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Pleiotropic Costs of Niche Expansion in the RNA Bacteriophage Φ6

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ABSTRACT

Natural and experimental systems have failed to universally demonstrate a trade-off between generalism and specialism. When a trade-off does occur it is difficult to attribute its cause to antagonistic pleiotropy without dissecting the genetic basis of adaptation, and few previous experiments provide these genetic data. Here we investigate the evolution of expanded host range (generalism) in the RNA virus Φ6, an experimental model system allowing adaptive mutations to be readily identified. We isolated 10 spontaneous host range mutants on each of three novel Pseudomonas hosts and determined whether these mutations imposed fitness costs on the standard laboratory host. Sequencing revealed that each mutant had one of nine nonsynonymous mutations in the Φ6 gene P3, important in host attachment. Seven of these nine mutations were costly on the original host, confirming the existence of antagonistic pleiotropy. In addition to this genetically imposed cost, we identified an epigenetic cost of generalism that occurs when phage transition between host types. Our results confirm the existence in Φ6 of two costs of generalism, genetic and environmental, but they also indicate that the cost is not always large. The possibility for cost-free niche expansion implies that varied ecological conditions may favor host shifts in RNA viruses.

ECOLOGISTS and evolutionary biologists have long sought to explain and predict the evolution of generalist and specialist populations (Wilson and Yoshimura 1994). Specialization (*i.e.*, using a relatively narrow niche) reduces competition and facilitates contact with suitable mates, but also confines the population to a small set of resources (Futuyma and Moreno 1988). Meanwhile, the advantages of generalism seem obvious; a generalist that exploits two resources as efficiently as either specialist should be favored under varying resource availability. However, observations of universally successful generalists are rare (Fry 1990; Kassen 2002; Caley and Munday 2003). Thus, many theories assume a cost of generalism, in keeping with the adage that a "jack-of-all-trades" tends to be a master of none (Levins 1968; Lynch and Gabriel 1987; for a review see Wilson and Yoshimura 1994).

The cost of generalism, the trade-off between niche width and fitness in particular niches, is widely regarded as a cost of adaptation (Levins 1968). Adaptation to one niche may be costly for performance in a second niche because of antagonistic pleiotropy, a negative genetic correlation between performance in the two niches (Levins 1968; Rausher 1984; Lynch and Gabriel 1987; Elena and Lenski 2003). However, the accumulation of neutral mutations that are deleterious in alternative niches may also explain the cost of generalism (Kawecki 1994). In microbes, fitness trade-offs across different niches are commonly observed during development of live-attenuated vaccines; adaptation of a virus to growth in nonhuman host cells can often result in a clinically attenuated genotype. Thus, virus attenuation and other microbial evolution experiments provide indirect evidence that antagonistic pleiotropy contributes a cost of adaptation; however, these studies often cannot rule out a role for mutation accumulation (Novella *et al.* 1995; Reboud and Bell 1997; Weaver *et al.* 1999; Cooper and Lenski 2000; Crill *et al.* 2000; Turner and Elena 2000; MacLean and Bell 2002; for a review see Ebert 2000; but see Kassen 2002). Some experiments directly confirmed the role of antagonistic pleiotropy by identifying particular mutations that improve performance on one host (*i.e.*, one niche), but reduce performance on another host (*e.g.*, Itoh *et al.* 1997; Crill *et al.* 2000; Hanley *et al.* 2003; Song *et al.* 2005). Our goal was to investigate more thoroughly the relative frequency with which antagonistically pleiotropic mutations arise during the initial shift to a novel host, where the effects of mutation accumulation are minimized.

