Investigating the effects of genetic variation and environmental fluctuations on the evolution of laboratory populations of *Escherichia coli*

A thesis submitted in partial fulfilment of the requirement

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by

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CERTIFICATE

Certified that the work incorporated in thesis titled '**Investigating the effects of genetic variation and environmental fluctuations on the evolution of laboratory populations of *Escherichia coli***', submitted by Selveshwari S was carried out by the candidate, under my supervision. The work presented here or any part of it has not been included in any other thesis submitted previously for the award of any degree or diploma from any other university or institution.

Dr. Sutirth Dey
Supervisor

Date: 02-Feb-2022

DECLARATION

I declare that this written submission represents my ideas in my own words and where others ideas have been included, I have adequately cited and referred to original sources. I also declare that I have adhered to all principles of academic honesty and integrity and I have not misinterpreted or fabricated or falsified any idea/data/fact/source in my submission. I understand that violation of the above can cause disciplinary action by the institute and evoke penal action from the sources which have thus not been properly cited or from whom proper permission has not been taken when needed.

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CONTENTS

Synopsis

Thesis title: Investigating the effects of genetic variation and environmental fluctuations on the evolution of laboratory populations of *Escherichia coli*

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Chapter 1: Introduction

Inherited variation is central to evolution by natural selection. In asexual organisms, mutations, arising from erroneous replication of the genetic material, are the ultimate source of variation. An increase in mutation rate which, increases the supply of variation, can influence the rate and extent of adaptation (Sniegowski *et al.* 1997; Taddei *et al.* 1997; Stich *et al.* 2010; Wielgoss *et al.* 2013). A number of genetic and environmental factors have been studied as mutation rate modifiers (reviewed in (Tenaillon *et al.* 2004; Galhardo *et al.* 2007; Ram & Hadany 2012). Ultraviolet (UV) radiation is one such extensively studied environmental mutagen. Extensive studies have shown that factors such as bacterial physiology and environmental fluctuations influence the microorganisms' response to UV radiation (Child *et al.* 2002; Dantur & Pizarro 2004; Sukhi *et al.* 2009; Bucheli-Witschel *et al.* 2010). Such factors can also influence adaptation by shaping UV induced selection and mutagenesis.

While mutations are a major source of variation, migration is an important factor that influences the distribution as well as maintenance of variation. Studies on migration in simple/constant environment have shown that migration can promote (Bell & Gonzalez 2011; Lagator *et al.* 2014) as well as impede (Morgan *et al.* 2005; Vogwill *et al.* 2011; Lawrence *et al.* 2016) adaptation. However, its effect on populations evolving in temporally fluctuating environment is less explored. Interestingly, fluctuating environments are also important in the maintenance of variation (Hallsson & Björklund 2012; Canino-Koning *et al.* 2019; Nguyen *et al.* 2021). While the predictability and frequency of fluctuations is expected to influence the evolutionary outcomes, few empirical studies have directly compared the effects of the nature of fluctuation on adaptation. In my thesis, I have used a combination of experimental evolution of laboratory populations of *Escherichia coli* and whole genome sequencing to address some of these lacunae. The key results are summarized below.

Chapter 2: Genomic signatures of UV resistance evolution in *Escherichia coli* depend on the growth phase during exposure

Physiological states and growth conditions of a microbial cell can influence its ability to handle stress (Gilbert *et al.* 1990; Lindqvist & Barmark 2014; Lin & Kussell 2016). I observed this in laboratory populations of *E.coli* where exponential phase cultures were more sensitive to UV radiation induced mortality than lag phase cultures. However, replicate populations exposed separately to UV radiation for 100 cycles in the two growth phases, showed no difference in their sensitivity to UV radiation. Interesting differences were observed in the genome of the evolved populations. Mutations in different functional groups were accumulated in populations subjected to lag and exponential growth phases. Genes involved in transcriptional and translational regulation was mutated in lag exposed

populations whereas, signal transduction and cell adhesion genes were mutated in exponential phase exposed populations. However, these genomic differences did not translate into phenotypic difference in fitness assayed in a number of novel environments. The results suggested that physiological differences influence selection pressures resulting in genomic signatures of adaptation, without observable phenotypic differences.

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Chapter 3: Fluctuating exposures to UV radiation and Erythromycin result in increased mean and variance in fitness in novel environments

UV radiation is known for its GC→AT transition bias in mutagenesis (Brash 2015). Such mutations biases have been shown to influence evolutionary trajectories (Stoltzfus & McCandlish 2017; Payne *et al.* 2019; Storz *et al.* 2019; Cano & Payne 2020; Gomez *et al.* 2020). In line with this expectation, when *E.coli* populations were exposed to UV radiation during evolution of resistance to erythromycin, they evolved to be genotypically different from the control (i.e. unexposed to UV) populations. While both UV exposed and control populations had fixed for an efflux pump mutation (*acrB*), the un-exposed control populations had an additional target site mutation (*rplD*) whereas, the UV exposed population had an additional efflux pump regulator mutation (*acrR*). However, this was not accompanied by phenotypic difference in resistance to erythromycin. I also studied the effect of constant vs. fluctuating exposures to UV. Interestingly, populations subjected to fluctuations in UV

and erythromycin exposures had evolved the highest mean and variation in fitness to novel antibiotic environments. Genetically distinct subpopulations, each resistant to a different antibiotic, coexisted in the fluctuating treatment. My results are in line with prior studies on the effect of mutation rates in fluctuating environments (Ishii *et al.* 1989; Travis & Travis 2002; Carja *et al.* 2014) and highlight the role of interactions between mutation rate modifiers and temporal fluctuations in shaping genetic variation and evolutionary trajectories.

Chapter 4: The effect of migration and variation on populations of *Escherichia coli* adapting to complex fluctuating environments

Migration, a key evolutionary force, has been shown to promote (Bell & Gonzalez 2011; Lagator *et al.* 2014) as well as impede (Morgan *et al.* 2005; Vogwill *et al.* 2011; Lawrence *et al.* 2016) adaptation. Unfortunately, most studies on the evolutionary effects of migration have been limited to simple and /or constant environments. Very little is known about the effects of migration on adaptation to complex as well as fluctuating environments. I used replicate population of *Escherichia coli*, to study the effect of migration on adaption to complex and unpredictably fluctuating environments. Here, I subjected populations to different proportions of clonal ancestral immigrants. Contrary to the results from simple/constant environments (Perron *et al.* 2007), clonal immigrants resulted in the reduction of all measured proxies of fitness. However, migration from a source population with larger variation for fitness resulted in little or no change in fitness w.r.t the no-migration control. Thus, the presence of variation in the immigrants could counter the negative effects of migration in complex and unpredictably fluctuating environments. My results demonstrate that the effects of migration are strongly dependent on the nature of the destination environment as well as the genetic makeup of immigrants. These results enhance our

understanding of the influences of migrating populations, which could help better predict the consequences of migration.

Chapter 5: Frequency and predictability of fluctuations have very little effect on adaptation in *Escherichia coli* populations

Adaptation in fluctuating environments has been shown to differ from adaptation in constant environments (Gilchrist 1995; Kassen 2002; Condon *et al.* 2014; Kassen 2014; Haaland *et al.* 2020). However, adaptation in fluctuating environment may not be consistent but is expected to be influenced by the predictability and frequency of fluctuation. I subjected laboratory populations of *Escherichia coli* to environments that fluctuated every 12 or 24 or 72 hours, either predictably or unpredictably. When fitness was assayed after 240 generations and 720 generations of evolution, I observed no strong effect of either predictability or frequency of fluctuation on adaptation. The only exception was predictable environments fluctuating every 12 hours. However, the observation was limited to fitness measured as growth rate in only one of the environments. Moreover, the trend of fitness differences was reversed between 240 generations and 720 generations. My results are consistent with prior studies that report no effects of predictability (Turner & Elena 2000; Karve *et al.* 2018) and frequency of fluctuation (Kassen & Bell 1998; Scheiner & Yampolsky 1998; Buckling *et al.* 2007) on adaptation.

Chapter 6: Conclusions and Future directions

I summarise the key findings, discuss their implications and possible future directions.

References

Bell, G. & Gonzalez, A. (2011). Adaptation and evolutionary rescue in metapopulations experiencing environmental deterioration. *Science*, 332, 1327-1330.

Brash, D.E. (2015). UV signature mutations. *Photochemistry and photobiology*, 91, 15-26.

Bucheli-Witschel, M., Bassin, C. & Egli, T. (2010). UV-C inactivation in Escherichia coli is affected by growth conditions preceding irradiation, in particular by the specific growth rate. *Journal of applied microbiology*, 109, 1733-1744.

Buckling, A., Brockhurst, M.A., Travisano, M. & Rainey, P.B. (2007). Experimental adaptation to high and low quality environments under different scales of temporal variation. *Journal of evolutionary biology*, 20, 296-300.

Canino-Koning, R., Wiser, M.J. & Ofria, C. (2019). Fluctuating environments select for short-term phenotypic variation leading to long-term exploration. *PLoS computational biology*, 15, e1006445.

Cano, A.V. & Payne, J.L. (2020). Mutation bias interacts with composition bias to influence adaptive evolution. *PLoS computational biology*, 16, e1008296.

Carja, O., Liberman, U. & Feldman, M.W. (2014). Evolution in changing environments: Modifiers of mutation, recombination, and migration. *Proceedings of the National Academy of Sciences*, 111, 17935-17940.

Child, M., Strike, P., Pickup, R. & Edwards, C. (2002). Salmonella typhimurium displays cyclical patterns of sensitivity to UV-C killing during prolonged incubation in the stationary phase of growth. *FEMS microbiology letters*, 213, 81-85.

Condon, C., Cooper, B.S., Yeaman, S. & Angilletta Jr, M.J. (2014). Temporal variation favors the evolution of generalists in experimental populations of Drosophila melanogaster. *Evolution*, 68, 720-728.

Dantur, K.I. & Pizarro, R.A. (2004). Effect of growth phase on the Escherichia coli response to ultraviolet-A radiation: influence of conditioned media, hydrogen peroxide and acetate. *Journal of Photochemistry and Photobiology B: Biology*, 75, 33-39.

Galhardo, R.S., Hastings, P.J. & Rosenberg, S.M. (2007). Mutation as a stress response and the regulation of evolvability. *Critical reviews in biochemistry and molecular biology*, 42, 399-435.

Gilbert, P., Collier, P.J. & Brown, M. (1990). Influence of growth rate on susceptibility to antimicrobial agents: biofilms, cell cycle, dormancy, and stringent response. *Antimicrobial agents and chemotherapy*, 34, 1865-1868.

Gilchrist, G.W. (1995). Specialists and generalists in changing environments. I. Fitness landscapes of thermal sensitivity. *The American Naturalist*, 146, 252-270.

Gomez, K., Bertram, J. & Masel, J. (2020). Mutation bias can shape adaptation in large asexual populations experiencing clonal interference. *Proceedings of the Royal Society B*, 287, 20201503.

Haaland, T.R., Wright, J. & Ratikainen, I.I. (2020). Generalists versus specialists in fluctuating environments: a bet-hedging perspective. *Oikos*, 129, 879-890.

Hallsson, L.R. & Björklund, M. (2012). Selection in a fluctuating environment leads to decreased genetic variation and facilitates the evolution of phenotypic plasticity. *Journal of evolutionary biology*, 25, 1275-1290.

Ishii, K., Matsuda, H., Iwasa, Y. & Sasaki, A. (1989). Evolutionarily stable mutation rate in a periodically changing environment. *Genetics*, 121, 163-174.

Karve, S.M., Bhave, D. & Dey, S. (2018). Extent of adaptation is not limited by unpredictability of the environment in laboratory populations of Escherichia coli. *Journal of evolutionary biology*, 31, 1420-1426.

Kassen, R. (2002). The experimental evolution of specialists, generalists, and the maintenance of diversity. *Journal of evolutionary biology*, 15, 173-190.

Kassen, R. (2014). *Experimental evolution and the nature of biodiversity*. Roberts, Colorado, United States.

Kassen, R. & Bell, G. (1998). Experimental evolution in Chlamydomonas. IV. Selection in environments that vary through time at different scales. *Heredity*, 80, 732-741.

Lagator, M., Morgan, A., Neve, P. & Colegrave, N. (2014). Role of sex and migration in adaptation to sink environments. *Evolution*, 68, 2296-2305.

Lawrence, D., Bell, T. & Barraclough, T.G. (2016). The effect of immigration on the adaptation of microbial communities to warming. *The American Naturalist*, 187, 236-248.

Lin, W.-H. & Kussell, E. (2016). Complex interplay of physiology and selection in the emergence of antibiotic resistance. *Current Biology*, 26, 1486-1493.

Lindqvist, R. & Barmark, G. (2014). Specific growth rate determines the sensitivity of Escherichia coli to lactic acid stress: implications for predictive microbiology. *BioMed research international*, 2014.

Morgan, A.D., Gandon, S. & Buckling, A. (2005). The effect of migration on local adaptation in a coevolving host–parasite system. *Nature*, 437, 253-256.

Nguyen, J., Lara-Gutiérrez, J. & Stocker, R. (2021). Environmental fluctuations and their effects on microbial communities, populations and individuals. *FEMS microbiology reviews*, 45, fuaa068.

Payne, J.L., Menardo, F., Trauner, A., Borrell, S., Gygli, S.M., Loiseau, C. *et al.* (2019). Transition bias influences the evolution of antibiotic resistance in Mycobacterium tuberculosis. *PLoS biology*, 17, e3000265.

Perron, G.G., Gonzalez, A. & Buckling, A. (2007). Source–sink dynamics shape the evolution of antibiotic resistance and its pleiotropic fitness cost. *Proceedings of the Royal Society B: Biological Sciences*, 274, 2351-2356.

Ram, Y. & Hadany, L. (2012). The evolution of stress-induced hypermutation in asexual populations. *Evolution*, 66, 2315-2328.

Scheiner, S.M. & Yampolsky, L.Y. (1998). The evolution of Daphnia pulex in a temporally varying environment. *Genetics Research*, 72, 25-37.

Sniegowski, P.D., Gerrish, P.J. & Lenski, R.E. (1997). Evolution of high mutation rates in experimental populations of E. coli. *Nature*, 387, 703-705.

Stich, M., Manrubia, S.C. & Lázaro, E. (2010). Variable mutation rates as an adaptive strategy in replicator populations. *PLoS One*, 5, e11186.

Stoltzfus, A. & McCandlish, D.M. (2017). Mutational biases influence parallel adaptation. *Molecular biology and evolution*, 34, 2163-2172.

Storz, J.F., Natarajan, C., Signore, A.V., Witt, C.C., McCandlish, D.M. & Stoltzfus, A. (2019). The role of mutation bias in adaptive molecular evolution: insights from convergent changes in protein function. *Philosophical Transactions of the Royal Society B*, 374, 20180238.

Sukhi, S.S., Shashidhar, R., Kumar, S.A. & Bandekar, J.R. (2009). Radiation resistance of Deinococcus radiodurans R1 with respect to growth phase. *FEMS microbiology letters*, 297, 49-53.

Taddei, F., Radman, M., Maynard-Smith, J., Toupance, B., Gouyon, P.-H. & Godelle, B. (1997). Role of mutator alleles in adaptive evolution. *Nature*, 387, 700-702.

Tenaillon, O., Denamur, E. & Matic, I. (2004). Evolutionary significance of stress-induced mutagenesis in bacteria. *Trends in microbiology*, 12, 264-270.

Travis, J. & Travis, E. (2002). Mutator dynamics in fluctuating environments. *Proceedings of the Royal Society of London. Series B: Biological Sciences*, 269, 591-597.

Turner, P.E. & Elena, S.F. (2000). Cost of host radiation in an RNA virus. *Genetics*, 156, 1465-1470.

Vogwill, T., Fenton, A. & Brockhurst, M.A. (2011). Coevolving parasites enhance the diversity-decreasing effect of dispersal. *Biology letters*, 7, 578-580.

Wielgoss, S., Barrick, J.E., Tenaillon, O., Wiser, M.J., Dittmar, W.J., Cruveiller, S. *et al.* (2013). Mutation rate dynamics in a bacterial population reflect tension between adaptation and genetic load. *Proceedings of the National Academy of Sciences*, 110, 222-227.

Chapter 1: Introduction

Natural environments are hardly ever constant. Instead, organisms are often faced with spatial and/or temporal heterogeneity in the environment resulting in fluctuations in both intensity and direction of natural selection. Evolution in heterogeneous environments can influence both mean and variance of fitness (Via & Lande 1987; Van Tienderen 1991; Kassen 2002; Byers 2005). Spatially heterogeneous environments, connected by migration, have been shown to limit/slowdown adaptation (Cuevas *et al.* 2003). This is because repeated introduction of locally maladapted individuals/alleles by migration can prevent the fixation of locally fit genotypes, diluting the strength of selection (Kawecki & Holt 2002; Lenormand 2002; Kawecki & Ebert 2004; Yeaman & Guillaume 2009). In temporally fluctuating environments, populations are subjected to one environment followed by another. Such fluctuating selection pressures between the different environments can prevent the fixation of any single genotype, slowing the rate of adaptation (Kassen 2014). However, the increased time to fixation in both spatially and temporally heterogeneous environments can promote the maintenance of greater variance in fitness within the populations (Kassen 2014). In this case, genetic variation may be transiently maintained, which may eventually be lost due to the fixation of a generalist phenotype that is fittest in all constituent environments. Conversely, variance in fitness can increase as a result of the evolution of phenotypic plasticity or bet-hedging strategies which has been shown to be favoured in heterogeneous environments (Kussell *et al.* 2005; Kussell & Leibler 2005; Acar *et al.* 2008; Beaumont *et al.* 2009; Patra & Klumpp 2014). Although both these mechanisms give rise to multiple phenotypes from the same genotype, phenotypic plasticity is expected to be favoured in predictable environments and bet-hedging in stochastic environments (Simons 2011). Other studies have shown that depending on the scale of variation, heterogeneous environments may promote the stable co-existence of specialist phenotypes (Rodríguez-Verdugo & Ackermann 2021). In short, organisms respond to environmental heterogeneity in multiple ways, influenced by different

elements of heterogeneity such as the nature (spatial vs. temporal), predictability (predictable vs. stochastic), and scale of variation (fine vs. coarse). Consequently, understanding evolution in heterogeneous environments is a fundamental question in evolutionary biology.

Of all the factors influencing adaptation in heterogeneous environments, migration is a key evolutionary force, particularly in spatially heterogeneous environments. Gene flow resulting from the migrating individuals can have contrasting effects on adaptation. On one hand, migration can limit adaptation and phenotypic divergence by altering allele frequencies shaped by local selection (Lenormand 2002; Kawecki & Ebert 2004). In extreme cases, high migration load can swamp local adaptation, resulting in loss of genetic variation in the population (Lenormand 2002; Kawecki & Ebert 2004). On the other hand, migration is an important source of variation, particularly in mutation limited populations (Holt & Gomulkiewicz 1997; Hermsen & Hwa 2010). It can facilitate rapid adaptation by promoting the spread of beneficial alleles between subpopulations. Thus, migration can have contrasting effects on adaptation influenced by the nature of environments. Migration between same/similar environments has been observed to facilitate faster adaptation (Dennehy *et al.* 2010; Bell & Gonzalez 2011) whereas, migration between unrelated /antagonistic environments limits evolution (Cuevas *et al.* 2003; Dennehy *et al.* 2010). Understanding the effects of gene flow in the context of pleiotropy between environments and/or fitness effects of alleles in the different environments may provide some explanation for the contrasting effects of migration on adaptation. However, most empirical studies on the effects of migration on adaptation have used constant single environments (Morgan *et al.* 2005; Dennehy *et al.* 2010; Ching *et al.* 2013; Lagator *et al.* 2014; Lawrence *et al.* 2016). The effect of migration on adaptation in the presence of multiple stress environments and temporally fluctuating environment has received relatively less attention (however see (Perron *et al.* 2007).

Pleiotropy between environments and traits may influence the evolution of specialist and generalist phenotypes in spatial and temporally heterogeneous environments (Van Tienderen 1991; Reboud & Bell 1997; Kassen 2002; Wang & Dai 2019). Additionally, the predictability of fluctuations is known to play a crucial role in influencing adaptation in temporally fluctuating environments. When the environment fluctuates predictably evolution of phenotypic plasticity may be favoured as it can lead to rapid generation of alternate phenotypes (West-Eberhard 1989; Kussell & Leibler 2005; Murren *et al.* 2015). Unpredictable fluctuations, on the other hand, are expected to favour the evolution of diversifying bet-hedging strategy (Acar *et al.* 2008; Beaumont *et al.* 2009; Simons 2011). In addition to the predictability of fluctuation, the scale of fluctuation is another important factor known to influence adaptation. For example, when organisms are exposed to fine-scale fluctuations (fluctuation within an organism's lifetime) or intermediately fluctuations (once every few generations), a single genotype with the highest fitness across all constituent environments i.e., generalist, are expected to evolve (Kassen 2002; Wang & Dai 2019). Conversely, coarse-grained fluctuations may promote the sequential evolution of specialist phenotypes (Crill *et al.* 2000; Tufto 2015). Despite these predictions, empirical studies on the effects of these factors have not been conclusive. While some studies have shown that predictable and unpredictable fluctuations result in qualitative differences in evolutionary outcomes (Hughes *et al.* 2007; Alto *et al.* 2013), other studies find no effect of the predictability of fluctuations (Turner & Elena 2000; Karve *et al.* 2018). Additionally, the few empirical studies on the effects of the scale of fluctuations (Kassen & Bell 1998; Scheiner & Yampolsky 1998; Buckling *et al.* 2007) showed no differential effect on adaptation.

Microbial evolution under heterogeneous environments depends on an intricate interaction between selection and generation of de novo variation through mutation. Another environmental factor that can influence both selection and genetic variation is ultraviolet

radiation (UV). Since the first microbial life during the Archean eon, all terrestrial organisms have been subjected to ultraviolet radiation. All of UV-C radiation (100-280nm) and most of UV-B radiation (280-315nm) is absorbed by the ozone layer of the atmosphere. However, the small portions of UV-B radiation and all of UV-A radiation (315-400nm) that reach the earth's surface have damaging effects on all organisms: from prokaryotes to eukaryotes including plants, animals and humans (Sinha & Häder 2002). Although UV-C radiation has the highest germicidal property, it is irrelevant in the context of natural environments, as it is completely absorbed by the ozone layer. Nonetheless, artificial UV-C is extensively used for disinfection in laboratories (Wedum *et al.* 1956; Gefrides *et al.* 2010), clinical microbiology (Rutala *et al.* 2010; Ramos *et al.* 2020), food packing industry (Ansari & Datta 2003; Guerrero-Beltr· n & Barbosa-C· novas 2004) as well as in strain engineering in biotechnology (Hashimoto *et al.* 2005; Tillich *et al.* 2012). Exposure to UV radiation causes widespread damages to the cell including lesions in nucleic acids, damages to proteins and membranes and inactivation of enzymes (Cadet *et al.* 2005; Goosen & Moolenaar 2008) and references therein).

Organisms have evolved multiple, robust mechanisms for dealing with UV induced damages. The best-known example is the naturally radio-resistant species of the genus *Deinococcus*. *Deinococcus radiodurans* can survive up to 5000 Gy of radiation with no loss of viability (Battista 1997). In contrast, exposure to 30Gy of radiation is lethal in *Escherichia coli* (Battista 1997). The extreme radio-resistance in *Deinococcus* sp. has been attributed to the extensive clean-up of damaged cellular products and reactive oxygen species, proteome protection, and efficient repair of DNA damages (Blasius *et al.* 2008; Jin *et al.* 2019). While damaged DNA maybe accurately repaired via photoreactivation, excision repair, and recombination repair (reviewed in (Sinha & Häder 2002), extreme damage, that cannot be repaired, can result in cell-cycle arrest and death. UV induced mortality is a strong agent of

selection and repeated exposures to UV can select for increased resistance to it. Evolution of UV resistance, in the laboratory, has been observed in multiple species: *Moraxella spp*. (Keller & Maxcy 1984), *Lactobacillus spp*., (Hastings *et al.* 1986), *Halobacterium spp*. (Kottemann *et al.* 2005; DeVeaux *et al.* 2007), yeast (Fabre 1970), *Escherichia coli* (Morton & Haynes 1969; Dantur & Pizarro 2004; Bucheli-Witschel *et al.* 2010), *Cronobacter sakazakii* (Arroyo *et al.* 2012), and *Salmonella typhimurium* (Child *et al.* 2002). However, huge variations in UV resistance have been observed in natural as well as experimental populations. Multiple factors like growth rate (Keller & Maxcy 1984; Berney *et al.* 2006; Bucheli-Witschel *et al.* 2010), DNA content (Fabre 1970; Bucheli-Witschel *et al.* 2010), and nutrient availability (Child *et al.* 2002; Sukhi *et al.* 2009) have been shown to influence an organisms' response to UV radiation. However, little is known about the effects of these factors on long-term exposures to UV radiation and the evolution of resistance to it.

