THE INFLUENCE OF GENERAL AND INDUCIBLE HYPERMUTATION ON ADAPTATION DURING EXPERIMENTAL EVOLUTION

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ABSTRACT

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Hypermutator (or mutator) strains of bacteria have been observed in a variety of clinical, environmental, and laboratory populations with up to 1000-fold increases in spontaneous mutation rates. Defects in DNA repair machinery responsible for general hypermutation most commonly include the inactivation of methyl-directed mismatch repair that result in constitutive increases in mutation rate. Alternatively, mutagenic DNA repair only transiently raises mutation rates through the activation of low-fidelity polymerases in response to DNA-damaging stress conditions. The widespread existence of both general and inducible mutator genotypes suggests that evolutionary strategies of bacteria include mechanisms for increasing mutability. This work investigates the influence of hypermutation on adaptation through experimental evolution with the contextually relevant model organisms *Pseudomonas cichorii* 302959 and *P. aeruginosa* PAO1. Following ~500 generations of growth, both model organisms exhibited comparable improvements in fitness, independent of mutator status, suggesting that hypermutation does not impede adaptation through mutation accumulation. Both general and inducible hypermutation facilitated genotypic diversification that was not observed in non-mutator lineages. The mechanistic differences underlying general and inducible hypermutation were reflected in unique spectra of nucleotide substitutions but did not restrict access to parallel adaptive traits despite considerable variation in gene expression profiles. The diversity in colony morphologies and gene expression traits observed in mutator lineages may represent a broad exploration of sequence space that is no doubt a favorable strategy for adaptation.

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CHAPTER 1: Literature Review

I. The Microbiology of Mutability

This section has been modified from a publication of the same title in FEMS Microbiology Letters (2007, 277:11-20), co-authored by George W. Sundin.

A. Introduction

Current dogma states that most mutations are deleterious, and that the accumulation of deleterious mutations, in the absence of recombination, will ultimately result in the extinction of a cell lineage (Muller's ratchet –Muller 1964, Haigh 1978). Thus, it has been argued that asexual populations will evolve a mutation rate that is as low as possible (Drake 1991) – a phenotypic trait that is limited only by the relative fitness costs of maintaining systems involved in error-avoidance and error-correction during DNA replication. However, asexual populations evolve. Evolutionary change can occur rapidly through the acquisition and incorporation of foreign DNA, enabling expansion into previously unoccupied ecological niches, or at a relatively slower pace through point mutation. Change engendered through point mutation enables evolution, through the generation of variation and the subsequent action of natural selection favoring novel, better-adapted alleles. Spontaneous mutants have an increased chance for selection in bacterial populations due to a variety of factors including the relatively small genome size of most bacteria, the limited amount of noncoding DNA in bacterial genomes, and the relatively large population size bacterial cells can achieve in certain environments. It should be noted that this 'slower evolutionary pace' is relative; spontaneous mutation can be critically important in the generation of antibiotic-resistance mutants presenting a continual clinical problem.

It has become apparent that the evolutionary strategies of bacteria include several mechanisms to increase cellular mutability. In some cases, mutability increases in response to

DNA damage or extreme starvation stress, and hence it is not yet clear if increased mutability is a byproduct of a 'survival at any cost' strategy or a strategy in itself to increase survival. There are three major cellular pathways responsible for invoking increased mutability in bacterial populations. The first pathway is constitutively active due to the loss of DNA proofreading capabilities during replication (Figure 1-1). Defects in genes of the methyl-directed mismatch repair (MMR) system can result in greater than a 100 × increase in the genomic mutation rate (Nohmi 2006). The second pathway is transient and inducible through the action of the specific low-fidelity DNA polymerase enzymes DNA polIV and polV, encoded by the *dinB* and *umuDC* loci of *Escherichia coli*, respectively, and orthologous genes in other organisms (Figure 1-1). The third pathway occurs in starved cells, is dependent on the induction of the σ^S (RpoS) regulon, and usually requires the activity of low-fidelity DNA polymerases (Figure 1-1). The biochemical basis of these bacterial mutability systems has been well characterized, but the role of increased mutation rates in the ecological and evolutionary biology of these organisms has received less attention.

Because most mutations are deleterious, organisms possessing an elevated mutation rate, whether constitutive or transient, must be primed to capitalize on the occurrence of rare, beneficial mutations. For example, bacteria exhibiting elevated mutation rates are overrepresented in pathogen populations or organisms inhabiting rapidly changing environments. Dobzhansky (1950), in a seminal statement on adaptation to diverse environments, wrote 'Changeable environments put the highest premium on versatility rather than on perfection in adaptation'. In this minireview, an examination of the role of systems conferring increased mutability in microbial ecology and evolution and the organisms and environments where such systems are selected will be carried out.

B. Mutators

Bacterial mutators (or hypermutators) are strains that possess a mutation rate that is elevated up to 100-fold due to defects in genes of the MMR system such as *mutS, mutL, mutH,* or *uvrD* (Miller 1996, Oliver *et al*. 2002). Mutators have been readily isolated from populations of a variety of human pathogens at frequencies ranging from 2% to as high as 57% (Table 1-1). More recently, results from a second wave of sampling experiments have shown an association between mutators and the evolution of spontaneous antibiotic resistance. For example, larger proportions of mutator strains of *Salmonella enterica* and *Staphylococcus aureus* with resistance to quinolines (Levy *et al*. 2004, Trong *et al*. 2005) and of *Staphylococcus aureus* with resistance to erythromycin (Prunier *et al*. 2003) were found compared with the proportion of mutators in antibiotic-susceptible strains. Experiments with *Pseudomonas aeruginosa* mutators in a mouse model of lung infection demonstrated that treatment with ciprofloxacin resulted in resistance selection in mutator but not in wild-type strains colonizing the mouse lungs (Maciá *et al*. 2006). In contrast, treatment of the mice with tobramycin did not result in resistance selection in either the mutator or wild-type *P. aeruginosa* pathogen populations (Maciá *et al*. 2006). The increased occurrence of mutators among some bacterial pathogens is now generally accepted; however, the link between mutators and enhanced evolution of antibiotic resistance is a more recent finding that remains to be fully resolved (Woodford and Ellington 2006).

A common hypothesis explaining the apparent success of mutators among bacterial pathogens suggests that the resulting increased mutation rate can enhance adaptability through the generation of variability in the rapidly changing host environment (LeClerc *et al*. 1996). Thus, the evolvability of mutators requires multiple steps, and the increased frequency of

mutator loci within populations is due to hitchhiking with other favorable mutations, whose chance of occurrence is increased in the mutator background (Tenaillon *et al*. 1999). Furthermore, the MMR system also acts as an inhibitor of interspecies recombination, indicating that mutators can more readily acquire and incorporate horizontal DNA (Matic *et al*. 1995). Most analyses of mutators, however, have focused on the elevated mutation rate in these strains, as the role of mutators in adaptive evolution is thought to be dependent on their effect on the overall mutation rate (Taddei *et al*. 1997).

i. The ecology of mutator bacteria

In laboratory culture-based experiments, mutators are disadvantageous when initially rare ($<10^{-5}$), and are unable to invade populations because of the greater likelihood that a beneficial mutation(s) will occur in the larger, non-mutator population (Chao and Cox 1983). However, mutators have arisen in some experiments, such as in *E. coli* adapting to a glucose-limited environment (Sniegowski *et al*. 1997, Notley-McRobb *et al*. 2002, Shaver *et al*. 2002). In one case, the enrichment of mutator strains resulted from hitchhiking with known beneficial mutations (Notley-McRobb *et al*. 2002), and evidence from other studies supported similar conclusions (Chao and Cox 1983, Shaver *et al*. 2002). Lethal selection in bacterial populations through exposure to antibiotics can also hasten the occurrence and increase the frequency of mutators in a population (Mao *et al*. 1997). Factors contributing to the persistence of mutators in constant environments are less clear as theoretical analyses suggest that the continued generation of deleterious mutations would ultimately reduce fitness (Taddei *et al*. 1997) implying that there is an interplay or trade-off between the possible benefits of mutation and the costs. The cost of a mutator phenotype was amply demonstrated by Funchain *et al*. (2000) who used bottleneck

experiments to encourage the accumulation of mutations in *E. coli* mutator lineages. These researchers found that passage through a bottleneck resulted in large losses of fitness and extinction in some cases for *E. coli* mutators (Funchain *et al*. 2000).

Reports of increased numbers of *P. aeruginosa* mutators associated with chronic lung infections in cystic fibrosis (CF) patients and the availability of different infection models have made this organism an attractive model to study the consequences of mutator status in a pathogen. *Pseudomonas aeruginosa* is an important pathogen that causes chronic lung infections in patients with CF; *P. aeruginosa* is also a ubiquitous environmental organism with a large genome and is known as a metabolically versatile and highly adaptable organism (Stover *et al*. 2000). The establishment of chronic infections requires adaptations of the pathogen to the CF lung environment including a transition from nonmucoid to mucoid, alginate-producing strains (Govan and Deretic 1996). The *mucABD* locus, involved in the regulation of alginate biosynthesis, is a frequent target for mutations resulting in the transition to mucoidy with mutations in *mucA* being the most common (Martin *et al*. 1993). Recently, Moyano *et al*. (2007) determined that the emergence of mucoid variants was more frequent among *P. aeruginosa* mutator than among non-mutator strains and that the resulting *mucA* mutations in the mutator populations were similar mainly to those found in natural CF isolates.

Genetic and genomic studies have shown that *P. aeruginosa* strains establishing chronic infections exhibit various adaptations including the loss of many virulence traits important for invasive infections (Figure 1-2). Selection for mutator strains accompanies the adaptations seen. The distinction between chronic and acute *P. aeruginosa* infections is apparent because mutators have not been detected in examinations of strains isolated from acute infections (Oliver *et al*. 2000).

The evolution of pathogenic *P. aeruginosa* strains in the CF lung has been suggested as an example of adaptive radiation (Oliver *et al*. 2000), a process whereby genetically uniform *Pseudomonas* populations rapidly diverge into phenotypic variants (Rainey and Travisano 1998). For example, comparative whole-genome sequencing of two *P. aeruginosa* isolates from a single patient separated by 7.5 years revealed 68 mutations with a high ratio of nonsynonymous to synonymous mutations that occurred during establishment of the chronic infection (Smith *et al*. 2006). The chronic infection strain was a *mutS* mutator with mutations inactivating virulence traits such as motility, type III secretion, and exotoxin and protease production, similar to virulence traits lost in other CF mutator strains (Smith *et al*. 2006, Hogardt *et al*. 2007). The loss of invasive virulence traits in chronic infection strains favors persistence in the original host, but disfavors transmissibility to new hosts, as shown for mutator strains in a CF mouse model (Mena *et al*. 2007, Montanari *et al*. 2007). However, in another study, where two predominant clones were found among Danish CF patients sampled over 20 years, these reduced-virulence clones were still found to be transmissible among patients (Jelsbak *et al*. 2007). In addition, other factors such as oxidative stress conditions that are prevalent in the CF lung also seem to select for mutator strains (Ciofu *et al*. 2005). Thus, the ability to establish a chronic infection requires alterations to the genotype of the environmental strains causing the initial infection (Figure 1-2). The extent of the mutations required for chronic infection seems to favor mutator strains that could acquire the necessary mutations rapidly.

A few other studies have been performed with mutator strains of enterobacterial pathogens. A significant early study by Giraud *et al*. (2001) demonstrated that a high mutation rate was initially beneficial to *E. coli* cells colonizing the gut of germ-free mice. However, once adapted to this environment, the mutator cells accumulated mutations that, although neutral in

the current environment, were costly in secondary environments (Giraud *et al*. 2001). A similar result indicating reduced fitness in secondary environments was shown for *Salmonella typhimurium* mutators adapted to mice (Nilsson *et al*. 2004). Furthermore, virulence is often reduced in mutator compared to non-mutator strains (Zahrt *et al*. 1999, Picard *et al*. 2001) in pathogenesis experiments, although there is one report of an increase in ability to cause a chronic infection of *E. coli* mutators that are urinary tract pathogens (Labat *et al*. 2005).

In summary, constitutive mutators with deficiencies in MMR are overrepresented in populations of some bacterial pathogens. That these mutators are not widely prevalent in all bacterial populations indicates that few ecological niches can be colonized by organisms exhibiting a high mutation rate. During pathogenesis, mutator cells appear to have advantages in conquering the host environment; however, these mutator strains are typically compromised in secondary environments. Chronic-infection strains, such as *P. aeruginosa*, may be unique in that these organisms are genetically distinct from corresponding invasive pathogen strains and may be able to withstand the accumulation of additional deleterious mutations.

C. SOS Response and Inducible Mutability

First described in 1970, the SOS response controls the expression of over 40 genes via the LexA repressor protein in response to DNA damage that threatens genome replication (Courcelle *et al*. 2001). Briefly, DNA lesions inhibit replication fork progression producing long stretches of single-stranded DNA, which is bound by RecA protein forming a nucleoprotein filament and reversibly converting RecA to the active $RecA^*$. $RecA^*$ then stimulates the autoproteolytic cleavage of the LexA repressor protein allowing the transcription of SOS genes, including specialized DNA polymerases that can bypass the DNA lesions, relieving the stalled

replication fork, and allowing further genome replication (Bridges 2005). Specialized polymerases capable of translesion DNA synthesis (TLS) belong to the Y-family class of DNA polymerases (Ohmori *et al*. 2001). The genetic and biochemical characteristics of Y-family polymerases and their role in SOS mutability have been reviewed extensively (Tippin *et al*. 2004, Nohmi 2006, Jarosz *et al*. 2007, Schlacher and Goodman 2007). Here, only a brief discussion is provided of such topics in preparation for the main focus on the broader effects of Y-family polymerases on the ecology and evolution of microorganisms.

Transcription of genes encoding two Y-family DNA polymerases, polIV (*dinB*) and polV *(umuDC*), is induced as part of the SOS regulon in a highly coordinated manner due to RecA filament formation and variation in LexA-binding affinity (Friedman *et al*. 2005, Krishna *et al*. 2007). A further posttranslational processing step, the proteolytic cleavage of UmuD to UmuD$'$ mediated by RecA* (Burckhardt *et al*. 1988), is required in the ultimate production of the active polV UmuD$'_2$C (Tang *et al*. 1999). The error-prone nature of TLS polymerases produces a transient increase in mutation rate during the activation of the SOS response. The contribution of each SOS-inducible polymerase to mutagenesis was evaluated by creating SOS-polymerase gene deletions in a *mutD5* background – deficient in polIII proofreading and therefore constitutively SOS active. Deletion of each polymerase has a different effect on the rate and type of mutation in the *mutD5* strains and interestingly depends on whether the bacteria were in the exponential or stationary phase (Nowosielska *et al*. 2004). Although both polIV and polV are responsible for the SOS mutator activity in strains that carry a constitutively activated RecA protein, deletion of polV completely abolishes mutagenesis to near wild-type levels. However, polIV is responsible for as much as 70% of mutations in these strains and over production in uninduced strains

produces a mutator phenotype independent of polV induction (Kuban *et al*. 2006). Instead of simply providing functional redundancy, the SOS-induced DNA polymerases likely perform complimentary functions in mutagenesis in which their activity and participation depends on the sequence and type of damage responsible for the block in replication.

Escherichia coli possesses several mechanisms to limit the extent of cellular mutability incurred during the SOS response. Translesion DNA synthesis in *E. coli* is tightly controlled on at least four levels: (1) transcriptional regulation by the RecA-LexA proteins; (2) regulation of the posttranslational modification of UmuD to UmuD$'$ by RecA* resulting in a cell cycle DNA damage checkpoint (Opperman *et al*. 1999); (3) subunit exchange of UmuD with UmuD$'$ leading to the dissolution of UmuD$'_2$C complexes following TLS (Shen *et al*. 2003); and (4) enhanced degradation of UmuD and UmuC by Lon protease (Frank *et al*. 1996). The posttranslational regulation of TLS is thought to allow added time for error-free nucleotide excision repair (NER). The NER protein apparatus (Sancar 1996) also acts in concert with the Mfd protein in transcription-coupled repair, a process in which the template strand of actively transcribed genes is preferentially repaired relative to the coding strand after DNA damage is incurred (Mellon and Hanawalt 1989). This process, initially termed mutation frequency decline, also acts to limit genome mutability following UV irradiation (Witkin 1994).

Nevertheless, UV-induced mutations occur as a consequence of TLS, and the observation of increased mutability in *E. coli* and *P. aeruginosa* containing *mucAB* relative to *umuDC* (Hauser *et al*. 1992, Kulaeva *et al*. 1995, Kim and Sundin 2000) suggests that the quantity of lesions bypassed via TLS can be altered based on the activity of the system. Mutagenesis mediated via TLS can also occur in the apparent absence of DNA damage; this process, termed

untargeted or spontaneous mutagenesis, occurs when polV competes with the replicative polymerase holoenzyme polIII for access to undamaged DNA (Fijalkowska *et al*. 1997).

Y-family polymerase function may not be limited to error-prone DNA damage repair induced by lesions blocking replication fork progression. They may also contribute to double-strand break repair, D loop primer extension during homologous recombination, nucleotide excision repair, stationary-phase mutagenesis and survival, mutation frequency decline, and nucleotide starvation (Lehmann 2006). The best studied alternative role for a Y-family polymerase is the stationary phase, adaptive mutation mediated by polIV (*dinB*) under control of the stress responsive sigma factor RpoS (McKenzie *et al*. 2001, Roth *et al*. 2006). The presence of such a system would suggest a mechanism for increasing the cellular mutation rate in response to stressful conditions, potentially accelerating evolution. Because the increased mutation rate is mediated by the low-fidelity alternative DNA polymerase polIV, adaptation results from the selection of stochastic, beneficial mutations and is not directed based on the applied stress. The resulting increase in mutation rate occurs throughout the entire genome and therefore many deleterious mutations may also be generated during the process (Hersh *et al*. 2004).

i. Impact of Y-family polymerase activity on cellular fitness

The Y-family of DNA polymerases includes representatives from all three domains of life (Ohmori *et al*. 2001). It is not immediately clear as to why these polymerases are maintained within the genome when used only transiently and with low fidelity, given that more efficient repair pathways also exist. However, their wide conservation across phylogenetic barriers suggests a fixed, beneficial function. A phylogenetic analysis of genes encoding all five of the *E. coli* polymerases (*polB*, *dinB*, *umuC*, *polA*, and *dnaE*) from a set of 30 strains indicated that these

genes exhibited low levels of recombination and sequence diversity similar to housekeeping genes (Bjedov *et al*. 2003). Therefore, the loci coding for TLS polymerases in *E. coli* must be under functional constraints and essential for bacterial cell survival and fitness similar to other DNA polymerases.

Although orthologs of TLS polymerases encoded by genes such as *umuDC* and *mucAB* have been mostly characterized for their functional role in TLS, many of these determinants also function in increasing host survival to DNA-damaging agents such as UV radiation (UVR). Perhaps because the first systems were present in *E. coli* and other enteric bacteria, the ecological ramifications of increased UVR survival were not examined extensively. Nevertheless, determinants such as *umuDC*, *mucAB*, and *impCAB* conferred 10 ×–100 × increases in UVR survival when expressed in *Salmonella typhimurium* (Sedgwick *et al*. 1991) and plasmids from eight of 20 incompatibility groups tested were shown to increase the UVR survival of an *E. coli* strain (Pinney 1980).

The TLS polymerase ortholog *rulAB* from the plant pathogen *Pseudomonas syringae* was the first system in which the phenotype of enhanced UVR survival was shown to be ecologically important (Sundin and Murillo 1999). *Pseudomonas syringae* is an important plant pathogen that, as a prerequisite to successful infection, must develop large ($>3 \times 10^5$ cells leaflet) populations on leaves (Hirano *et al*. 1995). The leaf surface or phyllosphere habitat is normally exposed to high doses of incident solar radiation since leaves, as the principal photosynthetic organs of plants, are typically positioned for optimal exposure to sunlight. *rulAB* was cloned from an indigenous plasmid as a determinant of UVR tolerance in *P. syringae* (Sundin *et al*. 1996), and shown to be widely distributed among different disease-causing pathovars of *P. syringae* (Sundin and Murillo 1999). Within the time-frame of colonization, establishment, and

the initiation of pathogenesis on a host plant (5–7 days), *rulAB* was required for the maintenance of population size, and *rulAB*-mediated mutability occurred at readily detectable levels when strains were inoculated on bean leaves that were subsequently irradiated (Kim and Sundin 2000). This work demonstrated the importance of the UVR survival phenotype to *P. syringae* ecology and that mutability could occur at significant levels in *P. syringae* populations on leaves.

The impact of *rulAB*-mediated mutability on cellular fitness was further examined in experiments where *rulAB*+ and *rulAB*− cells were passaged through cycles of UVR exposure and population bottlenecks (Zhang and Sundin 2004). After 60 cycles, the resulting lineages were compared to the ancestral strain for the occurrence of a suite of mutations. Newly arising mutations were detected in 60% of the UVR-exposed *rulAB*+ lineages; however, cellular fitness, measured as growth rate in a minimal medium, was not significantly different from the ancestral strains (Zhang and Sundin, 2004).

ii. The SOS response and the evolution of antibiotic resistance

As mentioned earlier, the linkage between MMR-deficient mutator cells and the evolution of antibiotic resistance remains controversial (Woodford and Ellington 2006). In contrast, there are clear linkages between certain synthetic antibiotics such as ciprofloxacin, the induction of the bacterial SOS response, and the mutational evolution of resistance. Ciprofloxacin is a quinoline antibiotic that functions by interfering with the essential type II topoisomerases gyrase and topoisomerase IV (Drlica and Zhao 1997). The impact of ciprofloxacin action in cells is the production of free double-strand ends of DNA and blockage of DNA replication forks, both resulting in the induction of the SOS response. The results of an elegant study by Cirz *et al*. (2005) in *E. coli* demonstrated that RecBC-mediated homologous

recombination, functioning in the repair of ciprofloxacin-mediated DNA damage, also induced LexA cleavage and the activation of the SOS response. This in turn induced the expression of polII, polIV, and polV, which did not affect survival but were essential for the evolution of mutational resistance to ciprofloxacin (Cirz *et al*. 2005). These results were extended to demonstrate that the SOS response was also required for the evolution of mutational resistance to ciprofloxacin in MMR-deficient mutator bacteria (Cirz and Romesberg 2006). Similar results were found in other organisms including studies demonstrating that antibiotic-induced SOS responses influenced virulence at the pathogen population level by affecting the mobilization and horizontal transfer of a pathogenicity island in *Staphylococcus aureus* or of the integrative conjugative element SXT in *Vibrio cholerae* (Beaber *et al*. 2004, Ubeda *et al*. 2005).

D. RpoS Regulon and the Growth Advantage in Stationary Phase (GASP) Phenotype

The RpoS regulon in *E. coli* comprises over 50 genes and is triggered by many different stresses including starvation and transition to stationary phase, high osmolarity, high and low temperature, and low pH (Hengge-Aronis 2000). Some of the responses mediated by RpoS are mutagenic. For example, studies of mutagenesis in aging colonies of *E. coli* show that this phenomenon is controlled by RpoS and is dependent on RecA and polII, resulting in error-prone DNA repair (Bjedov *et al*. 2003). RpoS also regulates the production of polIV in the stationary phase (Layton and Foster 2003), which in turn affects adaptive mutation processes, as described above.

i. GASP

The GASP phenotype is observed in laboratory cultures maintained for long time periods (usually ~10 days) in stationary phase without replenishment of nutrients (Zambrano and Kolter 1996). The GASP phenotype confers 'aged' cultures with a growth advantage over 'younger' cultures under direct competition, and depends on the appearance of new mutations affecting the ability to catabolize one or more amino acids that provide a competitive advantage to individuals allowing them to overtake the population (Finkel 2006). There is evidence to suggest that SOS polymerase genes are actively transcribed during the stationary phase and contribute to the evolution of a GASP phenotype. *Escherichia coli* strains deficient in SOS polymerase genes show significantly decreased relative fitness when cocultured with aged wild-type strains exhibiting a GASP phenotype (Yeiser *et al*. 2002). Likewise, the plasmid pWW0 in *P. putida* encodes a homolog of *rulAB* and significantly enhanced bacterial fitness under long-term starvation conditions with the transcription of *rulAB* increasing 5–6 × in the late-stationary phase (Tark *et al*. 2005). The presence of either pWW0 or chromosomally located pWW0-derived *rulAB* conferred a strong GASP phenotype, allowing young *rulAB*+ strains to out-compete older, *rulAB*-strains (Tark *et al*. 2005). The GASP phenotype seems to be dependent on a high rate of genetic diversity realized in long-term stationary phase cells; mutational occurrence in such cells has been estimated to be as high as one mutation per 600 bp of genome sequence (Finkel and Kolter 1999). Survival of GASP cells is also dependent on the transient polyploidy observed in starving cells (Akerlund *et al*. 1995), which would allow the tolerance of potentially lethal mutations. Perhaps the GASP phenotype underlies a specialized function of SOS polymerases in generating sequence diversity. Indeed, because natural bacterial populations are almost

constantly dwelling under nutrient limitation, the GASP phenotype may be a significant ecological process.

E. Synthesis

Stressful conditions, changing environments, and extreme nutrient limitation are all factors that place microbial populations at risk. Many examples are now emerging that demonstrate that, faced with a situation with a predictably poor outcome, bacteria respond through a mutational alteration of genotype. Such genetic variation enables a sampling of the 'sequence space' of the organism and increases the chances of natural selection acting on better-adapted alleles. Elevated mutation rates are clearly not universally beneficial. However, understanding the regulatory features underlying mutational survival strategies will have a significant impact on the ability to manage important clinical disease problems in the future as well as provide a more focused glimpse into microbial ecology in general.

II. Understanding Adaptation Though Experimental Evolution

A. Introduction

The process of evolution requires the generation of variation within a population and natural selection in favor of certain variants over others. Sewall Wright described evolving organisms as existing on an adaptive landscape that represented fitness in a multi-dimensional genetic space where natural selection pushed populations 'up hill' towards peaks of higher fitness (Wright 1932). The topology of the adaptive landscape depends not only on the complexity of the environment but also on epistatic gene interactions such that several fitness peaks likely exist separated by valleys. These different peaks represent local fitness optima

accessible based on the order in which beneficial mutations emerge and become fixed. Therefore, the rugged nature of the adaptive landscape suggests that more than one adaptive solution may exist in an environment and that replicate populations may diverge to distinct adaptive peaks due to the stochastic nature of mutation. The 'hill climbing' process of adaption is gradual and spans many generations rendering few biological models useful for the experimental study of evolution.

Microorganisms have long been recognized for their utility in enabling researchers to experimentally observe evolution in action. In the late 19th century, Rev. William Dallinger attempted to 'superinduce' adaptive changes on organisms with short life cycles in what is widely recognized as the first laboratory evolution experiment with microorganisms (Haas 2000). Over the course of a seven year study, Dallinger gradually raised the temperature of his incubated culture of 'monads' up to 158°F before his custom apparatus was accidentally destroyed. Not only did the organisms adapt to growth at high temperature, but they could no longer survive under the original 60°F conditions. Dallinger's simple study yielded experimental support for fundamental evolutionary processes and highlighted the value of microorganisms for observing adaptation over feasible timescales.

Bacteria, and other microorganisms, are well suited for the direct experimental study of evolutionary change for very practical reasons, including their short generation time, ability to found replicate isogenic populations, large population sizes, and the ease with which they can be propagated in controlled and reproducible laboratory environments (Elena and Lenski 2003). Furthermore, evolution experiments with bacteria permit the establishment of a 'fossil record' by periodically freezing cells in a non-evolving state. Adaptation to an experimental environment can later be quantified in terms of relative fitness changes by directly competing revived cells

from different generations with their ancestor. The haploid, asexual nature of bacteria also allows for the manageable genetic analysis of adapted strains.

The Long-Term Evolution Experiment of Richard E. Lenski and colleagues has maintained 12 replicate populations of *E. coli* since 1988 (Lenski *et al*. 1991). Over the course of more than 20 years and 50,000 generations, this celebrated project has significantly advanced our understanding of evolutionary patterns by taking advantage of a well characterized bacterial model system. Others have also contributed to the vibrant field of experimental evolution including studies of bacterial adaptation for metabolic engineering (Ibarra *et al*. 2002), 'undomesticated' soil bacteria (Riley *et al*. 2001), and yeast (Zeyl 2006) to name only a few. Some recurring themes have emerged from the various laboratory studies of evolution with different microorganisms. Parallel changes in fitness are often observed in the adaptation of replicate lineage and once clonal populations often diversify to occupy different niches in seemingly simple laboratory environments. Parallel evolution and diversification are hallmarks of natural selection. Understanding the dynamics of these processes has been a prominent focus of evolution experiments with microorganisms and these studies have provided some of the best direct evidence in support of evolutionary theories. Furthermore, the application of genomic analyses has opened the door for characterizing the process of adaptation in greater detail. The following is a brief review of how evolution experiments with microorganisms, and with bacteria in particular, have contributed to the theoretical concepts of adaptation and diversification.

B. Lessons of Adaptation

i. Parallel improvements in fitness

Fitness refers to the ability of an organism to survive and reproduce in the environment. The reward for high fitness is genetic as only those organisms surviving and producing offspring can contribute genes to the next generation (Orr 2009). The process of evolution requires that particular variants exhibit higher fitness than others and that these differences in fitness have a heritable, genetic basis. Without differences in fitness, natural selection cannot drive adaptation by the spread and substitution of more successful genotypes. Absolute fitness refers to a genotype's probability of survival and accounts for performance at every level of resource consumption, metabolic efficiency, viability, fecundity, etc., but is difficult to define experimentally (Orr 2009). However, because selection acts on differences in performance, the relative fitness of a genotype compared to its sympatric competitors is more informative. In evolution experiments with bacteria, the relative fitness of closely related strains is easily quantified by co-culturing to allow direct competition for resources and monitoring of changes in population frequency. Differences in relative fitness between individuals can be subdivided into distinct physiological components and defined by divergent performance among these metrics (Lenski *et al*. 1998, Sleight and Lenski 2007). Improvement in any of these components may increase fitness.

Genomic methods of analysis continue to become more pervasive and have been implemented for the comprehensive characterization of laboratory derived strains following experimental evolution. Gene expression profiles represent a composite phenotype of thousands of 'expression traits' that can be queried to assess adaptation. Studies that have employed gene expression profiling to investigate adaption during laboratory evolution have observed largely

parallel changes in expression patterns concurrently with parallel changes in fitness (Ferea *et al.* 1999, Cooper *et al*. 2003, Riehle et al 2003, Fong *et al*. 2005). Many adaptive changes observed have been reductions in gene expression compared to the ancestor suggesting the importance of repressing nonessential functions to improve fitness. The close association between expression phenotype and genotype can also identify candidate genes for investigating sequence changes responsible for adaptation.

Adaptation during experimental evolution ultimately stems from changes in DNA sequence. The increasing accessibility of whole-genome sequencing methods has had broad implications for the field of microbiology, especially studies of experimental evolution (MacLean 2009). Genome sequencing technology can reveal the complete genetic basis of adaptive differences between a derived strain following experimental evolution and its ancestor (Herring *et al*. 2006, Hegreness and Kishony 2007, Smith *et al*. 2008, Conrad *et al*. 2009). The contribution of each genetic change underlying adaptation can be observed clearly (Applebee *et al*. 2008) and their dynamics within the population can be tracked over time (Barrick *et al*. 2009). Furthermore, individual identified mutations have been introduced by allelic replacement into the ancestral genetic background to compare their direct influence on fitness (Conrad *et al.* 2009). Mutations at different nucleotide positions within a gene or distant nodes in a regulatory network often exhibit similar fitness phenotypes suggesting a degree of flexibility in adaptation (Applebee *et al*. 2008, Conrad *et al.* 2009).

The parallelism among adapting bacterial populations under experimental evolution is striking. Parallel phenotypic changes in fitness, gene expression, cell size, sugar specialization, and DNA supercoiling have been reported (Ferea *et al*. 1999, Philippe *et al*. 2007). Parallel cultures often exhibit analogous, fitness-enhancing changes due to different mutations that inflict

the same phenotypic outcome (Applebee *et al*. 2008). This parallelism should not be viewed as evidence in support of adaptation by directed mutation. Although mutation does not occur at equal rates at all loci in all environments, mutation is random with respect to its adaptive consequences for individual organisms and the production of variation always precedes selection (Sniegowski and Lesnki 1995). Some studies have supported the process of directed mutation and have been reviewed elsewhere (Sniegowski and Lenski 1995, Roth *et al*. 2006). Theories of directed mutation are fundamentally flawed because they fail to separate the stochastic phase of random mutation and deterministic phase of selection that make up the process of evolution. Both mutation rate and selection can influence mutant frequency in a population and their effects can be difficult to separate (Sniegowski and Lenski 1995, Roth *et al*. 2006). The observation of parallel populations with mutations in the same gene but at different nucleotide positions confirms the random, independent nature of mutation and the power of selection to champion adaptive improvements (Applebee *et al*. 2008).

During experimental evolution, improvements in fitness are often initially rapid due to intense selection triggered by sudden environmental changes but later slow dramatically and often plateau (Lenski and Travisano 1994, De Visser and Lenski 2002). This does not suggest that these populations have already ascended to the summit of a fitness peak. Rather, the non-linear fitness trajectories of experimental lineages reflect the distribution of fitness effects among beneficial mutations. Independent of the sorting environment, this distribution is always exponential such that most beneficial mutations have a very small effect on fitness and only a few have a large effect (Kassen and Bataillon 2006). The time required for a beneficial mutation to spread throughout a population is inversely proportional to its selective advantage. Therefore, because many beneficial mutations carry only a small fitness advantage, they are lost due to

random drift and only those few with a larger advantage become fixed quickly (Elena and Lenski 2003). In large asexual populations, clonal interference between different beneficial mutations that appear simultaneously also influences the ordered substitution and rate of mutation fixation (Gerrish and Lenski 1998, De Visser and Rozen 2005). Ultimately, the exponential distribution of beneficial mutations means that adaptation through mutations with progressively smaller effect can continue indefinitely at an increasingly slower pace without depleting the supply of useful improvements (Elena and Lesnki 2003).

In finite populations, mutation supply rate is a limiting factor for evolution and the time required for beneficial mutations to emerge in large part dictates the rate of adaptation (Tenaillon *et al*. 1999). Although much attention is given to the deleterious nature of mutation in favor of lower mutation rates, a recent review points out that the propensity for mutation to diminish or reduce the function of coded elements does not intrinsically lead to negative consequences (Behe 2010). A loss of function in a regulator or unneeded protein may yield a net fitness gain as seen in transcriptional studies of experimentally evolved organisms (Ferea *et al*. 1999, Cooper *et al*. 2003, Fong *et al*. 2005). Furthermore, higher rates of mutation can contribute to the number of variants within a population and, therefore, increase the probability that at least one will prevail. Computer models of bacterial populations suggest that the presence of mutator alleles can accelerate the rate of adaptation without being fixed in the population (Taddei *et al*. 1997). Even at low frequency mutator alleles can allow rapid fitness increases in the population by increasing genetic diversity and the supply rate of beneficial mutations. However, the parameters of these models required reversion of the mutator allele to avoid hitchhiking with beneficial mutation, something not yet experimentally observed. Mutator genotypes have emerged in long-term experimental populations of *E. coli* and risen to high frequency such that all subsequent isolates

exhibit an increased mutation rate (Sniegowski *et al*. 1997). In an *in vivo* selection experiment with *P. aeruginosa* populations grown in caterpillar hosts, only mutator populations evolved increased virulence, improved growth rate, and social cheating of public goods (Racey *et al*. 2010). The benefits of cheating during host infection may contribute to the high frequency of mutators in clinical populations of *P. aeruginosa* (Hall and Henderson-Begg 2006).

Adaptation implies specialization and corresponding reduced fitness in some disparate environment. Pleiotropic fitness tradeoffs have been reported in evolution experiments with *E. coli* (Cooper and Lenski 2000) and *P. fluorescens* (MacLean *et al*. 2005). Yet, wild organisms do not exist in static environments. A variant fixed through adaptation is not guaranteed to enjoy sustained fitness as the fitness landscape in nature is always changing. To combat this limitation, some bacteria have developed contingency, phase variation mechanisms in response to niche selection in fluctuating environments. The non-random nature of this variation reduces the risk of deleterious mutation by ensuring that only a subset of genes is hypermutable (Moxon *et al*. 2006). Alternatively, experimental evolution of *P. fluorescens* in a fluctuating environment has given rise to bet hedging strategies that generate stochastic phenotypic switching to ensure persistence in future environments (Beaumont *et al*. 2009).

ii. Diversification by adaptive radiation

The competitive exclusion principle dictates that the number of coexisting genotypes cannot exceed the number of limiting resources and asexual populations evolving under simple laboratory conditions should proceed by a series of selective replacements (Hardin 1960). Even though many mutations occur in large bacterial populations, low phenotypic diversity is expected due to periodic selective sweeps involving fitter mutants. The complete linkage of all genetic

loci purges variation throughout the genome with each effective bottleneck maintaining monomorphism within the population. However, diversity exists in nature, despite the apparent conflict with theoretical predictions that natural selection should eliminate all but the fittest variant in any environment (Kassen and Rainey 2004). The process of adaptive radiation entails the emergence and simultaneous evolution of distinct descendent lineages from a single, ancestral line (MacLean 2005).

The emergence and maintenance of diversity by adaptive radiation is well documented in evolution experiments with bacteria and these studies have shed light on the relationship between natural selection and diversification (Turner *et al.* 1996, Rainey and Travisano 1998, Rozen and Lenski 2000, Rainey *et al*. 2000, Riley *et al*. 2001, Kassen and Rainey 2004). In serial transfer experiments with *E. coli*, a stable polymorphism was identified as a new colony morphotype that coexisted in the population with the ancestral morphotype for thousands of generations (Elena and Lenski 1997, Rozen and Lenski 2000, Rozen *et al*. 2005). Serial transfer lineages involve temporal fluctuations in nutrient concentration and therefore opportunities for specialization to subtle, transient niches exist. A stable coexistence can emerge in such an environment provided one genotype has an advantage when resources are abundant and the other has an opposing advantage when resources are limited (Rainey *et al.* 2000, Kassen and Rainey 2004). However, diversification has also been observed in populations of *E. coli* grown in the constant environment of a chemostat when initially clonal populations diverged into collections of subpopulations exhibiting different adaptive changes (Maharjan 2006).

In the spatially heterogeneous laboratory environment of a stationary Erlenmeyer flask, *P. fluorescens* SBW25 has been observed to diversify into three dominant morphological variants, each occupying a different physical niche (Rainey and Travisano 1998). However,

when the physical heterogeneity of the environment was disrupted, diversification was not observed. Other similar studies have also observed the same dependence of phenotypic variation on environment structure (Riley *et al*. 2001). The balanced coexistence of divergent morphotypes in the serial transfer, chemostat, and stationary flask experiments were all maintained by negative frequency dependent selection such that individual types have a selective advantage when rare preventing their extinction. Furthermore, the repeated appearance and persistence of divergent morphological variants in each study indicated that multiple adaptive opportunities existed for the ancestral genotype.

The activities of organisms themselves can promote the emergence and coexistence of competing genotypes by contributing to environmental heterogeneity as a means of relieving competition (Rainey *et al*. 2000, Rozen and Lenski 2000, Rozen *et al*. 2005). In a well mixed, and otherwise homogeneous, environment the secretion of metabolites can change the chemical composition of the environment and allow for a cross-feeding relationship to develop through adaptation to exploit new compounds (Rozen and Lenski 2000, Rozen *et al*. 2005). Growth of bacterial biofilms also promotes diversity by created physical separation (Boles and Singh 2008). The resulting isolation increases the likelihood that cells will occupy alternate ecological niches and experience different selective pressures. The presence of phenotypic variants may contribute to biofilm function as it lessens the detrimental effect of environmental stress by increasing the chance that one variant will be more fit.

It is clear from these examples of evolution experiments that natural selection favors innovations that lead to divergent evolutionary trajectories as a general feature of adaptive evolution (MacLean 2005). Competition among niche specialists drives adaptive radiation in response to disparate ecological opportunities available in heterogeneous environments (Rainey

and Travisano 1998). Specialists, by definition, are expected to sacrifice opportunities in other niches in exchange for a competitive advantage in one particular niche (Kassen and Rainey 2004). Naturally, complex environments with more niches are expected to harbor great diversity as they provide additional opportunities for variation (Barrett and Bell 2006, Mahrjan *et al*. 2006). When variants formed by adaptive radiation diverge sufficiently to follow independent evolutionary trajectories, they can even fulfill a conceptual definition of asexual species (Cohan 2002). Interactions that drive adaptive radiation, either with the environment or other sympatric genotypes, are easily disrupted by environmental changes in resource availability or physical structure (Rainey *et al*. 2000). Many of the studies reviewed here relied on colony morphology to identify divergent subpopulations and because this method often underestimates the extent of genotypic variation, bacterial populations under laboratory evolution likely accommodate even more diversity (MacLean 2005, Tyerman *et al*. 2005).

