Repeatability and Contingency in the Evolution of a Key Innovation in Phage Lambda

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The processes responsible for the evolution of key innovations, whereby lineages acquire qualitatively new functions that expand their ecological opportunities, remain poorly understood. We examined how a virus, bacteriophage λ, evolved to infect its host, *Escherichia coli*, through a novel pathway. Natural selection promoted the fixation of mutations in the virus's host-recognition protein, J, that improved fitness on the original receptor, LamB, and set the stage for other mutations that allowed infection through a new receptor, OmpF. These viral mutations arose after the host evolved reduced expression of LamB, whereas certain other host mutations prevented the phage from evolving the new function. This study shows the complex interplay between genomic processes and ecological conditions that favor the emergence of evolutionary innovations.

Throughout the history of life, evolving lineages have acquired qualitatively new functions that enable organisms to expand their ecological opportunities and, in many cases, to undergo further diversification (*1*). Explaining how these transitions have occurred is usually difficult, both because the responsible events typically occurred in the distant past and because their rarity suggests that they might involve atypical evolutionary processes. For example, natural selection is critical for the process of adaptation, yet its role in producing key innovations is less clear because, by fixing variants that improve existing functions, selection might strand populations on local adaptive peaks and thereby prevent them from discovering new functions (*2*). Darwin was well aware of the difference between improving an existing trait and evolving a new one (*3*), and he reasoned that new traits originate by co-opting previously existing structures and functions. Without an understanding of genetic mechanisms, however, he could not provide a detailed account of how this process happens. Since then, others have proposed more explicit models of the origin of new functions that vary in two main respects: the structure of the adaptive landscape—including its dimensionality (*2*), genetic connectivity (*4*), and fluctuations caused by changing environments (*5*), including interactions with coevolving species (*6*)—and the relative importance of natural selection and random drift (*2*, *4*, *7*). However, no consensus has been reached owing, at least in part, to the paucity of cases with sufficient genetic and ecological data (*8*).

To that end, we examine the evolutionary forces responsible for the emergence of a novel trait in a microbial system, including data bearing on the genetic architecture of the adaptive landscape in which the novel capacity arose. Microbes are well suited for such research because their evolution can be observed in real time, experiments are easily replicated, and transitional states can be studied by reviving samples stored at different times during an experiment (*9*, *10*). We investigated how a virus evolved the ability to infect its host through a new receptor that the ancestral virus cannot use. We tested competing hypotheses about the evolution of this new trait by determining the conditions that promoted its evolution, the mutations that conferred the new function, and the evolutionary forces that drove its emergence.

Study system. Viruses are genetically and morphologically diverse, and they infect all groups of organisms (*11*, *12*). Viruses initiate infections by binding to receptors on the surface of host cells. The physicochemical properties of viral ligands determine which receptors they target and thereby influence the host range and ecological niche of the virus (*13*). Mutations in viral genes that encode the production of ligands can cause shifts in host range and thus are often associated with emerging diseases (*14*, *15*). The evolution of the ability to infect through a new receptor represents a key innovation for a virus.

The virus we studied, a strictly lytic derivative of phage λ called cI26 (*16*) (table S1), is only known to infect one bacterial species, *Escherichia coli*, and has a specialized ligand, the J protein, at the end of its tail (*17*). J targets a single protein, LamB, on the *E. coli* outer membrane (*17*, *18*). Phage λ requires only LamB to attach (*19*), and LamB is the only outer-membrane protein that affects λ reproduction (*20*).

Given interest in the fundamental question of how organisms evolve novel traits and in the practical problem of how emerging pathogens evolve to target new host receptors, we sought to determine whether λ could evolve to infect through an alternative receptor. Phage λ is well studied and amenable to experimentation (*21*). A related phage (Ur-λ) has side-tail fibers and can infect *E. coli* through a second receptor OmpC (*22*, *23*), which suggested that other receptors might be accessible to evolving λ populations. Moreover, we identified conditions that seemed suitable for promoting the use of a novel receptor. In particular, when *E. coli* and λ were cultured