UV radiation is also one of the most important and well-studied natural mutagens. UV induced mutagenesis plays an important role in evolution since mutations are the raw material for natural selection. An organism's persistence through multiple environmental changes may be dependent on the generation of novel variation via mutation. Spontaneous mutation rates are small, of the order of 10^{-10} mutations per nucleotide per generation or 10^{-3} per microbial genome per generation (Lee *et al.* 2012). Moreover, only a small proportion of all mutations are beneficial and contribute to fitness increase (Eyre-Walker & Keightley 2007). However, given the large population sizes, microbial populations adapting to a new environment typically involve 1 to 4 mutations with a median of 3 mutations (Kassen 2014). Exposure to mutagens, such as UV radiations, can increase mutation rates and the number of mutations within a population, influencing the rate and extent of adaptation (Sniegowski *et al.* 1997; Taddei *et al.* 1997; Stich *et al.* 2010; Wielgoss *et al.* 2013).

UV induced mutations are known for their bias in the mutational spectrum GC → AT transitions (Brash 2015). Differences in the mutations spectrum have been suggested to be an important factor driving evolutionary trajectories (Yampolsky & Stoltzfus 2001; Stoltzfus & McCandlish 2017; Cano *et al.* 2021) and the references listed within). Analysis of adaptive substitutions from ~50 natural and experimental populations revealed that transitions were over-represented compared to transversions (Stoltzfus & McCandlish 2017). Differences in the occurrence and/or supply of the different mutational classes can result in fixation biases (Streisfeld & Rausher 2011) which can in turn influence fitness landscapes and the direction of adaptation (Yampolsky & Stoltzfus 2001). Studies on T7 bacteriophage (Cunningham *et al.* 1997) and *E.coli* (Couce *et al.* 2015) have shown that manipulation of mutational biases can influence the genomics of adaptive evolution. Despite the extensive studies on UV induced mutagenesis, the evolutionary effects of the mutational biases of UV have received less attention (Shibai *et al.* 2017).

In my thesis, I attempt to address some of the above-mentioned lacunae using experimental evolution in *Escherichia coli*. Experimental evolution is an extremely powerful and popular tool for studying evolutionary processes and testing evolutionary theories in real-time (Kawecki *et al.* 2012; Van den Bergh *et al.* 2018; McDonald 2019). In particular, microbial populations are ideal model systems for experimental evolution studies. They are small, easy to maintain, have short generation time, and can be propagated in large population sizes and replicates. Moreover, microbial systems offer precise and powerful control over factors that can influence adaptation. To begin with, the causes and consequences of environmental heterogeneity can be studied by easy experimental manipulation of the selection environment within smaller culture volumes. Precise control of the genetics of the founding populations is easy in microbial populations. In addition, whole-genome sequencing of the evolved populations is a relatively new but powerful technique to study the genomics underlying

evolutionary changes (Dettman *et al.* 2012; Cooper 2018). In the following chapters, I will discuss the results of five selection experiments that I conducted with the aim to understand the significance of genetic and environmental variation on adaptation.

Bacterial populations show variation in their sensitivity to UV induced mortality depending on the phase of growth during exposure (Morton & Haynes 1969; Fabre 1970; Dantur & Pizarro 2004; Sukhi *et al.* 2009; Bucheli-Witschel *et al.* 2010). However, no prior study has compared the effects of long-term exposures to UV radiation at different growth phases. In chapter 2, I investigate the effects of UV exposure during different growth phases on the evolution of UV resistance. Populations of *Escherichia coli* were evolved under two different conditions, namely exposure during the lag and the exponential growth phases. Initially, populations in the two treatments exhibited differential survival, with exponential phase exposures resulting in greater UV sensitivity. However, there were no phenotypic differences between the two treatments at the end of 100 cycles of exposure and growth. Interestingly, there were strong growth phase specific signatures of UV exposure at the level of the genome. Different functional groups were found mutated in the lag and exponential UV treatment. In the former, genes involved in transcriptional and translational regulations and cellular transport were mutated, whereas the latter treatment showed mutations in genes involved in signal transduction and cell adhesion. Curiously, the genomic differences did not translate into fitness differences, measured in a number of novel environments.

In chapter 3, I focus on UV induced mutagenesis to investigate the effects of UV induced mutation bias on the evolution of antibiotic (erythromycin) resistance. Additionally, given that increased mutation rates are favoured in heterogeneous environments (Ishii *et al.* 1989; Travis & Travis 2002; Carja *et al.* 2014), I attempted to understand the interaction between UV mutagenesis and temporally fluctuating environment. To this end, I studied the evolution of erythromycin resistance in replicate populations of *Escherichia coli* in the absence of UV

radiation as well as constant and fluctuating exposures to UV. While all populations evolved similar levels of resistance to erythromycin, exposure to UV radiation, both constant and fluctuating, resulted in alternate genomic routes to resistance. While both UV exposed and unexposed populations had fixed for efflux pump mutation (*acrB*), the un-exposed control populations had an additional target site mutation (*rplD*) whereas, the UV exposed population had an additional efflux pump regulator mutation (*acrR*). Fixation of target-site vs. regulator mutations is expected to result in trade-offs between MIC and growth rate (Andersson & Hughes 2010; Hughes & Andersson 2017; Santos-Lopez *et al.* 2019). However, all populations had evolved similar MIC and growth rates in erythromycin. Another key result from chapter 3 is that fluctuating exposures to UV and erythromycin resulted in increased mean and variation in fitness in novel antibiotic environments. Genomic analysis revealed that the fluctuation populations had fixed mutations in global regulator genes. It is possible that these genes promote noisy gene expression, introducing and aiding the maintenance of genetically distinct subpopulations, resistant to different antibiotics.

In chapter 4, I study the effects of migration in populations adapting to complex and unpredictably fluctuating environments. Replicate *Escherichia coli* populations were subjected to complex and unpredictably fluctuating environments as well as one-way migration. Populations were subjected to different levels of migration from a source population that was either clonal or had accumulated variation in fitness. In contrast to adaptation in a simple/constant environment (Perron *et al.* 2007), I saw that clonal migration impeded adaptation in complex unpredictably fluctuating environments. The extent of reduction in fitness was influenced by the rate of migration. In the second selection experiment, where migrants originated from a source population with a larger variance in fitness, migration resulted in little to no change in fitness, w.r.t the no migration control. This suggests that variation in the migrating individuals can alleviate some of the negative effects

of migration. It also highlights the significance of the nature of the environment in determining the effect of migration.

In chapter 5, I study the effects of the frequency and predictability of temporal fluctuations on adaptation. Replicate populations of *E.coli* were subjected to environments that fluctuated either predictably or unpredictably every 12, 24, or 72 hours. In line with previous studies (Kassen & Bell 1998; Scheiner & Yampolsky 1998; Buckling *et al.* 2007), the frequency of fluctuation had no effect on the extent of adaptation. Predictability of fluctuations also had no effect on adaptation. However, a minor effect was observed in populations exposed to predictable 12-hour fluctuations. But this effect was limited to fitness measured as growth rate in only one of the environments.

In the final chapter, I summarise the results, highlight key findings and discuss their implications. I also discuss possible follow-up studies, build on the current work, to further our understanding of the effects of mutation, migration and temporally fluctuating environments.

Chapter 2: Genomic signatures of UV resistance evolution in *Escherichia coli* depend on the growth phase during exposure

2.1. Introduction

Environmental stresses can strongly influence the growth and physiology of microbial cells (Aertsen & Michiels 2004; Schimel *et al.* 2007). At the same time, antecedent growth conditions and physiology of the cells can influence a microbe's response to environmental stresses (Gilbert *et al.* 1990; Lindqvist & Barmark 2014; Lin & Kussell 2016). For example, it has been shown that the response of bacteria to ionizing radiations is influenced by both the growth phases during which the exposure happens as well as the physiology of microbial cells. The typical batch culture involves bacterial growth through three distinct growth phases: lag (where cells adapt to the new growth conditions), exponential (characterized by rapid cell division), and stationary phase (where growth plateaus as a result of nutrient depletion) (Monod 1949). Among the three growth phases, the fast-dividing cells (in exponential phase) have been shown to be more resistant to ionizing radiations than growth-arrested and non-dividing cells (in stationary phase) in *Moraxella spp*. (Keller & Maxcy 1984), *Lactobacillus spp*. (Hastings *et al.* 1986), *Halobacterium spp*. (Kottemann *et al.* 2005; DeVeaux *et al.* 2007), and yeast (Fabre 1970). However, the converse has also been observed in species like *Escherichia coli* (Morton & Haynes 1969; Dantur & Pizarro 2004; Bucheli-Witschel *et al.* 2010), *Cronobacter sakazakii* (Arroyo *et al.* 2012), and *Salmonella typhimurium* (Child *et al.* 2002). Although the effects of radiation on the exponential phase and the stationary phase have been explored in detail in the microbial literature, the same cannot be said about another important part of the microbial growth curve, namely the lag phase. Lag phase represents the adaptive period where the cells are metabolically active, but cell division has not begun (Rolfe *et al.* 2012). Study of this phase of growth has a number of implications including food preservation (Sun 2011), bacterial infections, and antibiotic resistance (Frimodt-Møller *et al.* 1983) as well as in maintaining laboratory cultures. Several environmental and physiological attributes that are unique to the lag phase (Bertrand 2019

and the references therein) can influence the response to radiation stress (Gayán *et al.* 2014). For example, microbes in lag phase express distinctive transcriptomic and proteomic profiles which include components essential for metabolism and growth (Rechinger *et al.* 2000; Hornbæk *et al.* 2004; Larsen *et al.* 2006) as well as genes and proteins involved in repair of stasis induced macromolecular damages (Rolfe *et al.* 2012). This increased expression of DNA repair machinery in the lag phase might influence the cells' response to radiation. Consequently, the physiological state of the cell can influence its sensitivity to radiation both in the short term as well as over longer, evolutionary timescales.

Experimental evolution of resistance to ultraviolet radiation have been demonstrated in several microbial species including *Escherichia coli* (Ewing 1995; Alcantara-Diaz *et al.* 2004; Goldman & Travisano 2011; Shibai *et al.* 2017), *Salmonella typhimurium* (Davies & Sinskey 1973), *Bacillus subtilis* (Wassmann *et al.* 2010), *Pseudomonas cichorii* (Weigand & Sundin 2009), and T7 bacteriophage (Tom *et al.* 2018). Although all these studies found that repeated exposures to UV resulted in increased ability to survive UV stress, there were several differences in the correlated responses to selection. For example, the increase in UV resistance was accompanied by increased cell size (Goldman & Travisano 2011), increased tolerance to osmotic, oxidative and desiccation stress (Wassmann *et al.* 2010), and the appearance and maintenance of rare colony morphologies due to increased rate of UV induced mutations (Weigand & Sundin 2009). These studies on evolution of radiation resistance looked at the effects of exposure either during the lag phase (Ewing 1995; Weigand & Sundin 2009; Goldman & Travisano 2011; Shibai *et al.* 2017) or the stationary phase (Davies & Sinskey 1973; Alcantara-Diaz *et al.* 2004; Wassmann *et al.* 2010). Consequently, there is little understanding of the evolutionary effects of exposure during the exponential phase. At the same time, these existing studies have primarily focused on the evolution of resistant phenotypes and the correlated phenotypic changes (but see Shibai *et al.*

2017; Tom *et al.* 2018). The genomics of radiation resistance has been studied in detail in radio-resistant species such as Deinococcus spp. (White *et al.* 1999; Blasius *et al.* 2008), Rhodobacter spp. (Perez *et al.* 2017). However, there is relatively less work on this aspect in the context of non-radio-resistant species (although see Bruckbauer *et al.* 2019). It is not obvious that the insights gained about the genomic correlates of exposure to UV in the above radio-resistant species would be applicable to other microbes. Thus, in order to unearth the putative mechanisms underlying the evolution of radiation resistance, it is important to extend the genetic studies conducted in the radio-resistant species to other microbes.

In this study, we attempt to address some of these lacunae in our understanding of the evolution of radio-resistance, particularly ultraviolet radiation. First, we observe that our ancestral population of *Escherichia coli* is more sensitive to UV in the exponential phase than in lag phase. To study the evolutionary response to UV radiation in these two phases, we subjected two sets of replicate populations to 100 rounds of UV exposure and growth in the lag and the exponential phases. Both treatments showed a significant reduction in sensitivity to UV compared to control populations. However, growth phase differences in UV sensitivity were no longer observed. To investigate the genomic correlates of evolution of UV resistance, the UV-treated and control populations were further subjected to whole-genome whole-population sequencing. Genes associated with DNA repair pathways, RNA polymerase, and cell membrane structure were commonly mutated in both UV-treated populations. Additionally, mutations in the populations exposed to UV in the lag phase were grouped in genes involved in transcription / translation regulation and cellular transport. On the other hand, only the populations that faced UV in the exponential phase contained mutations in signal transduction and cell adhesion. The genes and pathways that were mutated during UV resistance evolution may also have led to correlated changes in growth and survival in novel environments such as antibiotics, heavy metal salts, and minimal media

with a single carbon source. Contrary to our expectation, these differences in the genome did not translate to phenotypic differences between lag and exponential treatments in either the selection or the novel environments.

2.2. Materials and Methods

We used an *Escherichia coli* K12 MG1655 strain in which the lacY gene had been replaced with a Kanamycin resistance gene. Colonies of this bacterium are white coloured on MacConkey's agar as opposed to the red coloured colonies produced by other *Escherichia coli*. All cultures were maintained at 37^{o}C and 150 RPM throughout the selection and assays, except where stated otherwise.

2.2.1 Experimental evolution

Six populations were initiated from six independent *E. coli* colonies picked from a nutrient agar streaking of the ancestral *E. coli* strain. These six are henceforth referred to as the ancestor populations. From each ancestor, we derived three replicate populations and assigned them randomly to one of the three selection regimes, namely lag, exponential, and control. Both lag and exponential treatments were subjected to UV during the respective growth phases while the control was devoid of UV exposure. The populations were exposed to UV in a custom-built dark chamber where the only source of light was an 8W UV-C tube-light (Philips TUV 8W) with an emission peak at 254 nm. The lamp was placed at a height of 18 inches from a platform shaker, which results in a constant irradiance of 100.5 μW/cm^2. At the beginning of the experiment, the duration of exposure was 15 seconds, which resulted in 2 – 3 $\log_{10}$ reduction (100 – 1000 fold) in colony forming units (CFUs); see section 2.2.2 for more details regarding estimation of $\log_{10}$ reduction. To allow sufficient time for the populations to adapt, the duration of UV exposure was increased gradually i.e., once every 5 days. For the full trajectory of exposure duration throughout selection, from day 0 – 100 (see table 2.1). By the end of 100 days of evolution, the exposure time was 370 seconds, i.e. an increase of ~25 times.

Table 2.1. Exposure duration during selection. The duration of exposure to UV was increased every five days.

Days	UV exposure duration (seconds)
1 - 5	15
6 - 10	20
11 - 15	25
16 - 20	35
21 - 25	45
26 - 30	60
31 - 35	75
36 - 40	90
41 - 45	110
46 - 50	130
51 - 55	160
56 - 60	200
61 - 65	220
66 - 70	240
71 - 75	270
76 - 80	300
81 - 85	330
86 - 90	340
91 - 95	350
96 - 100	370

All cultures were grown in 2ml nutrient broth with kanamycin (NB^{Kan}) in six-well tissue culture plates. The use of six-well plates allowed for a larger surface area to volume ratio for better UV penetration. Growth of all populations reached a plateau within 24 hours. Every 24 hours, 20 µl of the grown culture was sub-cultured into fresh NB^{Kan}. Control populations (C) were sub-cultured in NB^{Kan} with no UV exposures, whereas populations designated as Lag (L) treatment were exposed to UV during the lag phase, immediately after subculture (0h). Exponential (E) treatment populations were exposed to UV during the exponential phase by monitoring their growth/OD_{600} in a plate reader (Synergy HT, BIOTEK Winooski, VT, USA) at 20-minute intervals. Based on pilot experiments, the exponential phase was deemed to have been reached when the OD_{600} of the culture was greater than 0.11, and the difference between two consecutive OD measurements were greater than 5% for the first time, i.e., $OD_{t+1} > 1.05 \times OD_t$, where the subscript t and t+1 refer to two successive time points. Populations in the exponential treatment were exposed to UV only when both the conditions were met. The 6-well plates containing the cultures were placed on the shaker platform directly under the UV lamp without the lid. The cultures were shaken at room temperature and 150 RPM during exposure. Since bacteria possess the photoreactivation pathway, which can induce error-free reversal of UV induced damages and reduce the efficiency of UV radiation (Thoma 1999; Sinha & Häder 2002), the cultures were maintained in darkness throughout the process of selection, except during sub-culturing. The UV induced mortality makes it impossible to accurately estimate the dilution ratio, and consequently, the number of generations in the UV-treated populations. However, at the end of 100 days, the UV-treated populations would have experienced more generations, on average, than control populations which underwent ~667 generations of evolution (6.67 doublings/transfer x 100 days). Every five days, 15% glycerol stocks (300 µl of 50% glycerol + 700 µl culture) of all populations were prepared and stored at -80^{o}C. Due to logistic reasons, the selection was interrupted once

at day 60. Selection was reinitiated by reviving 10µl glycerol stocks of all populations (UV-treated and controls) in 90 µl NB^{Kan} in a 96 well plate for 12 hours. 20 µl of this revived culture was inoculated in 2ml NB^{Kan} to initiate day 61. Glycerol stocks prepared at the end of 100 days, were used for all assays.

2.2.2 Measuring UV sensitivity

Sensitivity to UV induced mortality was measured as the log of change in the number of colony forming units (CFUs) before and after UV exposure ($\log_{10}$(CFUs before exposure/CFUs after UV exposure)) (Koivunen & Heinonen-Tanski 2005). The populations to be assayed were revived by inoculating 5 µl of the corresponding glycerol stocks in 2ml NB^{Kan} and incubating them overnight at 37°C. Assays were conducted in six well plates with 20µl of the revived culture inoculated in 2ml NB^{Kan}. UV sensitivities of all populations were assayed in both lag and exponential phases, where the UV exposures were carried out in a manner similar to the selection procedure. CFU counts before and after exposure were determined by serially diluting the cultures and plating 100 µl of the appropriate dilutions (to obtain a countable number of colonies) on 2% nutrient agar containing kanamycin (NA^{Kan}). The serial dilution and plating after UV exposure was carried out in a dark room illuminated by red light to prevent photo-reactivation. The NA^{Kan} plates were incubated in darkness at 37^{o}C, overnight and the number of colonies were counted manually and multiplied by the dilution factor to obtain the number of CFUs.

To measure the UV sensitivities of the ancestors, all six ancestral populations were assayed at both lag and exponential phase and four exposure durations: 15, 60, 200, and 370 seconds. After 100 rounds of selection for UV resistance, we measured the UV sensitivity of the evolved populations L, E, and C along with the ancestors. Log_{10} reduction in CFUs during both lag and exponential growth phases was measured at exposure duration of 370 seconds.

Two independent measurement replicates of the UV sensitivities of the evolved populations were obtained by conducting the entire assay from revival to CFU counts, twice. The relative change in UV sensitivity due to selection was obtained by scaling the sensitivities of the evolved populations by the sensitivity of the ancestors in the corresponding growth phases.

2.2.3 Whole-genome sequencing and analysis

To understand the genomics of repeated exposures to UV and selection for UV resistance, we randomly chose two replicates (rep 1 and 3) of the evolved populations (L, E, C), and their corresponding ancestors, and subjected them to whole-genome whole-population sequencing. 5 µl of the corresponding glycerol stocks were inoculated in 4ml NB^{Kan} and incubated overnight at 37°C. 3ml of the revived cultures were centrifuged down, washed twice in phosphate buffer saline (PBS), air dried, and shipped for genome sequencing to a commercial service-provider. They performed paired-end whole-genome sequencing on NextSeq500 platform (Illumina, USA) at ~100X (range: 98.8X – 139.2X) coverage and 150bp read length. The service-provider provided us with high quality reads after removing adaptor sequence, ambiguous reads (reads with unknown nucleotide "N" of more than 5%), and low-quality sequences (reads with more than 10% having a phred score < 20) using Trimmomatic v0.38. We then used *Breseq* version 0.33.2 pipeline (Deatherage & Barrick 2014) at default parameters for sequence alignment and variant calling. First, mutations in the ancestral genome were identified (in consensus mode) by aligning it to the reference genome of *Escherichia coli* MG1655 (Genbank accession: NC_000913.3). Breseq's gdtools package was used to incorporate the predicted mutations and update the ancestral genome. This updated ancestral genome sequence was then used as reference for alignment and variant calling (in polymorphism mode) in the evolved populations. Following previous studies (Bailey *et al.* 2015; Sandberg *et al.* 2017; Santos-Lopez *et al.* 2019), the list of predicted

mutations were further curated by removing all variants present at frequencies less than 10%. We also aligned the ancestral sequence in the polymorphism mode. To limit our analysis to the genomic changes that evolved *de novo* in response to selection, all polymorphic variants common between the ancestral and evolved populations were also removed from the dataset. This curated list of mutations was used for computing the number of SNPs and indels, SNPs in coding vs intergenic regions, synonymous vs nonsynonymous SNPs, as well as the mutational spectrum of all mutations. We estimated the dN/dS ratio as the number of non-synonymous mutations per non-synonymous sites (dN) to the number of synonymous mutations per synonymous sites (dS). The total number of non-synonymous and synonymous sites in the genome was estimated using Breseq's gdtools package. Note that the relationship between dN/dS ratio and the selection coefficient can be an issue when comparing two populations sharing a number of fixed mutations. However, this issue is avoided in our study as we compare evolved populations with their ancestors (Chen & Zhang 2020). Functional annotations and enrichment analysis of the genes were carried out on DAVID v.6.8 (Huang *et al.* 2009b, a), a web-based bioinformatics application. The list of mutated genes was classified into functional groups (based on UniProtKB keywords) followed by manual curation.

2.2.4 Measuring fitnesses in novel environment

We observed that the UV-treated populations had accumulated numerous mutations across the genome. These UV induced mutations could affect the fitness of the populations under environmental conditions not faced during selection (see Discussion for details). Therefore, after 100 days of selection for UV resistance, we assayed the fitness of the evolved populations (L, E, C) as change in minimum inhibitory concentration (MIC) and growth rate in a suite of environments.

Measuring minimum inhibitory concentrations

MICs of the evolved populations were measured in four antibiotic environments and three heavy metal environments. Antibiotics (ampicillin, chloramphenicol, nalidixic acid, and rifampicin) from four different classes were chosen for their different mechanisms of action (Kohanski *et al.* 2010). Similarly, the heavy metal salts (cobalt chloride, copper sulphate and nickel chloride) were chosen for the different ways in which they disrupt metabolic processes (Dupont *et al.* 2011; Macomber & Hausinger 2011; Majtan *et al.* 2011). To the best of our knowledge, evolution of resistance to UV has no known association with fitness in these environments.

Populations were grown in increasing concentrations of the assay environment (antibiotics or heavy metals), and the minimum concentration at which no visible growth was obtained was taken as MIC. The evolved populations and their corresponding ancestor were revived as mentioned before. A gradient of the assay environments was prepared by serial two-fold dilutions in 96 well plates. The revived cultures were inoculated in each concentration of the assay environment at a dilution of 1/1000 in triplicates. After 48 hours of growth, each plate was scored for absence of growth either visually (for antibiotic environments) or when OD_{600} < 0.2 (for heavy metals). A concentration was considered the MIC of the population only if at least two out of the three replicates showed no growth. The entire assay from revival to MIC determination was repeated twice and served as measurement replicates. MIC of all the evolved populations were scaled by the MIC of their corresponding ancestor to obtain the change in MIC due to selection.