C. Synthesis

Despite their relative simplicity, numerous studies of experimental evolution with bacteria have successfully confirmed long-standing theories of the dynamics of adaptation and diversification. The availability of genome sequencing technology permits the elucidation of the most fundamental genetic mechanisms of adaptation and their corresponding fitness effects. The experimental power of bacteria for these applications is unmatched. Furthermore, it should not be surprising that complex interactions evolve even in seemingly simple laboratory environments in light of the inexhaustible supply of innovation present in nature. True, single niche laboratory environments may not be possible in practice as selection always seems to exploit subtle niches not present by design. Bacterial populations are clearly not limited by the competitive exclusion principle as adaptive radiation can take place in near-constant environments by seizing

evolutionary opportunities created by organisms themselves to relieve selection pressure. Employing microorganisms as models for evolution has captured the gradual process of adaptation in manageable, experimental timeframes and will likely continue as a successful strategy for the future.

III. Project rationale, goals, and significance

There are increasingly more examples of bacterial strains that thrive in various environments despite maintaining an increased mutation rate. Constitutive mutator genotypes exhibit increased mutation rates due to defects in DNA repair processes and commonly result from the inaction of MMR. Alternatively, inducible mutator genotypes transiently increase mutation rates by implementing specialized, low-fidelity DNA polymerases activated in response to specific stress conditions. The widespread existence of mutator genotypes in environmental, clinical, and laboratory populations suggests that mutability contributes to the enduring success of bacteria. This project is founded on the central hypothesis that evolutionary strategies of bacteria include mechanisms for increasing mutability. Previous research in the field of bacterial hypermutation has focused on the underlying genetic and biochemical characteristics but has fallen short of understanding the broader role mutability plays in the biology of the organism. The production of variation by mutation permits adaptation through the action of natural selection. Therefore, mutability may contribute to adaptive evolution by expanding an organism's ability to gain fitness in an environment through better access to beneficial sequence alterations. If an organism can tolerate, or even avoid, the risk of accumulating deleterious mutations, the increased supply of beneficial mutations may provide additional opportunities for adaptation. The field of experimental evolution has employed bacteria for the study of

evolutionary processes over relatively short timescales with great success. The experiments outlined in this dissertation will address the influence of hypermutability on adaptation through experimental evolution with contextually relevant model bacteria.

The first objective of this project is to understand the long-term effects of inducible mutability in the form of mutagenic DNA repair. Inducible mutability may provide positive opportunities for adaption in response to stress by increasing variation. Alternatively, the pleiotropic hypothesis suggests that inducible mutagenesis is simply the unfortunate by-product of a last-ditch repair system designed to enhance survival following DNA damage (Tenaillon *et al.* 2004). The potential reduction in fitness of asexual lineages following an accumulation of deleterious mutations (Muller's ratchet – Muller 1964) also presents a critical opposition to an argument for the evolutionary advantage of mutagenic DNA repair. Inducible mutagenesis either provides positive opportunities for adaption or reduces fitness out of desperation for survival due to the predominantly deleterious nature of mutation. Experiments in this objective are designed to address the hypothesis that long-term mutagenic DNA repair activation contributes to adaptation and phenotypic diversification.

Historically, the *E. coli umuDC* system has been the most widely studied example of inducible hypermutability. However, enteric bacteria are poorly suited for investigating the evolutionary implications of inducible mutability because they do not routinely incur UVR-mediated DNA damage, the best recognized elicitor of the SOS response. Alternatively, epiphytic plant pathogens like *P. syringae* harbor the *umuDC*-homologue *rulAB* and inhabit leaf surfaces optimized for solar UVR exposure (Sundin *et al.* 1996). Translesion DNA synthesis mediated by *rulAB* confers both UVR tolerance and inducible mutability in these organisms, intimately linking survival and mutation (Sundin and Murillo 1999). Plant-associated

Pseudomonas species harboring *rulAB* display varied phenotypic levels of UVR tolerance and mutability (Sundin *et al.* 2000). In a survey of plant-associated *Pseudomonas* isolates, the epiphytic celery pathogen *P. cichorii* 302959 emerged as exceptionally UVR-mutable owing to a chromosomal copy of *rulAB* (Zhang and Sundin 2004a). The ecological relevance and pronounced transient mutator phenotype make *P. cichorii* 302959 an ideal model for investigating the impact of inducible mutability on adaptive evolution.

Previous work with *P. cichorii* 302959 has shown that during short, single-cell bottleneck lineages repeated UVR exposure and *rulAB* activation increase the occurrence of mutations but did not significantly reduce fitness (Zhang and Sundin 2004a, 2004b). This project has conducted ~500 generations of experimental evolution with parallel lineages of *P. cichorii* 302959 in a serial transfer regime with and without daily activation of *rulAB* in the form of UVR exposure. In Chapter 2, the experimental details of the *P. cichorii* 302959 UVR and non-UVR lineages are described along with relative fitness improvements of derived isolates and the emergence of a new colony morphotype. The results therein suggest that inducible mutability can contribute to gains in fitness and diversification in a stressful environment. Chapter 3 addresses patterns of adaptive specificity exhibited during experimental evolution with *P. cichorii* 302959 that correlated with discrete improvements in growth, independent of *rulAB* activation. Taken together, the results presented in Chapters 2 and 3 suggest that increased mutation rate in the form of inducible mutability does not impede adaptation by mutation accumulation. Rather, UVR and non-UVR lineages preferentially acquired adaptive growth improvements in a similar manner and additional fitness gains by UVR lineages may have been due to greater access to beneficial mutations.

The second objective of this project aims to compare the adaptive influences of general, *mutS*-deficient and inducible, *rulAB*-mediated hypermutabililty. The MMR system protects the genome against a wide variety of mutations and MutS recognizes single-base mismatches and insertion/deletion loops during repair initiation (Schofield and Hsieh 2003). Among environmental and clinical isolates, *mutS* inactivation is the most widespread defect responsible for hypermutation (Hall and Henderson-Begg 2006). Deletion of *mutS* leads to an increased occurrence of point and frameshift mutations and a hyper-recombinogenic phenotype (Smania *et al*. 2004). Alternatively, *rulAB*-mediated hypermutation results from template-independent, translesion DNA synthesis prone to point mutations and frameshifts (Maor-Shoshani *et al*. 2000). Experiments in this project address the hypothesis that the mechanistic differences between *mutS*-deficient and *rulAB*-mediated mutability produce unique mutation spectra and therefore unequal access to beneficial mutations.

P. aeruginosa is a ubiquitous Gram-negative, gamma-proteobacterium and the plasticity of its large genome permits growth in disparate natural environments and opportunistic infection of immunocompromised patients. Comparative genome sequencing suggests that the evolution of the *P. aeruginosa* genome has been driven by selection for a capacity to expand its environmental niches (Mathee *et al*. 2008). During chronic CF infection, initially clonal populations of *P. aeruginosa* accumulate genetic variants that promote long-term survival, reduced virulence, and clonal expansion as they adapt to the stressful host environment (Figure 1-2) (Ciofu *et al*. 2005, Smith *et al.* 2006). The prevalence of hypermutators in *P. aeruginosa* populations of chronic CF lung infections is one of the most illustrative examples of the importance of mutators in natural bacterial evolution (Oliver *et al.* 2000, Mena *et al*. 2008). There is a growing body of evidence to support the role of hypermutability in facilitating the

adaptive transitions associated with chronic infection (Ciofu *et al*. 2005, Hogardt *et al*. 2007, Moyano *et al*. 2007, Mena *et al*. 2008, Ciofu *et al*. 2010, Oliver and Mena 2010). Furthermore, the predicted amplification of a mutator genotype by hitchhiking with adaptive mutations has been clearly documented during the course of human infection, demonstrating the positive effects of hypermutation on *P. aeruginosa* evolution during chronic infection (Mena *et al*. 2008).

The laboratory strain *P. aeruginosa* PAO1 serves as an appropriate model to investigate the influence of hypermutation on adaptation given the clinical importance of mutators in chronic CF infection and the extensive body of previous research with this organism (Winsor *et al.* 2009). The genome of PAO1 has been fully sequenced (Stover *et al*. 2000) and transposon insertion mutants are available (Jacobs *et al*. 2003) allowing for the advanced study of defined hypermutable genotypes. The *P. aeruginosa* PAO1 genome lacks a *umuDC* homologue (Simonson *et al.* 1990) but plasmid-encoded homologues have been reported in native isolates of *P. aeruginosa* (Stokes and Krishnapillai 1978, Lehrbach *et al*. 1979, Jacoby *et al.* 1983, Simonson *et al.* 1990) and *rulAB* confers UVR tolerance and inducible mutability to *P. aeruginosa* PAO1 when introduced on a plasmid (Kim and Sundin 2000, 2001). This project has conducted ~500 generations of experimental evolution with parallel lineages of *mut*-deficient and *rulAB*-mediated mutators of *P. aeruginosa* PAO1. In Chapter 4, the experimental details of the *P. aeruginosa* PAO1 lineages are outlined along with relative fitness measurements of derived populations and a detailed analysis of point mutation frequency to rifampicin resistance. The results therein, confirm the divergent point mutation spectra of *mutS*-deficient and *rulAB*-mediated hypermutation and suggest that these differences in variation at the nucleotide level may not restrict access to adaptive phenotypes. Chapter 5 describes the comparison of transcriptome profiles that reveal a shared repression of flagellar biosynthesis and chemotaxis

that was linked to mutation in the central regulator FleQ in general and inducible mutator lineages. Together, the results presented in Chapters 4 and 5 suggest that despite unique point mutation spectra and divergent gene expression profiles, *mutS*-deficient and *rulAB*-mediated hypermutation can still provide access to common adaptive mutation targets. Such patterns support the stochastic nature of mutation and the power of selection to champion adaptive improvements.

The information gathered from the studies outlined in this dissertation will contribute to the fundamental understanding of how bacteria utilize mutation rates during adaption. The widespread existence of mutator genotypes suggests that evolutionary strategies of bacteria include mechanisms for increased mutation rates. While previous research has focused on the genetic and biochemical characterization of hypermutation, there exists a lack of information concerning the impact of mutability on the general biology of bacteria. Homologs of the low-fidelity, repair polymerases responsible for inducible mutability are found in eukaryotes, bacteria, and archaea but their function in such divergent backgrounds is not understood (Ohmori *et al*. 2001). However, the numerous direct and indirect triggers of the SOS response suggest that inducible mutability likely influences bacterial evolution in a wide variety of environments (Erill *et al*. 2007). Likewise, constitutive mutator strains with defects in MMR have been observed at varied frequencies within natural populations of bacteria, including many important human pathogens (Table 1-1).

Bacterial pathogens face an onslaught of stresses during host infection and inducible mutability may contribute to the emergence of antibiotic resistance, creating therapeutic challenges for control (Cirz and Romesberg 2007). These challenges are further complicated by the observation that application of some synthetic antibiotics can induce the SOS response and

lead to the evolution of antibiotic resistance (Cirz *et al*. 2005). Similarly, in CF lung populations of *P. aeruginosa* there is a clear link between hypermutation and the evolution of antibiotic resistance (Oliver *et al*. 2000). The development of multidrug resistance also correlates with the accumulation of mutators suggesting that intense antibiotic treatment strategies likely provide selective pressure in favor of the maintenance and amplification of hypermutable genotypes during chronic infection (Ciofu *et al*. 2010). This project employs experimental evolution with *P. cichorii* 302959, a plant pathogen, and *P. aeruginosa* PAO1, a closely related model of an opportunistic human pathogen, to understand the role of hypermutability in the adaptation of bacterial populations. A better understanding of the influence of hypermutability on the growth and adaptation of bacterial pathogens could lead to more effective control strategies that mitigate the contribution of hypermutation to the development of antibiotic resistance.

Table 1-1. Frequency of mutator or hypermutable strains of bacterial pathogens and accompanying analysis of gene defects in the methyl-directed mismatch repair system associated with mutator status.

Species	Frequency	Gene defect[a]	Reference
E. coli	4/212 (1.9%)	*mutS* – 4	LeClerc *et al*. (1996)
Salmonella spp.	5/137 (3.6%)	*mutS* – 3 *mutH* – 1 *uvrD* -- 1	LeClerc *et al*. (1996)
Neisseria meningitidis	54/95 (56.8%)	*mutL* – 16 *mutS* – 5 UNK –33	Richardson *et al*. (2002)
Staphylococcus aureus	13/89 (14.6%)	*mutS* – 5 UNK – 6	Prunier *et al*. (2003)
Haemophilus influenzae	14/500 (2.8%)	*mutS* – 4	Watson *et al*. (2004)
P. aeruginosa	33/62 (53.2%)	*mutS* – 11 UNK – 6	Macia *et al*. (2005)
P. aeruginosa	25/128 (19.5%)	*mutS* – >4[b] *mutY* – 4	Oliver *et al*. (2000)
S. aureus	5/124 (4.0%)	*mutS* + *mutL* – 1 UNK – 4	Trong *et al*. (2005)
N. meningitidis	4/18 (22.2%)	*mutS* – 1 *mutS* + *mutL* – 1 *mutL* – 1 UNK – 1	Colicchio *et al*. (2006)

a. The MMR-system gene mutated shown as well as the total number of mutator strains evaluated from each study. UNK, unknown mutation. These strains were examined, but the relevant MMR mutation was not discovered.

b. *mutS* was identified as mutated in at least four strains from this study, but the total number of *mutS* strains determined was not given.

Figure 1-1. Bacterial mutability pathways. (I) Constitutive mutability present in mutator strains with defects in the MMR system. The MMR system includes MutS, MutL, MutH, and UvrD, and functions in proofreading during replication; mutations accumulate when the MMR system is nonfunctional. (II) Inducible mutability through translesion synthesis occurring in response to DNA damage. Induction of the SOS regulon in response to DNA damage includes the expression of the error-prone DNA polymerases polV ($UmuD'_2C$) and polIV (DinB). Translesion synthesis can result in the misincorporation of nucleotides opposite lesions in the DNA template. (III), GASP mutability occurs in response to stresses such as starvation and involve the non-SOS induction of polIV via RpoS and untargeted mutagenesis. For interpretation of the references to color in this and all other figures, the reader is referred to the electronic version of this dissertation.

Figure 1-1 (cont'd).

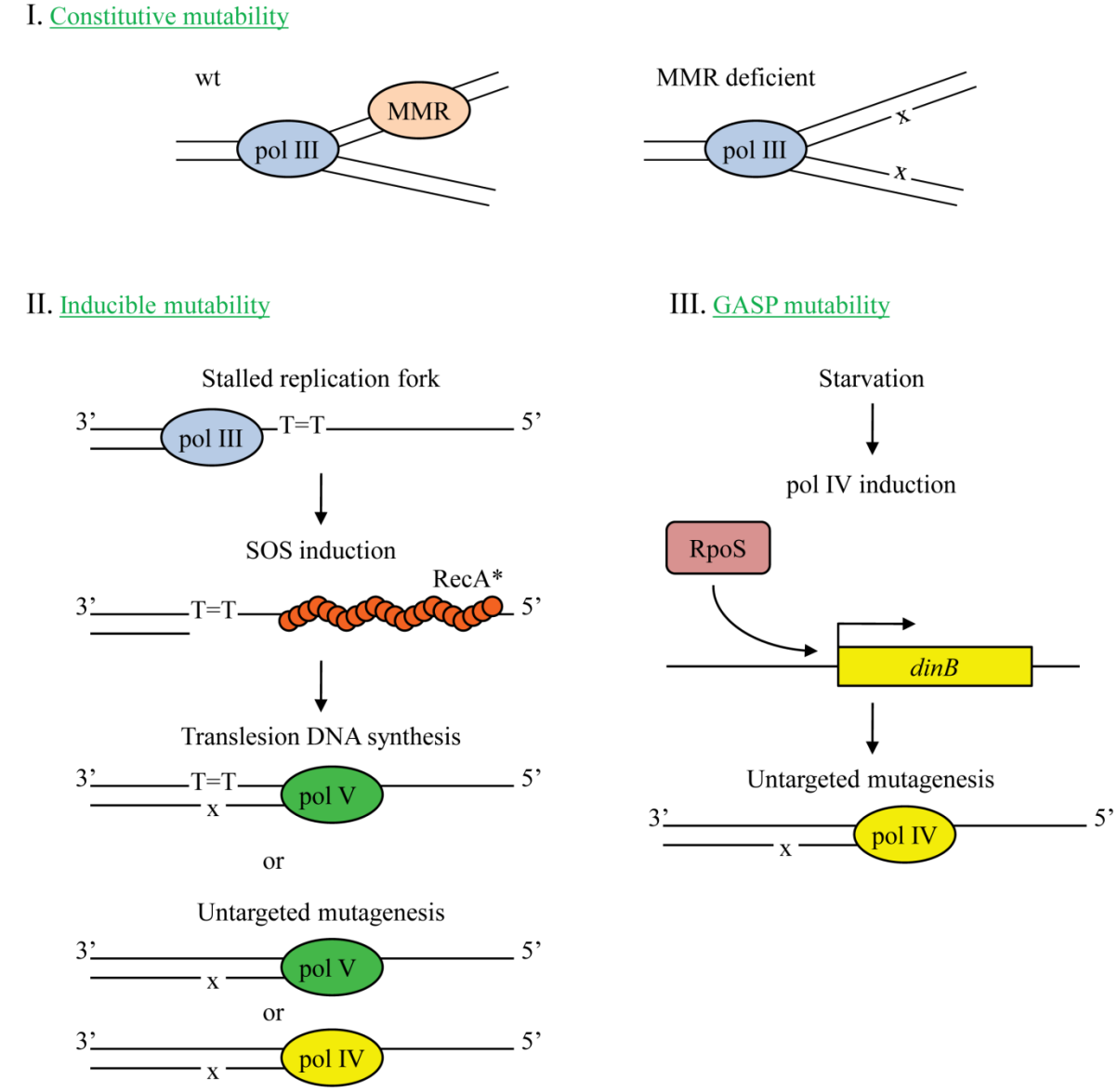
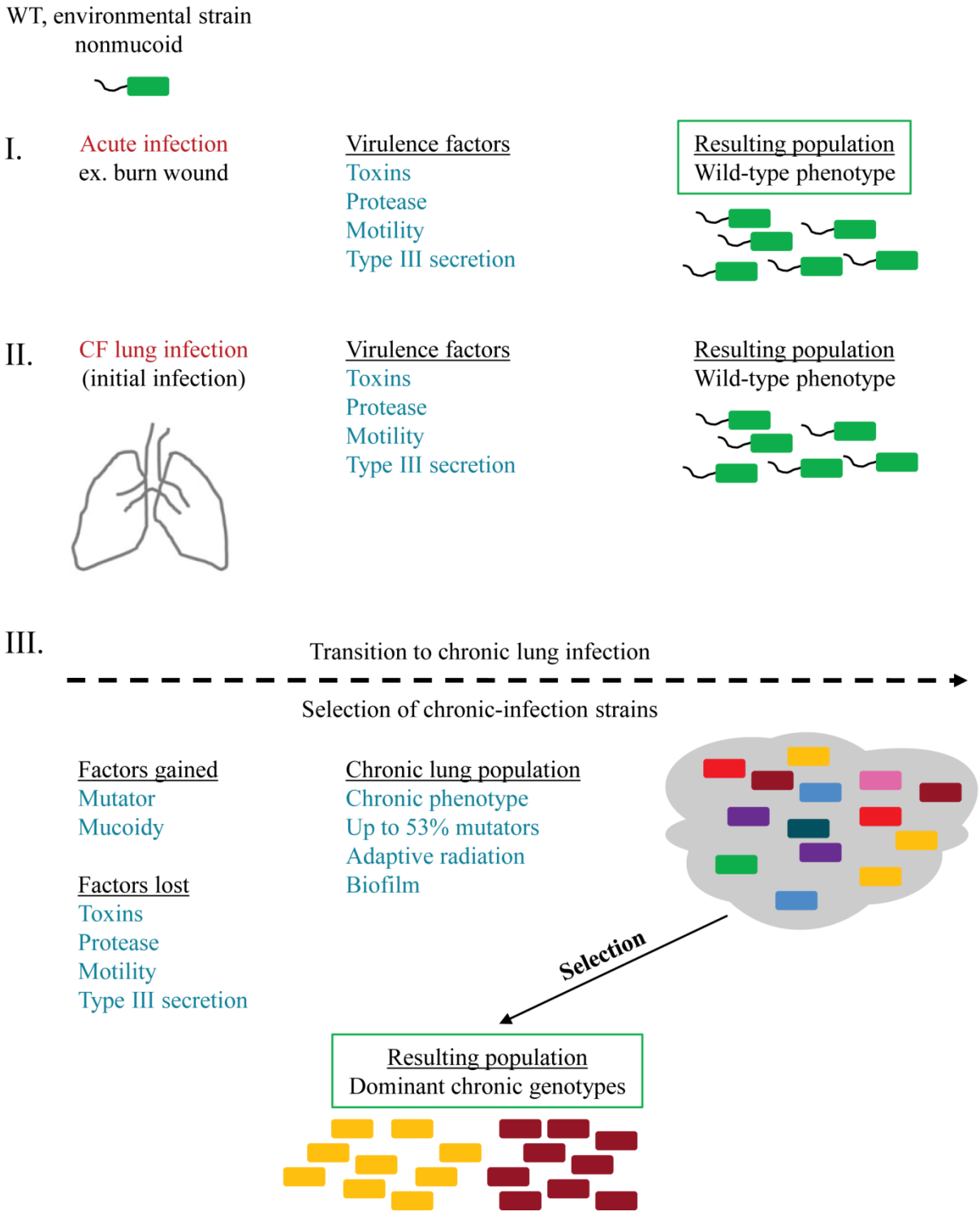


Figure 1-2. Role of methyl mismatch repair-deficient mutator cells in the establishment of chronic infections by *Pseudomonas aeruginosa* in the lungs of CF patients. (I) Wild-type (WT) environmental strains are nonmucoid and motile. Strains responsible for acute infections resemble environmental strains and express a variety of virulence factors during pathogenesis. When the course of infection is completed, the strains are disseminated back to the environment in an essentially genetically unchanged state. (II) CF lung infections caused by *Pseudomonas aeruginosa* are initiated by nonmucoid strains that resemble environmental strains and express a variety of virulence factors. (III), As *P. aeruginosa* CF lung infections transition to chronic infections, pathogen strains become mucoid and a majority of strains become mutators. In addition, virulence factors necessary for acute infections are lost as the mutator strains undergo adaptive radiation in the lung environment increasing the genotypic diversity of the population. Final selection results in a small number of dominant, chronic-infection genotypes**.**

Figure 1-2 (cont'd).



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CHAPTER 2: Long-term effects of inducible mutagenic DNA repair on relative fitness and phenotypic diversification in *Pseudomonas cichorii* 302959

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ABSTRACT

Mutagenic DNA repair (MDR) employs low-fidelity DNA polymerases capable of replicating past DNA lesions resulting from exposure to high-energy ultraviolet radiation (UVR). MDR confers UVR tolerance and activation initiates a transient mutator phenotype that may provide opportunities for adaptation. To investigate the potential role of MDR in adaptation, we have propagated parallel lineages of the highly mutable epiphytic plant pathogen *Pseudomonas cichorii* 302959 with daily UVR activation (UVR lineages) for ~500 generations. Here we examine those lineages through the measurement of relative fitness and observation of distinct colony morphotypes that emerged. Isolates and population samples from UVR lineages displayed gains in fitness relative to the ancestor despite increased rates of inducible mutation to rifampicin resistance. Regular activation of MDR resulted in the maintenance of genetic diversity within UVR lineages, including the reproducible diversification and coexistence of "round" and "fuzzy" colony morphotypes. These results suggest that inducible mutability may present a reasonable strategy for adaptive evolution in stressful environments by contributing to gains in relative fitness and diversification.

INTRODUCTION

Sequence variation in the form of mutation supplies the fuel for natural selection. However, boosting variation by raising the mutation supply rate does not automatically accelerate adaptation, as only a small minority of mutations is expected to be advantageous. The propensity for deleterious mutations maintains continual selection pressure in favor of lower genomic mutation rates (Sniegowski *et al.* 2000, de Visser 2002, de Visser and Rozen 2005). This notion is reinforced by the conservation of multiple DNA error-avoidance and error-repair processes. Therefore, bacteria must balance a mutation supply rate adequate to permit adaptation with the maintenance of genome stability, as both are necessary for long-term population survival (Rainey 1999).

There are an increasing number of examples of bacterial strains that do not maintain low genomic mutation rates and still manage to survive. General mutators (or hypermutators) exhibit increased mutation rates, as much as 100-1000 times greater than wild-type, due to defects in DNA proofreading and repair functions. Mutator alleles themselves have not been shown to confer any direct benefit to organismal fitness. Their advantage arises from an increased probability of producing rare, beneficial mutations compared to a wild-type population of the same size (de Visser *et al.* 1999, Tenaillon *et al.* 1999, Giraud *et al.* 2001). Mutator abundance within a population is likely the result of hitchhiking when the benefit from secondary adaptive mutations counterbalances the cost of accumulating deleterious mutations (Sniegowski *et al.* 2000, Tenaillon *et al.* 2001, de Visser 2002). The widespread existence of mutator genotypes in both environmental and clinical populations suggests that the evolutionary strategies of bacteria include mechanisms for increasing cellular mutability (Hall and Henderson-Begg 2006, Sundin and Weigand 2007).

Similarly, an inducible mutator phenotype can transiently increase mutability in response to specific stress conditions. One such transient mutator strategy is mutagenic DNA repair (MDR), executed by specialized low-fidelity DNA polymerases in response to UVR exposure. DNA lesions in the form of cyclobutane pyrimidine dimers and 6-4 photoproducts result from direct UVR exposure and distort the helical structure of DNA. These lesions disrupt replication fork progression and induce expression of the SOS response. The SOS regulon is a genotoxic stress response that coordinates the control of approximately 40 unlinked genes involved in DNA repair, recombination, and cell cycle control (Sutton *et al*. 2000, Michel 2005, Erill *et al.* 2007, Schlacher and Goodman 2007).

Among those genes induced in the SOS regulon are those encoding low-fidelity DNA polymerases polIV (*E. coli dinB*) and polV (*E. coli umuDC*) from the Y-family of DNA polymerases (Jarosz *et al*. 2007). The genetic and biochemical nature of the Y-family class of DNA polymerases has been the subject of intense study (Goodman 2002, Rattray and Strathern 2003). These repair polymerases exhibit high processivity and low-fidelity by nature of their more relaxed active sites, permitting them to perform translesion DNA synthesis (TLS) across damaged regions of DNA in a template-independent manner where normal, replicative DNA polymerases cannot (Goodman 2002, Rattray and Strathern 2003). TLS activity confers UVR tolerance by rescuing stalled replication forks and allowing complete replication of the genome but carries the cost of an increased mutation rate.

Members of the Y-family of DNA polymerases can be found across all three domains of life (Ohmori *et al*. 2001), yet their function in such divergent backgrounds is not well understood. According to the second-order selection hypothesis, the genetic diversity associated with an inducible increased mutation rate facilitates adaptation in stressful environments

(Radman 2001, Tenaillon *et al*. 2004). Alternatively, the pleiotropic hypothesis suggests that inducible mutability is simply the unfortunate by-product of a last-ditch effort to enhance survival in the face of DNA damage (Tenaillon *et al*. 2004). Inducible mutagenesis either provides positive opportunities for adaptive evolution under stressful conditions or reduces fitness out of desperation for survival due to the predominantly deleterious nature of mutation.

Presently, the *E. coli umuDC* system remains the most widely studied example of MDR. However, enteric bacteria are not routinely exposed to UVR-induced DNA damage, the best recognized elicitor of MDR, making them unsuitable for investigating the ecological or evolutionary implications of MDR. Alternatively, the *umuDC* homologue *rulAB* has been identified on an indigenous plasmid in the plant pathogen *Pseudomonas syringae* (Sundin *et al.* 1996). Epiphytic plant pathogens like *P. syringae* require large populations on host leaf surfaces for successful infection. Since leaves are optimized for solar UVR exposure, MDR-mediated UVR tolerance provides a critical ecological advantage to these organisms (Sundin and Murillo 1999). Plant-associated *Pseudomonas* species harboring *rulAB* display varied phenotypic levels of UVR tolerance and mutability, suggesting a beneficial but likely not essential function (Sundin *et al.* 2000). In a survey of plant-associated *Pseudomonas* isolates, *P. cichorii* 302959, an epiphytic pathogen of celery, has emerged as exceptionally UVR-mutable owing to a chromosomal copy of *rulAB* (Zhang and Sundin 2004a). *P. cichorii* 302959 *rulAB* provides an ecologically relevant context and a pronounced transient mutator phenotype ideal for investigating the potential role of inducible mutability in adaptive evolution.

We have designed a bacterial lineage experiment modeled after the *E. coli* Long-Term Experimental Evolution project (Lenski *et al*. 1991) using the highly mutable *P. cichorii* 302959. The *Pseudomonas* MDR determinant *rulAB* within the experimental evolution model provides a

controllable system that is both ecologically relevant and amenable to genetic manipulation in which to observe the adaptive influence of MDR. Here we report a long-term study to test our hypothesis that regular activation of MDR has a positive influence on adaptation to stressful environments without reducing fitness in a control environment in *P. cichorii* 302959. We maintained parallel cultures in a serial transfer regime with and without a daily dose of UVR and quantified changes in fitness relative to the ancestor. Among those lineages exposed to UVR, we observed distinct colony morphotypes, each of which exhibited increased fitness under the experimental conditions despite a transiently elevated mutation rate due to MDR induction.

MATERIALS and METHODS

Bacterial strains, growth conditions, and general molecular biology techniques

The ancestral 'round' (R) strain of *P. cichorii* 302959 was originally isolated in Japan (Zhang and Sundin 2004a). All evolved isolates were derived from the ancestral genotype following 500 generations of selection in the lineage experiment described below. Evolved isolates are denoted by lineage replicate and morphotype (e.g. 25R refers to an isolate from lineage 25 that exhibits the 'round' colony morphotype).

Strains of *P. cichorii* were cultured at 28° in Luria-Burtani broth (LB; Difco, Detroit, MI), in Davis Minimal broth supplemented with 25 mg L^{-1} glucose (DM25; Difco, Detroit, MI), or on King's medium B agar (KB; King *et al.* 1954). *Escherichia coli* DH5α was cultured at 37° in LB or on LB agar. Antibiotics were used where appropriate at the following concentrations: ampicillin 50 µg ml^{-1}, carbenicillin 50 µg ml^{-1}, gentamicin 10 µg ml^{-1}, rifampicin 250 µg ml^{-1}.

Competent cell preparation was performed according to Sambrook *et al.* (1989) for *E. coli* and according to Choi *et al.* (2006) for *P. cichorii*. Plasmid DNA was isolated from *E. coli*

and *P. cichorii* using either QIAprep (Qiagen, Valencia, CA) or Wizard Plus SV Miniprep (Promega, Madison, WI) kits and genomic DNA was isolated with the DNeasy Tissue Kit (Qiagen, Valencia, CA). Transformation by electroporation, standard agarose gel electrophoresis, and other recombinant DNA techniques were performed according to Sambrook *et al.* (1989).

PCR amplification and DNA sequencing

Primers were designed based on a consensus comprised of *P. aeruginosa, P. cichorii, P. fluorescens, P. putida,* and *P. syringae* sequences for 16S rDNA (small ribosomal RNA subunit), *atpD* (catalytic subunit of F_0F_1-ATPase), and *recA* (SOS recombinase) genes (Table 2-1). PCR amplifications were performed in a 20-µl reaction volume containing 1x PCR buffer, 2 mM $MgCl_2$, 125 µM of each deoxynucleoside triphosphate (Invitrogen, Carlsbad, CA), 0.4 µM of each primer, 1.0 U Taq polymerase (Invitrogen, Carlsbad, CA), and 40 ng genomic DNA. PCR amplifications were carried out as follows: one cycle at 94° for 3 min; 35 cycles at 94° for 1 min, 55° for 16S rRNA or 59° for *recA* and *atpD* for 1 min, 72° for 1.5 min; and a final extension at 72° for 7 min. PCR products were purified using the PCR Clean-Up Kit (Qiagen, Valencia, CA), and the purified fragments were sequenced at the Research Technology Support Facility at Michigan State University using the PCR primers listed in Table 2-1.

Long-term bacterial lineage experiment

Sixteen parallel lineages of *P. cichorii* 302959 were founded from a single colony and propagated in a manner similar to the *E. coli* Long-Term Experimental Evolution Project (Lenski *et al.* 1991). Cultures were maintained in 10 ml DM25 in 50-ml Erlenmeyer flasks and

incubated at 28° and 150 rpm in the dark. These culture conditions resulted in a stationary phase density of ~5 x 10^8 colony forming units per ml. Every 24 hours, eight UVR lineages (numbered 25-32) were individually mixed 1:1 with saline (0.85% NaCl) in a glass Petri dish and exposed to ~40 J m^{-2} of UVC (254 nm) radiation from an XX-15 UV lamp (UVP Products, San Gabriel, CA) placed horizontally at a fixed height above the cell suspension. The lamp was turned on 15 min prior to use to allow for stabilization of the UV output. The energy output of the lamp was monitored with a UV-X radiometer fitted with a UV-25 sensor (UVP Products, San Gabriel, CA) and determined to be 1.5 J m^{-2} s^{-1}. Following UVR irradiation, cultures were diluted 1:100 into fresh media and incubated under dark conditions to minimize photoreactivation. The other eight non-UVR lineages (numbered 33-40) were diluted 1:100 in saline and then transferred 1:10 into fresh media without UVR. Both transfer strategies result in 1000-fold daily growth of each population, representing ~10 generations of binary fission. Cultures were grown in this manner with daily transfers for approximately 500 generations. Population samples from each lineage were preserved in a non-evolving state in 10% glycerol at -80° every 10 generations for the first 200 generations and then every 50 generations thereafter for later analysis.

UVR mutability assays

Three lineages, two UVR and one non-UVR, were chosen at random, and preserved population samples at 100 generation intervals were thawed and approximately 10^8 cells were inoculated into 5 ml LB medium. Following overnight growth, 2 ml of culture was pelleted, washed with 1 ml saline, and resuspended in 1 ml saline on ice. Cell suspensions were mixed with 9 ml saline in a glass Petri dish and irradiated with a single dose of ~40 J m^{-2} as described

above. Before irradiation, cell suspensions were plated on KB and KB + rifampicin to determine the frequency of rifampicin resistant (Rif^R) cells in each population. Following irradiation, 1 ml of cell suspension was mixed with 1 ml 2x LB broth, incubated overnight in the dark, and plated on KB and KB + rifampicin to determine UVR-inducible mutability. The number of spontaneous Rif^R mutants in the absence of UVR was subtracted and the frequency of mutation to Rif^R due to UVR-inducible mutability was calculated as the number of Rif^R mutants per 10^8 surviving cells.

Competition experiments and relative fitness calculations

The relative fitness of isolates from each evolved lineage was determined by direct competition with the ancestor under the same non-UVR and UVR conditions as the long-term lineage experiment. Strain differentiation was accomplished by using a marker plasmid encoding catechol 2,3-dioxygenase (*xylE*) and gentamicin acetyltransferase-3-1 (*aacC1*) genes. Catechol dioxygenase-expressing colonies turn yellow when sprayed with 0.5M catechol for easy discrimination on agar media. The marker plasmid was constructed by inserting the *xylE-aacC1* cassette from pX1918GT (Schweizer and Hoang 1995) into the multiple cloning site of pUCP19 (Schweizer *et al.* 1991) resulting in pMRW2. A control plasmid, pMRW3, was constructed by removing an *Eco*RV restriction fragment required for both *xylE* and *aacC1* gene function. In preparation for competition assays, clones of each isolate were transformed with either the marker or control plasmid. Six replicates of each competition assay were conducted with three replicates of each marker pair (e.g. three replicates of ancestor/pMRW2 paired with 25R/pMRW3 and three replicates of ancestor/pMRW3 paired with 25R/pMRW2).

To ensure that competing isolates were comparably acclimated to the competition environment, isolates were simultaneously removed from the freezer, individually grown in LB broth over night, and then individually grown for 24 h in the competition environment. Under non-UVR conditions, competitors were mixed at a 1:1 volumetric ratio, diluted into fresh DM25, and incubated in the dark at 28° and 150 rpm for 24 h. Appropriate dilutions were plated on KB agar in triplicate at 0 h and 24 h to estimate the initial and final density of each competitor. Relative fitness (W) under non-UVR conditions was calculated as the ratio of the Malthusian parameters (m) of the two competing strains as determined by the following equations:

$$m = \ln[N(24)/N(0)]$$

$$W_{(\text{non-UVR})1:2} = m_1/m_2$$

where $N(0)$ and $N(24)$ are the population sizes of the strains at 0 and 24 h, respectively (Lenski *et al.* 1991). For competitions under UVR conditions, individual cultures were acclimated as described above, mixed at a 1:1 volumetric ratio, and then diluted in saline, irradiated, and transferred to fresh medium as described for the long-term lineage experiment. Dilutions were plated before UVR-irradiation (0 h) and after 24 h of growth. Because the 0 h density is measured before dilution into fresh medium, W must be calculated as the ratio of each competitor's realized (net) growth rate (r):

$$r = \ln[(N(24) \times 100)/N(0)]$$

$$W_{(UVR)1:2} = r_1/r_2$$

where $N(0)$ and $N(24)$ still represent the population sizes of the strains at 0 and 24 h, respectively. This was necessary to account for the 100-fold dilution into fresh medium following UVR (Sleight and Lenski 2007). When the two strains are equally fit in the competition environment, $W=1$.

The six replicate relative fitness measurements were analyzed by two-tail, independent *t*-tests against the hypothesis of mean equal fitness ($W=1$). Fitness measurements of each isolate under non-UVR and UVR conditions were compared by two-tailed paired *t*-test to determine any adaptation to daily UVR. Two-tailed *t*-tests were used because it was not known beforehand if UVR-induced mutability would produce an increase or reduction in relative fitness.

Similarly, the relative fitness of population samples from evolved UVR lineages was determined under both non-UVR and UVR conditions with a few modifications. Preserved lineage samples were thawed and approximately 10^8 cells were inoculated into 5 ml LB medium and grown overnight before acclimation to the competition environment. In order to maintain diversity, population samples were not transformed with either pMRW2 or pMRW3 and all six replicate competitions were conducted with the ancestor bearing pMRW2.

UVR tolerance assays

Bacterial isolates were grown overnight in LB broth and 2 ml of culture was pelleted, washed with 1 ml saline, and resuspended in 1 ml saline on ice. The cell suspensions were mixed with 9 ml saline in a glass Petri dish and irradiated as described above. Increases in the radiation dose delivered were accomplished by increasing the time of exposure. Cell

suspensions were mixed continuously while receiving UVR doses to eliminate survival as a result of shading. Following irradiation, surviving cells were enumerated by dilution plating conducted under dark conditions.

RESULTS

UVR mutability

To confirm MDR activity during lineage growth, preserved population samples from three lineages taken at 100 generation intervals were thawed and the frequency of UVR-induced mutation to rifampicin resistance was measured in each population. The results illustrated in Figure 2-1A show that the rate of UVR-inducible mutability fluctuated within UVR lineages 27 and 31 over the course of the lineage experiment. Conversely, non-UVR lineage 35 exhibited a constant rate of UVR-induced mutation to rifampicin resistance that was comparable to that of the ancestral population (Figure 2-1A). The total frequency of Rif^R cells within these three lineages is plotted in Figure 2-1B. Rif^R cells appeared and varied in frequency within UVR lineages 27 and 31 but were not present in non-UVR lineage 35.

Emergence of a 'fuzzy' morphotype in long-term bacterial lineages

To track population structure and identify possible contamination, each lineage population was plated daily on KB agar. After 60 generations, all eight of the UVR lineages contained a rare 'fuzzy' (F) colony morphotype (or morph) in addition to the ancestral 'round' (R) colonies (Figure 2-2A). Colonies with the F morphotype exhibited enlarged but variable size, a grainy surface, and rough edges only when grown on KB agar (Figure 2-2B) but not when grown on LB agar (Figure 2-2C).

PCR amplification and fragment sequencing was completed with the three housekeeping sequences 16S rDNA, *atpD*, and *recA* to determine if the F colonies were the result of a systematic contamination or represented a new morphotype of *P. cichorii* 302959. Aligned sequence fragments from R and F morphs from each lineage and the ancestor shared 100% sequence identity (data not shown). These F morphs never appeared in any of the non-UVR lineages or in a sterile medium control that was irradiated and serially transferred in parallel. We never detected any contamination in this sterile medium control throughout the duration of the experiment.

Relative abundance of the 'fuzzy' morphotype

Daily dilution plating of lineage populations on KB agar allowed for tracking of F morph abundance relative to ancestral R morphs. Figure 2-3 shows the percentage of total colonies that exhibited the F morphotype in the eight UVR lineages over the course of the long-term experiment. The relative abundance of the F morphotype fluctuated stochastically over time, exhibiting a mixture of local peaks and short periods of apparent stable population distribution. However, F morph abundance never exceeded 10% in lineage 26 and appeared to reach a plateau at ~94% in lineage 27.

Relative fitness of isolates from evolved lineages

Single colony representatives of R and F morphs were isolated from UVR lineages and R morphs were isolated from non-UVR lineages. Relative fitness of these 24 isolates was determined by competition with the *P. cichorii* 302959 ancestor and the results are illustrated in Figure 2-4. The average relative fitness of the ancestor competed against itself under both the

non-UVR and UVR conditions was not significant (both $P>0.32$, two-tailed independent *t*-tests, df=5, μ=1), confirming that pMRW2 and pMRW3 did not influence competitive growth (data not shown).