To accomplish this goal, we investigated the consequences of viral host range expansion, a type of generalism with particular relevance to disease emergence. Specifically, we measured the fitness costs associated with single mutations that caused host range expansion in the RNA virus Φ6, a bacteriophage of *Pseudomonas syringae* that was developed as a model system for

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studying segmented RNA viruses that infect humans (Mindich and Bamford 1988). Taking advantage of Φ6's ability to readily generate mutants capable of infecting a variety of novel Pseudomonas hosts (Cuppels *et al.* 1981), we isolated a large number of spontaneous mutants with expanded host ranges. By using a genetic model system for this study, we not only determined the proportion of these mutations that impose a growth rate cost on the standard host, but also identified the genetic bases of host range expansions by sequencing candidate genes. Our data suggest that antagonistic pleiotropy is a common but not universal property of mutations that extend the viral niche, implying that host range expansion in RNA viruses may be favored under a wide variety of ecological conditions.

MATERIALS AND METHODS

Strains and culture conditions: We obtained from the American Type Culture Collection (Bethesda, MD) wild-type phage Φ6 [American Type Culture Collection (ATCC) no. 21781-B1] and its standard laboratory host, *P. syringae* pathovar *phaseolicola* strain HB10Y (ATCC no. 21781; Vidaver *et al.* 1973). G. Martin (Cornell University, Ithaca, NY) generously provided the following 13 *P. syringae* pathovars, identified by the following Martin strain catalog nos.: 2231, *atrofaciens*; 2232, *coronofaciens*; 171, *glycinea*; 2227, *mori*; 2228, *morsprunum*; 2229, *persicae*; 177, *phaseolicola*; 169, *pisi*; 2230, *savastanoi*; 248, *solanacearum*; 2210, *syringae*; 2237, *tagetis*; and 113, *tomato*. L. Mindich (Public Health Research Institute, Newark, NJ) kindly provided the distantly related (Yamamoto *et al.* 2000) bacterium *P. pseudoalcaligenes East River* isolate *A* (ERA) (Mindich *et al.* 1976), which has been routinely used to isolate host range mutants of Φ6 (*e.g.*, Chao 1990; Turner and Chao 1998).

We used LC media (LB broth at pH 7.5) (Mindich *et al.* 1976) to grow all bacteria. Phage were grown by mixing viruses with a bacterial lawn in 3 ml of LC top agar (0.7%), overlaid onto an LC plate (1.5% agar) (Turner and Chao 1998). All cultures and plates were incubated at 25°.

Niche breadth of wild type: A high-titer lysate [$\sim 10^{11}$ plaque-forming units (PFU)/ml] of wild-type Φ6 was grown and titered on the original host, *P. syringae* pv. *phaseolicola*. We removed the bacteria by filtration (0.22 μm; Millipore, Bedford, MA) and serially diluted the resulting lysate using LC broth. Host range of wild-type Φ6 was determined by plating $\sim 10^2$ PFU onto lawns of the 14 other bacterial hosts, an approach similar to that previously employed for testing Φ6 host range (Cuppels *et al.* 1981). To ensure equal host density across the assay, we seeded lawns of each challenge host using 200 μl from a stationary-phase culture that had been adjusted with LC to have the same optical density (absorbance at 600 nm) as a stationary-phase culture of the standard lab host, *P. syringae* pv. *phaseolicola* ("original host"). After an ~18-hr incubation we looked for plaques formed on the plates; an additional 24-hr incubation ensured that slow-growing plaques were discerned. This screen was repeated three times, using independently grown overnight cultures. Bacterial hosts that allowed wild-type Φ6 plaque formation were designated "permissive hosts." We note that variation in phage sensitivity among strains of the same pathovar caused an apparent difference between our data and the previously described host range of Φ6 (Cuppels *et al.* 1981). This difference is explained by the practice of naming *P. syringae* pathovars by the plant used in strain isolation, rather than by the phylogenetic relatedness of distinct bacterial clades (Sarkar and Guttman 2004).