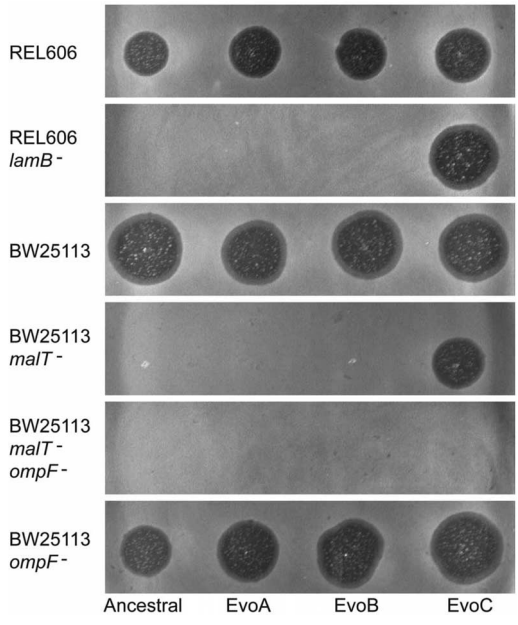

Fig. 1. Infection assay for four λ isolates tested on six *E. coli* strains. Each panel shows a bacterial lawn with aliquots of the phage applied to it; darker regions indicate successful infections that clear the lawn. The phage isolates include the ancestor and three clones isolated from the same population on day 8 of the initial evolution experiment, including one, EvoC, that can use the OmpF receptor. The bacterial strains include mutants that differ in the expression of LamB and OmpF porins on two genomic backgrounds, REL606 (the ancestral strain in the evolution experiments) and BW25113 (a derivative of K12). The *malT*⁻ strains do not express LamB at appreciable levels.

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together in a glucose-limited environment, the bacteria evolved resistance by mutations in *malT*, which interfered with its role as a transcription factor that promotes *lamB* expression (*23*). The mutants arose and fixed within 8 days, generating highly resistant populations (fig. S1), although the phage did not go extinct but instead persisted at densities of about 10^6 to 10^7 phage per ml or about one phage per 10^2 to 10^3 host cells (fig. S2). The phage evidently persisted on a subpopulation of cells that, despite their *malT* mutations, experienced spontaneous induction of the LamB protein (*24*). This explanation was confirmed by showing that phage were also sustained when they were grown on a *malT* mutant, whereas the phage went extinct when cultured with a *lamB* mutant that lacks the potential to produce the LamB protein (fig. S3). We reasoned that mutant viruses able to infect through some protein other than LamB would be favored after *malT* mutants arose because they could infect the entire host population rather than a small minority of cells.

Initial evolution experiment. We cocultured a virulent (nonlysogenic) derivative of λ and *E. coli* B in 10 ml of a minimal glucose medium in six replicate flasks for 28 days with daily transfers of 1% of each community into a flask containing fresh medium, and we preserved samples weekly by freezing 10% of the mixture (*16*). We tested whether the phage could infect cells through a new receptor by taking samples of the phage populations (typically ~5×10^4 virions) and inoculating them onto the surface of agar plates infused with a *lamB* mutant that does not produce the LamB receptor. A spot of clearing (lysis of host cells) provided evidence that some phage had evolved the ability to infect through a receptor other than LamB (Fig. 1). Such spots were observed in only one population, but this ability evolved quickly, such that ~0.01% of the phage could infect the *lamB*-negative mutants by day 8, including the isolate designated EvoC, and the majority did so by day 15.

Identification of the novel receptor. We used seven knockout strains (derivatives of K12 BW25113), each missing a gene encoding a different outer-membrane protein, to identify the new receptor (*16*, *25*). We then introduced *malT* mutations to these strains so that they also would not express the native LamB receptor. We inferred which one was the new receptor by testing the ancestral and evolved λ against these double mutants to see which ones were resistant to the various phage isolates. The only host that was resistant to the EvoC isolate was the *ompF malT* double mutant that lacked expression of both OmpF and LamB (Fig. 1 and table S2), which indicated that OmpF was the new receptor. This evolved phage could still infect the host strain expressing LamB but not OmpF, which showed that the phage retained the ability to infect through its native receptor (Fig. 1).