The R morphs isolated from non-UVR lineages exhibited increased fitness under non-UVR conditions (all $P<0.038$, two-tailed independent *t*-test, df=5, μ=1) and UVR conditions (all $P<0.022$, two-tailed independent *t*-test, df=5, μ=1) relative to the ancestor (Figure 2-4A). When comparing fitness gains under the two conditions by two-tailed paired *t*-test, only the relative fitness of isolate 39R varied significantly, showing an increase in fitness under UVR conditions that exceeded the gain under non-UVR conditions (Figure 2-4A). Isolates from the other seven non-UVR lineages did not exhibit significantly different increases in fitness between the non-UVR and UVR conditions (Figure 2-4A).

Six of the eight R morphs isolated from UVR lineages gained fitness under non-UVR conditions (all $P<0.038$, two-tailed independent *t*-test, df=5, μ=1) and all eight gained fitness under UVR conditions (all $P<0.009$, two-tailed independent *t*-test, df=5, μ=1) as seen in Figure 2-4B. The relative fitness of R morphs from UVR lineages, with the exception of isolate 29R, was significantly different under the two conditions when compared by two-tailed paired *t*-tests (Figure 2-4B).

F morphs emerged in all eight UVR lineages and these isolates also demonstrated significant changes in fitness relative to the ancestor under both non-UVR (all $P<0.037$, two-tailed independent *t*-test, df=5, μ=1) and UVR (all $P<0.009$, two-tailed independent *t*-test, df=5, μ=1) conditions (Figure 2-4C). One F morph isolate (28F) lost fitness relative to the ancestor when competed under both conditions. With the exception of isolates 27F and 29F, F morphs exhibited significantly different changes in relative fitness between the two growth conditions

(Figure 2-4C). R and F morphs from UVR lineages exhibited significantly greater gains in fitness under the UVR conditions in which they evolved but neither morphotype as a group exhibited higher relative fitness compared to the other. The reduced fitness of 28F relative to the ancestor under both non-UVR and UVR conditions was unique among all isolates.

UVR tolerance of isolates from evolved lineages

The UVR tolerance of isolates from a subset of evolved lineages was compared to the *P. cichorii* 302959 ancestor and the results are shown in Figure 2-5. Competitions under UVR conditions indicated that isolates 30R, 30F, and 32R all gained fitness relative to the ancestor (Figure 2-4) and these isolates displayed corresponding increases in UVR tolerance (Figure 2-5A). Isolate 40R was taken from a non-UVR lineage and exhibited a UVR tolerance phenotype comparable to the ancestor (Figure 2-5B). Isolates 26R and 32F were both taken from UVR lineages and displayed significantly higher fitness under UVR conditions (Figure 2-4), but did not exhibit increased UVR tolerance (Figure 2-5B). Isolate 28F displayed reduced fitness relative to the ancestor (Figure 2-4C) and also demonstrated reduced UVR tolerance (Figure 2-5B).

Relative fitness of populations from evolved lineages

Fitness changes in lineages were also assessed at the population level. Preserved population samples from the eight UVR lineages were thawed and sub-cultured. The relative fitness of these populations was determined by competition with the *P.cichorii* 302959 ancestor and the results are displayed in Figure 2-6. All eight UVR lineage populations exhibited increased fitness under non-UVR (all $P<0.0012$, two-tailed independent *t*-test, df=5, $\mu=1$) and

UVR (all $P<0.0055$, two-tailed independent *t*-test, df=5, μ=1) conditions relative to the ancestor. When compared by two-tailed paired *t*-tests, the relative fitness of these populations under UVR conditions was significantly greater than under non-UVR conditions.

DISCUSSION

The results of this long-term study suggest that inducible mutability can contribute to gains in fitness and diversification in a stressful environment. Using the highly UVR-mutable *P. cichorii* 302959, we maintained parallel cultures in a serial transfer regime with and without UVR exposure. The relative fitness of isolates and diverse populations from evolved lineages and the emergence of a new colony morphotype were investigated to evaluate the influence of MDR on adaptive evolution.

Before looking closely at the outcome of the long-term lineages, it was necessary to confirm the activity of UVR-inducible mutability in UVR lineages over the course of our experiment. To this end, we have observed the frequency of Rif^R cells following UVR irradiation in population samples taken from three randomly chosen lineages. UVR lineages 27 and 31 exhibited increased and varied rates of UVR-inducible mutability observed as a higher frequency of rifampicin resistance following UVR exposure (Figure 2-1A). UVR lineages not only maintained inducible MDR activity, but mutability to rifampicin resistance increased following repeated activation. The total number of Rif^R cells within UVR lineages 27 and 31 fluctuated over time (Figure 2-1B) similar to studies conducted previously (Zhang and Sundin 2004a). Because the total frequency of Rif^R cells varied rather than simply accumulating over time, this suggests the presence of other linked mutations which dictated the relative frequency

of rifampicin resistance in the population. Conversely, non-UVR lineage 35 maintained a constant level of UVR mutability similar to that of the ancestor (Figure 2-1A) and a very low overall frequency (<3 per 10^8 cells) of Rif^R cells (Figure 2-1B). This indicates that within non-UVR lineages inducible mutability does not decline in the absence of activation while maintaining a lower mutation rate compared to UVR-lineages.

During the course of the long-term experiment, all 16 lineages were plated daily to monitor for potential contamination. The emergence of the F morphotype in our UVR lineages was unexpected and originally believed to be a contaminant resulting from our daily UVR transfer scheme (Figure 2-2). The competitive-exclusion principle dictates that the number of coexisting genotypes cannot exceed the number of limiting resources and the evolution of asexual populations in an environment containing a single, limiting resource is expected to proceed by a series of clonal replacements (Rainey *et al.* 2000). However, the emergence and maintenance of diversity by adaptive radiation is well documented in evolution experiments with bacteria in simple environments (Turner *et al.* 1996; Rozen and Lenski 2000). The diversification of *P. fluorescens* SBW25 after three days of growth in a spatially heterogeneous environment represents the most notable example (Rainey and Travisano 1998). Rainey and Travisano (1998) found three morphotypes occupying different physical niches and maintaining their balanced coexistence through negative frequency dependent selection.

Our serial transfer regime, although spatially homogenous, exhibited temporal resource heterogeneity as nutrient availability fluctuated during each 24 hour growth cycle. A stable coexistence can emerge in such an environment provided one genotype has an advantage when resources are abundant and the other has an opposing advantage when resources are limited (Rainey *et al.* 2000, Kassen and Rainey 2004). Additionally, organisms themselves are capable

of creating metabolic heterogeneity within a well mixed, and otherwise homogeneous, environment in order to relieve resource competition (Rozen and Lenski 2000, Rozen *et al.* 2005). The F morphotype emerged rapidly and reproducibly in all eight UVR lineages suggesting both strong selection pressure in the environment and high adaptive potential of the ancestral *P. cichorii* 302959 genotype. Such divergent natural selection produces diversity as a general feature of adaptive evolution within a heterogeneous environment (MacLean 2005).

Although the F morph emerged reproducibly, abundance fluctuated in all UVR lineages (Figure 2-3) suggesting the F determinant itself may not carry a strong selective advantage. Fluctuations in F morph abundance likely correspond to relative changes in fitness between the R and F subpopulations as beneficial mutations occur at alternate loci. Linkage with a highly beneficial mutation likely lead to the high relative abundance of F and R morphs in UVR lineages 27 and 26, respectively (Figure 2-3). Interestingly, in these two extreme cases the lesser abundant morphotype is never eliminated, suggesting a frequency dependent relationship between the two subpopulations.

We have not pursued the underlying genetic loci responsible for the F morphotype. Although studies in *P. fluorescens* SBW25 have identified the genetic basis for the 'wrinkly spreader' (Spiers *et al.* 2002), the genetic bases of the 'fuzzy spreader' morph, which bears likeness to our *P. cichorii* 302959 F morph, remains unknown. Further characterization of the phenotype may provide candidate genes for investigation. The disappearance of the phenotype during growth on LB agar suggests a nutritional requirement, and previous studies of MDR in *P. syringae* identified a link between diversification in colony morphology and motility (Zhang and Sundin 2004b).

Our primary method for evaluating adaptation was through measuring changes in relative fitness. Non-UVR lineages were expected to gain relative fitness through adaptation to the culture conditions based on the results from similar studies conducted previously (Lenski *et al*. 1991). The increased relative fitness of isolates from non-UVR lineages (Figure 2-4A) indicate that 500 generations of growth by *P. cichorii* 302959 in this simple environment are adequate to produce measurable and statistically significant changes and therefore, validate our experimental system. These isolates displayed comparable fitness gains under non-UVR and UVR conditions such that any gains in relative fitness can be attributed to adaptation to the culture medium only.

The accumulation of deleterious mutations reduces organismal fitness in asexual lineages (Muller's ratchet – Muller 1964) and presents a critical opposition to an argument for the evolutionary advantage of MDR. The pleiotropic hypothesis suggests that inducible mutagenesis is simply the unfortunate by-product of a last-ditch repair system designed to enhance survival following DNA damage (Tenaillon *et al.* 2004). If this is true, the increased mutability associated with long-term induction of MDR, as seen in Figure 2-1, could result in population extinction due to mutation accumulation. The results in Figure 2-4B and 2-4C indicate that isolates from evolved lineages of *P. cichorii* 302959, with the exception of 28F, exhibited increased fitness relative to the ancestor after approximately 500 generations of growth with daily induction of MDR. Similarly, relative fitness gains by theoretical and experimental mutator populations have been reported previously (Sniegowski *et al.* 1997, Taddei *et al.* 1997). To our knowledge, this is the first report of changes in relative fitness following prolonged activation of inducible mutagenesis in the form of MDR. Isolates from UVR lineages (Figure 2-4B and 2-4C) not only gained relative fitness through adaption to the culture medium, as seen under non-UVR conditions, but most exhibited greater increases in relative fitness under UVR

conditions. These results suggest a specificity of adaptation in the eight UVR lineages that includes the UVR treatment.

Measures of relative fitness indicate the overall advantage of one strain over another but tell nothing of the potential underlying physiological differences. In a similar experimental evolution study with *E. coli* in a stressful freeze-thaw environment, Sleight and Lenski (2007) investigated changes in growth dynamics to gain insight into changes in relative fitness. Improved freeze-thaw survival, faster physiological recovery from freeze-thaw stress, and faster exponential-phase growth were identified as distinct components of overall gains in fitness. By competing isolates from UVR lineages with the ancestor under both non-UVR and UVR conditions, we have observed an adaptive specificity toward UVR irradiation (Figure 2-4B and 2-4C). To investigate the underlying physiology of fitness gains under UVR conditions, we compared the UVR tolerance of isolates from evolved lineages. Isolates 30R, 30F, and 32R displayed both greater relative fitness under UVR conditions (Figure 2-4B and 2-4C) and higher UVR tolerance (Figure 2-5A). However, isolates 26R and 27F, as well as others (data not shown), displayed UVR tolerance levels similar to the ancestor (Figure 2-5B) despite increased relative fitness under UVR conditions (Figure 2-4B and 2-4C). Therefore, increased UVR tolerance may contribute to fitness gains under UVR conditions for some, but not all, isolates from UVR lineages. Isolate 28F displayed reduced fitness relative to the ancestor under UVR conditions (Figure 2-4C) and exhibited a corresponding decrease in UVR tolerance (Figure 2-5A). However, isolate 28F also displayed reduced fitness under non-UVR conditions (Figure 2-4C) suggesting other factors likely contributed to lower relative fitness in this isolate. This suggests that changes in UVR tolerance can contribute to altered relative fitness under UVR

conditions but further characterization of growth dynamics is required as other components to fitness likely exist.

Colony morphology provides an underestimate of diversity and we can be certain that our UVR lineages harbor a much greater level of genetic diversity not reflected in colony morphology variation. Therefore, population level measures of fitness in UVR lineages were also performed. Measurements of relative fitness under non-UVR conditions at the population level (Figure 2-6) were comparable to R and F isolate fitness measurements with the exception of lineage 28 (Figure 2-4B and 2-4C). However, under UVR conditions, the relative fitness of population samples from UVR lineages (Figure 2-6) was greater than or equal to the relative fitness of individual R and F isolates (Figure 2-4B and 2-4C). The design of our long-term experiment permitted the maintenance of population diversity within a lineage by nature of the large number of cells transferred during each cycle. The structure and diversity of these populations may influence relative fitness gains in UVR lineages such that individual isolates sampled from the population are not as fit. Additionally, these UVR populations exhibited the same specificity of adaptation as UVR lineage isolates observable as significantly greater gains in fitness under UVR conditions compared to non-UVR conditions.

In summary, the transient rise in mutation rate associated with MDR activation may provide an opportunity for adaptive evolution only if the advantage of an increased supply of beneficial mutations can outweigh the cost of accumulating deleterious mutations. We have tested the hypothesis that regular activation of MDR has a positive influence on adaption to stressful environments without reducing fitness in a control environment. Our results suggest that inducible mutability can contribute to gains in relative fitness and diversification in a stressful environment. Isolates from UVR lineages exhibited significantly greater gains in

relative fitness under UVR conditions and this adaptive specificity cannot be attributed to changes in UVR tolerance alone. In tracking the frequency of two mutations we have shown that complex interactions between these mutations, their genomic backgrounds, and sympatric genotypes dictate the fluctuations in their abundance.

The emergence and maintenance of genotypic diversity in UVR lineage populations is a general feature of adaptive evolution within a heterogeneous environment and a relevant strategy for pathogenic bacteria. In microbial communities assembled by adaptive radiation, fitness relies on the ecological interactions of sympatric genotypes (MacLean 2005). The ecological interactions between genotypes in this study significantly influenced relative fitness under UVR conditions such that diverse populations were more fit than individual isolates. Additional components of relative fitness gains in evolved isolates and populations of *P. cichorii* 302959 and fitness relationships between isolates within lineages are currently being addressed. In the future, we will continue our investigation of the potential role of MDR in adaptive evolution with the use of this and other genetic systems.

Table 2-1. Pseudomonas PCR primers.

Gene	Sequence[a]	Fragment Length
16S rRNA	5'- GAGCGGCGGACGGGTGAGTAATG 5'- AGGTGATCCAGCCGCAGGTTCC	1400 bp
atpD	5'- AAGGGCGGTAAAGTCGGTCTGTTC 5'- GAGCGGTGTCGTAGTGTTCCTG	650 bp
recA	5'- GATGCCGACCACGCACTGGAC 5' - TGCCCTTGCCGTAAAGAATCTGGA	520 bp

a. Consensus based on aligned *P. aeruginosa, P. cichorii, P. fluorescens, P. putida,* and *P. syringae* gene sequences.

Figure 2-1. (*A*) UVR inducible mutability of *P. cichorii* 302959 lineages 27 (*white*), 31 (*grey*), and 35 (*black*) assessed from culture samples taken at 100-generation intervals during the long-term lineage experiment. The UVR dose applied was 40 J m^{-2}. The number of spontaneous mutations conferring rifampicin resistance in the absence of UVC irradiation has been subtracted. (*B*) Frequency of rifampicin resistant mutants in *P. cichorii* 302959 lineages 27 (●), 31 (▲), and 35 (■). Values are means and error bars represent standard error of the mean. If error bars are not visible it is because they are smaller than the size of the marker.

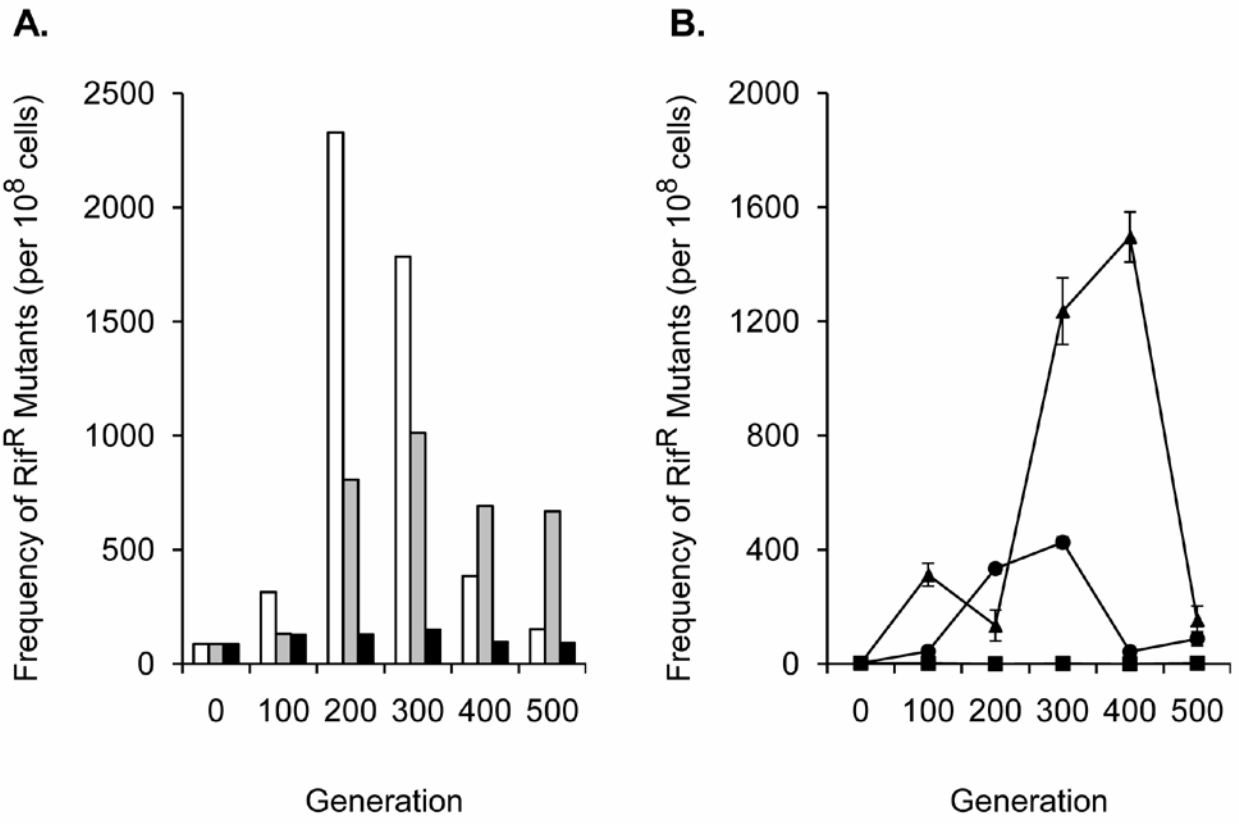


Figure 2-2. Phenotypic diversification among colonies of *P. cichorii* 302959 evolved in UVR lineages. (*A*) A 'fuzzy' (F) colony morphotype emerged reproducibly within the first 60 generations and coexisted with the ancestral 'round' morphotype in all lineages exposed to daily UVR. Colonies with the F morphology exhibited enlarged but variable size, grainy surface, and rough edges when plated on (*B*) KB agar but not (*C*) LB agar.

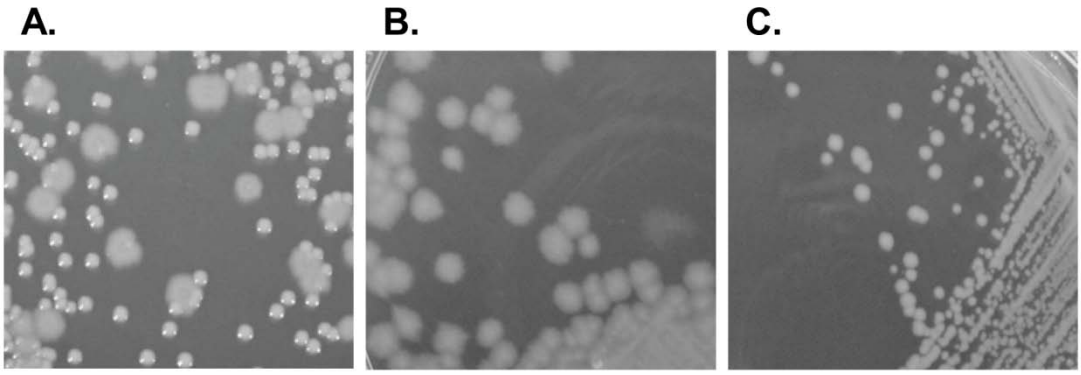


Figure 2-3. Relative abundance of ‘fuzzy’ (F) morphs in the eight replicate UVR lineages over the course of the experiment. Lineages are sorted in descending order by highest recorded abundance of F morphs. Sampling was conducted every 10 generations with the exception of a gap between generations 310 and 360. Although F morphs emerged reproducibly within the first 60 generations of all eight UVR lineages, their abundance fluctuated relative to the ancestral ‘round’ (R) type.

Figure 2-3 (cont'd).

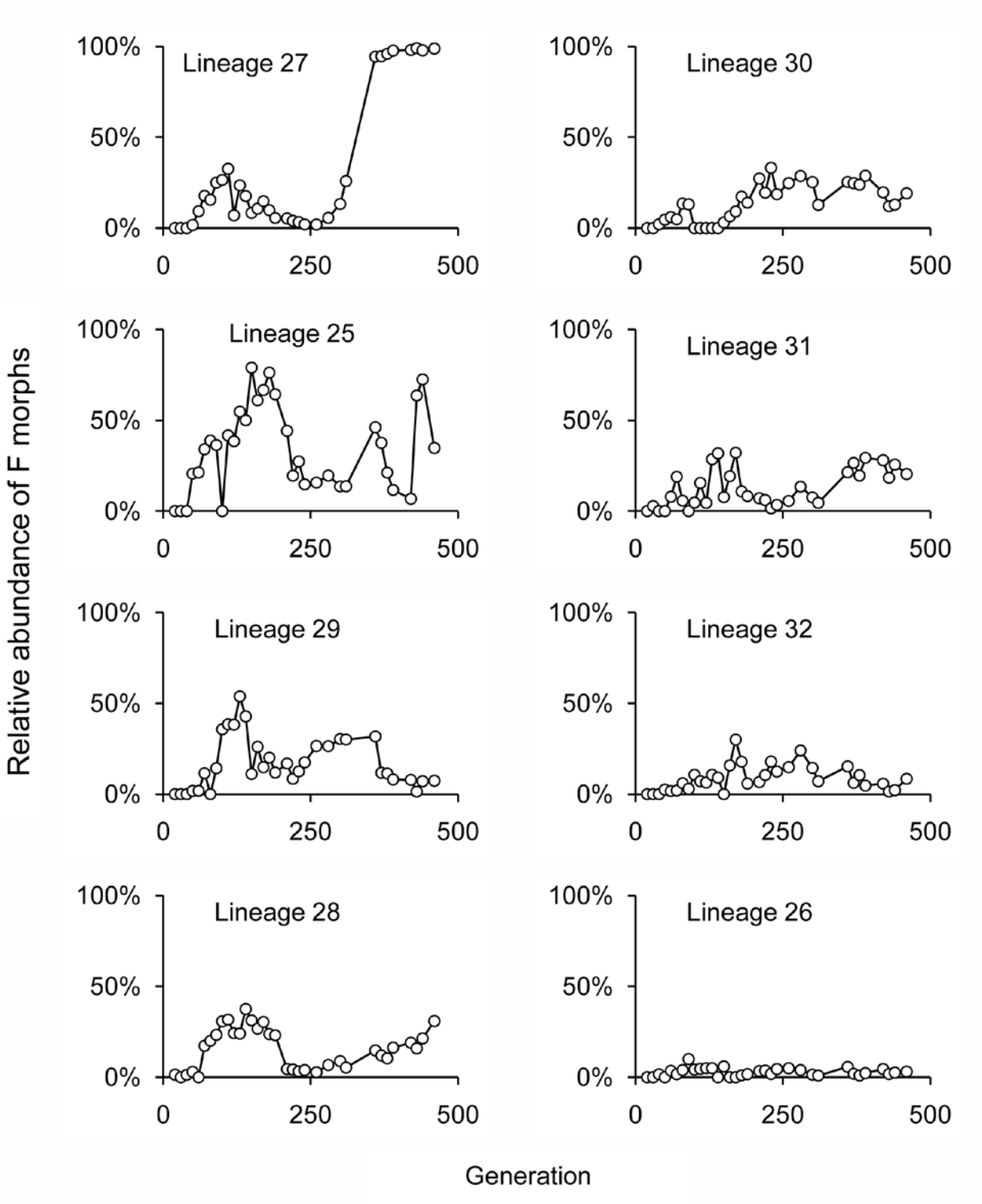


Figure 2-4. Relative fitness of isolates from evolved lineages of *P. cichorii* 302959 after 500 generations. Fitness values were measured in competition with the ancestor under non-UVR (*white*) and UVR (*grey*) conditions. (*A*) Lineages 33-40 were maintained without UVR and only contained the 'round' (R) morphotype. Lineages 25-32 received daily doses of UVR and gave rise to both (*B*) R and (*C*) 'fuzzy' (F) morphs. Relative fitness values are means and error bars represent 95% confidence intervals. Values are significant (two-tailed independent *t*-test, df=5, α=0.05) where indicated (* = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$). *P* Paired values are from two-tailed paired *t*-tests (df=5, α=0.05) between non-UVR and UVR relative fitness values for each isolate.

Figure 2-4 (cont'd).

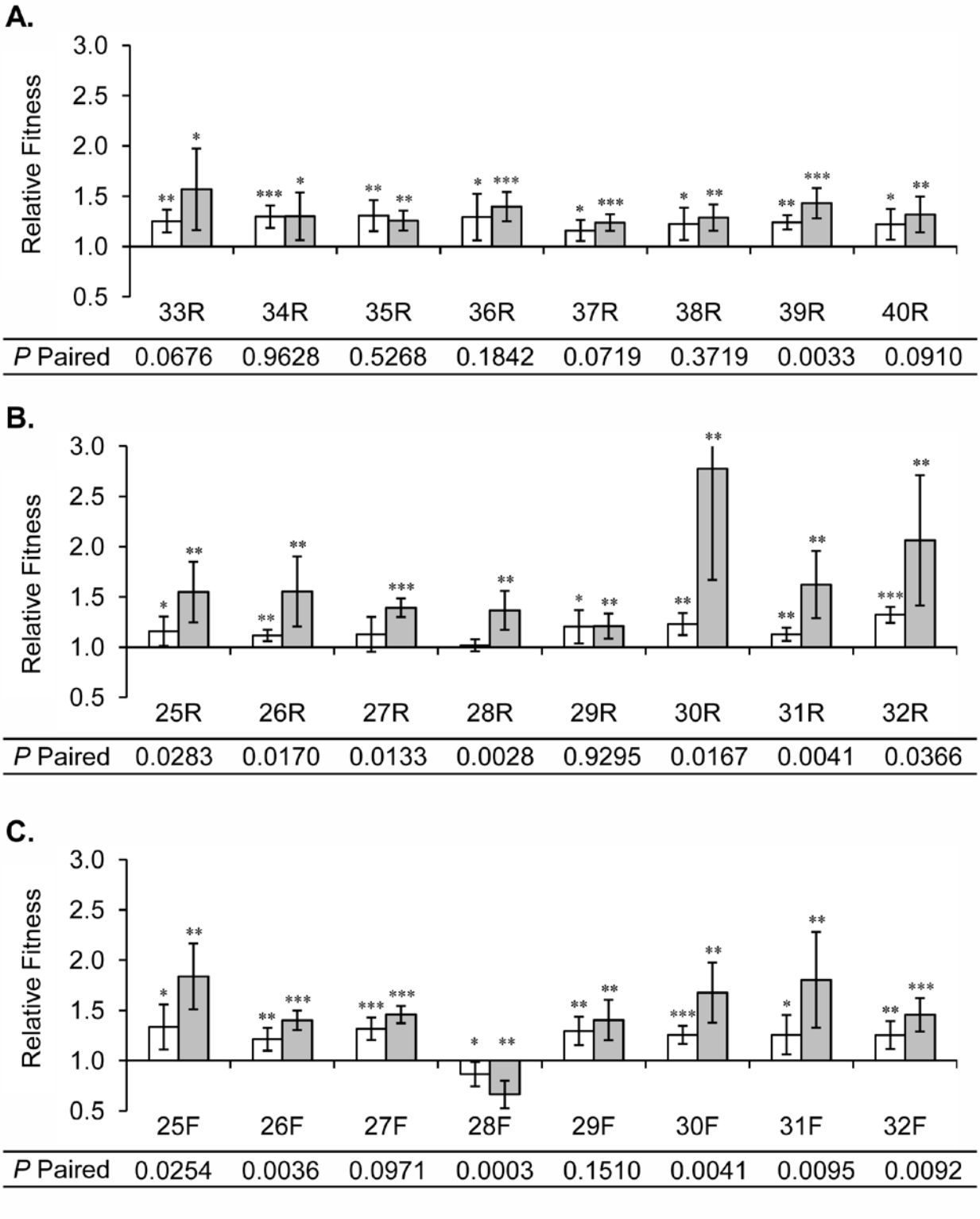


Figure 2-5. UVR tolerance of 'round' (R) and 'fuzzy' (F) isolates from evolved lineages of *P. cichorii* 302959 after 500 generations. (*A*) Survival of *P. cichorii* 302959 ancestor (●), 30R (□), 30F (Δ), and 32R (○). (*B*) Survival of *P. cichorii* 302959 ancestor (●), 26R (□), 28F (Δ), 32F (○), and 40R (◊). Values are means from three replicates and bars represent standard error of the mean. If error bars are not visible it is because they are smaller than the size of the marker.

Figure 2-5 (cont'd).

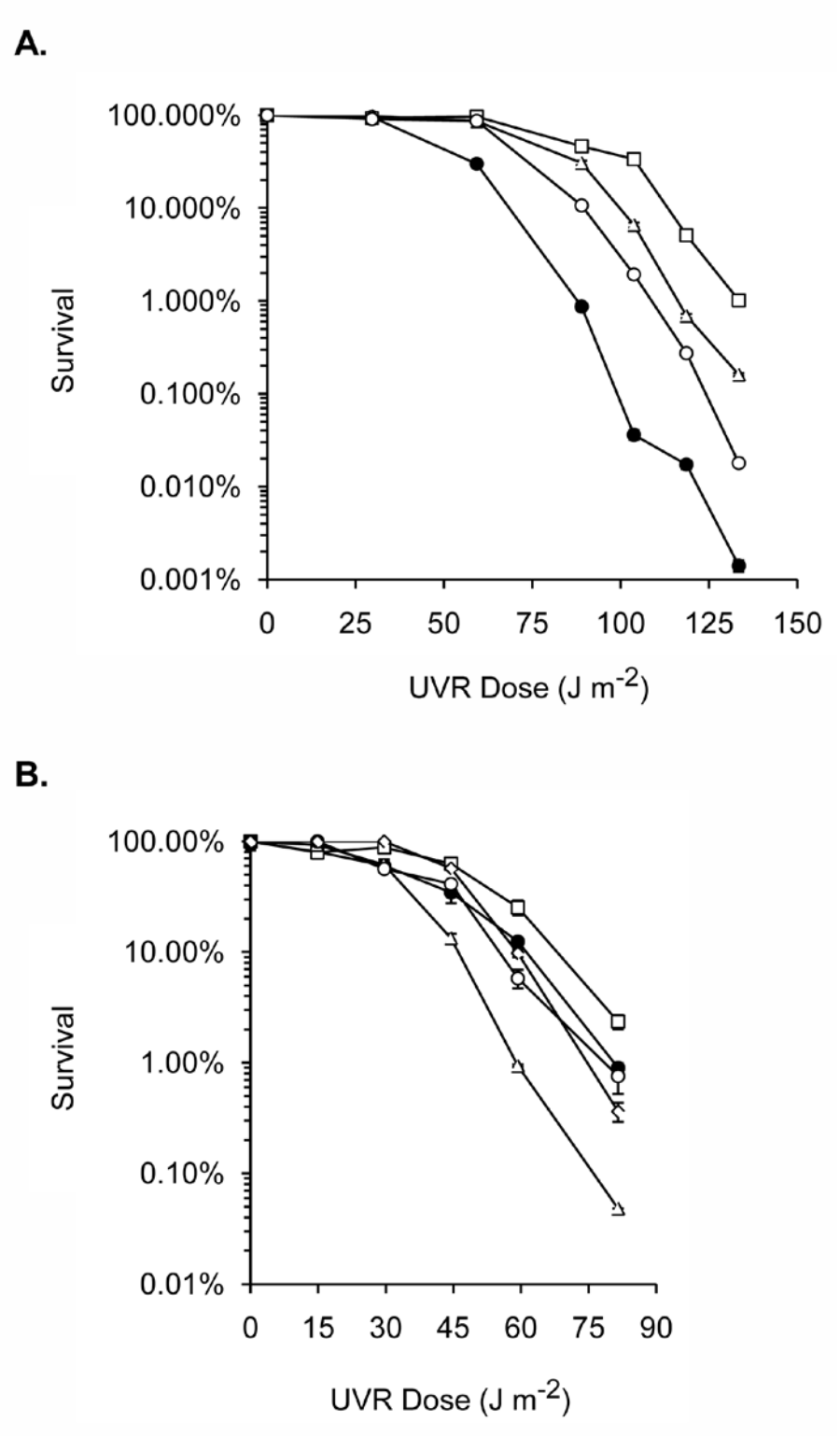
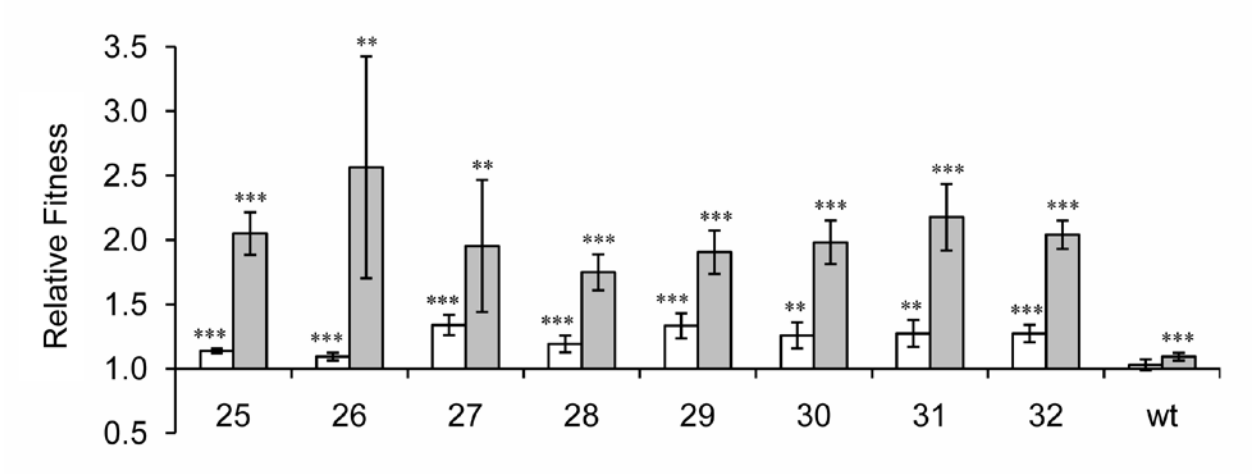


Figure 2-6. Relative fitness of populations from evolved UVR lineages of *P. cichorii* 302959 after 500 generations. Fitness values were measured in competition with the ancestor marked with pMRW2 under non-UVR (*white*) and UVR (*grey*) conditions. Relative fitness values are means and error bars represent 95% confidence intervals. Values are significant (two-tailed independent *t*-test, μ=1, df=5, α=0.05) where indicated (* = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$).



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CHAPTER 3: Growth parameter components of adaptive specificity during experimental evolution of the UVR-inducible mutator *Pseudomonas cichorii* 302959

This chapter has been modified from a publication of the same title in PLoS ONE (2011, vol. 6: e15975), co-authored by Vinh N. Tran and George W. Sundin.

ABSTRACT

Mutagenic DNA repair (MDR) transiently increases mutation rate through the activation of low-fidelity repair polymerases in response to specific, DNA-damaging environmental stress conditions such as ultraviolet radiation (UVR) exposure. These repair polymerases also confer UVR tolerance, intimately linking mutability and survival in bacteria that colonize habitats subject to regular UVR exposure. Here, we investigate adaptive specificity in experimental lineages of the highly UVR-mutable epiphytic plant pathogen *Pseudomonas cichorii* 302959. Relative fitness measurements of isolates and population samples from replicate lineages indicated that adaptive improvements emerged early in all lineages of our evolution experiment and specific increases in relative fitness correlated with distinct improvements in doubling and lag times. Adaptive improvements gained under UVR and non-UVR conditions were acquired preferentially, and differentially contributed to relative fitness under varied growth conditions. These results support our earlier observations that MDR activation may contribute to gains in relative fitness without impeding normal patterns of adaptive specificity in *P. cichorii* 302959.

INTRODUCTION

Mutation is the most fundamental source of variation on which natural selection may drive evolution. However, the predominantly deleterious nature of mutation maintains selection pressure in favor of lower mutation rates as evidenced by the conservation of multiple DNA error-avoidance and error-repair processes (De Visser and Rozen 2005). Yet hypermutator (or mutator) strains of bacteria have been observed in a variety of clinical, environmental, and laboratory populations with mutation rates 100-1000 times greater than wild-type due to defects in DNA proofreading and repair machinery (De Visser 2002). The widespread existence of both constitutive and inducible mutator genotypes suggests that evolutionary strategies of bacteria include mechanisms for increasing mutability (Hall and Henderson-Begg 2006, Sundin and Weigand 2007). These mutator genotypes have not been shown to confer an intrinsic fitness advantage and their abundance within natural populations likely results from hitchhiking with secondary, beneficial mutations which counterbalance the cost of accumulating deleterious mutations (Giraud *et al*. 2001, Tenaillon *et al*. 2001).

Inducible mutability in the form of mutagenic DNA repair (MDR) transiently increases mutation rate through the activation of low-fidelity repair polymerases in response to specific environmental stress conditions such as ultraviolet radiation (UVR) exposure. UVR directly damages DNA causing lesions, including cyclobutane pyrimidine dimers and 6-4 photoproducts, that distort the helical structure, interrupting replication fork progression and inducing the SOS response. The SOS regulon coordinates the control of more than 40 unlinked genes involved in DNA repair, recombination, and cell cycle control (Courcelle *et al.* 2001, Schlacher and Goodman 2007). Among these, the Y family DNA polymerases polIV (*Escherichia coli* DinB) and polV (*E. coli* UmuDC) rescue stalled replication forks and permit DNA synthesis across

damaged regions of DNA in a template-independent manner by nature of their high processivity and low fidelity (Jarosz *et al.* 2007). Secondary mutations caused by these error-prone polymerases comprise the majority of sequence alterations derived from UVR exposure and define the inducible mutator phenotype. This translesion DNA synthesis activity confers both UVR tolerance and inducible mutability, intimately linking survival and mutation in habitats subject to regular UVR exposure.

MDR-mediated UVR tolerance provides a critical ecological advantage to epiphytic plant pathogens harboring Y family polymerases that reside in habitats optimized for solar UVR exposure (Sundin and Murillo 1999, Kim and Sundin 2000). We have previously reported our studies of experimental evolution with the highly UVR-mutable celery pathogen *Pseudomonas cichorii* 302959 (Weigand and Sundin 2009). The major contributor of UVR-induced mutability in this strain is *rulAB*, a homolog of *umuDC* found in various plant-associated *Pseudomonas* species (Zhang and Sundin 2004). This system is both ecologically relevant and experimentally tractable, making it ideally suited to investigate the influence of MDR on adaptation. In our previous study, parallel lineages of initially isogenic *P. cichorii* 302959 were maintained in a serial transfer regime for 500 generations during which half received daily MDR activation in the form of UVR exposure (Weigand and Sundin 2009). During that evolution experiment, mutations that conferred any competitive advantage under the lineage conditions were favored by natural selection.

The results of our initial study suggested that regular activation of inducible mutability in the form of MDR was not detrimental to fitness, but rather could contribute to adaptation and genetic diversity (Weigand and Sundin 2009). Another recent study has also suggested that when fitness improvements are available, adaptation may be facilitated by moderately enhanced

mutagenesis (Loh *et al.* 2010). In our previous study, relative fitness measurements of UVR-exposed lineages indicated a specificity of adaptation observable as higher fitness gains under UVR conditions than under non-UVR conditions that could not be attributed to increases in UVR tolerance alone. Conversely, lineages not exposed to daily UVR displayed comparable improvements in relative fitness under both UVR and non-UVR conditions. Such specialization is a general feature of adaptive evolution (Cooper and Lenski 200, MacLean *et al.* 2004). However, given the common growth medium in both the UVR and non-UVR lineage environments, we expected that beneficial mutations would contribute to enhanced growth under both conditions.

In the present study, we investigated adaptive specificity in experimental lineages of *P. cichorii* 302959 by examining physiological components of relative fitness improvements. Relative fitness measurements indicate an overall growth advantage that we hypothesized could be subdivided into discrete improvements in bacterial physiology to reveal a more detailed understanding of the adaptation of *P. cichorii* 302959 to specific laboratory environments. Our results suggest that improvements in doubling and lag times contributed to specific relative fitness gains by both UVR and non-UVR lineages in their respective environments. Surprisingly, specific fitness improvements appeared relatively early in all experimental lineages, independent of regular MDR activation.

