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Supplementary Information for

Coevolutionary phage training leads to greater bacterial suppression and delays the evolution of phage resistance

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Supplementary Information Text

λtrn Resistance. The mechanisms by which some mutations affected λtrn resistance were unclear. All bacterial isolates from the coevolution experiment possessed a 12,090 bp deletion comprising 14 unannotated genes. Moreover, all populations (but not all isolates) were also found to have a 4 bp CCAG duplication in the coding sequence of another unannotated gene. And thirdly, the λtrn-sensitive isolate P1-T30-1 had a 777 bp IS element excision of loci *insB-22*, *insA-22* and *ECB_02825* that have previously been found to confer λtrn resistance through epistasis with mutations in *malT* (2). However, no *malT* mutations were found in any of the 12 strains isolated from day 30. These results beg the question: How did these bacteria reach high densities in flasks with λtrn if they were sensitive? The mechanism by which the 777 bp deletion interacts with mutations in *malT* is unknown and it is possible that this deletion also interacts with mutations at other loci. Although we were unable to detect growth of day-30 isolates when cocultured with λtrn, it is likely that these isolates are more resistant than their ancestor REL606—but that our assay was not sensitive enough to discriminate these differences.

Lastly, we point out two instances where λtrn recombined into the host genome. In λtrn Population 2, the completely resistant isolate P2-T25-9 had 18 mutations in *ybcD*, a predicted prophage replication protein fragment gene, from positions 539,643 to 539,915. Additionally, the completely resistant isolate P3-T15-2 from Population 3 had 4 mutations in *ybcT*, a phage lysis gene, from positions 549,746 to 549,772. BLASTX of mutant sequences to REL606 and λ reference genomes revealed a 100% match with λ, whereas they showed 89% and 96.88% identity with REL606, respectively, confirming that these sequences originated from the phage. Although both recombinations from phage were found in bacteria that evolved complete resistance, we have no evidence that these recombinations carry any selective benefit. More likely, recombination occurred due to the endogenous lambda red system which recombines homologous sequences at a high rate.

Phage Adaptation and Suppression of Resistance. For λtrn Population 1, comparisons were drawn from cultures where phage inoculums were not significantly different (p=0.09, two-sample t-test) and the mean density of λtrn ($\bar{x}$=1.17×10^8 pfu/mL) was higher than λtrn-P1-T25 ($\bar{x}$=6.25×10^7 pfu/mL). For Population 2, λtrn-P2-T15 led to greater suppression (p=0.02, two sample t-test, Fig. 5D, 5E) when phage inoculums were not significantly different (p=0.22, two-sample t-test) and the mean density of λtrn-P2-T15 ($\bar{x}$=8.13×10^7 pfu/mL) was higher than λtrn ($\bar{x}$=5.39×10^7 pfu/mL). We note that when mean density of λtrn was higher than λtrn-P2-T15 (albeit not significantly, p=0.24) we found no difference in suppression. Lastly, for Population 3, λtrn+ phage led to greater suppression (p=0.034, two sample t-test, Fig. 5G, 5H) when the mean density of λtrn ($\bar{x}$=3.19×10^8 pfu/mL) was higher than for λtrn-P3-T5 ($\bar{x}$=9.69×10^7 pfu/mL). Notably, when significantly more λtrn was used ($\bar{x}$=6.38×10^8 pfu/mL compared to $\bar{x}$=3.88×10^8 pfu/mL, p=0.02, two-sample t-test), λtrn-P3-T5 still showed significantly more suppression of host P3-T5-1-P (p=0.02, two-sample t-test).

