

# CHAPTER 14

## Experimental Evolution of Pathogens

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Why are certain microbes predisposed to causing disease, while others are apparently incapable of this shift? How fast, and by what genetic mechanisms, does this transition to pathogenesis occur? Is the evolution of greater or lesser virulence reversible? Could we have predicted the emergence of a coronavirus capable of causing SARS (Chapter 9, this volume)?

Modern biology typically probes these questions in a step-wise, “bottom-up” fashion: one hypothesizes that an interesting phenotype Y of pathogen A is associated with candidate genetic locus X, the locus is mutated, which affects some part of the phenotype, and then mutant and wild-type strains are evaluated in a well-defined model environment. If results from this analysis are as expected, we typically conclude that *gene X, which produces phenotype Y, contributes to the virulence of organism A*. We also usually infer that organism A is more virulent than other organisms *because* it has somehow acquired gene X, perhaps by horizontal gene transfer or by mutation of preexisting genes. We then conclude that the predisposition of organism A towards pathogenicity results from gaining gene X and similar elements.

But why organism A in the first place, and not other organisms? And why gene X, but not other loci? Although the bottom-up approach is exceptionally useful up to this point, here it fails. Understanding the origin and predicting the future response of incipient pathogens requires a different strategy. One method, comparative genomics, attempts to associate various microbial lifestyles with the presence or absence of large clusters of genes. This more complex, multivariate analysis extends the one-gene, one-phenotype approach and could help predict future evolutionary potential. Another method, experimental evolution, allows the investigator to continuously monitor the genetic and phenotypic response of microbial populations to a controlled laboratory environment. Quite literally, this is “evolution in

action,” in which the timing, effects, and interdependence of each underlying genetic change can be observed.

Those interested in the evolution of pathogen virulence, for example, might choose a susceptible host model or some other aspect of the pathogenic environment in which to perform experimental evolution. Although each laboratory environment likely favors its own set of adaptive genetic variants, it remains the environment itself and not some heavy-handed manipulation by the investigator that performs the selection. Experimental evolution can therefore be considered a top-down approach that is less biased than a bottom-up, locus-specific manipulation, and better reflects the natural process of genetic adaptation to a new environment or host.

Laboratory evolution experiments involving microbial pathogens (or incipient pathogens) have typically focused on one or more of the following questions: (i) How rapidly will a population of a given microbe evolve increased or reduced virulence? (ii) What are the consequences of this transition to pathogenicity? (iii) What factors promote or retard the emergence of new, more fit or virulent genotypes? (iv) What genetic changes underlie the changes in virulence, and do such changes occur consistently across replicate populations? These four topics are by no means exhaustive, and tend to exclude the complication of introducing foreign genetic material, but represent a cross section of current research.

This chapter focuses on three outstanding, representative examples of the growing number of studies involving experimental evolution of pathogens: one using a virus, one using a bacterium, and one using a primitive eukaryote, yeast. Each example explores multiple factors that could affect the population biology of pathogens and ultimately favor certain genotypes over others in direct competition. More important, these studies describe fundamental properties of microbial evolution that likely extend well beyond the particular organism or infectious process.

## 14.1 EXPERIMENTAL DESIGN

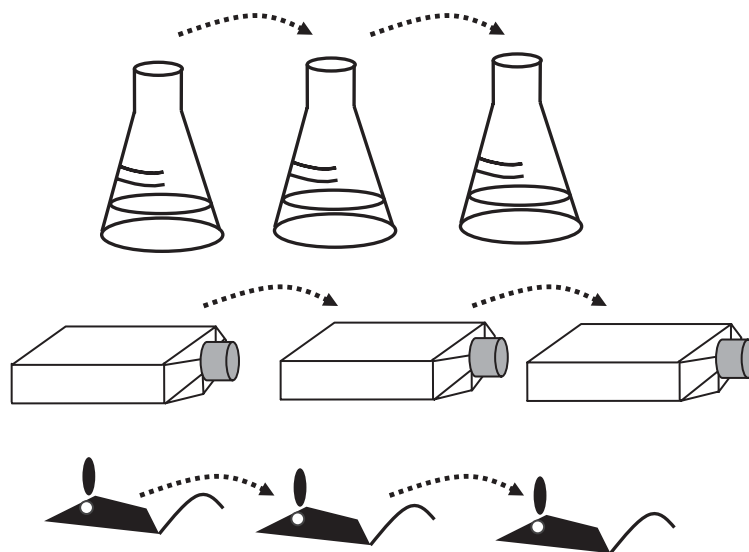
In addition to the unique opportunity to study evolutionary processes as they happen, experimental evolution is also appealing because of the significant control it affords the experimenter over both the outcome and the means of analysis. For example, evolutionary pathways can effectively be “replayed” by replicating initially isogenic populations under identical conditions, in effect, “rewinding life’s tape” [12]. Even better, given the speed with which microbial populations change relative to human schedules, intermediate steps of evolving lineages can be frozen and reconstituted for analysis at a later date. This “frozen fossil record” [17] permits ancestors and derived lineages to be compared head-to-head without relying on some indirect measure. Finally, genetic markers can be inserted that allow precise counts of different competitors as well as identification or exclusion contaminants from the long-term process.