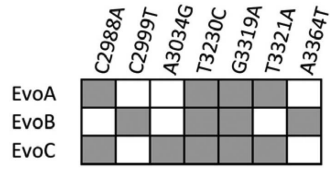

Fig. 2. Mutations in the λ gene encoding the J protein in three isolates from the same population on day 8 of the initial evolution experiment. The isolates are shown in rows and the mutations in columns, with the first letter being the ancestral nucleotide, the number the nucleotide position, and the last letter the evolved nucleotide. The gray fill indicates that the phage isolate has the corresponding mutation.

Both LamB and OmpF form trimeric porins composed of three identical β barrels (*26*, *27*). This overall structure is probably essential for the J protein in the λ tail to bind because J, too, forms a trimer and is thought to attach with radial symmetry across the three pore domains (*28*). Although OmpF has the most similar crystal structure to LamB of any *E. coli* protein determined to date (*29*), they are not the most similar pair by amino acid sequence (table S3). This discordance suggests that the overall structure is at least as important for λ binding as the identity of specific amino acid residues. Also, OmpF is the sole major porin in the *E. coli* B strain used in this study, and B expresses it constitutively during growth (*30*, *31*). Hence, OmpF provided a substantial ecological opportunity to phage that evolved the ability to target it.

Genome evolution. We sequenced the genome of the evolved phage EvoC in order to identify the mutations that allowed it to use the OmpF receptor (*16*). There were five mutations in total, and all of them were in the J gene (Fig. 2). Targeted sequencing of J showed that a single substitution (A to G at position 3034) differentiated EvoC from another evolved isolate from the same time point, EvoA, that could use only the ancestral LamB receptor, which indicated that mutation contributed to the new receptor function. Another LamB-dependent phage from the same day, EvoB, differed from EvoC at five sites in the J protein.

Large-scale evolution experiment. We repeated our first experiment with 96 more communities to identify general principles of how λ evolves the capacity to target an alternative receptor (*16*). We sampled daily for finer resolution of the evolutionary dynamics. As before, only some phage populations (24 out of 96) evolved the ability to use a second receptor. This ability emerged about the same time (median 12 days; range 9 to 17 days) (fig. S4), and all isolates with altered receptor function infected hosts through the OmpF protein.

Parallel molecular evolution. We sequenced J alleles from 24 phage isolates that independently evolved the ability to target OmpF during the large-scale evolution experiment to determine whether the mutation at position 3034 or any others were required to use that receptor. The isolates were taken the same day the new function was detected (*16*). For comparison, we sequenced phage from 24 populations that never evolved this trait; these isolates were sampled on the same days as those that evolved the new trait, so that the elapsed times were the same.

In total, there were 241 single-nucleotide polymorphisms (SNPs) across the 48 J alleles, but no insertions or deletions (Fig. 3). However, there were only 40 unique mutations because many arose repeatedly in replicate populations. Moreover, all of them were nonsynonymous. The alleles for phage able to target OmpF had on average 6.63 [± 0.51 95% confidence interval (CI)] SNPs, whereas the phage that required LamB had only 3.42 (± 0.50 95% CI) SNPs. This difference is highly significant on the basis of a paired comparison between the two types of phage matched for the day of their isolation and, in the case of multiple equivalent pairs, matched arbitrarily by position in the experiment ($t = 9.144$, 23 df, two-tailed test; $P < 0.0001$) (table S4). Also, across both classes of phage, more than 97% of the mutations were in the last 25% of the protein (C-terminal end), the region known to interact with LamB (*32*).

There are four striking cases of parallel evolution of the J protein in the phage that target OmpF. In two cases, the mutations were identical across all 24 populations; in two others, there were slight variations (Fig. 3). In particular, all J alleles from phage able to infect through OmpF had the A-to-G mutation at nucleotide position 3034 and G-to-A mutation at position 3319. Also, all of them had a mutation at either position 3320 or 3321, affecting the same codon (amino acid residue 1107) as the mutation at position 3319. Finally, all J alleles had at least one mutation between positions 2969 and 2999 (amino acid residues 990 to 1000).