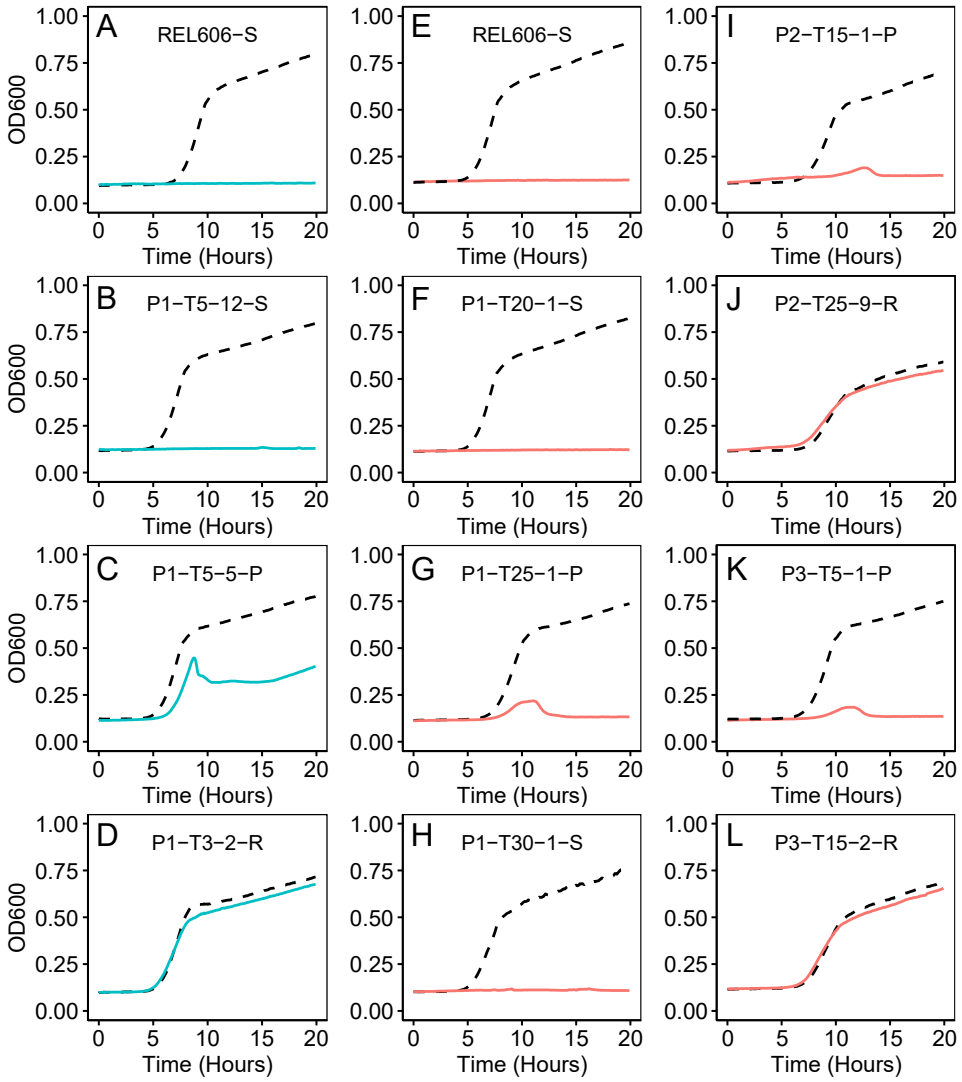


Figure S1. Diversity of resistance profiles determined from OD growth trajectories of bacterial isolates growing without (black, dotted) and with phage (solid, teal for λunt, red for λtrn) for 20 h. Plot titles indicate the bacterial isolate and resistance status. Apart from the ancestor REL606, isolates are labeled as [Population]-[Day Isolated]-[Isolate #]-[Resistance Status], where S = Sensitive, P = Partial Resistance, and R = Complete Resistance. Isolates were deemed sensitive if no growth was observed in the presence of phage, partially resistant if there was growth and inhibition, and completely resistant if there appeared to be no inhibition of growth by phage. Panels A-D correspond with phage λunt and panels E-L with λtrn.