Nearly all studies begin with a genetically homogeneous bacterial or viral population founded by only a few cells. All subsequent evolutionary change therefore depends on new variation arising within the population. This mutational variation is essential because it provides the grist for the evolutionary mill, so the experimental design must preserve at least a portion of this variation as each population is propagated. Each of the experiments presented below explicitly considers the effect of the “bottlenecking” that occurred during each transfer on the subsequent evolution. The stochastic effects of mutation and population bottlenecks also ensures that each replicate population is an independent evolutionary lineage almost certainly unlike any other. As a result, any commonality, or convergence, among the changes arising in replicate populations is worth noting and can be strong evidence of adaptation to the new environment [2,3,14].

Because during experimental evolution the environment itself is the agent of selection, choice of culture conditions is critical. These can range from liquid broth media, to tissue culture, to complex continuous passage in susceptible animals (Fig. 14.1). Though automated culture devices such as chemostats have been used with great success [9], most experiments employ manual serial batch transfer, in which an aliquot of each culture is introduced into a fresh, uninfected environment on a regular basis. Transfer typically occurs once the population has exhausted the resources of the current environment, which allows the population to enter a period of slow growth or stasis, called stationary phase in bacteria. Once introduced into new medium, the organisms then undergo a delay before resuming exponential growth, called lag phase for bacteria. Despite these varying growth conditions during serial passage, maximum growth rate is often the trait under the strongest selection, as opposed to performance during lag phase or stationary phase [28]. (More complicated culture methods, such as chemostats or turbidostats, may select more strongly on different traits such as resource affinity, or growth efficiency, because in these devices reproduction never ceases.)

## 14.2 MEASURING ADAPTATION

One of the best ways to evaluate the products of experimental evolution is by competing samples of the evolved populations against their ancestor(s). If the competition environment mimics that of the evolution experiment, we expect the evolved isolates to prevail. But how one exactly quantifies the margin of improvement can be a challenge. Each of the studies presented below applies a different method, each with its own merit.



**Fig. 14.1.** Experimental evolution strategies: serial culture in broth (top); continuous tissue culture (middle); or serial culture in laboratory animals (bottom). Examples of each are presented herein.

The simplest method involves measuring the growth rate of each genotype separately, and then either subtracting the ancestral value to yield a difference, or dividing by the ancestral value to produce a ratio. This frequently provides a sufficient picture, but sometimes adaptation is only detectable in the presence of competitors, which could be overlooked by this technique. For example, one genotype may evolve the ability to antagonize other competing genotypes by secreting a toxin or sequestering a key nutrient, and such effects would only be visible in head-to-head competition.

In direct competition, however, a marker must be introduced to distinguish the ancestor from the evolved isolates. Unfortunately, markers usually affect the growth of the marked genotype and usually require enumeration on different plates (e.g., one containing and one lacking an antibiotic), which introduces an extra measure of experimental variance. The best markers, on the contrary, affect fitness in few if any environments and can be visualized in both marker states on the same plate. Given one of these markers, evolved and ancestral genotypes of different markers can be introduced into the same environment, counted at time = 0, and then counted again at a later time. Here, the genotype that undergoes greater reproduction during the competitive interval is by definition the one with greater *fitness*. The key parameter, relative fitness, can be calculated as the ratio of the realized growth rates (doublings) of the two competitors using the following equation:

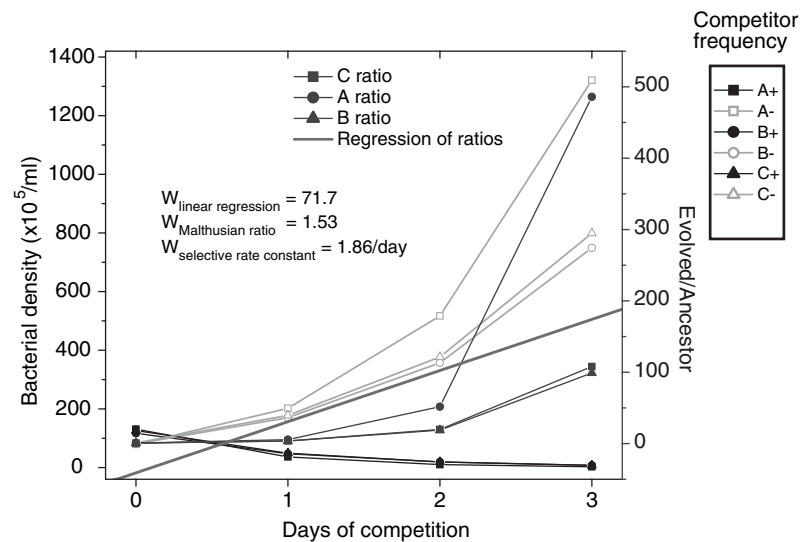
$$W_{ij} = \frac{\ln[N_i(1) / N_i(0)]}{\ln[N_j(1) / N_j(0)]}$$

Here the fitness of strain *i* relative to strain *j*,  $W_{ij}$ , is the ratio of the genotype densities,  $N_i$  and  $N_j$ , at times 0 and 1 day. A slightly different fitness measure, the *selection rate constant*, can also be calculated:

$$s = \frac{\ln[N_i(t) / N_i(0)] - \ln[N_j(t) / N_j(0)]}{t}$$

Here, the selection rate constant, *s*, is the *difference* in the rates of growth (per unit time), whereas relative fitness *W* is the corresponding *ratio*. Because fitness depends on the ratio of rates, it is dimensionless and thus may be more readily compared across different environmental or genetic contexts.

