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THE DISTRIBUTION OF MUTATIONAL FITNESS EFFECTS OF PHAGE ϕX174 ON DIFFERENT HOSTS

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Adaptation depends greatly on the distribution of mutation fitness effects (DMFE), but the phenotypic expression of mutations is often environment dependent. The environments faced by multihost pathogens are mostly governed by their hosts and therefore measuring the DMFE on multiple hosts can inform on the likelihood of short-term establishment and longer term adaptation of emerging pathogens. We explored this by measuring the growth rate of 36 mutants of the lytic bacteriophage ϕX174 on two host backgrounds, *Escherichia coli* (EcC) and *Salmonella typhimurium* (StGal). The DMFE showed higher mean and variance on EcC than on StGal. Most mutations were either deleterious or neutral on both hosts, but a greater proportion of mutations were deleterious on StGal. We identified two mutations with beneficial fitness effects on EcC that were neutral on StGal. Host-specific differences in fitness were associated with particular functional classes of genes involved in the initial stages of infection in accordance with previous studies of host specificity. Overall, there was a positive correlation between the effects of mutations on each host, suggesting that most new mutations will have general, rather than host-specific fitness effects. We consider these results in light of simple fitness landscape models of adaptation and discuss the relevance of context-dependent DMFE for multihost pathogens.

KEY WORDS: **Adaptation, bacteriophage, context dependent, distribution of fitness effects, fitness landscape, multihost pathogens.**

The adaptation of populations to novel environments is a central question in evolutionary biology (Fisher 1930; Orr 2005a). Mutations provide the fuel for adaptation by generating genetic variation, and the effect of each mutation on fitness determines the strength and direction of selection (Charlesworth and Charlesworth 1998; Lynch et al. 1999; Eyre-Walker and Keightley 2007). Making predictions about population adaptation or extinction therefore relies on knowledge about the distribution of mutation fitness effects (DMFE) (Eyre-Walker and Keightley 2007). The theoretical study of DMFE, specifically within the framework of Fisher's geometric model (Fisher 1930), has generated numerous predictions about the expected mean and variance in fitness according to the nature of the fitness landscape. Generally, when populations are close to a fitness peak it is expected that beneficial mutations are rare and deleterious ones are common, and that the variance in fitness effects should increase with the distance from the peak (Fisher 1930; Charlesworth and Charlesworth 1998; Lynch et al. 1999; Orr 2005a,b; Martin and Lenormand 2006a; Eyre-Walker and Keightley 2007; Patwa and Wahl 2008).

EVOLUTION
INTERNATIONAL JOURNAL OF ORGANIC EVOLUTION

Experiments have largely supported theory. For example, the emergence of fitness plateaus during experimental adaptation (Lenski and Travisano 1994; Silander et al. 2007) requires either that beneficial mutations become rarer, or that the overall effect sizes of mutations become smaller as a population approaches a local fitness peak. Empirical measures of DMFE in several biological systems have directly quantified the fitness effect of mutations using either mutation accumulation experiments (Chao 1990; Keightley 1994; Kibota and Lynch 1996; Halligan and Keightley 2009; Brito et al. 2010), or by introducing specific mutations using genetic engineering (Wloch et al. 2001; Sanjuán et al. 2004; Domingo-Calap et al. 2009; Peris et al. 2010). This latter class of experiments has permitted the study of single mutations at known genomic sites, making them particularly powerful in linking genotype, phenotype, and fitness (Elena and Sanjuán 2007; Sanjuán 2010). Altogether this work has shown that beneficial mutations are generally rare; deleterious mutations are relatively more common, and have a complex multimodal distribution that usually includes a large proportion of lethal mutations (Eyre-Walker and Keightley 2007).

Beyond these generalities, the DMFE may vary with the environment, especially if different environments represent a change in the position of the fitness peak and/or the shape of the landscape around those peaks. Recent reviews of experimental tests of mutational effects across environments support this idea, as the mean effect of deleterious mutations has been found to vary with the quality of the environment (Agrawal and Whitlock 2010). Stressful environments (defined as environments where organisms experience lower fitness) are also associated with increases in the variance of mutational fitness effects (Martin and Lenormand 2006a,b), an observation that is in line with predictions from simple fitness landscape models, where the variance in mutational effects is predicted to increase with the distance to the fitness peak (Fisher 1930; Martin and Lenormand 2006a; Orr 2005b). Furthermore, it has been suggested that beneficial mutations may either be "specific" (that is, beneficial only in a particular environment), or "universal" (that is, beneficial in all environments) (Elena and Sanjuán 2007).

Direct and simultaneous empirical measurements of the effect of single mutations in different environments are, however, not common. In the reviews mentioned above (Agrawal and Whitlock 2010), almost all cases measured the deleterious effect of mutations arising either during mutation accumulation experiments or due to exposure to a mutagen, neither of which allows the effect of single mutational effects to be gauged. One exception is a study by Remold and Lenski (2001), where the fitness effect of 26 random insertion mutations conferring antibiotic resistance (each carried on the same isogenic *Escherichia coli* background) was measured for four combinations of temperature and nutrient environments. This study did not focus on the DMFE per se, but highlighted how even a single insertion mutation can generate environment-dependent fitness effects (Remold and Lenski 2001). Wang et al. (2009) studied the fitness effect of 20 single mutant alleles in *Drosophila melanogaster* in environments of high and low nutrition, and found that the effect of deleterious mutations increased under the low food (Wang et al. 2009). Unlike the *E. coli* study, these mutations were not carried on an isogenic background, but the mutations (a mix of single nucleotide substitutions, deletions, and translocations) were crossed into a large, outbred fly population. It could be argued that in both these studies, the insertion or the deletion of a functional gene could have different effects on fitness compared to single nucleotide substitutions, which occur frequently during replication. We are aware of only one study where the effect of single nucleotide mutations engineered onto a common genetic background was measured across several environments. Lalić et al. (2011) tested a collection of 20 single nucleotide mutants of tobacco etch potyvirus (TEV) during infection of eight host plant species (each representing a different environment for the virus) and found host species-specific differences in the DMFE, such that the viral DMFE on any given host plant would not be predictable from the DMFE measured on its native host species (Lalić et al. 2011).

