*ompF746::kan*
JW3368-1	*ompR* knockout in Keio collection	Δ*ompR739::kan*
JW3603-2	*rfaB* knockout in Keio collection	Δ*rfaB739::kan*
JW3596-1	*rfaC* knockout in Keio collection	Δ*rfaC733::kan*
JW3594-1	*rfaD* knockout in Keio collection	Δ*rfaD731::kan*
JW3024-1	*rfaE* knockout in Keio collection	Δ*rfaE745::kan*
JW3595-2	*rfaF* knockout in Keio collection	Δ*rfaF732::kan*
JW3606-1	*rfaG* knockout in Keio collection	Δ*rfaG742::kan*
JW3818-1	*rfaH* knockout in Keio collection	Δ*rfaH783::kan*
JW3602-1	*rfaI* knockout in Keio collection	Δ*rfaI738::kan*
JW3601-3	*rfaJ* knockout in Keio collection	Δ*rfaJ737::kan*
JW3597-1	*rfaL* knockout in Keio collection	Δ*rfaL734::kan*
JW3605-1	*rfaP* knockout in Keio collection	Δ*rfaP741::kan*
JW3607-2	*rfaQ* knockout in Keio collection	Δ*rfaQ743::kan*
JW3604-2	*rfaS* knockout in Keio collection	Δ*rfaS740::kan*
JW3600-1	*rfaY* knockout in Keio collection	Δ*rfaY736::kan*
JW3599-1	*rfaZ* knockout in Keio collection	Δ*rfaZ735::kan*
Phage		
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[1]
Phage 132	2.75×10^{-7}	8/10
Phage U136B	3.20×10^{-6}	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^R**	**U136B^R**	***tolC* 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^{R} 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