Another way to analyze the outcome of head-to-head competition is by plotting the ratio of the two competitors over time and then determining the slope of this function over time from a linear regression. This method is especially useful when only the ratio of the two competitors, and not the actual number of total individual genotypes, can be calculated; this is common in experiments using viruses that grow in inherently variable environments such as tissue culture. Fortunately, given a data set with known starting and final numbers and frequencies, we can calculate relative fitness, selective rate constant, and fitness based on linear regression simultaneously (Fig. 14.2), and choose the best of these for our experiment.

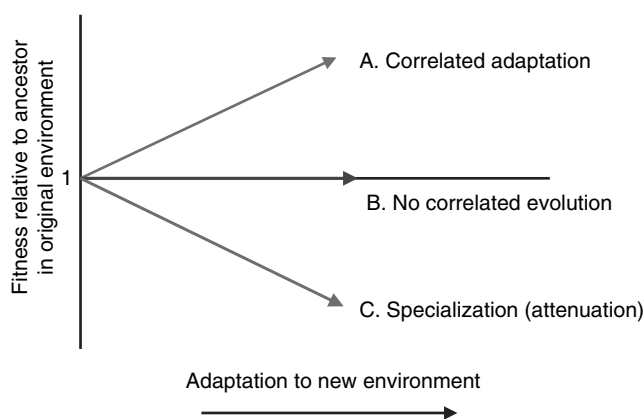


**Fig. 14.2.** Competition between evolved and ancestral genotypes derived from a 20,000 generation evolution experiment involving *E. coli* growing in a simple salts medium with glucose as the sole carbon source [3,16,17]. Relative frequencies of the ancestor and evolved genotypes are plotted as raw counts (left ordinate) and as ratios (right ordinate) over time. Fitness of the evolved genotypes is calculated relative to the ancestor by each of the three different methods described in the text.  $W$  = fitness.

### 14.3 EXPERIMENTAL EVOLUTION OF VESICULAR STOMATITIS VIRUS (VSV)

One of the best studied models of viral evolution uses vesicular stomatitis virus (VSV), a negative-sense RNA virus with a small genome (11.2 kb) that is capable of infecting a broad range of insect vectors and vertebrate hosts [19,23]. VSV is typically cultured in one of a few well-defined tissue culture systems. One cell type is baby hamster kidney cells (BHK-21), which serves as an analog of a mammalian host; another is derived from the sand fly (LL-5) and mimics the vector host. VSV researchers benefit from a well-defined marker, resistance to a monoclonal antibody (MARM), which does not alter the outcome of direct competition with an unmarked relative. Changes in various populations of evolving VSV can therefore later be compared relative to this marked progenitor.

When VSV populations have been serially transferred in a single tissue culture system, astounding rates of adaptation to that environment have been frequently observed [19,23]. In some cases, relative fitness may approach values five- or even 10-fold higher than the ancestor, which is a considerably greater margin than found in other systems [19]. One unresolved problem is whether viral adaptation to a particular cell type is specific to that environment, or more precisely, whether adaptation to a certain cell type either enhances or compromises growth in different cell types. Because one underlying assumption of vaccine design is that prolonged culture in a foreign environment will attenuate virulence in susceptible hosts, controlled studies of this process are especially useful. One potential outcome is that selection in a foreign environment actually enhances fitness in the susceptible host environment (Fig. 14.3A). This correlated adaptation may actually reflect adaptation to growth in the laboratory in general, and not a certain cell type in particular. Another pos-



**Fig. 14.3.** Hypothetical outcomes of adaptation to a new environment or host when fitness is quantified in the original environment or host. (A) Red line: correlated adaptation; (B) blue line: no correlated change; (C) green line: evolutionary specialization or attenuation.

sibility is that fitness in the original host is unaffected by adaptation to the new cell type (Fig. 14.3B). A final, perhaps more desired outcome from the perspective of the vaccine designer is reduced fitness in the susceptible host environment, caused by a genetic trade-off (Fig. 14.3C).

Trade-offs may be caused by either of two mechanisms: antagonistic pleiotropy or mutation accumulation. Under antagonistic pleiotropy, mutations that enhance fitness in the selected environment (cell type) also reduce fitness in other environments (cell types); thus, specialization is caused directly by selection. Under mutation accumulation, however, different sets of mutations improve performance in the selected environment and reduce performance elsewhere. The underlying cause is the genetic drift and ultimate fixation of mutations that harm traits no longer under selection in the favored environment; thus, loss of fitness in alternative environments results from an absence of selection. In theory, specialization by mutation accumulation may yield a more stably attenuated vaccine candidate, because such evolution may involve multiple mutational steps and prove less reversible, unlike the forward-and-back see-saw of single pleiotropic mutations.

Several groups have explored the potential for VSV to adapt to specific tissues and encumber trade-offs in other environments. Novella et al. [22] demonstrated one extreme example of a trade-off by culturing VSV populations for 10 consecutive months in sand fly cells, and then evaluating their competitive fitness in two mammalian cell environments (BHK-21 tissue culture and mouse brains). They found that sand fly-evolved VSV were at least 10 times as fit as the ancestor in sand fly cells, but the ancestral virus was at least 10-fold as fit as sand fly-evolved viruses in both mammalian environments (represented by Fig. 14.3C). Surprisingly, this clear trade-off did not persist once the sand fly-evolved VSV were passaged only once at high density in BHK-21 tissue culture, which restored virulence in both mammalian environments. Clearly, this process of attenuation was unstable and alarmingly reversible, which does not bode well for certain vaccine design strategies.