Measuring growth rate

Growth rates of the evolved populations were assayed under two kinds of conditions: a) in nutrient broth, and b) in M9 minimal media containing a single carbon source at

concentration of 4g/L. Growth rate was measured in five different carbon sources: fructose, glucose, glycerol, mannose and thymidine, all of which feed into the glycolysis pathway via different intermediates (Voet & Voet 2011). Mutations in the intermediate enzymatic steps can likely alter the efficiency of metabolism of the carbon sources and consequently growth. The revived evolved and ancestral populations were inoculated in 200 µl of the assay environment (i.e. M9 media containing one of the five carbon sources) at a dilution of 1/1000 in 96 well plates. These cultures were subjected to automated growth measurements using a plate reader (Synergy HT, BIOTEK Winooski, VT, USA). OD_{600} was measured every 20 minutes for 24 hours at 37^{o}C and slow continuous shaking, at 17 cycles per second. Following previous studies (Karve *et al.* 2015; Sprouffske *et al.* 2018; Chavhan *et al.* 2019; Rodríguez-Rojas *et al.* 2020), we computed the growth rate as the maximum slope of the curve over a moving window of 10 readings. Two measurement replicates were obtained by repeating the entire assay twice. The growth rates of the evolved populations were scaled by the ancestral growth rate to obtain the relative change in growth rate due to selection.

2.2.5 Statistical analysis

Ancestral UV sensitivities were compared using a three-way mixed model ANOVA with randomized complete block design (RCBD) (Rohlf & Sokal 1995). Here, we used growth phase (lag and exponential) and exposure duration (15s, 60s, 200s, and 370s) as fixed factors crossed with each other and replicate populations (6 populations) as an independent random factor (neither crossed nor nested in other factors). Cohen's *d* (Cohen 2013) was computed to assess the effect sizes of the differences between the two growth phases. The effects sizes were interpreted as small, medium and large for $0.2 < d < 0.5$, $0.5 < d < 0.8$ and $d > 0.8$, respectively (Sullivan & Feinn 2012). UV sensitivities of the evolved populations after 100 days of selection were compared in a three-way mixed model ANOVA with selection (lag,

exponential and control) and assay environment (lag and exponential) as fixed factors and replicate populations (6 populations) as a random factor in a full factorial design. Fitness in novel environments weas analyzed as separate two-way mixed model ANOVAs for each fitness measure and environment. Selection (lag, exponential and control) as fixed factors and replicate populations (6 populations) as a random factor were analyzed in a full factorial design. Correction for inflation of family-wise error rate was done for the two fitness measurements, independently, using the Holm–Šidák procedure (Abdi 2010).

We compared the dN/dS ratio of the evolved populations to the expected ratio (0.754, ratio of possible synonymous sites to nonsynonymous sites in the genome computed using Breseq) using a binomial test on R 3.6.1.

All the ANOVAs were performed on STATISTICA v7.0 (Statsoft Inc.). Cohen's *d* statistics were estimated using the freeware Effect Size Generator v2.3.0 (Devilly 2004).

2.3. Results

2.3.1 Ancestral UV sensitivity in lag and exponential growth phases

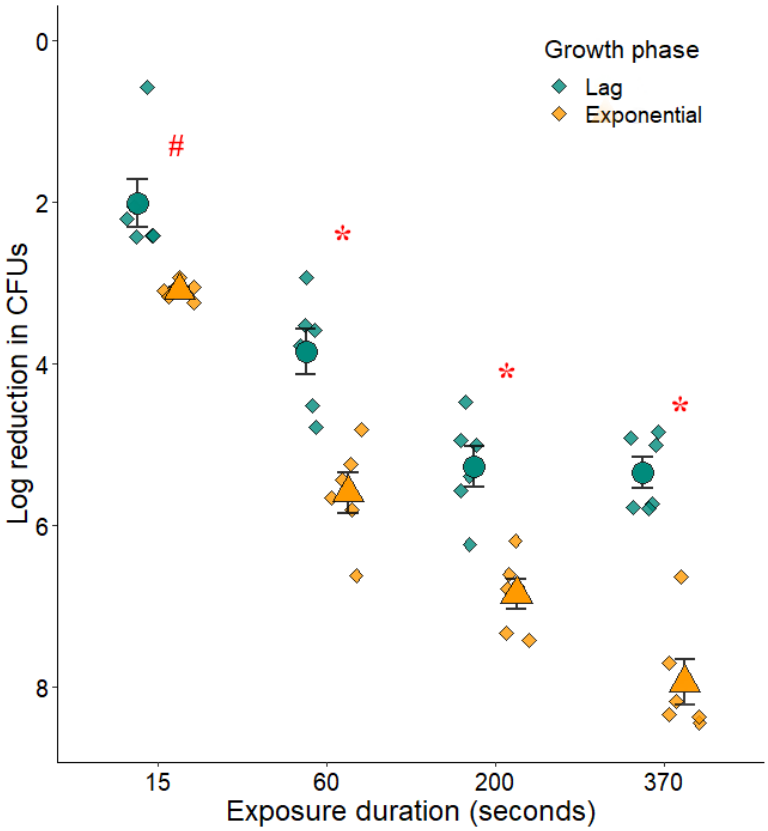

Figure 2.1. UV induced log_{10} reduction in CFUs during lag and exponential growth phases. UV sensitivity of the ancestral *E.coli* was measured at four exposure durations (15, 60, 200, and 370 seconds) and two growth phases. Mean sensitivity of the six ancestral populations are plotted as circles (lag phase) and triangles (exponential phase). Whiskers represent ±SE. The scatter of the six ancestral value is represented by diamonds (♦). * denote p value < 0.05 in Tukey's post-hoc analysis at that exposure value and # denotes p = 0.065

We first measured the UV sensitivity of the ancestral *Escherichia coli* during the lag and exponential phases at four exposure durations. We found a significant interaction between growth phase and exposure duration ($F_{3,35}$ = 3.34, P = 0.03). The exponential phase sensitivity was significantly larger than the lag phase sensitivity in all but one comparison in Tukey's post hoc analysis (Fig. 2.1; 60s: p=0.0005; Cohen's d = 2.69 (large), 200s: p=0.002; Cohen's d = 2.93 (large), 370s: p=0.0001; Cohen's d = 4.4 (large)). At 15 seconds of exposure, the difference between lag and exponential exposure was marginally non-significant but with a large effect size (Fig. 2.1; 15s: p=0.065; Cohen's d = 2.09 (large)). Additionally, the six ancestral populations show no significant differences in their UV sensitivity ($F_{5,35}$ = 0.36, P = 0.87). Together, this shows that our ancestral strain of *E.coli* was more sensitive to UV during exponential phase of growth. However, as the interaction of growth phase and exposure was significant, we refrain from interpreting the main effects.

2.3.2 UV sensitivity of evolved populations

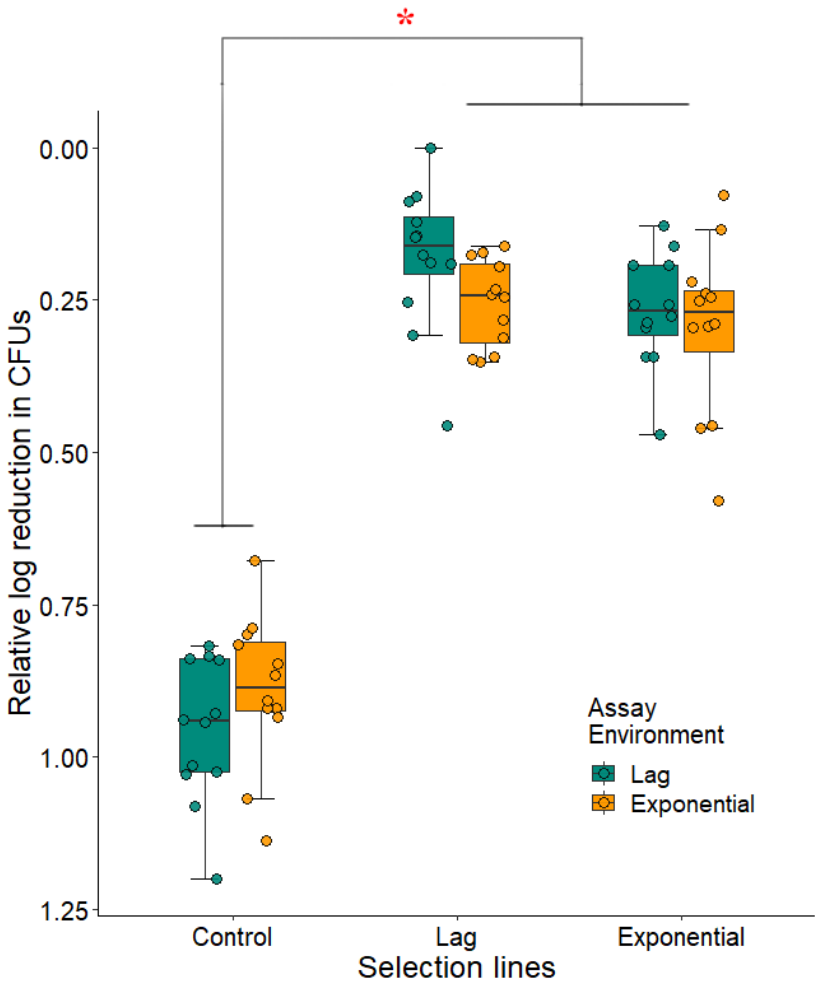

Figure 2.2. UV sensitivity of evolved populations relative to the ancestor's sensitivity. The decline in the relative $\log_{10}$ reduction (sensitivity to UV) in lag and exponential populations signifies evolution of resistance. Each box plot represents data from 12 values i.e., 6 replicate populations assayed twice. Solid lines represent median, upper whisker denotes the largest data point lesser than or equal to 1.5 * IQR (inter-quartile range) and similarly for the lower whisker. * denotes that the control population has significantly ($p < 0.05$) lower resistance to UV than both lag and exponential populations in Tukey's post-hoc analysis.

UV sensitivity of the evolved populations in the two growth phases were compared after scaling them by the corresponding ancestral values. At the end of 100 rounds of UV exposure and selection, the populations significantly differed in their relative UV sensitivities ($F_{2,36}$ = 249.72, P = 2.91E-09). Tukey's post hoc analysis showed that both lag (L) and exponential (E) populations had evolved significantly higher resistance compared to control (C) populations but were not different from each other (Fig. 2.2; L and C P = 0.0001; Cohen's d = 6.23 (large), E and C P = 0.0001; Cohen's d = 5.34 (large), and L and E P = 0.13; Cohen's d = 0.58 (medium)). Interestingly, the interaction between selection lines and assay environments was also not significant ($F_{2,36}$ = 3.82, P = 0.059). This also suggests that the populations had evolved similar extents of resistance in both growth phases irrespective of the selection environment.

2.3.3 Genomics of UV resistance evolution

To investigate the genomic changes accumulated during selection for UV resistance, we carried out whole-genome whole-population sequencing of two replicate populations from each selection regime (L, E, C) and their corresponding ancestors. Both our ancestral populations had nearly identical sequences. However, our lab strain differed from *E.coli* K-12 reference genome (NC_000913.3) at several genome locations. Therefore, we used the assembled sequences of the corresponding ancestor as reference for identifying mutations in the evolved populations. Exposure to UV in both growth phases resulted in a marked increase in the total number of mutations. The two replicates of lag (193 and 317) and exponential (60, 319) treatments had much greater number of single nucleotide polymorphisms (SNPs) compared to the control populations (8, 13). A histogram showing the distribution of mutation frequencies of all SNP in the four UV-treated populations is given in the figure 2.4. Very few indels (insertions and deletions) were identified in all three evolved populations (2, 2 in lag; 4,12 in exponential; and 3,7 in control). Some of these indels, 0 (L1), 1 (L3, E1, E3), 2 (C1) and 5 (C3), were found to be mediated by IS2 insertional elements.

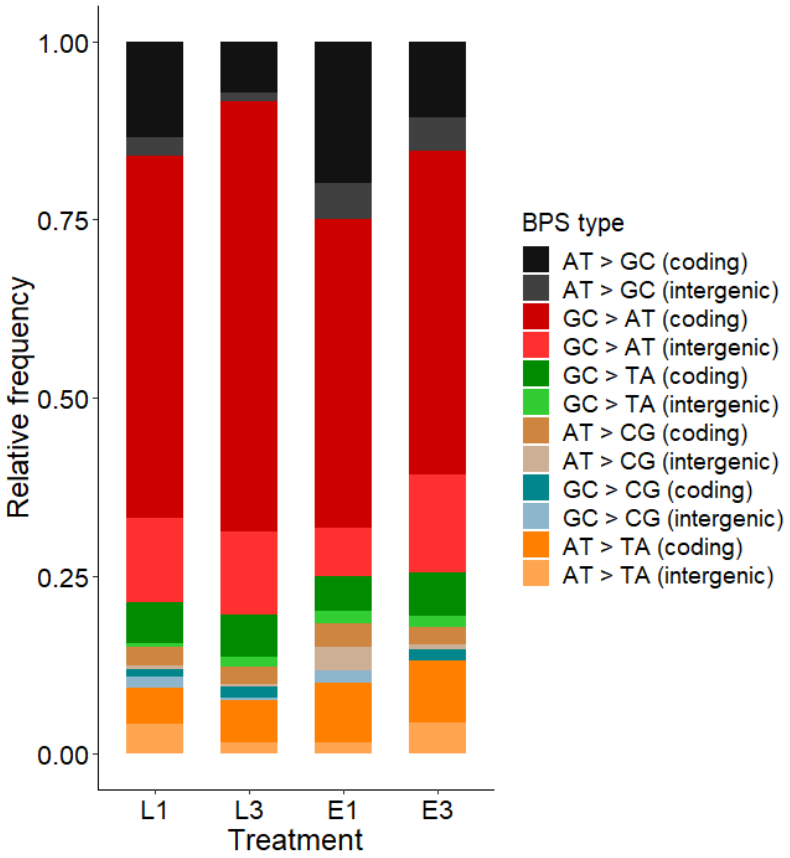

Figure 2.3: Mutational spectrum of SNPs accumulated during lag and exponential UV exposure. Colours in the stacked bar represent the relative frequency of each of the six type of base-pair substitutions (BPS), in the coding and intergenic regions.

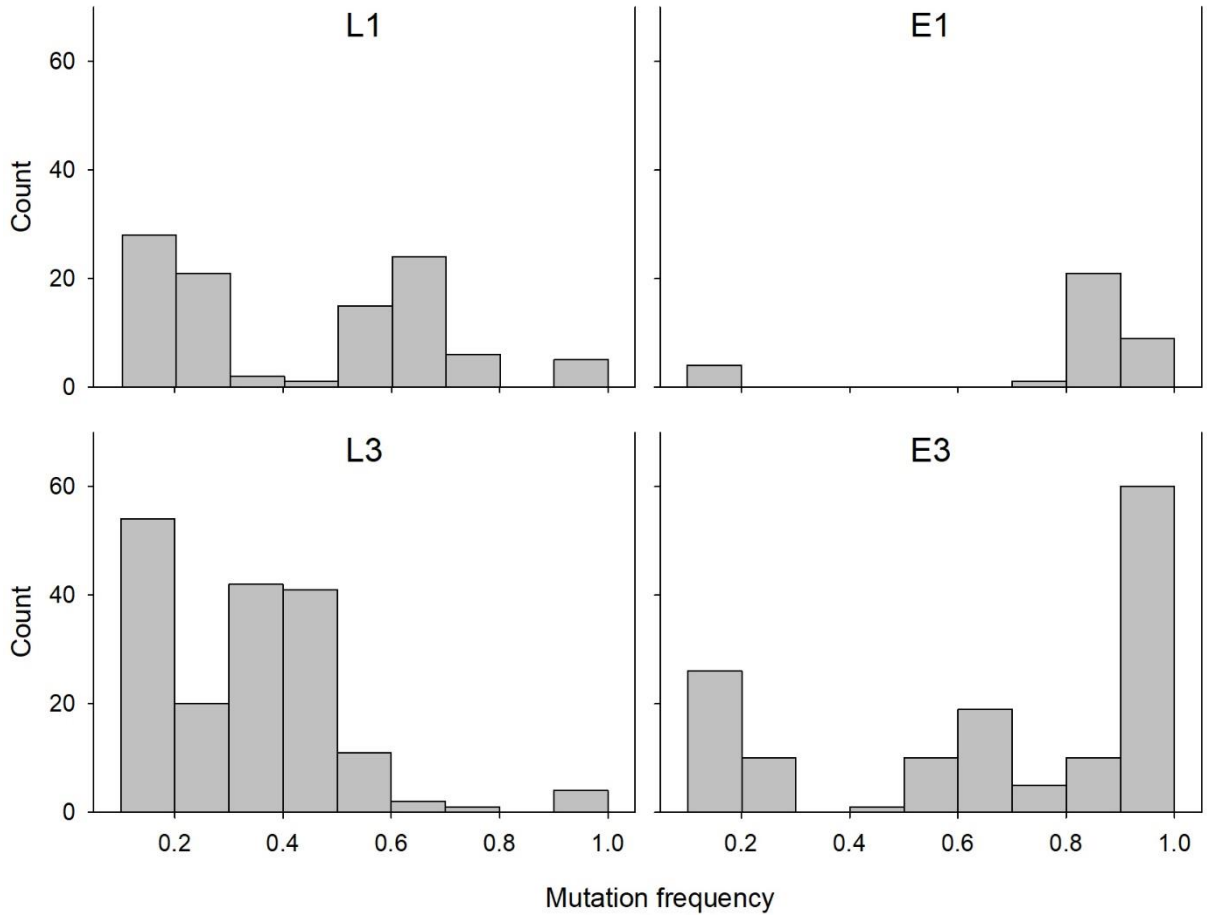

Figure 2.4. A histogram showing the distribution of mutation frequency in the UV selected populations.

We also found that the mutational spectra of both lag and exponential treatments were transition-biased as GC→AT transitions accounted for at least 50% of all mutations, a confirmed signature of exposure to UV (Fig. 2.3) (Griffiths *et al.* 2005; Brash 2015). In spite of the bias, all six mutations types were represented in both UV treatments. Mutations in the control populations were confined to three or four types only (see table 2.2).

Table 2.2. Mutational spectrum of all SNPs. The mutational spectrum of all SNPs identified in the selected populations including coding and non-coding regions and synonymous and non-synonymous mutations. The spectrum of two replicates (1 and 3) of lag (L), exponential (E) and control (C) populations are listed below.

Mutation type		**L1**	**L3**	**E1**	**E3**	**C1**	**C3**
Transition	A→G	17	14	6	25	0	0
	T→C	14	13	9	24	0	2
	G→A	52	129	20	111	2	2
	C→T	69	99	10	78	2	1
Transversion	G→T	7	12	1	11	0	0
	C→A	5	11	3	13	0	0
	A→C	3	2	3	4	3	2
	T→G	3	7	1	6	0	2
	G→C	1	2	1	1	0	0
	C→G	4	4	0	4	0	0
	A→T	13	8	2	20	0	2
	T→A	5	16	4	22	1	2
Total		193	317	60	319	8	13

We notice that 75 – 80% of all SNPs in the UV treatments were found in the coding regions which typically makes up about 88% of the *Escherichia coli* genome (Rogozin *et al.* 2002). We estimated the dN/dS ratio (ratio of non-synonymous mutations per non-synonymous sites to synonymous mutations per synonymous sites) to infer the selection pressure that led to the genomic changes. Table 2.3 summarizes the dN/dS ratios in the evolved populations. The ratios were statistically significant in both the lag treatments (L1, L3) and one of the exponential treatment (E3). In all three cases, the ratios were less than unity, indicating that these populations were primarily subjected to purifying selection (Yang & Bielawski 2000).

Table 2.3: Estimates of non-synonymous mutation to synonymous mutation ratio in the evolved populations. P value denotes the binomial probability that the observed dN/dS ratio differs from the expected ratio (0.754), the ratio of possible synonymous sites to nonsynonymous sites in the genome)

Population	dN/dS ratio	p value
L1	0.665	0.023
E1	0.877	0.737
C1	0.652	0.571
L3	0.632	6.0E-4
E3	0.469	2.75E-8
C3	0.489	0.274

Next, we investigated the genetics of UV resistance evolution by considering only the nonsynonymous mutations in the UV-treated populations. To begin with, the genes that were mutated differed considerably between lag and exponential treatments (see jeb13764-sup-0002-Supinfo2.xlsx for file containing the full list of non-synonymous mutations). There were only two genes (*recA* and *mepS*) that had mutated in both treatments but not in the control populations. Our ancestral populations had a single mutation in *recA* (G161D) with respect to the reference MG1655 strain. Reversion of this mutation had fixed in all UV-treated populations but not the controls. We note that this reversion in *recA* (D161G) is the only consistent mutation where all treatments have the same amino acid change. In addition to this, one other mutation (P314L) in *recA* was present at low frequency in L3. *recA* is involved in DNA recombination and repair (Smith *et al.* 1987; Schlesinger 2007) and plays an integral role in the mediation of SOS response (Markham *et al.* 1985; Maslowska *et al.* 2019). The second commonly mutated gene was *mepS* with four different mutations: Y82I (in E1), R83C (in L1), P111L (in L3, E3), and T114I (in L3). *mepS* is known to be involved in peptidoglycan biosynthesis during cell growth (Singh *et al.* 2012). Populations L1 and E3 had mutations in recJ and L3 had mutation in recQ, both components of the recFOR recombination pathway. Similarly, RNA polymerase genes rpoC was mutated in L1 and E3 population, while mutations in rpoB was observed in L3.

Besides these commonly mutated genes, we also identified 9 genes that were mutated in both the replicates of lag treatment. Of these, a functional cluster of 5 genes was identified. *crp*, *deoR*, *fadR*, *hfq*, and *lexA* are known to be involved in DNA-binding, transcription and translational regulation. Additionally, *lexA* in association with *recA*, is known to mediate the SOS response. The remaining four genes show no obvious clustering (see table 2.4 for full functional annotations). Similarly, in the populations exposed to UV during the exponential

phase, we identified four genes that were mutated in both replicates. However, these genes too showed no obvious clustering of function (table 2.4).

Table 2.4: List of genes consistently mutated in both replicates of lag and exponential treatments. The genes along with their functional annotations are listed.

Lag treatment	
Gene	**Functional annotation**
crp *deoR* *fadR* *hfq* *lexA*	DNA-binding proteins, Repressors, Transcription and translational regulation
lexA (recA)	SOS response
adiA	Arginine catabolic processes
fdoG	Cellular respiration
frlD	Carbohydrate phosphorylation
ycbK	Uncharacterised protein
Exponential treatment	
Gene	**Functional annotation**
fimA	Cell adhesion
phoR	Response to phosphate starvation and signal transduction
ygfS	Oxidation-reduction process
yoaE	Membrane component

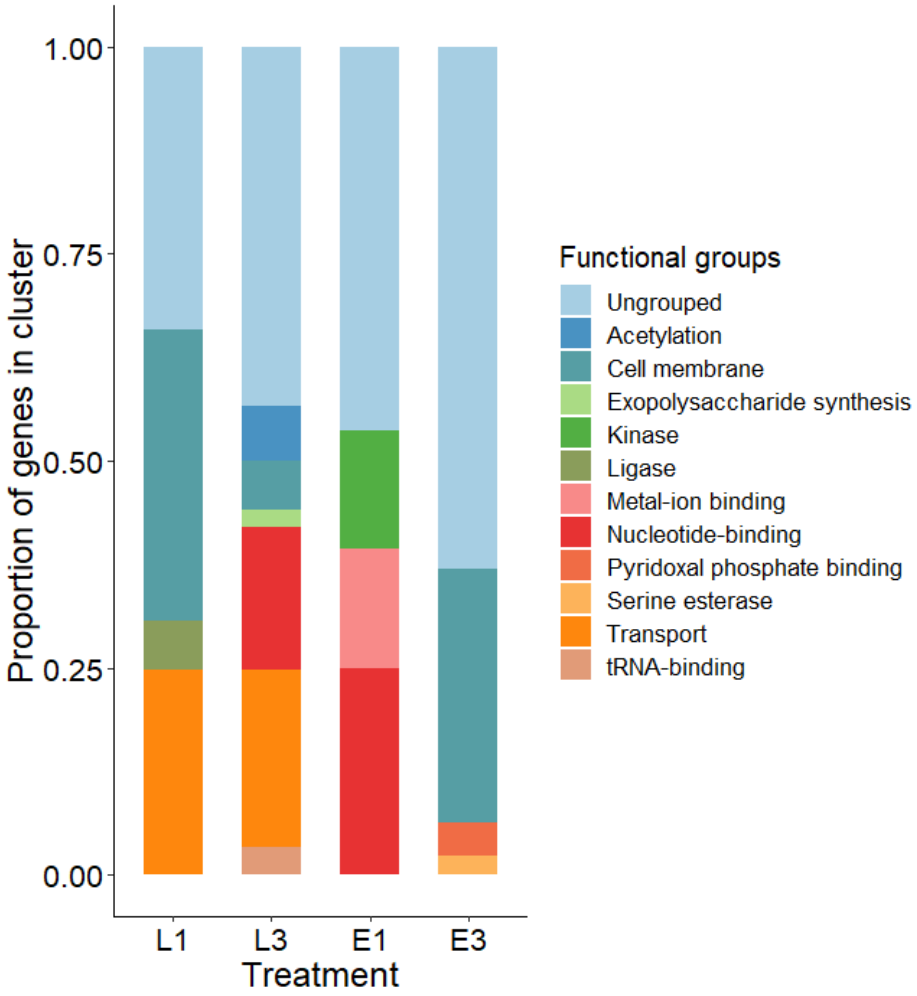

Figure 2.5: Functional grouping of non-consistent mutations. The functional categorizations are based on uniprot keywords and GO terms. Height of each stack represents the proportion of genes in that particular functional group. Functional annotation was done using DAVID v.6.8 followed by manual curation. See supplementary material Table S6 for list of genes in each group.