This last result highlights how the study of DMFE across environments can assist the study of pathogen emergence and adaptation. While only beneficial mutations will lead to an increase in pathogen fitness on novel hosts, the likelihood of pathogen emergence in heterogeneous host environments will depend on the whole DMFE, and whether these effects are host specific (Holmes 2009; Pepin et al. 2010). Host-specific infection success is well described in many host-pathogen systems, and commonly framed in terms of genotype-by-genotype (G×G) interactions (Lambrechts et al. 2006). Beyond these mostly phenotypic measures, however, the underlying genetic basis of host-specific pathogen fitness has remained elusive. In the present study we aimed to learn about how the distribution of fitness effects of new mutations in a DNA virus may vary in different hosts. We measured the fitness effects of 36 engineered single mutations randomly distributed along the genome of the bacteriophage ϕX174 on two susceptible, gram-negative, bacterial hosts, *E. coli* and *Salmonella typhimurium*. The well-described biology of this virus allowed us to determine the magnitude of mutational effects on specific genes according to their known function during the infection cycle.

Methods

BACTERIAL STRAINS AND VIRAL MUTANTS

The bacteriophage ϕX174 used in the present study was originally provided by Dr. James J. Bull (University of Austin, Texas). In a previous study, 45 single nucleotide ϕX174 mutants were

Table 1. φX174 mutants used in the study.

Mutant ID	Mutation	Amino acid substitution	Gene	Function
P1	c357a	None	C	Stage III replication
P3	a402t	Thr402Ser	D	External scaffolding and capsid morphogenesis
P5	t825a	Leu146Ile/Asn86Lys	D/E	External scaffold protein/Host cell lysis
P6	a827t	Leu146Ile/Tyr87Phe	D/E	External scaffold protein/Host cell lysis
P7	g915c	Gly23Ala	J	DNA binding protein; DNA packaging
P9	t1006g	None	F	Major coat protein
P10	c1023a	Ala8Asp	F	Major coat protein
P11	c1174t	None	F	Major coat protein
P12	c1217t	Pro73Ser	F	Major coat protein
P14	g1238c	Glu80Gln	F	Major coat protein
P15	a1251c	Lys84Thr	F	Major coat protein
P16	a1295c	Asn99Thr	F	Major coat protein
P17	t1432a	None	F	Major coat protein
P18	t1516c	None	F	Major coat protein
P19	t1663g	None	F	Major coat protein
P23	t1870g	None	F	Major coat protein
P25	g2511t	None	G	Major spike protein
P26	t2562a	Phe56Leu	G	Major spike protein
P27	t2630a	Ile79Asn	G	Major spike protein
P29	t3233g	Asp101Glu	H	DNA injection, piloting
P30	g3236a	None	H	DNA injection
P32	g3377t	Glu149Asp	H	DNA injection
P33	g3422c	Glu164Asp	H	DNA injection
P35	t3569c	None	H	DNA injection
P36	g3599t	Gln223His	H	DNA injection
P37	g3683c	Met251Ile	H	DNA injection
P39	t4201a	Val74Asp	A	Stage II and Stage III replication
P41	a4458c	Ile160Leu	A	Stage II and Stage III replication
P42	t4628g	Asn216Lys/Asn44Lys	A	Stage II and Stage III replication
P43	t4643g	None /None	A	Stage II and Stage III replication
P45	c4972g	Pro331Arg/Pro159Arg	A	Stage II and Stage III replication
P47	a5287g	Lys436Arg/None	A/B	Replication/Internal scaffolding protein
P48	t5344g	Phe455Cys/Ile90Met	A/B	Replication/Internal scaffolding protein
P49	a510g	Lys41STOP	D	External scaffolding and capsid morphogenesis
P52	t2350a	None	Intergenic	Primosome binding site, transcription terminator
P54	c5192g	Cys404Try/Cys232Try/Leu40Val	A	Stage II and Stage III replication

engineered using site-directed mutagenesis (Domingo-Calap et al. 2009). We used the subset of 36 nonlethal mutants from that study (see Table 1) and tested the distribution of fitness effects on two gram-negative host strains: *Escherichia coli* C (DSMZ 13127; hereafter referred to as EcC), a laboratory derived host of ϕX174, which has a specific rough lipopolysaccharide (LPS) recognized as the receptor (Feige and Stirm 1976), and another susceptible host *S. typhimurium* GalE (Hone et al. 1987) hereafter referred to as StGal (generously offered by Dr. James J. Bull, University of Austin, Texas).

FITNESS ASSAYS

The fitness effect of each mutation was estimated as the phage growth rate on each host. Growth assays were carried out in round-bottom 2.5 mL 96-well assay blocks (Dominique Dutscher, France). Mutant and wild-type phages were assayed in the same experimental plate, using a paired design, where the growth rate of each mutant was expressed relative to the growth rate of the wild-type phage in the adjacent well. Four replicate plates were setup per host, using the same host culture to inoculate all four replicates to minimize variance in fitness among replicates.

To measure growth rates, $\sim 10^4$ plaque forming unit (pfu) of either the mutant or the wild-type ϕX174 were inoculated into 500 μl of LB medium (supplemented with 5 mM $CaCl_2$ and 10 mM $MgCl_2$) containing either EcC or StGal growing in exponential phase ($OD_{600\ nm} = 0.20$–0.25; $\sim 4 \times 10^7$ cfu). The multiplicity of infection (MOI) was therefore maintained at a low level (approximately 10^{-4} at the beginning of the growth phase). An equivalent volume of each phage lysate was also aliquoted into 96-well plates containing 90 μl sterile water (10-fold dilution), and stored at -80°C for subsequent determination of the starting titers, T_0. The plates containing infected cultures were covered with Aeraseal ® porous adhesive covers (Ref 760215, Dominique Dutscher, France), incubated at 37°C in agitation (650 rpm) on a microplate shaker (Titramax 101, Heidolph Instruments, Germany), and harvested when the wild-type reached a density of $\sim 10^6$ pfu. The incubation time needed to reach these titers differed between host cultures, being 60 min for EcC and 90 min for StGal (see Fig. S1A). After this time, bacterial cells were removed by centrifugation (3 min at 6000 rpm/5790 g) and supernatants were stored for determination of final titers (T_f) as described for T_0.

