

Chapter II: The study of microbial adaptation by long-term experimental evolution

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Abstract

Microbial pathogens emerge or re-emerge by adapting to a novel host environment. Molecular mechanisms of virulence that differentiate pathogenic from commensal organisms — including secretion systems, toxins, adhesion and invasion strategies, and the like — are often expressed with exquisite precision, and seem to be obvious adaptations. But the evolutionary processes that gave rise to these traits are still poorly understood in any detail. One challenge is that natural selection acts on the phenotype of the entire organism and not on isolated genes, so locus-specific approaches fall short. A second challenge involves the potentially substantial contributions of interactions among genes and historical contingencies to the evolutionary process. A third major challenge is to define and measure fitness precisely in the context of an evolving pathogen. Fitness reflects survival and reproductive success; it is always dependent upon the environment and often sensitive to small variations. Therefore, obtaining accurate measures of microbial fitness in nature is daunting. In principle, a detailed temporal sequence of all the genetic and phenotypic changes leading to the emergence of a pathogen might overcome these challenges, but these data are rarely attainable in natural systems.

Experimental evolution permits the study in the laboratory of the fundamental processes of adaptation that underlie microbial evolution, in general, and the evolution of pathogens, in particular. However, this approach has not, to date, emphasized the evolution of pathogens, so there is much still to be done. Far from being equivalent to biological bean-counting, long-term evolution experiments with microbes have displayed numerous elements of the complexity of natural systems while addressing fundamental questions in evolutionary biology, ecology, physiology, and genetics. This chapter highlights some key examples of experimental evolution, emphasizing long-term, open-ended studies while also mentioning other relevant studies along the way. Despite many excellent studies using fungal and viral models (2, 10, 14, 16, 50, 78, 96, 120, 134, 135),

this chapter is focused primarily on bacterial systems with occasional mention of viral systems. Specific topics that are addressed include the dynamics of adaptation, the genetic and physiological bases of fitness, causes of specialization and functional losses, evolution of mutation rates, models of the evolution of virulence, and prospects for future experiments on the evolution of pathogens.

Introduction

To the evolutionary biologist or ecologist typically concerned with large-scale processes, microbial model systems have several distinct advantages. Many bacteria and viruses are easy to grow and count, especially in comparison with most higher eukaryotes. Hundreds of generations of reproduction can be observed in some bacteria during the time required for a single generation of fruit flies. Furthermore, many bacteria have easily accessible genetics and tools for manipulation, especially in the case of the most commonly used species, *Escherichia coli*. In the view of some, however, the simplicity of microbes somehow limits their utility or relevance for ecological and evolutionary studies. Their limited genetic exchange, uncomplicated laboratory environments, and simple behaviors fuel arguments that conclusions derived from microbial models cannot be extrapolated to higher systems.

These criticisms are debatable in general and most are irrelevant for microbiologists concerned with properties unique to bacteria or viruses. Realizing the inherent interest in pathogens, microbiologists typically have not sought to extrapolate, but rather to dissect. By engineering knockout mutants (just one example of a litany of techniques) in well-behaved genetic backgrounds, countless mechanisms of survival, invasion, physiology, and pathogenesis have been described, as reviewed in other chapters in this volume. However, the evolutionary origin of these mechanisms and their contributions to reproductive fitness remain poorly described. As a result, the evolutionary success of the complete reproductive unit — the microbe as a whole — cannot be predicted by these methods.

A fusion of these two perspectives is therefore ongoing that employs long-term laboratory selection to provide insight into evolutionary processes of general importance,

but which also focuses on well-studied microorganisms. Using the arsenal of molecular-genetic techniques developed for more reductionist studies, competing bacteria can be marked, engineered to perform certain functions, or reconstituted to an ancestral state. Microbiologists using this approach do not perform the artificial selection of plant and animal breeders because handling individual cells is difficult and, moreover, not a reasonable model of evolution in nature. Instead, some environment (often simplified or otherwise novel) is constructed in the laboratory, and it is this environment that is the agent of natural selection on a population of bacteria. Even with substantial differences in the performance of different genotypes, many generations and a patient investigator are required to observe evolutionary change. But the open-ended nature of the selection (as opposed to schemes focusing on a specific trait) raises a second problem: even in a highly specific environment, to what exactly do the bacteria adapt? The uncertainties surrounding the basic outcome of these experiments — the genetic and phenotypic targets of adaptation — highlight certain limitations and argue for further refinement of microbial selection experiments. I will begin by describing the general methods in greater detail.

Culture techniques

The easiest method involves serial batch culture of bacterial populations growing in flasks or tubes. At the end of a defined period of time, an aliquot from the current population is introduced into a new flask containing fresh media that permits further growth. In principle, these transfers could occur at any time during the growth phase of the bacterial population, but in practice, most transfers occur once the population has exhausted the resources in the media and entered stationary phase. As a result, the freshly diluted population begins each transfer with a lag phase prior to entering an exponential growth phase. The population size and resource concentration therefore fluctuate during each cycle. Despite the complex population dynamics of batch culture, selection typically acts most strongly during the exponential growth phase, and less strongly on the duration of lag phase or behavior during stationary phase (32, 68, 122),

although if the interval between transfers is very long, then selection to survive starvation can also be important (41, 123).

A different form of selection occurs in continuous culture (33, 34, 55, 105), in which chemostats maintain the bacterial population size, growth rate, and nutrient concentration at a predetermined, constant level. In chemostats, the rate of input of fresh media and the rate of removal of exhausted media and living cells are equal. As a result, the culture neither ever reaches stationary phase nor grows maximally, resulting in selection for maximum competitive ability for substrates at low concentration. One challenge of continuous culture is contamination, because the constant influx of fresh media and efflux of exhausted media and bacteria opens the system to unwanted microbes. An additional complication (or benefit, depending on the interpretation) of chemostat experiments is the potential for environmental partitioning and subsequent divergence of bacterial subpopulations that grow on the surfaces of the vessels. Because batch culture discards the exhausted culture vessel at regular intervals, such “wall growth” is selected against under that regime.

In unshaken liquid culture or on solid agar surfaces, genetically uniform populations may rapidly evolve heterogeneity (60, 61, 85, 99, 112, 117). One example involving *Pseudomonas fluorescens* is discussed in detail below. Because static or solid cultures have environmental structure and prevent mixing, adaptation to different microenvironments is possible. These mutants may occasionally be distinguished on the basis of colony morphology, and the spatial structure of distinct colonies may be preserved and propagated by replica plating. Unfortunately, enumerating the total population size and the relative proportion of various mutants still requires that all cells be resuspended in liquid, which destroys the spatial structure.

A growing trend is to replace flasks, chemostats, or petri dishes with laboratory plants or animals as the venue for selection, e.g. (48). Even if the starting animal is a germ-free mouse, however, these systems may prove far more complicated and less predictable than a chemostat. For all culture methods and these host models in particular, the construction and use of clear, selectively neutral markers is essential to differentiate among competing genotypes and to identify contaminants. Truly neutral markers are surprisingly rare, so this first step is often limiting.

Estimating fitness

The most important currency in the majority of these experiments is reproductive *fitness*, a property that is frequently discussed but rarely measured. Because fitness is always dependent upon environment, methods for its estimation vary, but typically depend on the relative growth rate of the competing genotypes. In batch culture, fitness can be estimated directly by inoculating separately marked genotypes in fresh medium and allowing them to compete for resources during one or more transfer periods. Relative fitness is simply the ratio of the growth rates of the two competitors, which can be inferred using plate counts at inoculation and at the end of the competition. (See (33) and (69) for details.) This equation can be described as:

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

Here, the fitness of strain i relative to strain j , W_{ij} , is the ratio of the strain densities, N_i and N_j , at times 0 and 1 day. A slightly different fitness measure, the *selection rate constant*, can be calculated for both batch and continuous cultures (33).

