Cite as: J. R. Meyer *et al.*, *Science* 10.1126/science.aai8446 (2016).

Ecological speciation of bacteriophage lambda in allopatry and sympatry

Justin R. Meyer,[1]* Devin T. Dobias,[2,3] Sarah J. Medina,[1] Lisa Servilio,[1] Animesh Gupta,[4] Richard E. Lenski[5]

[1]Division of Biology, University of California San Diego, La Jolla, CA 92093, USA. [2]Department of Biology, Washington University, St. Louis, MO 63130, USA. [3]Department of Biology, Loyola University Chicago, 1032 W. Sheridan Road, Chicago, IL 60660, USA. [4]Department of Physics, University of California San Diego, La Jolla, CA 92093, USA. [5]Department of Microbiology and Molecular Genetics, and BEACON Center for the Study of Evolution in Action, Michigan State University, East Lansing, MI 48824, USA.

*Corresponding author. Email: justin.raymond.meyer@gmail.com

Understanding the conditions that allow speciation to occur is difficult because most research has focused on either long-lived organisms or asexual microorganisms. We propagated bacteriophage λ, a virus with rapid generations and frequent recombination, on two *Escherichia coli* host genotypes that expressed either the LamB or OmpF receptor. When supplied with either single host (allopatry), λ improved its binding to the available receptor while losing its ability to use the alternative. When evolving on both hosts together (sympatry), the viruses split into two lineages with divergent receptor preferences. Although the level of divergence varied among replicates, some lineages evolved reproductive isolation via genetic incompatibilities. This outcome indicates that, under suitable conditions, allopatric and sympatric speciation can occur with similar ease.

Studies of extant and extinct species provide evidence that biological evolution can promote diversification rather than mere replacement of one form by another (*1*). Moreover, asexual microbes frequently diversify when cultured in the laboratory (*2*), even in simple conditions that would seem to favor homogeneity (*3*). Sympatric diversification in sexual populations has long been thought to be difficult because constant recombination prevents an interbreeding population from splitting into genetically distinct lineages that occupy different ecological niches (*4*, *5*). However, putative examples of sympatric speciation have advanced the argument that speciation in a recombining population may occur under certain circumstances (*6*, *7*), although other studies have disputed these claims (*8*). This debate is important because the process of speciation is fundamental to biological evolution.

To shed light on this question, we conducted evolution experiments with viruses to examine the effect of recombination on their divergence into distinct ecological niches. We studied a virulent (i.e., strictly lytic) derivative of phage λ, which infects *Escherichia coli*. Most λ can use only a single outer-membrane protein, LamB, as a receptor, but the strain we studied, EvoC, can also exploit a second receptor, OmpF (*9*). The ability to use OmpF arose via five point mutations in the host-recognition gene, *J*. To examine λ diversification, we propagated virus populations on one or both of two strains of *E. coli* that differ only in whether they possess the *lamB* or *ompF* genes, which encode LamB and OmpF, respectively (*10*). Phages tend to exploit specific receptors (*11*), leading us to predict that populations of the generalist phage EvoC would evolve toward increased receptor specificity. Whether or not two receptor specialists evolved, however, would likely also depend on the effects of recombination. When two or more λ viruses infect the same cell, their genomes can recombine (*12*).

We use λ as a model for analyzing the mechanisms of speciation. Of course, viruses are diverse (*13*), and their rates and mechanisms of speciation will depend on their life histories, just as the rates and mechanisms vary in plants and animals. However, some salient features of speciation are shared even across such distant taxa. For example, viruses that infect the same species and cell types are thought to have evolved mechanisms to limit recombination, including divergences in nucleotide composition and RNA structure that are analogous to pre-zygotic barriers in plants and animals (*14*–*16*). One feature of our system that might promote sympatric speciation is the connection between the viruses' ecological niches (i.e., host cells) and where genetic exchange occurs. Specialists that use the same receptor will naturally tend to recombine because they are more likely to infect the same host. Therefore, reproductive isolation can evolve as a byproduct of ecological divergence, without requiring other mutations that govern recombination. Such dual-effect mutations have been described in other systems and are important because their simultaneous effects on ecological niches and mating propensities promote speciation when resources are patchily distributed (*17*–*19*).