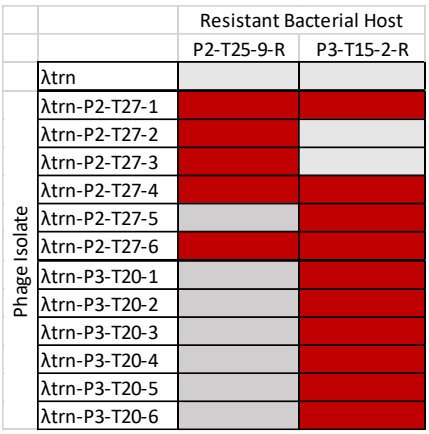
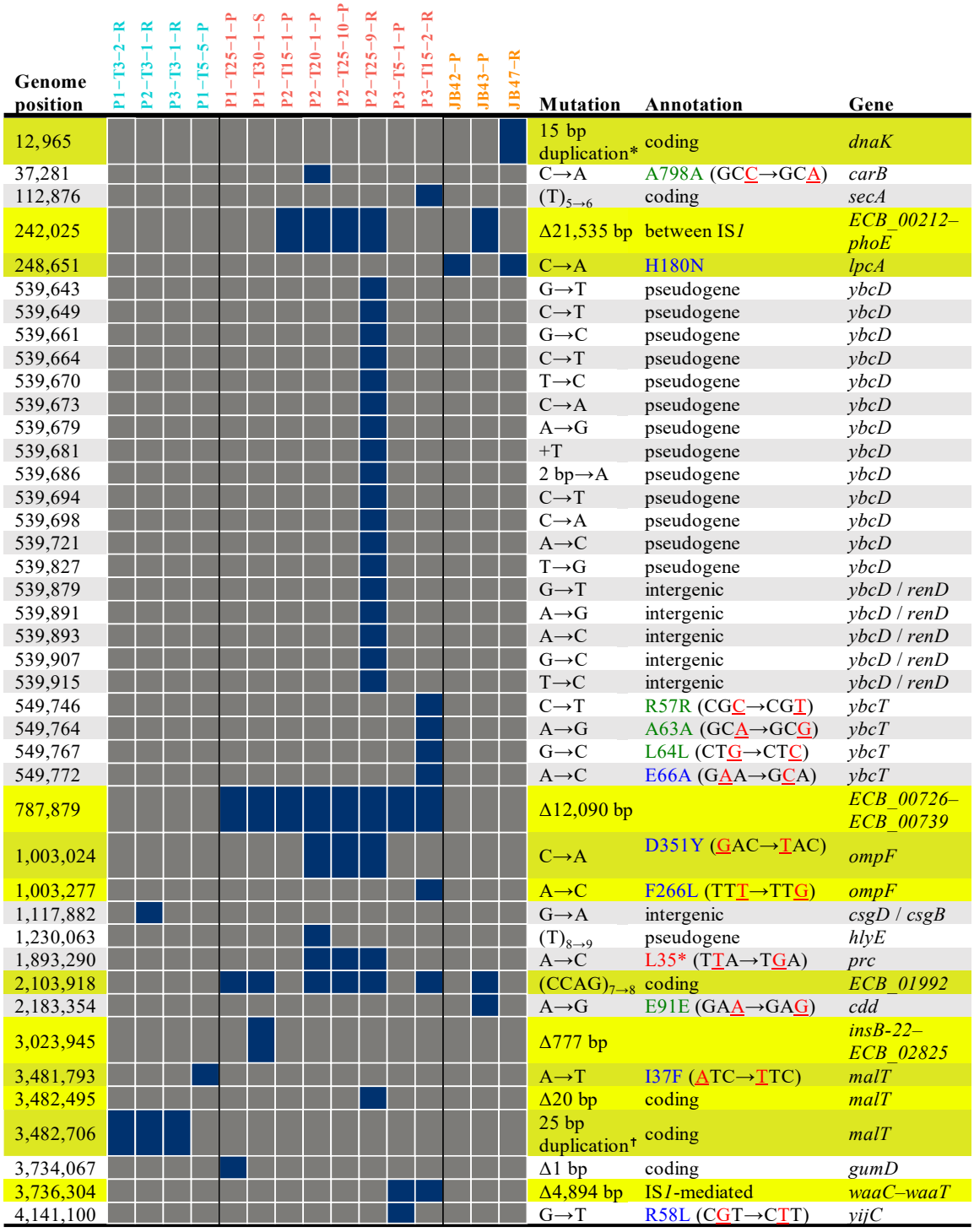


Figure S2. Infectivity of λtrn and descendant phages on bacterial hosts isolated from the coevolution experiment. Hosts (columns, labeled as in Fig. S1) were completely resistant to λtrn due to *malT* and *OmpF* mutations (see Fig. S3). Lysates of λtrn and descendant phage isolates were spotted onto host infused LB agar. Fill indicates resistance/infectivity (grey = resistant, red = sensitive).