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

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

Several fitness components can be used as surrogates of organismal fitness, if the experimental design prevents these absolute measures. Maximum growth rate during exponential phase, the duration of lag phase, death rate, estimates of nutrient affinity, total biomass, and other factors can each describe different elements of fitness. Nonetheless, let the experimenter beware: when fitness components are measured in isolated isogenic cultures and not in head-to-head competition, important differences in relative fitness may be undetected. Significant relative growth differences may be

observed between strains that exhibit identical growth curves in isolation. These need not require direct antagonism between competitors, either; subtle reductions in the duration of lag or increased resource affinity can produce substantial relative advantages.

Experimental design: ecological exclusion

Many microbial population biology experiments have determined which of a small number of defined genotypes is the superior competitor in a particular environment, and some have identified the mechanisms responsible for the ecological superiority. Because these experiments focus more on the ecological *fate* of genetic and phenotypic variation, as opposed to its evolutionary *origin*, they are not the focus of this chapter. Nonetheless, several are worth mentioning as examples. Zamenhof and Eichhorn (133) demonstrated that tryptophan auxotrophic mutants of a parental prototrophic strain of *Bacillus subtilis* are competitively superior in chemostat environments containing tryptophan. Derepressed mutants that produced unneeded tryptophan constitutively were at a clear competitive disadvantage in supplemented media. When the authors added indole, a precursor that auxotroph mutants could use to convert to tryptophan, the relative advantage of tryptophan auxotrophy was reduced but not eliminated. From these results, the authors concluded that the mechanism responsible for competitive exclusion was some form of energy conservation, which might eventually lead to the selective elimination of extraneous structural genes. Further, they argued that the reduced advantage of mutants in the presence of indole was a product of the extra energy expended to convert indole to tryptophan.

Dykhuisen (31) refuted this “energy conservation hypothesis” in a series of competitions between various mutants in chemostats. The key assumption of this hypothesis is that the rate of selection against producers is equivalent to the amount of energy saved by mutants that eliminate unneeded processes. Dykhuisen estimated these quantities under conditions similar to those employed by Zamenhof and Eichhorn, but found that auxotrophs were favored at a rate three orders of magnitude greater than the theoretical percentage of the energy budget devoted to tryptophan production. Moreover, the discrepancy in the two estimates was consistent across growth phases and population

demography, and even among mutants that varied in the degree of peptide synthesis from the *trp* operon. The energy conservation hypothesis also predicts that polar nonsense mutants (which produce no transcript) should grow faster than missense mutants (which produce malfunctioning transcripts), but this was not observed. Because all measurements suggested that the combined cost of peptide synthesis and the production of intermediate metabolites was vastly less than the observed advantage of auxotrophy, some other unknown selective advantage must have been favored. Thus, the “motive force” of regressive evolution, or the disabling and loss of unused coding sequence, may only rarely be energy conservation. Despite the power of these experiments, many evolutionary arguments still assume that natural selection acts frequently and powerfully to conserve energy expenditure. Microbial evolutionists are advised to pay closer attention to these findings in future studies.

Bacteria may also actively exclude ecological competitors by producing toxins, which are best exemplified by the colicin family in *Escherichia* (102, 103). Colicins pose a paradox, because the capacity to produce toxin usually slows cell growth and the actual production of toxin is lethal to the cell. How such a trait evolved is mysterious, because natural selection must have favored a trait lethal to individuals that manifested it. Yet another problem is that benefits from killing competitors, such as increased access to nutrients, are accessible to all neighbors, and not just clonemates. However, when the environment is structured, colicin producers may kill sensitive neighbors and allow their slower-growing relatives to reproduce without being overwhelmed by fast-growing sensitive competitors. Chao and Levin (15) studied this phenomenon in structured, soft-agar habitats, and found that colicin producers indeed were at an advantage. Surprisingly, clones resistant to colicins did not subsequently evolve because of their especially slow growth, resulting in a stable balance of sensitive and producing cells. These experiments resolved one aspect of prokaryotic altruism and also highlighted the importance of environmental structure in determining evolutionary outcomes.

Another key series of experiments by Hartl et al. (53) investigated whether naturally occurring allozyme variation in *E. coli* is selectively neutral in a common genetic background. While no study has yet resolved the “neutralist-selectionist” debate, this one demonstrated that extant “wild” variation in the enzyme 6-phosphogluconate

dehydrogenase (6PGD, encoded by *gnd*) affected fitness of the common strain by no more than one percent, the limit of experimental detection. However, these alleles had far greater fitness effects in different genetic backgrounds, where they apparently interacted strongly with other loci. The authors concluded that most variation is selectively neutral in any particular environment, but that certain alleles may potentiate adaptation in changing environments. Together, these studies emphasize the potential abundance of epistasis for any given trait, and suggest that *a posteriori* manipulation of a trait assumed to adaptive might not exactly reflect the previous object of selection. Later, in a different set of strains, considerable epistatic interactions were found among novel combinations of single gene knockout mutations (39). On average, these mutations tended to act additively, but roughly equivalent numbers of double and triple mutant assemblies reduced fitness either significantly more or less than additively. These systematic investigations of gene-by-gene interaction bolster the argument that individual gene effects may be less important than their contributions to genetic networks (43).

Experimental design: open-ended evolution

Laboratory microbial populations are typically founded from a single clone without any initial genetic variation. However, genetic and phenotypic homogeneity does not last long in most large microbial populations. With a typical per-genome, per-generation mutation rate among bacteria of 3×10^{-3} (29), a bacterial population founded by a single cell will produce more than a million mutations at a typical stationary-phase density of 10^9 . All subsequent phenotypic and genetic change therefore is the product of natural selection among newly occurring mutations. Tens or hundreds of generations might be required for beneficial mutants to reach high frequency in the population and appreciably affect the population mean phenotype, however, so the evolutionary response is understandably slower than in populations with initial genetic variation. After several hundred or even thousand generations of propagation in the laboratory, numerous properties of the evolutionary process may be studied in an open-ended (and not necessarily goal-directed) manner.

Because I will refer repeatedly to two focal long-term experiments throughout this chapter, I first provide a brief overview. The first involves the long-term experimental evolution of twelve populations of *Escherichia coli* B, originally founded by Richard Lenski in 1988. This is the longest continuous microbial selection experiment of which I am aware, and it remains ongoing. The ancestral strain is prototrophic, but it is unable to use arabinose owing to mutation in the *ara* operon (60, (65)). It also lacks any transmissible plasmids and viruses; as a result, all subsequent evolution was strictly asexual. A spontaneous mutant of the ancestor capable of using arabinose (Ara⁺) was used to found six of the populations, whereas the other six were founded with the Ara⁻ ancestor. This trait can be used to distinguish between populations on indicator plates, but it is neutral in the selective environment. The populations are maintained by the daily transfer of 0.1 ml of culture into 9.9 ml of fresh Davis minimal media supplemented with 25 µg/ml of glucose (DM25). These conditions allow $\ln_2(100) = \sim 6.6$ generations per day and $\sim 5 \times 10^7$ cells/ml at stationary phase. Growth occurs in a constant 37°C shaking incubator, but samples of each population were stored in a glycerol suspension at -80°C at 100-generation intervals through 2,000 generations, and at 500-generation intervals thereafter. These lines, hereafter referred to as the Lenski long-term lines (LT), have now undergone more than 20,000 generations of evolution, though only 20,000 have been studied comprehensively (21).

The second focal experiment of this review was derived from this first one as follows. After 2,000 generations of evolution, a clone from one LT population was used to found five separate experimental groups each consisting of six independent populations. Each group was subsequently maintained for an additional 2,000 generations at a different temperature: 20°, 32°, 37°, 42°, and temperatures of 32° and 42°C that alternated daily. This experiment was designed by Bennett and Lenski to investigate the dynamics and consequences of thermal adaptation, and the populations are hereafter referred to as the BL lines (5, 6).