Using DAVID v.6.8, a web-based bioinformatics application, we carried out functional annotation and enrichment analysis of the list of non-consistent mutations. Only about half the list of genes fell into clusters of groups having similar functions (Fig. 2.5). This could have resulted in the observed lack of significance in enrichment analysis (table 2.5). However, functional clusters comprising genes involved in or a part of cell membrane, were represented in three out of four populations (L1, L3, and E3). This, in addition to the fact that mutations of *mepS* were consistent across treatments, can suggest that cell membrane modification may play an important role in evolution of UV resistance. A second major group, that was found to be common between the two replicates of lag treatment, consisted of genes involved in transmembrane transport. Aside from these, mutations in lag treatment were grouped by genes involving in ligase activity, acetylation, tRNA-binding, nucleotide binding, and exopolysaccharide synthesis (Fig. 2.5 and table 2.5). Similarly, mutations in exponential treatments were grouped by genes involved in metal-ion binding, nucleotide binding, pyridoxal phosphate binding, kinase, and serine esterase. These differences imply that selection for UV resistance at the two growth phases can result in differences in how and where mutations accumulate in the genome.

To summarize, repeated exposure to UV resulted the accumulation of a large number of mutations despite the effects of purifying selection. While genes such as *recA* and *mepS* were commonly mutated in all UV-treated populations, genes of different functional classes were mutated in lag and exponential treatments. This led us to the investigation of how these mutations affected the fitness of the populations under other environmental conditions.

Table 2.5. Functional categorization using DAVID. The list of genes that were unique to each treatment population categorized into functional groups using DAVID.

	UniProt_KEYWORD	No. of genes	Gene name	p-Value
L1	Transport	21	YEHB, DCTA, YFDV, PLAP, HYCB, FRVA, ADIC, CAIT, AMPG, MURJ, MLAE, XAPB, FTSW, YTFR, YJEH, PROV, ARAE, GLNP, CUSC, MACB, ACRD	0.04873
	Membrane	30	YBHM, YQJA, AMPG, YEAQ, MURJ, MLAE, FTSW, YAFT, ATOS, YJEH, PROV, BSMA, GLNP, YIAT, MACB, ACRD, YEHB, DCTA, YFDV, CBRB, PLAP, ADIC, CAIT, DAMX, PLSB, XAPB, YEJM, ARAE, EVGS, CUSC	0.06482
	Ligase	5	PANC, GLUQ, GLYS, QUEC, FOLC	0.09765
	Ungrouped	47	AEGA, AHPF, ASTD, BISC, CAS3, CHIA, DADX, DGT, DSBA, ELFD, FUMA, FUSA, HCAB, HSDS, INSG, LPXC, LRHA, MENC, MRP, NADC, NAGC, PPNN, PRIC, PUTA, RECJ, RFBA, RPOC, SMG, YAAI, YABR, YACH, YAIL, YCJW, YDDL, YDEP, YEBB, YEBK, YFDE, YGGF, YGID, YHJJ, YIAU, YIHM, YJFJ, YLBG, YPHB, ZINT	-
L3	Acetylation	10	ALAS, RPLL, GUAB, THRS, RPOB, RPLE, ADHE, PYKA, MANX, SODB	0.0051
	tRNA-binding	5	ALAS, MNMA, TMCA, RPLE, DUSC	0.00673
	Exopolysaccharide synthesis	3	WZA, RFFH, WZC	0.01062
	Nucleotide-binding	26	TDCD, LON, DNAC, YJIA, CLPA, SERS, RTCR, GLPK, ALAS, THRS, RECQ, ASNB, NORR, PYKA, OPPD, ACKA, MNMA, WZC, TYRR, UUP, XYLG, ZRAR, TMCA, PRIA, ARGG, KEFC	0.0197

	Transport	32	SFMD, UHPT, BGLH, RCNA, YFEO, DDPA, WZA, DTPB, AAEB, FECA, ACRB, FECB, MANX, PANF, SETA, OPPD, CHBC, MNGA, EMRA, OPPA, YEJE, YICJ, URAA, UUP, FIMD, XYLG, CHIP, YGCQ, TEHA, RSXA, EXBB, KEFC	0.08821
	Cell outer membrane	9	FIMD, SFMD, WZA, CHIP, BLC, FECA, OMPX, BGLH, MIPA	0.0075
	Ungrouped	85	ACEB, ALLS, AMIA, APPB, AROC, ARPA, ASTB, BCSB, CASC, CREA, CREB, CUEO, CYTR, CYUP, DADA, DCP, DLD, DNAN, DNAQ, FTSL, GATD, GFCD, GHRA, GLGP, GOR, HYFI, IADA, IDNR, LIPA, MLTD, NAC, NIRD, OTSB, PABA, PANB, PDEF, PDXI, PFLD, PPPA, QUEG, RBFA, RIMP, RSMC, SAD, SDAB, SELU, SLT, TCYN, TDCA, TDCG, THYA, UXAC, WAAF, WBBI, YAEH, YAER, YCAC, YDCC, YDFH, YEAG, YEGQ, YEHI, YEJA, YFBU, YGFB, YGFM, YGGT, YGIM, YHCO, YHFS, YHHH, YHHX, YHIJ, YIDP, YIGF, YIHU, YJBM, YJGL, YJGN, YMGE, YPEC, YPJB, YPJC, YQGA, YTFI	-
E1	Metal-ion binding	4	AMYA, CHAA, MGLB, YDEN	2.81E-04
	Nucleotide-binding	7	CYAA, NARQ, DNAB, HRPA, ARCB, HISS, YIHV	0.05411
	Kinase	4	NARQ, ARCB, YIHV, FRYA	0.06819
	Ungrouped	16	CUEO, CYUA, GHXQ, GSIC, LEPB, MDTE, RSXC, SAD, SBCD, TDCE, YAAA, YDDL, YDEE, YDHU, YHDP, YPAB	-
E3	Serine esterase	3	YPFH, YQIA, YEIG	0.02751
	Cell membrane	39	YDDG, ALAE, AMPG, ENVZ, FETB, ELAB, YDDW, YJGN, UIDB, CSGD, NFRB, EPTC, YBBW, CDH, YJEM, ATPA, UACT, YJHB, NUOM, YAEF, WCAD, CYSA, RHTB, UGPE, HYFF, LIVH, GFCB, CCMA, YDGA, LIVM, MRDB, SDHC, NUPX, FLIG, POTB, HFLK, PNUC, KEFC, KCH	0.09658
	Pyridoxal phosphate binding	5	LTAE, ADIA, CYSK, ALR, YGGS	0.09696

	Ungrouped	80	ACEF, ARAF, ASMA, BAER, CAIC, CAIE, CARB, CASA, CHBF, CLPB, CSPH, DAPB, DGOD, DNAE, ELAD, FABR, FDNG, FIMH, FUCR, GLMS, GLMU, GLPD, GLTB, GSHB, HMP, HXPB, LPTD, MALQ, METF, MQO, OPGG, PDHR, PHEM, PNP, PURN, RAPA, RDGB, RECJ, RHAB, RHSB, RIMJ, RLMA, RNB, RNK, RPMG, RPOC, SELU, SRLR, STPA, TORZ, TREA, UUP, VIAA, XSEA, YBHH, YCCT, YCHS, YCIA, YCIE, YCIF, YCJT, YCJW, YCJX, YDBD, YDIF, YDJO, YEEJ, YEJK, YFBK, YGIF, YHCO, YHHX, YIAJ, YIGL, YJGL, YJJQ, YKFM, YMGD, YPFJ, ZAPA	-

2.3.4 Fitness in novel environment

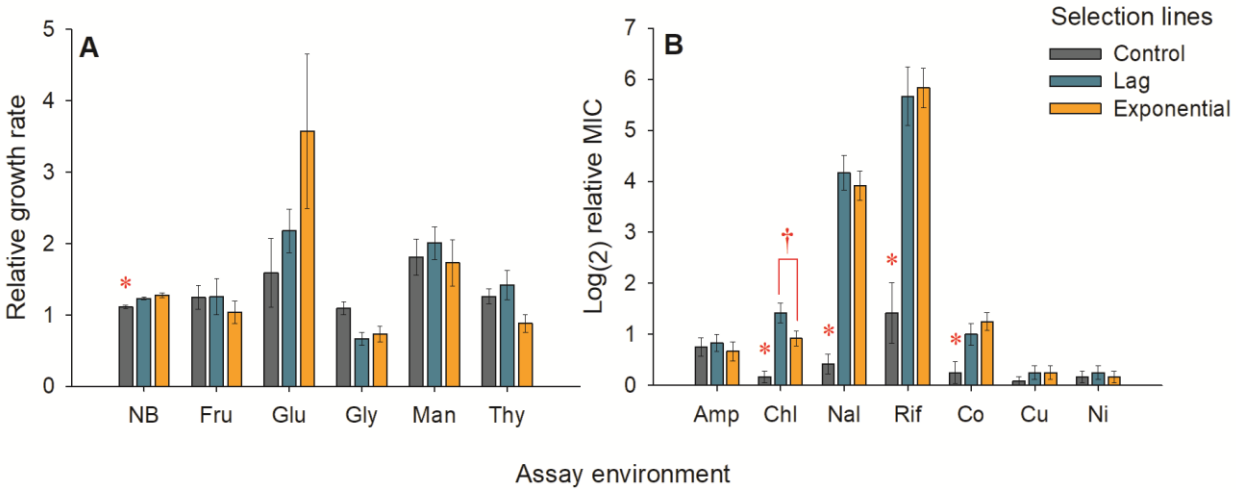

Figure 2.6: Relative fitness of the evolved populations in novel environment. Fitness is measured as (A) relative growth rate and (B) resistance, measured as minimum inhibitory concentration (MIC). Growth rate of the UV-treatedand control populations were assayed in nutrient broth (NB), M9 minimal media containing fructose (Fru), glucose (Glu), glycerol (Gly), mannose (Man) and thymidine (Thy). Resistance of all three evolved populations were measured in ampicillin (Amp), chloramphenicol (Chl), nalidixic acid (Nal), rifampicin (Rif), cobalt chloride (Co), copper sulphate (Cu), and nickel chloride (Ni). Fitnesses of the evolved populations were scaled by the ancestral fitness. Bars represent mean of 12 values i.e., 6 replicate populations assayed twice. Whiskers represent ±SE. * denote control is significantly (p value < 0.05) less than lag and exponential in Tukey's post-hoc analysis. † denotes significant differences between lag and exponential treatments.

Fitness of the evolved populations in novel environments was measured in two ways. Growth rate was measured in nutrient broth (NB^{Kan}) and M9 minimal media in the presence of 5 different carbon sources: fructose (Fru), glucose (Glu), glycerol (Gly), mannose (Man) and thymidine (Thy). After correction for inflation of family-wise error rate (Holm–Šidák's correction) only NB showed a significant effect of selection (Fig. 2.6A: $F_{2,18} = 17.04$, $P = 0.0036$). Tukey's post-hoc analysis show that both lag and exponential treatments have increased growth rate in NB^{Kan} compared to control populations but did not differ w.r.t each other. We also measured the resistance of evolved population in stress environments. Resistance was measured as change in MIC in 4 antibiotic environments: ampicillin (Amp), chloramphenicol (Chl), nalidixic acid (Nal), and rifampicin (Rif) and 3 heavy metal environments: cobalt chloride (Co), copper sulphate (Cu), and nickel chloride (Ni). After correction for multiple testing (Holm–Šidák's correction), main effect of selection was significant in chloramphenicol (Fig. 2.6B: $F_{2,18} = 16.76$, $P = 0.0037$), nalidixic acid ($F_{2,18} = 70.33$, $P = 9.02E-06$), rifampicin ($F_{2,18} = 16.37$, $P = 0.0035$), and cobalt chloride ($F_{2,18} = 7.22$, $P = 0.045$). In all four cases, Tukey's post hoc tests suggested that lag and exponential treatments had increased MIC w.r.t control populations. Only in the case of chloramphenicol, there was a difference between lag and exponential populations: lag had higher MIC than exponential treatment ($P = 0.046$ in Tukey's post hoc analysis). To further explore the difference in resistance between lag and exponential treatments, we also measured their growth rate at sub-MIC concentration (0.5 µg/ml) of chloramphenicol. The main effect of selection was significant (Fig. 2.7: $F_{2,18} = 49.67$, $P = 6.4E-06$) and both lag and exponential treatments had higher growth rate than control populations. But interestingly, populations in the exponential treatment had higher growth rate than those in the lag treatment ($P = 0.0005$ in Tukey's post hoc analysis).

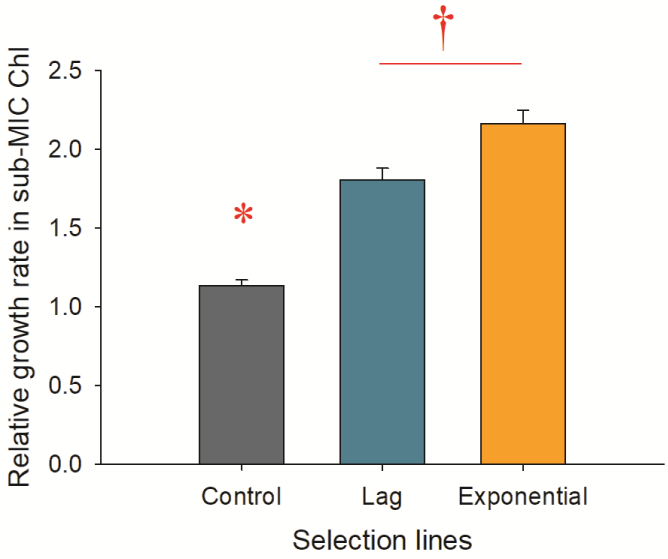

Figure 2.7: Relative growth rate of evolved populations in sub-MIC chloramphenicol. Bars represent mean of 12 values, i.e., 6 replicate populations assayed twice. Whiskers represent ±SE. * denote control is significantly (p value < 0.05) less than lag and exponential in Tukey's post-hoc analysis. † denotes significant differences between lag and exponential treatments in Tukey's post-hoc analysis.

To summarize, the genomic changes due to selection for UV resistance did not result in fitness changes, between the treatments, in all but one novel environment. Interestingly, the genomic changes induced by selection under UV, never led to a decrease in fitness, w.r.t control populations, in the novel environments. In the case of chloramphenicol, where there was an observable phenotypic difference between the lag and the exponential treatments, the effect was not consistent as lag treatment had higher MIC whereas exponential treatment had higher growth rate. In other words, genomic differences between the populations that experienced selection in different growth phases did not translate into observable differences in their phenotypes, either in the selection environment (Fig. 2.2), or in the novel environments (Fig. 2.6).

2.4. Discussion

Bacterial growth phases show considerable variations in their resistance to ionizing radiations. While some studies have demonstrated that the exponential phase can have increased sensitivity to ionizing radiations (Morton & Haynes 1969; Child *et al.* 2002; Dantur & Pizarro 2004; Bucheli-Witschel *et al.* 2010; Arroyo *et al.* 2012), others have shown that this phase can have greater resistance to such radiations (Keller & Maxcy 1984; Hastings *et al.* 1986; Kottemann *et al.* 2005; DeVeaux *et al.* 2007; Sukhi *et al.* 2009). In this study, we compared the UV sensitivity of our ancestral strain of *Escherichia coli* MG1655 in lag and exponential phase. Ancestral cells in the exponential phase were more sensitive to UV, which resulted in larger reduction (~ 1-2 $\log_{10}$ fold more reduction) in colony forming units (CFUs) compared to lag phase (Fig. 2.1). The two growth phases differ in a number of factors that can influence UV sensitivity. Factors such as changes in growth rate (Keller & Maxcy 1984; Berney *et al.* 2006; Bucheli-Witschel *et al.* 2010), growth environment (particularly nutrition availability post irradiation) (Child *et al.* 2002; Sukhi *et al.* 2009), and the quantity of genetic material (Fabre 1970; Bucheli-Witschel *et al.* 2010), have been suggested to explain the differential UV sensitivity in different growth phases. These differences can influence the organisms' ability to repair UV induced damages and consequently the dynamics of resistance evolution.

We subjected our *E.coli* populations to selection under UV exposures during the lag and the exponential phase. After 100 rounds of exposure and growth, the UV exposed populations evolved increased resistance to UV compared to the non-exposed control populations (Fig. 2.2). 370 seconds of UV exposure in the ancestor and the control populations resulted in 5-7 $\log_{10}$reduction in viable CFUs whereas the UV-treated populations experienced only 1-2 $\log_{10}$reduction in CFUs. Contrary to our expectations, the differences between growth phases

observed in the sensitive strains (ancestor and control populations) did not translate into significant differences in the evolved response. Both the treatments had evolved resistance to UV in both growth phases, irrespective of the selection environment. These results are comparable with previous studies in *Bacillus subtilis* (Wassmann *et al.*, 2011) where it was shown that populations that evolved UV resistance in the stationary phase, also showed similar increased UV resistance in all growth phases. Interestingly, in that study, this was observed in spite of the fact that their ancestral and control populations showed growth phase dependent sensitivity to UV (Wassmann *et al.* 2011). Considering the results from our study and Wassman et al., (Wassmann *et al.* 2011), it is tempting to hypothesize that there are only a few routes to UV resistance, which might not be influenced by the growth phases. To investigate this possibility, we employed evolve and re-sequence (E&R) technique on replicate populations to characterize the genomics of UV resistance at different growth phases.

Both the treatments saw the accumulation of a large number of mutations with an overrepresentation of GC→AT transitions (at least 50% of all mutations). GC→AT transitions are the signature mutations of UV (Griffiths *et al.* 2005; Brash 2015) which are a result of the repair of UV-induced oxidative damages to the DNA (Wang *et al.* 1998). This demonstrates the strong influence of UV radiation in shaping the genome of the UV-treated populations.

To investigate the genomic changes associated with UV resistance, we focused on the non-synonymous mutations in the populations. We found that a single amino acid change in *recA* (D161G) was convergent across all four UV-treated populations. Our ancestor had a mutation in *recA* (G161D) with respect to the reference strain, MG1655 (NC_000913.3). It is known that D161 is an extremely conserved amino acid and it plays an important role in determining

the preference of recA for single stranded DNA over double stranded DNA (Shinohara *et al.* 2015). The fact that all UV-treated populations, but not the controls, had fixed for reversion to the wild type form, suggests that this region is highly essential for the functioning of *recA* in the presence of UV stress.

While *recA* protein is a key regulator of recombinational repair of UV induced damages (Smith *et al.* 1987) we did not find any other prominent change in its sequence. Interestingly, we found mutations in *recJ* and *recQ* genes, which are components of the recFOR recombination machinery that is regulated by *recA* in response to DNA damage (Morimatsu & Kowalczykowski 2014). Additionally, mutations in the RNA polymerase (RNAP) genes, *rpoB* and *rpoC*, were also observed in UV-treated populations. Although mutation in rpoB gene are major effectors of rifampicin resistance, it was not observed in all populations. Since rifampicin resistance had evolved in both UV-treated populations, resistance cannot simply be explained by rpoB mutation. We also note that the mutation M1243L (in L3) is not one of the 69 known rpoB mutations conferring rifampicin resistance (Garibyan *et al.* 2003). However, mutations in RNAP have previously been shown to confer radiation resistance in *Deinococcus* (Bruckbauer *et al.* 2019). Studies show that RNAP and DNA repair proteins can interact when replication is stalled in rapidly dividing cells (Trautinger *et al.* 2005; Baharoglu *et al.* 2010). In our populations, we see an interesting combination of mutations in the recFOR pathway and RNAP genes. Mutations in recJ and rpoC were found together and at similar frequency in L1 and E3 populations while mutations in recQ and rpoB occurred together in L3, at similar frequency (see jeb13764-sup-0002-Supinfo2.xlsx for file containing the full list of non-synonymous mutations). It is likely that the interaction between the different components of the two mechanisms (recFOR pathway and RNAP) is crucial for UV resistance. However, these components can interact in multiple ways and figuring out the details of these interactions is a challenge that is outside the scope of this study.

Although DNA/nucleotides are considered to be the primary target of UV radiation, there is growing evidence that cell membrane (Alper 1977; Schwarz 1998; Kumar *et al.* 2016) are also indirect targets of UV damage via generation of reactive oxygen species (ROS). Prevention and/or tolerance to damages to the cell membrane and protein can be an alternate strategy of UV resistance. It has been shown that ROS generated by UVB stress causes lipid peroxidation resulting in cell membrane damage (Gomes *et al.* 2013; Santos *et al.* 2013). Thus, maintaining cell membrane integrity is probably one of the first priorities under UV stress. For instance, in *Enterobacter cloacae*, outer membrane protein (*ompC*) and periplasmic oligopeptide binding protein (*oppA*) were among the differentially expressed genes when exposed to UVB (Kumar *et al.* 2016). Although a causal link between cell membrane structure and radiation resistance has not been experimentally shown, the highly radio-resistant *Deinococcous* spp. is well known for its unique multilayered (six layers) cell membrane (Makarova *et al.* 2001). Thus, it is possible that the structure and composition of the cell membrane might influence radiation resistance. Consistent with this, we see that all our UV-treated populations, but not the controls, carry mutations in *mepS*, an endopeptidase which is a part of cell wall biogenesis (Singh *et al.* 2012). Additionally, mutation clusters in genes involved in or a part of cell membrane structure were consistent among UV-treated populations (Fig. 2.5). With the present data, it is not possible for us to comment on whether these mutations directly led to increased UV resistance or were neutrally accumulated as a consequence of increased mutation rate in the UV-treated populations. However, this opens potential avenues for investigating the mechanism of UV resistance from the point of view of cell membrane structure and composition. It is also possible that the increased mutation supply in the UV treated populations allowed them to explore the mutation landscape for mutations that could be beneficial in the laboratory growth conditions. This is evident from

the fact that both UV-treated populations had significant increase in growth rate in nutrient broth than the control populations (Fig. 2.6A).

One of the mechanisms of radiation resistance in *Deinococcus* is the export of damaged/degraded DNA following radiation (Battista 1997; White *et al.* 1999). Transport of the damaged oligonucleotides can prevent them from being reincorporated during repair. While the cells export damaged DNA, nutrients such as amino acids, sugars and phosphates may also be imported into the cell (Makarova *et al.* 2001). This increased nutrition is essential for the energy expensive DNA repair process (Venkateswaran *et al.* 2000). Consequently, radio-resistance has also been attributed to efficient transport of nutrients into the cell (Makarova *et al.* 2001; Child *et al.* 2002; Sukhi *et al.* 2009). In line with this, we observe clustering of mutations in genes involved in cellular transport (Fig. 2.5). These mutations are unique to the lag treatment and were not present in the exponential treatment. Another unique characteristic of the genome of the lag treatment is the fixation of mutations in *lexA* genes. *lexA* is a transcriptional repressor of SOS response (Maslowska *et al.* 2019). Additionally, mutations in transcriptional regulators such as *crp*, *deoR*, *fadR*, *hfq* were also common in the lag populations (Table 2.4). Taken together, this suggests that in addition to the direct response to radiation, UV resistance in lag treatment can comprise of protection/tolerance mechanisms as well as indirect response via regulation of other genes.