Turner and Elena [27] also demonstrated that adaptation to new cell types by VSV was typically associated with a loss of fitness on BHK cells. Specifically, populations evolved on human epithelia carcinoma (HeLa) cells and those alternating between HeLa and Madin-Darby canine kidney (MDCK) were, on average, only one-quarter as fit as the ancestor on BHK cells, but anywhere from two- to eight-fold more fit than the ancestor in their selective environment (as in Fig. 14.3C). However, populations evolved in MDCK cells alone were typically more fit (1.4–3.8-fold) than the ancestor on BHK cells, an example of correlated adaptation to an unseen environment (Fig. 14.3A). Adaptation by VSV to specific tissues may therefore either result in reduced fitness or increased fitness in alternative environments, and these alternatives are difficult to predict lacking previous experimentation.

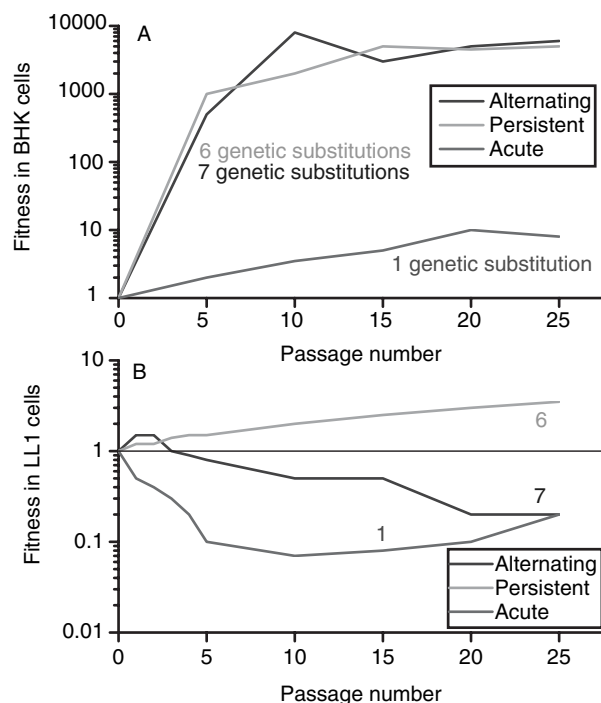
Given the obvious adaptive potential of VSV populations, Zarate and Novella [29] asked why natural populations of

VSV are evidently stable from year to year, varying only between distinct ecotypes. They hypothesized that the VSV life cycle alternating between insect vector and vertebrate constrains the evolution of viral populations and favors stable intermediate phenotypes capable of growth in both hosts. Because Turner and Elena [27] had previously shown that acute infection in alternating cell types promoted adaptation to both environments, Zarate and Novella [29] changed the infection dynamic and studied the more realistic effect of persistent (non-acute) infections in sand fly cells alternating with acute infections in BHK cells. Thus, they established three sets of VSV lineages: (i) alternating between persistent replication in LL-5 sand fly cells and acute infection in mammalian BHK cells, (ii) continuous persistent infection in LL-5 cells, and (iii) repeated acute infections in BHK cells. Fitness was assessed by calculating the slope from the ratio of the two competitors over time from a linear regression.

First, Zarate and Novella [29] found that viruses replicating in alternating environments and those replicating solely in LL-5 cells were several-fold better than the ancestor in LL-5 cells, but significantly worse on BHK cells (Fig. 14.4). Only repeated acute infections in BHK led to significant adaptation to that cell type, and any persistent infection in LL1 cells compromised adaptation to BHK cells. We can therefore infer that persistent infection in insect cells, and not the mammalian

environment, clearly dominates the selective forces acting on VSV; otherwise, populations experiencing alternating environments would have adapted to both cell types. This implies that acute infections of vertebrates may be less relevant to the evolution of the larger VSV population, though these infections certainly serve to amplify viral populations to huge numbers.

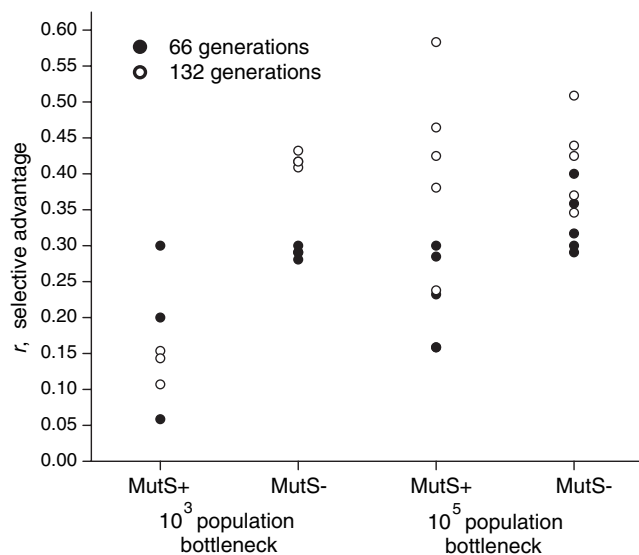
Second, to better characterize the molecular mechanisms underlying this asymmetrical trade-off, Zarate and Novella sequenced the entire RNA genome of selected evolved and ancestral isolates from the experiment. They observed only one replacement substitution in the isolate from the BHK-restricted population, whereas they found six replacement substitutions in the LL1-restricted population. Interestingly, those populations alternating between the two environments acquired nearly the same set of substitutions as the LL1-restricted population, and most of these mutations became common early in the course of the 25-passage experiment. The lack of substantial genetic change in the final 10 passages coincided with a lack of further adaptation during this interval. Thus, despite the long history of passaging VSV in a variety of mammalian cell types and observing drastic rates of adaptation, it appears that in the wild, VSV evolution is dominated by long-term chronic infection of insect populations, whose environment constrains viral populations to a relatively narrow range of genotypes. Such knowledge of the selective forces that act on viral populations can certainly inform future vaccine design by illustrating those strategies that should be most efficient in producing attenuated, host-specialized genotypes.