Host range mutants: A high-titer lysate of wild-type Φ6 was grown and titered on the original host. Approximately 2.5×10^9 PFU were plated onto lawns of the bacterial strains that were nonpermissive for growth of the wild type. Plates were incubated for 18 hr and then examined for plaques indicating spontaneous host range mutants. We arbitrarily chose plaques from lawns of each nonpermissive host and streaked these onto a fresh lawn of the appropriate nonpermissive host to plaque purify isolates. Streaked plates were incubated for 18 hr and a single plaque was arbitrarily chosen, excised, and stored in 500 μl of sterile 40% glycerol/60% LC broth at −20°. Nonpermissive hosts allowing isolation of host range mutants were designated "novel hosts."

Niche breadth of the host range mutants: A high-titer lysate of each host range mutant was obtained by plating a portion of the frozen stock on the novel host used to isolate the strain. We tested the host range of these mutants by spotting each mutant ($\sim 10^3$ PFU in 5 μl) onto lawns of the 13 *P. syringae* pathovars and *P. pseudoalcaligenes* ERA, consistent with previous methods for host range determination in Φ6 (Cuppels *et al.* 1981). We repeated this assay three times, using independently grown overnight cultures of each host. Any inconsistent results across the three trials were resolved by standard plating on the host in question.

Sequencing: We extracted genomic RNA from high-titer lysates, using QiaAMP viral RNA extraction kits (QIAGEN, Valencia, CA). RNA was converted into cDNA, using RT-PCR with Superscript polymerase and random hexamer primers (Invitrogen, Carlsbad, CA). Standard PCR methods were used to amplify portions of the genome, and PCR reactions were purified for sequencing with ExoSAP-It (United States Biological, Swampscott, MA). Sequencing was performed using standard methods by the University of North Carolina, Chapel Hill Automated Sequencing Facility. The Φ6 genome consists of three segments, designated small, medium, and large (Mindich and Bamford 1988). The entire genome (except the single-stranded packaging regions at the ends of each segment) of the wild-type ancestor was sequenced and entered into GenBank (DQ201338–DQ201340). The expanded host range mutants had >3000 bp, or ~75%, of their medium segment sequenced. This region included the genes involved in host attachment (P3) and fusion to the host membrane (P6), the best candidate genes for host range expansion (Gottlieb *et al.* 1988). The gene for P3 was previously identified as the site of host range mutations that allow infection of ERA (L. Mindich, personal communication).

Fitness of the host range mutants on the original host: Paired growth assays (PGAs) (Chao 1990) measure the relative growth of two competing phage strains during a 24-hr period (Chao *et al.* 1997; Turner and Chao 1998, 1999, 2003). We competed each host range mutant against the wild-type Φ6 virus (*i.e.*, the nonmutated common ancestor) on the original host, *P. syringae* pv. *phaseolicola*. The numbers of mutant and wild-type viruses were counted at the beginning of the assay ($t = 0$) and then again after a day's competition ($t = 24$). Fitness (W) is the ratio of the relative proportions of the competing genotypes at the start of the experiment and after 24 hr; $W = R_{24}/R_0$. All mutants were competed six times against the ancestor.

We then tested whether there was a "maternal effect" of the host used to prepare the lysate on the outcome of the PGAs. High-titer lysates of 12 mutants (representing five different mutations) were prepared on either the original host or the appropriate novel host (total of 24 lysates). Triplicate PGAs were then conducted on the original host.

Statistical analysis: All analyses were conducted in Microsoft (Redmond, WA) Excel 2004. One-tailed *t*-tests were used to

determine whether mutations imposed a significant fitness cost on the original host. A two-tailed *t*-test was used to compare the fitness of different mutants with the same nonsynonymous mutation. For comparisons of the host used to prepare the phage lysates, we used a two-factor ANOVA (maternal host times expanded host range mutant).

RESULTS

Niche breadth of wild-type Φ6: To establish the host range of the wild-type Φ6, ~100 PFU of the phage were plated onto 15 Pseudomonas strains. Wild-type Φ6 could infect three *P. syringae* pathovars with roughly the same efficiency as its original host, *P. syringae* pv. *phaseolicola*. These hosts, *P. syringae* pv. *persicae*, *savastanoi*, and *tagetis*, were designated permissive hosts. Together with the original host, these four strains make up the host range (niche breadth) of wild-type Φ6 for the purposes of this study.