The mutations in the exponential treatment, except for those in genes related to repair and cell membrane structure and function, could not be associated with any known pathway associated with UV resistance (Table 2.4 and Fig. 2.5). Characterizing the role of signal transduction, cell adhesion, metal-ion and nucleotide binding proteins, and enzymes such as kinases, and serine esterase, in the context of UV resistance, might suggest the association of novel pathways of UV resistance.

Horizontal gene transfer (HGT), by natural transformation of exogeneous DNA from the environment, is known to be induced by stress (Claverys *et al.* 2006; Prudhomme *et al.* 2006), particularly UV radiation (Charpentier *et al.* 2011). Additionally, bacterial competence is also known to be influence by multiple factors including, growth phase (Szostkova *et al.* 1999). However, exploring the influence of HGT on the genome evolution of our UV-treated populations was out of the scope of this study.

UV as a mutagen is expected to increase the genome wide mutagenesis. A large number of the resulting mutations are expected to be deleterious in the selection environment and therefore likely to be purged by purifying selection. The observed dN/dS ratio of mutations in the coding region being less than one suggests that the UV-treated populations were indeed subjected to purifying selection (Table 2.3). The mutations that escaped being purged and accumulated to high frequencies were either beneficial or neutral in the selection environment. However, it is possible that some of these neutral mutations are contextually neutral, i.e. have an effect on fitness when the environment is altered (Wagner 2005). To investigate this possibility, we studied the fitness of the UV-treated populations under various antibiotics, heavy metals, and carbon sources. Despite the large genetic variation, fitness of the UV-treated populations was significantly different from the control populations in only four out of 12 novel environments namely, chloramphenicol, nalidixic acid, rifampicin, and cobalt chloride (Fig. 2.6). One possible way by which the UV-treated populations could have acquired resistance to these environments is via the UV induced alterations in the cell membrane permeability. Outer membrane permeability has previously been implicated in the evolution of antibiotic resistance (Delcour 2009; Ghai & Ghai 2018; May & Grabowicz 2018). On the other hand, exposure to UV could have also resulted in the introduction of antibiotic resistance mutations in smaller subpopulations which could allow them to grow at higher concentrations of antibiotics (Band & Weiss 2019). Interestingly, the lag and

exponential populations showed significant differences only in resistance to chloramphenicol. Even in terms of chloramphenicol resistance, the differences between lag and exponential treatments were not consistent across MIC and growth rate (Figs. 2.6 and 2.7). Thus, taken together, the genomic signatures of UV adaptation in lag and exponential treatment populations did not result in any major phenotypic differences between them in both the selection as well as novel environments.

2.5. Conclusion

Experimental evolution in combination with high-throughput sequencing (evolve and resequence) is an extremely powerful tool to study the genomics of adaptation (Long *et al.* 2015; Schlötterer *et al.* 2015). While it is known that the phenotype-genotype map can be degenerate, molecular parallelisms can be found at different levels of genome organization ranging from same nucleotide substitution to similar gene networks (Rosenblum *et al.* 2014; Hao *et al.* 2019). Evidence for this comes from previous studies where huge diversity in the beneficial mutations have been reported at the level of nucleotides but convergence was observed at the level of genes and functional groups (Woods *et al.* 2006; Tenaillon *et al.* 2012). Similarly, in our study, although the large number of mutations initially seemed to be randomly distributed in the genome, we observed some convergence of functional groups. DNA repair, RNA polymerase and genes associated with cell membrane structure were some of the convergent changes observed in the UV-treated populations. However, the exposure to UV during different growth phases also led to some unique genomic signatures. It is likely that the two growth phases represent different physiological and biochemical environments inside the cell, which could have constrained the UV induced mutations and consequently the amount and nature of genetic variation available for selection to act. Nevertheless, mutations in genes for mechanisms besides DNA repair systems that have been observed in the UV-

treated populations are suggestive of the role of other indirect pathways involved in UV resistance. These results are reminiscent of mechanisms of extreme radio-resistance in *Deinococcus radiodurans* R1 where resistance has been shown to rely more on indirect mechanisms such as cellular cleansing, signal transduction and transcriptional regulation than on extensive damage repair mechanisms (Makarova *et al.* 2001; Galperin *et al.* 2006; Blasius *et al.* 2008). In addition to recognizing the different possible mechanisms of UV resistance, this study highlights the influence of physiology in shaping genomic evolution. We see that mutational profiles are dependent on the growth phase of exposure. Very little is known about such growth phase specific effects of most mutagens. Such physiological biases of mutagenesis can have important implications in industrial strain improvement studies. Additionally, as UV radiation is widely used as a disinfectant, it is important to acknowledge the evolution of antibiotic resistance as a correlated response. To better manage this, further experiments need to be done to understand the relationship between growth physiology, UV response and antibiotic resistance evolution.

Chapter 3: Fluctuating exposures to UV radiation and Erythromycin result in increased mean and variance in fitness in novel environments

3.1. Introduction

Variation in fitness within a population is one of the prerequisites of evolution by natural selection. The amount of available genetic variation determines the rate of fitness increase and consequently evolution (Fisher 1930). Evolution in asexual organisms such as bacteria, viruses, and other microbes, is largely dependent on *de novo* variation arising from mutations. While organisms are equipped with robust replication machinery, un-repaired errors in replication results in mutations. The rate at which such mutations arise, vary between organisms but are strongly regulated within a species. For example, the spontaneous mutation rate in divergent strains of *Escherichia coli* were nearly identical (Foster *et al.* 2015), at a rate of 10^{-3} per genome per generation (Lee *et al.* 2012). However, a number of factors can influence mutation rates including loss-of-function mutations in replication and/or repair mechanisms. Such hypermutator phenotypes, with tens to few thousand fold higher mutation rates have been routinely observed in natural, clinical and experimental populations (reviewed in (Sniegowski *et al.* 1997; Eliopoulos & Blázquez 2003; Oliver 2005; Jolivet-Gougeon *et al.* 2011)). While the increased mutational supply may not always be adaptive, it can increase the probability of beneficial mutations. Such mutator phenotypes can quickly spread and fix within a population by hitchhiking with beneficial mutations. The evolution of mutator phenotypes, as well as their effects on evolution is still a dynamic field of study (reviewed in (Raynes & Sniegowski 2014; Natali & Rancati 2019)).

In addition to intrinsic genetic changes, many environmental factors have been identified as strong regulators of mutation rate. Factors such as temperature, pH, nutritional state, chemicals and radiations have been studied for their mutagenic properties (reviewed in (Tenaillon *et al.* 2004; Galhardo *et al.* 2007; Ram & Hadany 2012)). Typically, stress environments, that negatively affect the growth rate and/or fitness of an organism exerts

direct or indirect influence over mutation rates. For example, Liu & Zhang (Liu & Zhang 2019) showed that yeast populations grown in seven different environments show varying mutation rates with high mutation rates usually accompanying slow growth rate. More recent studies have shown that in addition to mutation rates, environments also influence mutational biases (Maharjan & Ferenci 2017; Liu & Zhang 2019). Maharanja & Ferenci (Maharjan & Ferenci 2017) showed that iron and oxygen limitation resulted in higher transposition events compared to greater incidence of base-pair substitutions and indels when populations were phosphate-limited. Such mutational differences may be due to the fact that different environmental conditions induce differences in the nature of DNA damage such as double strand breaks (DSBs) and incorporation of oxidized bases. Additionally, the mechanisms/pathways employed to repair the damaged DNA can also vary resulting in unique mutational signatures. For example, UV radiations induces pyrimidine dimers and photoproducts which are repaired by excision repair mechanisms resulting in a majority of GC→AT transitions (Brash 2015). In contrast, knockout of *mutT* which, repairs mispairing due to mutagenic 8-oxo-dGTP, results in greater incidences of A:T → C:G transversions (Sekiguchi 1996; Fowler & Schaaper 1997). Thus mutational biases are ubiquitous in adaptive evolution ((Stoltzfus & McCandlish 2017; Cano *et al.* 2021) and references within), which can be shaped by the type of both DNA damage and DNA repair (Volkova *et al.* 2020).

An increasing number of empirical and observational studies have shown that mutational biases strongly influence the evolutionary trajectories (Stoltzfus & McCandlish 2017; Payne *et al.* 2019; Storz *et al.* 2019; Cano & Payne 2020; Gomez *et al.* 2020). For example, Couce et. al., (Couce *et al.* 2015) showed that mutator strains with different mutational spectra evolved antibiotic resistance via divergent mutational pathways (i.e., divergence in genomic evolution). De novo mutations are central to antibiotic resistance evolution (Woodford &

Ellington 2007) and the effects of mutator strains have been well studied (Eliopoulos & Blázquez 2003; Perron *et al.* 2010; Gifford *et al.* 2019). Mutator strains may provide a selective advantage in the presence of antibiotics by increasing the probability of resistance mutations. On the other hand, exposure to antibiotic can itself induce higher mutation rates by induction of stress response mechanisms resulting in mutagenesis. Long et. al., (Long *et al.* 2016) showed that antibiotic (norfloxacin) concentration and mutation rates have a strong linear relationship. As a result of both these scenarios, elevated mutation rates have often been observed accompanying resistance evolution. However, a large majority of studies on the relationship between resistance evolution and mutation rates have focused only on the genetic mutation rate modifiers. Environmental mutagens and their role in the evolution of antibiotic resistance evolution has received relatively less attention.

UV radiation, an environmental mutagen, is both an agent of selection as well as mutagenesis. It has been previously shown that outcome of selection for UV resistance as well as the mutational profile depends on the growth phase during which the bacteria are exposed to the radiation (Selveshwari *et al.* 2021). In this study, we aim to understand how UV induced mutational biases influence the evolution of antibiotic resistance. Another important factor that affects the maintenance of variation is environmental heterogeneity, both temporal and spatial (Hallsson & Björklund 2012; Canino-Koning *et al.* 2019; Nguyen *et al.* 2021). Environmental heterogeneity, resulting in divergent selection, could promote the maintenance of polymorphism for traits that show a negative genetic correlation between components of the heterogeneous environments (Levene 1953; Kassen 2014). Other factors such as negative frequency-dependent selection, in heterogeneous environments, have also been cited as mechanisms influencing the extent of genetic variation (Dykhuizen & Dean 2004; Friesen *et al.* 2004; MacLean *et al.* 2005). Additionally, it is interesting to note that multiple theoretical as well as empirical studies have shown that increased mutation rates are

maintained and even favored in fluctuating environments (Ishii *et al.* 1989; Travis & Travis 2002; Carja *et al.* 2014). Thus, it would be interesting to investigate the interaction between mutation rate modifications and fluctuating environments, and how that in turn influences genetic variation and evolutionary trajectories.

To address the aforementioned issues we used the technique of experimental evolution on laboratory populations of *Escherichia coli*. Replicate populations were subjected to increasing concentrations of the antibiotic (erythromycin) with or without UV exposures. Moreover, when the populations were exposed to UV radiation, they were also subjected to either constant or fluctuating treatments. Experimental evolution was followed by whole genome sequencing to further understand the genomics of evolution. After 60 rounds of selection, we saw that populations exposed to UV and/or erythromycin had evolved similar extents of resistance to their respective stress environments. However, there were major differences between UV exposed and unexposed populations in terms of the genes associated with erythromycin resistance. Erythromycin specific target-gene mutation (rplD) were observed in populations that were selected in only erythromycin. Exposure to UV radiation (both constant and fluctuating) resulted in mutation in efflux pump regulator gene (acrR) and the absence of target gene mutation (rplD). Additionally, fluctuating exposures to UV radiation and erythromycin resulted increased fitness in other antibiotics. Follow-up experiments revealed that the greater variance in fitness in these populations was likely due to the maintenance of large proportions of genetically distinct subpopulations.

3.2. Materials and Methods

Escherichia coli K12 MG1655 strain with kanamycin resistance was used in this study. Cultures were maintained at 37ºC and 150 RPM, throughout selection and assays, except where stated otherwise.

3.2.1 Experimental evolution

Six independent *E.coli* colonies were picked from nutrient agar streaks to initiate six replicate ancestral populations. Four selection regimes: only UV, only antibiotic (erythromycin), constant exposure to both UV+antibiotic, and fluctuating exposures to UV and antibiotic; were initiated from each of the replicate ancestral populations. It is known that SOS response is one of the primary responses to DNA damage due to UV radiation (Sinha & Häder 2002). SOS response is also known to be induced on exposure to a number of antibiotics (Kohanski *et al.* 2010). Therefore, in order to independently study the effect of exposure to UV radiation as well as antibiotics, we used erythromycin. It belongs to the macrolide class of antibiotics which inhibits protein synthesis by binding to the 50s ribosomal complex (Weisblum 1995). Therefore, it has no known link to DNA damage and induction of SOS response.

The UV control populations were exposed to UV as described in a previous study (Selveshwari *et al.* 2021). Briefly, they were exposed to a constant irradiance of 100.5 $\mu W/cm^2$ in a custom-built UV chamber with a 254nm UV-C tube-light (Philips TUV 8W). All populations were grown in six-well tissue culture plates in 2ml NB^{Kan}, until they reached the stationary phase. After 20 hours from inoculation, the populations were exposed to UV at room temperature and 150 RPM orbital shaking. After every 5 exposures, the duration of exposure to UV was increased. This ensured that all populations were allowed sufficient time to adapt. At the beginning of the experiment, populations were exposed to 15 seconds of UV,

which was gradually increased up to 3 minutes and 20 seconds by the end of 60 exposures. The antibiotic control populations were grown in NB^{Kan} containing erythromycin. They were initially exposed to a sub-lethal concentration of erythromycin which resulted in ~75% reduction in growth rate compared to growth rate in NB (personal observation). This concentration was also increased every 5 days, from 15µg/mL to 200µg/mL. Populations subjected to constant UV + antibiotic treatments were grown in NB^{Kan} containing erythromycin and every 20 hours from subculture they were exposed to UV radiation. The fluctuating treatment populations, on the other hand, were subjected to daily alternation between the two stresses, starting with growth in erythromycin. For direct comparisons between treatments, the number of exposures to each stress was kept constant in all treatments. Therefore, the selection in fluctuating treatment lasted twice as long as the other treatments. For the full trajectory of exposure duration and antibiotic concentration see Table 3.1. At the end of every 5 rounds of exposures to stress, before the intensity of stress was increased, all populations were stored as 15% glycerol stocks at -80^{o}C. Fitness of the evolved populations were assayed at the end of 60 rounds of exposures to stress, either UV or erythromycin or both. Throughout selection and assays, except during subculturing, all populations were maintained in dark. This prevents induction of photoreactivation, the first and most efficient, error-free repair mechanism (Sinha & Häder 2002).

Table 3.1. Exposure duration during selection. The duration of exposure to UV was increased every 5 days.

Days	UV exposure duration (seconds)
1 - 5	15
6 - 10	20
11 - 15	25
16 - 20	35
21 - 25	45
26 - 30	60
31 - 35	75
36 - 40	90
41 - 45	110
46 - 50	130
51 - 55	160
56 - 60	200

3.2.2 Sensitivity to UV radiation

Sensitivity to UV exposure was measured as described in a previous study (Selveshwari *et al.* 2021). Briefly, resistance to UV was estimated as the log of the ratio of the number of colony forming units (CFUs) before and after UV exposure ($\log_{10}$(CFUs before exposure/CFUs after UV exposure)) (Koivunen & Heinonen-Tanski 2005). Glycerol stocks of the evolved and ancestor populations were revived in 2ml NB^{Kan}, overnight. 20µl of the revived culture was inoculated in 2ml NB^{Kan} and allowed to grow till stationary phase (for 20 hours) before being exposed to 3 minutes and 20 seconds of UV radiation. CFUs of the populations were determined, both before and after exposure, by plating appropriate dilutions on 2% nutrient agar containing kanamycin (NA^{Kan}). The serial dilution and plating after UV exposure were performed in a dark room illuminated by red light. The number of colonies on the plates were counted after 24 hours of incubation in darkness at 37ºC. The average CFUs of two independent measurement replicates, obtained by repeating the entire assay from revival to CFU counts twice, were compared.

3.2.3 Resistance to erythromycin

Fitness of the evolved populations in erythromycin was measured in two ways: growth rate and minimum inhibitory concentration (MICs).

Measuring growth rate

Since the populations were exposed to sub-lethal concentrations during selection, growth rates of the evolved and ancestral populations were measured at the final concentration of erythromycin. The revived evolved and ancestral populations were inoculated in 200µl NB^{Kan} with 200µg/mL erythromycin at a dilution of 1/1000 in 96 well plates. These cultures were incubated at 37ºC and continuous shaking and monitored for 24 hours using a plate reader

(Synergy HT, BIOTEK Winooski, VT, USA). The OD_{600} was measured every 20 minutes. Growth rate was computed as maximum slope of the curve over moving windows of 3 hours (Karve *et al.* 2015; Sprouffske *et al.* 2018; Chavhan *et al.* 2019; Rodríguez-Rojas *et al.* 2020). The average of two measurement replicates obtained by repeating the entire assay twice, was compared between populations.

Measuring minimum inhibitory concentrations

The resistance of the evolved and ancestor populations were also estimated as the Minimum Inhibitory Concentration or MIC. The revived populations were diluted to $1/1000^{th}$ initial concentration and inoculated in serial two-fold dilutions of erythromycin, in triplicates. After 48 hours, the populations were visually scored as growth or no-growth. The minimum concentration where at least two replicates showed no growth was determined as the MIC of the populations. MICs of the evolved populations were scaled by their corresponding ancestral MIC and the average of two independent experiments were analyzed.

3.2.4 Fitness in novel environment

To understand how exposures to UV or erythromycin influence cross resistance to novel antibiotics, we measured growth rate and MIC of the evolved and ancestor populations in three antibiotics: rifampicin, nalidixic acid and chloramphenicol, belonging to different classes of antibiotics. Nalidixic acid and rifampicin inhibit DNA and RNA synthesis by binding to DNA gyrase and RNA polymerase, respectively (Kohanski *et al.* 2010). Chloramphenicol inhibits protein synthesis by binding to the 50s ribosome (Kohanski *et al.* 2010) which is similar to the mechanism of action of erythromycin. However, the antibiotics have different sites of inhibition on the ribosome. Both growth rate and MICs of all populations were measured as described above (section 2.3). Growth rate estimations were carried out at concentrations that reduced growth rate by ~50%. The sub-lethal concentrations

used were as follows: 4µg/mL rifampicin, 25µg/ml nalidixic acid, and 0.5µg/ml chloramphenicol.

3.2.5 Population analysis profile

To further explore antibiotic resistance of the evolved populations, we performed a population analysis profile (PAP). PAP is traditionally performed on clonal populations to study the presence of resistant sub-populations within an otherwise isogenic susceptible population. However here, we use the PAP protocol to examine the distribution of variation in resistance phenotype within our evolved populations. The protocol from (Sherman *et al.* 2019) was used with modifications. Briefly, the number of CFUs of the evolved and ancestor populations were estimated on NA^{Kan} plates with or without a gradient of antibiotics. Antibiotic plates were prepared with two-fold increment of antibiotic starting from 0.25x where 1x corresponds to the MIC of the ancestor in broth. The assay was performed for erythromycin as well as the three novel antibiotics: rifampicin, nalidixic acid and chloramphenicol. Revived cultures were diluted and spotted (20µl) and spread undiluted (100µl), at each concentration of the antibiotic and incubated for 48 hours. CFU estimations were made at appropriate dilution in increasing concentration until either no growth or only a single colony were observed in 100µl of undiluted culture. In rifampicin, our observations were restricted to 256x (or 4096µg/ml) as the fluctuating treatment populations were able to grow at all assayed concentrations. The experiments were replicated twice and the average proportion of resistant cells at each concentrations were estimated. One of our populations (C2) repeatedly failed to revive. In this case, the PAP assay was performed on a pseudostock; cultures revived from D60 stock, stored as a second glycerol stock for backup.

Resistance and the proportion of resistant cells were interpreted using a modified protocol based on (Andersson *et al.* 2019; Sherman *et al.* 2019; Maeda *et al.* 2020). Populations were

considered susceptible when a particular concentration of antibiotic resulted in less than $1x10^{-7}$ CFUs. This ensures that the observed resistance is not a result of spontaneous resistance mutants which is expected to arise at a rate $<10^{-7}$ per cell per generation (Williams 2014). This concentration was termed as the inhibitory concentration and the concentration prior to it as the highest non-inhibitory concentration. Homogeneous resistance was identified when at least 50% of the populations survive concentration at least eight fold higher than the highest non-inhibitory concentration of the ancestors. Populations exhibited variation in resistance when the proportion of population surviving at least 8X non-inhibitory concentration of the ancestors was between 10^{-7} and 50%.

3.2.6 Cross resistance in subpopulations

Using PAP assay, we observed that resistance in F populations were distributed as small proportions of resistant cells. To further understand the resistance phenotypes of these subpopulations, we checked the cross-resistance of these resistant subpopulations in all novel environments. Resistant subpopulations were obtained by inoculating the revived F population in 8X concentration of nalidixic acid and rifampicin, and 2X concentration of chloramphenicol. These correspond to 1024µg/mL nalidixic acid, 128µg/mL rifampicin, and 8µg/mL chloramphenicol. After 48 hours of growth at these antibiotic concentrations, the resulting resistant subpopulations were assayed for their resistance (MIC) in all three antibiotics, as described before. The ancestors, revived 24hour prior, were also assayed alongside.

3.2.7 Whole-genome sequencing and analysis

To understand the genomics of adaptation, we performed whole-genome sequencing at two levels. Firstly, we performed whole-population whole-genome sequencing on two randomly-chosen replicates (rep 3 and 5) and their corresponding ancestors. Secondly, we sequenced

only the nalidixic acid and rifampicin resistant subpopulations of the corresponding replicates of fluctuating treatments; F3 and F5. Whole-population sequencing was performed on the glycerol stocks revived in 4ml NB^{Kan}. After incubation for ~16 hours, the culture was pelleted, twice washed in phosphate buffer saline and shipped for genome sequencing with a commercial service-provider (Eurofins Genomics India Pvt. Ltd., Bengaluru). For subpopulation sequencing, the revived populations were inoculated in 1024µg/mL nalidixic acid and 128µg/mL rifampicin i.e., 8X concentration of two antibiotics (where 1X is the ancestral MIC). The cultures were pelleted after 48 hours of growth. Cell pellets were washed in PBS and shipped to a genome-sequencing service provider for sequencing (Genepath Diagnostics India Pvt Ltd., Pune). The whole-population sequencing was performed on NextSeq500 platform (Illumina, USA) at an average depth of ~135X (range: 83X – 200X) and 150bp read length. The subpopulations were sequenced on MiSeq platform (Illumina, USA) at average depth of ~91X (range: 87X – 96X) and 150bp read length. The sequence reads were trimmed of adaptors, ambiguous and low-quality reads using Trimmomatic v0.38 (Bolger *et al.* 2014). The resulting high quality reads were aligned and variants called using *Breseq* version 0.33.2 pipeline (Deatherage & Barrick 2014) at default parameters. We used a custom reference genome for alignment. The NCBI reference (Genbank accession: NC_000913.3) was updated by incorporating mutations identified in our ancestral sequence. The whole population and subpopulations were aligned to their corresponding ancestor sequence and mutations were predicted in polymorphism mode. Only the mutations that were present at a frequency greater than 10%, and those that were unique in our evolved populations, were used in our analysis.

3.2.8 Statistical analysis

UV sensitivity, and erythromycin MIC and growth rate of the evolved and ancestral populations were compared in three independent two-way mixed model ANOVAs and randomized complete block design (RCBD) (Rohlf & Sokal 1995). Treatment (5 levels) was taken as fixed factor and replicates (6 levels) as an independent random factor (neither crossed nor nested in other factors). The evolution of correlated fitness in populations exposed to single stress (controls) and combination of stress (constant or fluctuating) were independently compared to the ancestral fitness. The growth rate and MIC in the three novel antibiotics were analyzed in separate two-way mixed model ANOVAs, as before, followed by Holm–Šidák correction (Abdi 2010) for inflated family-wise error rate. When the main effect of treatment was significant after correction, Tukey's post-hoc analysis was performed for all pairwise comparisons. The MIC of the subpopulations in the three novel antibiotics were also analyzed in separate two-way mixed model ANOVAs followed by Holm–Šidák correction. Subpopulation identity (3 levels) and replicates (6 levels) were taken as fixed and independent random factors, respectively. Tukey's pairwise comparisons were performed for significant main effects, after correction. We also computed the Cohen's *d* as a measure of effect sizes of fitness differences between pairs of treatment populations (Cohen 2013). The effect sizes were interpreted as small, medium and large for $0.2 < d < 0.5$, $0.5 < d < 0.8$ and $d > 0.8$, respectively (Sullivan & Feinn 2012).