Dynamics of adaptation

Evolution by natural selection requires two processes: 1) the production of heritable variation, and 2) nonrandom differential survival and reproduction. If microbial populations are of the large sizes typical of laboratory culture, the production of variation is rarely limiting (but see references (27, 46) for a more detailed discussion). The efficiency of the second step, on the other hand, can vary widely and ultimately depends upon the mean and variance in fitness of the population in its current environment. Populations whose phenotype is far from some optimum may adapt more rapidly, whereas those close to their optimum may adapt more slowly. Also, genetically diverse populations should respond more rapidly to natural selection. Theory predicts that genetic mixis (sexual recombination) and increased mutation rate may also accelerate adaptation, at least under certain conditions; tests of these predictions are discussed below.

Microbial populations introduced to novel laboratory environments typically undergo a period of rapid adaptation, but then the rate of adaptation slows. For example, the LT lines became ~25% better than the ancestor over the first 1,000 generations, but only improved an additional 12% during the second 1,000 generations (69). The rate of adaptation continued to slow in these populations, such that an equivalent improvement of required an additional 18,000 generations (21). While this degree of improvement is substantial by any measure, other populations introduced to drastically novel environments have undergone even more rapid improvement. For example, populations of a soil isolate of *Ralstonia eutropha* selected in laboratory environments containing 2,4-dichlorophenoxyacetic acid as the sole carbon source adapted roughly twice as quickly in the early going (61). Higher rates of adaptation have also been observed in populations founded by strains with initially low fitness, such as those carrying deleterious mutations or bearing side-effects of conditionally beneficial traits such as resistance to antibiotics or phage (4, 9, 64, 87). Finally, viral populations (and especially RNA viruses) may exhibit extreme rates of adaptation that, perhaps simply because of their short generation times, are more rapid than any bacterial system (38, 91, 92).

{Figure 1 here}

On the other hand, the BL lines achieved relatively modest fitness increases during 2,000 generations of selection in novel temperatures, owing to the previous adaptation by the progenitor to all other environmental conditions besides the novel temperature. The greatest adaptation among the BL lines occurred in the extreme temperature groups because the ancestor was least fit in these environments. In summary, the distance from the optimum phenotype and thus maximal fitness for a population in a defined environment, combined with the availability of beneficial genetic variance, together govern the rate of adaptation. While these two properties are difficult to predict *a priori*, it seems reasonable to assume that freshly-isolated “wild” microbes will usually adapt more dramatically to laboratory conditions than more “domesticated” strains owing to this “distance” effect.

The force of natural selection on a population may be buffered by events in its previous history or by chance effects. Travisano et al. (116) asked whether “replaying life’s tape” (49) repeatedly would result in distinct evolutionary outcomes in microbial populations, thus disentangling the contributions of chance and history. Single clones from each of the 12 LT lines were used to found three new populations in an environment that differed only its carbon source: maltose was substituted for glucose. Because the fitness of each new ancestor varied in the novel environment, the effect of evolutionary history (or starting genetic condition) could be quantified. Further, any variation that arose subsequently among the replicates of each clone could be attributed to chance. Travisano et al. (116) quantified the fitness of each line after 1,000 generations in the novel environment, and found that each of the lines had converged on a nearly equivalent value of fitness (Figure 2). Thus, natural selection had mostly obscured the effects of previous history and chance events during the experiment.

However, when the authors measured change in cell size, a morphological trait only loosely correlated with fitness, they found substantial effects of adaptation, chance, and history. For example, populations founded with small cells tended to remain relatively small, though significant variation among replicates from the same ancestor evolved. The authors emphasized two key conclusions. First, natural selection has the potential to erase the effects of prior evolutionary history and stochastic events when the object of selection (here, fitness itself) is tightly related to reproductive success. Second,

in contrast, traits that arose for reasons other than natural selection (e.g. genetic drift, mutation rates, or recombination) may remain etched in the organism and may contribute significantly to future evolution. This basic framework might be instructive when evaluating the potential for various microbial populations to respond to ecological opportunities or pressures.

{Figure 2 here}

Evolution of genetic and ecological diversity

One of the key objectives of the LT lines was to determine the shape of the “fitness landscape,” or in more formal terms, to determine whether evolutionary dynamics are better explained by a Fisherian or a Wrightian model (42, 131). A Fisherian landscape is theoretically dominated by one common adaptive peak, so different populations are expected to achieve this common solution through multiple small adaptive steps that eventually converge (42). On the other hand, a Wrightian landscape is much more structured and features multiple fitness optima separated by maladaptive intermediates, or valleys (131).

{Figure 3 here}

According to Wright (132), different populations are expected to achieve distinct adaptive solutions of varying relative fitness as a product of stochastic events in their history. Numerous long-term evolution experiments have demonstrated that genetic variance among replicate populations is sustained over time, which supports a Wrightian interpretation (6, 21, 71, 122). This prolonged variance almost certainly results from different adaptations to the laboratory environment early in their history that establish distinct pathways of subsequent evolution. One explanation contends that the asexual reproduction of bacterial populations is biased towards a Wrightian interpretation, because favorable mutations arising in separate populations cannot assemble. Thus, promoting recombination and inter-lineage competition might conceivably yield a much

more Fisherian outcome, or a common adaptive peak. A separate but certainly relevant issue is whether bacteria typically evolve in clonal isolation, which remains a matter of debate (51).

Similar Wrightian results were found in other long-term evolution experiments (61, 99, 125), and especially in those involving structured environments. Environmental structure is expected to subdivide populations and increase the probability that heterogeneous lineages will evolve within and among replicate populations. Korona et al. (61) investigated whether liquid or solid agar environments would favor greater diversity among experimental populations of *Ralstonia*. Significant variance evolved in both environments, but as expected, the spatial structure afforded by the agar surface generated considerably more diversity in both mean fitness and colony morphology.

Bacteriophage can be potent capacitors of evolutionary diversification because resistance to infection is typically costly (8, 74). Lenski (65, 66) quantified the trade-off between fitness in phage-free environments and resistance to bacteriophage T4, and found that all resistant mutants were inferior to their susceptible ancestor. The effects of these resistant mutations nonetheless varied widely because of pleiotropic and epistatic effects (65, 66). With this data, one may predict that susceptible bacteria will be maintained in nature despite the presence of phage because of their relative growth advantage, which in turn could lead to fluctuating cycles between the subpopulations of susceptible individuals, resistant individuals, and bacteriophage (67, 73). In other words, phage are neither expected to eliminate susceptible hosts or select completely for resistant mutants, and thus may maintain heterogeneous populations.

Bohannan and Lenski (7) tested these predictions in chemostat microcosms of phage and bacteria, and also studied the effects of altering the resource concentration on the dynamics of these subpopulations. Increasing resource concentration led to: 1) an increased equilibrium size of the bacteriophage population; 2) only slight increases in the number of susceptible bacteria; 3) more rapid evolution of resistant bacteria, and 4) greatly destabilized population cycles between bacteriophage and susceptible bacteria. These empirical findings matched the predictions of an elegant mathematical model that describes the behavior of a simple, prey-dependent (or host-dependent) ecosystem food chain. In this model, predator density is tied directly to prey density, as opposed to a

more complex ratio of predators to prey. Studies such as this one are especially useful not only because they explain the coexistence of phage and bacteria in microbial populations, but also for their potential as models of ecosystem composition.

Remarkably, the evolution of different phenotypes from a single ancestor is sometimes predictable. Rainey and Travisano (99) studied the adaptive response of a strain of *Pseudomonas fluorescens* to novel opportunities created by static (as opposed to continuously shaken) culture tubes. Three different genotypes, described by their morphology as smooth (SM), wrinkly spreader (WS) and fuzzy spreader (FS), predictably and repeatedly emerged in replicate populations. These genotypes form a stable polymorphic population by exploiting distinct niches — the broth itself, the air-broth interface, and the tube walls. Further, each genotype was able to invade these complex communities when rare. The genetic changes responsible for these heritable phenotypes appear to be highly repeatable among replicate experiments, but remain incompletely resolved. In the case of the WS morph, at least, the expression of an operon involved in biofilm formation consistently becomes upregulated and undergoes altered cell cycle control (112).