**Fig. 14.4.** Fitness of VSV populations assayed in (A) mammalian cells (BHK) or (B) insect cells (LL1). Populations were propagated as persistent infections of insect cell lines (persistent, green line), as acute infections of mammalian cells (acute, red line), or alternating between the two cell types (alternating, blue line). Total number of genetic substitutions identified in each population is displayed under each trajectory. Adapted from Zarate and Novella [29].

#### 14.4 IN VIVO EVOLUTION OF *SALMONELLA TYPHIMURIUM*

A wide variety of estimates exist for rates of bacterial adaptation *in vitro*, but far less exist for *in vivo* conditions. To fill this gap, and to quantify the effects of mutation rate and the size of the transferred population on adaptation, Nilsson et al. [20] serially passaged 18 independent lineages of *Salmonella typhimurium* LT2 in BALB/C mice by intraperitoneal injection and subsequent recovery of the infected spleen. The 18 lineages comprised four groups: (i) wild-type mutation rate ( $5 \times 10^{-8}$  for nalidixic acid resistance) and low population size ( $10^3$ ), (ii) wild-type mutation rate and high population size ( $10^5$ ), (iii) 700-fold increased mutation rate ( $\sim 7 \times 10^{-5}$ ) and low population size, and (iv) increased mutation rate and high population size. Both theory [8,10,26] and empirical evidence [8,11,13] suggest that these two experimental variables, mutation rate ( $\mu$ ) and effective population size ( $N_e$ ), are key determinants of the rate of microbial adaptation to a new environment, but that neither variable can predict this rate alone. Together, mutation rate and population size determine the supply rate of beneficial mutations – the lower  $\mu$  or  $N_e$ , the fewer favorable mutations arise in each population, and hence the slower adaptation proceeds.



**Fig. 14.5.** Fitness of *Salmonella typhimurium* populations following 66 or 132 generations of growth in serial murine infections. Populations were either MutS proficient (wild type) or deficient (mutator) and experienced population bottlenecks between infections of  $10^3$  or  $10^5$  cells. Fitness was quantified as the selective rate constant expressed relative to the ancestor; see text for details. Adapted from Nilsson et al. [20].

After fewer than 200 generations of adaptation to the endothelial cell environment of the mouse, Nilsson et al. [20] observed selective coefficients, or relative advantages, ranging from 0.11 to 0.58 per generation. Adaptation occurred more quickly for the lineages with higher mutation rates at both population sizes, but lineages with lower mutation rates eventually narrowed the gap in relative fitness in later generations. The small  $N_e$ , low  $\mu$  group was the least well adapted, whereas the small  $N_e$ , high  $\mu$  group was nearly as fit as those groups passed with higher  $N_e$  (Fig. 14.5). These results are consistent with the findings of deVisser et al. [8], who observed that  $N_e$  and  $\mu$  combine to set a “speed limit” on adaptation, which results from a saturation of the number of competing favorable mutations. These findings clearly apply to pathogens that undergo population bottlenecks during the infectious process. During such bottlenecks, genetic variation among individuals will be low, so increased genomic mutation rate may provide a short-term advantage to the population by generating more adaptive variation that may help elude host defenses.

However, increased mutation rate may also be costly to a microbial population. Because most mutations are deleterious, populations with an elevated mutation rate generate more unviable offspring as well as a much larger pool of less fit individuals. Most of these unfit offspring are weeded out by natural selection, but “mutator” populations also accumulate mutations in unused traits by the persistent force of genetic drift, thus accelerating the evolution of specialization. To test the effect of increased mutation rate on mouse-adapted populations of *S. typhimurium*, Nilsson et al. [20] quantified the

metabolic capacity and frequency of auxotrophy in each population. They also quantified fitness relative to the ancestor in simple broth at three temperatures, predicting that *in vivo* selection would compromise *in vitro* growth. Somewhat surprisingly, they found no overall loss of fitness under laboratory conditions, and some non-mutator lineages even increased their ability to grow in broth. On the contrary, all mutator lineages harbored a variety of auxotrophs, ranging from 0.7% to 39% of the total population, whereas auxotrophs were undetected in non-mutator populations. Further, all of the mutator lineages had lost at least one of the metabolic functions screened, whereas none of the wild-type lineages acquired any defects. Clearly, increased mutation rate accelerated adaptation to the mouse environment, but this reduced the overall functionality of the mutator populations, which could conceivably compromise their long-term viability outside a particular host.