MATERIALS and METHODS

Bacterial strains, growth conditions, and general molecular biology techniques

The ancestral 'round' strain of *P. cichorii* 302959 was originally isolated in Japan (Zhang and Sundin 2004). In a previous study, sixteen populations of *P. cichorii* 302959 were derived

from a single ancestral colony and propagated by serial transfer for 500 generations in minimal liquid media (Weigand and Sundin 2009). Eight UVR lineages (numbered 25-32) received a single, daily dose of UVC (254 nm) radiation to activate mutagenic DNA repair. Cultures were individually mixed 1:1 with saline (0.85% NaCl) in a glass Petri dish and exposed to ~40 J m^{-2} of UVC (254 nm) radiation from an XX-15 UV lamp (UVP Products, San Gabriel, CA) that resulted in ~10% survival by each lineage population. The energy output of the lamp was monitored with a UV-X radiometer fitted with a UV-25 sensor (UVP Products, San Gabriel, CA) and determined to be 1.5 J m^{-2} s^{-1}. Following UVR irradiation, cultures were diluted 1:100 into fresh medium and incubated under dark conditions to minimize photoreactivation. These lineages diversified into a fluctuating coexistence of 'round' (R) and 'fuzzy' (F) colony morphotypes. Eight non-UVR lineages (numbered 33-40) were diluted daily into fresh medium without MDR activation by UVR exposure and contained only the R morphotype. The culture transfer strategies for both UVR and non-UVR lineages resulted in a 1000-fold daily increase in the growth of each population, representing ~10 generations of binary fission.

Samples from each lineage were periodically preserved in a nonevolving state in 10% glycerol at -80°C. All evolved isolates in this study were derived from the ancestral genotype following either 250 or 500 generations of selection in the lineage experiment (Figure 3-1). We define "lineage isolates" as single-colony isolates from any of the replicate lineages in this experiment. Evolved isolates are denoted by lineage replicate and morphotype (*e.g.*, 25R refers to an isolate from lineage 25 that exhibits the round colony morphotype). Each lineage contained ~10^{10} total *P. cichorii* 302959 cells at stationary phase, and the relative fitness of these communities has been shown to rely on the ecological interactions of sympatric genotypes

(Weigand and Sundin 2009). Therefore, lineage communities were also analyzed in this study as population samples; these samples contained ~10^8 cells thawed from preserved UVR lineages to maintain the assemblage of diverse genotypes. Isolates and population samples from generation 500 characterized here are identical to those reported previously (Weigand and Sundin 2009).

Strains of *P. cichorii* were cultured at 28°C in Luria–Burtani (LB) broth (Difco, Detroit, MI), in Davis Minimal broth supplemented with 25 mg l^{-1} glucose (DM25) (Difco), or on King's medium B (KB) agar (King *et al.* 1954). Antibiotics were used where appropriate at the following concentrations: carbenicillin 50 µg ml^{-1}, gentamicin 10 µg ml^{-1}. Competent cell preparation was performed according to Choi *et al.* (2006). Plasmid DNA was isolated using the QIAprep Miniprep kit (QIAGEN, Valencia, CA). Transformation by electroporation, standard agarose gel electrophoresis, and other recombinant DNA techniques were performed according to Sambrook *et al.* (1989).

Competition experiments and relative fitness calculations

The relative fitness of all isolates and population samples was determined by direct competition with the ancestor under both UVR and non-UVR conditions to identify patterns of specific or general adaptation as described previously (Weigand and Sundin 2009). Briefly, competitions were performed under the same UVR and non-UVR conditions as the lineage experiment described above. To ensure that competitors were comparably acclimated to the competition environment, isolates were simultaneously removed from glycerol stocks maintained at -80°C, individually grown in LB broth overnight, and then individually grown for 24 hr in the competition environment. Competitors were mixed at a 1:1 volumetric ratio, and appropriate dilutions were spread on KB agar in triplicate at 0 and 24 hr to estimate the initial and final

density of each. Strain differentiation was accomplished by plasmid-encoded catechol 2,3-dioxygenase (*xylE*) which causes expressing colonies on agar media to turn yellow when sprayed with 0.1 M catechol. The relative fitness (W) of the two competitors was calculated as the ratio of their realized growth rate as described previously (Weigand and Sundin 2009). When the two competitors are equally fit in the competition environment, $W = 1$.

The six replicates of each relative fitness measurement were analyzed by two-tailed, independent *t*-tests against the hypothesis of mean equal fitness ($W = 1$). Fitness measurements were compared using two-tailed paired *t*-tests to determine any significant differences in adaptation between growth intervals (generations 250 vs. 500) or growth environments (UVR vs. non-UVR conditions).

Growth curves and calculations

Before optical density (OD) measurement, lineage isolates and population samples were grown overnight in LB broth and subcultured into 10 ml DM25 at 28°C for 24 hr acclimation. Cultures were diluted 1000-fold into fresh DM25, and 100 µl samples were removed for OD measurement at 600-nm in 96-well microtiter plates using a Tecan Safire (Tecan US, Inc., Durham, NC). Samples were removed for measurement at 0 hr and at 1 hr intervals from 4 hr to 15 hr. Growth curves for each lineage isolate and population sample were repeated in triplicate under both UVR and non-UVR conditions.

OD_{600} values from growth curves were standardized by dividing by the initial OD_{600} (0 hr) and then $\log_2$ transformed. The transformed values were plotted, and the window of exponential growth was identified by linear regression (Lenski *et al.* 1994). Lag time was

calculated simply as the x-intercept of the linear equation. Doubling time (g) was determined by the equation:

$$g = 3.3(\log_{10}(N_2)-\log_{10}(N_1)) / t$$

where N_1 and N_2 are raw, untransformed OD values corresponding to the window of exponential growth observed in the $\log_2$ transformation plot and t is the time interval between them (Madigan and Martinko 2006).

UVR tolerance assays

Lineage isolates and population samples were grown overnight in LB broth and 2 ml of culture were pelleted, washed with 1 ml saline, resuspended in 1 ml saline, and held on ice. The cell suspensions were mixed with 9 ml saline in a glass petri dish and exposed to a single dose of approximately 140 J m^{-2} UVC as described above. Cell suspensions were mixed continuously while receiving UVR doses to eliminate survival as a result of shading. Following irradiation, surviving cells were enumerated by dilution plating conducted under dark conditions.

RESULTS

Relative fitness of evolved lineages

The relative fitness of all lineages was determined by competition with the *P. cichorii* 302959 ancestor under both UVR and non-UVR conditions to examine adaptation during experimental evolution. We evaluated the relative fitness of each lineage by competing both population samples (a subset of the entire lineage maintaining the assemblage of diverse genotypes) and single-colony isolates. In our previous study, we analyzed changes in relative

fitness in replicate lineages after 500 generations (Weigand and Sundin 2009). In this current study, we report on alterations in relative fitness occurring after 250 generations with a focus on underlying mechanistic changes responsible for enhancement of relative fitness.

Isolates from UVR lineages expressed two different morphologies, R and F, and single-colony representatives of each from generations 250 and 500 were characterized. Neither morphotype group exhibited a significant fitness advantage under either UVR or non-UVR conditions (all $P > 0.320$). Therefore, measurements of the two groups have been combined when comparing isolates from UVR and non-UVR lineages but are also presented individually to provide additional sampling of the genetic diversity present in UVR lineages. Relative fitness measurements were also conducted using population samples to observe any influence of genotype community structure in UVR lineages. We have combined the relative fitness values determined at generation 250 in this study with previously published measurements at generation 500 (Weigand and Sundin 2009) in our data analysis and illustrations to highlight differences in adaptive improvements at the two time points. The average relative fitness of all isolates and population samples is summarized in Figure 3-2 and individual measurements of each lineage are reported in Figures 3-3 to 3-6. Each relative fitness change was analyzed by an independent *t*-test against the hypothesis of mean fitness equal to that of the ancestral *P. cichorii* 302959 ($W = 1$) and is significant where indicated in the figures. Paired *t*-tests were also used to determine significant differences in relative fitness between growth environments (UVR vs. non-UVR conditions) or growth intervals (generations 250 vs. 500) and these *P* values are listed at the bottom of each figure.

Population samples of ~10^8 cells from UVR lineages at generation 250 exhibited increased relative fitness under non-UVR conditions and further improvements under UVR

conditions (Figure 3-2A). Likewise, single-colony isolates from these UVR lineage communities exhibited increased relative fitness at generation 250 in favor of the UVR conditions (Figure 3-2A). Similar adaptive specificity was observable in population samples and isolates from UVR lineages at generation 500 (all $P < 0.0013$, Figure 3-2B). The specific differences in relative fitness exhibited by UVR lineages under the two conditions were significant at both generations 250 and 500. Conversely, isolates from non-UVR lineages generally displayed comparable fitness gains under both non-UVR and UVR conditions (Figure 3-2A). The average relative fitness of population samples and isolates from all lineages at generation 500 was increased under UVR conditions compared to generation 250 (Figure 3-2B).

Population samples taken from individual UVR lineages at generation 250, with the exception of lineage 28, displayed a 15% average improvement in relative fitness under non-UVR conditions (all $P < 0.026$, Figure 3-3A). Under UVR conditions, individual population samples from UVR lineages at generation 250 exhibited a 61% average improvement in relative fitness (all $P < 0.002$, Figure 3-3B). Only population samples from lineages 28 and 32 exhibited significant differences in relative fitness under non-UVR conditions between generations 250 and 500 (all $P < 0.017$, Figure 3-3A). However, relative fitness under UVR conditions exhibited by population samples from half of the UVR lineages differed significantly between generations 250 and 500 (all $P < 0.002$, Figure 3-3B).

As described previously, the ancestral *P. cichorii* 302959 displayed a 'round' colony morphology that gave rise to complex mixtures of R and F morphotypes during the experimental evolution of all UVR lineages [12]. Under non-UVR conditions, individual R morphotype isolates recovered at generation 250 from only three UVR lineages (25, 26, and 27) exhibited improvements in relative fitness (all $P < 0.039$, Figure 3-4A). However, all R isolates from

UVR lineages at generation 250 gained relative fitness under UVR conditions by an average of 30% (all $P < 0.021$, Figure 3-4B). Only an R morphotype isolated from lineage 28 at generation 250 displayed reduced fitness under non-UVR conditions ($P = 0.004$, Figure 3-4A). When compared to isolates taken at generation 500, only the relative fitness of R morphotype isolates from UVR lineages 30 and 32 differed significantly under non-UVR conditions (all $P < 0.02$, Figure 3-4A). Under UVR conditions, the relative fitness of R morphotypes isolated from lineages 30, 31, and 32 differed significantly between generations 250 and 500 (all $P < 0.040$, Figure 3-4B).

Nearly all F morphotypes isolated from UVR lineages at generation 250 exhibited increased fitness under non-UVR conditions by an average of 17% (all $P < 0.010$, Figure 3-5A). Only an F morphotype isolated from lineage 27 at generation 250 exhibited reduced fitness under non-UVR conditions ($P = 0.00005$, Figure 3-5A). Under UVR conditions, all F isolates from UVR lineages at generation 250 gained relative fitness by an average of 35% (all $P < 0.005$, Figure 3-5B). When compared to isolates taken at generation 500, the relative fitness of F morphotype isolates from UVR lineages 27, 28, 29 and 30 all differed significantly under non-UVR conditions (all $P < 0.025$, Figure 3-5A). Under UVR conditions, the relative fitness of F morphotypes isolated from lineages 27, 28, and 30 differed significantly between generations 250 and 500 (all $P < 0.043$, Figure 3-5B).

Non-UVR lineages 33-40 contained only R morphotypes. Single-colony isolates from generation 250 displayed a 24% average improvement in relative fitness under non-UVR conditions (all $P < 0.020$) with the exception of the isolate from lineage 37 (Figure 3-6A). Isolates from non-UVR lineages at generation 250 also exhibited a 19% average improvement in relative fitness under UVR conditions (all $P < 0.006$, Figure 3-6B). Only the relative fitness of

the isolate from lineage 36 varied significantly between the two conditions ($P = 0.029$). When compared to isolates from generation 500, there was no significant difference in relative fitness under non-UVR conditions (Figure 3-6A) and only isolates from non-UVR lineages 36 and 39 differed significantly from their generation 500 counterparts under UVR conditions (Figure 3-5B).

Growth dynamics

Growth parameters were examined to elucidate components of adaptation observed in evolved isolates and population samples in an attempt to determine mechanisms responsible for specific changes in relative fitness. Growth curves of each population sample and single-colony isolate from generations 250 and 500 were plotted to calculate changes in doubling and lag times under both non-UVR and UVR conditions. Isolates and population samples from all evolved lineages displayed improvements in both doubling and lag times. The ancestral *P. cichorii* 302959 strain exhibited doubling times of 1.45 ± 0.09 hours and 1.68 ± 0.10 hours at 28°C under non-UVR and UVR conditions, respectively. Population samples from UVR lineages improved their doubling time under non-UVR conditions by an average reduction of 15% at generation 250 and 17% at generation 500 (Figure 3-7A and B). Doubling times of population samples were further reduced under UVR conditions by an average of 24% and 37% at generations 250 and 500, respectively (Figure 3-7A and B). Isolates from UVR lineages, including both R and F morphotypes, exhibited similar patterns of doubling time improvement, with greater average reductions under UVR conditions at both generation 250 and 500 (Figure 3-7A and B). However, differences in doubling time improvements under the two conditions were only significant at generation 500 for both population samples and isolates from UVR lineages.

Conversely, the average doubling time of isolates from non-UVR lineages was more analogous under non-UVR and UVR conditions and reductions were only significant at generation 500 (Figure 3-7A and B).

Similar improvements in lag time (i.e. reductions in lag time) were also observed. The ancestral *P. cichorii* 302959 strain exhibited analogous lag times of 10.27 ± 0.32 hours and 10.47 ± 0.26 hours under non-UVR and UVR conditions, respectively. Population samples from UVR lineages improved their lag times under non-UVR conditions by an average reduction of 16% at generation 250 and 21% at generation 500 (Figure 3-8A and B). Lag times of population samples were further reduced under UVR conditions by an average of 32% at generation 250 and 34% at generation 500 (Figure 3-8A and B). The lag times of isolates from UVR lineages again exhibited similar patterns of improvement significantly with greater average reductions under UVR conditions at both generation 250 and 500 (Figure 3-8A and B). Conversely, isolates from non-UVR lineages exhibited comparable reductions in lag times under non-UVR and UVR conditions at both generation 250 and 500 (Figure 3-8A and B). A complete list of doubling and lag times for each population sample and single-colony isolate is available in Tables 3-1 and 3-2.

UVR Tolerance

Changes in UVR tolerance could lead to changes in relative fitness under UVR conditions. Therefore, the UVR tolerance of population samples and isolates from each UVR and non-UVR lineages taken at generations 250 and 500 was determined following a single dose of UVC. An elevated dose of ~140 J m^{-2} s^{-1} was used to resolve subtle differences in UVR tolerance that could not be distinguished at the lower dosage used during lineage propagation. The ancestral *P. cichorii* 302959 strain exhibited a 0.7% survival rate at the elevated dose.

Population samples from UVR lineages displayed the most consistent improvements in UVR tolerance with average survival rates of 4.3% and 4.0% at generations 250 and 500, respectively. R and F morphotype isolates exhibited a mixture of UVR tolerance phenotypes that were higher, lower, or comparable to that of the ancestor. A complete list of UVR tolerance measures for each population sample and isolate is available in Table 3-3.

Relative fitness trajectories

To track changes in relative fitness during lineage growth over the course of 500 generations, preserved population samples from four randomly selected lineages were obtained at different intervals and competed against the *P. cichorii* 302959 ancestor under both non-UVR and UVR conditions. The resulting fitness trajectories are plotted in Figure 3-8. UVR lineages 26 and 30 first exhibited fitness gains under their native, UVR conditions before later gaining fitness under the alternate, non-UVR conditions (Figure 3-9A and B). The relative fitness of UVR lineage 26 remained comparable under both non-UVR and UVR conditions until additional fitness gains were later achieved under UVR conditions by generation 250 (Figure 3-9A). UVR lineage 30 displayed improved fitness only under UVR conditions for a much longer period until later gaining fitness under non-UVR conditions (Figure 3-9B). However, even after gaining fitness under non-UVR conditions, UVR lineage 30 continued to exhibit significantly higher fitness under UVR conditions.

Similarly, non-UVR lineages 36 and 39 exhibited fitness gains under their native, non-UVR conditions first (Figure 3-9C and D). Neither lineage 36 nor 39 exhibited a significant increase in non-UVR fitness following the initial improvements observed in the first 100

generations. However, additional fitness improvements under UVR conditions were achieved before generation 500 in both non-UVR lineages examined (Figure 3-9C and D).

DISCUSSION

Our results suggest that adaptive specificity in laboratory evolved populations of the highly UVR-mutable *P. cichorii* 302959 emerged early and correlated with discrete growth improvements but was not dependent upon MDR activation. In a previous study, we maintained parallel cultures of *P. cichorii* 302959 for 500 generations with daily MDR activation in the form of UVR exposure and observed higher relative fitness particularly under UVR conditions (Weigand and Sundin 2009). In the present study, changes in relative fitness and underlying physiological characteristics were analyzed at intermediate points (in particular, at generation 250) in the experimental evolution of *P. cichorii* 302959 to evaluate this observed adaptive specificity.

The relative fitness of all 16 lineages was determined at the generation 250 midpoint of our evolution experiment with the intent to gain further information on the acquisition of fitness observed previously at the generation 500 endpoint. Fitness measurements at the midpoint can offer some indication of the timing, number, magnitude, and parallel nature of adaptive improvements achieved by each lineage. Measurements of relative fitness at generation 250 reflected the same patterns of adaptive specificity observed previously at generation 500 (Weigand and Sundin 2009). The interaction between sympatric genotypes within UVR lineages was previously shown to contribute to relative fitness under UVR conditions (Weigand and Sundin 2009). Such ecological interactions were established in UVR lineages by generation 250 and population samples exhibited greater fitness gains than individual isolates. A comparison of

relative fitness values at different time points indicated that most relative fitness improvements observed in *P. cichorii* 302959 lineages emerged within the first 250 generations of growth. Additional fitness gains attained between generations 250 and 500 were primarily achieved under UVR conditions, even in non-UVR lineages. The dramatic changes in fitness exhibited by F isolates from lineages 27 and 28 demonstrate that UVR lineage populations contained a dynamic balance of genotypes, and that isolates sampled at generation 500 were not necessarily derived from those sampled at generation 250. Together, these results emphasize the complexity of adaptation in the UVR lineage populations due to the high adaptive potential and plasticity of the ancestral *P. cichorii* 302959 inducible mutator genotype. Additionally, the increase in relative fitness observed only under UVR conditions between generations 250 and 500 in some non-UVR lineages offers the first indication that beneficial mutations differentially contribute to fitness under the two growth conditions.

Measures of relative fitness indicate the overall advantage of one bacterial strain or population over another but provide no information about the underlying physiological differences responsible for the advantage. Changes in growth dynamics have been identified as distinct components of relative fitness improvements in similar studies conducted previously (Lenski *et al.* 1998, Sleight and Lenski 2007). We plotted optical density changes during growth of isolates and population samples from lineages of *P. cichorii* 302959 obtained at generations 250 and 500 under both UVR and non-UVR conditions. Growth dynamic improvements correlated well with changes in relative fitness such that nearly all evolved lineages with increased fitness displayed corresponding reductions in both doubling and lag times compared to the ancestor. The average reductions in both lag and doubling times exhibited by UVR lineages were greatest under UVR conditions while non-UVR lineages displayed comparable reductions

under both conditions. These results are consistent with the observed patterns of adaptive specificity in relative fitness measurements suggesting that specific growth improvements are responsible for the characteristic gains in relative fitness displayed by UVR lineages under UVR conditions.

Improvements in lag time could be indicative of a faster metabolic transition from stationary phase to exponential growth due to adaptation to the culture media or to the cyclic nature of the serial transfer regime. Improvements in lag time under UVR conditions specifically could result from increased UVR tolerance or more efficient DNA repair in the form of improved SOS induction or polymerase processivity. The complex SOS regulatory network is comprised of both transcriptional and post-transcriptional regulators that tightly control the temporal activity of its gene products (Courcelle *et al.* 2001, Friedman *et al.* 2005, Shimoni *et al.* 2009). Beneficial adaptations targeted to any of these regulators that contribute to the timing or efficiency of the SOS network could be observable as a reduction in lag time particularly under UVR conditions.

We explored changes in UVR tolerance as an obvious candidate for improved fitness and lag time under UVR conditions but only observed increased UVR tolerance concurrently with fitness gains in a limited number of instances. In general, there was no pattern to suggest that greater UVR tolerance was primarily responsible for increased relative fitness observed under UVR conditions. Alternatively, improved fitness under UVR conditions could result from enhanced nutrient scavenging. Approximately 90% of the population died as a result of UVR exposure during each cycle of the UVR lineage regime. The death of these individuals could release additional nutrients in the form of cell lysate that is transferred with surviving cells to fresh medium for the next cycle of growth. Any mutation that would enhance the utilization of

these nutrients could lead to greater fitness exclusively under UVR conditions. In evolution experiments with *E. coli*, adaptive mutations that contribute to survival through catabolism of cellular detritus (Finkel 2006) or secreted metabolites (Turner *et al.* 1996) have been reported. However, we were unable to observe growth by the ancestral *P. cichorii* 302959 or any evolved lineages in glucose-free Davis Minimal medium amended with a bacterial suspension killed either by prolonged heat or UVR exposure (data not shown). Therefore, increased relative fitness under UVR conditions and the adaptive specificity of UVR lineages that is reflected in improved growth likely did not result from either increased UVR tolerance or a heightened ability to scavenge nutrients as a carbon source.

We previously observed the reproducible emergence of an F morphotype that coexisted with the ancestral, R morphotype in all UVR lineages but never in non-UVR lineages (Weigand and Sundin 2009). In the current study, single-colony representatives of both morphologies have been examined for changes in relative fitness and physiology at generation 250 and 500 under both UVR and non-UVR conditions. We have found no evidence to suggest that either morphotype possesses an inherent adaptive advantage observable as improved relative fitness, growth dynamics, or UVR tolerance. In our analyses we found only subtle differences in doubling times between the two groups. The results of the current study are consistent with our previous observations of the stochastic fluctuations of F morphotype abundance (Weigand and Sundin 2009) suggesting the F determinant itself likely does not carry a strong selective advantage. Therefore, measurements of the two groups can be combined and interpreted as additional sampling of the genetic diversity present in the UVR lineages.

It is clear from our relative fitness measurements that adaptive changes in both non-UVR and UVR lineages were concentrated in the first 250 generations, confirming both the strong

selection pressure of the experimental environments and the high adaptive potential of the ancestral *P. cichorii* 302959 genotype. Furthermore, the early fitness improvements by UVR lineages are consistent with the emergence of F morphotypes and spontaneous rifampicin-resistant colonies reported previously (Weigand and Sundin 2009). For a more detailed view of adaptation, we tracked the acquisition of relative fitness under UVR and non-UVR conditions in four randomly selected lineages. The resulting fitness trajectories are somewhat punctuated, suggesting adaptive evolution due to the successive acquisition of beneficial mutations consistent with similar studies conducted previously (Elena *et al.* 1996). Rapid changes in relative fitness interrupted periods of apparent stasis throughout the history of each lineage. However, these populations remained quite dynamic, as the frequency of F morphotypes and rifampicin-resistant colonies continued to fluctuate (Weigand and Sundin 2009) without affecting the overall fitness of the population. The fitness trajectories in this study confirm that within communities assembled by adaptive radiation, such as those present in our UVR lineages, changes in individual fitness produce fluctuations in genotype frequency, and the interactions of those sympatric genotypes influence the relative fitness of the population (MacLean 2005). Furthermore, complete selective sweeps are not required for adaptive improvements to influence the fitness of a population.

The fitness trajectories in this study also suggest that adaptive improvements differentially contributed to relative fitness gains under UVR and non-UVR conditions. The ordered acquisition of improvements in the two environments favored the respective conditions under which each lineage was propagated such that UVR lineages first exhibited specific fitness gains under UVR conditions and non-UVR lineages under non-UVR conditions. Based on our previous observations of relative fitness at generation 500, we concluded that non-UVR lineages

exhibited comparable fitness gains under UVR and non-UVR conditions due to their adaptation to the shared culture medium (Weigand and Sundin 2009). We expected that any phenotypic traits contributing to success under non-UVR conditions should likewise be beneficial during growth under UVR conditions. However, it is clear from fitness trajectories of non-UVR lineages that increases in fitness under non-UVR conditions were not mirrored by comparable increases in fitness under UVR conditions. These results suggest that adaptive specificity does not depend on MDR activation but is in fact a common pattern of adaption in *P. cichorii* 302959 exhibited by both UVR and non-UVR lineages. Such adaptive specialization can result in fitness trade-offs due to either antagonistic pleiotropy or mutation accumulation resulting in lower fitness under different environmental conditions (Travisano and Lenski 1996, Cooper and Lenski 2000, MacLean *et al.* 2004). However, in this study the adaptive specificity of both UVR and non-UVR lineages did not include any observable negative effect on fitness in the respective alternate environments.

Interestingly, when fitness did increase in the alternate environment, corresponding improvements in fitness were not observed in the native environment. It is unlikely that fitness improvements under UVR and non-UVR conditions are independent. Rather, mutations that contribute to fitness observable only in the alternate environment may represent general improvements of smaller effect in the native environment. After gaining fitness in the alternate environment, UVR lineages continued to gain fitness specifically under their native conditions while non-UVR lineages did not, producing the illusion of adaptive specificity exclusively in UVR lineages. We can propose three possible explanations for this discrepancy: (1) MDR activation provides access to more beneficial mutations by altering the mutation spectrum of UVR lineages; (2) by increasing the overall mutation rate, MDR activation increases the rate at

which beneficial mutations appear in the population and non-UVR lineages would gain additional relative fitness if propagated for a longer time; or (3) the ancestral *P. cichorii* 302959 genotype is more poorly adapted to the UVR conditions and therefore more adaptive opportunities exist than under non-UVR conditions. Although the first two possibilities lend support to the proposed positive influence of inducible mutability on adaptive evolution, we do not have sufficient information to distinguish them from each other or from the third possibility.

In summary, we have investigated the adaptive specificity of *P. cichorii* 302959 during experimental evolution by measuring relative fitness and corresponding changes in physiology. Our results suggest that adaptive specificity in this organism correlates with discrete growth improvements but does not depend on MDR activation. Adaptive improvements in all lineages were concentrated in the first 250 generations of experimental evolution and specific increases in relative fitness correlated with distinct improvements in doubling and lag times. Furthermore, UVR lineages exhibited additional gains in fitness after generation 250 exclusively under UVR conditions that were reflected in further doubling and lag time improvements but likely not greater UVR tolerance or scavenging of nutrients to support the growth of new biomass. Fitness trajectories of select lineages clearly indicate that adaptive improvements under UVR and non-UVR conditions were acquired preferentially and differentially contributed to relative fitness under varied growth conditions.

The results of this study lend support to our earlier observations that suggest increased mutation rate in the form of inducible mutability does not impede adaptation by mutation accumulation. Rather, UVR and non-UVR lineages preferentially acquired adaptive growth improvements in a similar manner and additional fitness gains by UVR lineages may have been due to greater access to beneficial mutations. Our future work will compare the influence of

different mechanisms of mutability on adaptation in an advanced genetic system equipped with genomic tools for analysis.

Table 3-1. Doubling time (in hrs) of population samples and isolates from lineages of *P. cichorii* 302959 under non-UVR and UVR conditions.[a]

	Generation 250		Generation 500	
	Non-UVR	UVR	Non-UVR	UVR
UVR Lineage Population Samples				
25	1.10 ± 0.06	1.32 ± 0.07	1.25 ± 0.30	1.04 ± 0.04
26	0.94 ± 0.08	1.60 ± 0.19	1.19 ± 0.10	0.94 ± 0.08
27	1.11 ± 0.14	1.30 ± 0.03	1.20 ± 0.15	1.11 ± 0.14
28	0.98 ± 0.14	1.04 ± 0.05	1.09 ± 0.11	0.98 ± 0.14
29	0.96 ± 0.25	1.24 ± 0.05	1.20 ± 0.07	0.96 ± 0.25
30	0.86 ± 0.14	1.26 ± 0.13	1.26 ± 0.18	0.86 ± 0.14
31	1.23 ± 0.10	1.12 ± 0.05	1.26 ± 0.16	1.23 ± 0.10
32	1.37 ± 0.15	1.27 ± 0.17	1.19 ± 0.12	1.37 ± 0.15
Round UVR Lineage Isolates				
25R	1.18 ± 0.08	1.05 ± 0.30	1.58 ± 0.04	1.88 ± 0.27
26R	1.19 ± 0.10	1.07 ± 0.06	1.65 ± 0.04	1.29 ± 0.12
27R	1.28 ± 0.23	1.14 ± 0.09	2.32 ± 0.11	1.02 ± 0.17
28R	0.89 ± 0.20	1.23 ± 0.12	1.61 ± 0.05	1.10 ± 0.23
29R	1.05 ± 0.18	0.69 ± 0.20	1.40 ± 0.05	1.73 ± 0.32
30R	1.13 ± 0.05	1.62 ± 0.23	1.07 ± 0.07	1.37 ± 0.04
31R	1.25 ± 0.06	1.18 ± 0.13	1.42 ± 0.08	1.68 ± 0.10
32R	1.93 ± 0.19	1.41 ± 0.07	1.12 ± 0.07	1.32 ± 0.07
Fuzzy UVR Lineage Isolates				
25F	1.34 ± 0.09	1.47 ± 0.33	1.19 ± 0.11	1.13 ± 0.16
26F	1.33 ± 0.16	1.10 ± 0.03	1.57 ± 0.11	1.31 ± 0.11
27F	1.09 ± 0.20	1.47 ± 0.20	1.17 ± 0.03	1.30 ± 0.11
28F	1.10 ± 0.11	1.26 ± 0.04	1.47 ± 0.07	1.44 ± 0.21
29F	1.24 ± 0.17	1.34 ± 0.05	1.34 ± 0.20	1.29 ± 0.31
30F	1.59 ± 0.04	1.39 ± 0.25	1.44 ± 0.08	1.12 ± 0.08
31F	0.84 ± 0.18	1.34 ± 0.19	1.55 ± 0.10	1.12 ± 0.11
32F	1.00 ± 0.09	1.81 ± 0.13	1.43 ± 0.06	1.25 ± 0.10
Non-UVR Lineage Isolates				
33R	1.18 ± 0.07	1.62 ± 0.19	1.20 ± 0.11	1.44 ± 0.16
34R	1.38 ± 0.26	1.84 ± 0.06	1.38 ± 0.02	1.39 ± 0.18
35R	1.66 ± 0.08	1.94 ± 0.07	1.38 ± 0.07	1.58 ± 0.03
36R	1.71 ± 0.14	1.13 ± 0.12	1.49 ± 0.04	1.21 ± 0.08
37R	1.70 ± 0.15	1.51 ± 0.02	1.37 ± 0.03	1.69 ± 0.20
38R	1.96 ± 0.08	1.45 ± 0.16	1.30 ± 0.06	1.03 ± 0.13
39R	0.97 ± 0.23	2.03 ± 0.37	0.93 ± 0.09	1.12 ± 0.18
40R	0.99 ± 0.13	1.06 ± 0.06	1.24 ± 0.03	1.07 ± 0.07

a. Doubling times of the *P. cichorii* 302959 ancestor were 1.45 ± 0.09 hrs and 1.68 ± 0.10 hrs under non-UVR and UVR conditions, respectively.

Table 3-2. Lag time (in hrs) of population samples and isolates from lineages of *P. cichorii* 302959 under non-UVR and UVR conditions.[a]

	Generation 250		Generation 500	
	Non-UVR	UVR	Non-UVR	UVR
UVR Lineage Population Samples				
25	9.83 ± 0.19	7.24 ± 0.09	6.86 ± 0.28	6.36 ± 0.04
26	8.18 ± 0.17	6.93 ± 0.20	8.20 ± 0.29	7.42 ± 0.05
27	8.43 ± 0.10	6.80 ± 0.07	7.46 ± 0.31	7.01 ± 0.16
28	9.85 ± 0.22	8.33 ± 0.09	9.48 ± 0.11	7.36 ± 0.17
29	8.16 ± 0.21	8.02 ± 0.10	8.62 ± 0.13	6.99 ± 0.21
30	8.53 ± 0.11	6.38 ± 0.21	7.63 ± 0.30	7.10 ± 0.20
31	8.04 ± 0.32	6.63 ± 0.06	8.45 ± 0.07	6.56 ± 0.11
32	7.76 ± 0.20	6.80 ± 0.25	8.05 ± 0.35	6.12 ± 0.06
Round UVR Lineage Isolates				
25R	10.01 ± 0.27	7.08 ± 0.23	8.83 ± 0.02	5.48 ± 0.23
26R	9.19 ± 0.27	7.65 ± 0.25	10.24 ± 0.08	6.88 ± 0.08
27R	7.78 ± 0.36	7.45 ± 0.34	9.60 ± 0.23	7.66 ± 0.14
28R	10.04 ± 0.11	6.93 ± 0.10	8.26 ± 0.07	6.70 ± 0.10
29R	9.94 ± 0.33	9.45 ± 0.24	8.14 ± 0.08	6.72 ± 0.35
30R	7.74 ± 0.34	7.00 ± 0.22	7.70 ± 0.08	6.46 ± 0.09
31R	7.83 ± 0.18	6.63 ± 0.19	8.25 ± 0.10	6.21 ± 0.16
32R	7.74 ± 0.24	7.24 ± 0.29	7.59 ± 0.06	6.02 ± 0.10
Fuzzy UVR Lineage Isolates				
25F	8.55 ± 0.06	7.59 ± 0.05	7.62 ± 0.07	6.46 ± 0.32
26F	7.41 ± 0.29	7.15 ± 0.29	8.79 ± 0.01	6.57 ± 0.20
27F	9.86 ± 0.05	7.30 ± 0.25	8.17 ± 0.02	7.04 ± 0.16
28F	9.48 ± 0.37	6.84 ± 0.09	10.23 ± 0.08	7.86 ± 0.04
29F	8.64 ± 0.15	6.79 ± 0.13	8.44 ± 0.18	5.94 ± 0.12
30F	7.89 ± 0.03	7.86 ± 0.03	8.37 ± 0.08	6.88 ± 0.32
31F	8.38 ± 0.22	6.26 ± 0.16	8.79 ± 0.09	7.25 ± 0.17
32F	8.05 ± 0.30	6.42 ± 0.02	8.27 ± 0.11	7.10 ± 0.16
Non-UVR Lineage Isolates				
33R	8.24 ± 0.44	7.28 ± 0.39	8.58 ± 0.07	7.80 ± 0.08
34R	7.26 ± 0.15	7.64 ± 0.18	7.06 ± 0.14	7.24 ± 0.25
35R	7.42 ± 0.05	7.75 ± 0.18	7.02 ± 0.09	6.92 ± 0.10
36R	7.54 ± 0.23	7.99 ± 0.09	6.80 ± 0.34	7.45 ± 0.13
37R	7.55 ± 0.25	8.47 ± 0.19	6.68 ± 0.06	6.55 ± 0.34
38R	7.90 ± 0.17	8.20 ± 0.04	7.44 ± 0.25	6.96 ± 0.14
39R	7.89 ± 0.12	7.43 ± 0.24	7.93 ± 0.07	7.75 ± 0.06
40R	7.10 ± 0.23	8.19 ± 0.18	7.24 ± 0.11	9.10 ± 0.21

a. Lag times of the *P. cichorii* 302959 ancestor were 10.27 ± 0.32 hrs and 10.47 ± 0.26 hrs under non-UVR and UVR conditions, respectively.

Table 3-3. Percent survival of population samples and isolates from lineages of *P. cichorii* 302959 following ~140 J m^{-2} UVC.[a]

	Gen 250	Gen 500
UVR Lineage Population Samples		
25	6.1%	5.0%
26	5.0%	1.5%
27	6.0%	0.4%
28	1.3%	0.7%
29	8.0%	5.1%
30	5.6%	6.5%
31	0.9%	1.4%
32	1.4%	11.1%
Round UVR Lineage Isolates		
25R	1.3%	1.2%
26R	2.5%	0.5%
27R	5.7%	0.6%
28R	1.7%	0.1%
29R	0.5%	0.9%
30R	1.3%	0.9%
31R	0.9%	0.01%
32R	8.4%	0.9%
Fuzzy UVR Lineage Isolates		
25F	8.1%	0.5%
26F	1.1%	0.1%
27F	7.0%	0.4%
28F	8.7%	0.04%
29F	2.7%	0.5%
30F	1.2%	0.2%
31F	2.3%	0.1%
32F	0.4%	< 0.01%
Non-UVR Lineage Isolates		
33	1.1%	0.1%
34	0.8%	2.9%
35	0.9%	0.1%
36	0.6%	0.7%
37	0.8%	0.1%
38	1.2%	0.2%
39	2.3%	0.2%
40	3.9%	1.6%

a. Percent survival of the *P. cichorii* 302959 ancestor was 0.7%.

Figure 3-1. Evolutionary history of isolates and population samples derived from experimental lineages of *P. cichorii* 302959 and characterized in this study. Sixteen parallel lineages of *P. cichorii* 302959 were derived from a single colony and propagated by serial transfer. Eight UVR lineages received daily UVR exposure and diversified into mixtures of 'round' and 'fuzzy' colony morphotypes. Eight non-UVR lineages did not receive UVR exposure and contained only the ancestral, 'round' colony morphotype. All 16 lineages were propagated for 500 generations and samples from each were periodically preserved. Isolates and population samples in this study were derived from the ancestor following either 250 or 500 generations of selection in the evolution experiment.

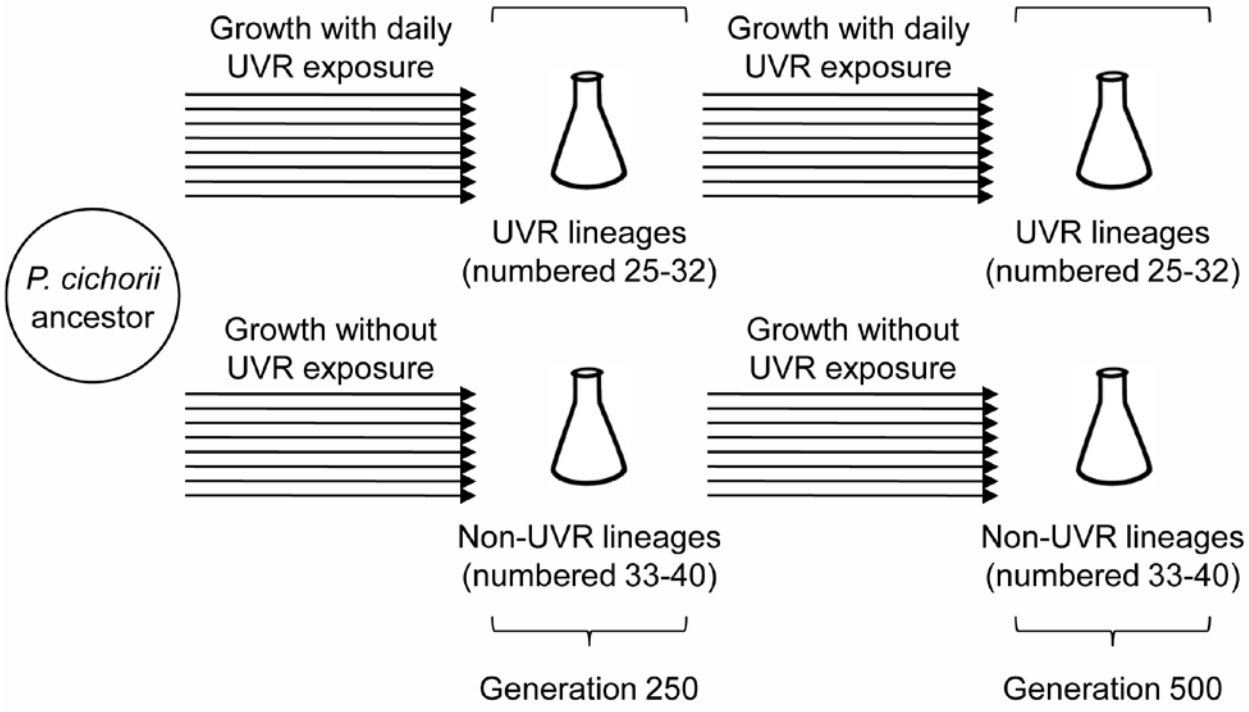
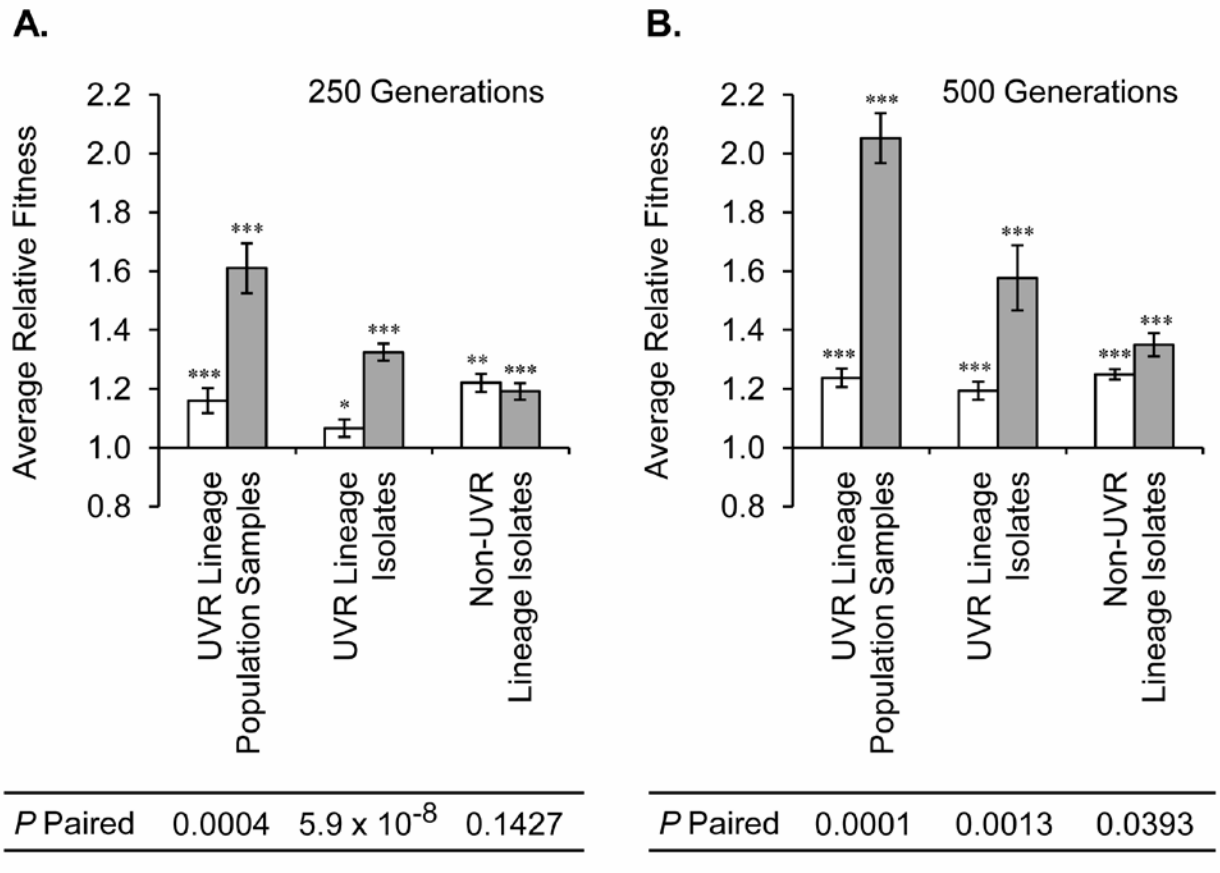


Figure 3-2. Average relative fitness indicates adaptive specificity in evolved lineages of *P. cichorii* 302959. Relative fitness of UVR lineage population samples, UVR lineage isolates, and non-UVR lineage isolates was measured by direct competition with the ancestor under non-UVR (open bars) and UVR (shaded bars) conditions after (A) 250 and (B) 500 generations of experimental evolution. Fitness values are means and error bars represent standard error of the mean. n = 8 for UVR lineage population samples and non-UVR lineage isolates but n = 16 for UVR lineage isolates. Values are significant by two-tailed independent *t*-test ($\alpha = 0.05$) where indicated (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$). *P*-paired values correspond to two-tailed paired *t*-tests ($\alpha = 0.05$) between average relative fitness values under the two conditions.