In other cases, the bacteria themselves generate environmental complexity despite an apparently uniform environment. One of the best examples involves the evolution of a population founded by a single clone of *E. coli* K12 in a glucose-limiting chemostat over 773 generations, described in detail by Adams and colleagues (55, 62, 105, 119). This population rapidly became polymorphic as genetic variability arose in the secretion and uptake of the secondary metabolites glycerol and acetate. “Wasteful” genotypes capable of rapid glucose uptake because they exported secondary substrates were the first to evolve, and were then followed by specialists that used these secretions. Thus, a single resource effectively became partitioned into three separate niches capable of harboring unique specialists for a long period. Rozenzweig et al. (105) and Kurlandska et al. (62) later inferred the evolutionary sequence of these genotypes from their ecological and physiological roles and also demonstrated fundamental tradeoffs related to resource specialization.

Rozen and Lenski (106) described the evolution of a sustained two-component polymorphism in the LT lines over a longer evolutionary period. Two clones were

isolated on the basis of their differing colony morphology (small, S and large, L) after 6,000 generations of evolution, and studied over the subsequent 14,000 generations. S clones were competitively inferior to L clones in fresh medium. However, in media “conditioned” by the growth of L clones, which were removed and replaced by an appropriate amount of glucose, S clones were the superior competitors. This suggested that L clones excreted a substrate that promoted S growth; in addition, S clones were also shown to increase L death rate. At any given evolutionary timepoint, the coexistence of these two clones was maintained by the reciprocal ability to invade when rare (frequency-dependent selection), but their equilibrial frequencies fluctuated over time.

The mechanisms responsible for this fluctuating equilibrium are unclear, but could illuminate alternative mechanisms of the co-evolutionary process. One possibility, in which S or L cells compete almost exclusively against fellow S or L cells for the limiting nutrients, requires no direct interaction between the sub-populations. Given a shared pool of nutrients, fluctuations in their relative frequency merely result from a time lag between adaptations accumulating in each population. Another, more intriguing explanation involves true co-evolution and even antagonism (perhaps a “Red Queen” interaction (121)). Under this scenario, consumer S cells evolve increasingly efficient strategies of using L secretions, perhaps even by promoting L cell lysis. L cells counter this interaction by evolving defenses to S antagonism or by limiting the quantity of their secretions. Distinguishing between these alternatives would illustrate the poorly understood mechanisms of co-evolution, which are clearly important to understand ecological complexity. As the number of infections caused by the coordinate action of multiple species grows, such complexity begs further study.

Periodic selection and the effect of recombination

One key distinction between the population genetics of prokaryotes and many eukaryotes is the amount of sexual, or homologous, recombination that occurs. Most long-term experimental evolution has permitted little if any recombination between asexual clones, which confines adaptations to their source lineage and prevents mixing. This produces a pattern known as *periodic selection*, which is actually nothing more than

the sequential replacement of clonal lineages (much like “leap-frog”). However, interesting patterns can emerge if markers are linked to the competing lineages and followed over time. Atwood et al. (3) first described fluctuations in the frequency of histidine auxotrophs during long-term transfer of *E. coli* populations. When beneficial mutations arose by chance on either the *his*⁺ or *his*⁻ genetic background, one type increased and the other decreased in frequency. If one lineage were able to completely exclude the other, one might expect complete elimination of the alternative *his* genotype, but in fact the constant rate of mutation to the alternative *his* genotype combined with selection for beneficial mutations on both genetic backgrounds prevents this exclusion. As a result, secondary loci can act as markers for the “selective sweeps” of novel adaptive mutations.

This pattern of periodic selection proved especially obvious in one LT population (37). Lenski and Travisano (71) had previously demonstrated that a model of stepwise improvement best described the pattern of adaptation in this population, as opposed to a continuous linear or hyperbolic function. The stepping pattern resulted from two factors: intermediate sampling intervals (47) and the large population size typical of most laboratory bacterial cultures. In large populations, even highly beneficial alleles require several hundred generations to rise to a detectable frequency, but the selective “sweep” occurs relatively rapidly. Sampling both alleles (winner and loser) is therefore possible only during a short interval, so when populations are sampled infrequently, they appear to change instantaneously. In this particular case, the “marker” for adaptation was cell size because of a strong correlation between cell volume and fitness (80, 116). Each beneficial allele that rose to fixation in this asexual population therefore produced a “punctuated” increase in cell size. This experiment demonstrated that simple population genetic processes such as periodic selection can account for relatively complex morphological evolution, and suggests that complicated macroevolutionary patterns may have simple explanations. Periodic selection may even explain punctuated evolution in sexually recombining populations if beneficial variation is limiting; under these conditions, adaptive mutations would arise separately in time and space and produce sudden phenotypic changes.

{Figure 4 here}

The larger question of whether recombination (as opposed to strict asexuality) accelerates adaptation in bacteria is more contentious, though the apparent ubiquity of horizontal genetic exchange illustrated by genome sequencing projects has led some to contend that recombination is a critical force (17, 51, 52). We have seen that populations undergoing evolution in a constant environment typically experience a decelerating rate of adaptation. Souza et al (111) investigated whether recombination could lift these restrictions and enhance adaptation. They periodically introduced donor *E. coli* K-12 Hfr+ strains to *E. coli* B populations that were evolving in a simple environment. Contrary to their predictions, regular recombination opportunities did not accelerate adaptation, but only increased the genetic variance among populations for fitness. One possibility why this experiment defied predictions was that the donor K-12 strain had not adapted previously to the selective environment, thus reducing the possibility of introducing beneficial variation. Another possibility is that beneficial variation in these populations was never limiting, so recombination would add no benefit; this hypothesis is discussed in greater detail below.

Evolution of mutation rates

Is the mutation rate optimal or minimal? This long-standing question asks whether the amount of variation produced during reproduction is ideal for the long-term success of the lineage, or simply held at the limits of physiochemical boundaries (109). The fact that mutation rates vary across taxa has been advanced as support for optimality, but these comparisons often lack phylogenetic foundation. In recent years, however, genomic mutation rates of related lineages within the same species of bacteria have been found to vary (63, 77). These “mutator” bacteria have often been pathogens, but this association has been challenged as a product of biased sampling because pathogens are studied more intensely (77, 97). Nonetheless, pathogenic bacterial populations seem to contain a greater frequency of mutator clones: both *E. coli* derived from urinary tract infections and *Pseudomonas aeruginosa* derived from cystic fibrosis patients seem to be composed of a high frequency of mutators (48, 94). Before we may properly evaluate the

association between pathogenicity and high genomic mutation rates, we must first consider the selective forces acting on mutation rate.

Mutation rate evolves primarily through genetic hitchhiking, or physical linkage to other beneficial alleles. When beneficial genetic variation is limiting in a population, any genetic change that increases the mutation rate, and hence the supply of adaptive mutations, may become genetically linked to a different beneficial mutation. Natural selection should not directly increase mutation rates, because on average, the only outcome will be reduced offspring quality. Thus, mutators should only evolve when beneficial genetic variation is limiting, which may occur when population sizes are prohibitively small (so-called “bottlenecking”) or after a long period of sustained adaptation to a constant environment.

These hypotheses were subject of two elegant experiments by de Visser et al (27) and by Giraud et al (48), who collectively demonstrated that 1) mutators were only at an advantage in small or initially well-adapted populations 2) the selective advantage of mutator lineages disappeared over time as the non-mutator lineages achieved similar levels of fitness, and 3) mutator lineages tended to accumulate defects when passaged *in vivo* that precluded survival in minimal environments. Further, de Visser et al (27) found that a “speed limit” on adaptation exists in asexual populations, no matter how abundant the beneficial genetic variation or how rapid the mutation rate. This speed limit arises from “clonal interference,” which occurs when different beneficial lineages arise simultaneously in a population, compete with one another, and slow the eventual fixation of the best-adapted lineage (46). To conclude, mutator lineages should only be favored when beneficial genetic variation becomes limiting, and perhaps then only transiently.