Isolation of host range mutants: Of the remaining 11 Pseudomonas strains, host range mutants of Φ6 could be readily isolated on three hosts: *P. pseudoalcaligenes* ERA and *P. syringae* pathovars *atrofaciens* and *tomato*. We observed no host range mutants on the other eight *P. syringae* pathovars in triplicate trials, indicating that if spontaneous host range mutations occur on these hosts the rate lies below 10^{-10} (the number of plaque forming units plated), well below the most conservative estimated single mutation rate of Φ6 (2.7×10^{-6}/nucleotide) (Chao *et al.* 2002). Ten single-plaque isolates from each of the three novel hosts were preserved for further study (30 mutants total) and designated by the host used in isolation—A (*P. syringae* pv. *atrofaciens*), E (*P. pseudoalcaligenes* ERA), or T (*P. syringae* pv. *tomato*)—and serially numbered 1–10.

Niche breadth of host range mutants: We used the set of 15 Pseudomonas strains to determine the niche breadth of each host range mutant. All of the host range mutants retained the ability to infect the original host and all three of the permissive hosts, *P. syringae* pv. *persicae*, *savastanoi*, and *tagetis*, but remained unable to form plaques on the eight pathovars on which no host range mutants arose. For the purpose of this study, we defined these expanded host range mutants as generalists because they exploited more of the 15 hosts than the wild type.

Strengthening this claim, 29 of the 30 host range mutants also gained the ability to infect one of the unselected novel hosts (Table 1). The complete host range of each mutant depended on the host used to isolate the mutant, and the pattern was asymmetric. Mutants isolated on *P. syringae* pv. *atrofaciens* or *tomato* could infect *P. pseudoalcaligenes* ERA, while all but 1 of the mutants isolated on *P. pseudoalcaligenes* ERA could form plaques on either *P. syringae* pv. *atrofaciens* or *tomato*, but never on both.

Genetic changes: The genes that encode the attachment protein P3 and the membrane-fusion protein P6 were sequenced for the 30 mutants (>3000 bases or ~75% of the medium genome segment) (Gottlieb *et al.* 1988). We found no mutations in P6, but all 30 mutants had a nonsynonymous mutation in P3. Each mutant had one of nine distinct nonsynonymous mutations in the P3 attachment gene and 1 mutant also had a synonymous mutation (Table 1); the nonsynonymous changes in P3 were deemed likely responsible for the expanded host ranges. Five of the nonsynonymous mutations were associated with an expanded host range into *P. syringae* pv. *tomato* and *P. pseudoalcaligenes* ERA (E8K, E8G, E8A, G515S, and D554G), three were associated with an expanded host range into *P syringae* pv. *atrofaciens* and *P. pseudoalcaligenes* ERA (D35A, A133V, and S246T), and the remaining nonsynonymous mutation (K311T) was associated with the ability to infect *P. pseudoalcaligenes* ERA. Because our data are sensitive to a "jackpot" effect (Luria and Delbrueck 1943), where multiple descendants of the same mutational event could appear in our 30 host range mutants, the frequency with which individual mutations appeared in our data set probably does not reflect differences in mutation rates among sites. However, this potential jackpot effect does not contradict the conclusion that many mutations are available that expand the host range of Φ6.

TABLE 1

Expanded host range mutations in the attachment gene P3

Mutant IDs[a]	Mutations[b]	Host range[c]
E1, E3, E4, E8	E8K	T + E
E5, T1–T8	E8G	T + E
E6	E8A	T + E
A1, A8	D35A	A + E
E2, A2–A7, A9	A133V	A + E
A10	A133V (S381S)	A + E
E9, E10	S246T	A + E
E7	K311T	E
T10	G515S	T + E
T9	D554G	T + E

[a] Uppercase letters designate the host strain (A, *P. syringae* pv. *atrofaciens*; E, *P. pseudoalcaligenes* ERA; or T, *P. syringae* pv. *tomato*). ID, identification.