P Paired	0.0004	5.9 x 10^{-8}	0.1427

P Paired	0.0001	0.0013	0.0393

Figure 3-3. Relative fitness of individual population samples from UVR lineages 25-32. Relative fitness was measured by direct competition with the ancestor under (A) non-UVR and (B) UVR conditions. Population samples contained ~10^8 cells of *P. cichorii* 302959 after 250 (open bars) and 500 (shaded bars) generations of experimental evolution. Fitness values are means and error bars represent standard error of the mean. Values are significant by two-tailed independent *t*-test (d.f. = 5, $\alpha = 0.05$) where indicated (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$). *P*-paired values correspond to two-tailed paired *t*-tests (d.f. = 5, $\alpha = 0.05$) between relative fitness values at generation 250 and 500.

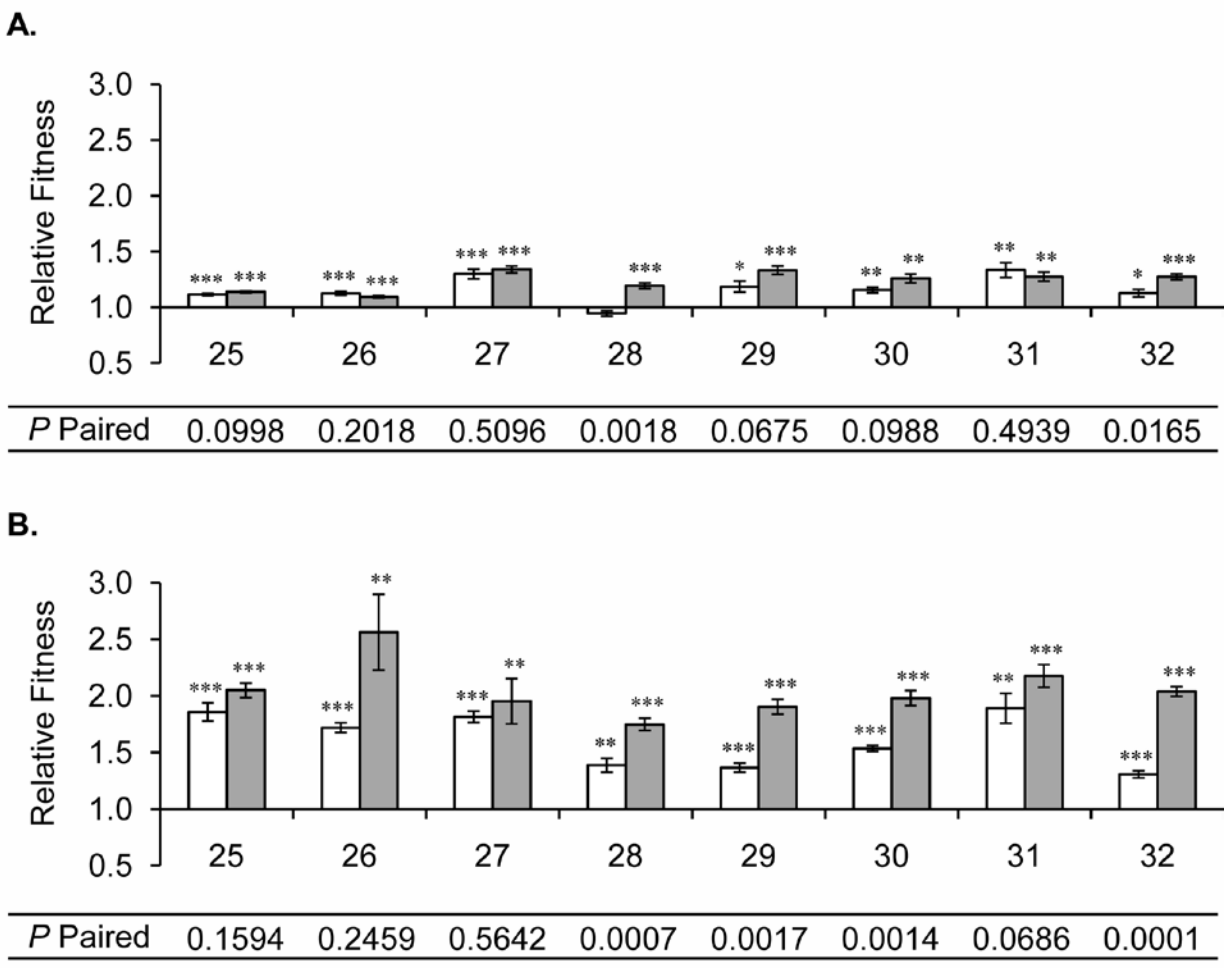


Figure 3-4. Relative fitness of 'round' (R) isolates from UVR lineages 25-32. Relative fitness was measured by direct competition with the ancestor under (A) non-UVR and (B) UVR conditions. Single isolates of *P. cichorii* 302959 exhibiting the 'round' colony morphology were taken after 250 (open bars) and 500 (shaded bars) generations of experimental evolution. Fitness values are means and error bars represent standard error of the mean. Values are significant by two-tailed independent *t*-test (d.f. = 5, $\alpha = 0.05$) where indicated (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$). *P*-paired values correspond to two-tailed paired *t*-tests (d.f. = 5, $\alpha = 0.05$) between relative fitness values for isolates from generation 250 and 500.

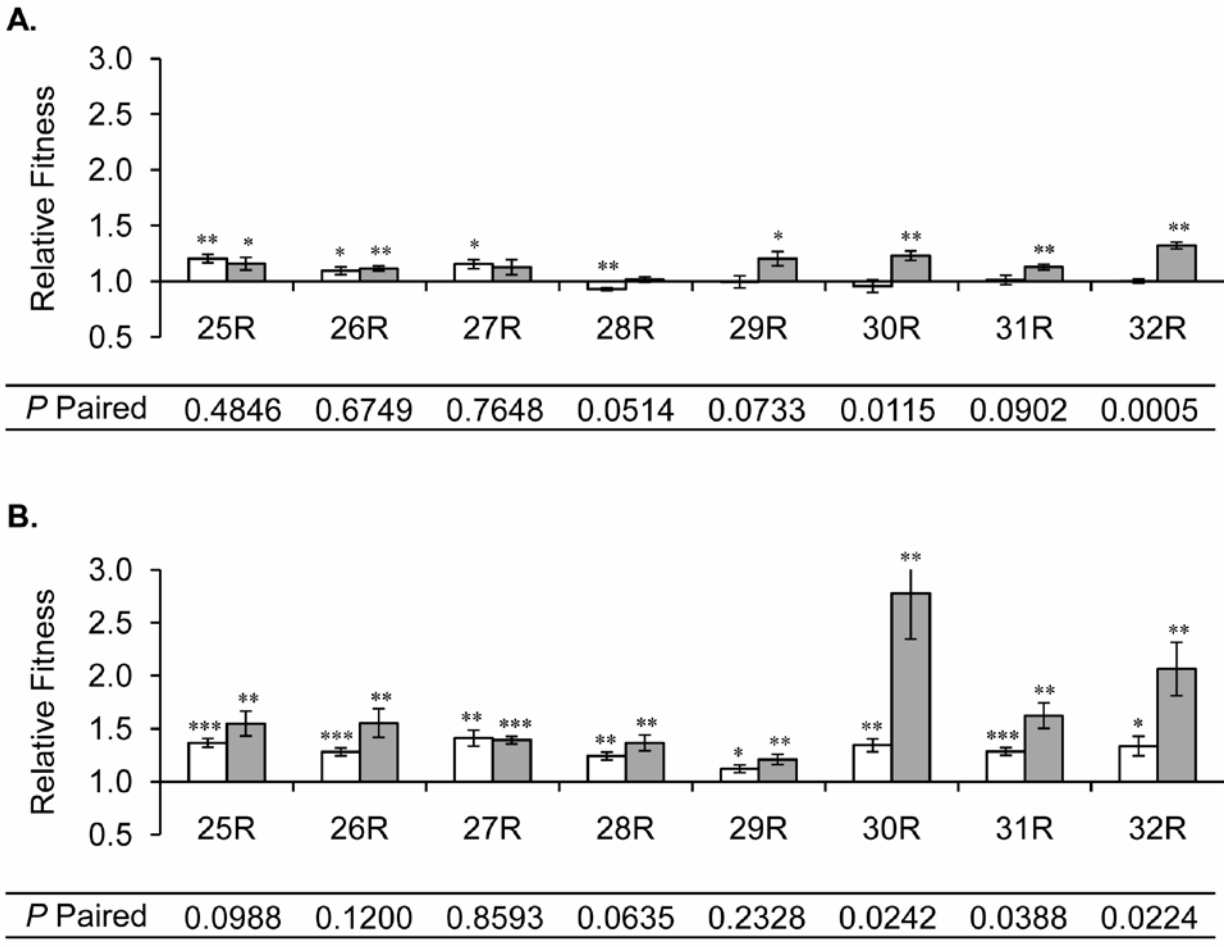


Figure 3-5. Relative fitness of 'fuzzy' (F) isolates from UVR lineages 25-32. Relative fitness was measured by direct competition with the ancestor under (A) non-UVR and (B) UVR conditions. Single isolates of *P. cichorii* 302959 exhibiting the 'fuzzy' colony morphology were taken after 250 (open bars) and 500 (shaded bars) generations of experimental evolution. Fitness values are means and error bars represent standard error of the mean. Values are significant by two-tailed independent *t*-test (d.f. = 5, $\alpha = 0.05$) where indicated (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$). *P*-paired values correspond to two-tailed paired *t*-tests (d.f. = 5, $\alpha = 0.05$) between relative fitness values for isolates from generation 250 and 500.

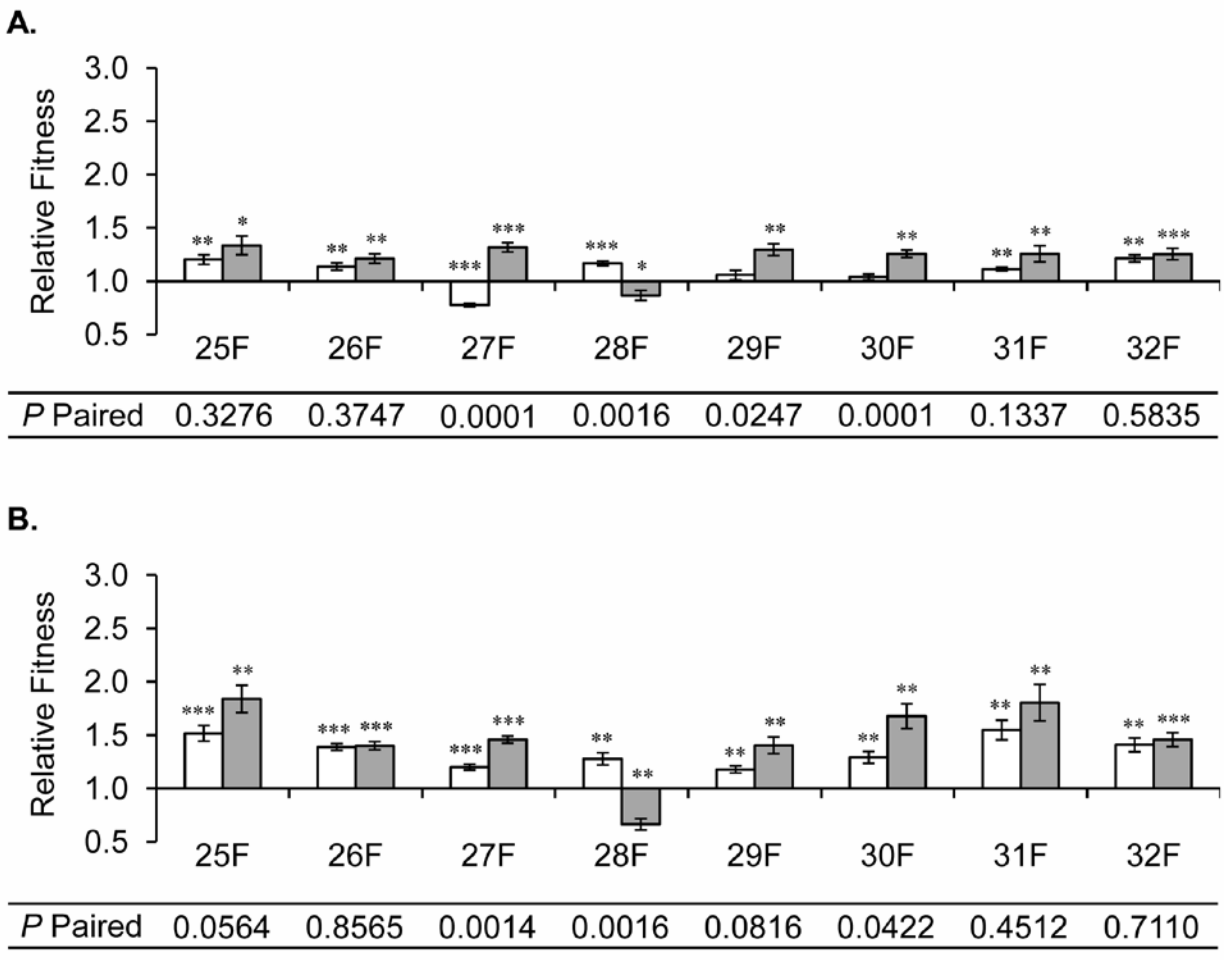


Figure 3-6. Relative fitness of isolates from non-UVR lineages 33-40. Relative fitness was measured by direct competition with the ancestor under (A) non-UVR and (B) UVR conditions. Non-UVR lineages contained only colonies with the 'round' morphology and single isolates of *P. cichorii* 302959 were taken after 250 (open bars) and 500 (shaded bars) generations of experimental evolution. Fitness values are means and error bars represent standard error of the mean. Values are significant by two-tailed independent *t*-test (d.f. = 5, $\alpha = 0.05$) where indicated (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$). *P*-paired values correspond to two-tailed paired *t*-tests (d.f. = 5, $\alpha = 0.05$) between relative fitness values for isolates from generation 250 and 500.

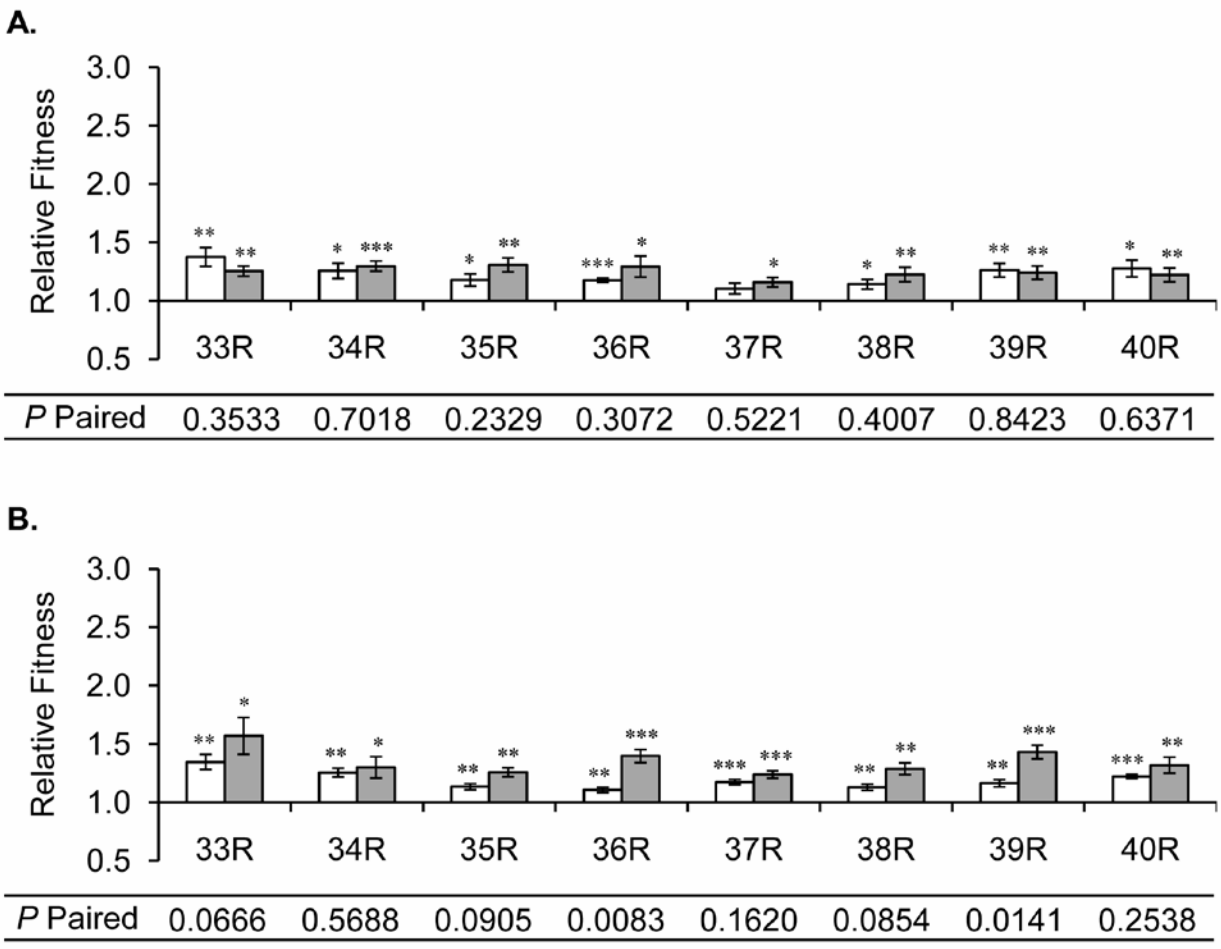


Figure 3-7. Average percent reduction in doubling time. Doubling time of UVR lineage population samples, UVR lineage isolates, and non-UVR lineage isolates after (A) 250 and (B) 500 generations of experimental evolution was measured under non-UVR (open bars) and UVR (shaded bars) conditions. Values represent the average percent improvement compared to the *P. cichorii* 302959 ancestor doubling times of 1.45 ± 0.09 hrs and 1.68 ± 0.10 hrs under non-UVR and UVR conditions, respectively. Error bars represent standard error of the mean. n = 8 for UVR lineage population samples and non-UVR lineage isolates but n = 16 for UVR lineage isolates. Values are significant by two-tailed independent *t*-test (α = 0.05) where indicated (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$). *P*-paired values correspond to two-tailed paired *t*-tests (α = 0.05) between non-UVR and UVR conditions.

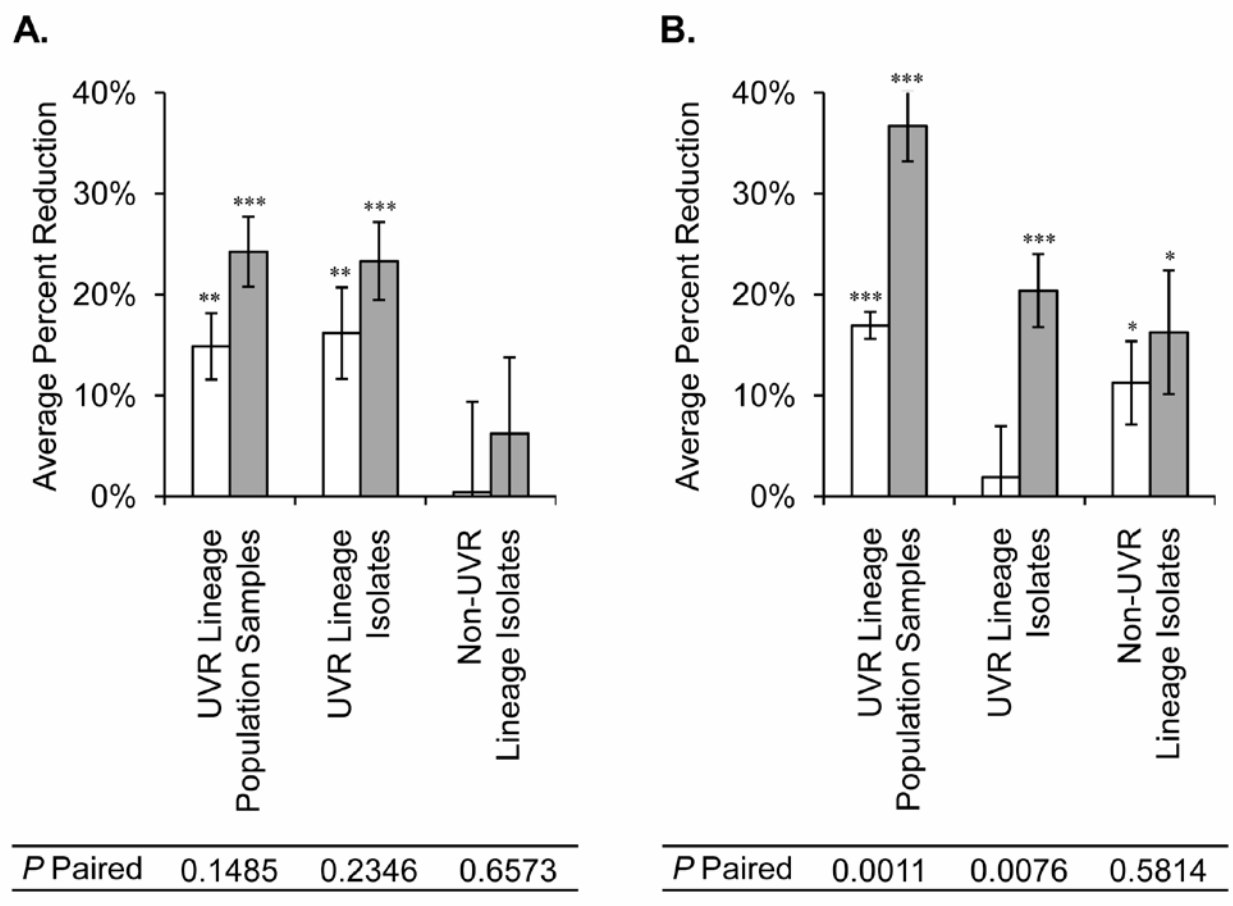


Figure 3-8. Average percent reduction in lag time. Lag time of UVR lineage population samples, UVR lineage isolates, and non-UVR lineage isolates after (A) 250 and (B) 500 generations of experimental evolution was measured under non-UVR (open bars) and UVR (shaded bars) conditions. Values represent the average percent improvement compared to the *P. cichorii* 302959 ancestor doubling times of 10.27 ± 0.32 hrs and 10.47 ± 0.26 hrs under non-UVR and UVR conditions, respectively. Error bars represent standard error of the mean. n = 8 for UVR lineage population samples and non-UVR lineage isolates but n = 16 for UVR lineage isolates. Values are significant by two-tailed independent *t*-test ($\alpha = 0.05$) where indicated (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$). *P*-paired values correspond to two-tailed paired *t*-tests ($\alpha = 0.05$) between non-UVR and UVR conditions.

Figure 3-8 (cont'd).

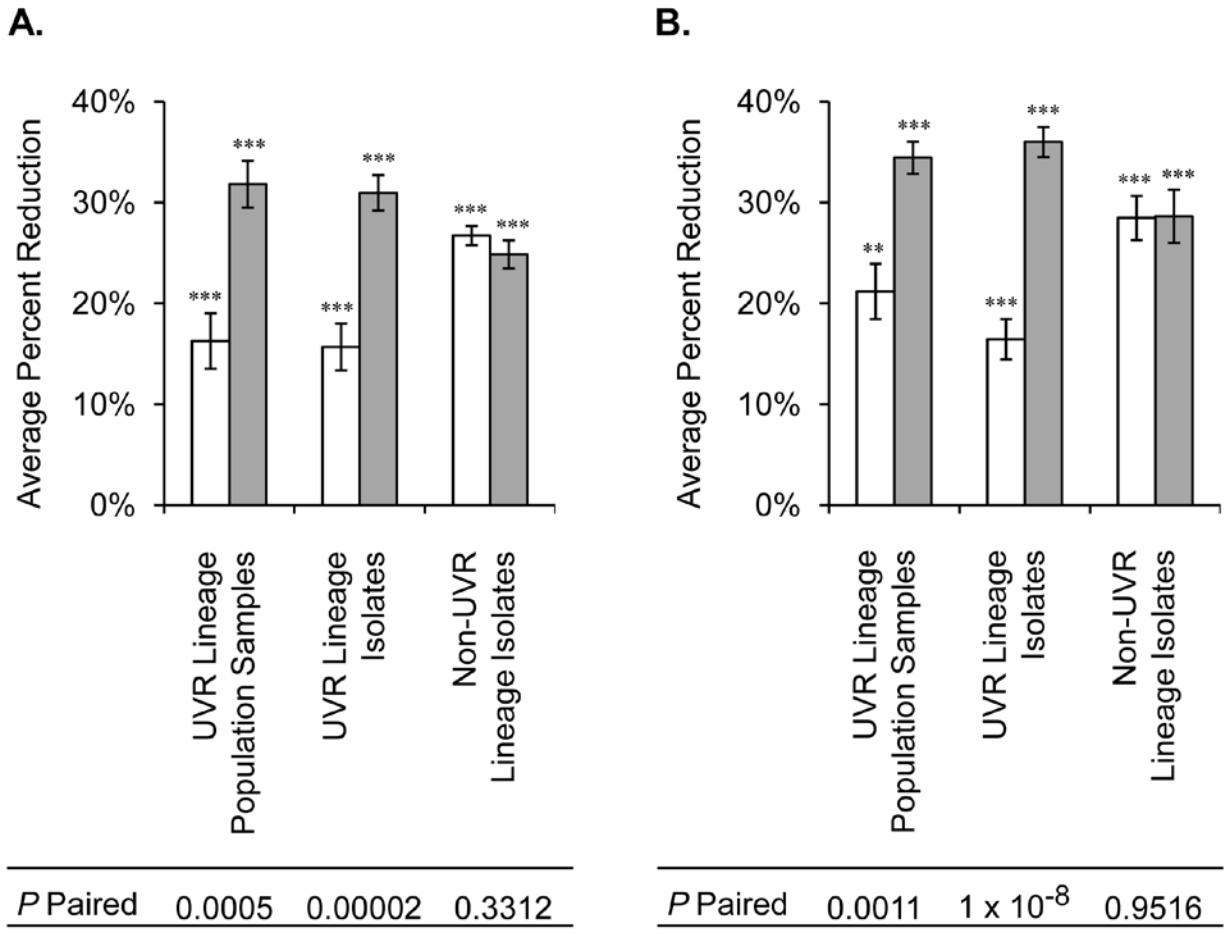
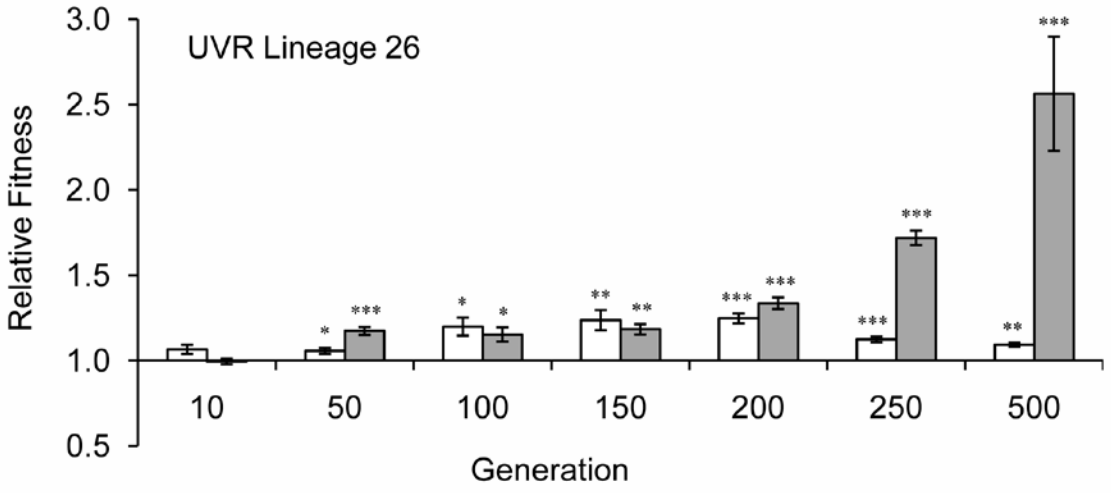


Figure 3-9. Relative fitness trajectories of population samples from select lineages of *P. cichorii* 302959. Relative fitness of population samples containing ~10^8 cells from UVR lineages (A) 26 and (B) 30 and non-UVR lineages (C) 26 and (D) 39 under non-UVR (open bars) and UVR (shaded bars) conditions was measured by direct competition with the ancestor. Fitness values are means and error bars represent standard error of the mean. Values are significant by two-tailed independent *t*-test (d.f. = 5, $\alpha = 0.05$) where indicated (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$). *P*-paired values correspond to two-tailed paired *t*-tests (d.f. = 5, $\alpha = 0.05$) between non-UVR and UVR relative fitness values.

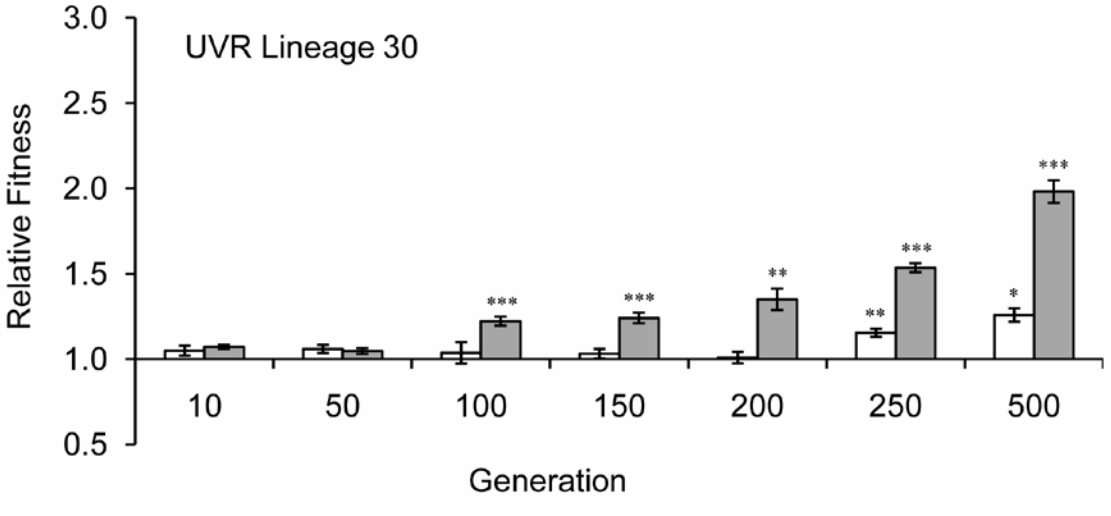
Figure 3-9 (cont'd).

A.



P Paired	0.1077	0.0033	0.4979	0.5024	0.1956	0.0001	0.0068

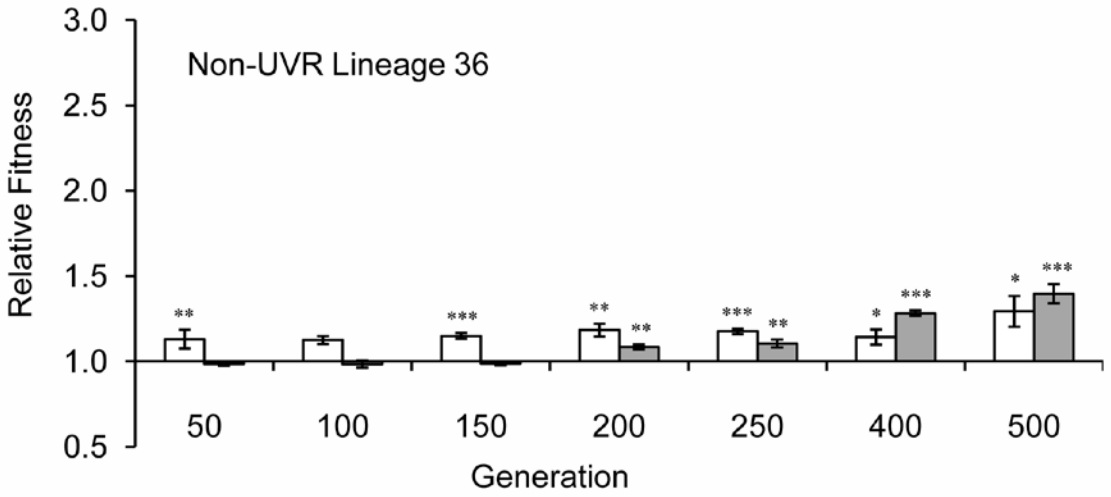
B.



P Paired	0.4633	0.4681	0.0363	0.0004	0.0014	0.0001	0.0007

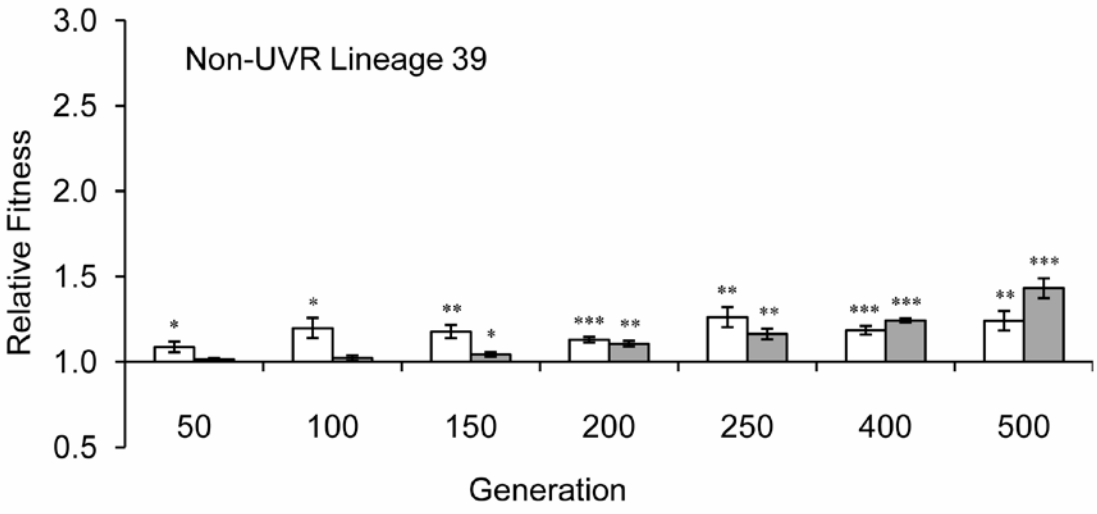
Figure 3-9 (cont'd).

C.



P Paired	0.0720	0.0070	0.0003	0.0559	0.0288	0.0404	0.1843

D.



P Paired	0.1019	0.0272	0.0136	0.4431	0.0508	0.1413	0.0033

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CHAPTER 4: Point mutation bias of *mutS*-deficient and *rulAB*-mediated hypermutation does not restrict adaptation in *Pseudomonas aeruginosa* PAO1

ABSTRACT

Hypermutators (or mutators) have been observed in a wide variety of clinical, environmental, and laboratory populations suggesting that evolutionary strategies of bacteria include mechanisms for increasing mutability. The opportunistic pathogen *Pseudomonas aeruginosa* colonizes the lungs of patients with cystic fibrosis, and DNA repair-deficient hypermutation facilitates chronic respiratory infection. Similarly, the Y-family DNA polymerase *rulAB* transiently increases mutability under stressful conditions as part of the SOS response and contributes to survival of plant-pathogenic bacteria. I hypothesized that the mechanistic differences between *mutS*-deficient (general) and *rulAB*-mediated (inducible) hypermutation likely produce unique mutation spectra and therefore unequal access to beneficial mutations. General and inducible mutators favored divergent spectra of single base mutations within a gene sequence to achieve antibiotic resistance. However, derived general and inducible mutator populations exhibited comparable fitness phenotypes following experimental evolution. Our results suggest that the different mechanisms of *mutS*-deficient and *rulAB*-mediated mutability are reflected in unique nucleotide substitutions that may not restrict access to adaptive phenotypes.

INTRODUCTION

A mutation rate optimized to ensure the long-term survival of bacterial populations should be sufficiently high to permit adaptation through sequence variation. However, simply raising the mutation supply does not necessarily accelerate adaptation. The conservation of multiple DNA error-avoidance and error-repair processes suggests a bias towards lower mutation rates to avoid the primarily deleterious nature of mutation (Sniegowski *et al.* 2000, De Visser 2002, De Visser and Rozen 2005). Yet hypermutator (or mutator) strains of bacteria have been observed in a variety of clinical, environmental, and laboratory populations with defects in DNA proofreading and repair machinery that exhibit spontaneous mutation rates 100-1000 times greater than wild-type (De Visser 2002).

The most commonly affected genes responsible for hypermutability are those belonging to the methyl-directed mismatch repair (MMR) system, and the inactivation of *mutS* is the most widespread defect in both environmental and clinical isolates (Hall and Henderson-Begg 2006). Within clinical populations of bacterial pathogens, mutator strains with defects in MMR can account for up to 57% of the total population (Sundin and Weigand 2007). MMR-defective mutator strains have also arisen during laboratory evolution experiments with *Escherichia coli* (Sniegowski *et al*. 1997, Notley-McRobb *et al*. 2002, Shaver *et al*. 2002). The abundance of mutator genotypes within natural populations likely results from hitchhiking with secondary, beneficial mutations that counterbalance the cost of accumulating deleterious mutations (Giraud *et al.* 2001, Tenaillon *et al*. 2001).

The opportunistic pathogen *Pseudomonas aeruginosa* colonizes the airways of patients with cystic fibrosis (CF) and chronic infection is the primary cause of morbidity and mortality in effected individuals. The presence of MMR-deficient strains of *P. aeruginosa* in chronic CF

infections represents the best studied example of the abundance and importance of hypermutators within a natural population (Oliver *et al.* 2000, Mena *et al*. 2008). A strong link between hypermutability and the emergence of multidrug resistance in CF populations of *P. aeruginosa* has been observed and presents therapeutic challenges (Blázquez 2003, Chopra *et al*. 2003, Cirz and Romesberg 2007, Henrichfreise *et al.* 2007). There is also growing evidence to support the role of hypermutation in facilitating adaptation of *P. aeruginosa* to the CF lung during chronic infection (Ciofu *et al*. 2005, Smith *et al.* 2006, Hogardt *et al*. 2007, Moyano *et al*. 2007, Mena *et al*. 2008, Ciofu *et al*. 2010, Oliver and Mena 2010).