Each of these mutations or classes of mutations were also found in at least one of the phage that retained the ancestral host-range, although none of them had all four together (Fig. 3). Two LamB-dependent isolates, F2 and H4, had three of the mutations, as did EvoA from the initial experiment (Fig. 2), yet none produced clearing on lawns of *lamB* mutants.

The correspondence between the use of the OmpF receptor and the presence of these four mutations, coupled with the observation that phage having only three of the four cannot use OmpF, provides evidence that all four are required for λ to infect through OmpF. We performed two additional assays to confirm that only phage with all four mutations can infect *lamB* mutants (*16*). The assays were performed using isolates EvoA, F2, and H4 that each had three of the four canonical mutations and D7 that had all four and no others. Only D7 exhibited a measurable adsorption rate on *lamB* mutant cells (fig. S5), and it was also the only one that reproduced on *lamB* mutants in the medium used in the evolution experiments (fig. S6). These findings indicate an "all-or-none" form of epistasis among the four mutations responsible for the novel receptor phenotype.

Role of natural selection. In the λ population that evolved to use OmpF in the initial experiment, the A3034G mutation was the fourth

and final step, and it was clearly advantageous because it conferred the ability to infect the entire cell population. However, the all-or-none epistasis among the mutations means that selection for that new capacity per se was not responsible for the rise of the three prior mutations. Nonetheless, there are several lines of evidence that selection drove their rise. First, all 248 independent mutations in the 51 sequenced J alleles were nonsynonymous, whereas the expected ratio of nonsynonymous to synonymous changes is 3.19:1 under the null model for the ancestral J sequence (*16*). This great excess is evident even if we include only the 82 nonsynonymous mutations in the 24 isolates that did not evolve the new receptor function. Second, the mutations are highly concentrated in the region of the J protein that interacts directly with LamB (*18*). Third, there was parallel evolution at the genetic level across the populations. For those phage that evolved to exploit OmpF, an average of 61% (4.06 out of 6.63) of mutations were shared across independently derived pairs (fig. S7), which greatly exceeds the fraction expected under a conservative randomization test (*16*) that used only the variable sites in J ($P < 10^{-5}$). Pairs of phage that remained dependent on LamB shared on average 17% (0.58 out of 3.42) of their mutations (fig. S7), and this fraction is again significant under the same test ($P < 10^{-5}$). Thus, it is clear that selection acted on the J protein even before the new capacity evolved. This selection presumably improved the interaction of the phage tail with LamB.

Stochasicity and contingency. All of the λ populations had the same ecological incentive to exploit an alternative receptor, but only some evolved that ability. Why were some populations successful and others not? One possibility is that all of them would eventually have evolved that function but that there was insufficient time to do so. This explanation is consistent with the facts that the LamB-dependent isolates had fewer mutations than those able to target OmpF, and that the two groups shared many mutations. Alternatively, the evolution of the new receptor function might have been contingent on earlier events (*33*–*35*), such that particular changes in the phage or the host promoted or impeded the subsequent evolution of that function. To test these hypotheses, we replayed evolution (*35*, *36*) using various combinations of phage and bacteria.

First, we tested whether certain mutations in the phage that might enhance performance on LamB would impede the evolution of the new OmpF function. We inoculated flasks with the ancestral bacteria and one of six phage isolates. Three of the six phage isolates had different sets of three mutations that were present in multiple isolates that evolved to use the OmpF receptor, including one, zero, and two of the four canonical mutations. The other three isolates had three, two, and one mutations that were not observed in any isolate that previously evolved the ability to use OmpF; these isolates also each had one of the canonical mutations. The first set provided a positive control; the second set had candidate mutations for impeding the evolution of the new function. For each phage, we propagated 12 communities for 10 days and surveyed daily the phage's ability to lyse *lamB* mutant cells. There were as many or more successes in evolving the new function among the three phages that had the potentially interfering mutations as among the positive controls (Fig. 4, top). This experiment thus provides no evidence that some phage failed to evolve the new function because they had mutations that prevented them from doing so.

Next, we asked if the outcome was contingent on mutations in the evolving bacteria. To that end, we performed a similar replay experiment except that the initial phage type was held constant while the starting bacterial isolate was varied. For the phage, we used EvoA, an isolate that was one mutation away from using OmpF (Fig. 2).