We estimated viral titers using ϕX174-specific quantitative PCR (detailed below). Most studies of DMFE measure phage growth rates by growing mutants in liquid medium and then determine differences in initial and final viral densities by plating on a solid lawn of bacterial hosts and counting pfus. By using ϕX174-specific qPCR we endeavored to determine phage replication by measuring phage genome units (pgus) in the same liquid environment in which the growth assays were carried out. In addition this method also allowed us to assay all the experimental replicates simultaneously, which helped to keep experimental error low in the measurement of s. Initial and final titers were used to determine the growth rate (r) of all mutants and wild types on each host background, calculated as the increase in log titer per hour. Relative fitness was obtained as $W = r_{mut}/r_{wt}$, where r_{mut} is the mutant growth rate and r_{wt} is the growth rate of the paired wild type in the adjacent well. The selection coefficient of each mutant was then calculated as $s = W - 1$. We also considered an alternative measure of fitness based on the relative difference (and not the ratio) in growth rates between mutants and wild type (Chevin 2011), but this did not qualitatively change any of the results.

QUANTITATIVE PCR

Phage titers were determined using SYBR Green® Real-Time Quantitative RT-qPCR (Roche Diagnostics GmbH, Penzberg, Germany), which allows quick and precise measurements to be carried out simultaneously on a large number of samples. Using the sequenced genome of ϕX174 (Sanger et al. 1977); Refseq NC_001422), qPCR primers were designed using LC Probe Design Software 2 (Roche). These amplified the genomic region between base positions 2675 and 2776, a fragment of gene G that codes for the surface spike protein and that was identical in both wild-type and all mutant phages. Primer sequences were Forward 5′–TTGAGTCTTCTTCGGTTCCGACTA–3′ and Reverse 5′–TCACACAGTCCTTGACGGTATAAT–3′. Prior to the growth rate assays, we established a standard curve by plating a 10-fold dilution series of wild-type ϕX174 lysate (in duplicate) and we used these titers to relate their qPCR crossing point (CP) values to numbers of pfu (see Fig. S2). One intermediate dilution was titrated by plating ($\sim 6.6 \times 10^3$ pfu) and was stored at -80°C. This was later included as an internal control of qPCR efficiency in each experimental qPCR, allowing an accurate and independent estimation of pgu based on the original calibration. For the experimental determination of titers at T_0 and T_f, qPCR was carried out in white 384-well LC Multiwell plates (Roche) using a LightCycler® 480 thermocycler (Roche Diagnostics GmbH, Germany). Reagents per 10 μl reaction were 2.4 μl of sterile PCR-grade water, 5 μl of LC 480 SYBR Green I Master Mix (Roche) (Final concentration 1×), 0.3 μl of each 10 μM primer solution (final concentration 0.3 μM), and 2 μl of phage DNA sample (these were thawed from -80°C storage by placing them at 4°C for 2–3 h before each qPCR run). We used the following PCR program: 95°C, 10 min, followed by 40 cycles for 15 s at 95°C, 20 s at 60°C, and 30 s at 72°C. We considered any amplification only detectable after cycles 40 to be a false positive, as this is on the limit of detection of a single DNA copy. Analysis of raw CP data from each qPCR run and calculation of corresponding number of pgu was performed using the second derivative method for absolute quantification, using LightCycler® 480 Software release 1.5.0 (Roche).

Quantitative PCR is commonly used to quantify titers of viral pathogens of plants and animals (Halfon et al. 2006; Carrasco et al. 2007; Lalić et al. 2011), but most studies of bacteriophages measure viral densities by counting pfus on a solid bacterial lawn (but see [Rose et al. 1997; Edelman and Barletta 2003; Refardt and Rainey 2010) for examples of bacteriophage titer determination using qPCR]. It is possible that low-fitness mutants may be erroneously classified as lethal based on plating assays because they form microscopic and thus hardly detectable plaques (Fig. S3), or conversely, that low fitness mutants in a solid environment might yield higher viral densities when measured directly in liquid medium with qPCR (Fig. S4). However, by measuring viral densities in the same liquid environment in which the growth assays were carried out, we used an assay method that reflects the fitness of phage mutants in their growth environment, and obtained precise values of relative fitness for a large number of mutants simultaneously.

STATISTICAL ANALYSES

To test for differences in fitness between ϕX174 mutants on each host background, we fitted a linear mixed model with the selection coefficient s as the response variable, host, mutant, and their interaction as fixed effects, and included assay replicate and qPCR replicate as random effects, using restricted maximum likelihood (REML) to estimate their variance components. The standard errors of the mean (SEM) s value for each mutant from this model were used to test (t-test) whether mean s values were significantly different from 0 (neutrality) and −1 (lethality, indicating growth rate was effectively 0). Values significantly higher than −1 and lower than 0 were classified as deleterious and values significantly positive were classified as beneficial. Pearson correlation coefficient between mean fitness values for each mutant on the different host backgrounds was also calculated. Standard errors of the mean were also taken into account in these calculations by randomly sampling, for each mutation, from a normal distribution with the corresponding mean and SEM. We performed 10,000 such random samplings and considered the mean of the 10,000 obtained correlation coefficients. Finally, we tested if the variance in s values differed between hosts using Levene's homoscedasticity (homogeneity of variance) test, which performs an ANOVA where the response is the absolute value of the difference between each observation and the group mean (Brown and Forsythe 1974).