Nonetheless, the pathogenic lifestyle may include conditions favorable for the evolution of mutator lineages. A small number of pathogens are thought to found most infections, and establishing a successful infection probably requires an initial period of rapid adaptation. Thus, favorable variation may be limiting at the outset and repair-deficient lineages should produce a greater frequency of better-adapted offspring. The association between high mutation rates and pathogenicity may therefore be expected in certain situations. Lineages with lower mutation rates might eventually replace mutators over the longer term because they better avoid accumulating genetic defects.

Non-pathogenic bacteria may also evolve high mutation rates. For example, four of the twelve benign LT populations evolved 50- to 100-fold greater genomic mutation rates over the course of 20,000 generations, having acquired defects in methyl-directed mismatch repair (110). Empirical and mathematical models have set boundaries on the conditions under which mutators should be advantageous, and have boiled the evolution of mutators down to basic population parameters (27, 113). In the LT populations, the effective population size and the rate of beneficial mutation combine to produce a “boundary condition” in which mutators may, but need not, evolve. This accounts for the observation that only one-third of the populations became mutators, and permits several comparisons between mutator and wild-type populations (46).

One obvious question is whether mutator LT populations achieved greater adaptation, but measures of relative fitness between mutator and non-mutator LT populations have remained too close to resolve any significant differences over time. While certain mutator populations may have acquired transient advantages, no sustained advantage has been found (Cooper and Lenski, unpublished data). Given no measurable fitness benefit of high mutation rates, one may wonder whether these populations actually accumulated more mutations. However, multi-locus sequencing of mutator LT clones confirm that they have accumulated more point mutations than non-mutator clones. Extrapolating from the 10 changes found in ~18,000 sequenced base pairs from both mutator and non-mutator clones, roughly 250 substitutions have occurred in each mutator population, as compared with roughly three substitutions per non-mutator population after 20,000 generations (72). All of the 10 observed mutations were synonymous substitutions, but it is likely that at least some of the estimated 250 may have compromised unused functions.

Several long-term evolution experiments, including the LT lines, and numerous other studies have demonstrated the importance of transposable elements in generating adaptive mutations (see, for example: (23, 62, 79, 95, 107)). Clearly, mobile elements, typically in the form of insertion sequences (IS), represent an important class of mutations, and unusually active elements may significantly increase the raw material upon which natural selection may act. “Bursts” of IS activity may conceivably ease limits on beneficial genetic variation. The relative importance of IS mutations remains a

matter of debate, however. IS mobilizations are more readily detectable at the level of the genome than single substitutions, insertions, or deletions and may as a result be over-represented. Further, it is unclear whether IS are most mobile when adaptation is most rapid, as an adaptive theory of IS mutations might suggest. In the case of the LT populations, most IS activity appeared once the rate of adaptation slowed, and the rapid adaptation may in fact have been caused mostly by single substitutions (107). In other evolving chemostat *E. coli* populations, adaptive mutations were only partly the product of IS movement (62, 79). Thus, the contribution of IS mobilization to adaptation is currently compelling but incompletely resolved. Improvements in single nucleotide screening methods in bacteria should better describe the effects of single substitutions and clarify this issue soon.

Evolutionary specialization and genetic trade-offs

Correlated responses to adaptation, as opposed to the direct adaptive response, have been the focus of much experimental evolution (104, 118). Several groups have explored whether adaptation to the selective environment is accompanied by loss of adaptation to alternative environments; that is, whether specific adaptation produces ecological specialization (19, 21, 45, 115, 118). One might predict that the LT populations, evolving in a simple glucose-only medium, have undergone tremendous genetic streamlining to eliminate needless functions. Such a prediction is based on the premise that unused functions, and especially constitutively expressed ones, are energetically and physiologically quite costly to the cell. Bear in mind, however, that the cost of producing of unused peptides is typically much too small to explain the large benefits measured when traits are eliminated in novel environments (31). The energy conservation hypothesis therefore is poorly supported.

Genetic tradeoffs caused by antagonistic pleiotropy provide a more realistic explanation of evolutionary specialization. Under antagonistic pleiotropy, the same mutation that produces adaptation in the selective environment also reduces performance in alternative environments. For example, mutations that increase the transfer efficiency of a particular resource into the cytoplasm might decrease efficiency for less important or

absent resources. Specialization by antagonistic pleiotropy is therefore driven by natural selection, and loss of function and adaptation are expected to be quantitatively and temporally correlated.

An alternative cause of specialization is mutation accumulation, in which unused genes that are hidden from selection accumulate substitutions by genetic drift. Because genetic drift is a stochastic process, losses of function are expected to accumulate randomly (and slowly) for any given locus, and log-linearly over time across all unused loci. When specialization is caused by mutation accumulation, the mutations responsible for functional decay and those causing adaptation are distinct groups.

Travisano and others began the study of specialization in the LT populations by measuring fitness in a variety of growth media after 2,000 generations of evolution in minimal glucose medium (115, 118). They concluded that the populations had adapted to growth on glucose in particular, because they grew better when the carbon source was transported using the glucose pathway, but worse and more variably when the carbon source was transported by different mechanisms. I expanded upon this study by measuring the performance of each population on 95 different carbon sources over 20,000 generations (21). The longer evolutionary history not only provided greater resolution, but also allowed me to evaluate the relative importance of antagonistic pleiotropy and mutation accumulation in causing specialization. Average performance for the collection of all foreign substrates decayed over time in all populations, but importantly, not significantly more in mutator populations. Because an accelerated mutation rate increases the rate of substitutions by genetic drift, but mutator populations were not more specialized, mutation accumulation could not explain much of the observed specialization in this system. On the other hand, when the same loss occurs repeatedly in replicate populations adapting to a common environment, we can infer that this convergent evolution is tied to adaptation. In fact, 16 functions were lost or significantly impaired in all 12 replicate populations in the first 10,000 generations, and nine of these accumulated during the first 2,000 generations of rapid adaptation. The tight association between adaptation and functional decay suggests that antagonistic pleiotropy produced much of the resource specialization in these populations, and a growing number of mechanistic studies (see below) support this inference (18, 23).

We (20) also quantified the evolution of thermal niche in the LT populations, which are maintained at a constant 37°C. Maximum growth rate increased for all “moderate” temperatures surrounding the selected temperature, but tended on average to decrease at extreme temperatures (20°C, >40°C). Because these two trends evolved coincidentally during the first few thousand generations (impaired growth at extreme temperatures mirrored adaptation to moderate temperatures almost exactly), antagonistic pleiotropy also likely shaped the thermal niche. The loss of any particular trait by genetic drift in populations containing 10^7 or more individuals typically requires tens of thousands of generations, so mutation accumulation was unlikely to constrain the thermal niche by generation 2,000.

The loss of a particular function, the ability to catabolize D-ribose, in all 12 LT populations allowed (23) a more precise dissection of the population and molecular genetic mechanisms responsible. A genetic screen for insertion sequences in a single LT populations revealed that one element, *IS150*, had been lost from the region containing the genes that allow ribose catabolism, the *rbs* operon, along with a 2.7kb fragment. Further analysis proved that some or all of the operon was eliminated in all populations by IS duplication, transposition, and recombination. This highly mobile element explained the extreme mutation rate of this locus in ancestral populations, which became *rbs*- at rates 10^3 – 10^5 times faster than for other traits. Thus, the most obvious explanation was loss by mutation accumulation. However, complete loss of ribose function in these populations occurred roughly ten times too rapidly if only mutation accumulation were involved.