We propagated six initially homogeneous and identical populations of λ strain EvoC for 35 cycles of dilution into host populations containing an equal mix of genotypes ex-

pressing either the LamB or OmpF receptor (*20*). The high initial ratio of viruses (~10^8) to bacteria (~2×10^6) increases the opportunity for co-infection and recombination. After 8 hours, surviving bacteria were killed to stop the host population from evolving resistance, and a new cycle of viral reproduction was initiated by transferring 1% of the phage into a fresh population of naive bacteria. Twelve other phage populations were also cultured with a single host type (half expressing only LamB, and half expressing only OmpF). We describe these single-host populations as allopatric because the separate flasks prevent viruses that use different hosts from recombining at any point in time. We call the mixed-host treatments sympatric because viruses that infect the different host types in one generation may infect the same host and recombine in the next generation. One caveat to this terminology is that viral recombination can only occur within host cells, thereby introducing a subtle fine-scale spatial element to the sympatric treatment.

Strong receptor specialization evolved in all 12 single-host populations (Fig. 1). All six phage populations (7-12) that evolved with only the LamB-expressing hosts could no longer produce plaques on OmpF cell lawns (*20*). Five of six populations (1-5) propagated on only the OmpF-expressing cells evolved similar specialization; one population (6) continued to produce plaques on LamB hosts, but at lower levels than on OmpF hosts. The ancestral phage EvoC produced plaques on both hosts, although more on the type expressing the LamB receptor (Fig. 1).

To determine whether phage performance on the two hosts also diverged when they evolved on mixed hosts, we isolated six phages that produced plaques on LamB hosts and six on OmpF hosts from each sympatric-treatment population. We performed the same assay on these 72 isolates as on the single-host populations (Fig. 1). Four populations (13, 14, 17, 18) evolved both OmpF and LamB specialists, although most were not as specialized as those that evolved in allopatry. Two populations (15, 16) contained a mixture of LamB specialists and generalists that, like the ancestral phage, could infect both LamB and OmpF hosts. Therefore, diversification occurred in sympatry as well as in allopatry.

Models of adaptive radiations indicate that ecological speciation requires a tradeoff in the ability to use different resources (*21*). Moreover, the relation must be convex, such that a diminished ability to use one resource is outweighed by an improvement on the alternative. We measured the adsorption rates for the ancestor and two isolates from each sympatric population, one isolated on OmpF-only cells, and the second on LamB-only cells (Fig. 2). In all six cases, the ancestral values fell below the line connecting the evolved isolates (sign test, one-tailed $P = 0.0156$). This pattern indicates that mutations that increased specialization provide greater gains in adsorption rate on the focal receptor than losses on the alternative one. Natural selection should, all else equal, favor specialization and drive diversification. Adsorption rate is an important component of phage fitness, but not every adsorption event necessarily leads to a productive infection. Nonetheless, the correlation in receptor specialization based on adsorption rates and plaque formation is highly significant (*20*) (fig. S1).

We sequenced the host-recognition gene, *J*, from one phage isolated on each host from the mixed-host populations, one from each OmpF-only population, and one from five of the six LamB-only populations (*20*). (Population 8 was not represented because the sample failed to preserve.) In total, we found 67 substitutions at 28 different positions. All were nonsynonymous and in the reactive region of the host-recognition J protein (*22*), indicating that they were likely adaptive (Fig. 3A). The LamB specialists had fewer mutations than OmpF specialists (means of 1.41 and 4.54, respectively; $t = 9.93$, df = 21, two-tailed $P < 0.0001$). The fewer mutations in the LamB specialists is unsurprising, given that the most recent ancestor (EvoC) favored LamB, and the ancestor of EvoC (λ strain cI26) only used LamB. In general, the evolved LamB specialists did not reacquire that preference via reversion; only one of the 12 sequenced alleles reverted at a single site of the 5 that distinguish EvoC from cI26 (a2988c in population 17). Only 2 sites had mutations in both LamB and OmpF specialists, whereas 10 sites had parallel substitutions across independently evolved specialists of the same type.