[b] Notation indicates the amino acid position and change associated with each mutation. The single synonymous second-site mutation is shown in parentheses.

[c] Mutants acquired the ability to infect the indicated hosts and retained the ability to infect all four of the previously permissive hosts.

Fitness on the original host: Fitness data from the 30 expanded host range mutants were pooled by the nine nonsynonymous mutations, and it is clear that most of the mutations impose a fitness cost on the original host (Figure 1). Expanded host range mutants carrying seven of the nine nonsynonymous mutations were significantly less fit on the original host than their more specialized ancestor ($P < 0.05$, one-tailed *t*-test). The

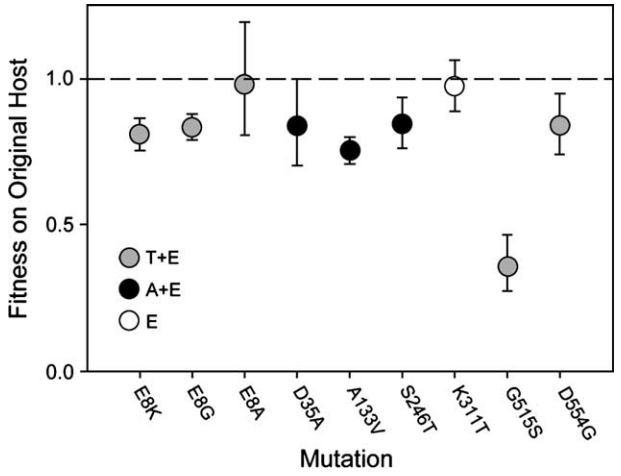

FIGURE 1.—Frequent antagonistic pleiotropy of expanded host range mutation. The relative fitness of the 30 host range mutants of Φ6 on the original host, grouped by their mutations in P3. The fitness of the common ancestor ATCC Φ6 is shown by the dashed line. Each point represents the grand mean fitness (±95% confidence interval) of the collected mutants bearing the indicated mutation. The killing spectrum of each mutation across the novel hosts is indicated as follows: solid circles, *P. syringae* pv. *atrofaciens* and *P. pseudoalcaligenes* ERA; shaded circles, *P. syringae* pv. *tomato* and *P. pseudoalcaligenes* ERA; open circle, *P. pseudoalcaligenes* ERA.

two expanded host range mutants with mutations E8A or K311T were not significantly different in fitness relative to the ancestor on the original host, and we concluded that these mutants experienced genuinely cost-free host range expansions. The synonymous second-site mutation S381S that was found in a strain with the A133V mutation did not affect the fitness of that strain; the fitness of the double mutant was equal to the fitness of the viruses that carried only the nonsynonymous mutation A133V ($P = 0.66$, two-tailed *t*-test).

Effect of maternal host: We also determined whether the novel host from which the mutants emerged (*i.e.*, the maternal host) contributed to the fitness costs measured on the original host. Each one of a subset of 12 mutant strains, representing five of the nine nonsynonymous mutations, was grown separately on lawns of the novel and standard laboratory hosts. We then measured the fitnesses of phage harvested from these different maternal hosts, using paired growth assays against wild-type phage that had been harvested from the standard laboratory host. The results were analyzed through ANOVA and showed a small but significant effect of the maternal host. Phage genotype ($P = 1.65 \times 10^{-6}$, d.f. = 11, $F = 6.533$) and maternal host ($P = 0.0113$, d.f. = 1, $F = 6.949$) both had significant effects on fitness, explaining 53 and 5.1%, respectively, of the variance in the data. There was no significant interaction between these effects ($P = 0.652$, d.f. = 11, $F = 0.786$). Thus, although the magnitude of the fitness costs shown in Figure 1 was determined primarily by the identity of the expanded host range mutation carried by each genotype, the use of novel hosts as maternal hosts also contributed to the observed costs.