Next, the fitness effect of the *rbs* deletions was quantified; seven independent mutations improved competitive ability relative to the ancestor by 1-2%. Natural selection for these deletion mutants combined with their high rate of appearance would certainly lead to their eventual fixation, but this process would require an estimated 3000 generations. To explain the complete loss of ribose function by generation 500 in seven populations, we must presume that *rbs* mutations became linked genetically with other mutations of greater benefit. Thus, the first “selective sweeps” in these experimental populations likely involved double-mutants, in which small-benefit mutations hitchhiked to fixation with distinct large-benefit mutations. In summary, antagonistic pleiotropy,

mutation accumulation, and genetic hitchhiking were all responsible for functional loss in this instance; this argues that the study of specialization (e.g. by a pathogen to a particular host) may frequently defy assumptions and require empirical evaluation.

{Figure 5 here}

The flip side to specialization is the retention of function and phenotypic flexibility in the face of directional selection. The LT lines actually maintained most of the diet breadth of the ancestor, and even improved in head-to-head competition versus the ancestor in some foreign environments (19). Because these populations were forced to manufacture all cellular components using only glucose as a carbon source, auxotrophs could not evolve. If *E. coli* B were allowed instead to evolve in a resource-rich, ecologically complex environment, more extensive specialization may have evolved. Lacking a direct test at the moment, this hypothesis remains supported by plenty of folk microbiological wisdom (auxotrophs are commonly isolated during serial passage in rich broth) and by a compelling study of *E. coli* evolution in a mouse (48), in which 25% of mutator populations evolved auxotrophy after 300 days of *in vivo* passage. A more direct test of the relationship between ecological complexity and specialization is certainly worthy of further study. To summarize, the weight of evidence suggests that natural selection, and not an absence of selection, causes ecological specialization in large, evolving bacterial populations.

In small or bottlenecked populations, such as the founders of new infections, the case may be somewhat different. Stochastic effects may operate more strongly and fix deficient mutants in the population, perhaps with long-term consequences for the pathogen. For example, mutants that grow less efficiently outside the host or in alternative hosts may randomly become numerically dominant, which could erode survival outside the host. Such specialization could also be favored by natural selection (antagonistic pleiotropy), but the smaller the population size, the less deterministic the evolutionary process and the more rapid the effects of drift. In small, asexual populations, the process of Müller's ratchet can increase the mutation load and decrease relative fitness. This process has been confirmed during repeated population bottlenecks

of laboratory *E. coli* populations, which have become functional specialists and inferior competitors (44, 58). Such experiments may reflect the processes that have generated the extreme specialization observed in obligate intracellular symbionts and pathogens (82-84). For example, the *Buchnera* symbionts of aphids are derived from Enterobacteriaceae but retain less than one-half the genome of their free-living relatives. Another extreme example of specialization is *Mycobacterium leprae*, whose closest relative is *M. tuberculosis*. Both are obligate pathogens, but *M. leprae* has barely half the functional genome of *M. tuberculosis*. At the moment, it is unclear whether the tissue specificity of *M. leprae* or its simplified genome evolved first, but a combination of antagonistic pleiotropy, mutation accumulation, permissive environments, and small effective population sizes should provide the explanation.

Mechanisms of adaptation

The more we understand how adaptation proceeds *in vitro*, the better we will understand how pathogens and specialists evolve. The following section discusses selected examples in which the mechanisms and consequences of genetic adaptation were discovered. In many cases, the comparative method provided the key inference, and convergent evolution highlighted specific targets of adaptation to a defined environment.

Following prolonged serial transfer, adapted genotypes exhibit the following general trends. First, maximum growth rate (V_{\max}) increases; this is often the most significant component of fitness in both batch culture and chemostats (34, 122). Second, the duration of lag phase typically shortens in batch culture to permit an earlier start of exponential growth (122). Third, the resource affinity, which can be measured using Michaelis-Menton kinetics as the resource saturation constant K_s (this essentially describes the ability of the strain to uptake a limiting nutrient from the medium) has been shown to improve in chemostats but actually decrease slightly in batch culture (34, 122). This seeming contradiction is a product of more intense selection for maximal growth in batch culture and selection for resource affinity at submaximal growth in chemostats. Lastly, and less predictably, a number of correlated responses to selection may evolve, including the secretion of metabolic by-products (55, 105, 106), increased cell size (71,

80), decreased phage susceptibility (8), variable death rate in stationary phase (122), and widely divergent fitness in alternative environments (19, 115, 118). While the direct physiological response to experimental evolution is often quite repeatable among replicates within a particular environment, the correlated responses are far less predictable.

Heterogeneous correlated responses to selection arise either from variation in the adaptive mechanisms or from separate, neutral substitutions that accumulate independently of direct selection. Even if the direct and correlated responses are temporally correlated, secondary mutations producing the correlated response could have hitchhiked along with selected mutations. These alternative scenarios complicate finding the genes that enhance fitness: is the phenotype in question the direct or the correlated response? The best solutions employ screens of multiple characters, use evolutionary convergence to select candidate loci, and take advantage of whole-genome technologies.

Adams and colleagues were among the first to identify genetic changes responsible for adaptation in *E. coli* selection experiments (55, 62, 105, 119). They found that glucose-limited populations in an apparently uniform chemostat environment repeatedly evolved polymorphisms, which contradicts the ecological theory of competitive exclusion. Biochemical analysis of the growth medium implicated the metabolic by-product acetate as a factor, and subsequently over-producers and consumer genotypes of this substance were found. In each case, acetate crossfeeders evolved by overexpressing acetyl CoA synthetase (*acs*), which enhances acetate uptake. Mutations consistently occurred in the regulatory region upstream of *acs*, but some were single base substitutions while others were caused by insertion sequences (105, 119). Thus, the mysterious evolution of polymorphism in a uniform environment was explained by biochemical analysis and the genetic analysis of candidate loci.

Following similar methods, Notley-McRobb and Ferenci identified different adaptive mutations in *E. coli* chemostat populations (76, 88-90). Adaptation consistently arose from mutations in the *mgl* operon, which increased the binding protein-dependent transport of glucose. However, the nature of these mutations differed across replicates: some were caused by base substitutions, and others by short duplications, small deletions, or IS1 insertions in the ~1Kb *mgl* repressor *mglD*. The majority were actually found in

short *mgl* operator (*mglO*), involving single substitutions that greatly increased glucose affinity at low concentrations (90). Because each population harbored several beneficial lineages with distinct adaptive mutations, Ferenci and colleagues argued that this complexity is in conflict with the more conventional model of periodic selection (see above) that purges genetic variation (90). It remains to be seen whether this diversity could be sustained over a longer period of time (by means of fine-scale environmental partitioning, for example) or whether the genetic variance amounts simply to unresolved competition between different adapted lineages (e.g. clonal interference (27, 46)).

More recently, the genetic bases of adaptation in the LT populations have been illuminated by means of DNA expression arrays (18). Parallel changes in two replicate populations after 20,000 generations of evolution were sought as an indicator of adaptation and to highlight genes for further study. When compared to the ancestor, the expression of 59 genes had changed significantly in the same direction in both populations, and most of these were members of the cAMP-cAMP receptor protein (CRP) and guanosine tetraphosphate (ppGpp) regulons (18). The authors then sequenced several candidate genes and identified a nonsynonymous mutation in *spoT*, which is involved in the phosphorylation of ppGpp, in one population. When this mutation was introduced into the ancestral background, it increased fitness and changed expression in some other genes of the 59-member group. However, the same mutation had no effect on fitness when introduced into the other evolved population, indicating that a mutation of similar effect was present already and confirming the hypothesis that multiple genetic solutions produced adaptation in these populations (18). A survey of all 12 populations revealed eight separately evolved *spoT* alleles, and illustrates its general importance in adaptation of the LT populations (Figure 6).