In addition to MMR-deficient hypermutation, inducible mutability can transiently increase mutation rates in response to specific stress conditions. One such system, mutagenic DNA repair (MDR), is executed by specialized low-fidelity DNA polymerases following ultraviolet radiation (UVR) exposure as part of the SOS response (Schlacker and Goodman 2007, Courcelle *et al*. 2001). The Y-family DNA polymerases polIV (*E. coli* DinB) and polV (*E. coli* UmuDC) permit synthesis across damaged regions of DNA in a template-independent manner (Jarosz *et al*. 2007). Secondary mutations caused by these error-prone polymerases comprise the majority of sequence alterations derived from UVR exposure. Y-family polymerases also confer UVR tolerance and are, therefore, critical components of epiphytic growth in plant pathogenic bacteria harboring the *umuDC*-homolog *rulAB* that reside in habitats optimized for solar UVR exposure (Kim and Sundin 2000, Sundin and Murillo 1999).

Hypermutation has been observed in a wide variety of clinical, environmental, and laboratory populations suggesting that evolutionary strategies of bacteria include mechanisms for increasing mutation rate. Fortunately, bacteria are well suited for the study of adaptation through experimental evolution (Elena and Lenski 2003) and have aided in the observation of

fundamental evolutionary processes (Lenski *et al*. 1991). Observable phenotypes arising through adaptation ultimately stem from changes in DNA sequence and current sequencing technology has the power to reveal the complete genetic basis of adaption following experimental evolution (Herring *et al*. 2006, Hegreness and Kishony 2007, Conrad *et al*. 2009). Furthermore, the contribution of each genetic change can be evaluated (Applebee *et al*. 2008) and their dynamics within the population can be tracked over time (Barrick *et al*. 2009) to provide a clear, genetic view of evolution.

Although different hypermutable systems may increase mutation rate by the same magnitude, their influence on the genome is likely not equal. I hypothesize that the mechanistic differences between *mutS*-deficient (general) and *rulAB*-mediated (inducible) hypermutability likely produce unique mutation spectra and therefore unequal access to beneficial mutations. In this study, I have serially propagated parallel lineages of the model organism *P. aeruginosa* PAO1 for ~500 generations to compare the influence of general and inducible hypermutation on adaptation. I have observed comparable relative fitness improvements in general and inducible mutator lineages despite divergent point mutation spectra. Our results suggest that the different mechanisms of *mutS*-deficient and *rulAB*-mediated mutability are reflected in unique nucleotide substitutions but such divergence may not restrict access to adaptive phenotypes.

MATERIALS and METHODS

Bacterial strains, growth conditions, and general molecular biology techniques

The bacterial strains and plasmids used in this study are described in Table 4-1. Strains of *P. aeruginosa* PAO1 were obtained from the two-allele library at the University of Washington (Jacobs *et al*. 2003). The MMR-deficient strain PW7148 (*mutS*-C10::IS*lacZ*/hah) is

a general mutator with a spontaneous mutation frequency to rifampicin resistance (Rif^R) of 3.2×10^{-6}. Wild-type PAO1 was transformed by electroporation with the *rulAB*-encoding construct pJJK25 (Kim and Sundin 2000) to generate a UVR-inducible mutator because the PAO1 genome lacks a *umuDC* homolog (Simonson *et al*. 1990). Plasmid-encoded homologs in native isolates of *P. aeruginosa* have been reported (Stokes and Krishnapillai 1978, Jacoby *et al*. 1983, Simonson *et al*. 1990) and *rulAB* confers UVR-inducible mutability to PAO1 when introduced on a plasmid (Kim and Sundin 2000). All evolved bacterial populations in this study were derived from these three ancestral genotypes following ~500 generations of selection in the lineage experiment described below (Figure 4-1). Each lineage contained ~10^{10} total cells at stationary phase. These communities were analyzed in this study as population samples containing ~10^8 cells thawed from preserved freezer stocks to maintain the assemblage of diverse genotypes. Sympatric individuals were taken as multiple single-colony isolates from a given lineage spread on agar plates.

Strains of *P. aeruginosa* were cultured at 37° and *P. syringae* pv. *syringae* B86-17 was cultured at 28°, all in Luria-Burtani (LB) broth (Difco, Detroit, MI), in Davis Minimal broth (Difco) supplemented with 25 mg l^{-1} glucose (DM25), or on King's medium B (KB) agar (King *et al*. 1954). Antibiotics were used where appropriate at the following concentrations: carbenicillin 50 µg ml^{-1}, rifampicin 250 µg ml^{-1}. Competent cell preparation was performed according to Choi *et al.* (2006). Standard agarose gel electrophoresis and other recombinant DNA technologies were performed according to Sambrook *et al.* (1989).

Experimental evolution

Eighteen parallel lineages of *P. aeruginosa* were serially propagated for ~500 generations in a manner similar to another study conducted previously (Weigand and Sundin 2009). Cultures were maintained in 10 ml DM25 in 50-ml Erlenmeyer flasks incubated at 37° and 150 rpm in the dark. These culture conditions resulted in a stationary phase density of ~9 x 10^8 colony-forming units per ml. Every 24 hr, inducible mutator lineages of PAO1/pJJK25 (numbered 41-46) were individually mixed 1:1 with saline (0.85% NaCl) in a glass petri dish and exposed to ~75 J m^{-2} of UVC (254 nm) radiation from an XX-15 UV lamp (UVP Products, San Gabriel, CA) placed horizontally at a fixed height above the cell suspension. The lamp was turned on 15 min prior to use to allow for stabilization of the UV output. The energy output of the lamp was monitored with a UV-X radiometer fitted with a UV-25 sensor (UVP Products) and determined to be 1.5 J m^{-2} s^{-1}. The UVR dosage delivered to lineages of PAO1/pJJK25 was optimized to closely match the rate of Rif^R mutants generated by PW7148. Following UVR irradiation, cultures were diluted 1:100 into fresh media and incubated under dark conditions to minimize photoreactivation. General mutator lineages of PW7148 (numbered 47-52) and non-mutator lineages of PAO1 (numbered 53-58) were diluted 1:10 in saline and then 1:100 into fresh DM25 broth without UVR exposure. Both transfer strategies resulted in 1000-fold daily growth of each population, representing ~10 generations of binary fission. Population samples from each lineage were preserved in a nonevolving state in 10% glycerol at -80° at regular intervals for later analysis. Lineages were spread on KB agar every 10 generations and on KB+Rif agar every 50 generations to monitor population structure and possible contamination.

Following experimental evolution, strain identity was confirmed by PCR amplification and fragment sequencing of multiple random colonies from each lineage. Primers were designed based on the published *P. aeruginosa* PAO1 genome sequence (Stover *et al.* 2000) for amplification of genes PA3620 (*mutS*, DNA mismatch repair) and PA3617 (*recA*, SOS recombinase). Universal *Pseudomonas* primers based on a consensus of *P. aeruginosa, P. cichorii, P. fluorescens, P. putida,* and *P. syringae* gene sequences were used for amplification of the 16S rDNA (small ribosomal RNA subunit) gene. Primer sequences are listed in Table 4-1. PCR amplifications were performed in a 20-µl reaction volume containing 1x PCR buffer, 2 mM $MgCl_2$, 125 µM of each deoxynucleoside triphosphate (Invitrogen, Carlsbad, CA), 0.4 µM of each primer, 1.0 U Taq polymerase (Invitrogen), and 40 ng genomic DNA. PCR products were purified using the QIAquick PCR Purification Kit (QIAGEN, Valencia, CA), and the purified fragments were sequenced at the Research Technology Support Facility at Michigan State University.

DNA sequence analysis of Rif^R mutants

The frequency and location of spontaneous Rif^R mutations was determined from 10 independent cultures each of PAO1/pJJK25 and PW7148 and four independent cultures of B86-17. Cultures of PAO1/pJJK25 and B86-17 were grown overnight in LB broth and 2 ml of culture were pelleted, washed with 1 ml saline, and resuspended in 1 ml saline on ice. The cell suspensions were mixed with 9 ml saline in a glass petri dish and exposed to UVC radiation as described above to activate mutagenic DNA repair. Following irradiation, 1 ml of the cell suspension was mixed with 2× LB broth and incubated overnight under dark conditions.

Cultures of PW7148 were simply grown over night in LB broth to generate mutants. In both cases, resulting cultures were then spread on KB+Rif agar and incubated for 48 hrs. Rif^R colonies were picked up with sterile toothpicks, suspended in 50 µl H_2O, and cells were lysed by boiling for 10 mins. To identify responsible Rif^R mutations, the region of *rpoB* (β–subunit of RNA polymerase) corresponding to Rif clusters I, II, and III (Jatsenko *et al.* 2010) was amplified by PCR. Fragments were amplified from strains of *P. aeruginosa* using primers PAO1rpoB1 and PAO1rpoB3 and from B86-17 using primers PAO1rpoB7 and PAO1rpoB4 listed in Table 4-1. PCR products were purified using the QIAquick PCR Purification Kit (QIAGEN) and then sequenced at the Research Technology Support Facility at Michigan State University. Sequences were compared using Lasergene SeqMan Pro (DNASTAR, Madison, WI) for mutation identification.

Relative fitness assays

The relative fitness of population samples from each evolved lineage was determined by direct competition with the respective ancestor under both UVR and non-UVR conditions as described previously (Weigand and Sundin 2009). Briefly, competitions were performed under the same UVR and non-UVR conditions described above. To ensure that competitors were comparably acclimated to the competition environment, strains were simultaneously removed from glycerol stocks maintained at -80°, individually grown in LB broth overnight, and then individually grown for 24 hr in the competition environment. Competitors were mixed at a 1:1 volumetric ratio, and appropriate dilutions were spread on KB agar in triplicate at 0 and 24 hr to estimate the initial and final density of each. Strain differentiation was accomplished by

plasmid-encoded catechol 2,3-dioxygenase (*xylE*) on pMRW2 which causes expressing colonies to turn yellow when sprayed with 0.1 M catechol on agar media (Weigand and Sundin 2009).

The relative fitness (*W*) of the two competitors was calculated as the ratio of their realized growth rate as described previously (Weigand and Sundin 2009). When the two competitors are equally fit in the competition environment, $W = 1$. The six replicates of each relative fitness measurement were analyzed by two-tail, independent *t*-tests against the hypothesis of mean equal fitness ($W = 1$). Fitness measurements were compared using two-tailed paired *t*-tests to determine any significant differences between growth conditions.

Draft genome sequence

Genomic DNA was extracted from a single isolate of inducible mutator lineage 44 using the DNeasy Blood and Tissue Kit (QIAGEN) and RNA was degraded with 400 µg RNase A in 10 mM Tris-HCl (pH = 8.0). A prepared shotgun library was sequenced using the GS-FLX Titanium platform (454 Life Sciences, Branford, CT). The resulting reads were both mapped directly to the PAO1 reference genome (AE004091) with GS Reference Mapper software (454 Life Sciences) and assembled with GS De Novo Assembler v2.5.3 (454 Life Sciences) at the Research Technology Support Facility at Michigan State University. The physical distribution of identified variations was visualized with the CGView Server (Grant and Stothard 2008).

RESULTS

Frequency of Rif^R mutants

All lineages were spread on KB+Rif agar during propagation to monitor the frequency of Rif^R mutants in each population. The average frequency of inducible mutator, general mutator,

and non-mutator lineages is plotted in Figure 4-2. The average frequency of Rif^R mutants in inducible and general mutator lineages fluctuated slightly but largely remained stable throughout the course of lineage growth. Although the mutability rates of PAO1/pJJK25 (1.7×10^2) and PW7148 (3.2×10^2) were standardized at the beginning of the evolution experiment, inducible mutator lineages maintained a 10-fold greater average frequency of Rif^R mutants. In non-mutator lineages, the frequency of Rif^R mutants was effectively zero and only a few resistant colonies were ever recovered.

DNA mutations in Rif^R mutants

The distribution and frequency of mutations in Rif^R colonies of the ancestral PAO1/pJJK25 and PW7148 strains were compared to identify differences in point mutation bias between *rulAB*-mediated and *mutS*-deficient hypermutation. Mutations responsible for Rif^R in bacteria map almost exclusively to *rpoB* and produce amino acid substitutions in three primary regions of the β subunit of RNA polymerase (Garibyan *et al.* 2003). These regions, termed clusters I-III, are highly conserved among bacteria (Campbell *et al.* 2001) and were recently well characterized in *P. aeruginosa* by Jatsenko *et al.* (2010). The region of *rpoB* containing clusters I-III from Rif^R mutants of PAO1/pJJK25 and PW7148 was investigated by sequencing PCR products. In total, I observed point mutations in Rif^R mutants at 16 unique nucleotide positions in *rpoB* clusters I and II. The *rpoB* sequence from PAO1 is illustrated in Figure 4-3 with the observed mutations in PW7148 and POA1/pJJK25 indicated above and below, respectively. For

comparison, the same region of *rpoB* in Rif^R mutants of B86-17 was also analyzed because this strain harbors an indigenous *rulAB*-encoding plasmid. There are only 10 nucleotide differences between the PAO1 and B86-17 sequences in clusters I and II, none of which produces a difference in amino acid sequence. None of the mutations in *rpoB* observed in Rif^R mutants of B86-17 were located at any of these unique nucleotide positions and many were identical to alterations observed in *P. aeruginosa*.

The frequency of each single nucleotide substitution and corresponding amino acid change observed in *rpoB* clusters I and II from Rif^R mutants in all replicate cultures are listed in Table 4-2. All sequenced *rpoB* fragments from Rif^R mutants of *P. aeruginosa* and *P. syringae* contained only one point mutation each. In *P. aeruginosa*, both *mutS*-deficient and *rulAB*-mediated hypermutation produced Rif^R mutants with single base substitutions at multiple positions. In general, more Rif^R mutants contained transitions than transversions in both PW7148 and PAO1/pJJK25. Although each mechanism favored mutation at a few specific sites, some overlap was observed. Almost 75% of Rif^R PW7148 mutants contained an A → G substitution at either position 1553, 1562, or 1592 (Table 4-2). However, in PAO1/pJJK25 only the point mutation at position 1553 was observed. Conversely, 16% of Rif^R PAO1/pJJK25 mutants had a substitution at position 1607 that was observed only rarely in PW7148. In *rpoB* sequences from Rif^R mutants of B86-17, similar mutation patterns were observed with the same preference for transitions over transversions. Mutations at position 1607 were prevalent in Rif^R

mutants of both PAO1/pJJK25 and B86-17 but the frequency distribution of base substitutions in the two strains was otherwise divergent despite their homologous hypermutation mechanisms.

Relative fitness of derived lineages

The relative fitness of each derived lineage was determined by direct competition with its respective ancestor (Figure 4-1) under both UVR and non-UVR conditions to examine adaptation during ~500 generations of experimental evolution. Many of the inducible and general mutator lineages diversified into mixtures of distinct colony morphologies and so I evaluated the relative fitness of lineage population samples comprising a subset of the entire lineage to maintain the assemblage of sympatric genotypes. All derived lineages of inducible mutators, general mutators, and non-mutators exhibited improvements in fitness under their native conditions relative to their respective ancestor (Figure 4-4).

Two inducible mutator lineages (44 and 46) displayed an exceptional 234% average improvement in relative fitness under their native, UVR conditions (all $P < 0.004$, Figure 4-4A). The remaining inducible mutator lineages exhibited a 51% average improvement in relative fitness under UVR conditions (all $P < 0.007$, Figure 4-4A). When assayed under non-UVR conditions, all inducible mutator lineages exhibited an 18% average improvement in fitness compared to PAO1/pJJK25 (all $P < 0.02$, Figure 4-4A). All inducible mutator lineages exhibited significantly higher fitness under UVR conditions compared to non-UVR conditions (Figure 4-4A).

General mutator lineages displayed a 19% average improvement in relative fitness under their native, non-UVR conditions (all $P < 0.002$, Figure 4-4B). Under UVR conditions, general mutator lineages displayed a 57% average improvement in fitness compared to PW7148 (all $P <$

0.002, Figure 4-4B). Furthermore, the relative fitness exhibited under the UVR conditions compared to non-UVR conditions was significantly greater in all general mutator lineages except lineage 51 (Figure 4-4B).

Non-mutator lineages displayed a 27% average improvement in relative fitness under non-UVR conditions (all $P < 0.008$, Figure 4-4C). When assayed under UVR conditions, only non-mutator lineage 54 exhibited an improvement in fitness relative to PAO1 ($P = 0.028$, Figure 4-4C). The relative fitness of non-mutator lineages was significantly greater under the non-UVR conditions in which they were propagated compared to UVR conditions in all but lineage 57 (Figure 4-4C).

Draft genome sequence of an inducible mutator isolate

The genome of a single isolate from inducible mutator lineage 44 was sequenced to determine the complete list of mutational differences relative to the published PAO1 reference sequence (AE004091, Stover *et al.* 2000). Inducible mutator lineage 44 was chosen because of its pronounced relative fitness under UVR conditions (Figure 4-4A). A single clone with similarly high fitness was selected from a group of randomly tested sympatric isolates exhibiting varied levels of relative fitness (data not shown). Sequencing of the shotgun library generated 383,834 total passed filter reads that, when mapped to the published PAO1 reference sequence, produced 26 contigs totaling 6,227,769 bp in length and covering 99.4% of the reference sequence. This strategy has identified 402 high confidence variations including single base substitutions, multiple base substitutions, and insertions or deletions. Twenty-two variations have been observed between the ancestral PAO1 strain used in this study and the published PAO1 reference sequence (Klockgether *et al*. 2010). All 22 of the ancestral variants were

present in the draft genome sequence leaving 380 new variations summarized in Table 4-3. Of these, 205 result in some form of amino acid substitution affecting over 200 unique open reading frames. Nonsense mutations were detected in 13 genes listed in Table 4-4. Genes with nonsynonmyous or frameshift mutations with annotated functions in DNA metabolism or motility and attachment are listed in Table 4-5.

The physical distribution of all 380 mutations mapped to the reference sequence is illustrated in Figure 4-5. This map highlighted three large regions devoid of mutation at positions 537,954 – 667,028 (129.1 kb), 3,198,266 – 3,328,641 (130.4 kb), and 4,942,047 – 5,033,101 (91.1 kb). Depth of sequencing coverage, gene density, and %GC in these regions was not significantly divergent from the rest of the genome.

DISCUSSION

The results of this study confirm the divergent point mutation spectra of *mutS*-deficient and *rulAB*-mediated hypermutation but suggest that these differences in variation at the nucleotide level may not restrict access to adaptive phenotypes. I maintained parallel lineages of general and inducible mutator genotypes of the model organism *P. aeruginosa* PAO1 in a serial transfer regime. The mutation spectra of antibiotic-resistant clones, relative fitness of derived populations, and the draft genome of an inducible mutator isolate were investigated to compare the mechanistic differences of *mutS*-deficient and *rulAB*-mediated hypermutation during adaptation.

During the course of propagation, I monitored the frequency of Rif^R mutants in each lineage to observe population structure dynamics through fluctuations in a readily measurable phenotype associated with a known genetic determinant. The 10-fold, stable difference in the

average frequency of Rif^R mutants between general and inducible mutator lineages suggests that that *mutS*-deficient and *rulAB*-mediated mutability are not equal at the *rpoB* locus. The UVR dose admitted daily to lineages of PAO1/pJJK25 was designed to produce approximately the same number of Rif^R mutants as PW7148 so that any dissimilarity between general and inducible mutator lineages could be linked to mechanistic differences in hypermutation. This normalization corresponded to a single cycle of the serial transfer regime and, after five cycles, (~50 generations) general and inducible mutator lineages had already established divergent frequencies in Rif^R mutants. I expect that rates of Rif^R mutability remained constant throughout lineage propagation such that the disparity in Rif^R mutant frequency does not reflect divergent supply rates.

In both general and inducible mutator lineages, Rif^R mutants did not accumulate over time, but rather the average frequency of Rif^R mutants remained low and fairly constant. This stability in Rif^R mutant frequency could indicate that mutations in *rpoB* come at a fitness cost in the absence of rifampicin preventing them from increasing in frequency. Indeed, some Rif^R mutations have been shown to reduce RNA polymerase function, and Rif^R isolates of *E. coli* and *P. aeruginosa* with mutations in *rpoB* exhibit varied defects in relative fitness when grown in the absence of rifampicin (Reynolds 2000, MacLean and Buckling 2009, Barrick *et al*. 2010, Hall *et al*. 2011). The mutagenic activities of PW7148 and PAO1/pJJK25 may have generated a constant supply of new Rif^R mutants from the predominantly sensitive population while the fitness costs associated with mutation in *rpoB* continually remove them from the population. A

balance between the supply of new Rif^R mutations and the loss of mutants with fitness defects could produce the stable average frequency of Rif^R mutants observed in both general and inducible mutator lineages. Mutations in *rpoB* can also be neutral or only weakly deleterious and even contribute to adaptation by increasing evolvability (Barrick *et al*. 2010, Hall *et al.* 2011). However, because the frequency of Rif^R mutants remained very low in all mutator lineages, I suspect that mutations in *rpoB* derived from *mutS*-deficient and *rulAB*-mediated hypermutation are at least weakly deleterious.

The disparity in Rif^R mutant frequency could result from the biased mutation of *rpoB* imposed by the unique mechanisms of *mutS*-deficient and *rulAB*-mediated hypermutation. The molecular characterization of mutations responsible for resistance to rifampicin in *P. aeruginosa* identified 38 possible single base substitutions spread over 26 nucleotide positions in *rpoB* (Jatsenko *et al*. 2010). Given the diverse mutational possibilities available to confer resistance, it is unlikely that any bias exists towards the point mutation spectra of general or inducible hypermutation. However, not all point mutations in *rpoB* are equal with respect to their pleiotropic fitness costs due to defects in RNA polymerase function (Barrick *et al*. 2010, Hall *et al*. 2011). I investigated the potential for bias in *rpoB* mutation in PW7148 and PAO1/pJJK25 by sequencing clusters I-III in Rif^R colonies. It is clear from the frequency distribution of point mutations observed in Rif^R mutants that *mutS*-deficient and *rulAB*-mediated hypermutation favor different nucleotide substitution at the *rpoB* locus. The point mutation spectra of PW7148 may favor *rpoB* mutations that carry greater fitness defects and thus lead to a lower standing frequency of Rif^R mutants in general mutator lineages compared to inducible mutator lineages.

The function of RpoB is essential for cell survival and therefore the *rpoB* gene can only tolerate limited sequence variation. Although diverse nucleotide substitutions can confer resistance, measurement of Rif^R as a means of evaluating hypermutation ignores insertions, deletions, and other sequence polymorphisms that would eliminate RNA polymerase function. Therefore, Rif^R estimates do not represent a comprehensive assessment of how hypermutation alters the genome. Yet the frequency of Rif^R mutants is widely used to classify hypermutator phenotypes (Oliver *et al.* 2000). Both *mutS*-deficient and *rulAB*-mediated hypermutation can produce frameshift mutations and *mutS* deletion also leads to a hyper-recombinogenic phenotype (Schaaper and Dunn 1987, Smania *et al*. 2004). None of these mutations are detectable by measuring the appearance of Rif^R mutants. I suspect that *mutS*-deficient and *rulAB*-mediated hypermutation produce varied mutation spectra with respect to all forms of DNA polymorphisms. Indeed, the draft genome sequence of an inducible mutator isolate identified 34 insertions and deletions. Therefore, given the differences in Rif^R mutant frequency and distribution of *rpoB* sequence alterations, I can only conclude that PW7148 and PAO1/pJJK25 differ in their production of single base substitutions. Furthermore, the distribution of point mutations in Rif^R mutants of PAO1/pJJK25 differed from mutants of B86-17 which carries an endogenous *rulAB*-encoding plasmid. This dissimilarity suggests that among inducible mutators, host genetic background can influence *rulAB* hypermutation, consistent with the observation of varied levels of mutability in plant-associated Pseudomonas species harboring *rulAB* (Sundin *et al*. 2000).

To compare the influence of *mutS*-deficient and *rulAB*-mediated hypermutation on adaptation I measured changes in fitness relative to each lineage's respective ancestor. Inducible mutator lineages exhibited increased fitness relative to the PAO1/pJJK25 ancestor after ~500 generations of growth with daily induction of mutagenic DNA repair. Inducible mutator lineages favored the native, UVR conditions under which they were propagated and exhibited significantly higher relative fitness under UVR conditions compared to non-UVR conditions. These results indicate that inducible mutator populations acquired a combination of adaptive improvements specific to growth under UVR conditions and general improvements that also contribute to fitness under non-UVR conditions. Larger increases in fitness under UVR conditions may signify lower initial fitness under these conditions such that more adaptive opportunities existed for improvement. Both the magnitude and pattern of relative fitness changes exhibited by inducible mutator populations of *P. aeruginosa* are consistent with a similar experimental evolution study conducted previously with *P. cichorii* 302959 (Weigand and Sundin 2009). The exceptional fitness gains exhibited by lineages 44 and 46 under UVR conditions in this study may reflect additional adaptive improvements not yet realized by the other inducible mutator lineages.

General mutator and non-mutator lineages both gained similar levels of fitness compared to their respective ancestors under their native, non-UVR conditions. The adaptation of non-mutator lineages was specific to the non-UVR conditions and no improvements in fitness under UVR conditions were observed. These results differ from a similar study with *P. cichorii* 302959 in which lineages grown in the absence of UVR acquired general adaptive improvements that were also beneficial under UVR conditions (Weigand and Sundin 2009, Weigand *et al*. 2011). Conversely, lineages of general mutators exhibited improved fitness under UVR

conditions that were comparable to that of inducible mutator lineages despite an absence of previous exposure to UVR.

The genome of PAO1, and consequently PW7148, lacks a *umuDC* homolog (Simonson *et al.* 1990) and so I added a *rulAB*-encoding plasmid to confer UVR-inducible mutability. Addition of *rulAB* to PAO1 also increases UVR tolerance (Kim and Sundin 2000), and PAO1/pJJK25 exhibits considerably higher UVR tolerance than PW7148. In microorganisms, UVR tolerance is linked to direct and indirect mechanisms of DNA maintenance (Jacobs *et al.* 2005). General mutator lineages may have acquired improvements in DNA repair in response to *mutS* inactivation to mitigate the effects of an increased mutation rate and ensure genome stability. Such mutations would be compensatory under their native, non-UVR conditions, but could carry the added benefit of improving relative fitness following UVR exposure by better preparing cells to handle additional DNA damage.

To elucidate the genetic determinants underlying improved fitness under UVR conditions, I sequenced the genome of an isolate from inducible mutator lineage 44, chosen because of its extraordinary fitness phenotype. Of the 380 sequence variations identified, most were single base substitutions (85%) and the frequency distribution of transitions versus transversions was nearly identical to that observed in *rpoB* sequences from Rif^R mutants of PAO1/pJJK25. Both also indicated a high preference for C·G → T·A transitions in *rulAB*-mediated hypermutation. These results confirm that Rif^R frequency serves as a reasonable estimate for single base substitution rates throughout the genome (Garibyan *et al*. 2003). By extension, I predict that the *rpoB* mutation frequency distribution in PW7148 is also representative of single base substitutions resulting from *mutS*-deficiency.

The mutations identified in the draft genome sequence of an inducible mutator isolate were distributed rather evenly throughout the chromosome of *P. aeruginosa* PAO1. However, mutations were particularly dense near the origin of replication and more dispersed elsewhere. Variations were absent in three particularly large regions approximately 91-130 kb in length. I have not been able to identify any obvious characteristics about these three regions that would preclude them from mutation based on their sequence. A self blastn alignment of the PAO1 reference sequence indicated that these three regions contained only a few genes from well represented families, but other similar regions of the genome exist and bear mutation. High depth of sequencing and aligned blastn hits from *de novo* assembled contigs confirm that these three regions have not been deleted or rearranged. I have not thoroughly investigated what genome sequence or structural features may protect these regions from mutation.

The presence of nonsynonmyous mutations provides strong evidence for natural selection (Nielsen 2005). The open reading frames with amino acid sequence variation identified in the draft genome sequence included genes with annotated functions in energy production, amino acid biosynthesis, central metabolism, transcription, translation, and membrane transport, among others. Some of these likely represent adaptive improvements to the culture media that confer improved fitness under both UVR and non-UVR conditions. Others likely correspond to genes unused and under relaxed selection in the lineage environment. Those mutated genes with annotated functions in DNA processing or metabolism could be good candidates for improving fitness specifically under UVR conditions. Among these, *dnaE2* (PA0669) encodes a second copy of the DNA pol III α-subunit and shows the most promise.

The absence of *umuDC*-homologs in genomes from disparate bacterial species correlates with the presence of an *imuA-imuB-dnaE2* 'mutagenesis cassette' (PA0671 – PA0669) that likely

performs mutagenic DNA repair in the absence of DNA PolV (Erill *et al*. 2006). In *Mycobacterium tuberculosis* and *Caulobacter crescentus*, DnaE2 homologs act like error-prone DNA polymerases and confer an inducible mutator phenotype following UVR exposure (Boshoff *et al*. 2003, Galhardo *et al*. 2005). Specialization of DnaE2 may result from subtle structure variations that distinguish it from DnaE1, restricting interaction with other subunits of the PolIII holoenzyme to prevent incorporation during normal DNA replication (Warner *et al*. 2010). Although structurally similar to Y-family polymerases, ImuB lacks the necessary active-site residues for polymerase activity but rather likely coordinates with ImuA to mediate DnaE2 access to the replication fork for mutagenic lesion bypass (Warner *et al*. 2010). Alternatively, in *Pseudomonas putida*, DnaE2 displays antimutator activity during extended stationary phase growth suggesting that *imuA-imuB-dnaE2* module activity may be controlled by other factors (Koorits *et al*. 2007, Kivisaar 2010).

In *P. aeruginosa*, genetic studies have identified *dnaE2* as part of a putative SOS regulon under LexA repression (Cirz *et al.* 2006) and required for DinB-mediated UVR mutagenesis (Sanders *et al.* 2006). However, in preliminary experiments I did not detect UVR-induced hypermutability in PAO1 and Rif^R mutants were only observed in strains transformed with *rulAB*-encoding plasmids (Weigand, unpublished). If DnaE2 is active in response to UVR exposure, its participation in damage-induced genome replication would make it integral to growth under UVR conditions. The presence of an amino acid substitution in *dnaE2* suggests altered function that may represent a beneficial mutation. I cannot predict how the added presence of *rulAB* may create competition among damage-repair polymerases for stalled replication forks or the individual contributions of *rulAB* and *dnaE2* to repair. However,

nonsynonmyous mutation in *dnaE2* represents a strong candidate for improving fitness under UVR conditions given its role in DNA damage repair and warrants future consideration.

Increased cell aggregation could also contribute to fitness under UVR conditions through protection from UVR exposure. The draft genome sequence of an inducible mutator isolate also contained nonsynonmyous mutations in a variety of genes with annotated functions in attachment, lipopolysaccharide biosynthesis, and motility. During propagation, each lineage was spread on KB agar at regular intervals and diversity in colony morphology was observed in all inducible and general mutator lineages. Such diversification is a common feature of adaptive evolution (MacLean 2005) and has been observed in other evolution experiments (Turner *et al*. 1996, Rainey and Travisano 1998, Weigand and Sundin 2009). Mutations at the *wspR-wspABCDEF* locus (PA3702-PA3708) have been shown to produce changes in colony morphology on agar media and autoaggregation in broth culture (D'Argenio *et al.* 2002). Similarly, the RocR/PvrR response regulator has been shown to modulate phenotypic switching, antibiotic resistence, and biofilm formation in *P. aeruginosa* PA14 (Drenkard and Ausubel 2002). The draft genome sequence presented here contained mutations in both *rocR* (PA3947) and *wspE* (PA3704).

In summary, I have compared the influence of general and inducible hypermutability on adaptation through experimental evolution with the model organism *P. aeruginosa* PAO1. By sequencing *rpoB* from Rif^R colonies, I have confirmed the divergent mutation spectra of single base substitutions derived from *mutS*-deficient and *rulAB*-mediated hypermutation. The spectra of point mutations present in *rpoB* closely matched that of a draft genome sequence of an inducible mutator isolate. I expect that the divergence in mutation spectra between general and inducible hypermutation at *rpoB* can be largely extended to include the whole genome. However,

general and inducible mutator lineages exhibited comparable improvements in fitness compared to their respective ancestors, including larger fitness gains under DNA damage-inducing, UVR conditions. These results suggest that differences in point mutation spectra provide unequal access to beneficial mutations at the nucleotide level but may not restrict access to adaptive phenotypes. Derived lineages of general and inducible mutators either acquired different adaptive solutions through mutation at unique genetic loci with similar fitness effects or analogous mutations at the same locus but through divergent sequence alterations. I was not able to differentiate these two possibilities within the scope of this study but it is noteworthy that neither mechanism appeared to restrict adaptation to the lineage environment relative to the other.

The draft genome sequence presented in this study has identified the presence of nonsynonmyous mutations in numerous genes of varied annotated function and only a few have been mentioned here. Mutation in *dnaE2* (PA0669), a second copy of the DNA pol III α-subunit, provides the most promise for identifying a beneficial mutation that contributes to relative fitness under UVR conditions. Further experiments are needed to elucidate to fitness effects of the *dnaE2* variant both in an ancestral *dnaE2*-deficient background and parallel derived lineages of general mutators.

Table 4-1. Strains, plasmids, and primers used in this study.

Strain, plasmid, or primer	Relevant characteristics or sequence	Reference
Strains		
PAO1	Wild-type, laboratory strain *P. aeruginosa*	Jacobs *et al.* 2003
PW7148	PAO1 *mutS*-C10:: IS*lacZ*/hah	Jacobs *et al.* 2003
B86-17	Wild-type *P. syringae* pv. *syringae*	Legard *et al.*1993
Plasmids		
pJJK25	*rulAB*, confers inducible hypermutability	Kim *et al.* 2000
pMRW2	*xylE*, strain differentiation during competition	Weigand *et al.* 2009
Primers		
Ps16S.F	5'- GAGCGGCGGACGGGTGAGTAATG - 3'	Weigand *et al.* 2009
Ps16S.R	5'- AGGTGATCCAGCCGCAGGTTCC - 3'	Weigand *et al.* 2009
PA3620a.F	5'- CTTCATCACGCCGGAACT - 3'	This study
PA3620a.R	5'- GTACATGGTGCAGGAACACG - 3'	This study
PA3617.F	5'- CGGTACGGTAGATGCCCTTA - 3'	This study
PA3617.R	5'- CAACCGTTCGGAACATTCTT - 3'	This study
PAOrpoB1	5'- TCATCGATGTGCTCAAGACC - 3'	Jatsenko *et al.* 2010
PAOrpoB3	5'- GACCGAGTCGATCACACC - 3'	This study
PAOrpoB7	5'- CCGTCGTATCGGTCGTACCGA - 3'	This study
PAOrpoB4	5'- GGACGCTGGTTGATGCAGGTG - 3'	This study

Table 4-2. Frequency of Rif^R mutations observed in *P. aeruginosa* and *P. syringae*.

Position	nt change	aa change	*P. aeruginosa*[a]		*P. syringae*[b]
			PW7148	PAO1/pJJK25	B86-17
Cluster I					
1547	T → C	L516P	4	1	0
1550	C → T	S517F	0	0	16
1550	C → A	S517Y	0	0	1
1552	C → A	Q518K	3	0	0
1553	A → C	Q518P	1	0	0
1553	A → G	Q518R	13	14	0
1553	A → T	Q518L	0	2	0
1561	G → A	D521N	1	0	7
1561	G → T	D521Y	0	0	2
1562	A → G	D521G	34	0	0
1580	C → T	S527F	1	0	0
1591	C → A	H531N	1	6	0
1591	C → T	H531Y	0	17	0
1592	A → G	H531R	11	0	0
1592	A → T	H531L	0	2	3
1601	G → A	R534H	1	0	0
1607	C → A	S536Y	1	3	4
1607	C → T	S536F	4	11	19
1613	T → C	L538P	2	0	1
Cluster II					
1706	C → T	P569L	1	0	0
1729	A → T	I577F	0	7	3
1730	T → G	I577S	0	1	0
1736	C → T	S579F	0	4	0
Transitions			73 (94%)	47 (69%)	43 (77%)
Transversions			5 (6%)	21 (31%)	13 (23%)
Total number Rif^R mutants			78	68	56

a. Total number of mutants observed in all 10 replicate cultures each of PW7148 and PAO1/pJJK25.

b. Total number of mutants observed in all 4 replicate cultures of B86-17.

Table 4-3. Summary of the 380 variations identified in the draft genome sequence of an inducible mutator isolate from lineage 44.

Location	Type	Number
Coding	Single base substitution	269
	Nonsynonymous	171 (64%)
	Synonymous	98 (36%)
	Multiple base substitution	18
	Insertion/deletion	17
Non-coding	Single base substitution	58
	Multiple base substitution	6
	Insertion/deletion	18
Total	Single base substitution	322
	Transitions	218 (68%)
	Transversions	104 (32%)
	Multiple base substitution	24
	Insertion/deletion	34

Table 4-4. Nonsense mutations identified in annotated genes of *P. aeruginosa*.

ID	Gene	Annotation[a]	Product size[b]	
			Reference	Variant
PA0027		Hypothetical protein	429	132
PA0264		Hypothetical protein	139	134
PA0299	*spuC*	Putrescine aminotransferase	457	448
PA1174	*napA*	Periplasmic nitrate reductase	830	514
PA1247	*aprE*	Alkaline protease secretion protein	433	249
PA1371		Hypothetical protein	234	225
PA1785		Probable response regulator	193	101
PA2493	*mexE*	RND multidrug efflux membrane protein	415	159
PA3280	*oprO*	Pyrophosphate-specific outer membrane porin	439	359
PA3696		Hypothetical protein	249	168
PA3704	*wspE*	Probable chemotaxis fusion protein	770	521
PA3756		Hypothetical protein	167	16

a. Annotations are based on listings in the Pseudomonas Genome Database (Winsor *et al*. 2009).

b. Number of amino acids

Table 4-5. Select nonsynonymous and frameshift mutations in annotated genes of *P. aeruginosa*.

Function and ID	Gene	Annotation[a]	aa change
DNA metabolism			
PA0010	*tag*	DNA-3-methyladenine glycosidase I	A101V
PA0438	*codB*	Cytosine permease	L171F
PA0669	*dnaE2*	Damage-inducible polymerase	W512R
PA3344	*recQ*	ATP-dependent DNA helicase	E687K
PA3947	*rocR*	Probable response regulator	A299V
PA4316	*sbcB*	Exodeoxyribonuclease I	H56Y
PA5241	*ppx*	Exopolyphosphatase	T189N
Motility and attachment			
PA0006	*yaeD*	Probable LPS biosynthesis protein	I165M
PA0395	*pilT*	Type 4 fimbrial biogenesis protein	G91S
PA0869	*pbpG*	D-alanyl-D-alanine-endopeptidase	L11P
PA0928	*gacS*	Sensor/response regulator hybrid	-1
PA1080	*flgE*	Flagellar hook protein	D81N
PA1092	*fliC*	Flagellin type B	G367S
PA1097	*fleQ*	Transcriptional regulator	E214G
PA1456	*cheY*	Chemotaxis response regulator	R21Q
PA1461	*motD*	Flagellar motor protein	A211S
PA2794		Probable pseudaminidase	N401T
PA3153	*wzx*	O-antigen translocase	W400R
PA3649	*mucP*	Probable outer membrane protein	F382S
PA4082	*cupB5*	Probable adhesion	H676Y
PA4512	*lpxO1*	Lipopolysaccharide biosynthetic protein	F196S
PA4525	*pilA*	Type 4 fimbrial precursor	E77K
PA5000	*wapR*	Alpha-1,3-rhamnosyltransferase	E246K
PA5448	*wbpY*	Glycosyltransferase	D323N
PA5511	*mifR*	Two-component response regulator	A66V

a. Annotations are based on listings in the Pseudomonas Genome Database (Winsor *et al*. 2009).

Figure 4-1. Relationship of ancestral *P. aeruginosa* PAO1 genotypes and their derived experimental lineages. Transformation of wild-type PAO1 with pJJK25 produced a UVR-inducible mutator phenotype. Disruption of *mutS* by transposon insertion (Jacobs 2003) produced a general mutator phenotype. Six parallel lineages of each ancestral genotype were derived from a single colony and propagated by serial transfer. Population samples and isolates in this study were derived from their respective ancestors following ~500 generations of selection in the evolution experiment.