A cluster analysis performed on the basis of the genetic distance among *J* alleles shows the different pattern of substitutions between the LamB and OmpF specialists (*20*) (ANOSIM $R = 0.659$, $P = 0.0001$, Fig. 3B). Phage isolated on the same host genotype but from different flasks had more similar *J* alleles than phage isolated from the same flask but specialized on different hosts. By contrast, there are no compelling differences between phage specialized on the same host type, but evolved in allopatry versus sympatry (*20*) (ANOSIM $R = 0.075$, $P = 0.1336$). These patterns indicate that selection in the two-host sympatry treatment drove the generalist phage populations to diverge into two subpopulations with distinctive genomes and preferences for the different receptors.

Another layer of reproductive isolation may arise along with host preference, if the mutations for LamB and OmpF specialization are incompatible. The Dobzhansky-Muller model predicts that speciation will occur if hybrids have lower fitness than their parents, reducing effective gene flow (*23*). To examine this possibility, we focused on population 18, where the LamB and OmpF specialists had 1 and 4 mutations, respectively, in the *J* gene (Fig. 3A). Using Multiplexed Automated Genome Engineering (MAGE) (*20*, *24*) to edit a lysogenic strain of phage, cI857, we constructed alleles cor-

responding to the ancestral EvoC strain, the LamB specialist, the OmpF specialist, and a hybrid with all 5 mutations from the two specialists (Fig. 4, A and B). As expected, the constructed strains with the evolved alleles were specialists and the one with the EvoC allele was a generalist (Fig. 4C). These observations indicate that the mutations in *J* are responsible for most, if not all, of the divergence in receptor preferences. Whole-genome sequencing of the specialists 18O and 18L revealed four additional mutations, but they occur in genes unlikely to affect interactions with the host receptor (*20*). Sympatric speciation is more feasible when a single gene has a large effect on ecological differentiation because it reduces the extent to which recombination could prevent divergence. Other studies of newly formed species have also found that variation in one or a few genomic regions accounts for most reproductive isolation (*25*).

Our first attempt to construct a hybrid between the LamB and OmpF specialists produced no viable phage, suggesting the hybrid might be nonviable. Our second attempt, however, produced a few infectious phage. Given this discrepancy, we hypothesized that the viable phage may have resulted from reversion or compensatory mutations that occurred during the MAGE procedure. To test this, we sequenced the reactive region of *J* for five randomly chosen hybrids. All five had reverted the a2996t mutation to the ancestral state, removing the single LamB-specialization mutation and thereby recreating the OmpF-specialized allele. Thus, we found no viable true hybrids, providing evidence that the mutations that encode OmpF and LamB specialization are incompatible.

Our results show that λ likely evolved two mechanisms of reproductive isolation: (i) divergent host use leading to positive assortative recombination and (ii) genetic incompatibilities. These barriers satisfy the criteria for the Biological Species Concept (*5*, *26*). The distinctive ecological niches and genomic changes in the specialist phages also fulfill some other species definitions (*27*, *28*). However, the specialists fall far short of a widely used criterion in phage taxonomy, namely <70% sequence similarity (*28*). We take this discrepancy to mean that we have observed the essential processes that can lead to speciation, but they have not operated long enough for the specialists to be classified as distinct species.