3.3. Results

3.3.1 Fitness in selection environments

3.3.1.1 UV resistance is not influenced by selection for erythromycin resistance.

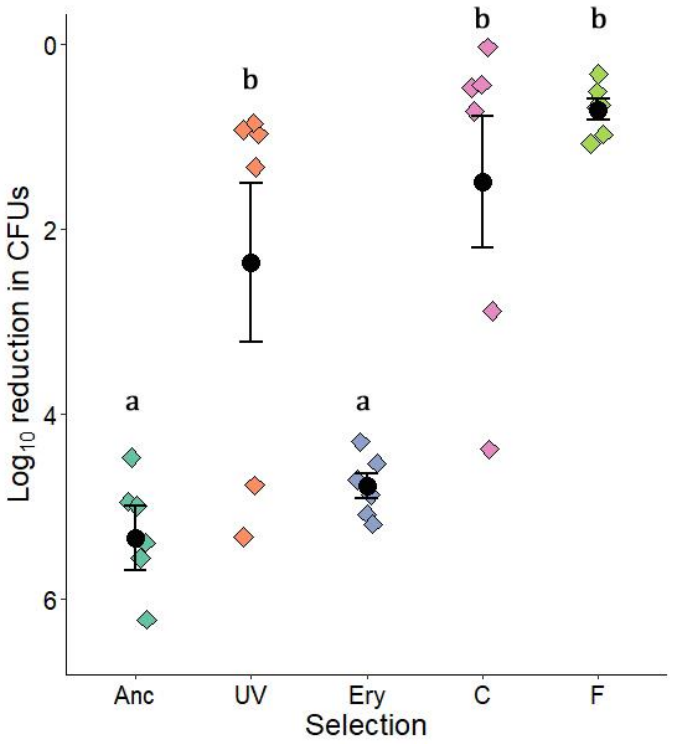

Fig. 3.1 Mean log_{10} reduction (±SEM) in CFUs. Sensitivity to UV induced reduction in CFUs was measured at 3 minutes and 20 seconds of exposure. Circles represent mean sensitivity and whiskers represent ±SE. The scatter of the six replicate populations is plotted as diamonds (♦). Populations denoted by different alphabets are significantly ($P < 0.05$, Tukey's HSD test) different from each other.

UV sensitivity, measured as the $\log_{10}$ reduction in CFUs, was significantly between the evolved populations and their ancestors (Fig. 3.1; $F_{4,20} = 18.69$, $P = 1.56E\text{-}06$). Tukey's pairwise comparisons showed that the three UV exposed populations, UV control, C (constant UV + erythromycin) and F (fluctuations between UV and erythromycin), show a significant reduction in sensitivity compared to the ancestor and Ery control populations (see Table 3.1 for Tukey values and their effect sizes). Ery and ancestors showed no significant differences (Tukey's $P = 0.91$) suggesting that selection in erythromycin did not result in UV resistance. Furthermore, C and F populations had evolved similar extents of UV sensitivity, compared to UV control populations (see Table 3.1). Taken together, these results suggest that the presence or the absence of erythromycin did not influence the evolution of resistance to UV.

Table 3.1. Summary of Tukey's *P* value and Cohen's *d* value of pairwise comparisons of UV sensitivity.

Pairwise comparison	*P* value (Tukey's post hoc)	Cohen's *d*	Interpretation
C vs. Anc	2.24E-04	2.800	Large
F vs. Anc	1.36E-04	7.172	Large
Er vs. Anc	0.915	0.850	Large
UV vs. Anc	0.002	1.858	Large
C vs. Er	0.001	2.627	Large
F vs. Er	1.70E-04	13.203	Large
UV vs. Er	0.013	1.613	Large
C vs. UV	0.688	0.453	Medium
F vs. UV	0.134	1.109	Large
C vs. F	0.765	0.628	Medium

3.3.1.2 Erythromycin resistance is not influenced by UV resistance

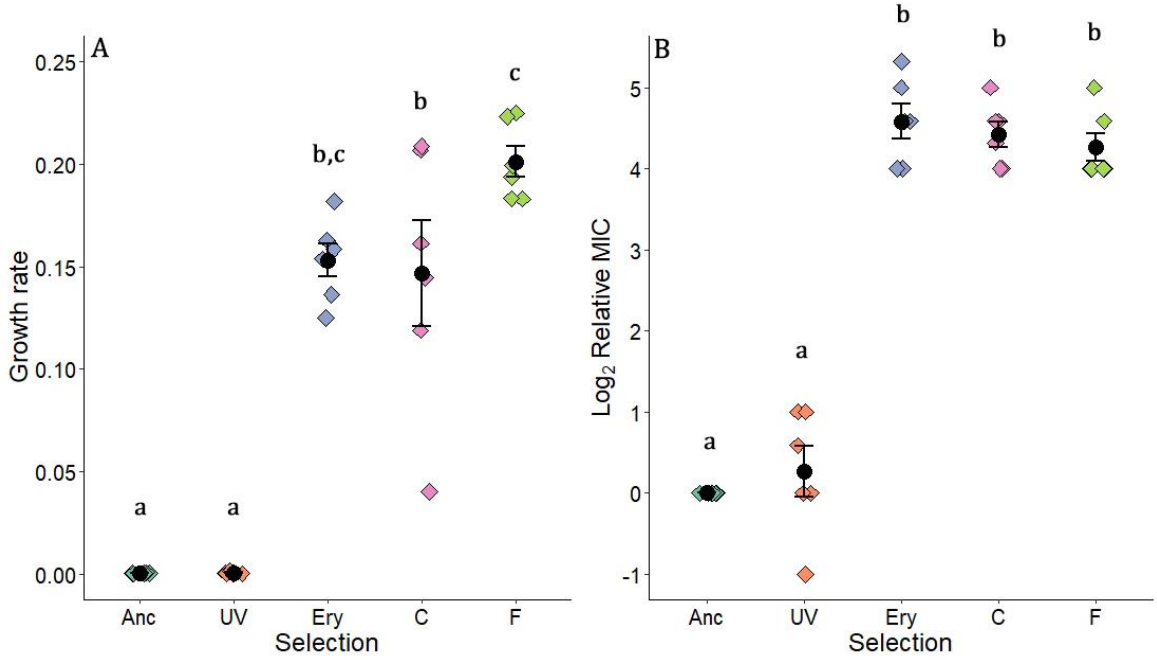

Fig. 3.2 Fitnesses of the four selection regimes and ancestor in erythromycin. (A). Mean growth rate at 200µg/ml of erythromycin (B). Mean of log transformed value of the change in MIC (Minimum Inhibitory Concentration), w.r.t the ancestor. Error bars represent SEM. The scatter of the six replicate populations is plotted as diamonds (♦) around the mean. Populations denoted by a different alphabets are significantly ($P < 0.05$, Tukey's HSD test) different from each other.

Fitness of the evolved populations and their ancestors, in erythromycin, was measured as change in minimum inhibitory concentration (MIC) as well as growth rate in 200µl/mL erythromycin (final concentration of erythromycin during selection). The populations showed a significant main effect for both measures of fitness: growth rate (Fig. 3.2A; $F_{4,20}$ = 55.75, P = 1.45E-10) and change in MIC (Fig. 3.2B; $F_{4,20}$ = 152.29, P = 1.12E-014). Tukey's post hoc pairwise comparisons showed that all treatments that faced erythromycin during selection (Ery control, C, and F) had evolved significantly higher growth rate as well as MIC than both ancestor and UV control populations (see Table 3.2 for exact Tukey's values and their effect sizes). Growth rate and MIC of C and F treatments were not significantly different from growth rate and MIC of Ery populations. However, the growth rate of F populations was marginally greater than C populations with large effect size. There was no significant difference between C and F populations in terms of their MICs. Finally, there was no significant difference between UV control and ancestors in terms of both growth rate and MIC. Taken together, these results suggest that exposure to UV does not influence fitness in erythromycin. However, fluctuations between the two stresses confer a marginal advantage, over constant exposures, in-terms of growth rate in erythromycin.

Table 3.2. Summary of pairwise comparisons of fitnesses in erythromycin.

Pairwise comparison	Growth rate			MIC		
	P value (Tukey's post hoc)	Cohen's *d*	Interpretation	*P* value (Tukey's post hoc)	Cohen's *d*	Interpretation
C vs. Anc	1.32E-04	3.290	Large	1.32E-04	16.089	Large
F vs. Anc	1.32E-04	15.157	Large	1.32E-04	14.032	Large
Er vs. Anc	1.32E-04	10.762	Large	1.32E-04	12.247	Large
UV vs. Anc	1.000	0.498	Small	0.861	0.489	Small
C vs. Er	0.996	0.136	Small	0.971	0.359	Small
F vs. Er	0.087	2.478	Large	0.763	0.659	Medium
UV vs. Er	1.32E-04	10.748	Large	1.32E-04	6.567	Large
C vs. UV	1.32E-04	3.286	Large	1.32E-04	6.846	Large
F vs. UV	1.32E-04	15.140	Large	1.32E-04	6.449	Large
C vs. F	0.043	1.174	Large	0.979	0.369	Small

3.3.3.2 Genomics of adaptation

Using whole genome analysis of two replicates of each treatment we identified 77 and 167 mutations in UV, 14 and 15 mutations in Ery, 33 and 32 mutations in C, and 66 and 26 mutations, in the respective replicates (see https://doi.org/10.5281/zenodo.5918401 for full list of mutations). To understand the genomics of resistance evolution we focused on non-synomymous SNP that were consistent in both replicates of a treatment. Table 3.3 summarizes the list of genes, substitutions, and frequency of mutations that were observed in both replicate of at least one treatment group (convergent mutations).

D161G substitution in *rec A* was fixed in all UV control populations; UV, C, and F. It is a reversion of an ancestral mutation that was also observed in our previous experiments (Selveshwari *et al.* 2021). The UV control had mutation in 5 other genes (Table 3.3) involved in transcriptional regulation, cell division, aerobic respiration, and protein processing. With the exception of *crp* and *yhjJ* mutation in UV3, all SNPs were found in smaller proportions in the populations (<40%).

When compared to UV control, exposures to erythromycin (Ery, C and F) resulted in fewer mutations. All erythromycin exposed populations, Ery, C and F, show fixation for L828S in *acrB*, a component of RND efflux system (Zgurskaya & Nikaido 1999). In addition, Ery control populations had fixed for G66A/D substitutions in *rplD*, a ribosomal protein with a known macrolide binding site (Arevalo *et al.* 1988). On the other hand, C and F treatments do not have the *rplD* mutations instead, they have an additional mutation, fixation of T5N substitution in *acrR*, a regulator of RND efflux (Ma *et al.* 1996). In addition to these common mutations, *ydcT* was convergent in C populations and *cyaA*, *nudE*, and *rpsG* were convergent in F populations. These genes may be involved in regulation of translation via amino acid

transport/metabolism, cAMP biosynthesis, purine metabolism, and ribosomal assembly (see discussion).

Table 3.3. Summary of genes consistently mutated in both replicate of at least one treatment populations. The treatments with mutations in both replicates have been highlighted in bold.

Gene	Codon substitution	Population	Proportion
recA	D161G (GAC→GGC)	**UV 3**	100%
		UV 5	100%
		C 3	100%
		C 5	100%
		F 3	100%
		F 5	100%
crp	T203I (ACC→ATC)	**UV 3**	100%
	H20Q (CAC→CAA)	**UV 5**	25.50%
	K45E (AAA→GAA)	**UV 5**	25.20%
	L74R (CTG→CGG)	C 5	100%
ftsQ	P116F (CCT→TTT)	**UV 3**	16.80%
	P116L (CCT→CTT)	**UV 5**	39.40%
rsxC	V707E (GTA→GAA)	**UV 3**	10.40%
		UV 5	10.90%
		F 3	22.20%
	Q575P (CAG→CCG)	F 3	11.90%
	Q582E (CAA→GAA)	C 5	12.70%
yacH	K487Q (AAG→CAG)	**UV 3**	10.60%
		F 3	11.30%
	R492S (AGA→AGC)	**UV 5**	10.00%
	K487Q (AAG→CAG)	C 5	10.60%
		Er 5	10.00%
yhjJ	W283* (TGG→TAA)	**UV 3**	92.90%

	E457K (GAA→AAA)	**UV 5**	18.80%
acrB	L828S (TTA→TCA)	**Ery 3**	100%
		Ery 5	100%
		C 3	100%
		C 5	100%
		F 3	100%
		F 5	100%
rplD	G66A (GGC→GCC)	**Ery 3**	100%
	G66D (GGC→GAC)	**Ery 5**	100%
		C 3	100%
		F 5	76.50%
acrR	T5N (ACC→AAC)	**C 3**	100%
		C 5	100%
		F 3	100%
		F 5	100%
ydcT	D166G (GAT→GGT)	**C 3**	100%
		C 5	100%
cyaA	R162C (CGT→TGT)	**F 3**	100%
		F 5	100%
	R160C (CGC→TGC)	C 3	100%
	R160S (CGC→AGC)	Ery 5	100%
nudE	D83Y (GAT→TAT)	**F 3**	100%
		F 5	100%
	P54L (CCG→CTG)	C 3	100%
rpsG	L157* (TTA→TGA)	**F 3**	100%
		F 5	100%
		UV 3	100%

3.3.3 Fitness in novel environments

3.3.3.1 Correlated fitness of exposure to single stress is limited

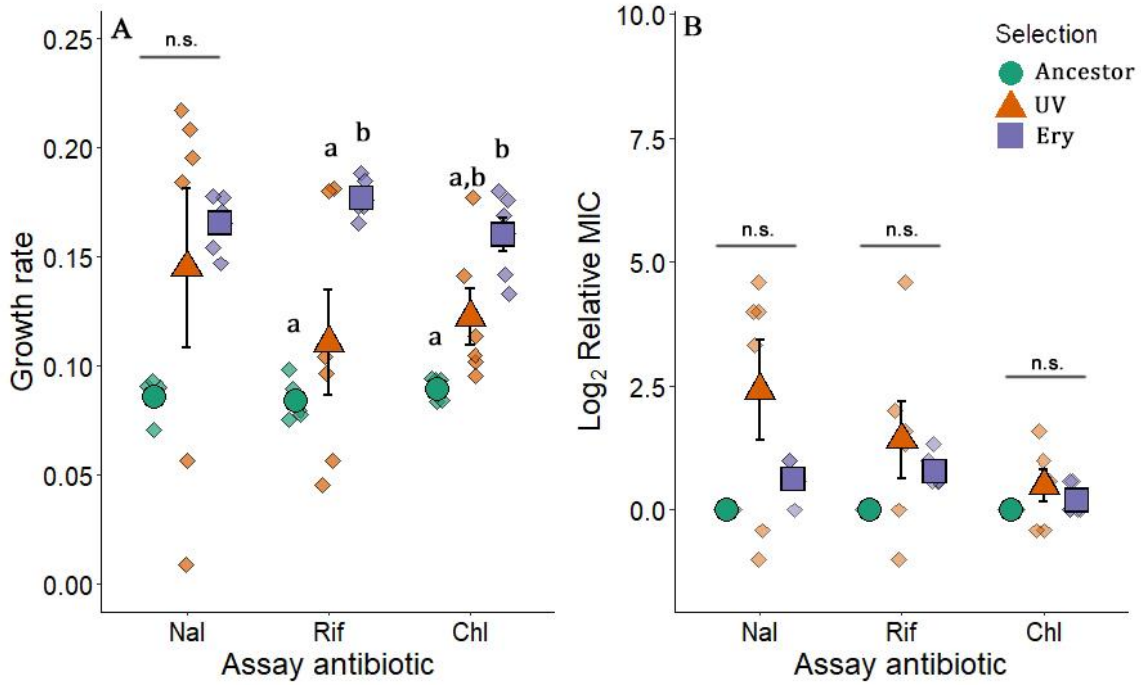

Fig. 3.3 Fitness of control populations in novel antibiotics. (A) Mean growth rate and (B) Mean MIC of control populations compared to ancestors in three antibiotics, Nalidixic acid (Nal), Rifampicin (Rif) and Chloramphenicol (Chl). Error bars represent SEM. The scatter of the six replicate populations is plotted as diamonds (♦) around the mean. Populations denoted by different alphabets are significantly ($P < 0.05$) different in Tukey's pairwise comparisons. Non-significant main effects are represented as n.s.

The correlated response of UV and erythromycin resistance was measured as growth rate and MIC in three novel antibiotics: nalidixic acid, rifampicin, and chloramphenicol. The control populations (UV and Ery) and ancestor showed significant difference only in terms of growth rate in rifampicin and chloramphenicol (Fig. 3.3A, Table. 3.4). Selection for erythromycin resistance resulted in higher growth rate in rifampicin and chloramphenicol with large effect sizes. Growth rate of the Ery populations was significantly greater than both UV selected and ancestor population, in rifampicin and significantly greater than ancestor but marginally non-significant w.r.t UV populations, in chloramphenicol (see table 3.4 for all pairwise comparisons). It is interesting to note that despite a large number of mutations in the UV control populations, they did not show any fitness difference in the three novel antibiotics.

Table 3.4. Summary of the pairwise comparisons of the control populations in novel environment

Growth rate

Assay environment	Holm–Šidák Corrected *P* value	Pairwise comparison	*P* value (Tukey's post hoc)	Cohen's *d*	Interpretation
Nalidixic acid	0.067	-	-	-	-
Rifampicin	0.007	UV vs. Anc	0.451	0.626	Medium
		Ery vs. Anc	0.004	10.834	Large
		Uv vs. Ery	0.026	1.574	Large
Chloramphenicol	0.007	UV vs. Anc	0.106	1.469	Large
		Ery vs. Anc	0.002	5.113	Large
		Uv vs. Ery	0.063	1.459	Large

MIC

Assay environment	Holm Sidak Corrected *P* value	Pairwise comparison	*P* value (Tukey's post hoc)	Cohen's *d*	Interpretation
Nalidixic acid	0.123	-	-	-	-
Rifampicin	0.292	-	-	-	-
Chloramphenicol	0.178	-	-	-	-

3.3.3.2 Fluctuating exposures to stresses result in broad collateral resistance

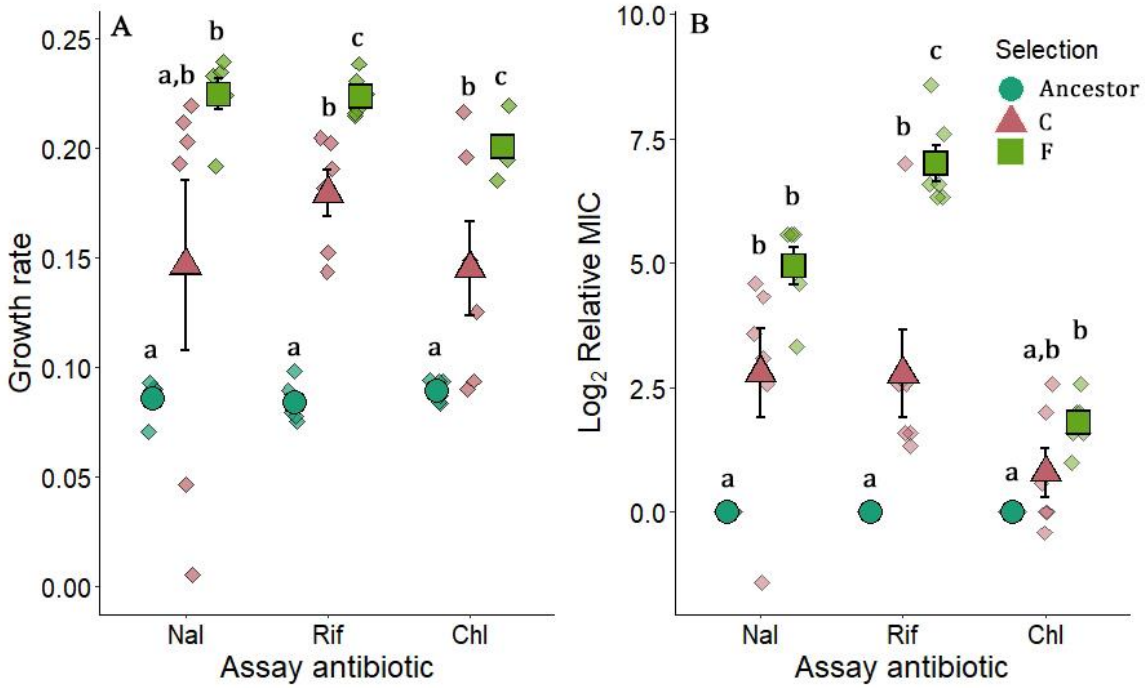

Fig. 3.4 Novel environment fitness of populations exposed to both stresses. (A) Mean growth rate and (B) Mean MIC w.r.t the ancestors in three antibiotics, Nalidixic acid (Nal), Rifampicin (Rif) and Chloramphenicol (Chl). Error bars represent SEM. The scatter of the six replicate populations is plotted as diamonds (♦) around the mean. Populations denoted by different alphabets are significantly ($P < 0.05$) different in Tukey's pairwise comparisons.

The growth rate and MIC of C and F treatment populations were compared to the ancestors in the three novel antibiotics. In contrast to the correlated effect of control populations, the main effect of selection was significant in all three antibiotics as well as both measures of fitness (Fig. 3.4 and Table 3.5). Tukey's post hoc analysis showed that the C populations had evolved significantly greater growth rate in rifampicin and chloramphenicol and greater MIC in nalidixic acid and rifampicin, with large effect sizes. However, F populations had evolved significantly higher growth rate and MIC, than ancestors, in all three antibiotics. Interestingly, the F populations had evolved higher fitness than C populations that was significant in three cases (G.R and MIC in rifampicin and G.R in chloramphenicol) and only marginally non-significant in the remaining three cases (G.R and MIC in nalidixic acid and MIC in chloramphenicol) (Table 3.5). Thus, taken together, fluctuating exposures to UV and erythromycin resulted in stronger correlated response than evolution of resistance to single stress as well as constant exposures to both stresses. A summary of these results have been presented in Table 3.6.

Table 3.5. Summary of the pairwise comparisons of C and F populations in novel environment

Growth rate

Assay environment	Holm–Šidák Corrected *P* value	Pairwise comparison	*P* value (Tukey's post hoc)	Cohen's *d*	Interpretation
Nalidixic acid	0.006	C vs. Anc	0.210	0.898	Large
		F vs. Anc	0.005	10.357	Large
		C vs. F	0.092	1.148	Large
Rifampicin	6.23E-07	C vs. Anc	2.01E-04	4.928	Large
		F vs. Anc	1.99E-04	15.366	Large
		C vs. F	0.003	2.280	Large
Chloramphenicol	3.79E-04	C vs. Anc	0.018	1.495	Large
		F vs. Anc	3.12E-04	12.758	Large
		C vs. F	0.018	1.464	Large

MIC

Assay environment	Holm Sidak Corrected *P* value	Pairwise comparison	*P* value (Tukey's post hoc)	Cohen's *d*	Interpretation
Nalidixic acid	8.15E-04	C vs. Anc	0.016	1.801	Large
		F vs. Anc	4.71E-04	7.821	Large
		C vs. F	0.057	1.286	Large
Rifampicin	2.39E-08	C vs. Anc	0.001	1.836	Large
		F vs. Anc	1.68E-04	10.904	Large
		C vs. F	1.85E-04	2.569	Large
Chloramphenicol	0.004	C vs. Anc	0.161	0.919	Large
		F vs. Anc	0.003	4.743	Large
		C vs. F	0.070	1.063	Large

Table 3.6 Summary of results in novel antibiotic environment. Growth rate and MICs were measured in Nalidixic acid (Nal), Rifampicin (Rif) and Chloramphenicol (Chl). ↑ denotes significant increase in fitness w.r.t ancestor ($P < 0.05$, Tukey's HSD test). ≈ represents no significant change from ancestor.

* denotes F population significantly better than C with $P<0.05$ from Tukey's HSD test and # denotes F population significantly better than C with $P<0.1$ from Tukey's HSD test.