Genome position	P1−T3−2−R	P2−T3−1−R	P3−T3−1−R	P1−T5−5−P	P1−T25−1−P	P1−T30−1−S	P2−T15−1−P	P2−T20−1−P	P2−T25−10−P	P2−T25−9−R	P3−T5−1−P	P3−T15−2−R	JB42−P	JB43−P	JB47−R	Mutation	Annotation	Gene
12,965																15 bp duplication*	coding	*dnaK*
37,281																C→A	A798A (GCC→GCA)	*carB*
112,876																$(T)_{5\rightarrow6}$	coding	*secA*
242,025																Δ21,535 bp	between IS*1*	*ECB_00212–phoE*
248,651																C→A	H180N	*lpcA*
539,643																G→T	pseudogene	*ybcD*
539,649																C→T	pseudogene	*ybcD*
539,661																G→C	pseudogene	*ybcD*
539,664																C→T	pseudogene	*ybcD*
539,670																T→C	pseudogene	*ybcD*
539,673																C→A	pseudogene	*ybcD*
539,679																A→G	pseudogene	*ybcD*
539,681																+T	pseudogene	*ybcD*
539,686																2 bp→A	pseudogene	*ybcD*
539,694																C→T	pseudogene	*ybcD*
539,698																C→A	pseudogene	*ybcD*
539,721																A→C	pseudogene	*ybcD*
539,827																T→G	pseudogene	*ybcD*
539,879																G→T	intergenic	*ybcD / renD*
539,891																A→G	intergenic	*ybcD / renD*
539,893																A→C	intergenic	*ybcD / renD*
539,907																G→C	intergenic	*ybcD / renD*
539,915																T→C	intergenic	*ybcD / renD*
549,746																C→T	R57R (CGC→CGT)	*ybcT*
549,764																A→G	A63A (GCA→GCG)	*ybcT*
549,767																G→C	L64L (CTG→CTC)	*ybcT*
549,772																A→C	E66A (GAA→GCA)	*ybcT*
787,879																Δ12,090 bp		*ECB_00726–ECB_00739*
1,003,024																C→A	D351Y (GAC→TAC)	*ompF*
1,003,277																A→C	F266L (TTT→TTG)	*ompF*
1,117,882																G→A	intergenic	*csgD / csgB*
1,230,063																$(T)_{8\rightarrow9}$	pseudogene	*hlyE*
1,893,290																A→C	L35* (TTA→TGA)	*prc*
2,103,918																$(CCAG)_{7\rightarrow8}$	coding	*ECB_01992*
2,183,354																A→G	E91E (GAA→GAG)	*cdd*
3,023,945																Δ777 bp		*insB-22–ECB_02825*
3,481,793																A→T	I37F (ATC→TTC)	*malT*
3,482,495																Δ20 bp	coding	*malT*
3,482,706																25 bp duplication†	coding	*malT*
3,734,067																Δ1 bp	coding	*gumD*
3,736,304																Δ4,894 bp	IS*1*-mediated	*waaC–waaT*
4,141,100																G→T	R58L (CGT→CTT)	*yijC*

Figure S3. Mutations of representative bacterial isolates. Teal and red labels correspond to isolates from λunt and λtrn coevolution treatments, respectively. Orange corresponds with isolates from fluctuation tests. Coevolution isolates are labeled as in Fig. S1. S, P, and R within the labels indicate resistance level (Sensitive, Partial Resistance, and Complete Resistance) relative to the phage ancestor with which they evolved. Mutations were classified as putatively resistant if they occurred in genes 1) known to be involved in λ infection or 2) evolved in multiple populations, fluctuation tests, or previous coevolution experiments (yellow rows). *(AAGCGGCAGAAAAAG) †(AGTGGGAACTGGCGGCGGAGCTGCC).