DISCUSSION

Our results demonstrate that the majority of mutations that confer an expanded host range in Φ6 impose a significant cost for fitness on the original host. Thus, antagonistic pleiotropy appears to be a common property of the first mutations to arise during a host shift. However, we also observed two mutations that imposed no significant cost, indicating that antagonistic pleiotropy is not a universal property of expanded host range mutations in this virus. Our study of a single bout of natural selection contrasts sharply with most previous studies of experimental and natural populations, which examine the outcomes of long-term evolution. Nonetheless, our conclusion that the evolution of increased generalism (adaptation to a new or wider host range) is often, but not always, accompanied by a performance cost on the original host is identical to the conclusion drawn from longer-term studies (Novella *et al.* 1995; Weaver *et al.* 1999; Crill *et al.* 2000; Turner and Elena 2000). Our data suggest several avenues for future short-term investigations into the evolution of host range, including examinations of the relative fixation probabilities of costly *vs.* cost-free mutations and of the potential for subsequent compensatory mutations to ameliorate the initial costs of expanded host range mutations.

Genetic basis of the cost of generalism: Although adaptation to a novel host often caused a reduction in fitness on other hosts in longer-term studies, the absence, or limited nature, of genetic data from those studies makes it difficult to ascribe whether the costs resulted from antagonistic pleiotropy. Even in cases where genetic data were collected (Bull *et al.* 1997; Weaver *et al.* 1999; Sala and Wain-Hobson 2000), numerous mutations were identified that contributed to long-term adaptation, often making it difficult to rule out alternative explanations, such as the accumulation of mutations that were neutral in the selected environment but costly in others (Kawecki 1994). By limiting adaptation to a timescale over which viruses usually acquired only single mutations, we were able to confirm the effects of antagonistic pleiotropy directly.

To identify and confirm that mutant viruses had acquired only single mutations, we sequenced candidate genes. In Φ6 the ability to infect a particular host is governed, in part, by the ability to attach to a cellular receptor, usually the type IV pilus (Bamford *et al.* 1987; Gottlieb *et al.* 1988). In contrast to the core genome of *P. syringae*, which is highly conserved among strains (Sarkar and Guttman 2004), the presence and expression of the genes for the type IV pilus are variable (Romantschuk and Bamford 1985). Thus, the phage proteins that interact with the type IV pilus and host cell

membrane to mediate attachment and entry, P3 and P6 (Gottlieb *et al.* 1988), were identified as the most likely targets for host range mutations. Sequencing only these genes, we identified nonsynonymous mutations in P3 in all 30 host range mutants and found only one synonymous mutation in P3. We found no mutations in P6.

We are confident that the identified nonsynonymous mutations were responsible for the expanded host ranges despite the lack of sequence data over the rest of the genome. Our sequence data covered 24% of the genome (3200 of 13,385 bp) and detected only two second-site mutations [one synonymous mutation each in P3 (Table 1) and in the nonessential gene P13]. Extrapolating to the rest of the genome, we expect to find only six additional second-site mutations among the entire collection of mutants. Our conclusion that the majority of host range mutants carry only a single mutation, combined with the observation that the second-site mutations were both synonymous, indicates that the observed costs of generalism resulted from antagonistic pleiotropy and cannot be explained by second-site deleterious mutations.