A fraction of the variance of the s values arises from measurement error. Following Peris et al. 2010, the influence of experimental variance on the distribution of the s values was removed by deconvoluting it with the distribution of measurement errors. Measurement error was estimated by repeated measures of s for the wild-type ϕX174 on EcC. Wild-type fitness was measured in all wells of two duplicate 96-well assay plates, and fitness was determined using relative hourly growth rates to a wild type in the adjacent well, and titrated in duplicate using qPCR, as described above for mutants. This yielded a total of 192 measures of wild-type fitness (48 independent growth rates per plate × 2 assay plates × 2 qPCR replicates). As all wild types are identical, and assuming that experimental error is independent of the host and mutant treatments, variation in s values measured this way reflects the total experimental error in s values attributable to position effects within assay plate, and qPCR replicates. The distribution of these 192 s values from the wild-type assays was satisfactorily approximated by a normal distribution ($r^2 = 0.995$) with mean −0.0028 and standard deviation 0.134. We modeled the deconvoluted distribution of the s values by trying four different distribution functions (normal, shifted gamma, log-normal, Weibull) that were truncated at −1 and whose parameters were estimated by maximum likelihood (Table 2). To do so, we built a likelihood function by convoluting one of the above four tested distribution functions with the distribution of measurement errors, as in Peris et al. 2010.

Results

We measured the effect of 36 independent mutations (Table 1) on the growth rate of bacteriophage ϕX174 on two hosts, *E. coli* C (EcC) and *S. typhimurium* GalE (StGal). From the outset, measured in our experimental conditions the wild-type ϕX174 had a growth rate of 4.95 (SD ±0.72) log titer per hour on EcC, and a lower growth rate of 3.6 (SD ±0.42) log titer per hour on StGal (Fig. S1B), suggesting that these two host species present different environments for the wild-type virus.

THE DMFE

The best-fitting statistical model for the distribution of fitness effects on both EcC and StGal, after accounting for sources of experimental error (see Methods), was a Weibull distribution (Table 2, Fig. 1). The mean selection coefficient varied between hosts ($F_{1,499} = 477.74$, $P < 0.001$), being more deleterious on average when viral mutants were grown on StGal (mean $s_{\mathrm{StGal}} = -0.53$; mean $s_{\mathrm{EcC}} = -0.29$) than on EcC. The variance in s also differed significantly between hosts (Levene's homogeneity of variance, $F_{1,571} = 26.62$, $P < 0.0001$) being higher on EcC ($\mathrm{var}_{\mathrm{EcC}} = 0.09$) than on StGal ($\mathrm{var}_{\mathrm{StGal}} = 0.04$). There was a positive correlation between the mean fitness effect of each mutation on EcC and StGal ($r = 0.64$, $P < 0.001$; Fig. 2)

DELETERIOUS, NEUTRAL, AND BENEFICIAL MUTATIONS

The set of mutations studied affected viral fitness to different degrees ($F_{35,499} = 49.55$, $P < 0.001$), and the rank order of their fitness effects differed depending on the host (Fig. 1C and D; mutant × host, $F_{35,499} = 11.91$, $P < 0.001$). On EcC, 26 out of 36 mutations were deleterious ($s_{\mathrm{deleterious}} = -0.447$), including one lethal mutation. Eight out of 36 were effectively neutral and two mutations showed a beneficial effect on fitness ($s_{\mathrm{beneficial}} = 0.382$; Fig. 1C). On StGal, this pattern differed, as no beneficial mutations were detected, and nearly all (34/36) were deleterious ($s_{\mathrm{deleterious}} = -0.578$), which included three lethal mutations (Fig. 1D).

SYNONYMOUS VERSUS NONSYNONYMOUS CHANGES

Twelve out the 36 mutations studied did not confer an amino acid change (Table 1), and the fitness effects of synonymous or nonsynonymous changes differed in a host-dependent way ($F = 12.22$, $P = 0.005$). On host EcC, nonsynonymous amino acid changes in the viral genome caused an average fitness effect of

Table 2. Maximum likelihood estimation of DMFE model parameters.

Distribution	Parameter estimates (95% CI)			Log-likelihood	Goodness of fit
E. coli					
Weibull	Shape	3.86	(3.44; 4.31)	−81.89581	0.9975
	Scale	1.12	(1.08; 1.16)		
Shifted gamma	Shape	30.60	(8.64; 43780.5)	−85.89	0.9945
	Rate	16.58	(8.30; 423.85)		
	Delta	1.14	(0.31; 68.18)		
Normal	Mean	−0.29	(−0.33; −0.25)	−87.91733	0.9969
	SD	0.31	(0.28; 0.34)		
Log-normal	Meanlog	−0.01	(NA; 0.031)	−114.1212	0.9927
	SDlog	0.32	(0.29; 0.36)		
S. typhimurium					
Weibull	Shape	5.04	(4.35; 5.88)	18.55887	0.9966
	Scale	0.85	(0.82; 0.88)		
Shifted gamma	Shape	8.65	(4.72; 25.46)	18.23547	0.9957
	Rate	12.64	(8.64;22.43)		
	Delta	0.214	(0.06; 0.68)		
Normal	Mean	−0.53	(−0.56; −0.50)	10.26762	0.9949
	SD	0.19	(0.17; 0.22)		
Log-normal	Meanlog	−0.22	(NA; −0.18)	−31.58279	0.9952
	SDlog	0.27	(0.24; 0.30)		

$s = -0.38$, while synonymous mutations had a smaller but still substantial effect of $s = -0.21$ ($F = 18.54$, $P < 0.001$) (Fig. 3A). The effect of either class of mutations was larger when the virus-infected StGal ($F = 113.32$, $P < 0.001$), but on this host there was no difference in the average fitness effect according to whether mutations changed the coded amino acid or not (mean $s = -0.58$; $F = 0.46$, $P = 0.50$; Fig. 3A).Therefore, despite not changing the coded amino acid, synonymous mutations still appear to cause a decrease in viral fitness in a host-dependent way.