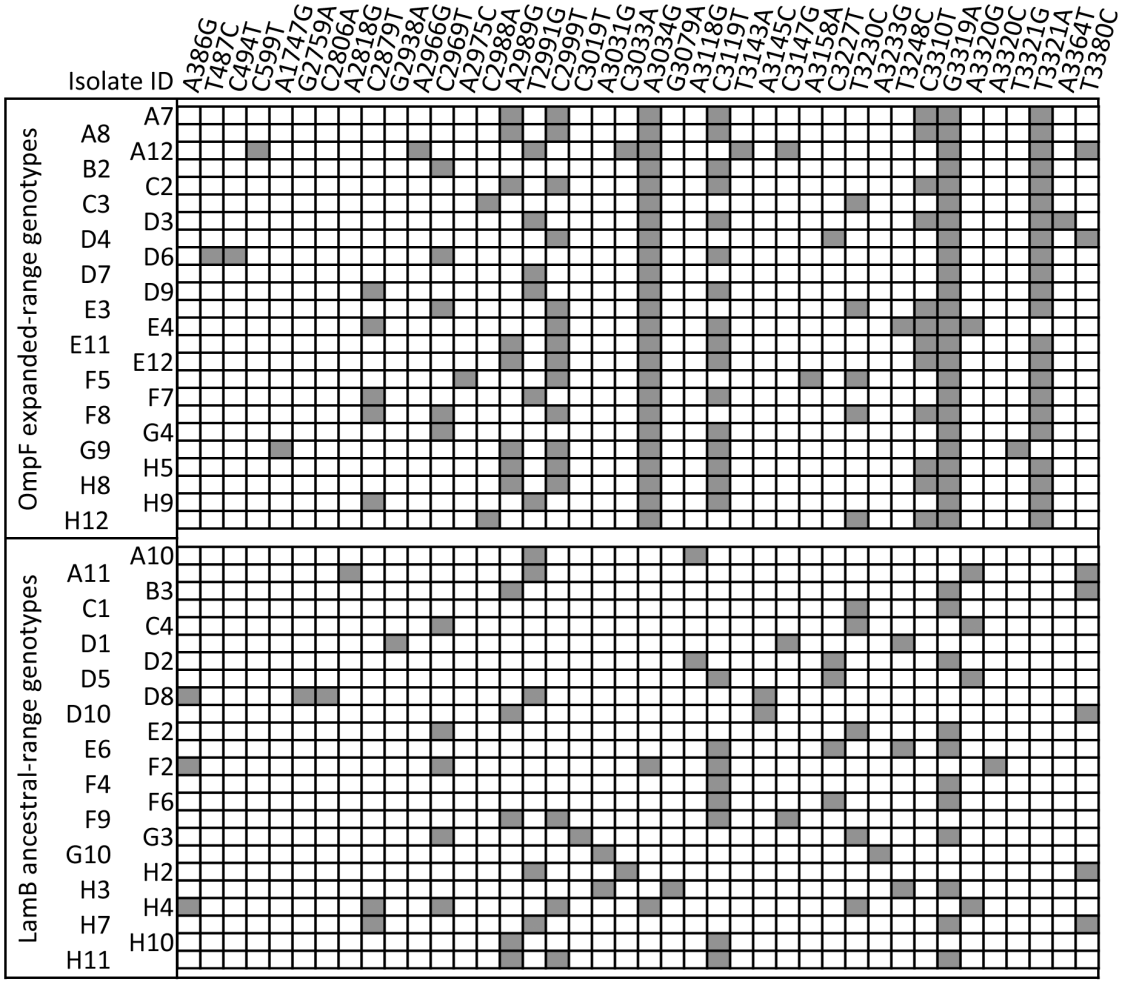

Fig. 3. Mutations affecting the J protein in phage isolates from 48 independent populations of the large-scale experiment. Isolates are shown in rows (with alternate labels offset for readability) and mutations in columns; gray fill indicates that an isolate has the mutation. The top 24 rows show phage isolates that can target the new OmpF receptor; the bottom 24 rows show phage that remain dependent on LamB.