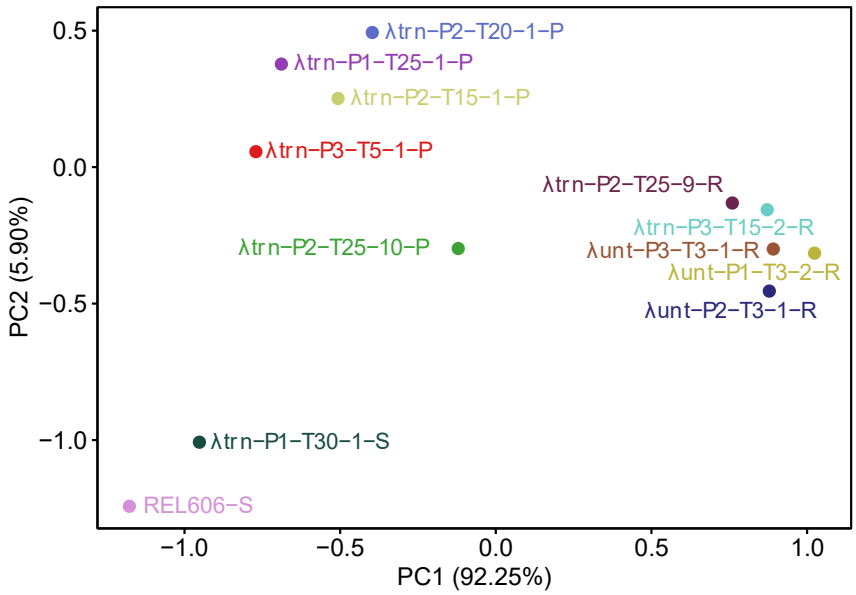


Figure S4. Principal Component Analysis (PCA) of the differences in OD trajectories of representative bacterial isolates growing without and with phages in plate reader experiments. PC1 distinguishes between sensitive, partial, and complete resistance against ancestral phages from respective coevolution treatments (λunt or λtrn). See Fig. S7 to assess the correlation between PC1 and a commonly calculated measure of resistance, Efficiency of Plating (EOP). Apart from the ancestor REL606, isolates are labeled adjacent to points as in Fig. S1 with their names additionally preceded with the phage treatment.

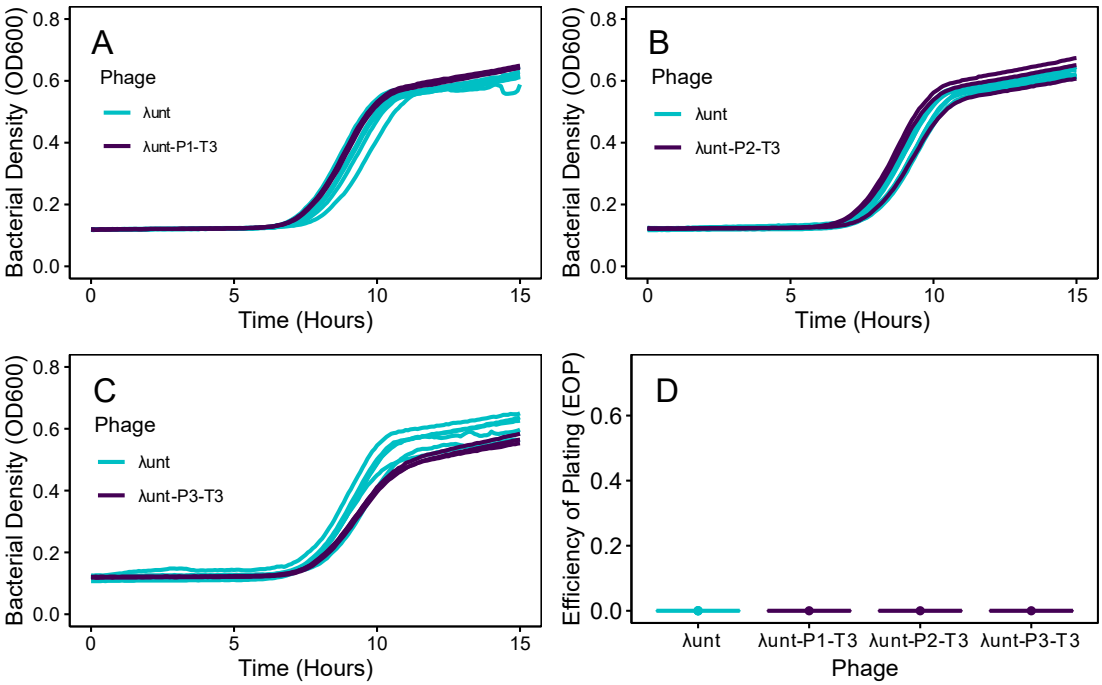


Figure S5. Coevolved λunt+ phages do not show improved suppression of resistant bacteria isolated on the very first day resistance was detected. Panels A-C: Growth trajectories of replicate wells were inoculated with ~1000 cells of the resistant host and either λunt (teal, n=6) or a contemporary λunt+ phage (purple, n=3) from the same population and time-point as the host. Panel A pertains to host P1-T3-2-R. Panel B pertains to host P2-T3-1-R. Panel C pertains to host P3-T3-1-R. Panel D: Efficiency of plating (EOP) of λunt and λunt+ phages on respective, resistant hosts compared to on REL606. One explanation for why λunt+ stains do not possess enhanced ability to suppress resistant bacteria is that they may not have had an opportunity to evolve yet. We can reject this explanation because each isolate gained multiple mutations in the reactive region of host recognition gene *J.* These mutations were identified in previous coevolution experiments and were shown to be adaptive; λunt-P1-T3: C2999T, G3319A. λunt-P2-T3 and λunt-P3-T3: C2999T, C3147A, T3380C (1,2). The conclusion is that despite gaining adaptive mutations, λunt+ strains were still unable to significantly suppress resistant bacteria.