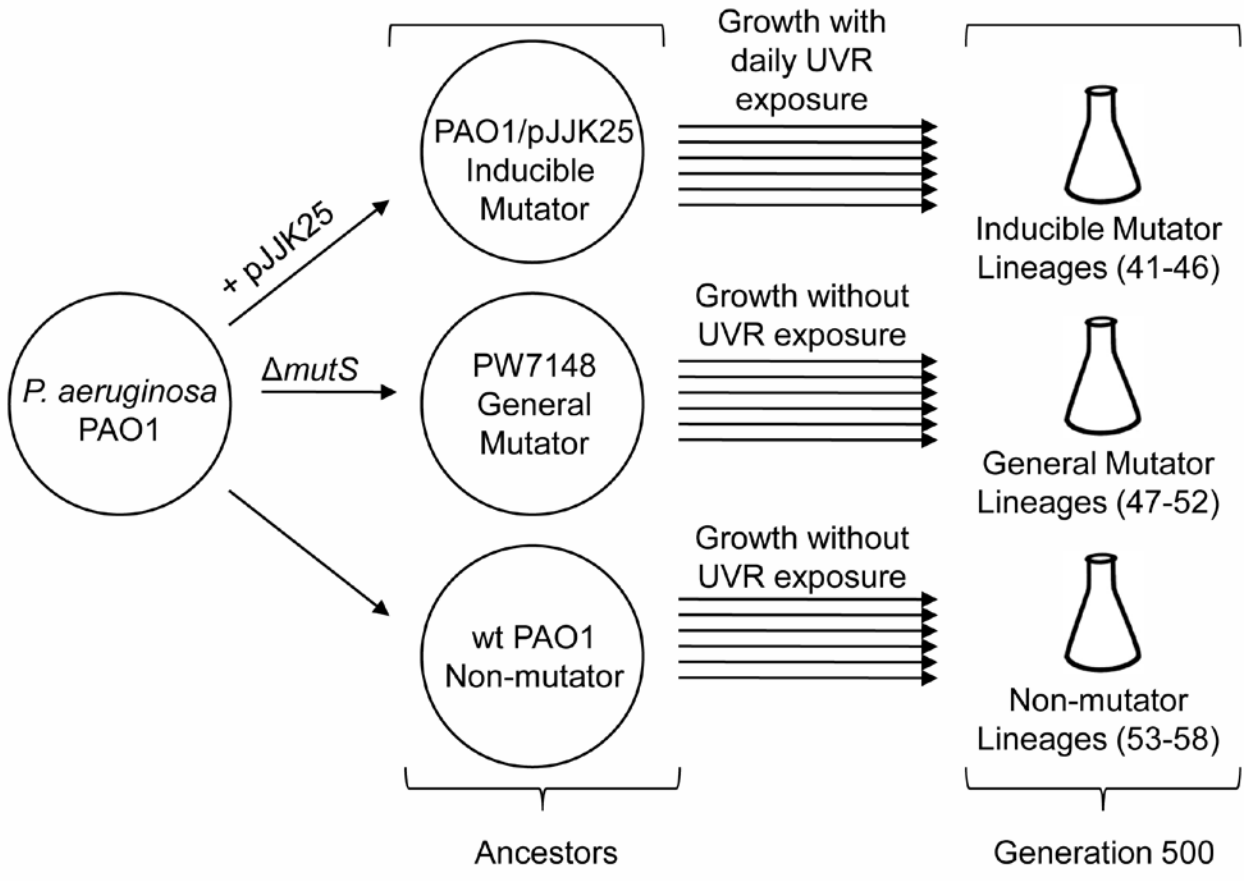


Figure 4-2. Average frequency of rifampicin-resistant mutants in lineages of *P. aeruginosa* PAO1 (●) inducible mutators, (■) general mutators, and (▲) non-mutators during experimental evolution. Values are means and error bars represent standard error of the mean. If error bars are not visible it is because they are smaller than the size of the marker.

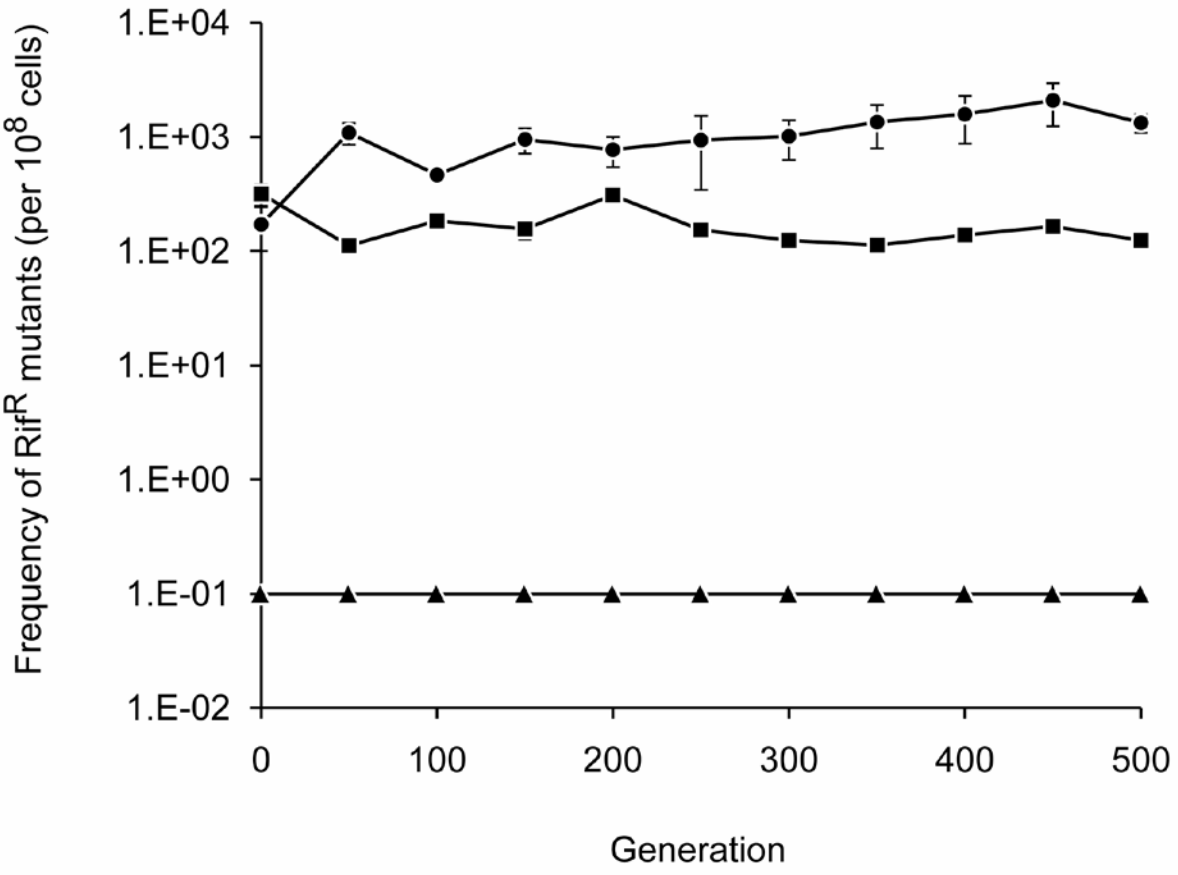


Figure 4-3. Spectra of point mutations responsible for Rif^R observed in clusters I and II of the *rpoB* gene of *P. aeruginosa*. Mutations observed in PW7148 are indicated above and mutations observed in PAO1/pJJK25 are indicated below. All mutations were nonsynonmyous and each Rif^R clone sequenced contained only one base substitution.

Figure 4-3 (cont'd).

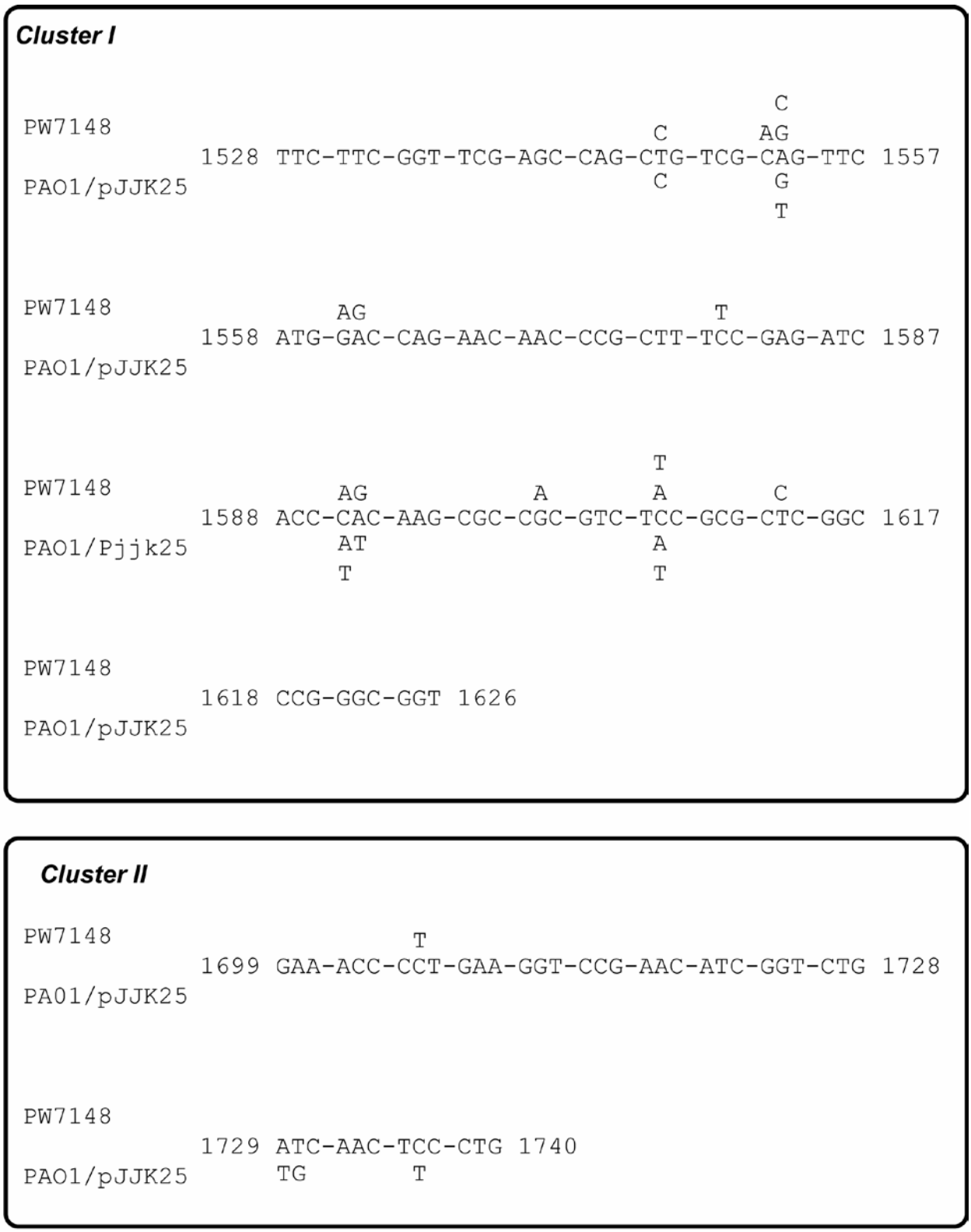


Figure 4-4. Relative fitness of population samples from derived lineages of *P. aeruginosa* PAO1 (A) inducible mutators, (B) general mutators, and (C) non-mutators after ~500 generations of growth. Relative fitness was measured by direct competition each lineage's respective ancestor under non-UVR (open bars) and UVR (shaded bars) conditions. Fitness values are means and error bars represent standard error of the mean. Values are significant by two-tailed independent *t*-test (d.f. = 5, $\alpha = 0.05$) where indicated (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$). *P*-paired values correspond to two-tailed paired *t*-tests (d.f. = 5, $\alpha = 0.05$) between relative fitness values under the two conditions.

Figure 4-4 (cont'd).

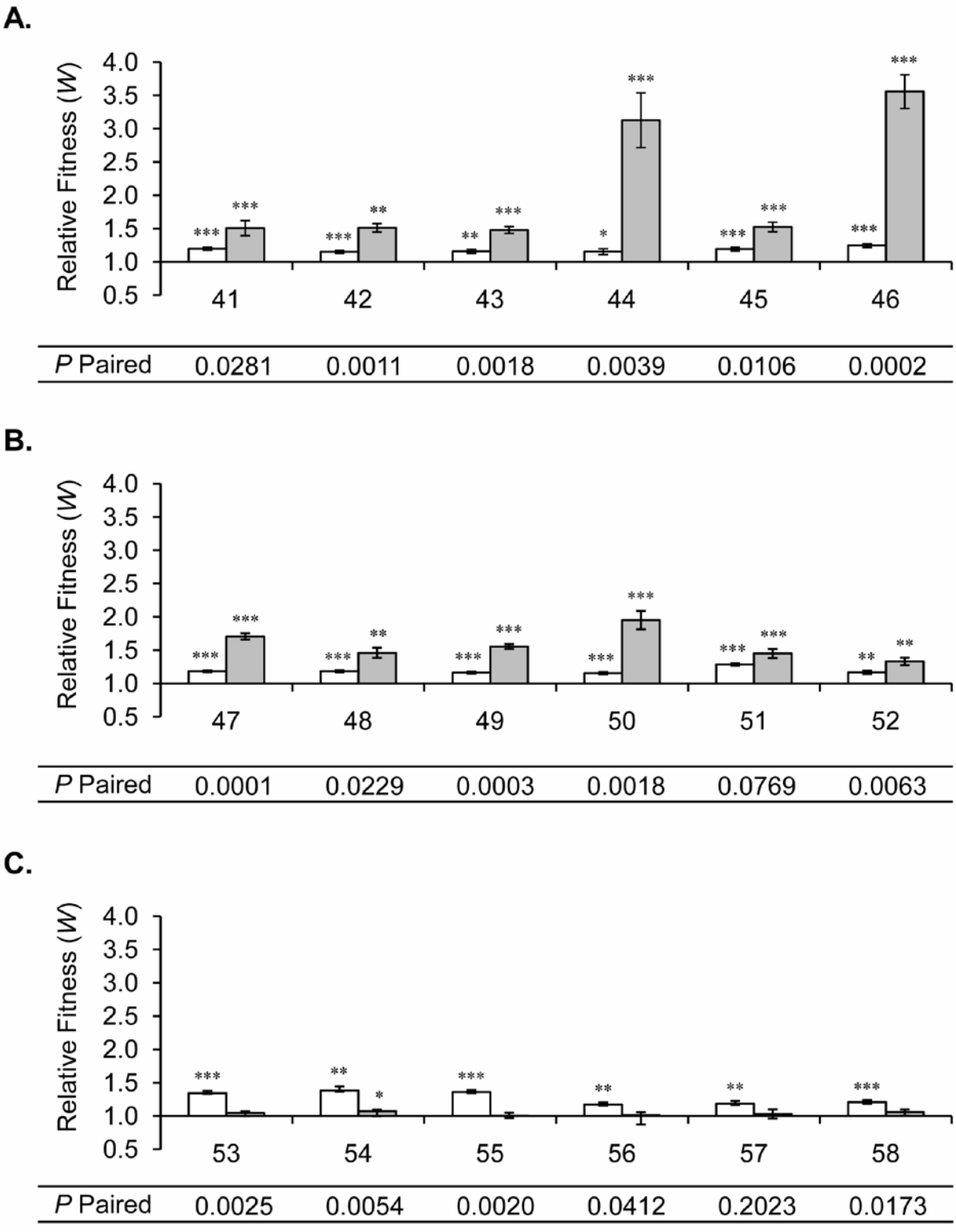
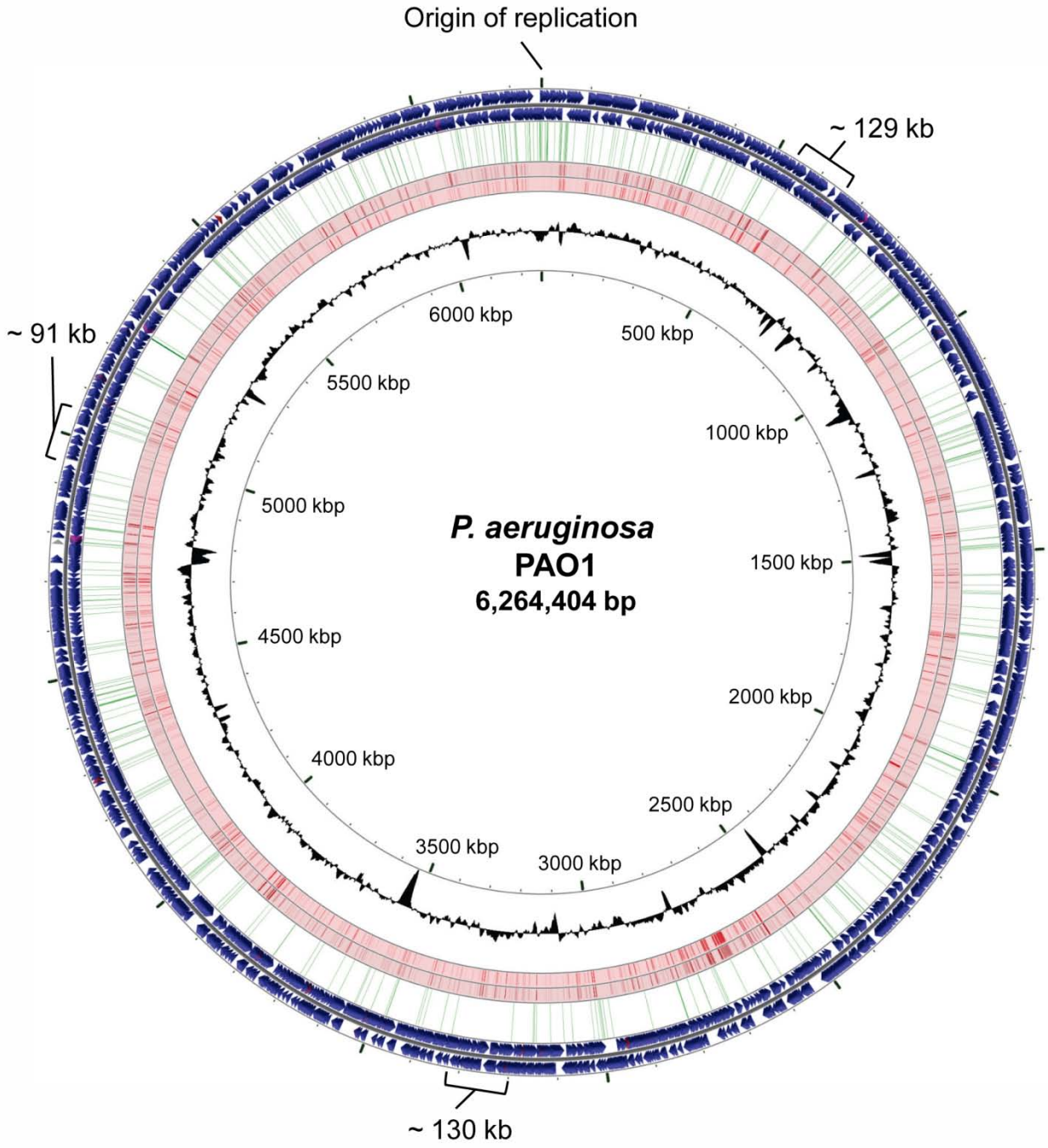


Figure 4-5. The chromosome of *P. aeruginosa* PAO1 based on the published reference sequence (AE004091, Stover *et al*. 2000). Starting with the outer most ring, rings one and two are CDS (blue), tRNA (red), rRNA (purple), and other (grey) ORFs on the forward and reverse strands, respectively. Ring three (green) indicates the position of 402 high confidence variations identified in an isolate from inducible mutator lineage 44. Rings four and five map pairwise blastn alignments of the PAO1 reference sequence and the *de novo* assembled contigs, respectively. Darker stripes indicate the presence of related gene families and duplications. Ring six shows G+C content (deviation from average) and ring seven indicates scale (in kbp). Three genomic regions > 91 kb that lack any mutations and the origin of replication are also indicated. The map was generated using the CGView Server (Grant and Stothard 2008).

Figure 4-5 (cont'd).



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CHAPTER 5: Parallel repression of flagellar biosynthesis in inducible and general mutator lineages of *Pseudomonas aeruginosa* PAO1 by mutation of the 'master regulator' FleQ

ABSTRACT

The opportunistic pathogen *Pseudomonas aeruginosa* establishes chronic respiratory infections in patients with cystic fibrosis characterized by broad phenotypic changes that promote long-term persistence. Late stage isolates of *P. aeruginosa* become increasingly diverse as they adapt to the host environment, a process facilitated by hypermutation. Hypermutator (or mutator) bacteria have been observed in a wide variety of clinical, environmental, and laboratory populations suggesting that evolutionary strategies of bacteria include mechanisms for increasing mutation rate. Here, I compare gene expression profiles between laboratory derived lineages of *rulAB*-mediated (inducible) and *mutS*-deficient (general) mutators. Although all lineages exhibited comparable fitness improvements, I hypothesize that their unique mutation spectra likely generated divergent adaptive gene expression profiles. I observed the universal down regulation in flagellum biosynthesis and chemotaxis. Underlying nonsynonymous mutations in the central regulatory gene *fleQ* were identified in random isolates from inducible and general mutator lineages but not a non-mutator control. Such mutations likely prohibit activation of the flagellar biosynthesis network in these populations. Our results suggest that, despite considerable variation in transcriptome profiles, laboratory evolved populations of *P. aeruginosa* PAO1 shared a subset of parallel expression traits independent of mutator status. Such parallelism is a hallmark of adaptive evolution.

INTRODUCTION

Pseudomonas aeruginosa is a ubiquitous, Gram-negative bacterium with a large genome that affords high adaptability and permits opportunistic infection of immunocompromised individuals. Cystic fibrosis (CF) patients are highly susceptible to bacterial pathogens, and chronic airway infection by *P. aeruginosa* is the primary cause of morbidity and mortality in affected individuals. Over the course of chronic CF infection, initially clonal populations of *P. aeruginosa* undergo dramatic phenotypic shifts that promote long-term survival, reduced virulence, and clonal expansion as they adapt to the stressful host environment (Ciofu *et al*. 2005, Smith *et al.* 2006, Hoboth *et al.* 2009). There is strong evidence to support the role of hypermutation in facilitating the adaptive transitions associated with chronic infection (Hogardt *et al*. 2007, Moyano *et al*. 2007, Mena *et al*. 2008, Ciofu *et al*. 2010, Oliver and Mena 2010).

Hypermutator (or mutator) strains of bacteria carry deficiencies in DNA proofreading and repair machinery that produce up to 1000-fold increases in spontaneous mutation rates; these strains have been observed in a variety of clinical, environmental, and laboratory populations (De Visser 2002, Sundin and Weigand 2007). Hypermutation arises most commonly through disruption of the methyl-directed mismatch repair (MMR) system, and the inactivation of *mutS* represents the most widespread defect in both environmental and clinical isolates (Hall and Henderson-Begg 2006). Similarly, inducible hypermutation can transiently increase mutation rates in response to specific stress conditions. Mutagenic DNA repair, one source of inducible hypermutation, is executed by specialized low-fidelity DNA polymerases as part of the SOS response following exposure to ultraviolet radiation (UVR) or other DNA-damaging agents (Schlacker and Goodman 2007, Courcelle *et al*. 2001). Secondary mutations caused by the Y-family polymerases polIV (*E. coli* DinB) and polV (*E. coli* UmuDC) during template-

independent synthesis of damaged DNA comprise the majority of sequence alterations derived from UVR exposure. Y-family polymerases also confer UVR tolerance and, therefore, contribute to epiphytic survival in plant pathogenic bacteria harboring the *umuDC*-homolog *rulAB* and residing in habitats optimized for solar UVR exposure (Kim and Sundin 2000, Sundin and Murillo 1999).

The widespread occurrence of hypermutators in various clinical, environmental, and laboratory populations suggests that evolutionary strategies of bacteria include mechanisms for increasing mutation rate. Bacteria are well suited for the study of adaptation (Elena and Lenski 2003) and I have previously conducted experimental evolution experiments with *P. aeruginosa* PAO1 to compare the influence of *rulAB*-mediated (inducible) and *mutS*-deficient (general) hypermutation on adaptation (Chapter 4). In that study, I observed comparable improvements in relative fitness among inducible and general mutator lineages despite divergent point mutation spectra (Chapter 4). Transcriptional profiling has been used in similar studies conducted previously to evaluate the adaptation of *in vitro* parallel lineages of *Escherichia coli* (Cooper *et al.* 2003) and *in vivo* CF lineages of *P. aeruginosa* (Huse *et al*. 2010). Both studies revealed parallel changes in gene expression during adaptation of replicate lineages.

In the present study, I have implemented transcriptional profiling to identify potential adaptive expression traits in select lineages of *P. aeruginosa* PAO1 inducible and general mutators. Although inducible and general mutator lineages exhibited comparable fitness improvements, I hypothesized that their unique mutation spectra likely generate divergent adaptive gene expression profiles. Our results indicated universal reductions in flagellum biosynthesis among the otherwise diverse gene expression traits exhibited by evolved lineages of *P. aeruginosa* PAO1 inducible mutators, general mutators, and non-mutators. Furthermore, I

have observed unique nonsynonmyous mutations within *fleQ* in inducible and general mutator lineage isolates that likely prevent activation of the flagellar biosynthesis regulatory network.

MATERIALS and METHODS

Bacterial strains, growth conditions, and general molecular biology techniques

The bacterial strains and plasmids used in this study are described in Table 1. Strains of *P. aeruginosa* PAO1 were obtained from the two-allele library at the University of Washington (Jacobs et al 2003). In a previous study, eighteen parallel lineages of *P. aeruginosa* were serially propagated for ~500 generations in minimal liquid medium (Chapter 4). Six inducible mutator lineages of PAO1/pJJK25 (numbered 41-46) received a single, daily dose of UVC (254 nm) radiation to activate plasmid-mediated mutagenic DNA repair. Cultures were individually mixed 1:1 with saline (0.85% NaCl) in a glass Petri dish and exposed to ~75 J m^{-2} of UVC radiation from an XX-15 UV lamp (UVP Products, San Gabriel, CA) that resulted in ~10% survival by each lineage population. The energy output of the lamp was monitored with a UV-X radiometer fitted with a UV-25 sensor (UVP Products, San Gabriel, CA) and determined to be 1.5 J m^{-2} s^{-1}. Following UVR irradiation, cultures were diluted 1:100 into fresh medium and incubated under dark conditions to minimize photoreactivation. Six general mutator lineages of PW7148 (numbered 47-52) and six non-mutator lineages of PAO1 (numbered 53-58) were diluted 1:10 in saline and then 1:100 into fresh DM25 broth without UVR. Both transfer strategies resulted in 1000-fold daily growth of each population, representing ~10 generations of binary fission. Population samples from each lineage were preserved in a nonevolving state in 10% glycerol at -80° at regular intervals for later analysis. The evolved lineages in this study represent a subset of lineage populations derived from the ancestral genotypes following ~500 generations of growth

selected because of relative fitness phenotypes reported previously (Chapter 4). Strains of *P. aeruginosa* were cultured at 37° in Luria-Burtani (LB) broth (Difco, Detroit, MI), in Davis Minimal broth (Difco) supplemented with 25 mg l^{-1} glucose (DM25), or on King's medium B (KB) agar (King et al 1954).

RNA isolation and gene expression analysis

Gene expression profiles were assessed for all three ancestral genotypes, two inducible mutator lineage populations, two general mutator lineages, and one non-mutator lineage. Ancestral strains and preserved population samples were first acclimated to the culture conditions for 24 hrs and then diluted 1:1000 into fresh DM25 and grown to mid-exponential phase for RNA extraction. In a previous study, changes in growth parameters were observed following experimental evolution (Weigand *et al.* 2011). Therefore harvesting times were determined by plotting growth curves and varied for each strain or population sample. Cells were mixed 1:1 with RNAProtect Bacteria Reagent (QIAGEN, Valencia, CA) and lysed by treatment with 200 µg lysozyme according to the manufacturer's recommendations. Total RNA was purified using the RNeasy Mini Kit (QIAGEN) including on-column DNase I digestion with the RNase-Free DNase Set (QIAGEN). RNA integrity was evaluated with an Agilent 2100 Bioanalyzer.

Custom array design was carried out using the Agilent Technologies eArray tool (http://eArray.chem.agilent.com/eArray). Triplicate probes, where possible, were designed to 5,571 annotated PAO1 open reading frames and eight additional plasmid or IS element sequences resulting in a set of 14,392 total probes. These were incorporated into an Agilent 8x15K format array design along with the appropriate Agilent QC probe set. RNA was labeled

using the Fairplay III labeling kit (Agilent Technologies, Santa Clara, CA). Hybridization and stringency washes were carried out with Agilent reagents according to the manufacturer's standard protocols. Slides were scanned on an Agilent G2505B Array Scanner and the resulting images were analyzed with the Agilent Feature Extraction Software (v9.5.1).

Analysis of probe expression intensities was carried out using the GeneSpring v11.0.1 software (Agilent Technologies). Derived lineages were compared to their respective ancestors (Chapter 4). Significance was determined by unpaired *t*-test and asymptotic *P*-value computation. False discovery was controlled by Benjamini-Hochberg multiple-testing correction. Genes with altered expression greater than 2.0-fold and a *P*-value ≤ 0.05 were selected for further analysis. Functional annotation clustering of differentially expressed gene lists was conducted using DAVID Bioinformatics Resources (Huang *et al.* 2009).

Transmission electron microscopy

Bacteria were examined by electron microscopy to confirm the presence or absence of flagella. Cells were grown overnight in LB broth, transferred to 10 ml DM25 for 24 hr, and then diluted to ~1×10^4 CFU/ml with sterile water. The cell suspension was negatively stained with 0.25% uranyl acetate (Electron Microscopy Sciences, Hatfield, PA) and a 2 µl aliquot was placed on a carbon-coated transmission electron microscopy grid. Samples were examined on a JEOL100 CXII microscope (Japan Electron Optics Laboratories, Tokyo, Japan).

Motility assay

Differences in motility were observed on media containing 10 g l^{-1} tryptone, 5 g l^{-1} NaCl, and 0.3% (w/v) agar as described previously (Rashid and Kornberg 2000, Linares *et al*. 2010). Plates

were allowed to solidify for ~45 min in a laminar flow hood before inoculation with 5 µl drops from 24 hr cultures in 10 ml DM25. Plates were incubated at 37° for 24 hr and then photographed.

DNA sequence analysis of the flagellum regulatory gene *fleQ*

To identify potential mutations underlying the down regulation of flagellar biosynthesis genes, the coding region and upstream 500 bp of *fleQ* (PA1097) were amplified by PCR. Eight random colonies were chosen each from preserved lineages 44, 46, 51, 52, and 55 as well as ancestors PAO1/pJJK25, PW7148, and PAO1. Colonies were suspended in 50 µl of water and disrupted by boiling for 10 mins. Fragments were amplified using primers fleQ-up1 and fleQ2 listed in Table 1. PCR products were purified using the QIAquick PCR Purification Kit (QIAGEN) and then sequenced at the Research Technology Support Facility at Michigan State University using primers fleQ-up1, fleQ1, and fleQ2 listed in Table 1. Lasergene SeqMan Pro (DNASTAR, Madison, WI) was used for sequence comparison and mutation identification.

RESULTS

Gene expression profiles of select lineage populations

I have previously conducted experimental evolution with the model organism *P. aeruginosa* PAO1 to compare the effects of *rulAB*-mediated and *mutS*-deficient hypermutation on adaptation (Chapter 4). In that study, I observed increased relative fitness in derived populations of inducible mutators (18%), general mutators (19%), and non-mutators (27%) under defined laboratory conditions in the absence of UVR exposure (Chapter 4). To determine if these fitness gains were the result of parallel or divergent adaptive improvements, I measured

changes in gene expression in select inducible mutator (44 and 46), general mutator (51 and 52), and non-mutator (55) lineages during growth under the non-UVR conditions of lineage propagation. Gene expression changes in each derived lineage were determined relative to the lineage's respective ancestor (Chapter 4) to highlight only those alterations associated with adaptation during experimental evolution rather than the subtle genetic differences in ancestral genotypes.

Differentially expressed genes in inducible mutator lineages 44 and 46 were compared to identify parallel changes in gene expression associated with the adaptation of inducible mutator populations and a total of 112 shared expression changes were observed (Figure 5-1A). Functional annotation clustering of the 75 shared genes with annotations revealed enrichment for genes involved in flagellum assembly, flagellum organization, quorum sensing, and glycolysis (Table 5-2). In addition to this overlap, the two populations also displayed unique gene expression changes that included differences in the total number of altered genes (Figure 5-1A).

Comparison of transcriptome profiles in general mutator lineages 51 and 52 identified 114 shared genes with altered expression (Figure 5-1B). Functional annotation clustering of the 59 shared genes with annotations indicated enrichment for genes associated with flagellum assembly, lipopolysaccharide biosynthesis, pyrrolquinoline quinone biosynthesis, and chemotaxis (Table 5-2). Similar to inducible mutator lineages, general mutator lineages 51 and 52 were also divergent in their total number of differentially expressed genes (Figure 5-1B).

Non-mutator lineage 55 displayed the fewest number of genes with altered expression (51) and functional annotation clustering indicated enrichment for flagellum assembly only (Table 5-2). These were compared to the parallel gene expression changes identified within inducible mutator populations (44 and 46, Figure 5-1A) and general mutator populations (51 and

52, Figure 5-1B) to identify a core set of expression changes associated with adaptation to the lineage environment (Figure 5-2). Sixteen genes displayed altered expression in all five lineages studied and all were down regulated relative to ancestral levels (Table 5-3). All 11 annotated genes were either experimentally or putatively associated with motility. These results suggest that regulation of flagellum-mediated motility is a common target for adaptation that likely carries a relatively large fitness effect. That flagellum assembly was universally down regulated in parallel lineages independent of the mutation supply mechanism provides strong indication for natural selection.

Evolved lineages lack flagella and exhibit altered motility phenotypes

All five lineages studied displayed decreased expression of flagellum assembly components. To confirm the absence of flagella in evolved lineages, cells were visualized using transmission electron microscopy. *P. aeruginosa* PAO1 produces a single, polar flagellum and ancestral PAO1/pJJK25, PW7248, and PAO1 all had flagella (Figure 5-3A and B). Conversely, of the 12 or more individual cells observed from each evolved lineage population, none were flagellated and no detached flagellar fragments were observed (Figure 5-3C and D). Cells from evolved lineage populations were often associated with a thick, extra cellular matrix that was not present on any of the ancestral cells. The density and structure of the surrounding material was unique to each lineage suggesting varied composition.

To investigate changes in flagellum-dependent motility, evolved lineage populations were spotted on 0.3% (w/v) agar motility media. The ancestral PAO1/pJJK25, PW7148, and PAO1 exhibited identical, uniform motility (Figure 5-4A). Evolved inducible mutator lineages 44 and 46 displayed severely reduced motility compared to the ancestral PAO1/pJJK25 (Figure

5-4B). General mutator lineages 51 and 52 were relatively motile but displayed phenotypes distinct from PW7148 (Figure 5-4C). Non-mutator lineage 55 was highly motile compared to PAO1 and also displayed altered morphology (Figure 5-4D). The motility observed in general mutator and non-mutator lineages may result from the presence of a few flagellated individuals within the population or other mechanisms of locomotion that do not require flagella. Replicate lineages of inducible mutator and general mutators exhibited discrete phenotypes suggesting that individual lineages possess distinct alterations in motility despite their shared down regulation of flagellar production.

DNA mutations in the flagellum master regulator *fleQ*

Flagellum biosynthesis and assembly involves approximately 50 genes in a complex and tightly controlled four-tiered regulatory cascade (Dasgupta *et al* 2003). The DNA sequence of select structural and regulatory components of flagella assembly were investigated to identify the underlying genetic changes responsible for the loss of flagellum observed in evolved lineages. Reduced expression of flagellin type-b (*fliC*) was observed in all five evolved lineages (Table 5-3) and also exhibited the largest fold change reduction in each individual lineage. Eight random isolates from inducible mutator lineage 44 contained a single, identical nonsynonymous mutation within the coding region of *fliC* (PA1092) and one random isolate from inducible mutator lineage 46 contained a C·G → T·A that was 310 nucleotides upstream of the translational start site (data not shown). However, in the eight random isolates each from general mutator lineages 51 and 52 and non-mutator lineage 55, no mutations were observed in *fliC* or the 500 bp upstream region. Similarly, no mutations were identified in *vfr* (PA0652), *fleN* (PA1454), *fliA*

(PA1455), *flgM* (PA3351), or their respective 500 bp upstream regions in any of eight random isolates from each evolved lineage.

The transcriptional activator FleQ sits atop the intricate flagellum regulatory hierarchy and this master regulator is required for the expression of nearly all known flagellar genes (Dasgupta *et al.* 2003). DNA sequence analysis of the *fleQ* locus (PA1097) revealed nonsynonymous point mutations in all random isolates from inducible and general mutator lineages. The three domain structure of FleQ is illustrated in Figure 5-5 along with the amino acid substitutions observed in different isolates. All observed missense mutations were in the ATP-binding region of the AAA+ ATPase domain which mediates interaction with RpoN (σ^{54}). All eight random isolates from inducible mutator lineage 44 were of the same *fleQ* genotype and bore the E214G mutation. The eight isolates from inducible mutator lineage 46 all contained a nonsense mutation at amino acid 154 (not shown) which falls between the REC and AAA+ domains.

Mutations observed in isolates from general mutator lineages at the *fleQ* locus were more heterogeneous. Seven different nonsynonymous mutations were observed among the eight isolates each from general mutator lineages 51 and 52. Nearly all *fleQ* genotypes were unique to one lineage and only the H218R mutation was observed in two isolates from different lineages. Only one isolate from non-mutator lineage 55 bore a nonsynonymous mutation in *fleQ* that produced the S36R mutation within the REC domain (not shown). The presence of nonsynonymous mutations within the ATP-binding region of FleQ in all isolates from inducible and general mutator populations strongly suggests that the observed down regulation of flagellum biosynthesis and associated defects in motility result from an inability to activate the flagellar regulatory network. Furthermore, 20 differentially regulated genes were shared among

inducible and general mutator populations only (Figure 5-2) and 14 of those down regulated genes have been shown previously to require FleQ for transcription (Table 5-4, Dasgupta *et al.* 2003).

DISCUSSION

Despite considerable variation in transcriptome profiles, laboratory evolved populations of *P. aeruginosa* PAO1 shared a subset of parallel expression traits independent of mutator status. In a previous study, I maintained parallel lineages of *P. aeruginosa* PAO1 to compare the influence of *rulAB*-mediated and *mutS*-deficient hypermutation on adaptation and observed comparable improvements in relative fitness despite divergent point mutation spectra (Chapter 4). Similar studies conducted previously have observed largely parallel changes in gene expression during adaptation of replicate lineages (Cooper *et al.* 2003, Huse *et al*. 2010). Alternatively, analogous, fitness-enhancing changes can result from different mutations that inflict the same phenotypic outcome (Applebee *et al.* 2008). Here, transcriptional profiles of select lineage populations were compared to highlight potential adaptive expression traits, and mutations in a central regulator of flagellum biosynthesis were identified.

I measured gene expression in two replicates each from a set of parallel lineages of inducible and general mutators. The large difference in the number of differentially expressed genes between replicate lineages was initially striking. Inducible mutator lineage 46 exhibited altered expression in more than twice as many genes as lineage 44 and general mutator lineage 52 more than six times as many as lineage 51. Clearly, parallel lineages of *P. aeruginosa* hypermutators were exploring divergent solutions for adaptation to the experimental evolution environment. Lists of differently expressed genes from replicate lineage pairs were compared to

identify shared expression traits, which were then used for subsequent comparison of inducible mutators, general mutators, and non-mutators.

All lineages experienced similar selection pressures imposed by the conditions used during propagation which were expected to produce some common expression traits. Not surprisingly, functional annotation clustering of expression profiles identified the universal down regulation of flagellar biosynthesis in all lineages. The homogenous, unstructured lineage environment likely provided little opportunity for individual cell motility and, therefore, selected against flagellum production. Parallel, convergent expression profiles in a similar study conducted previously likewise observed down regulation of numerous flagellum structural genes during experimental evolution under almost identical conditions (Cooper *et al*. 2003). Furthermore, among the genes down regulated in all lineage populations, the 11 annotated genes (out of 16 total) were all either experimentally or putatively associated with motility. This biased overlap in transcriptome profiles strongly suggests that repression of flagellum-mediated motility provides a highly favorable fitness effect and such parallelism is a hallmark of adaptive evolution.

Flagella facilitate colonization and biofilm formation during initial infection, and early stage CF isolates of *P. aeruginosa* are accordingly highly motile (Mahenthiralingam *et al.* 1994). Reduced motility is among the phenotypic transitions associated with chronic infection, in part because the loss of motility correlates with resistance to phagocytosis (Mahanthiralingam *et al*. 1994, Amiel *et al.* 2010). It has also been shown that flagellin, the structural subunit of the flagellar fiber, is recognized by host Toll-like receptors to mobilize innate defense responses (Hayashi *et al.* 2001). Interestingly, of the 16 genes differently regulated in all lineages here, five (PA1092, PA1423, PA3326, PA3722, and PA4310) were also down regulated in *P.*

aeruginosa challenged with respiratory fluid derived from chronically infected adult CF patients (Wolfgang *et al.* 2004). Transcriptional profiling of mutator isolates of *P. aeruginosa* recovered from end stage CF infection also identified reduced expression in genes associated with motility and chemotaxis (Hoboth *et al.* 2009), seven of which were likewise down regulated in all lineages described here (PA1092, PA1095, PA1423, PA2652, PA3349, PA4310, and PA4633).

Flagellar biosynthesis is well characterized and fairly conserved across distant bacterial phyla (Aldridge and Hughes 2002, McCarter 2006, Liu and Ochman 2007). Assembly of a functional flagellum depends on a highly complex and tightly governed regulatory cascade that coordinates the efficient, accurate, and ordered expression of approximately 50 genes encoding structural subunits, regulators, motor force generators, and chemosensors (Aldridge and Hughes 2002, Dasgupta *et al.* 2003, Renyi and Ochman 2007). Given the close association between expression phenotype and genotype, transcriptome profiling can identify candidate genes for investigating underlying sequence changes responsible for adaptation. I sequenced select structural and regulatory components of the flagellum biosynthesis pathway in random isolates from select *P. aeruginosa* lineages and uncovered parallel mutations in *fleQ* in inducible and general mutators.