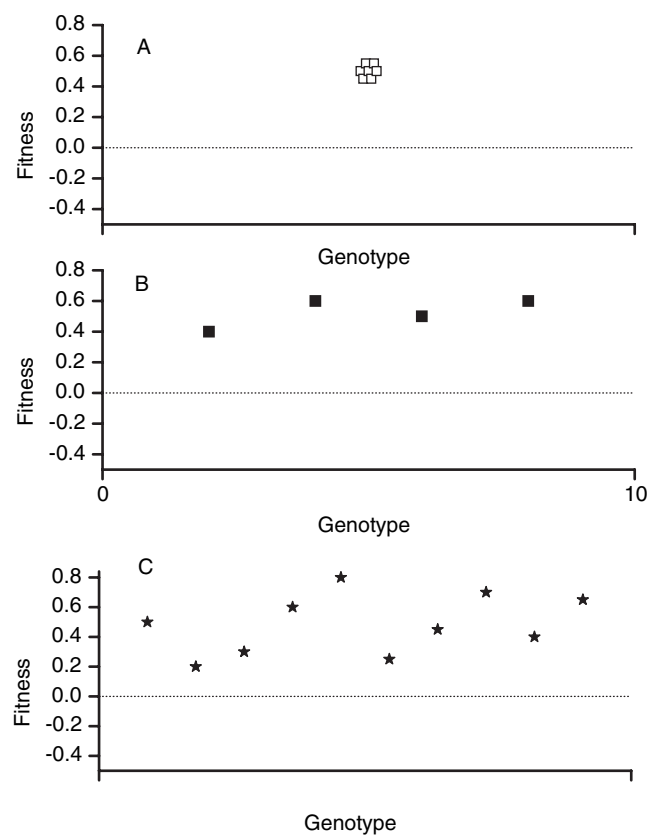
How exactly did these populations of *S. typhimurium* adapt to colonizing mice by intraperitoneal injection? Research is still ongoing to identify the specific mutations responsible for the ~10–50% improvement in competitive growth, but the authors were able to determine some important components of this adaptive process. Most notably, the pace and magnitude of adaptation was quite high, which provides strong evidence that numerous sites in the genome were capable of producing beneficial alleles. Had only a few nucleotide substitutions produced favorable variation, the rate of adaptation would have been much slower and the variation among replicates of each group likely would have been higher, owing to the stochastic appearance of these few rare mutations. Rather, Nilsson et al. estimated that  $10^4$  bp of the *S. typhimurium* genome or more could generate favorable variation in this system, which may reflect the complexity of the mouse environment. The course of infection involves uptake by macrophages at peripheral lymph nodes and then transport by blood and lymph to the liver and spleen, and it is likely that a variety of substitutions may increase the efficiency of each of these steps [20]. It is no wonder, then, that potential but not obligate pathogens such as *S. typhimurium* can cause successful infections from relatively few founders originating from a variety of environments [24]: even maladapted genotypes may rapidly become more efficient because of a wide variety of potential genetic adaptations.

## 14.5 EXPERIMENTAL EVOLUTION OF *CANDIDA ALBICANS* ANTIBIOTIC RESISTANCE

Relatively few experimental evolution projects have been performed with microbial eukaryotes (Zeyl et al. [13,30] and Luckinbill [18] are two of several notable exceptions), let alone microbial eukaryotic pathogens. Yet some microbial eukaryotes are among the world’s worst pathogens and thus further research into the factors that govern their evolution is essential. One series of studies by Cowen et al. [5–7] tackled this challenge by following adaptation by the pathogenic fungus

*Candida albicans* to inhibitory concentrations of the antimicrobial drug fluconazole. Six independent populations were exposed to either steady or increasing concentrations of the antibiotic, depending on the level of the most recently measured minimum inhibitory concentration (MIC). Six additional populations were passaged in the absence of antibiotic; these served as controls for adaptation to the laboratory environment in general and not the antibiotic. As in the previous studies, each of these populations was founded by a single clone with no capacity for genetic exchange between individuals, so all variation arose *de novo* by mutation during the experiment. This stochastic variation arising uniquely within each population allowed the authors to study how chance affects the evolution of azole resistance in *C. albicans*.

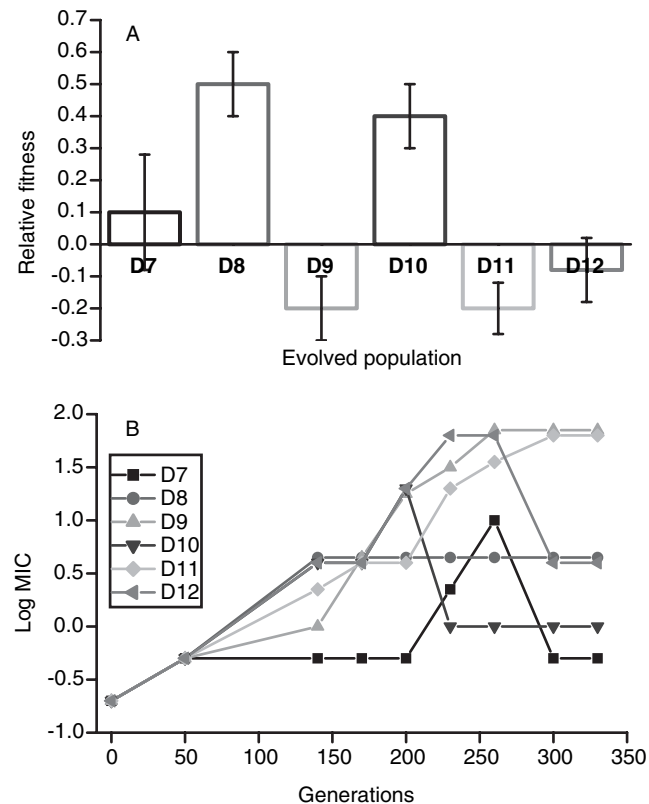
The authors predicted three possible genetic and phenotypic outcomes (Fig. 14.6): (A) all populations could adapt to the experimental environment by mutations in the same few loci and evolve identical phenotypes, which implies that only