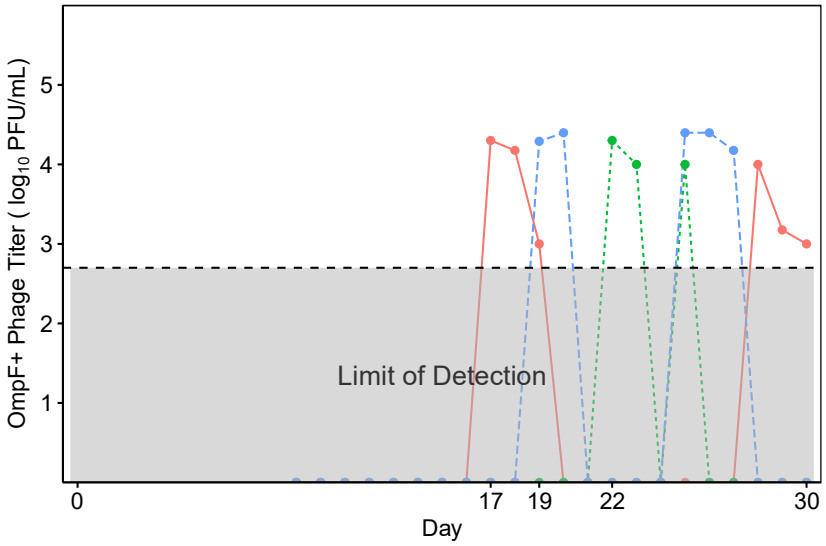


Figure S6. Emergence and density of OmpF$^+$ phages evolved *de novo* during the initial experiment (Fig. 1) in the λunt treatment. OmpF$^+$ phages in Population 1 (solid, red), Population 2 (dotted, green), and Population 3 (dashed, blue) were first detected on days 17, 22, and 19, respectively. OmpF$^+$ phages were detected and enumerated by spotting phage lysate on soft agar plates infused with cells of a LamB$^-$ REL606 mutant.

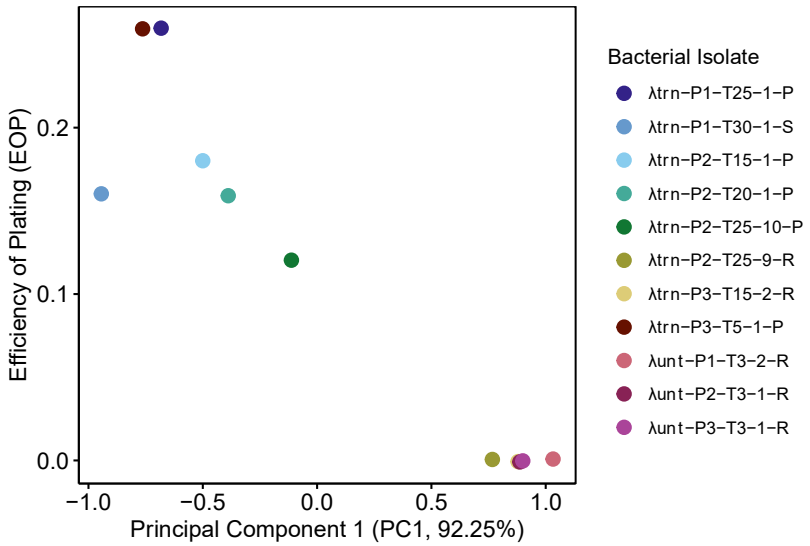


Figure S7. Correlation between Principal Component 1 (PC1, see Fig. S4) and Efficiency of Plating (EOP) of different representative resistant bacterial isolates (Spearman's rank correlation, n=11, p=0.0003, rho=-0.879). Higher values of PC1 and lower values of EOP correspond with greater resistance. Bacteria are labeled are as in Fig. S1 with their names additionally preceded with the phage treatment.

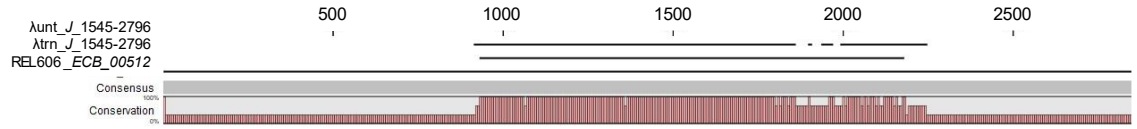


Figure S8. Alignment of recombination region (*J* positions 1545-2796) of λunt (no recombination) and λtrn (with recombination) with REL606 *ECB_00512* (genome positions 551811-554657). Sequence alignment was conducted in CLC Sequence Viewer 8.0.