In *P. aeruginosa* PAO1 the transcriptional activator FleQ sits atop a four-tiered regulatory hierarchy and is regarded as the 'master regulator' due to its requirement for the expression of nearly all known flagellar genes (Dasgupta *et al.* 2003). Based on sequence homology, FleQ belongs to the NtrC subfamily of transcriptional activators that work in concert with the alternate sigma factor RpoN (σ^{54}), and *fleQ*-deficient mutants of *P. aeruginosa* are non-motile (Arora *et al.* 1997). Expression of *fleQ* is σ^{70}-dependent and negatively regulated by Vfr, a homolog of the *E. coli* cyclic AMP repressor protein (Dasgupta *et al.* 2002), and indirectly by

AlgT-MucA (σ^{22} and anti- σ^{22}, Tart *et al.* 2005, 2006). Additionally, FleQ functions as a c-di-GMP responsive transcription factor to repress exopolysaccharide biosynthesis and biofilm formation, centrally positioning it in the regulatory switch between planktonic growth and biofilm development (Hickman and Harwood 2008). A few nonsynonymous *fleQ* mutations have also been observed in isolates from chronic CF infections (Smith *et al.* 2006).

The presence of nonsynonymous mutations within the same gene but at different nucleotide positions in parallel populations confirms the random, independent nature of mutation and the power of selection to champion adaptive improvements (Applebee *et al*. 2008). All the missense mutations I observed in *fleQ* were located within an ATP-binding region of the RpoN-interacting domain. The observed down regulation of flagellum biosynthesis and associated defects in motility likely resulted from an inability to activate the flagellar regulatory network. In CF isolates of *P. aeruginosa*, flagellation and mucoidy are often linked to mutation in the anti-sigma factor MucA (Boucher *et al.* 1997). MucA inactivation deregulates AlgT (σ^{22}), increasing alginate production but also indirectly decreasing flagellar biosynthesis (Tart *et al.* 2005, 2006). Therefore, the loss of flagella is commonly observed in CF isolates of *P. aeruginosa* linked to *mucA* mutation (Smania *et al.* 2004, Ciofu *et al*. 2010) likely resulting from fundamentally different regulatory changes than those observed in this study linked to FleQ inactivation.

Sequence heterogeneity in *fleQ* from sympatric isolates of general mutator lineages indicates adaptive radiation within these populations that was first suggested by diversity in colony morphology observed previously (Chapter 4). I observed four different *fleQ* alleles among eight random isolates from each lineage, suggesting that the beneficial alteration of FleQ appeared at least four independent times in the evolutionary history of both general mutator lineages 51 and 52. Conversely, within inducible mutator lineages 44 and 46, sympatric isolates

from each population all bore the same sequence variation. However, divergent colony morphologies and relative fitness of random sympatric isolates supports adaptive radiation and genetic heterogeneity at other loci in these lineages (data not shown). These results suggest that both the E214G in lineage 44 and nonsense mutation in lineage 46 either appeared very early in the evolution experiment or were part of a selective sweep that purged diversity at the *fleQ* locus.

No mutations in *fleQ* were recorded in random isolates from non-mutator lineage 55. A comparison of parallel gene expression among all lineages indicated additional flagellum structural genes with altered expression in inducible and general mutator lineages but not non-mutator lineage 55. Transcription of most of these shared down regulated genes has been shown to be FleQ dependent (Dasgupta *et al.* 2003) consistent with the predicted inactivation of FleQ exclusively in inducible and general mutator lineages. However, non-mutator lineage 55 displayed no flagellum when visualized by transmission electron microscopy and altered motility on 0.3% agar suggesting the presence of an alternate regulatory change. Parallel down regulation of flagellum and chemotaxis components in all lineages suggest that these modifications reflect beneficial mutations. In *P. aeruginosa* PAO1, flagellum structural and regulatory genes are distributed across three genomic regions and organized into 17 operons (Dasgupta *et al.* 2003). Within the three gene operon *fliD-fliS-fleP* (PA1094-PA1096), only *fliS* and *fleP* were down regulated in non-mutator lineage 55. A polar mutation in PA1095 (*fliS*) could explain this selective pattern of down regulation and could indirectly influence the expression of the *fliC-fleL* (PA1092-PA1093) operon. The close physical association of these genes should aid in the future elucidation of the underlying mutation(s) responsible for flagellum deficiency in nonmutator lineage 55.

Multiple solutions often exist for adaptation and have been observed previously at the level of gene expression (Fong *et al.* 2005, Huse *et al.* 2010). Individual expression variants also emerge in isolates of *P. aeruginosa* from end stage CF patients (Hoboth *et al.* 2009). Therefore, it is not surprising that I observed divergent gene expression profiles among replicate lineages of inducible and general mutator populations. Different lineages exhibited altered expression in genes associated with quorum sensing, lipopolysaccharide biosynthesis, exopolysaccharide production, siderophore secretion, and multi-drug efflux pumps. Each may reflect divergent loci in genotypic space where individual lineages have found adaptive mutations. Further analyses are needed to characterize these divergent expression traits and determine their relative influence on fitness.

In summary, I have implemented transcriptional profiling to identify potential adaptive expression traits in select lineages of *P. aeruginosa* PAO1 inducible and general mutators. A comparison of expression profiles from all lineages has identified a subset of genes associated with flagellum biosynthesis and chemotaxis that were universally down regulated independent of mutator status. Accordingly, all lineages lacked visible flagella and exhibited altered phenotypes in flagellar-dependent motility. This biased overlap indicated that repression of flagellum-mediated motility provided a highly favorable fitness effect under the experimental conditions and such parallelism is a hallmark of adaptive evolution. By sequencing the coding and upstream regulatory regions of key genes in the flagellum regulatory network, I identified several, independent nonsynonymous mutations in the 'master regulator' FleQ. These mutations were observed only in isolates from inducible and general mutator lineages and correlated with broader reductions in expression of flagellar gene in those populations compared to a non-mutator lineage. Future studies with *P. aeruginosa* PAO1 mutants deficient in FleQ and other

central flagellum regulators will further characterize the influence of flagellum repression on fitness in this system.

Table 5-1. Strains, plasmid, lineages, and primers used in this study.

Strain, plasmid, lineage, or primer	Relevant characteristics or sequence	Reference
Strains		
PAO1	Wild-type, laboratory strain *P. aeruginosa*	Jacobs *et al.* 2003
PW7148	PAO1 *mutS*-C10:: IS*lacZ*/hah	Jacobs *et al.* 2003
Plasmid		
pJJK25	*rulAB*, confers inducible hypermutability	Kim *et al.* 2000
Lineages[a]		
44	Inducible mutator, derived from PAO1/pJJK25	Chapter 4
46	Inducible mutator, derived from PAO1/pJJK25	Chapter 4
51	General mutator, derived from PW7148	Chapter 4
52	General mutator, derived from PW7148	Chapter 4
55	Non-mutator, derived from PAO1	Chapter 4
Primers		
fleQ-up1	5’ - AGGCCGGCAACTGGGATG - 3’	This study
fleQ1	5’ - GAGCGAGACCCATTCGATGC - 3’	This study
fleQ2	5’ - CAAAGCGTTGCGAAACGACCTGT - 3’	This study

a. Derived lineage populations following ~500 generations of experimental evolution.

Table 5-2. Functional annotation clustering of shared genes with altered expression.

Annotation Category[a]	Genes[b]	Enrichment Score[c]
Inducible mutators (44,46)[d]		
Flagellum assembly	19	12.8
Flagellum organization	9	8.3
Quorum sensing	4	4.4
Glycolysis	4	2.4
General mutators (51,52)[e]		
Flagellum assembly	14	9.92
Lipopolysaccharide biosynthesis	6	2.1
Pyrrolquinoline quinone biosynthesis	3	2.0
Chemotaxis	4	1.2
Non-mutator (55)		
Flagellum assembly	3	2.74

a. GO term, KEGG pathway designation, and/or SP PIR keyword.

b. Total number of differentially expressed genes in annotation cluster.

c. Geometric mean of all enrichment *P*-values for each analogous annotation term in the group. Higher scores indicate annotation categories that are more represented (Huang *et al*. 2009).

d. Based on the 75 annotated genes out of the 112 gene expression changes shared between lineages 44 and 46.

e. Based on the 59 annotated genes out of the 114 gene expression changes shared between lineages 51 and 52.

Table 5-3. Genes with altered expression in all lineages.

ID	Gene	Annotation[a]	Regulation
PA1092	*fliC*	Flagellin type-b	down
PA1093	*fleL*	Flagellar length control[b]	down
PA1095	*fliS*	Flagellar elongation[b]	down
PA1096	*fleP*	Flagellar attachment and type IV pili length control[b]	down
PA1423	*bdlA*	Biofilm dispersion MCP[c]	down
PA1679		Hypothetical protein	down
PA1913		Hypothetical protein	down
PA2652		Probable chemotaxis transducer	down
PA3349	*cheV*	Probable chemotaxis regulator[b]	down
PA3351	*flgM*	FliA-specific antisigma factor	down
PA3526	*motY*	Probable outer membrane protein	down
PA3662		Hypothetical protein	down
PA3720		Hypothetical protein	down
PA3722		Hypothetical protein	down
PA4310	*pctB*	Chemotaxis transducer	down
PA4633		Probable chemotaxis transducer	down

a. Annotated functions are based on listings in the Pseudomonas Genome Database (Winsor *et al*. 2009) unless otherwise referenced.

b. Dasgupta *et al*. 2003.

c. Morgan *et al.* 2006.

Table 5-4. Genes with altered expression in inducible and general mutator lineages only.

ID	Gene	Annotation[a]	Regulation
PA1077[b]	*flgB*	Flagellar basal-body rod	down
PA1078[b]	*flgC*	Flagellar basal-body rod	down
PA1079[b]	*flgD*	Flagellar basal-body rod modifier	down
PA1080[b]	*flgE*	Flagellar hook	down
PA1081[b]	*flgF*	Flagellar basal-body rod	down
PA1082[b]	*flgG*	Flagellar basal-body rod	down
PA1083[b]	*flgH*	Flagellar L-ring	down
PA1086[b]	*flgK*	Flagellar hook-associated protein	down
PA1087[b]	*flgL*	Flagellar hook-associated protein	down
PA1089		Conserved hypothetical protein	down
PA1090		Hypothetical protein	down
PA1094[b]	*fliD*	Flagellar cap	down
PA1100[b]	*fliE*	Flagellar hook-basal body complex	down
PA1458[b]	*cheA*	Probable two-component sensor	down
PA1462[b]		Probable plasmid partitioning protein	down
PA3193	*glk*	Glucokinase	up
PA3610	*potD*	Polyamine transport protein	up
PA4309	*pctA*	Chemotactic transducer	down
PA4324		Hypothetical protein	down
PA4326[b]		Hypothetical protein[b]	down

a. Annotated functions are based on listings in the Pseudomonas Genome Database (Winsor *et al*. 2009).

b. Open reading frames with FleQ-dependent regulation (Dasgupta *et al.* 2003).

Figure 5-1. Venn diagram comparison of genes with significant changes in expression between (A) inducible mutator lineages 44 and 46 relative to PAO1/pJJK25 and (B) general mutator lineages 51 and 52 relative to PW7148. Total number of differentially expressed genes are subdivided into up-regulated (+) and down-regulated (-) contributions. Genes listed in the overlapping regions were used to represent inducible and general mutator populations for comparison with non-mutator lineage 55 in Figure 5-2 and functional annotation clustering in Table 5-2.

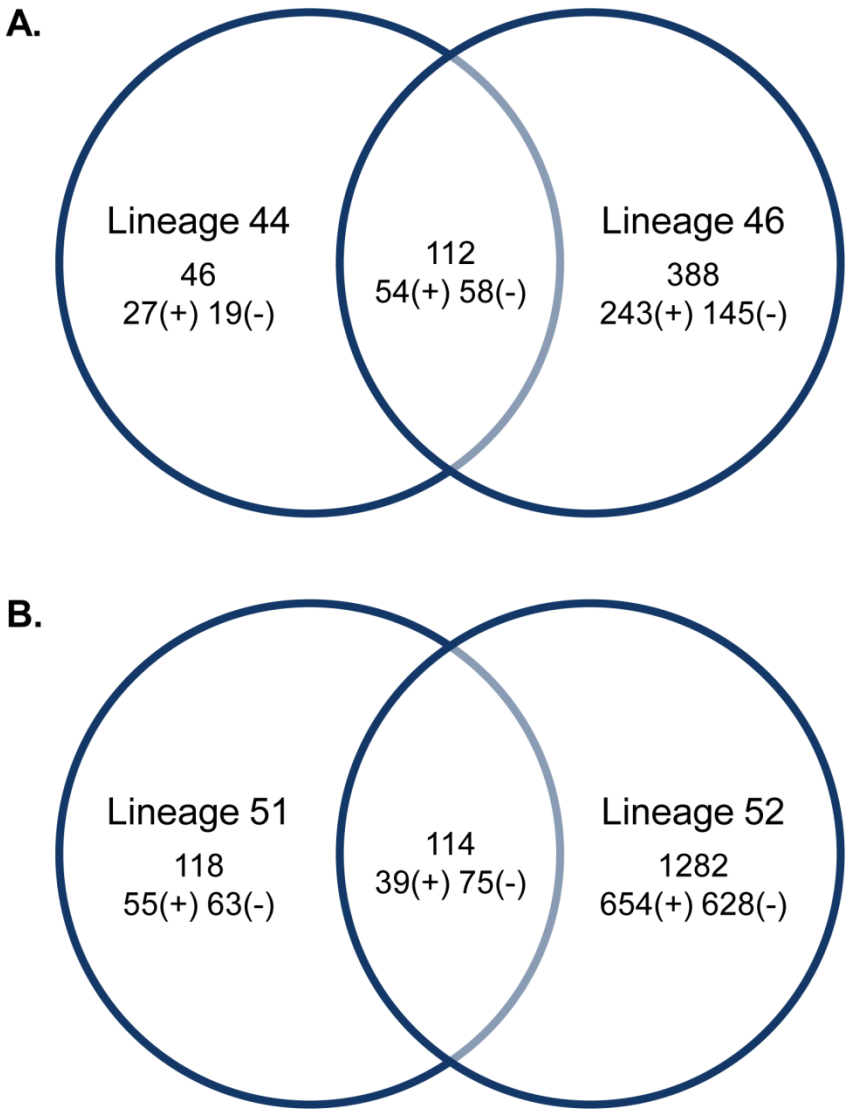


Figure 5-2. Venn diagram comparison of genes with significant changes in expression between non-mutator lineage 55 and those genes with shared expression patters within pairs of inducible (44 and 46) and general (51 and 52) mutator lineages. Total number of differentially expressed genes are subdivided into up-regulated (+) and down-regulated (-) contributions. Genes with altered expression in all lineages are listed in detail in Table 5-3 and those shared by inducible and general mutators only are listed in Table 5-4.

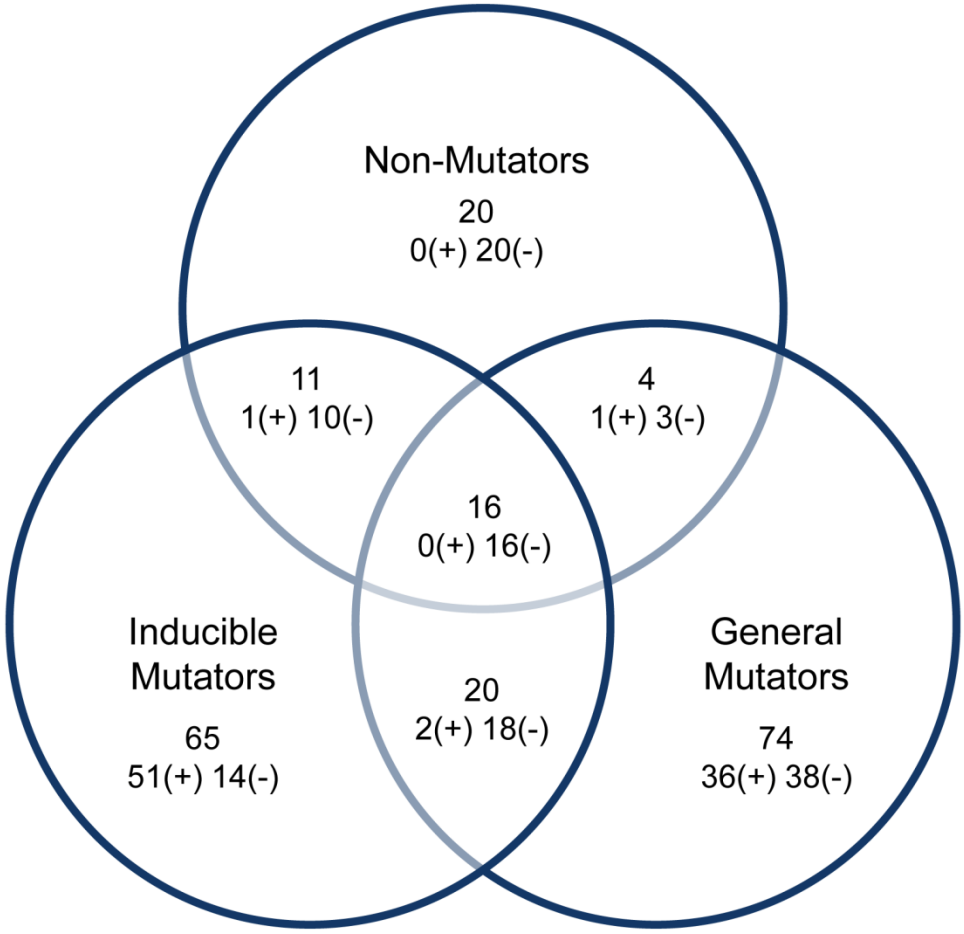


Figure 5-3. Flagellum visualization by transmission electron microscopy. (A and B) Ancestral genotypes of *P. aeruginosa* (PAO1/pJJK25, PW7148, and PAO1) all displayed a single, polar flagellum. After ~500 generations of experimental evolution inducible mutator, general mutator, and non-mutator cells alike (C and D) lacked flagella and were often associated with a thick extracellular matrix.

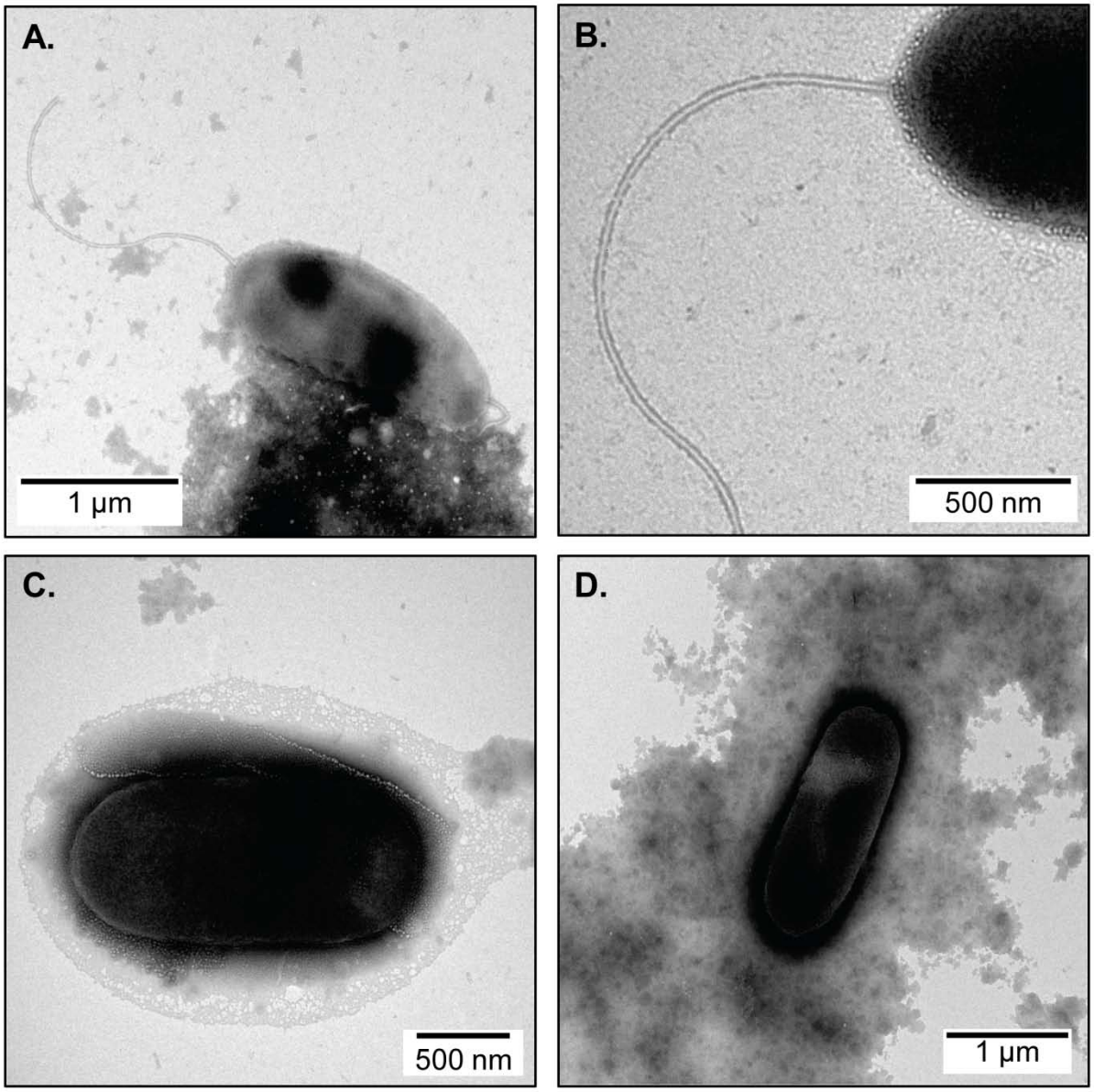


Figure 5-4. Derived populations display altered motility on 0.3% agar. (A) Motility was identical among ancestral genotypes PAO1/pJJK25, PW7148, and PAO1. (B) Inducible mutator lineages 44 and 46 were severely impaired in motility compared to PAO1/pJJK25. (C) General mutator lineages 51 and 52 and (D) non-mutator lineage 55 exhibited motility that differed from the canonical swimming of PW7148 and PAO1, respectively.

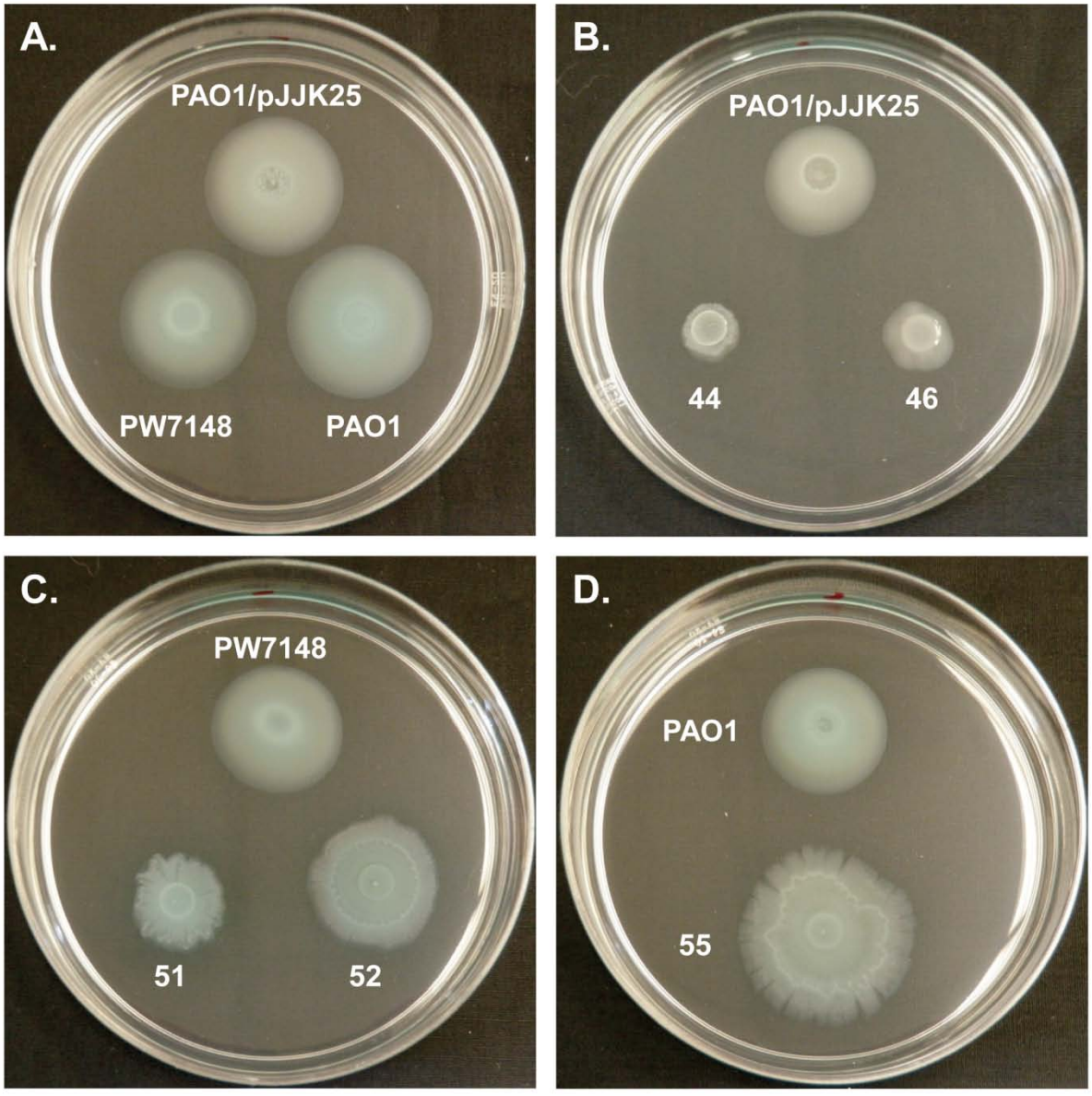
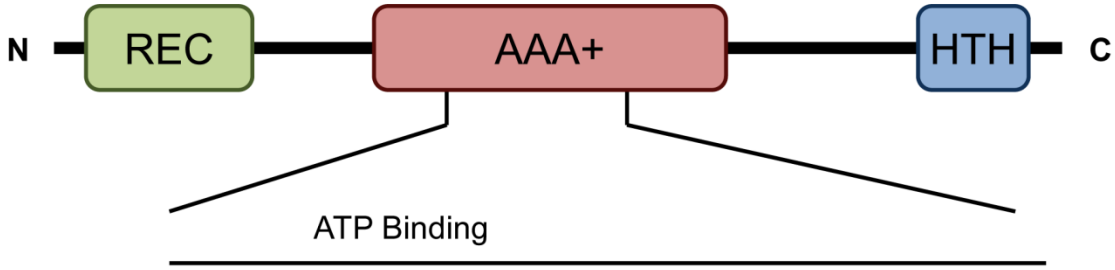


Figure 5-5. Nonsynonymous mutations in *fleQ* in isolates from inducible and general mutator lineages of *P. aeruginosa*. The three domain structure of FleQ includes an N-terminal REC signal receiving, an AAA+ ATPase, and a helix-turn-helix DNA binding domains. All observed mutations were located within the ATP binding region of the AAA+ domain. Only the variable amino acids are shown. The ancestral residues are listed at the top and the observed mutant substitutes are indicated below.



Genotype	Residue	↑	↑	↑	↑	↑	↑	↑	↑
Ancestral		N	A	L	E	H	D	R	H
44-1	214	.	.	.	G	.	.	.	.
51-1	202	S	.	.	.	.	.	.	.
51-2	218	.	.	.	.	R	.	.	.
51-3	260	.	.	.	.	.	.	C	.
51-4	287	.	.	.	.	.	.	.	P
52-1	205	.	V	.	.	.	.	.	.
52-2	210	.	.	P	.	.	.	.	.
52-3	218	.	.	.	.	R	.	.	.
52-4	245	.	.	.	.	.	G	.	.

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CHAPTER 6: Concluding Remarks

SUMMARY

Whether resulting from permanent defects in DNA repair or temporally regulated in response to external stress; the pervasiveness of hypermutation within bacterial populations is apparent. Yet, hypermutator genotypes have not been shown to confer a direct fitness advantage (De Visser 2002), but rather their abundance likely results from hitchhiking with secondary, beneficial mutations (Giraud *et al*. 2001, Tenaillon *et al*. 2001). Because many mutations are deleterious, organisms possessing elevated mutation rates, whether constitutive or transient, must be primed to capitalize on the occurrence of rare, beneficial mutations.

Not surprisingly, mutators are overrepresented in pathogenic bacteria which regularly experience host defense stress and fluctuating environmental conditions during infection cycles. The presence of hypermutable strains of *P. aeruginosa* in chronic airway infections of cystic fibrosis (CF) patients represents the best studied example of the abundance and importance of hypermutators within a natural bacterial population (Oliver *et al.* 2000, Oliver and Mena 2010). During chronic CF infection, initially clonal populations of *P. aeruginosa* undergo dramatic phenotypic diversification (Smith *et al.* 2006, Hoboth *et al.* 2009) and there is growing evidence to support the role of hypermutation in facilitating this adaptive transition (Hogardt *et al*. 2007, Moyano *et al*. 2007, Mena *et al*. 2008, Ciofu *et al*. 2010). Alternatively, inducible hypermutability in the form of mutagenic DNA repair is mediated by error-prone Y-family polymerases as part of the SOS response (Schlacher and Goodman 2007). Y-family polymerases also confer UVR tolerance, making them integral to the epiphytic survival of plant pathogenic bacteria residing in habitats optimized for solar UVR exposure (Kim and Sundin 2000, Sundin and Murillo 1999). Additional direct and indirect triggers of the SOS response suggest that

inducible hypermutation is likely active in bacterial populations dwelling in diverse ecological niches (Erill *et al.* 2007).

The widespread existence of mutator genotypes suggests that hypermutation contributes to the enduring success of bacteria. Additional variation supplied by an increased mutation rate offers fuel for natural selection. Therefore, hypermutation may contribute to adaptive evolution by expanding access to beneficial sequence alterations in order to gain fitness. Fortunately, bacteria are well suited for the study of adaptation through experimental evolution (Elena and Lenski 2003). The experiments presented in this dissertation have centered on laboratory-derived lineages of *P. cichorii* 302959 and *P. aeruginosa* PAO1 mutators. Subsequent experiments were performed to address the influence of hypermutation on adaptation within these contextually relevant and tractable model systems.

Experimental evolution with *P. cichorii* 302959, an epiphytic pathogen of celery, was conducted to examine the long-term effects of inducible hypermutation in the form of mutagenic DNA repair. Although the *E. coli umuDC* system has been well studied previously, enteric bacteria do not routinely incur UVR-mediated DNA damage, making them poorly suited for investigating the evolutionary implications of inducible hypermutation. Alternatively, epiphytic plant pathogens inhabit leaf surfaces where they experience high UVR exposure and inducible hypermutation contributes to survival by also conferring UVR tolerance (Sundin and Murillo 1999). The ecological relevance and pronounced transient mutator phenotype of *P. cichorii* 302959 make it an ideal model for investigating the impact of inducible hypermutation on adaptation.

Following propagation, parallel lineages of *P. cichorii* 302959 exhibited increased relative fitness, both in the presence and absence of UVR exposure, confirming their adaptation

to the laboratory environment. All lineages displayed a specificity of adaptation in favor of the native conditions, either UVR or non-UVR, under which they were propagated which correlated with discrete improvements in growth. In those lineages where inducible mutability was activated, a discrete colony morphology variant emerged and fluctuated in abundance over time. The experimental evolution of *P. cichorii* 302959 presented in this dissertation suggests that inducible hypermutation does not interfere with normal patterns of adaptation and may provide additional adaptive opportunities by increasing variation.

Experimental evolution with *P. aeruginosa* PAO1 was conducted to compare the adaptive influences of *mutS*-deficient and *rulAB*-mediated hypermutation. Inactivation of *mutS* is the most common defect associated with constitutive hypermutation (Hall and Henderson-Begg 2006) and leads to higher rates of base substitutions, frameshift mutations, and recombination (Smania *et al*. 2004). Conversely, template-independent, translesion DNA synthesis associated with *rulAB*-mediated mutagenic DNA repair produces base substitutions and frameshift mutations (Maor-Shoshani *et al*. 2000). These mechanistic differences likely produce unique mutation spectra and therefore unequal access to beneficial mutations. The laboratory strain *P. aeruginosa* PAO1 serves as an appropriate model to investigate the influence of hypermutation on adaption given the clinical importance of *P. aeruginosa* mutators in chronic CF infections. Furthermore, a completed genome sequence (Stover *et al.* 2000), transposon insertion mutant library (Jacobs *et al*. 2003), and extensive body of previous research (Winsor *et al.* 2009) provide tools for advanced genetic analysis.

All parallel lineages of *P. aeruginosa* PAO1 exhibited increased relative fitness following experimental evolution, independent of mutator status. The investigation of Rif^R mutations and a draft genome sequence confirmed the divergent point mutation spectra of *mutS*-deficient and

rulAB-mediated hypermutation. However, the comparison of transcriptome profiles from select lineage populations revealed a shared down regulation of flagellar biosynthesis and chemotaxis genes. Furthermore, changes in motility correlated with mutations in the central regulatory gene *fleQ* in general and inducible mutator lineages but not in a non-mutator lineage. The experimental evolution of *P. aeruginosa* PAO1 suggests that, despite unique point mutation spectra and divergent gene expression profiles, *mutS*-deficient and *rulAB*-mediated hypermutation can still provide access to similar beneficial phenotypes. Divergent expression traits exhibited by replicate general and inducible mutator lineages may represent a broad exploration of sequence space in search of beneficial mutations.

Taken together, the experimental evolution of *P. cichorii* 302959 and *P. aeruginosa* PAO1 presented in this dissertation suggest that hypermutation does not impede adaptation through mutation accumulation. In fact, both model organisms exhibited comparable, parallel improvements in relative fitness, independent of mutator status. Hypermutation in lineages of both *P. cichorii* 302959 and *P. aeruginosa* PAO1 facilitated the emergence of new colony morphologies that were not observed in non-mutator lineages. The diversity in colony morphologies and divergent gene expression profiles in mutator lineages likely represent an exploration of adaptive sequence space that is no doubt a beneficial strategy for bacterial pathogens during host infection. Such patterns of parallel adaptation and diversification are classic indicators of natural selection. Although the experiments presented here represent only a very short window in evolutionary time, the results of this dissertation confirm that such studies can still be informative. Finally, some adaptive traits were observed in common with chronic populations of hypermutable bacterial pathogens, confirming that *in vitro* adaptation to very simple, defined environments can offer insights into more complex, *in vivo* situations.

FUTURE CONSIDERATIONS

P. aeruginosa PAO1 provides an attractive model for the continued study of hypermutation and adaptation given its advanced genetic capabilities and relation to a deadly opportunistic pathogen. The prevalence of mutator strains in chronic CF infecting populations of *P. aeruginosa* has been well studied (Oliver and Mena 2010) and hypermutation likely facilitates the adaptive transitions necessary for chronic persistence of *P. aeruginosa* in the CF lung. Study of the laboratory strain *P. aeruginosa* PAO1 is facilitated by a completed genome sequence (Stover *et al.* 2000), well curated genome annotation database (Winsor *et al.* 2009), and comprehensive transposon mutant library (Jacobs *et al.* 2006). Laboratory-derived lineages of *P. aeruginosa* PAO1 studied in this dissertation have revealed positive signals of evolution including adaptive fitness phenotypes and genotypic diversification. The results presented here lay a solid foundation for numerous short- and long-term future studies focused on the further characterization of replicate lineage populations already established.

The draft genome sequence of an isolate from inducible mutator lineage 44 highlighted over 400 sequence variations relative to the published PAO1 reference and some of these likely represent beneficial mutations. However, the scope of this dissertation did not permit the correlation of these sequence variations with improved fitness under DNA-damaging, UVR conditions. Mutations in genes with annotated functions in DNA processing provide good candidates for improving fitness specifically under UVR conditions. Among these, a nonsynonymous mutation in *dnaE2* (PA0669), which encodes a second copy of the DNA polIII α-subunit, could represent a beneficial mutation given the predicted function of DnaE2 in response to DNA damage (Erill *et al.* 2006, Sanders *et al.* 2006). Relative fitness of a *dnaE2*-deficient mutant and a mutant complimented with the variant *dnaE2* could identify the adaptive

nature of this mutation. If the variant *dnaE2* is beneficial, its presence and distribution could be tracked throughout the evolutionary history of inducible mutator lineage 44 and correlated with improvements in relative fitness. Likewise, mutations in *dnaE2* could also be investigated in replicate lineages and the presence or absence of *dnaE2* mutation may correspond to variations in fitness under UVR conditions. Observed parallel mutation would strongly suggest selection in favor of mutation at the *dnaE2* locus.

More striking is the presence of three large genomic regions without any sequence alterations. Sequence or structural contexts that influence the distribution of mutations created by inducible hypermutation are not known and these regions could provide some clues. These observations highlight the limitations of making informed conclusions based on the genome sequence of only a single isolate. The power of comparative genome sequencing to identify parallel, adaptive mutational events has been demonstrated (Conrad *et al.* 2009). A comparison of inducible and general mutator isolate genomes could both further elucidate the divergent mutation spectra associated with specific mechanisms of hypermutation and aid in the identification of beneficial mutations.

Transcriptome profiling of select inducible and general mutator lineage populations revealed a shared down regulation of flagellum biosynthetic genes and individual cells lacked flagella when visualized by transmission electron microscopy. PCR fragment sequencing of key flagellum regulator genes led to the discovery of parallel, nonsynonymous mutation in the ATP-binding region of FleQ in a few inducible and general mutator lineages. Given the complex regulatory hierarchy controlling flagellar biosynthesis (Dasgupta *et al*. 2003), mutation at different nodes could likely produce similar down regulation of flagellar genes. However, mutations in genes at different levels in the flagellum regulatory cascade could have varied

impacts on fitness. Simply reducing the production of FliC, the structural subunit of the flagellum fiber, by deletion of genes directly related to *fliC* expression may be enough to increase fitness. However, deletion of higher-level regulators, like FleQ or RpoN, may provide additional fitness because without them fewer resources are invested in flagellar structural components. Comparing the fitness of insertion mutants deficient in different flagellum regulators could highlight the relative benefits associated with various levels of flagellum repression. Understanding how deficiency in each gene contributes to fitness may shed light on the observed differences in fitness between replicate lineages if they bear different flagellum regulatory mutations.

Mutations in *fleQ* were absent in isolates from non-mutator lineage 55. Other similar studies have observed parallel phenotypes in replicate lineages, each associated with unique regulatory mutations (Applebee *et al.* 2008). However, PCR fragment sequencing of other key regulators also failed to identify mutations in this lineage. An alternate approach to understanding the down regulation of flagellum biosynthesis may be to focus on the operon structure of flagellar genes. In *P. aeruginosa* PAO1, flagellum structural and regulatory genes are organized into 17 operons distributed across three genomic regions (Dasgupta *et al.* 2003). Coordinating gene expression patterns with the division of genes among operons could provide candidate genes or promoters for investigation. If mutated, these loci could block flagellum assembly without directly altering central regulatory proteins. For example, within the three gene operon *fliD-fliS-fleP*, only *fliS* and *fleP* were down regulated in non-mutator lineage 55. A polar mutation in *fliD* or *fliS* could explain this selective pattern of down regulation and may be sufficient to prevent formation of a functional flagellum.

During experimental evolution of both *P. cichorii* 302959 and *P. aeruginosa* PAO1, hypermutation facilitated the emergence of new colony morphologies that were not observed in non-mutator lineages. Such diversification is a common feature of adaptive evolution (MacLean 2005), but also creates new opportunities for social interaction within once clonal populations (West *et al.* 2006). In similar studies, metabolic cross-feeding (Turner *et al.* 1996) and exploitation of secreted 'public goods' (Harrison and Buckling 2011) have been reported following adaptive radiation. Furthermore, hypermutable populations of *P. aeruginosa* have been shown to develop social 'cheaters' more frequently than non-mutators during experimental evolution (Racey *et al.* 2010, Harrison and Buckling 2011). Through gene expression profiling of select populations of *P. aeruginosa* PAO1 in this dissertation, replicate lineages exhibited divergent expression of genes associated with quorum sensing, exopolysaccharide production, lipopolysaccharide biosynthesis, and biofilm formation. A thick extra cellular matrix surrounding cells of derived mutator populations was also visible by transmission electron microscopy. Colorimetric differences between lineage cultures grown in LB broth or on KB agar were also observed suggesting altered production of siderophores. Any of these traits has the potential to create complex social interactions that would coincide with the observation of higher fitness in population samples compared to individual isolates. That these interactions, and the requisite adaptive radiation, appeared to require hypermutation in this study is significant. This could highlight a fundamental role for hypermutation in the evolution of bacterial populations; a wider exploration of adaptive sequence space increases diversity and thus creates opportunities for social interaction that contribute to population fitness. Further studies are needed to address this hypothesis and the model system of experimental evolution with *P. aeruginosa* PAO1 hypermutators established in this dissertation is ideally suited for this purpose.

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