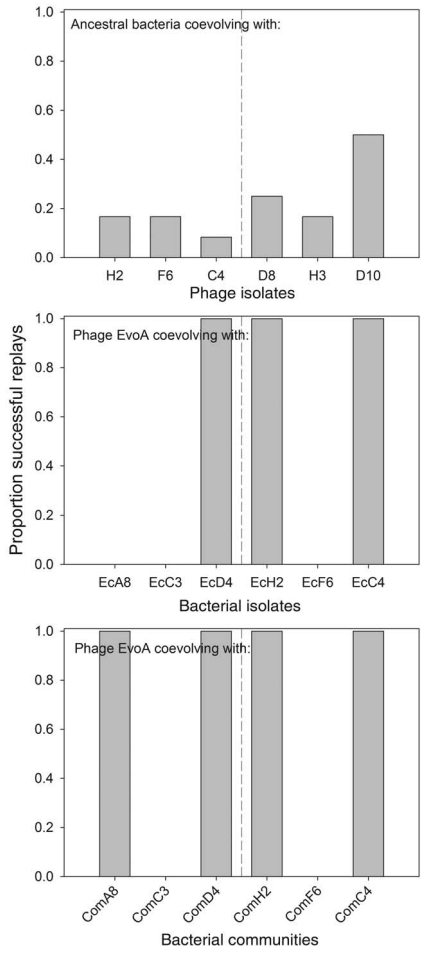

Fig. 4. Replay experiments using different combinations of phage and bacteria. For each panel, the *y* axis shows the proportion of replicate replays that produced phage able to target the new OmpF receptor. (**Top**) Replays were initiated with the ancestral bacteria and six phage isolates. Each combination was replicated 12-fold. Three of the phage (H2, F6, and C4) had mutations shared by multiple lineages that evolved the capacity to target OmpF in the large-scale experiment. The other three (D8, H3, and D10) had mutations that were never observed in phage that targeted OmpF. The latter mutations were candidates for impeding the evolution of the new function, but that hypothesis was not supported. (**Middle**) Replays were initiated with phage EvoA (which needs only one more mutation to use OmpF) and six bacterial clones. Each combination was replicated fourfold. Three clones (EcA8, EcC3, and EcD4) were isolated from flasks in which phage evolved the capacity to target OmpF in the large-scale experiment. The other three (EcH2, EcF6, and EcC4) came from flasks in which phage did not evolve that function. The replay outcomes did not support these categories, but sequencing the bacterial genomes identified mutations that uniquely determined whether the phage would evolve the OmpF function. See text for details. (**Bottom**) Replays were initiated using the same phage and bacteria used in the middle panel, except with full bacterial communities rather than individual clones. Each combination was replicated 12-fold. The different outcomes for one bacterial clone (EcA8, middle) and its source community (ComA8, bottom) show the effect of bacterial diversity on phage evolution.

For the bacteria, we used six clones: three from communities where λ evolved to use OmpF, and three where the phage retained their dependence on LamB. We observed a striking "all-or-none" pattern of outcomes, although not in accordance with our categories (Fig. 4). In particular, all 36 λ populations evolved the final mutation required to use OmpF in communities with three bacterial clones, whereas none of the phage evolved that ability in 36 communities with the three other clones. For two of the latter class (EcA8 and EcF6), the phage were unable to reproduce and went extinct; in the other case (EcC3), phage persisted but none of the replays yielded phage able to use OmpF. It is clear that bacterial characteristics determined whether the phage would evolve the new receptor function. However, the bacteria that promoted that outcome did not necessarily come from communities in which λ had previously evolved to exploit OmpF.