**Fig. 14.6.** Alternative evolutionary outcomes of replicate populations adapting to a common, novel environment. Fitness is expressed relative to the ancestor = 0 (dotted line). (A) Only one genetic solution exists to the challenges posed by the new environment, so all populations achieve the same level of fitness by the same mechanism. (B) Replicate populations converge upon relatively few genotypes that permit adaptation to the new environment, with some variation in relative fitness. (C) Each replicate population adapts to the new environment by different genetic combinations that produce different levels of relative fitness.

one adaptive solution to fluconazole exists for *C. albicans*; (B) each population could acquire one of a few different combinations of mutations, which implies that adaptation is constrained to a limited number of pathways; (C) each population could acquire its own unique combination of mutations with separate consequences, which implies that adaptation may proceed along many different pathways.

The initial findings were somewhat surprising and seemed to favor the last of these outcomes (Fig. 14.6C). Each population evolved different levels of drug resistance over distinctly different trajectories, which was accompanied by different growth rates in the absence of drug and different expression patterns in four genes known to be associated with azole resistance (Fig. 14.7A). In addition, the authors found genetically distinct lineages within the populations that were



**Fig. 14.7.** Relative fitness (A) and evolved level of fluconazole resistance (B) of six populations of *C. albicans* propagated for 330 generations in the presence of increasing levels of the antimicrobial drug fluconazole. Here, fitness was quantified in a medium lacking fluconazole and estimated as the difference in the numbers of doublings between the evolved population and the genetically marked progenitor, standardized by the total number of doublings in the assay, which varied across environments. The six populations achieved approximately three different levels of fitness relative to the ancestor. Some of the variation within populations in fluconazole resistance over time resulted from fluctuations in different genotypes that coexisted in the experimental environment. Note that the less fit populations are among the most resistant to fluconazole. From Cowen et al. [6].

maintained over time, rather than sequentially excluding the preceding lineage. When they analyzed several neutral genetic markers that were heterozygous in the ancestor, they found that some populations became homozygous at an intermediate sample, but then later reverted to the ancestral heterozygosity. Because the probability that two lineages differing in their genotype at this locus coexisted in the population is much greater than a forward- and back-mutation at the same site, they concluded that multiple competing genotypes persisted throughout the experiment. Thus, the evolution of azole resistance in *C. albicans* populations may involve variable combinations of distinct genotypes, rather than a predictable succession of mutational events in a given lineage.

However, when Cowen et al. [6] quantified the fitness of the six *C. albicans* populations evolved in the presence of fluconazole in an environment lacking antibiotic (Fig. 14.7B), they determined that the populations probably fell into three different groups with different levels of fitness. The number of genetic solutions to the experimental environment containing fluconazole was therefore apparently limited. The authors dug further into the genetic architecture of the evolved fluconazole resistance in *C. albicans* by performing expression microarray analysis on four of the six replicate evolved populations as well as the ancestral strain [7]. They identified 301 of more than 5000 ORFs with significantly altered expression, but these were reduced to only three distinct patterns of genetic adaptation over time. One pattern was unique to one population and involved upregulation of a known multidrug ATP-binding cassette transporter gene, CDR2. The other two patterns were found as successive steps in the other populations, one that occurred early in two populations, and another that occurred late in all three remaining populations. Both of these patterns were complexes of expressed changes that upregulated a multidrug major facilitator transporter, MDR1, which seems to represent a dominant program for the evolution of azole resistance. Even more notable was their finding that many fluconazole-resistant clinical isolates of *C. albicans* also express the same three suites of genetic expression. Thus, a clearer interpretation of this adaptive landscape, a metaphor describing the process by which different populations climb to levels of greater fitness, is that it is dominated by only a few peaks, or adaptive solutions, that all populations converge upon.

One hope in spite of the widespread use of antibiotics and the growing frequency of resistant pathogens is that antimicrobial resistance is typically costly to the resistant individual in environments lacking the antibiotic compound. This trend is borne out in a wide range of prokaryotic and eukaryotic microbes, though the magnitude of the cost varies substantially [1]. However, it is possible that *prolonged* antibiotic use could reinforce the stability of resistance in the absence of antibiotic by selection for alleles that compensate for any incurred cost. Cowen et al. [5,7] observed varying levels of fluconazole resistance among the populations evolving in the presence of this antibiotic, and also significantly more variation among these replicates than among populations evolved without

antibiotic. Apparently, the evolutionary trajectories of *C. albicans* populations become less predictable in simple culture once antibiotic is added. One notable pattern was a transient increase, and then a *reduction* in the MIC of fluconazole for several populations that evolved under increasing antibiotic concentrations (Fig. 14.7B). The first increase in MIC was typically accompanied by a cost in fitness relative to the sensitive ancestor, but the subsequent reduced MIC eliminated that cost. Even more intriguing was their finding that antibiotic-evolved populations could grow at fluconazole concentrations well beyond their measured MIC, which means that the selected genotypes had eluded an apparent trade-off between level of resistance and competitive ability. Such genotypes may underlie the clinical problem of failure to treat cases of *C. albicans* infection in which the genotype was originally typed as “sensitive” by standard means.