Four features of the experimental system contributed to the success of sympatric speciation and the speed with which it occurred. First, the convex tradeoff between exploiting the two hosts set the stage for specialists to evolve. Second, the homogenizing effect of recombination was limited because a few mutations in a single gene accomplished this specialization. Third, the connection between host use and reproduction generated a barrier to gene flow as a by-product of natural selection. Fourth, the mutations that improved use of the alternative receptors were incompatible, preventing gene flow between the lineages. Determining how widespread these features are in nature remains an important empirical challenge. Understanding which features, if any, can be relaxed without disrupting speciation is both a theoretical and empirical challenge.

REFERENCES AND NOTES

1. D. Schluter, *The Ecology of Adaptive Radiation* (Oxford Univ. Press, 2000).
2. R. Kassen, *Experimental Evolution and the Nature of Biodiversity* (Roberts, 2014).
3. P. B. Rainey, M. Travisano, Adaptive radiation in a heterogeneous environment. *Nature* **394**, 69–72 (1998). doi:10.1038/27900 Medline
4. S. Gavrilets, Models of speciation: Where are we now? *J. Hered.* **105** (suppl. 1), 743–755 (2014). doi:10.1093/jhered/esu045 Medline
5. E. Mayr, *Animal Species and Evolution* (Belknap Press, 1963).
6. M. Barluenga, K. N. Stölting, W. Salzburger, M. Muschick, A. Meyer, Sympatric speciation in Nicaraguan crater lake cichlid fish. *Nature* **439**, 719–723 (2006). doi:10.1038/nature04325 Medline
7. J. L. Feder, C. A. Chilcote, G. L. Bush, Genetic differentiation between sympatric host races of the apple maggot fly *Rhagoletis pomonella*. *Nature* **336**, 61–64 (1988). doi:10.1038/336061a0
8. C. H. Martin, J. S. Cutler, J. P. Friel, C. Dening Touokong, G. Coop, P. C. Wainwright, Complex histories of repeated gene flow in Cameroon crater lake cichlids cast doubt on one of the clearest examples of sympatric speciation. *Evolution* **69**, 1406–1422 (2015). doi:10.1111/evo.12674 Medline
9. J. R. Meyer, D. T. Dobias, J. S. Weitz, J. E. Barrick, R. T. Quick, R. E. Lenski, Repeatability and contingency in the evolution of a key innovation in phage lambda. *Science* **335**, 428–432 (2012). doi:10.1126/science.1214449 Medline
10. T. Baba, T. Ara, M. Hasegawa, Y. Takai, Y. Okumura, M. Baba, K. A. Datsenko, M. Tomita, B. L. Wanner, H. Mori, Construction of *Escherichia coli* K-12 in-frame, single-gene knockout mutants: The Keio collection. *Mol. Syst. Biol.* **2**, 0008 (2006). doi:10.1038/msb4100050 Medline
11. A. A. Lindberg, Bacteriophage receptors. *Annu. Rev. Microbiol.* **27**, 205–241 (1973). doi:10.1146/annurev.mi.27.100173.001225 Medline
12. A. R. Poteete, What makes the bacteriophage λ Red system useful for genetic engineering: Molecular mechanism and biological function. *FEMS Microbiol. Let.* **201**, 9–14 (2001). doi:10.1111/j.1574-6968.2001.tb10725.x Medline
13. A. T. Peterson, Defining viral species: Making taxonomy useful. *Virol. J.* **11**, 131 (2014). doi:10.1186/1743-422X-11-131 Medline
14. G. A. Schachtel, P. Bucher, E. S. Mocarski, B. E. Blaisdell, S. Karlin, Evidence for selective evolution in codon usage in conserved amino acid segments of human alphaherpesvirus proteins. *J. Mol. Evol.* **33**, 483–494 (1991). doi:10.1007/BF02102801 Medline
15. E. C. Bronson, J. N. Anderson, Nucleotide composition as a driving force in the evolution of retroviruses. *J. Mol. Evol.* **38**, 506–532 (1994). doi:10.1007/BF00178851 Medline
16. D. R. Forsdyke, Implications of HIV RNA structure for recombination, speciation, and the neutralism-selectionism controversy. *Microbes Infect.* **16**, 96–103 (2014). doi:10.1016/j.micinf.2013.10.017 Medline
17. S. Duffy, C. L. Burch, P. E. Turner, Evolution of host specificity drives reproductive isolation among RNA viruses. *Evolution* **61**, 2614–2622 (2007). doi:10.1111/j.1558-5646.2007.00226.x Medline
18. M. R. Servedio, G. S. Van Doorn, M. Kopp, A. M. Frame, P. Nosil, Magic traits in speciation: 'Magic' but not rare? *Trends Ecol. Evol.* **26**, 389–397 (2011). doi:10.1016/j.tree.2011.04.005 Medline
19. K. E. Filchak, J. B. Roethele, J. L. Feder, Natural selection and sympatric divergence in the apple maggot *Rhagoletis pomonella*. *Nature* **407**, 739–742 (2000). doi:10.1038/35037578 Medline
20. Materials and methods and supplementary text are available as supporting material on *Science* Online.
21. M. Doebeli, *Adaptive Diversification* (Princeton Univ. Press, 2011).
22. S. Chatterjee, E. Rothenberg, Interaction of bacteriophage λ with its *E. coli* receptor, LamB. *Viruses* **4**, 3162–3178 (2012). doi:10.3390/v4113162 Medline