GENE-SPECIFIC FITNESS EFFECTS OF MUTATION

The mean fitness effect of mutations varied according to mutated gene ($F_{9,553} = 22.23$, $P < 0.001$), and this effect depended on the host background (host × function $F_{9,553} = 86.34$, $P < 0.001$). Notably, mutations in genes associated with viral DNA replication reduced fitness to a similar extent regardless of the host, while those affecting initial DNA binding and injection, and also the major coat and spike proteins, caused host-specific differences in viral fitness (Fig. 3B). Examining the correlation of fitness effects between hosts for the two genes with the greatest number of mutations (gene F, 11 mutants and in gene H 7 mutants) illustrates this host-specific gene effect well: there is significant positive correlation for gene F ($r = 0.84$, $t = 4.58$, df = 9, $P = 0.0013$), but no significant correlation for gene H ($r = 0.42$, $t = 1.02$, df = 5, $P = 0.3537$).

Discussion

TWO HOSTS, TWO FITNESS LANDSCAPES

We aimed to compare the mean and variance of viral mutation effects on two hosts and in the process learn about the nature of the fitness landscapes they may present. Given that *E. coli* is the native host of the wild-type ϕX174 and that the wild-type strain used in this experiment had been previously passaged on another strain of *E. coli* (*E. coli piF*, see Domingo-Calap et al. 2009), we could expect ϕX174 to be closer to a fitness peak on the *E. coli* strain used in this experiment (EcC) than StGal. Based on this we had a priori predictions about the mean and variance in fitness in ϕX174 on either host. Specifically, if ϕX174 were closer to a fitness peak on EcC, we would expect fewer beneficial mutations relative to StGal and the overall DMFE on EcC to have lower variance, as expected under simple fitness landscape models (Martin and Lenormand, 2006a,b). However, the distribution of mutational fitness effects revealed a more complex adaptive scenario.

The differences in mean and variance in fitness between hosts offer the first clue about the nature of the fitness landscape that each host provides. Fitness landscape models predict that the variance in mutational fitness effects should be greater with increasing distance from the fitness peak (assuming that only the distance and not the shape of landscape changes) (Fisher 1930; Martin and Lenormand 2006a). The variance on EcC ($\text{var}_{\text{EcC}} = 0.09$) is more than double that of the variance in s on StGal

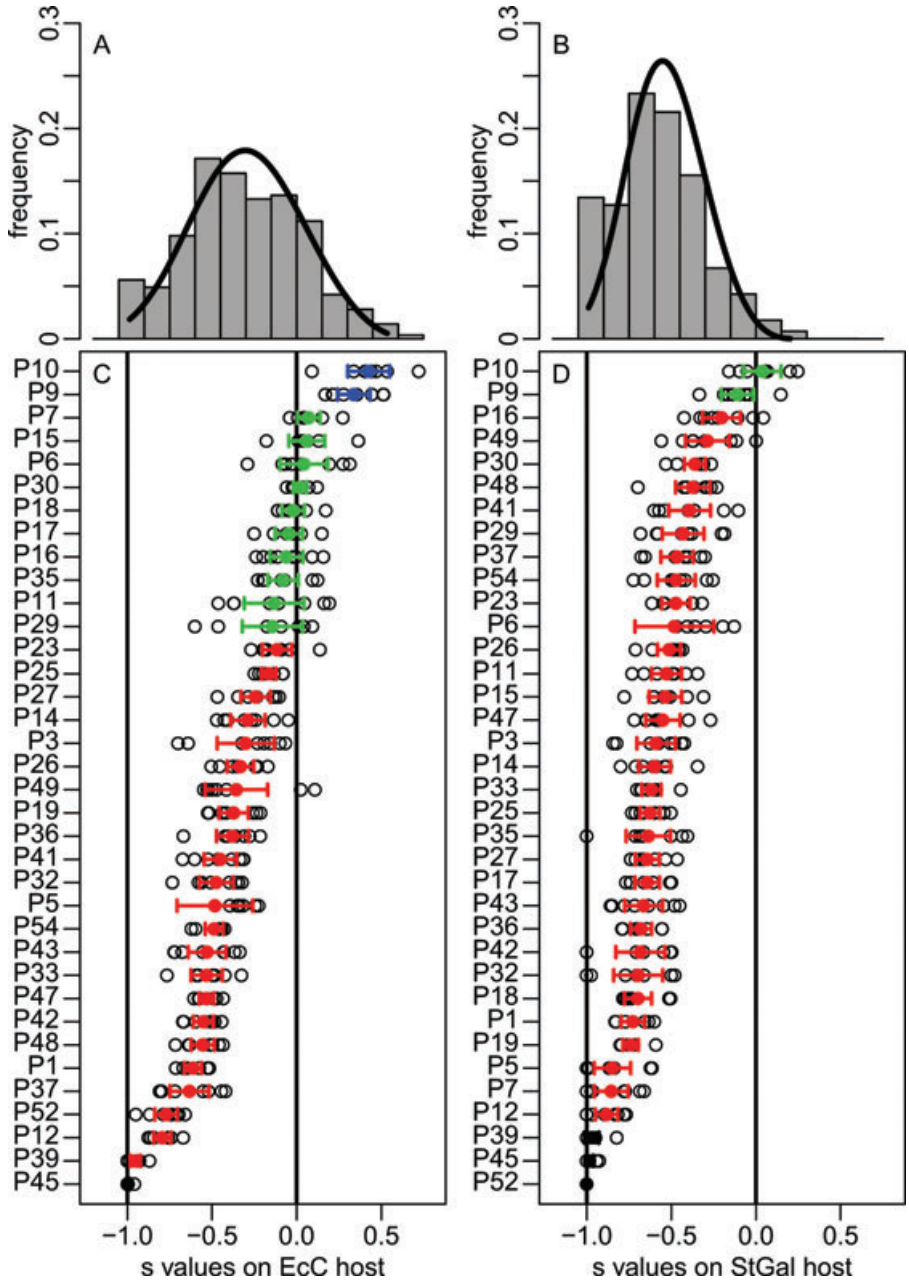

Figure 1. The distribution of fitness effects (DMFE) of new mutations on two hosts. The best fit distribution to the data on either host was a Weibull distribution (see Table 2). (A) On EcC the DMFE had mean $s = -0.29$ and variance 0.09. (B) On StGal the mean s was -0.53 and the variance 0.04. (C and D) The number of lethal, deleterious, neutral, and beneficial mutations varied between hosts (see text for details). On each panel, mutants are ranked by their mean s values; note that the rank order changes between hosts. Colors indicate lethal (black), deleterious (red), neutral (green), or beneficial (blue) effects of each mutation.