The finding that multiple genetic solutions exist for microbes confronted with antimicrobial compounds is certainly troublesome because it affects the design of public health strategies to manage the emergence and spread of resistance. Should most genetic causes of resistance cause that microbe to grow less efficiently than susceptible relatives in the absence of antibiotic, then resistant genotypes will wane and ultimately disappear from the population. However, other genotypes that balance resistance with overall growth efficiency have been shown to evolve *in vitro* [1,15,25], which makes the preservation of antibiotic susceptibility among pathogens a challenge indeed. It is presently unclear whether resistant genotypes can be formidable competitors in their natural environment, outside of test tubes or even the highly favorable setting of hospitals doused in antibiotics. Antimicrobial resistance may also interfere with the ability of the pathogen to compete within the larger natural microbial community, a dynamic that should be tested in more realistic microcosm experiments.

## 14.6 FUTURE PROSPECTS

What have these three examples of experimental evolution of pathogens taught us? First, following the evolution of pathogenic microbes in the laboratory allows us to quantify their rate of adaptation to challenging environments or hosts. Some organisms are evidently extremely capable of rapid adaptation to certain environments, such as VSV in BHK cells, whereas others are far more constrained, such as VSV in sand fly cells [29]. Second, replicate populations founded by the same starting genotype often evolve different solutions to the same environment as a result of alternative favorable mutations arising by chance. This variation among replicates was observed in all three examples presented here – in a virus, in a bacterium, and in yeast [6,20,29]. Thus, chance can have a considerable effect on the evolutionary outcome of pathogens introduced into a new environment. Further, the alternative genetic strategies may vary in their clinical significance or manifestation; for example, the variability among the *C. albicans* populations evolved in the presence of

antibiotic encompassed traits that have proven difficult to treat in susceptible patients. Third, advances in genome-wide screening techniques have made it possible to pinpoint each relevant substitution in evolving pathogenic populations, which was formerly far more laborious and uncertain. It is now possible to trace changes in populations of pathogenic microbes, from the coarsest phenotype to the most precise genetic consequence, within reasonable human time frames. Lastly, they illustrate the potential of experimental evolution as a method to better understand the biology of extant pathogens and new emerging infectious diseases. Properties of the evolution of many microbes can be studied in defined laboratory environments, which enables the pursuit of any of the following questions, each of which remains largely untested.

- (i) Are genes known to affect pathogen virulence, especially those that were evidently acquired recently from other organisms, selectively *optimal* in their various environments and *stable* in their current host? Do some environments make the possession or expression of these virulence traits deleterious, which could guide new strategies to combat the disease agent?
- (ii) Is the evolutionary transition to pathogenicity typically accompanied by specialization that compromises the ability to persist and grow in alternative environments? Evidence is growing that long-term selection in a given environment will affect the ability to grow elsewhere [3,4,20,21], but is this a general property of emerging pathogens?
- (iii) How does the introduction of mechanisms of inter-genomic recombination, such as phage, plasmids, and natural DNA transformation, affect the progress and outcome of experimentally evolved pathogens? Are incipient pathogens with greater ability for genetic exchange more adaptable, and thus potentially of greater concern?
- (iv) Can closely controlled and monitored populations of experimentally evolving pathogens provide insight into improving the efficiency of vaccine design, perhaps by highlighting fundamental mutational tendencies or consequences?

Given the global resurgence of infectious microorganisms as causes of morbidity and mortality, the need to streamline treatment and vaccine design is obvious, but integrating this industry with the much smaller but rigorous field of experimental evolution could prove extremely valuable. The contributors to this volume will hopefully provide a good start to this synthesis.

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## GLOSSARY

*Adaptive landscape*: A metaphor or three-dimensional image that describes the relative *fitness* of combinations of different sets of gene variants (*alleles*), some being of low value and producing valleys, and others being of high value and producing peaks.

*Allele*: Any particular gene variant.

*Attenuation*: The process by which pathogens are weakened in *pathogenicity* or *virulence*, especially for vaccine construction.

*Ecotype*: A species subset that persists as a distinct group as a result of specific environmental selection and isolation.

*Fitness*: The capability of an individual of certain *genotype* to reproduce, which typically reflects the proportion of the individual's *alleles* among all *alleles* present in the next generation.

*Genotype*: The totality of gene variants that describe an individual organism.

*Genomics*: The study of all of the nucleotide sequences of the chromosomes of an organism, as well as their organization.

*Isogenic*: Of exactly the same *genotype*.

*Pathogenicity*: The capacity to cause disease.

*Phenotype*: The visible or quantifiable properties of an organism that are produced by the interaction of the genotype with the environment.

*Stochastic*: Random, especially in a mathematical sense.

*Virulence*: The degree to which a parasite reduces the fitness or function of its host.

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