23. D. R. Matute, I. A. Butler, D. A. Turissini, J. A. Coyne, A test of the snowball theory for the rate of evolution of hybrid incompatibilities. *Science* **329**, 1518–1521 (2010). doi:10.1126/science.1193440 Medline
24. H. H. Wang, F. J. Isaacs, P. A. Carr, Z. Z. Sun, G. Xu, C. R. Forest, G. M. Church, Programming cells by multiplex genome engineering and accelerated evolution. *Nature* **460**, 894–898 (2009). doi:10.1038/nature08187 Medline
25. H. D. Bradshaw Jr., S. M. Wilbert, K. G. Otto, D. W. Schemske, Genetic mapping of floral traits associated with reproductive isolation in monkeyflowers (*Mimulus*). *Nature* **376**, 762–765 (1995). doi:10.1038/376762a0
26. Q. Wheeler, R. Meier, *Species Concepts and Phylogenetic Theory: A Debate* (Columbia Univ. Press, 2000).
27. D. J. Howard, S. H. Berlocher, *Endless Forms: Species and Speciation* (Oxford Univ. Press, 1998).
28. M. Achtman, M. Wagner, Microbial diversity and the genetic nature of microbial species. *Nat. Rev. Microbiol.* **6**, 431–440 (2008). Medline
29. R. W. Hendrix, *Lambda II* (Cold Spring Harbor Laboratory Press, 1983).
30. F. W. Studier, P. Daegelen, R. E. Lenski, S. Maslov, J. F. Kim, Understanding the differences between genome sequences of *Escherichia coli* B strains REL606 and BL21(DE3) and comparison of the *E. coli* B and K-12 genomes. *J. Mol. Biol.* **394**, 653–680 (2009). doi:10.1016/j.jmb.2009.09.021 Medline
31. E. Crozat, T. Hindré, L. Kühn, J. Garin, R. E. Lenski, D. Schneider, Altered regulation of the OmpF porin by Fis in *Escherichia coli* during an evolution experiment and between B and K-12 strains. *J. Bacteriol.* **193**, 429–440 (2011). doi:10.1128/JB.01341-10 Medline
32. K. A. Datsenko, B. L. Wanner, One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc. Natl. Acad. Sci. U.S.A.* **97**, 6640–6645 (2000). doi:10.1073/pnas.120163297 Medline
33. J. Sambrook, D. W. Russell, *Molecular Cloning: A Laboratory Manual*, 3rd edn. (Cold Spring Harbor Laboratory Press, 2001).
34. J. Weitz, *Quantitative Viral Ecology: Dynamics of Viruses and Their Microbial Hosts* (Princeton Univ. Press, 2015).
35. Ø. Hammer, D. A. T. Harper, P. D. Ryan, PAST: paleontological statistical software package for education and data analysis. *Palaeontol. Electron.* **4**, 1–9 (2001). http://palaeo-electronica.org/2001_1/past/issue1_01.htm
36. B. S. Powell, D. L. Court, Y. Nakamura, M. P. Rivas, C. L. Turnbough Jr., Rapid confirmation of single copy lambda prophage integration by PCR. *Nucleic Acids Res.* **22**, 5765–5766 (1994). doi:10.1093/nar/22.25.5765 Medline
37. H. H. Wang, H. Kim, L. Cong, J. Jeong, D. Bang, G. M. Church, Genome-scale promoter engineering by coselection MAGE. *Nat. Methods* **9**, 591–593 (2012). doi:10.1038/nmeth.1971 Medline
38. M. Baym, S. Kryazhimskiy, T. D. Lieberman, H. Chung, M. M. Desai, R. Kishony, Inexpensive multiplexed library preparation for megabase-sized genomes. *PLOS ONE* **10**, e0128036 (2015). doi:10.1371/journal.pone.0128036 Medline
39. D. E. Deatherage, J. E. Barrick, Identification of mutations in laboratory-evolved microbes from next-generation sequencing data using *breseq*. *Methods Mol. Biol.* **1151**, 165–188 (2014). doi:10.1007/978-1-4939-0554-6_12 Medline