There are theoretical and intuitive reasons to expect antagonistic pleiotropy to underlie the cost of generalism (reviewed by Wilson and Yoshimura 1994), but in the face of these reasons it is difficult to explain why antagonistic pleiotropy is not a universal property of mutations that confer increased niche breadth. In systems of historical interest to ecologists, such as the Galapagos finches, antagonistic pleiotropy is often imposed by visible mechanical constraints. For example, narrow beaks are mechanically better able to process small seeds than large seeds (Grant and Grant 1995; Herrel *et al.* 2005). In this case, mutations that change beak size necessarily impose a trade-off between the abilities to process small and large seeds. Apparently, the biochemical constraints that govern the ability of Φ6 to attach to its host differ from mechanical constraints, in that trade-offs between the abilities to attach to alternative hosts are not imposed universally. Other phenotypes that are governed by biochemical constraints show the same pattern. Among Gram-negative bacteria, for example, mutations have been identified that confer resistance to the antibiotic streptomycin without reducing growth rate in the absence of streptomycin (Schrag *et al.* 1997). Thus, the existence of a subset of mutations that do not exhibit antagonistic pleiotropy may be a common property of phenotypes that are governed by biochemical rather than mechanical constraints.

Maternal effects and the cost of generalism: Although the strongest cost of generalism found in this system had a genetic basis, we also found an environmentally imposed cost. Our results demonstrate that an RNA phage inherits something from its host that is costly if the same phage genotype next infects a different host type (see also Dennehy *et al.* 2006). It is surprising that this inheritance cost has not received more attention in the literature because it is a cost of generalism that may be difficult to overcome through genetic mutations. This effect is analogous to a maternal effect, an inherited trait that affects fitness but is not encoded in the genotype (Wade 1998), and will impose a small, but significant, cost of generalism on phage that switch between host types. Φ6 is a lipid-coated RNA bacteriophage (Mindich and Bamford 1988), and "maternal effects" in other enveloped RNA viruses are known to arise from the incorporation of host membrane components into the viral envelope (Bastiani *et al.* 1997). Accordingly, the observation that Φ6 discriminates in its incorporation of host lipids (Laurinavicius *et al.* 2004) may explain why the maternal effect we observed imposed only a small cost.

We can rule out an alternative explanation for the apparent cost of emerging from a novel host—that growth of the phage (before the fitness assay) on either the novel or the original host may have been accompanied by adaptation to that host. During growth on the original host, compensatory mutations may have arisen that ameliorated the cost of expanded host range. During growth on the novel host, additional pleiotropic mutations may have arisen that increased the cost of expanded host range. Although either one of these phenomena could produce an apparent cost of transitioning from a novel host to the original host, the cost would be only on the order of the phage mutation rate, 10^{-6}. Even extremely strong selection acting over the approximately five generations of growth that occurred before the fitness assay would only marginally increase the frequency of mutations that are initially present at frequencies near 10^{-6}.

Implications for RNA viruses: Using an RNA virus as a model for studying the evolution of niche breadth is particularly relevant for public health. RNA viruses readily produce mutations in their attachment proteins that change or expand their host ranges (Parker and Parrish 1997; Baranowski *et al.* 2001; Rainey *et al.* 2003; Brault *et al.* 2004), providing a partial explanation for why >50 RNA viruses, including human immunodeficiency virus, Ebola virus, SARS coronavirus, and several hantaviruses have shifted from nonhuman hosts to humans since World War II (Morse 1993). Although data such as ours cannot address the long-term outcome of evolution (*e.g.*, whether the evolving virus would eventually lose the ability to grow on the original host), our results do address an important step in viral emergence. Our data are critical for predicting the range of ecological conditions in which an expanded host range is likely to evolve and, therefore, for predicting the potential for disease emergence in novel hosts (Holmes and Rambaut 2004). In particular, the existence of antagonistic pleiotropy would limit the set of ecological conditions expected to favor expanded host range. However, our data suggest that antagonistic pleiotropy is not a universal property of mutations that

expand viral host range and, therefore, that the set of ecological conditions expected to favor expanded host range may be quite broad.

Of even greater concern is the observation that host range mutations in this study often conferred the ability to infect unselected novel hosts, which has previously been noted in other RNA viruses (Baric *et al.* 1997, 1999). These observations suggest that increasing exposure of zoonotic RNA viruses to any phylogenetically related novel host will increase the risk of emergence of viruses capable of infecting humans.

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