{Figure 6 here}

Riehle et al. (101) recently described the role of insertions and deletions in evolutionary adaptation to high temperature by six *E. coli* BL populations. Here, the insertion sequence (IS186) produced a hotspot that led to duplications in three of six lines and implicated four genes previously shown to enhance survival under stressful and starvation conditions. In addition, the timing of the duplications coincided with the timing of increased fitness at high temperature. It remains to be seen whether these

duplications alone improve high-temperature fitness in the naïve ancestor, and whether they also reduce fitness at moderate temperatures as a model of genetic tradeoffs might predict.

Both the costs of specific adaptation and its unpredictability were demonstrated after the single-stranded DNA bacteriophage ϕ X174 was grown on alternate bacterial hosts (24). Phage populations were cultured first on *Salmonella* and then on *Escherichia*, alternating every 11 days. Costs of adaptation were not reciprocal: adaptation to *Salmonella* reduced growth on the traditional *Escherichia* host, but the reverse was not the case. Moreover, continued host switching did not alter this pattern because two or three substitutions in a viral capsid gene caused all of the adaptation in *Salmonella*, but then reverted in *E. coli* (24). This repeated reversion might be more likely in viruses because their much smaller genomes have fewer targets, and because evidence suggests that fluctuating environments promote second-site, compensatory changes in bacteria, rather than reversion at the original mutational site (81).

With the exception of the examples presented in this chapter, genetic tradeoffs have proven surprisingly difficult to find (45). Tradeoff models provide the foundation of much of behavioral ecology, despite a paucity of mechanistic analysis. The primitive social interactions (or at least cell-to-cell interaction) among the bacterium *Myxococcus xanthus* were an ideal starting point. Velicer et al. (124-126) selected *M. xanthus* in conditions favoring asocial behavior — liquid batch culture — for 1,000 generations. All populations lost at least part of their social motility, losses that were partly explained by mutations in the *pil* gene cluster that forms a type IV pilus (126). When the ancestral *pil* genes were restored by complementation, social traits were regained and fitness in the asocial environment was reduced. Likewise, when defined *pil* mutants were constructed in the ancestor, asocial fitness improved (126). Because social interaction in *M. xanthus* is both evolutionarily labile and phenotypically costly when conditions favor asocial behavior, sociality is evidently under strong stabilizing selection in its natural environment. Otherwise, one might expect to find a high frequency of asocial *M. xanthus*.

Studying the mechanisms of evolutionary adaptation may never be more important than as a strategy to curb the epidemic of antibiotic resistance. It is widely assumed but poorly proven that antibiotic resistance is phenotypically costly and reduces

competitive fitness when antibiotics are absent. This presumption has been perhaps the sole source of comfort in the face of the increasing problem of antimicrobial resistance. Unfortunately, two contradictory examples are clear: one involving a tetracycline resistant plasmid (9, 70, 86), and the other involving chromosomal streptomycin resistance in *E. coli* (108). In both cases, resistance in a naïve host background was costly, but after only a few hundred generations, the cost of resistance was eliminated. Even worse, the mechanisms of compensatory adaptation actually precluded reversion to antibiotic sensitivity, because genetically engineered sensitivity was toxic to the evolved host (9, 70, 86, 108). These models suggest that prolonged antibiotic usage could irrevocably purge antibiotic sensitivity from pathogenic and commensal bacterial populations.

Summarizing this section, it seems that transposable elements are the primary source of microbial adaptation. Once again, this could simply be an artifact of the ease of IS detection, but their importance to date as mechanisms of microbial adaptation is compelling. We must now examine whether IS-related mutations produce a different phenotypic spectrum than single base-pair substitutions, and whether certain of these selfish elements are more adaptive or harmful than others. It is also clear from this microbial literature that genetic trade-offs are common, and if this generality holds, then specialization may be inevitable. Thus, the actual paradox may be the existence of phenotypic generalists like *Pseudomonas* and *Burkholderia*, for example. Finally, chance effects may play a large role in the early adaptation of isolated microbial populations, with all subsequent substitutions being contingent upon the first, randomly-arising beneficial mutation. These stochastic effects should be unique in every population and therefore may suggest that “rewinding life’s tape” will always yield a different genetic outcome.

Empirical models of the evolution of virulence

Some of the best studies of the evolution of pathogens are the fruits of vaccine research. Attenuated vaccine development is predicated on the assumption that serial passage of a pathogen in a novel host or culturing system will lead to loss of adaptation in

the affected host; in other words, that specialization and/or “genetic paralysis” will ensue in the vaccine population. (Readers interested in an overview of serial passage experiments using parasites, including many vaccine trials, should consult Ebert’s (35) fine review.) The success of several attenuated vaccines for humans, including Theiler’s yellow fever vaccine and Sabin’s polio vaccine, suggests that this underlying assumption may sometimes be valid (35).

However, focused searches for weakened vaccine candidates may have obscured some of the subtle evolutionary conflicts that pathogens face. Despite many challenges to various tradeoff models of pathogen evolution, it is probable that many directly transmitted pathogens face one fundamental life-history tradeoff (22): within-host competition versus between-host transmission. For example, competition among parasites for resources within a host can lead to escalating exploitation and hence increased virulence, whereas selection for transmission between hosts may (but not always) counter this escalation (25). More specifically, overly aggressive competition among pathogens may kill the host and prevent transmission between hosts, especially if they are rare or ecologically dispersed.

Thus, one reason why serial passage experiments have actually typically led to *increased* virulence in the selected host is that artificial transmission alleviates all requirements for transmission between hosts. Other possible reasons include the genetic homogeneity and stasis of the host model, high initial pathogen diversity, and large pathogen populations. Increased fitness in the model host sometimes restricts host range, as it did in one study of bacteriophage ϕ X174 (24). In addition, prolonged serial transfer of vesicular stomatitis virus greatly enhanced fitness in a tissue culture analogue of a human host while compromising survival in tissue culture analogues of mouse or canine hosts (91). Further, serial transfer of a nuclear polyhedrosis virus in a single moth species significantly reduced the infectious capacity of the virus in other insect species (35).

Somewhat surprisingly, few serial passaging experiments involving bacterial pathogens have been reported in detail in the English microbiology literature, but many have appeared in Russian and in specialist veterinary journals and have thus gone unrecognized. Two products of Russian bioweapons research deserve special mention

because of their intriguing potential for future study. The first reported the enhancement and stabilization of *Yersinia pestis* virulence after serial transfer in guinea pig macrophages (28), generating a scary pathogen indeed. The second described stable avirulence of a natural isolate of *Francisella tularensis* despite ten passages in laboratory mice (59), discovering perhaps an excellent candidate for vaccine research. Among the more conventional systems, the *in vitro* culture of *B. anthracis* to produce attenuated vaccines for humans and livestock has selected strains lacking particular plasmid-borne virulence determinants (13). In another case, Mekalanos demonstrated that *Vibrio cholerae* strains with duplicated cholera toxin genes rapidly achieve numerical superiority during a few transfers in a rabbit model, with concomitant increased toxin production (30). In this case, the duplication mechanism has since been shown to be mediated by transposition of a selfish element, which is a functional filamentous phage in El Tor strains but not in the classical strains used in these experiments (127). Remarkably, the ancestral strain used in this study was itself the product of serial transfer in rabbits, the original progenitor being hypovirulent (30). To conclude, despite this brevity of this overview of a large applied field, it is apparent that the experimental evolution of pathogens can illuminate both adaptive potential and genotypic constraints. More hypothesis-driven studies should therefore become essential components of the development of predictive models of the evolution of pathogens.

We will conclude this section by highlighting three projects that used experimental evolution to empirically test models of the evolution of virulence. The first involved the filamentous bacteriophage ϕ 1, which is F-pilus specific, and *E. coli* hosts that were either F+ or F-. Bull et al. (12) designed selection regimes to either enforce high host fidelity through infrequent opportunities for new infection, or low host fidelity by providing ample opportunities for new infections. High fidelity should favor vertical viral transmission and hence low virulence, whereas low fidelity should favor horizontal transmission and greater virulence. These predictions were confirmed: when evolved viruses competed in the absence of susceptible hosts, selection favored vertically transmitted viruses because they harmed the infected host significantly less. However, once susceptible hosts were introduced to the culture, the horizontally transmitted viruses gained a selective advantage. Thus, the mode of transmission (e.g. lysis versus lysogeny)

is critical to the virulence evolution of pathogens, and the greater the density of susceptible hosts, the more infectious and potentially severe the pathogen may evolve.