ACKNOWLEDGMENTS

The work was supported in part by the BEACON Center for the Study of Evolution in Action (NSF Cooperative Agreement DBI-0939454). We thank N. Hajela for help in the lab, I.-N. Wang and H. Wang for materials, and the UCSD IGM Genomics Center for genome sequencing. Sequence reads are available at the National Center for Biotechnology Information (accession number SRP093504) and other data at the Dryad Digital Repository (doi:10.5061/dryad.4qb79). Correspondence and requests for materials should be addressed to J.R.M. (justin.raymond.meyer@gmail.com). Sharing of biological material requires a Material Transfer Agreement, described at http://blink.ucsd.edu/research/conducting-research/mta/index.html.

SUPPLEMENTARY MATERIALS

www.sciencemag.org/cgi/content/full/science.aai8446/DC1
Materials and Methods
Supplementary Text
Fig. S1
Tables S1 and S2
References (*29*–*39*)

19 August 2016; accepted 9 November 2016
Published online 24 November 2016
10.1126/science.aai8446

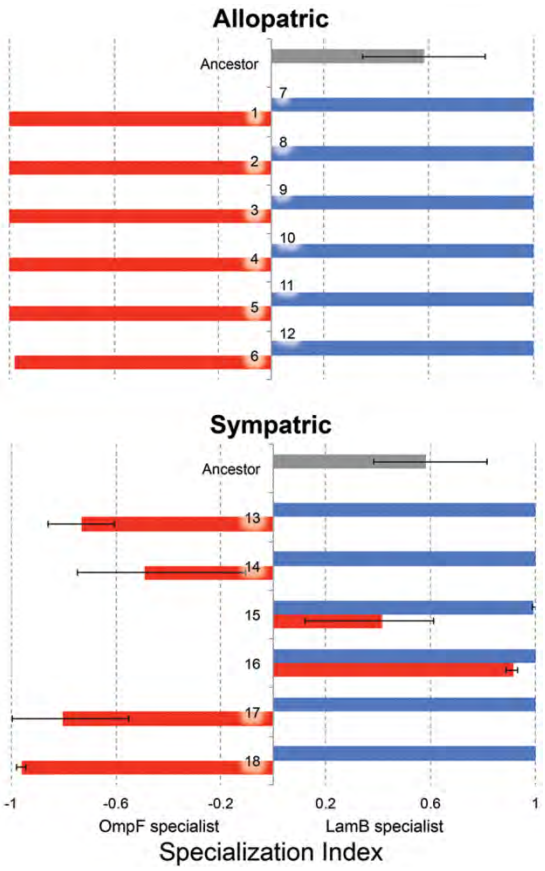