	Sub-Lethal (Growth rate)			**Lethal** (MIC)		
	Nal	Rif	Chl	Nal	Rif	Chl
UV	≈	≈	≈	≈	≈	≈
Ery	≈	↑	↑	≈	≈	≈
Constant UV+Ery	≈	↑	↑	↑	↑	≈
Fluctuating UV/Ery	↑#	↑*	↑*	↑#	↑*	↑#

3.3.4 Resistance in fluctuating treatment is due to resistant subpopulations

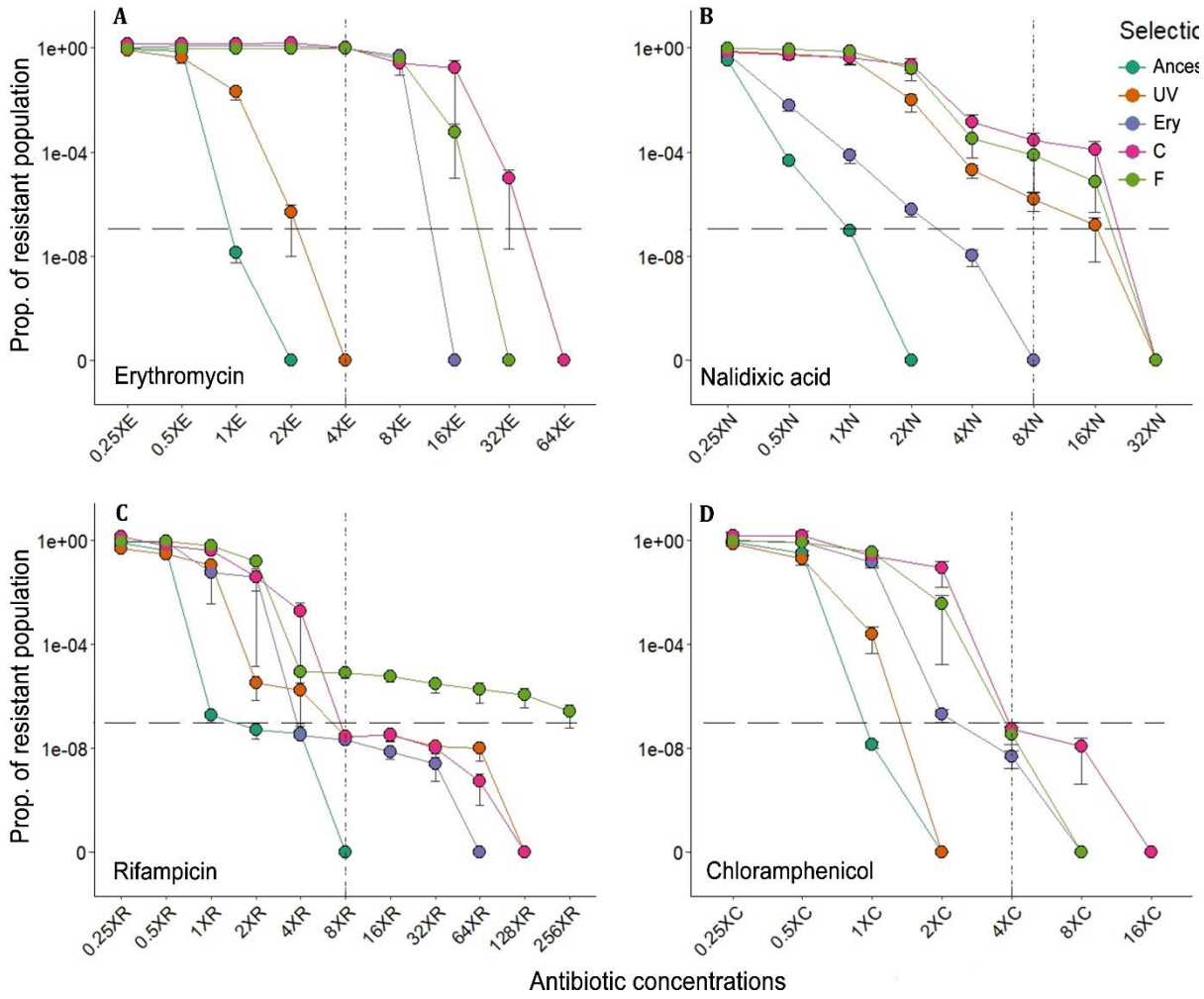

Fig. 3.5 Population analysis profile (PAP) of the four selection regimes and their ancestors. PAP of the 5 population were assayed in four antibiotics: (A) Erythromycin, (B) Nalidixic acid, (C) Rifampicin, and (D) Chloramphenicol. The horizontal line denote the threshold for detection of resistance: 1 X 10^{-7}. The vertical line denotes the concentration that is 8 times higher than the non-inhibitory concentration of the ancestral population.

We used a population analysis profile (PAP) to study the distribution of variation in resistance in the evolved populations. In erythromycin, all erythromycin selected populations survived concentrations that were at least 8 times higher than the highest non-inhibitory concentration of the ancestors. Since 50% of the total population showed growth at this concentration, the populations were considered to be genetically resistant to erythromycin (Maeda *et al.* 2020) (Fig. 3.5A). The only exception was replicate 1 of the C treatment where 50% of the total population was resistant to concentrations four-fold higher than ancestor. At eight-fold higher concentration, the proportion of the resistant populations dropped to 17%.

In chloramphenicol, Ery, C and F populations survived concentrations only four-fold higher than the ancestors (Fig. 3.5D) which corresponds to the resistance level observed in MIC assays. However, based on the resistance threshold typically used in PAP assays (El-Halfawy & Valvano 2015; Andersson *et al.* 2019) these populations were considered not resistant. We observe growth at eight-fold higher concentrations of nalidixic acid and rifampicin. Three treatments, UV, C, and F, were resistant in nalidixic acid (Fig. 3.5B) and only F treatment was resistant in rifampicin (Fig. 3.5C). The proportion of resistant cells of the populations in these antibiotics were much less than 50% but greater than $1x10^{-7}$. This is indicative of the presence of large variation in resistance phenotypes (heteroresistance) (El-Halfawy & Valvano 2015; Andersson *et al.* 2019) and that high resistance was due to a smaller proportion of resistant subpopulations. It is interesting to note that the F treatment had the largest fitness gains in the novel environment and that the high MIC was actually due to small subpopulations.

3.3.5 Resistance in fluctuating treatment is due to independent subpopulations

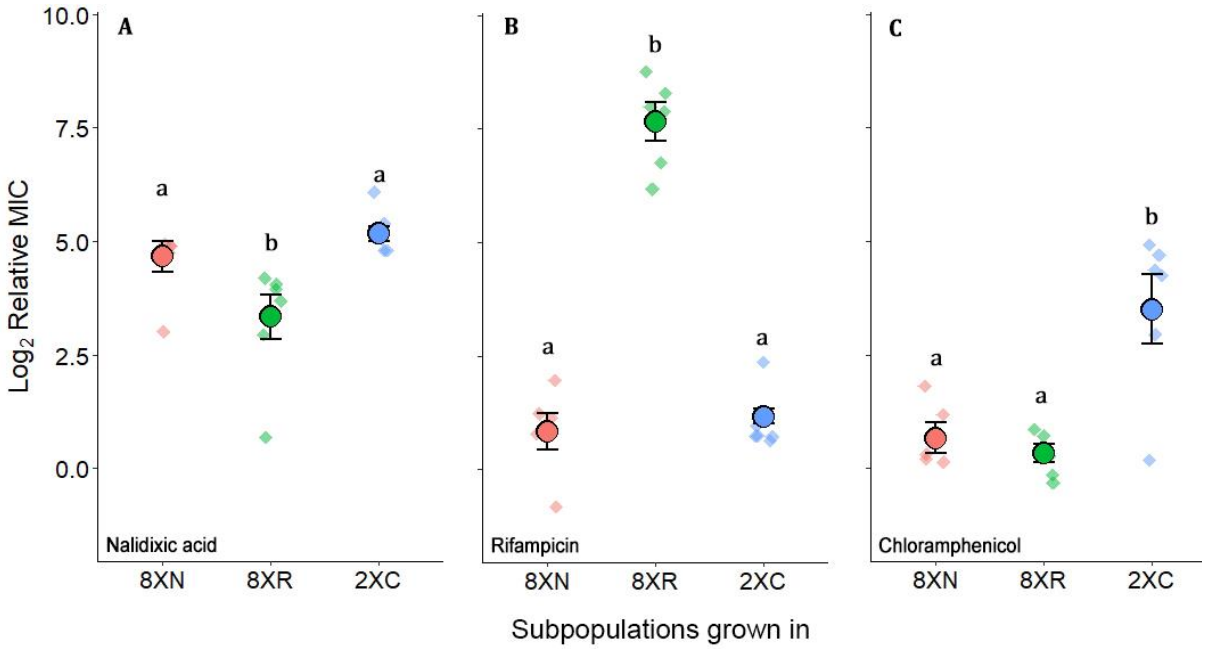

Fig. 3.6 Cross resistance profile of the subpopulation in F population. Subpopulations were enriched in nalidixic acid (8XN), rifampicin (8XR), and chloramphenicol (2XC) where, 1X corresponds to the ancestral MIC. The MICs of the resulting subpopulations were measured in (A) Nalidixic acid, (B) Rifampicin, and (C) Chloramphenicol. Mean MIC w.r.t the ancestors and SEM are plotted along with scatter of the six replicate values. Populations denoted by different alphabets are significantly ($P < 0.05$) different in Tukey's pairwise comparisons.

While we see that resistance in the F populations was due to resistant subpopulations, it is not clear whether this is due to a single subpopulation resistant to all antibiotics or multiple subpopulations each resistant to one antibiotic. To investigate this, we estimated the MIC of the resistant subpopulations from F population in all three novel antibiotics.

The resistant subpopulations show significant differences in their MICs in all three antibiotics: nalidixic acid (Fig. 3.6A; $F_{2,10} = 8.22$, $P = 0.008$), rifampicin (Fig. 3.6B; $F_{2,10} = 112.89$, $P = 4.12E-07$), and chloramphenicol (Fig. 3.6C; $F_{2,10} = 14.73$, $P = 0.002$). The nalidixic acid MIC of the Nal subpopulations were significantly greater than Rif subpopulations (Tukey's $P = 0.04$) but not significantly different from Chl subpopulations (Tukey's $P = 0.55$). The rifampicin MIC of the Rif subpopulations were significantly greater than both Nal subpopulations (Tukey's $P = 0.0002$) and Chl subpopulations (Tukey's $P = 0.0002$). Similarly, the chloramphenicol MIC of the Chl subpopulations were significantly greater than both Nal subpopulations (Tukey's $P = 0.004$) and Rif subpopulations (Tukey's $P = 0.002$). Thus, the subpopulations did not show a strong cross resistance to other novel antibiotics. It is possible that the resistance to multiple drugs was likely due to the coexistence of multiple subpopulations.

3.3.6 Resistant subpopulations vary in their resistance mechanism

To determine the genetics of resistance in the subpopulations of the F treatment, we sequenced the subpopulations resistant to at least 8X ancestral MIC in nalidixic acid and rifampicin. We used the same two replicate populations as used in the whole genome sequencing, F3 and F5 (see https://doi.org/10.5281/zenodo.5918401 for full list of mutations).

In addition to the six nonsynomyous mutations found in the whole population sequencing of F treatment (Table 3.3), both nalidixic acid and rifampicin subpopulations had fixed for T315P substitution in *glvC*. It is a putative PTS enzyme II component and likely a pseudogene (Reizer *et al.* 1994). No other mutations, nonsynonymous, synonymous, or intergenic, were common between the two replicate subpopulations in nalidixic acid. Additionally, no mutations were observed in the common quinolone resistance genes; gyrA and parC. On the other hand, rifampicin resistant subpopulations had accumulated multiple *rpoB* mutations: D516N (both replicates), P645L (both replicates), S531F (F3), and Q517H (F5).

Taken together, phenotypic variation in the F populations was also accompanied by variation in the genomics of resistance i.e., target-gene mutations vs. general mechanisms of resistance.

3.4. Discussion

UV radiation acts as both a mutagen and a selective agent. Exposure to UV is expected to increase the mutational supply thereby increasing variation available for selection. This can result in phenotypic and/or genomic variation in the populations. It has previously been shown that UV induces correlated fitness changes in novel environments including antibiotics (Zhang *et al.* 2017; Li *et al.* 2021; Selveshwari *et al.* 2021). Contrary to this earlier result, we found that populations were able to evolve similar extents of resistance to erythromycin, irrespective of whether or not they were exposed to UV radiation during selection (Fig. 3.1 and 3.2). Interestingly, constant or fluctuating exposures to UV and erythromycin had very little effect on the extent of fitness. However, fluctuating exposures resulted in a marginally higher growth rate in erythromycin than constant populations (Fig. 3.2A). These results are contrary to our *a priori* expectation that UV radiation would influence the evolutionary outcomes. The observed convergence of phenotypes could indicate a strong selection pressure for increased resistance. However, genomic variation could open up multiple routes to the same phenotype. Therefore, we next investigated the whole population whole genome sequences of the evolved and ancestor populations.

The number of mutations in the UV control populations was greater than those in the erythromycin control population as well as the constant and fluctuating treatment populations (see https://doi.org/10.5281/zenodo.5918401 for full list of mutations). UV exposures in both constant and fluctuating treatments may have increased the supply of mutations. However, we believe that the presence of the second selection pressure (erythromycin) reduced this pool of variation to only those that were either beneficial or neutral in the presence of erythromycin. Subsequently, to investigate the likely drivers of the evolved phenotypes, we limited our analysis to nonsynonymous mutations in genes that were observed in both

replicates of at least one treatment population. We identified six such genes in the UV control populations. Out of these six, only one mutation was also shared by the treatment populations (C and F): D161G *recA*. We have previously shown that this reversion occurs in UV exposed populations and discussed its importance in our previous study (Selveshwari *et al.* 2021). Briefly, although this mutation is a reversion to wild-type *recA*, the 161st amino acid is an important site for proper functioning of *recA* (Shinohara *et al.* 2015). Although exploring the functional effects of these mutations are currently out of scope for our study, we emphasize that a properly functioning *recA* is crucial under UV stress. *recA* is responsible for recombination repair and as an effector of SOS response in the presence of damaged DNA (Smith *et al.* 1987; Schlesinger 2007; Maslowska *et al.* 2019). Four of the remaining five mutated genes *crp, rsxC, yacH*, and *yhjJ* have been previously observed in at least one replicate of UV resistant populations (Selveshwari *et al.* 2021). These genes are involved in transcriptional regulation, regulation of transcription factors, membrane protein, and with no putative function, respectively (Keseler *et al.* 2021). The only exception is *ftsQ* which is an essential cell division protein (Carson *et al.* 1991). Genes related to *ftsQ*, such as *ftsW* and *ftsL* were observed in our previous study. Although there are few to no prior studies linking the genes and these specific mutation to UV resistance phenotypes, this opens up new avenues for research into novel UV resistance mechanisms.

The two convergent mutations in the Ery control populations are in the known erythromycin resistance genes: G66A/D *rplD* and L828S *acrB* (Table 3.3). Point mutation in the ribosomal protein L4, coded *rplD*, at G66 is known to modify the ribosomal complex and reduce the erythromycin binding capacity (Weisblum 1995; Gregory & Dahlberg 1999; Gomes *et al.* 2017). Indeed, substitutions in L4 protein has been recovered in macrolide resistance strains of multiple species including *Legionella pneumophila* (Descours *et al.* 2017), *Haemophilus influenza* (Clark *et al.* 2002; Peric *et al.* 2003), *Streptococcus pneumonia* (Tait-Kamradt *et al.*

2000; Schroeder & Stephens 2016). In addition to mutation in L4 protein, mutations in another ribosomal proteins, L22 and 23s rRNA are also known to confer resistance phenotypes (Gomes *et al.* 2017). However, we failed to detect mutations in these two genes in our Ery control populations. The fluctuating treatment, on the other hand, had mutations in other ribosomal proteins. Substitution that results in L157* in the S7 ribosomal subunit was fixed in the F populations. We also observed this mutation in one of the replicate of UV control populations (UV3). A truncated S7 protein is a natural variant that is common in all *E. coli* strains except K as well as other *Enterobacteriaceae* (Tritsch *et al.* 1977). Therefore, it is less likely that this mutation resulted in direct fitness changes in the selection environment. This mutation may have appeared at higher frequency in the fluctuating treatment and rose to fixation. We also observed small frequencies of other ribosomal mutations in the fluctuating treatment: F3: K90I (L22 protein), K60E (L20 protein) P22F (S12 protein) and F5: R95L/C (L6 protein). However, these genes were mutated in only one replicate and we refrain from discussing their potential fitness effects in the selection environments.

In addition to mutations to the macrolide binding sites on ribosomal proteins and 23s rRNA, epigenetic modifications and altered efflux activity have also been implicated in erythromycin resistance (Gomes *et al.* 2017). However, since WGS cannot identify epigenetic modifications, we refrain from discussing its role in resistance in our populations. On the other hand, we observe mutation in efflux genes that may have increased resistance to erythromycin. A mutation in *acrB* gene, a part of the RND family of efflux pumps, was fixed in our populations. Mutations in the *acrAB* system has been previously reported in erythromycin resistance in *E. coli* (Chollet *et al.* 2004; Li *et al.* 2015). Very interestingly, a single SNP (L828S) was fixed in the erythromycin control population as well as the constant and fluctuating treatment populations. L828 is a known binding site for erythromycin and

changes at this site can influence substrate binding and decrease susceptibility (Schuster *et al.* 2016). In addition to mutation in the *acrB* gene, the constant and fluctuating treatment populations had additional mutation in *acrR*, a repressor of *acrAB* genes. The SNP T5N was fixed in both treatments and is known to inactivate the repressor and increasing the expression of *acrA* by atleast 20% (Gerken & Misra 2004).

While *rplD* and *acrB* mutations were the only convergent mutations in Ery population, both C and F populations lacked the *rplD* mutation instead, they had a combination of mutations in *acrB* and *acrR* genes. The C treatment had an additional mutation in *ydcT* which is an ATP-binding component of a putative ABC transporter (Saier Jr *et al.* 2014) which likely functions as an additional efflux system in the presence of antibiotics. The F treatment on the other hand, had mutations in ribosomal subunit protein (*rpsG*) along with *cya* A and *nudE*. We have already discussed rpsG mutation in the context of erythromycin resistance above and will be discussing the relevance of cyaA and nudE genes in the context of antibiotic and UV resistance later in the discussion.

Altered efflux activity may or may not increase the MIC but will increase the chance of mutations in the antibiotic target sites (target altering mutations) resulting in high fitness (Ebbensgaard *et al.* 2020; Papkou *et al.* 2020). For example, the median change in ciprofloxacin MIC in strains with increased efflux activity results was orders of magnitude lower than strain with both target alteration and efflux mutations (van der Putten *et al.* 2019). We see a similar combination of target (*rplD*) and efflux (*acrB*) mutations which, can explain the high levels of resistance in Ery control populations. However, it is interesting that similar levels of erythromycin resistance in C and F treatments were achieved with mutations in the efflux gene (*acrB*) and its regulator (*acrR*) but without target altering mutations. More importantly, all three treatments were homogeneously resistant to erythromycin, as seen in

population analysis profile (PAP) assay (Fig. 3.5A). Thus we see that although exposure to UV radiation along with selection for erythromycin resistance in the C and F treatments had very little effect in terms of the phenotype, it resulted in divergent genomic pathways to resistance. These results are in line with Couce *et al.* (Couce *et al.* 2015) where differences in the spectrum of mutational supply influenced the trajectory of genomic evolution to cefotaxime resistance.

Although the genomic differences did not translate into phenotypic differences in the selection environment, the polymorphism may become apparent as fitness differences in other novel environments i.e., conditional neutrality (Wagner 2005). To study this, we investigated the correlated fitnesses of our evolved populations in the presence of other antibiotic environments. The key mutations in our evolved populations have been shown to also influence fitness in other antibiotic environments. While G66A/D *rplD* has no effect on fitness in the presence of rifampicin (Descours *et al.* 2017), L828S *acrB* may influence fitness in rifampicin (Nakashima *et al.* 2011). Nakashima *et al* (Nakashima *et al.* 2011) found that there was an overlap of erythromycin and rifampicin binding regions in the *acrB* protein. In addition, mutation at the 5th amino acid position of *acrR*, more specifically T5N substitution, is known to influence resistance to norfloxacin and chloramphenicol (Wang *et al.* 2001). Interestingly, although there was such overlap in terms of resistance mechanisms, all the three antibiotics belong to different classes with different sites of action.

The correlated effect of the evolved population in norfloxacin, rifampicin, and chloramphenicol, measured as growth rate and MIC revealed interesting interactions. The UV control population did not show change in correlated fitness in any of these environments, in both growth rate and MIC (Fig. 3.3). The lack of correlated effect of UV resistance is contrary to our a priori expectation that increased genetic variation may result in beneficial

variation for increased resistance. It is also in contrast to our previous study (Selveshwari *et al.* 2021) where exposure to UV radiation during lag and exponential phase resulted in increased MIC in nalidixic acid, rifampicin, and chloramphenicol. The discrepancy could be due to the fact that the populations in this study were subjected to 60 rounds of UV exposure during stationary phase as compared in 100 rounds during lag and exponential phase in our previous study. It is possible that 60 exposures were not long enough to elicit a strong collateral response. Secondly, UV induced mutations during the stationary phase may have very different functional effects resulting in little cross resistance. This further highlights the importance of studying the growth phase specific effects of UV mutagenesis.

Erythromycin resistance in erythromycin control populations on the other hand, was associated with increased growth rate in rifampicin and chloramphenicol but not MIC (Fig. 3.3). While the observed mutations and the resulting change in efflux activity may explain fitness in rifampicin, it does not explain fitness in chloramphenicol. One of the primary macrolide resistance mechanisms is target altering mutations of 23s rRNA which, is also known to confer chloramphenicol resistance (Ettayebi *et al.* 1985). While there is an interaction in the resistance mechanisms between chloramphenicol and erythromycin, we do not see this exact pathway being implicated in our population. It may be possible that there are other unknown pathways to chloramphenicol and erythromycin cross resistance that do not involve 23s rRNA mutations and/or *acrB* efflux protein.

While novel fitness in the Ery control populations were limited to increased growth rate in chloramphenicol and rifampicin, the C treatment had additional increase in nalidixic acid and rifampicin MIC (Fig. 3.4). The observed mutation in *acrB* and *acrR* genes (efflux protein) likely explain the increase in growth rate in the novel antibiotics in C treatment. However, we expect changes in MIC to be usually accompanied with target altering mutations such as

DNA gyrase and topoisomerase IV, and RNA polymerase w.r.t nalidixic acid and rifampicin resistance, respectively (Bearden & Danziger 2001; Ruiz 2003; Hopkins *et al.* 2005; Tupin *et al.* 2010; Goldstein 2014). However, we do not observe any target altering mutations in either of *gryA*, *parC*, or *rpoB* genes in the WGS of C treatment. The F treatment had the highest fitness increase in all three antibiotics in terms of both growth rate as well as MIC (Fig. 3.4). While mutations in *acrB* and *acrR* resulting in altered efflux activity, may explain the fitness changes in the three antibiotic environments, it is not clear why or how the F populations were able to evolve fitness greater than C populations which also share the same mutations. No additional target altering mutations that could be attributed to greater increase in MICs, were identified in the F treatment.

Further analysis (PAP) revealed that the evolved populations showed variation in resistance to the novel antibiotics. Mutations in the efflux proteins were fixed in these populations. Thus, if modified efflux activity were alone responsible for resistance to novel antibiotics, we would expect no differences in fitness. However among the treatment populations, C showed variation in resistance to nalidixic acid whereas F showed variation in both nalidixic acid and rifampicin resistance (Fig. 3.5B and 3.5C). Since the proportion of these resistant cells were orders of magnitude smaller, other genetic determinants for resistance, if any, may be missed in the whole population sequencing. Identification of other such mechanisms may explain the increase in fitness and variation in the F treatment. Therefore, we focused on WGS of the resistant subpopulations from the F treatment.