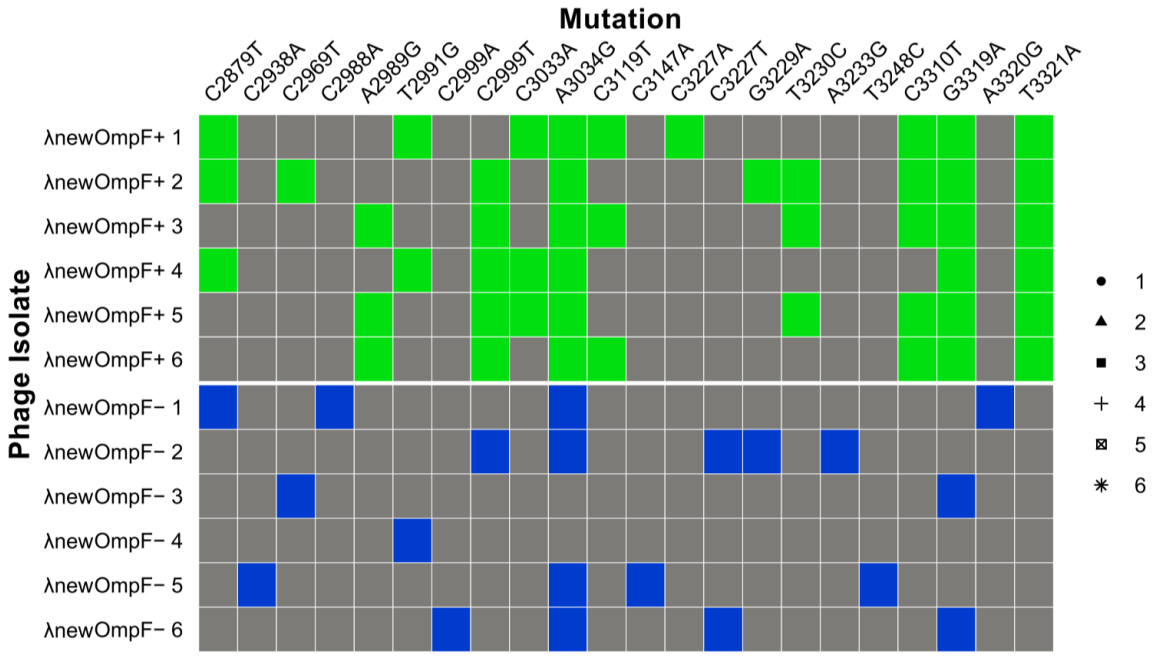


Figure S9. Mutations in the reactive region of host recognition gene *J* of new trained phage genotypes. λnewOmpF+ phages at top (mutations indicated by green) and λnewOmpF− phages at bottom (mutations indicated by blue). Symbols at right indicate how different genotypes correspond with lines in Fig. 7.

Table S1. Genomic differences between untrained phage λunt (λcI26) and trained phage λtrn. For genomic differences between λunt and the λ reference genome (GenBank: NC_001416) see Meyer et al. 2012 (1). Synonymous mutations are in green and nonsynonymous mutations in blue. Mutations from position 17,049 to 17,868 evolved via recombination with a relict prophage in the genome of REL606 (1).