Fig. 1. Specialization in allopatry and sympatry. Phage λ populations 1–6 evolved with OmpF-only host cells, populations 7–12 with LamB-only cells, and populations 13–18 with both types. Specialization Index = ($plaques_{LamB}$ – $plaques_{OmpF}$)/($plaques_{LamB}$ + $plaques_{OmpF}$). Index values of 1 and –1 indicate complete LamB and OmpF specialization, respectively. Values for populations 1–12 were measured using samples of the entire λ population. Using whole-population samples for 13–18 would hide the underlying diversity of specialists, so each bar shows the mean of six phage isolated on either OmpF-only (red bars) or LamB-only (blue bars) hosts. Error bars are 95% confidence intervals.

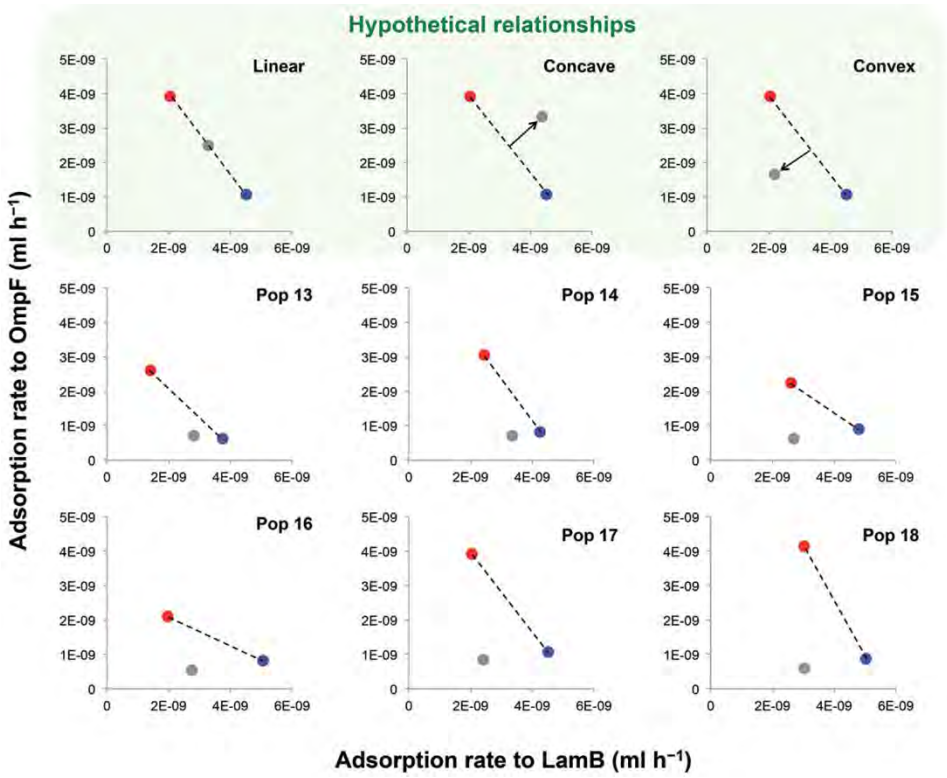

Fig. 2. Adsorption rates for two isolates from each sympatric population and the ancestor. The top panels show hypothetical locations of the ancestor relative to evolved isolates. The six panels below show the data for each population that evolved on mixed hosts. Red symbols indicate phages isolated on OmpF-only hosts, blue symbols indicate phages isolated on LamB-only hosts, and grey symbols show the ancestor (EvoC). The ancestral rates were separately measured for each panel to ensure independence. See text for statistical analysis.