($\text{var}_{\text{StGal}} = 0.04$), suggesting that the wild-type ϕX174 is closer to the fitness peak when growing on StGal. Furthermore, it is expected that beneficial mutations will be more common with increasing distance from the fitness peak (Sanjuán et al. 2004; Silander et al. 2007; Domingo-Calap et al. 2009). Two mutations had beneficial fitness effects when ϕX174 grew on EcC but were effectively neutral on StGal, and no mutation induced a beneficial effect on StGal. We also found that a greater proportion of mutations were deleterious on StGal relative to EcC. Hence, contrary to our simple predictions, the data from the DMFE on both hosts suggest that the wild-type ϕX174 is relatively closer to a fitness peak when growing on StGal than on EcC.

Further, we observed that the mean effect of mutations differs between hosts, suggesting that it may not only be the distance to the peak that differs. In single peak and quadratic fitness landscape models, the mean effect of mutations should be invariant

with the distance to the peak (Martin and Lenormand 2006a). Other work has explored different types of landscapes and found that, depending on the curvature of the landscape considered, the expected mean fitness effect of mutations may either increase or decrease with increasing distance to the fitness peak (see Fig. 2A in Gros et al. 2009). These predictions are especially hard to test because the distance to the peak and the precise curvature of the landscape is not easily measurable. However, it would be possible to use DMFE data obtained here, in combination with experimental adaptation of the virus to both hosts, to test the prediction that the distance to the peak is shorter on StGal than on EcC One such experiment showed that the absolute growth rate of ϕX174 evolving on StGal plateaus at a lower level relative to EcC (Bull et al. 1997). Those results would suggest that the fitness landscape presented by StGal has a lower fitness peak relative to EcC, which constrains the evolution of growth rate on the former, and thus reinforces the idea that EcC and StGal represent different fitness landscapes.

Our analysis of synonymous and nonsynonymous mutations revealed that even those mutations that do not change the amino acid sequence can result in considerable fitness loses, and this effect was host dependent (Fig. 3A). Curiously, these effects of synonymous changes were more pronounced than those reported in previous studies of ssDNA phages (Cuevas et al. 2012). We detected fewer lethal mutations than expected from previous reports of DMFE, although this was not entirely surprising given that the mutants used were all nonlethal on another *E. coli* host (*E. coli* piF) (Domingo-Calap et al. 2009). We detected two beneficial mutations on EcC in a set of 36, but none on StGal (Fig. 1A), and recent work characterizing the fitness effect of single viral mutants on several plant hosts found similar fractions of beneficial mutants, depending on the host species being infected (Lalić et al. 2011). In a separate section below, we discuss the relevance of such host-dependent fitness effect of new mutations on the emergence and evolution of multihost pathogens.

Overall, the results from the present experiment are consistent with previous studies of mutational fitness effects where most mutations have been found to have deleterious or nearly neutral fitness effects, and beneficial mutations are rare (Eyre-Walker and Keightley 2007; Halligan and Keightley 2009). However, the exact distributions described in these studies vary slightly. Sanjuan (2010) recently reviewed the properties of mutational fitness effects arising from site-directed mutagenesis in several DNA and RNA viruses. The DMFE for vesicular stomatitis virus (VSV) was well modeled by a log-normal distribution (Sanjuán et al. 2004), that of tobacco etch virus was better described by a beta distribution (Carrasco et al. 2007), while those of two bactriophages showed the best fits for either exponential (ϕX174) or gamma (Qβ) distributions (Domingo-Calap et al. 2009). Yet, another study of DMFE in the RNA virus causing foot and mouth disease found, as we did in the present study, that it was best described by a Weibull distribution. It is worth noting, however, that most alternative distributions tested in these studies showed very good fits to the data ($r^2 = 0.91$–0.98).

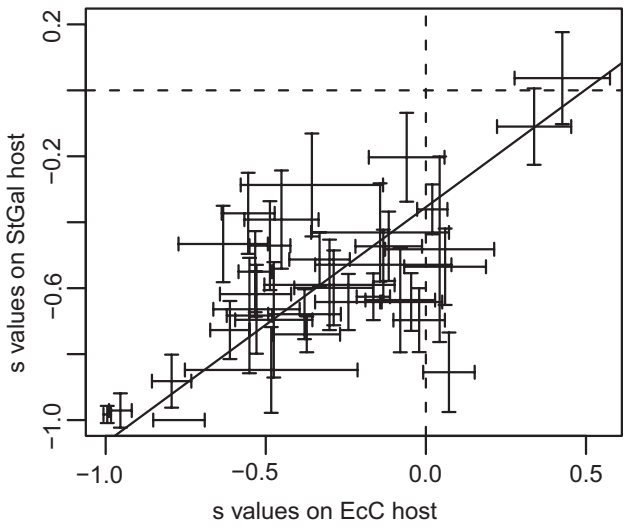

Figure 2. The correlation between mutation fitness effects on both hosts. Each datapoint is the mean *s* value for each phage mutant, error bars are standard errors (see Table S1 for details). The correlation coefficient $r = 0.64$, df = 35, $P < 0.001$. See text for further details.

LINKING GENOTYPE TO FUNCTION TO FITNESS

A great strength of microbial systems is that the ambitious goal of linking genotype, function, and fitness becomes attainable (Elena and Lenski 2003). Virus systems in general are powerful in this regard because the combination of relatively small genomes and well-honed molecular tools make it possible to target known genomic loci with well-described functions (Elena and Sanjuán 2007). ϕX174 is one of the best-characterized viruses on the genetic, biochemical, and structural levels (Fane et al. 2006; Wichman and Brown 2010). The set of ϕX174 mutants used in this study were engineered to introduce single mutations into a common genetic background, making it possible to enquire if the magnitude of fitness effects of these mutations is associated with specific genes, in light of their extensively well-described functions during the viral infection cycle (for a detailed review of the infection cycle we direct the interested reader to Fane et al. 2006).