We sequenced the full genomes of these six bacteria to identify the mutations responsible for the differences in phage evolution. The six genomes harbor a total of 15 mutations (table S5). Five have similar deletions that affect the *rbs* operon, which confers the ability to grow on ribose; previous work has shown that these deletions occur at an unusually high rate owing to a nearby insertion-sequence element in the ancestral strain (*37*). All 10 other mutations are directly related to the interaction with λ. As expected, all six genomes have mutations in *malT* that confer resistance to the ancestral phage, as described earlier. One genome from a community where λ evolved to use the OmpF receptor has a nonsynonymous mutation in the *ompF* gene, which might confer partial resistance to the evolved phage. The three remaining mutations disrupt *manY* or *manZ*, and they uniquely differentiate the three strains that prevented phage from evolving to use OmpF from the three strains that allowed that change (table S5). The *manY* and *manZ* genes encode the transmembrane channel of the ManXYZ mannose permease, which is required for λ DNA to cross the inner membrane (*38*–*40*). These mutations thus confer resistance by blocking a later step during infection, and they would render ineffective any phage mutations that altered the receptor function. Therefore, the evolution of phage that target OmpF is promoted by bacterial mutations in *malT* but impeded by mutations in *manYZ*, which indicated contingency dependent on the host-parasite coevolution.

After discovering the *manYZ* mutations, we screened all 96 bacterial populations from the large-scale experiment to determine how many harbored mannose-deficient mutants that would block λ infections (*16*). At least 80 populations (table S6) had such mutations, including many from communities in which λ evolved the ability to exploit the OmpF receptor. However, these mutations rarely fixed; instead, susceptible subpopulations persisted in 77 of the 80 communities that allowed the phage to continue to evolve. This finding suggests a complex interplay between coevolving phage and bacteria, one that depends on the entire community and its diversity. To test this hypothesis, we repeated the second replay experiment using the same phage and bacteria, except with bacterial communities instead of clones (*16*). Once again, some bacteria consistently impeded the evolution of phage that used OmpF while others consistently promoted that change (Fig. 4, bottom). Moreover, those outcomes differed for one clone (EcA8) (Fig. 4, middle) and its community (ComA8) (Fig. 4, bottom), which confirmed the effect of bacterial diversity on the phage's evolution.

Repeatability, contingency, and the evolution of a key innovation. Phage λ often, but not always, evolved the ability to infect its *E. coli* hosts by targeting a new receptor, OmpF. Figure 5 summarizes the important steps in this process, including some that promoted this key innovation and others that impeded it. The fact that several mutations are required for λ to use OmpF may explain why no previous studies have reported this change, despite decades of intense study of this phage. However, by running evolution experiments rather than mutational screens, we observed 25 cases in which this new function evolved. Our experiments are not the first to demonstrate evolutionary transitions in viruses (*13*, *41*–*43*); one study found that a single mutation allowed phage φ6 to infect a new host

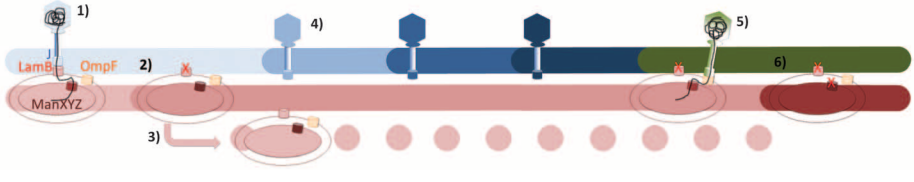

Fig. 5. Steps in the coevolution of phage λ and its *E. coli* host leading to the phage's ability to target a new receptor, OmpF. 1) The ancestral phage targets the LamB porin using the J protein and injects its DNA into the periplasm, then the DNA is transported into the cytoplasm via the ManXYZ permease. 2) The bacteria evolve resistance by mutations in *malT*, a positive regulator of LamB expression. 3) However, spontaneous inductions of LamB generate a subpopulation of phenotypically sensitive cells that can sustain the phage population. 4) The phage evolves mutations in the J protein that improve performance on the LamB receptor. Some of these mutations are also required for the phage to target OmpF. 5) The phage eventually evolves the four mutations that enable it to use OmpF. 6) However, the bacteria may evolve additional resistance by mutations in *manY* or *manZ* that prevent transport of the phage DNA into the cytoplasm. When these mutants become sufficiently common, there is little or no benefit to mutant phage that can use OmpF.

species (*13*), and a study of phage SBW25Φ2 showed that coevolution experiments were more effective at producing host-range shifts than were screens using new hosts (*43*). The rich and complex dynamics of coevolving species may thus sometimes facilitate key innovations (*6*, *44*).