Genome position	Mutation	Type	Amino acid change	Gene	Product
11,451	C → T	Substitution	A304V	*H*	Tail component
17,049	C → T	Recombination	S515S	*J*	Tail: host specificity protein
17,055	T → C	Recombination	G517G	*J*	Tail: host specificity protein
17,059	G → A	Recombination	A519T	*J*	Tail: host specificity protein
17,081	+G	Recombination	—	*J*	Tail: host specificity protein
17,082	A → C	Recombination	G526G	*J*	Tail: host specificity protein
17,085	Δ1 bp	Recombination	—	*J*	Tail: host specificity protein
17,088	C → G	Recombination	G528G	*J*	Tail: host specificity protein
17,090	A → G	Recombination	N529S	*J*	Tail: host specificity protein
17,136	A → G	Recombination	V544V	*J*	Tail: host specificity protein
17,160	T → C	Recombination	G552G	*J*	Tail: host specificity protein
17,183	A → G	Recombination	E560G	*J*	Tail: host specificity protein
17,200	C → T	Recombination	L566L	*J*	Tail: host specificity protein
17,211	A → C	Recombination	R569R	*J*	Tail: host specificity protein
17,280	G → A	Recombination	V592V	*J*	Tail: host specificity protein
17,328	A → C	Recombination	E608D	*J*	Tail: host specificity protein
17,334	T → C	Recombination	S610S	*J*	Tail: host specificity protein
17,343	G → A	Recombination	V613V	*J*	Tail: host specificity protein
17,391	T → C	Recombination	T629T	*J*	Tail: host specificity protein
17,409	T → C	Recombination	Y635Y	*J*	Tail: host specificity protein
17,421	G → C	Recombination	A639A	*J*	Tail: host specificity protein
17,424	A → C	Recombination	R640R	*J*	Tail: host specificity protein
17,430	C → T	Recombination	D642D	*J*	Tail: host specificity protein
17,433	A → G	Recombination	T643T	*J*	Tail: host specificity protein
17,457	T → C	Recombination	S651S	*J*	Tail: host specificity protein
17,466	C → T	Recombination	L654L	*J*	Tail: host specificity protein
17,469	T → C	Recombination	R655R	*J*	Tail: host specificity protein
17,478	+GG	Recombination	—	*J*	Tail: host specificity protein
17,487	C → T	Recombination	D661D	*J*	Tail: host specificity protein
17,494	A → C	Recombination	S664R	*J*	Tail: host specificity protein
17,502	G → A	Recombination	R666R	*J*	Tail: host specificity protein
17,535	A → T	Recombination	T677T	*J*	Tail: host specificity protein
17,547	G → A	Recombination	T681T	*J*	Tail: host specificity protein
17,556	G → T	Recombination	A684A	*J*	Tail: host specificity protein
17,586	G → A	Recombination	A694A	*J*	Tail: host specificity protein
17,613	T → G	Recombination	D703E	*J*	Tail: host specificity protein
17,652	A → G	Recombination	A716A	*J*	Tail: host specificity protein
17,659	2bp → CA	Recombination	—	*J*	Tail: host specificity protein
17,673	G → A	Recombination	T723T	*J*	Tail: host specificity protein
17,679	C → G	Recombination	G725G	*J*	Tail: host specificity protein
17,721	C → T	Recombination	D739D	*J*	Tail: host specificity protein
17,759	A → G	Recombination	Q752R	*J*	Tail: host specificity protein
17,775	A → G	Recombination	R757R	*J*	Tail: host specificity protein
17,788	+CA	Recombination	—	*J*	Tail: host specificity protein
17,795	Δ2 bp	Recombination	—	*J*	Tail: host specificity protein
17,805	T → C	Recombination	G767G	*J*	Tail: host specificity protein
17,862	C → T	Recombination	Y786Y	*J*	Tail: host specificity protein
17,868	T → C	Recombination	Y788Y	*J*	Tail: host specificity protein
18,285	C → A	Recombination	D927E	*J*	Tail: host specificity protein

18,297	4bp → ATAT	Recombination	—	*J*	Tail: host specificity protein
18,503	C → T	Substitution	A1000V	*J*	Tail: host specificity protein
18,538	A → G	Substitution	S1012G	*J*	Tail: host specificity protein
18,814	C → T	Substitution	H1104Y	*J*	Tail: host specificity protein
18,823	G → A	Substitution	D1107K	*J*	Tail: host specificity protein
18,825	T → A	Substitution	D1107K	*J*	Tail: host specificity protein
18,868	A → T	Substitution	I1122F	*J*	Tail: host specificity protein
19,260	T → C	Substitution	L99P	*lom*	Outer host membrane
20,661	A → G	Substitution	K338E	*Orf-401*	Tail fiber protein
45,176	$(G)_{5\rightarrow6}$	Insertion	—	*Orf-64*	Hypothetical prot./anti-holin

Table S2. Primers used to amplify segments of λ gene *J* for engineering alleles with the recombination.

Primer ID	Primer Position	Sequence (5' → 3')
J Forward	15 bp upstream	CTGCGGGCGGTTTTGTCATT
2906 J Forward	Intergenic (2906)	CTCCGGGACGCTCAGTA
J Reverse	318 bp downstream	ACGTATCCTCCCCGGTCATCACT

SI References

1. J. R. Meyer *et al.,* Repeatability and contingency in the evolution of a key innovation in phage lambda. *Science* **335,** 428-432 (2012).
2. A. Gupta *et al.*, Leapfrog dynamics in phage-bacteria coevolution revealed by joint analysis of cross-infection phenotypes and whole genome sequencing. *bioRxiv* doi: 10.1101/2020.10.31.337758