In actuality, virulence may increase in evolving pathogens for several reasons, contrary to the longstanding assumption that microbial virulence is maladaptive. Even if the frequency of available hosts remains constant, the timing of transmission may affect the evolution of virulence (22, 36). For example, early transmission may favor more rapidly reproducing pathogens and hence increased virulence, while late transmission may favor slower replicators and reduced virulence. An experiment in Paul Ewald's lab (22) tested this hypothesis by serially transferring a gypsy moth nuclear polyhedrosis virus in live larvae. One set of viral lineages was transmitted early, while another set was transmitted late during larval infections. Early-transmitted viruses evolved increased virulence, but they tended to kill their larval hosts sooner, when larvae were small, so total virus production declined. Late-transmitted viruses tended to be more benign and allowed additional larval growth and much greater viral production. Here, virulence evolution obeyed a tradeoff that is theoretically ecologically dependent: sparsely distributed hosts will select for reduced virulence, but high densities of susceptible hosts will favor rapid replicators and greater severity.

Evolutionary scenarios for pathogens may become far more complex if multiple infections per host (superinfection) are common. Under these conditions, competition among pathogens within a host may become maladaptive (see, for example: (25, 26, 40, 93)), and not only because overly aggressive host exploitation may prevent transmission. One of the most fascinating studies of intra-host pathogen competition propagated the RNA phage $\phi 6$ at either high or low ratios of phages to the host bacterium *Pseudomonas phaseolicola* (120). Phage grown at high rates of co-infection first evolved increased fitness, but then surprisingly became less fit. This pattern was clearly explained by a game theoretical model of "prisoner's dilemma," in which selfish viral strategies evolve despite the greater fitness rewards of cooperation. The reduced viral fitness, or defection, likely resulted from selfish use of gene products that are normally shared. This strategy evolved only among genetically unrelated competitors, and not among clonal, highly related sister clones. Even the simplest parasites may therefore follow evolutionary strategies typically observed in animals competing in complex social environments.

Prospectus: future directions

From the evidence presented here, the future of using long-term laboratory culture to understand the evolution of microbial pathogens is promising indeed. The rate-limiting steps of this approach remain unchanged: appropriate choice of ancestral genotypes, the definition of amenable and relevant experimental conditions, and sufficient evolutionary time for variation to arise *de novo* and predominate. However, technologies for strain detection, identifying selected mechanisms, and novel models continue to improve apace. High-density hybridization and expression arrays are becoming increasingly available to assess the genomics of adaptation, microsatellites (56, 100) and other novel marker systems enhance detection and quantification (57), and innovative host models are on the upswing. Here we highlight four areas for future research that should be of interest to readers of this book.

One assumption that largely remains untested is whether genes important for virulence are selectively optimal in particular hosts, genetic backgrounds, or environments. This is especially critical when virulence traits are borne on plasmids or phage, because suboptimality may favor an unstable relationship between the host and the mobile element, and thus only occasional pathogenicity. Suboptimal gene-by-genome interactions may also reflect inadequate coevolutionary history, as in the case of emerging or opportunistic pathogens, and thus should be followed closely. Optimality may be tested in defined laboratory culture by directly competing genotypes that differ only for these virulence genes. Further, culture conditions can and should be varied to reflect the changing environment of the pathogen. We can also examine whether the cost of carrying a pathogenic element is ameliorated over longer-term laboratory selection, which has already been demonstrated for elements encoding antibiotic resistance. Despite conventional thought that most human pathogens are well adapted to human hosts, predicting the evolutionary epidemiology of microbial pathogens hinges upon empirical estimates of the benefits of virulence mechanisms.

Of course, the success of this approach requires realistic models of the microbial environment, which is a tall order. Given the unwieldy complexity that evolves even in

the simplest systems, developing natural *in vivo* or “environmental” models has seemed intractable. Nevertheless, the future of experimental evolution must move beyond the simplest systems if the goal is to evaluate the role of a particular pathogen in nature. Indeed, “mice are not furry chemostats” (11), as some authors referenced in this chapter have demonstrated. Recently, a number of novel animal and plant models of pathogenesis have been used to assay different candidate genes, and these have often arrived at the somewhat surprising conclusion that mechanisms for virulence in *Arabidopsis* (for example) are frequently identical in animal models (mice or *C. elegans*; (1, 75, 98, 114)). My group is currently using these findings as the basis for *in vivo* experimental evolution of species of the *Burkholderia cepacia* complex, and similar applications are ongoing in other laboratories.

Such use of novel laboratory culture systems may permit the study of many poorly understood microbes and better address classic questions. For example, it is frequently presumed that the evolutionary transition to pathogenicity is accompanied by niche specialization that compromises growth in other environments (129, 130). Experiments presented here have shown that prolonged growth in laboratory culture produces specialization, but it is unclear whether the evolution of virulence also must lead to specialization. The fact that parasites exhibit some of the most extraordinarily specialized life histories is certainly suggestive, and may result from the extreme natural selection that occurs during host invasion and is in need of further study.

Finally, the evidence that important steps in microbial evolution have been fueled significantly, and perhaps mostly, by inter-species recombination continues to grow. Comparative genomics shows that only 40% of the genome of *E. coli* is shared by three different sequenced strains (128), and abundant evidence for recombination in pathogenic lineages exists. The most frequent objection to previous experimental evolution, an absence of recombination, may prove to one of the most exciting avenues in the future. Natural competence and plasmid exchange rates vary widely in bacteria and are obvious targets for natural selection, but some species that feature large “integron islands” (54) may experience extreme levels of recombination. Do these species harbor extraordinary evolutionary flexibility and the potential for rapid adaptation? What are the evolutionary processes that maintain generalism? How do bacteria preserve the capacity for horizontal

transfer, which on average should produce maladaptive offspring? These seemingly ponderous questions may prove in the near future to be excellent candidates for simple manipulations and yield to the power of long-term experimental evolution.

Summary bullets

- The most conspicuous product of evolution, adaptation, is surprisingly difficult to study in most systems. Experimental evolution using bacteria has shed light on this process by taking advantage of the large population sizes, rapid generation times, and relatively accessible genetics of microbial systems.
- Adaptation by bacterial populations to a novel environment is often initially rapid, and the sequential replacement of clones within the population can lead to punctuated dynamics known as periodic selection.
- Competitive exclusion is not inevitable, however, because stable polymorphisms may be maintained by cross-feeding, environmental partitioning, interactions with bacteriophage, or toxin production.
- Adaptation is frequently accompanied by genetic trade-offs that compromise fitness in alternative environments. Selection may favor the silencing or deletion of certain genes, which has been associated with the transition to pathogenicity.
- Insertion sequences and other transposable elements are responsible for a large proportion of the genetic mechanisms of adaptation published to date.
- Some trade-offs may be overcome by compensatory evolution, and in the case of antibiotic resistance, may preclude reversion to sensitivity.
- While increasing the mutation rate may lead to short-term advantages, it may also be detrimental in the long run, either because of enhanced mutation accumulation in small populations or the eventual loss of functionality in alternative environments.
- Experimental evolution remains a powerful but underutilized technique in the study of microbial pathogens and the evolution of virulence.

Acknowledgments: Thanks to the editors for their patience and encouragement, and to the Michigan Society of Fellows for financial support. Thanks to Richard Lenski for his help in organizing the material in this chapter and with editing a draft of this chapter, and whose funding from the NSF (currently DEB-9981397) supported much of the work that I have reviewed here.

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