Phage ϕX174 infects gram-negative bacteria like *E. coli* and *Salmonella* by attaching to LPS residues (Feige and Stirm 1976). Extracellular tropism has been well described, and several lines of evidence implicate proteins H, the major spike protein G, and the major coat protein F, as the main determinants of host-specific infection (Jazwinski et al. 1975; Fane et al. 2006; Wichman and Brown 2010). Indeed, Figure 3 shows that the fitness effect of mutations in these regions was host specific,

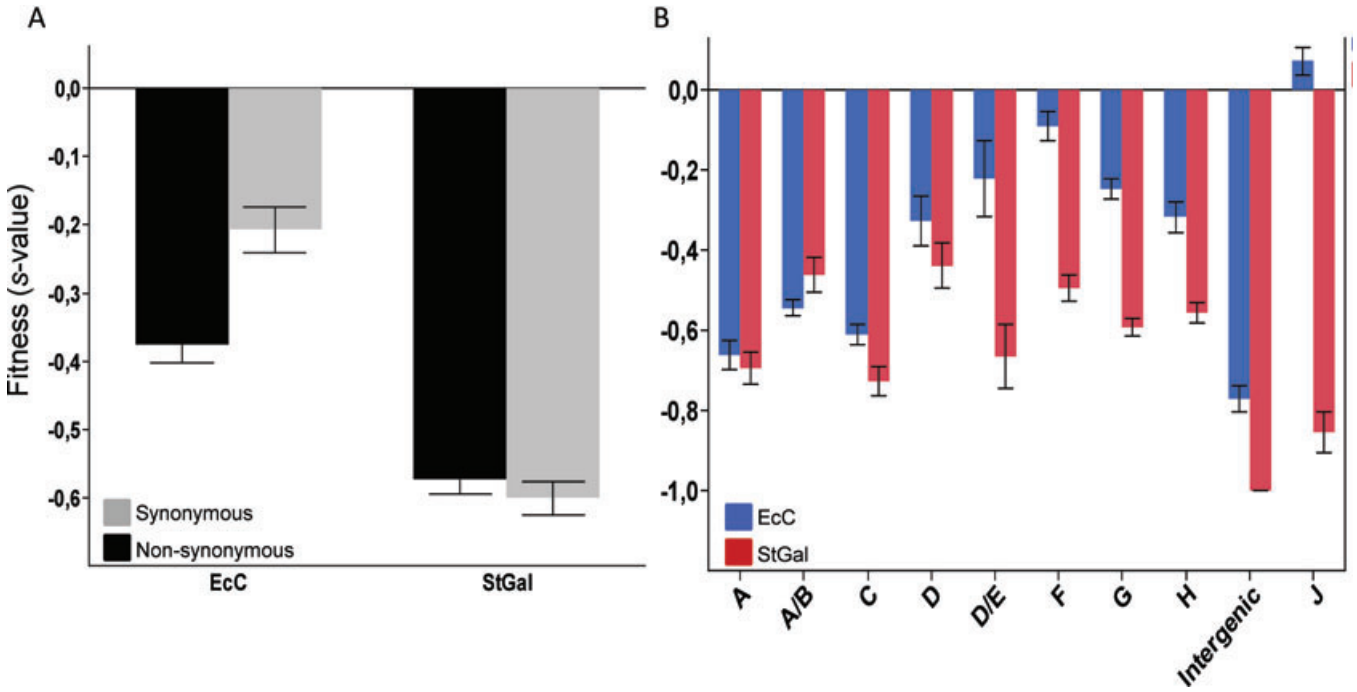

Figure 3. **Gene-specific fitness effects. The fitness effect of mutations varied according to which φX174 gene they were present on and whether these they changed the amino acid sequence or not. (A) The effect of synonymous and nonsynonymous changes on fitness. (B) Gene specific fitness effects of mutations. Bars show the mean (± standard errors) in the fitness effect of mutations. See text for details relating the functional importance of each gene during the phage infection cycle.**

being generally less deleterious on EcC. The major coat protein F has been especially well studied, frequently showing multiple and convergent nucleotide substitutions during experimental evolution of ϕX174 on both *E. coli* and *Salmonella* (Crill et al. 2000; Pepin and Wichman 2008; Pepin et al. 2008), as well as the evolution and then reversion of specific substitutions during host switching (Crill et al. 2000). Notably, both mutations we measured as beneficial on EcC (P9 and P10) map to the major coat protein F (Table 1). However, we should note that mutant P9 bears a synonymous change and P10 maps to a region that has not been described to be involved with host specificity. The effects of altering DNA-capsid interactions on infectivity and virion surface charge via mutations in protein J, have also been described (Bernal et al. 2004), which may explain the dramatic host-specific effect of the mutation mapping to protein J (mutant P7; Fig. 3).

Following entry into the host cell, the viral life cycle proceeds with single-stranded DNA replication, which may be classified into three separate stages. During Stage I DNA replication, the primosome complex is assembled in the region between genes F and G (Shlomai and Kornberg 1980). This is therefore a crucial step in the viral life cycle as no replication can occur if this step is disrupted, and we observed that even a synonymous mutation in this region (phage mutant P52) can cause a severe reduction in viral fitness (Table 1; Fig. 1). When this step is successful, Stage II replication follows, and proceeds through rolling-circle amplification of RF I DNA, resulting in RF II DNA. At this point, phage protein A cleaves the RF II DNA and binds to the host rep protein (Eisenberg et al. 1976). Without forming this A-rep-RF II DNA complex, stage III replication, where the single-stranded DNA genome is concurrently synthesized and packaged with the aid of protein C, cannot begin. Consistently, mutants with mutations in protein A or protein C showed severe reductions in fitness ($s > -0.60$) on either host backgrounds (Fig. 3).