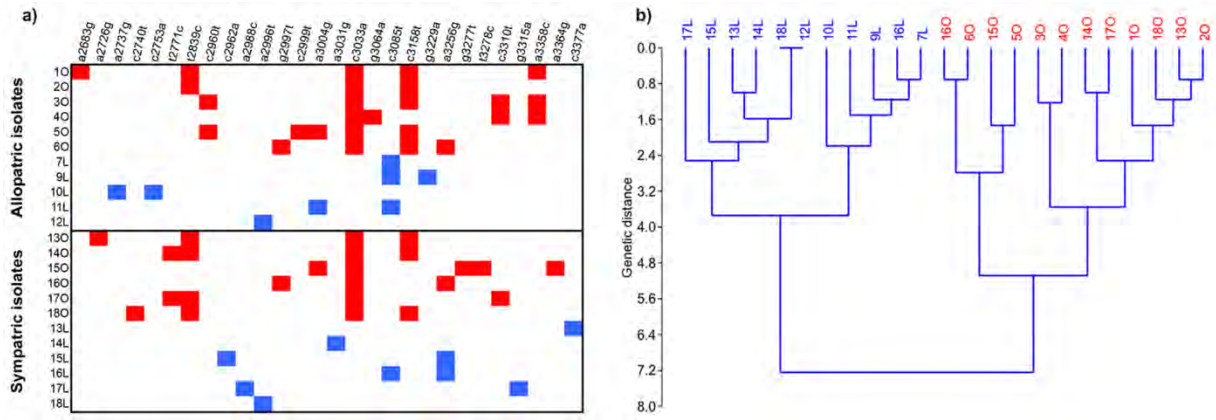

Fig. 3. Genetic signatures of ecological diversification. (**A**) Substitutions in *J* gene from λ isolated from allopatric and sympatric treatments. Rows are λ isolates; columns are mutations. Red and blue fill shows mutations in phage isolated on OmpF-only and LamB-only hosts, respectively. (**B**) Cluster analysis using Ward's method separates alleles from independently evolved phage isolated on OmpF-only and LamB-only hosts, regardless of allopatric or sympatric treatment.

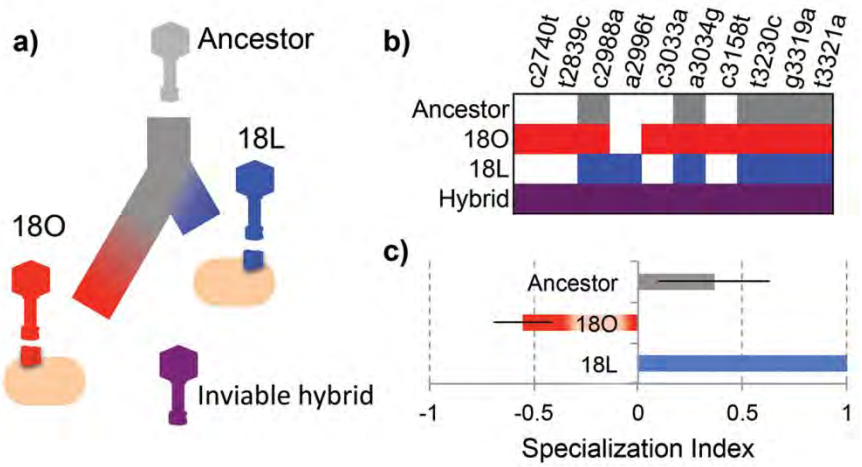

Fig. 4. Phage genotypes engineered to test interaction of mutations from OmpF and LamB specialists. (A) Schematic of phage diversification in population 18 and the engineered hybrid of the two specialists. (B) Engineered *J* alleles. Colored fill shows mutations in engineered phage relative to the lysogenic phage background, cI857, used to engineer the four alleles shown. (C) Specialization index from plaque assays for the ancestor and two engineered specialists; the engineered hybrid with 1 LamB- and 4 OmpF-specific mutations did not produce plaques on either host.