More generally, our study shows some of the challenges that make it difficult to observe and explain the origin of many new functions, including the requirement for multiple mutations, the complex interactions of mutations within and between species, and the resulting historical contingency that can enable or impede the outcome of interest depending on the order in which mutations occur. The "all-or-none" epistasis among the four canonical phage mutations implies that it would have been unlikely for the new function to evolve on the scale of our experiments, except for the lucky fact that some of the mutations were beneficial to the phage in performing their current function, thereby pushing evolution toward the new function. The mutations in the bacteria, and how they influenced the phage's evolution, were also important. In particular, the initial resistance mutation generated a physiological subpopulation of hosts that allowed the phage to persist and adapt to the original receptor and, thereby, to accumulate the required mutations. Yet, as fortuitous as these circumstances were, another mutation could—and often did—derail the emergence of the new function: Namely, a mutation that conferred an alternative mode of host resistance eliminated the advantage to the phage of targeting the OmpF receptor. The interactions between bacteria and phage, which contributed so much to the development of microbial genetics and molecular biology (*45*, *46*), continue to serve as powerful models to study ecology and evolution (*47*, *48*).

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Supporting Online Material

www.sciencemag.org/cgi/content/full/335/6067/428/DC1
Materials and Methods
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Crystal Structure of the Human Two–Pore Domain Potassium Channel K2P1

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Two–pore domain potassium (K^+) channels (K2P channels) control the negative resting potential of eukaryotic cells and regulate cell excitability by conducting K^+ ions across the plasma membrane. Here, we present the 3.4 angstrom resolution crystal structure of a human K2P channel, K2P1 (TWIK-1). Unlike other K^+ channel structures, K2P1 is dimeric. An extracellular cap domain located above the selectivity filter forms an ion pathway in which K^+ ions flow through side portals. Openings within the transmembrane region expose the pore to the lipid bilayer and are filled with electron density attributable to alkyl chains. An interfacial helix appears structurally poised to affect gating. The structure lays a foundation to further investigate how K2P channels are regulated by diverse stimuli.

The high resting permeability of the plasma membrane to potassium ions (K^+) was appreciated for nearly 50 years (*1*–*3*) before these "background" K^+ currents were partially attributed to a family of two–pore domain K^+ (K2P) channels (*4*). A total of 15 K2P channels in humans can be divided into 6 K2P subfamilies based on sequence identity and functional characteristics: TWIK, TREK, TASK, TALK, THIK, and TRESK (*4*). Under normal physiological conditions, the K^+ gradient across the cellular membrane (~150 mM inside and ~5 mM outside) causes the net efflux of K^+ ions through K2P channels, which stabilizes the negative electrical potential (resting potential) of the cell (*4*). Expression of the K2P channel K2P1 (TWIK-1) has been detected in several tissues and plays important roles in excitable cells in the heart and brain (*5*–*7*). K2P1 has the unusual property that it becomes permeable to sodium ions (Na^+) under physiological conditions when the extracellular

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Science

Repeatability and Contingency in the Evolution of a Key Innovation in Phage Lambda

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Natural Selection Caught in the Act

Understanding how new functions evolve has been of long-standing interest. However, the number of mutations needed to evolve a key innovation is rarely known, or whether other sets of mutations would also suffice, whether the intermediate steps are driven by natural selection, or how contingent the outcome is on steps along the way. **Meyer *et al.*** (p. 428; see the Perspective by **Thompson**) answer these questions for a case in which phage lambda evolved the ability to infect its host *Escherichia coli* through a novel receptor. This shift required four mutations, which accumulated under natural selection in concert with coevolution of the host. However, when **Tenaillon *et al.*** (p. 457) exposed 115 lines of *E. coli* to high temperature and sequenced them, adaptation occurred through many different genetic paths, showing parallelism at the level of genes and interacting protein complexes, but only rarely at the nucleotide level. Thus, epistasis—nonadditive genetic interaction—is likely to play an important part in the process of adaptation to this environment.