At the end of stage III replication, the internal (B) and external (D) scaffolding proteins that mediate the morphogenetic pathway are expressed. Previous work using ϕX174 mutants with nonfunctional B proteins showed that assembly of the spike and coat proteins still occurs (Siden and Hayashi 1974). In this case it becomes difficult to interpret the effect of mutation on gene B, as both mutations mapping to this region (P47, P48) also overlapped with gene A (Table 1). As for the external scaffolding protein D, it has at least a dual function in directing the placement of the major spike protein, and generally serves to stabilize the viral capsid (Dokland et al. 1999). We observed deleterious but less severe fitness effects in mutants of protein D relative to other mutations (Fig. 3), which suggests that it is still possible for some virions to complete the infection cycle even if the capsid is not perfectly stable. As expected, mutations in both scaffolding genes showed host-independent effects. Together with the internal and external scaffolding proteins the spike (G) and coat proteins (F) are also

expressed at this stage, and once these are assembled into a functional procapsid, protein J plays a second and essential functional role in aiding genome encapsidation (Bernal et al. 2004).

Finally, once the capsids are assembled and genomes are encapsidated, the lytic infection cycle culminates with the lysis of the host cell. Lysis is achieved by viral protein E, which attaches to the bacterial cell membrane where it inhibits peptidoglycan biosynthesis (Bernhardt et al. 2000), eventually resulting in cell lysis and the release of new virions. Our set of mutants included two nonsynonymous changes in the lysis protein E (P5 and P6). Interestingly, the deleterious effects of these mutations appeared to be less severe when infection occurred on EcC, although these effects could also arise from the nonsynonymous changes incurred by the same mutation on the overlapping regions of gene D.

THE DMFE AND THE EMERGENCE OF MULTIHOST PATHOGENS

Many studies of DMFE are carried out in environments to which wild-type populations are preadapted (but see Sanjuán et al. 2004). This ensures they are as close as possible to the fitness peak in that particular environment, which allows making clear predictions in light of landscape models of adaptation (Eyre-Walker and Keightley 2007). Arguably, studying the adaptive process is most relevant when considering situations of environmental change, or migration of species to novel, often harsh, habitats (Prentis et al. 2008; Visser 2008; Agrawal and Whitlock 2010; Pepin et al. 2010), and our study aimed to examine the common biological scenario when populations are not well adapted to their environments when setting off on adaptive walks.

Such information is especially valuable in understanding the impact of adaptation on the establishment of pathogens in a new host environment. Evolutionary emergence of pathogens occurs when the introduced pathogen is poorly adapted to the novel host and where adaptive mutations are required before epidemic spread (Antia et al. 2003; André and Day 2005). The frequency and size of the fitness effects of mutations on the original as well as on the novel host is key to making accurate predictions about the likelihood of pathogen evolutionary emergence (Holmes 2009; Alexander and Day 2010; Pepin et al. 2010). We investigated this by measuring the correlation between the mean fitness effect of each mutation on EcC and StGal, and found that there was a positive correlation between fitness effects on both hosts (Fig. 2). This indicates that an adaptive mutation in a new host may not necessarily pay a fitness cost or may even be beneficial in the original host. This type of pattern may thus promote evolutionary emergence and speed up host shifts.

It is important to note, however, that this type of pattern does not necessarily equate with the absence of trade-offs between adaptation to these two bacterial hosts. The present study focused on the effects of random mutations on the covariance in fitness between two hosts. In evolving populations, selection will act on the genetic variation introduced by mutation and mold the pattern of covariation in fitness between hosts. Here, the focus shifts from the effects of random mutations to the G matrix of genetic variances and covariances (Lande and Arnold 1983; Blows 2007). Knowledge of the G matrix during adaptation is therefore central to the study of pathogens that infect multiple host species (Lambrechts 2010). If the two host environments represent two different peaks in the fitness landscape, adaptation to one host will necessarily lead to maladaptation to the other host and thus to specialization. The evolution of specialization was observed in previous work that allowed ϕX174 adapt to either *E. coli* or *Salmonella* in alternation: evolution of ϕX174 on *Salmonella* always selected for specialist phage (only able to infect *Salmonella*), while evolution on *E. coli* increased phage host range (the ability to infect both *E. coli* and *Salmonella*) (Crill et al. 2000). This suggests that despite the presence of a positive correlation in the fitness effects of novel mutations, ultimate constraints may prevent the emergence of fully generalist pathogens able to maximize fitness simultaneously on all available hosts (Lambrechts et al. 2006).

CONCLUDING REMARKS

Despite evidence for context-dependent fitness effects, the distribution of fitness effects of new mutations is frequently only measured in a single environment. By measuring the DMFE of the same set of single nucleotide mutations of a virus on different hosts we found that they exhibit differences in the mean and variance in *s* that reflect how each host offers distinct fitness landscapes. A recent study of an RNA plant virus underlined this point by showing that the distribution of fitness effects of new mutations may be highly unpredictable in heterogeneous host populations (Lalić et al. 2011). Our study is the first to study such context dependence in a DNA virus. Together with a growing body of work on variable mutational fitness effects, we highlight how long-standing theoretical predictions about the adaptive process may offer a valuable framework to study more applied issues, such as the emergence (Lloyd-Smith et al. 2005; Yates et al. 2006) and evolution of multihost pathogens (Gandon 2004). Environment-dependent mutational effects are likely to influence rates of pathogen adaptation (Dennehy 2009; Pepin et al. 2010), and consequently generate variable rates of antagonistic adaptation and counteradaptation between hosts and their pathogens (Thompson 2005; Wolinska and King 2009). More empirical data on the DMFE in a variety of biotic and abiotic contexts is therefore clearly valuable to our understanding of pathogen emergence and evolution.

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Supporting Information

The following supporting information is available for this article:

Table S1. Mean fitness effect of each mutation on each host.
Figure S1. (A) Incubation times on each host were determined based on the time it took the wild-type ϕX174 to reach at least 10^6 pfu, after incubating host cultures with $\sim 10^4$ pfu.
Figure S2. qPCR standard curve.
Figure S3. Lethality on solid medium.
Figure S4. Measuring fitness.

Supporting Information may be found in the online version of this article.