The genomic sequences of the resistant subpopulations revealed divergent resistance mechanisms. Both replicates of the rifampicin resistant subpopulations had acquired mutations in the *rpoB* gene which codes for RNA polymerase (RNAP), the primary target of rifampicin. In total, four different mutations were observed in the *rpoB* genes of which, 3

SNPs have been previously shown to confer rifampicin resistance in *E.coli* (Garibyan *et al.* 2003) and the fourth SNP has been observed in rifampicin resistant *Mycobacterium sp*. (Wu *et al.* 2009; Zenteno-Cuevas *et al.* 2009). The nalidixic acid resistant subpopulations, on the other hand, do not have any convergent genomic changes between the two replicate populations which could be used to deduce the mechanism of resistance. More importantly, no additional target altering mutations in DNA gyrase (*gryA*) or DNA topoisomerase IV (*parC*) were identified even in the nalidixic acid resistant subpopulations. There is a rare pleotropic effect of rpoB mutations that is known to confer resistance to ciprofloxacin, a fluroquinolone similar to nalidixic acid (a quinolone) (Pietsch *et al.* 2016). However, we did not find any *rpoB* mutations in the nalidixic acid subpopulations which could point towards cross-resistance within the subpopulations. Interestingly, we found that the F population were actually composed of multiple subpopulations, each resistant to one antibiotic (Fig. 3.6). This is different from 'multi-drug resistance' where a single mutation (such as efflux regulation) makes the population resistant to multiple antibiotics (Gifford *et al.* 2019).

Phenotypic heterogeneity, similar to that observed in the F treatment, is a common phenomenon in microbial populations (Holland *et al.* 2014; Ackermann 2015; van Boxtel *et al.* 2017). Heterogeneity within microbial populations can originate due to multiple factors including variation in the environment (Holland *et al.* 2014; Smith *et al.* 2018), genome (Bódi *et al.* 2017; Carja & Plotkin 2017), epigenetic changes such as phase-variable genes, methylation and feedback regulatory networks (Smits *et al.* 2006; Ackermann 2015). Environment fluctuations are central to the F treatment which, is expected to maintain greater genetic variation (Kussell *et al.* 2005; Kussell & Leibler 2005; Acar *et al.* 2008; Beaumont *et al.* 2009; Carja *et al.* 2014; Patra & Klumpp 2014). However, the effect of heterogeneous environment on variation may be limited by the supply of variation itself (Bürger & Gimelfarb 2002). Since the F populations had increased MIC in rifampicin and nalidixic acid

we are unable to perform a fluctuation test to estimate the mutation rates. Therefore, we can only speculate the effects of increased mutation rates on fitness and variation in fitness. An increased intrinsic or UV induced mutation rate in the F treatment, may interact with environmental fluctuations resulting in the observed variation in fitness.

Another factor that could influence variation in fitness is epigenetic changes resulting in noisy gene regulation. It could promote variation by ensuring the survival of at least a small proportion of cells under stress conditions (Garcia-Bernardo & Dunlop 2015; Freddolino *et al.* 2018). Global regulator genes have been implicated in generation of variation antibiotic tolerance, resulting in persister cells (Mok *et al.* 2015; Uppal & Jawali 2016; Molina-Quiroz *et al.* 2018). Persisters increase survivorship as well as mutation rates, increasing the likelihood of acquiring antibiotic resistance mutations (Windels *et al.* 2019). Additionally, selection for increased growth rate in antibiotic free medium can also give rise to antibiotic resistance mutations such as *rpoB* mutations (Katz & Hershberg 2013; Knöppel *et al.* 2017). We observe this in the F treatment as significant increase in growth rate in the presence of erythromycin (Fig. 3.2A) as well as in its absence (growth in NB, figure 3.7).

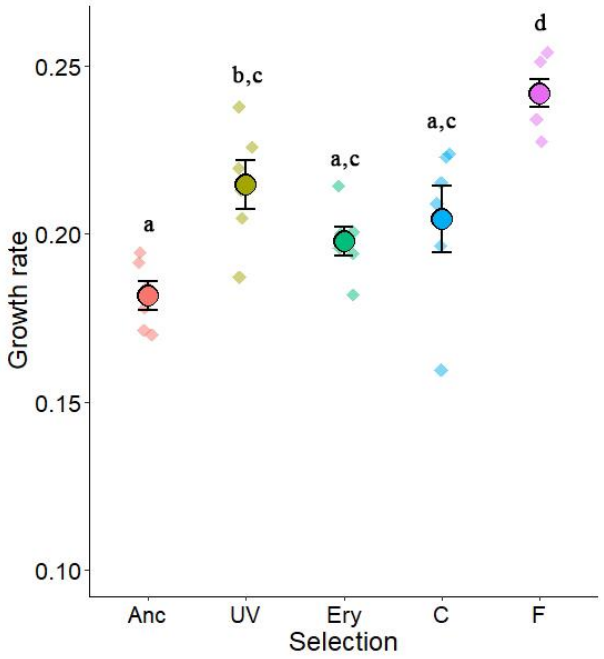

Figure 3.7. Mean growth rate (± SEM) of the evolved and ancestral population in nutrient broth. The scatter of the six replicate populations is plotted as diamonds (♦) around the mean. The main effect of selection in significant ($F_{4,20}$ = 14.94, P = 8.34E-06). Tukey's post hoc analysis show that F treatment has a significantly higher growth rate compared to all other populations (p values of pairwise comparisons with A: p=0.0001; UV: p=0.025; Ery: p=0.0004; C: p=0.002). Populations denoted by a different alphabets are significantly (P < 0.05, Tukey's HSD test) different from each other.

Additionally, we also observe fixation of two global regulator genes in our F treatment: *cyaA* and *nudE*. The product of *cyaA,* adenylate cyclase, catalyzes the synthesis of cyclic AMP (cAMP), an important metabolism-signaling molecule (Pastan & Perlman 1970). Additionally, cAMP bound to crp protein is responsible for the regulation of over half of E.coli genome (reviewed in (Soberón-Chávez *et al.* 2017). Mok et. al., (Mok *et al.* 2015) showed that expression of *cyaA,* in addition to other global regulator genes, leads to phenotypic variation and persisters within isogeneic populations. Although *nudE* gene has not been previously associated with antibiotic resistance, it is a part of the nudix hydrolase superfamily which regulate (p)ppGpp (Sanyal *et al.* 2020), which is a crucial regulator of metabolism in persisters (Liu *et al.* 2017). Changes in these global regulator genes could have resulted in the generation of increased variation in fitness, more specifically antibiotic tolerant persister cells. Such intermediate antibiotic tolerance can function as stepping stones towards resistance via genetic changes (Levin-Reisman *et al.* 2017; Barrett *et al.* 2019).

To summarize, evolution of erythromycin resistance resulted in correlated fitness increase in other novel antibiotics. However, exposure to UV radiation during erythromycin resistance evolution led to the increase in the extent of the correlated fitness. Further analysis revealed that fluctuation in exposure to UV and erythromycin resulted in the maintenance of subpopulations resistant to different antibiotics. Whole genome sequencing of the population and subpopulations suggests complex interactions between mutational spectrum and supply, and epigenetic changes, resulting in the observed variation in fitness in the F treatment.

Chapter 4: The effect of migration and variation on populations of *Escherichia coli* adapting to complex fluctuating environments

4.1. Introduction

Migration affects a number of ecological and evolutionary processes, such as a species' range (Kirkpatrick & Barton 1997; Barton 2001; Sexton *et al.* 2009), composition, and diversity in meta-populations and natural communities (Venail *et al.* 2008; Albright & Martiny 2018) and evolution of traits like virulence (Boots & Sasaki 1999; Lively 1999) and antibiotic resistance (Perron *et al.* 2007). Interestingly, when it comes to adaptation, migration can have contrasting effects. For example, migration has been shown to impede adaptation in a microbial community subjected to warmer temperatures (Lawrence *et al.* 2016) and in coevolving host-phage systems (Morgan *et al.* 2005; Vogwill *et al.* 2011). Similarly, in viruses, migration can reduce the extent of specialization to different tissue types (Cuevas *et al.* 2003). One of the ways migration negatively affects adaptation is by swamping the destination environment with alleles that are beneficial or neutral at the source environment but maladaptive at the destination (Kawecki & Holt 2002; Kawecki & Ebert 2004; Yeaman & Guillaume 2009). At the same time, several studies have demonstrated that migration can promote adaptation. For example, asexual populations of *Chlamydomonas* exposed to herbicides (Lagator *et al.* 2014) and yeast populations evolving in the presence of salt stress (Bell & Gonzalez 2011) adapted more rapidly in the presence of migration. Similarly, adaptation of Φ6 phage population to a novel host was favored by migration from populations that had the ability to infect this novel host (Ching *et al.* 2013). However, when migrants came from control populations unable to infect the novel hosts there was no effect on the absolute fitness (Ching *et al.* 2013). Migration can positively influence adaptation by increasing the supply of beneficial mutations, particularly when populations are mutation limited (Holt 2003; Sexton *et al.* 2009), such as in asexual microbes.

Interestingly, most empirical studies on how migration influences adaptation in microbes have been carried out only in constant environments, typically in the presence of a single selection pressure (Morgan *et al.* 2005; Dennehy *et al.* 2010; Ching *et al.* 2013; Lagator *et al.* 2014; Lawrence *et al.* 2016). However, in nature, organisms are often faced with heterogeneous environments that contain multiple stressors at the same time. To further complicate matters, the magnitudes of these stresses can fluctuate over time, either predictably or unpredictably. Adaptations in such spatially and/or temporally heterogeneous environments can be very different from what has been observed in simple constant environments (Levins 1968; Reboud & Bell 1997; Cooper & Lenski 2010; Karve *et al.* 2016). The effect of migration on adaptation under such complex and fluctuating environments has received relatively less attention (however see (Perron *et al.* 2007)).

Here, we present the results of our study on the effects of different rates of migration on adaptation, in replicate populations of *Escherichia coli* that were subjected to complex environments undergoing unpredictable fluctuations. We also looked at the effects of migrants that were either clonal or carrying variation. When the immigrants were clonal, the recipient populations evolved reduced fitness compared to the no migration control. Interestingly, the magnitude of fitness reduction varied positively with the fraction of immigrants received. However, treatments that received immigrants with variation showed little or no change in fitness compared to the no migration control. Thus, our results highlight the importance of considering the nature of the environment as well as the immigrants in studying the effects of migration on adaptation.

4.2. Materials and Methods

This study was conducted using *Escherichia coli* MG1655 with kanamycin resistance cassette. All cultures were maintained at 37^{o}C and 150 RPM throughout the selection and assays, except where stated otherwise.

4.2.1 Immigrant Populations

This study consisted of two selection experiments. In each selection experiment, we used two types of populations: the immigrant and the native. The native populations evolved in the complex fluctuating environments and experienced the effects of immigration. The immigrant populations were non-evolving cultures freshly revived every day (see section 2.2 Selection protocol). The two selection experiments differed only in terms of the nature of the immigrant populations. In experiment 1, we used a **C**lonal immigrant population (henceforth C). This was derived from a single *E.coli* colony and grown in 150ml NB with kanamycin (NB^{Kan}) for 18 hours. Multiple 1 ml glycerol stocks (15%) of this culture were prepared and stored at -80^{o}C. In the second experiment, we used the **V**ariation immigrant population (henceforth V), which was derived from the C population. V population was initiated by reviving 1ml glycerol stock of the C population in 10ml NB^{Kan} followed by inoculation of 1ml of this revived culture in 50ml NB^{Kan}. For the next 15 days, we sub-cultured (1/10th dilution) this population into 50ml NB^{Kan} every 12 hours. After 15 days (i.e. 30 transfers), 50ml of the grown culture was added to 50ml fresh NB^{Kan} and incubated for another 12 hours. Multiple 1ml glycerol stocks (15%) were prepared and stored at -80^{o}C. By this time, the V population had spent ~100 generations in benign environment, during which a number of spontaneous mutations were expected to have arisen in the culture. Since we maintained this population under optimal conditions, large culture volume (N_f: 50ml) and lenient bottlenecks (1/10), we expect most mutations arising in the population to accumulate. Thus,

the V population was expected to harbor greater genetic variation than the C population. To confirm this, we quantified the within-population variance in fitness in the C and V populations using the methodology of an earlier study (McDonald *et al.* 2012).

4.2.2 Quantification of within-population variance

To quantify the amount of variation accumulated in the V population, fitnesses of 72 individual colonies of both C and V populations was assayed in 6 environments and the within-population fitness variance is used as a proxy for genetic variation (McDonald *et al*. 2012). Single colony suspensions were made from 6 similar sized colonies of both C and V population. 10µL of the single colony suspension was inoculated in 200µL of six assay environments, assayed in a single 96-well plate. The environments included sub-lethal concentrations of all stress combinations and NB^{Kan}. Environment I: pH 5+Salt 3.5g%, Environment II: pH 8.5+Salt 2.5g%, Environment III: pH 5+4µl 0.3% H_2O_2, Environment IV: pH 8.5+2µl 0.3% H_2O_2, Environment V: Salt 2.5g%+1.5µl 0.3% H_2O_2 and Environment VI: NB^{Kan}. H_2O_2 was added, 2 hours after inoculation, where required. The populations were continuously monitored (OD_{600}) for 24 hours, from inoculation, using a plate reader (Synergy HT) at 37^{o}C and continuous medium shaking. This entire procedure was repeated over 12 days for a total of 72 colonies from each of the C and V populations. Growth rate and yield were estimates using a custom python script; see section 2.4 for details of estimation. The coefficient of variation in fitness (growth rate and yield) between the six colonies of each day was compared, between C and V and across each environment.

4.2.3 Selection Protocol

We initiated 48 replicate populations each from both the C and the V ancestor populations. 1ml of the corresponding glycerol stocks were revived overnight in 10ml NB^{Kan} and 20µL of the revived culture (OD_{600} 1.0 – 1.1) was inoculated in 2ml selection environment

Selection environment: The populations were subjected to selection for 30 days in environments that were complex (i.e., multiple stressors were present simultaneously) and fluctuated unpredictably. Selection regime similar to a previous study (Karve *et al.* 2015) was used. Briefly, selection involved three stresses: pH, osmotic (NaCl) and oxidative (H_2O_2) stress. Combinations of the stresses were created such that two of the three components was present at inhibitory concentration while the third was present at concentration as found in NB (i.e., pH=7, NaCl=0.5g% and H_2O_2=0). A number of such combinations at different concentrations were tested for their effect on growth of the WT in a pilot experiment. Combinations that resulted in ~ 40 – 70% reduction in growth as compared to growth in NB were chosen. A total of 28 combinations were chosen and a sequence of 30 environments were chosen from a uniform distribution with replacement. The sequence of environments used is listed in Table 4.1.

Migration treatment: We used four levels of migration, namely 0% (control), 10% (low migration), 50% (intermediate migration) and 90% (high migration). Addition of immigrant population as surplus to the native population can increase population sizes proportional to the extent of migration. This can result in large differences in population sizes across treatments, which can affect the evolutionary outcomes (Chavhan *et al.* 2019). To avoid the confounding effect of population size, we kept the inoculum size constant (~10^7 cells) and defined migration as the percentage of immigrants in the inoculum. For example, in the low (10%) migration treatment, 10% of the individuals in the subculture inoculum consisted of

immigrants while the remaining 90% were individuals from the native population (evolving under complex fluctuating environment). The proportions were adjusted based on the OD_{600} values of the immigrant and native populations at the time of subculture. When $OD_{600} = 1$, the culture contained ~10^9 cells / ml of NB (S Selveshwari, personal observations).

1ml glycerol stock of the C or V population were revived in 10ml NB^{Kan}, everyday. OD_{600} of the revived culture was adjusted to 1.0 – 1.1 and used as immigrant population. OD_{600} of the native populations were also measured and appropriate volume of culture containing the required inoculum size was used for the sub-culture. When the OD_{600} of these populations was less than 0.3, the populations were considered extinct and native population was obtained from the previous non-extinct population, stored at 4^{o}C. The selected populations were stored as glycerol stocks at the end of 30 day.

4.2.4 Fitness assays

Fitnesses of the evolving populations were measured during selection as well as post selection. OD_{600} in the selection environment was noted at the end of every 24 hours and the geometric mean of these values over the 30 days of selection was used as a measure of fitness under fluctuating stress (Orr 2009). Using the same data, we also estimated the probability of extinction ($OD_{600} < 0.3$) during selection.

Fitnesses post selection, was measured as growth rate and yield of the populations in three representative environments. Environment 1: pH 8.5+salt 4.5g%; Environment 2: pH 5+0.5µL H_2O_2; Environment 3: salt 2.5g%+0.7µL H_2O_2. All 48 replicate populations of each selection experiment were assayed twice in each assay environment. 4µL of glycerol stock was revived in 2ml NB^{Kan}, overnight. The OD_{600} of the revived culture was measured and a volume containing 10^7 cells (assuming 10^9 cells/2ml when $OD_{600} = 1$) was inoculated in 2ml assay environments in 24-well tissue culture plates. The OD_{600} was measured every 2 hours

for 24 hours at 37^{o}C and slow continuous shaking using a plate reader (Synergy HT BioTek, Winooski, VT, USA). Following previous studies, we used population growth rate and yield as fitness measures (Karve *et al.* 2015; Chavhan *et al.* 2019). The growth curve data was analyzed using a custom python script which fits overlapping straight lines over periods of 6 hours. Growth rate was computed as the maximum slope of the curve and yield as the maximum OD_{600} reached in 24 hours.

4.2.5 Statistical analysis

All fitness comparisons were performed independently for the two selections. Population fitness during selection (geometric mean of growth and extinction probability), were compared across the migration treatments using separate one way ANOVAs with migration treatment (0, 10, 50, 90) as a fixed factor. Fitnesses (growth rate and yield), post selection, were compared as independent two way mixed model ANOVAs in each of the assay environments. Migration treatment (0, 10, 50, 90) was taken as fixed factor. The growth rate and yield from the two rounds of assays was considered as measurement replicates and the biological replicates (12 levels) was taken as random factor and nested in migration treatment. To account for the inflation of family-wise error rate, the *P* values of the main effect of migration were subjected to Holm–Šidák correction (Abdi 2010). When the corrected *P* value was significant, pairwise comparisons were performed using Tukey's post hoc analysis. We also computed the Cohen's *d* statistics (Cohen 2013) as a measure of the effect sizes (Sullivan & Feinn 2012). The biological significance of the differences between the treatments were interpreted as small, medium and large for $0.2 < d < 0.5$, $0.5 < d < 0.8$ and $d > 0.8$, respectively. The within-population variance in fitness of the C and V populations was compared using paired t-Test in each environment. The inflation in family-wise error was controlled using Holm–Šidák correction.

All the ANOVAs were performed on STATISTICA v7.0 (Statsoft Inc.). Cohen's *d* statistics were estimated using the freeware Effect Size Generator v2.3.0 (Devilly 2004).

Table 4.1. Sequence of complex fluctuating environments used during selection.

Complex fluctuating environment			
Day	**pH**	**NaCl** (g/100ml)	**H_2O_2** (µL)
1	5	4	-
2	4.5	3	-
3	5	4.5	-
4	-	2.5	0.8
5	5	3.5	-
6	5	-	0.6
7	5	3	-
8	-	4	0.5
9	-	3	0.5
10	5	3	-
11	-	4	0.5
12	-	3	0.5
13	5	-	0.7
14	-	3	0.5
15	8.5	-	0.5
16	-	3	0.7
17	5	-	0.5
18	5	3	-
19	9	-	0.5
20	5	5	-
21	-	3	0.5
22	9	-	0.5
23	8.5	5	-
24	4.5	3	-
25	8.5	4	-
26	-	2	0.8
27	5	3	-
28	8.5	4	-
29	5	4	-
30	8.5	4	-

4.3. Results

4.3.1. Clonal immigration impedes adaptation in complex and unpredictable environment

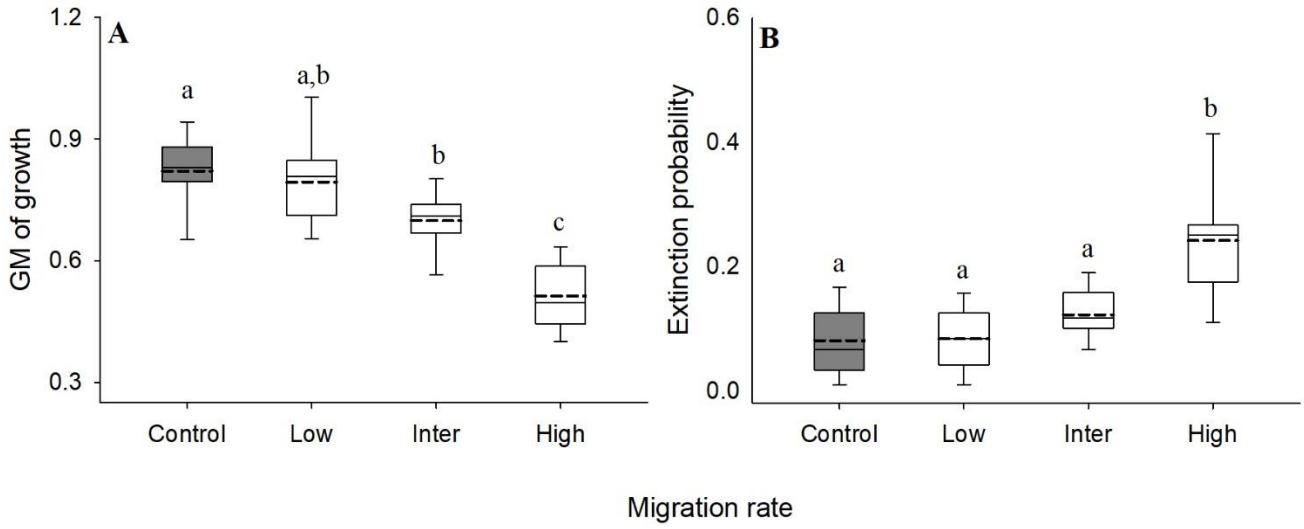

Figure 4.1. Effect of clonal migration during selection in complex unpredictable environments. Fitness was measured as A) Geometric mean of growth, during selection. B) Probability of extinction in the selection environments, during selection. Each box plot represents data from 12 replicate populations. Solid lines represent median, dotted lines denote mean, whiskers denote 10th and 90th percentiles and dots denote 5th and 95th percentile. Box plots denoted by different letters are significantly different from each other ($P < 0.05$ in Tukey's posthoc analysis).

After 30 rounds of clonal migration and selection, (corresponding to ~200 generations in the no migration control populations) we found that the control populations (i.e. those that did not receive any immigrants) were significantly better adapted than populations that received immigrants (from clonal source, C). The geometric mean of growth, during selection, was significantly different between the migration treatments (Fig. 4.1A; $F_{3,44}$ = 28.76, P = 1.88E-10). The no migration control populations had the highest GM of growth. However, this was only significantly higher than intermediate (Tukey's p = 0.01; Cohen's d = 1.49 (large)) and high (Tukey's p = 0.0002; Cohen's d = 3.56 (large)) migration treatments. The reduced GM of growth of high migration treatment was also significant w.r.t low (Tukey's p = 0.0002; Cohen's d = 2.88 (large)) and intermediate (Tukey's p = 0.0002; Cohen's d = 2.42 (large)) migration treatments. This reduction in growth was accompanied by a significant effect in terms of extinction probability (Fig. 4.1B; $F_{3,44}$ = 13.48, P = 2.25E-06). Tukey's post hoc indicated that the high migration treatment had significantly greater extinction probability compared to all treatment population (control: p = 0.0002; Cohen's d = 2.11 (large); intermediate: p = 0.0002; Cohen's d = 2.13 (large); high: p = 0.006; Cohen's d = 1.66 (large)). Thus, during selection, presence of migration reduced the populations' ability to survive and grow in the selection environment, resulting in significant increase in extinction probability at high migration. We next tested how this reduced survivability and growth affected the overall adaptation.

Table 4.2. Summary of P values of the effect of clonal migration on fitness measured during selection.

	ANOVA *P* value	Pairwise comparison	*P* value (Tukey's post hoc)	Cohen's *d*	Interpretation
GM of growth	1.88E-10	Cont. vs. Low	0.8840	0.265	Small
		Cont. vs. Inter	0.0097	1.490	Large
		Cont. vs. High	0.0002	3.557	Large
		Low vs. Inter	0.0613	1.017	Large
		Low vs. High	0.0002	2.879	Large
		Inter vs. High	0.0002	2.425	Large
Extinction probability	2.25E-06	Cont. vs. Low	0.9977	0.054	Small
		Cont. vs. Inter	0.1719	0.868	Large
		Cont. vs. High	0.0002	2.112	Large
		Low vs. Inter	0.2414	0.869	Large
		Low vs. High	0.0002	2.132	Large
		Inter vs. High	0.0058	1.657	Large

4.3.2. Presence of variation in the immigrant pool counters the negative effect of migration.

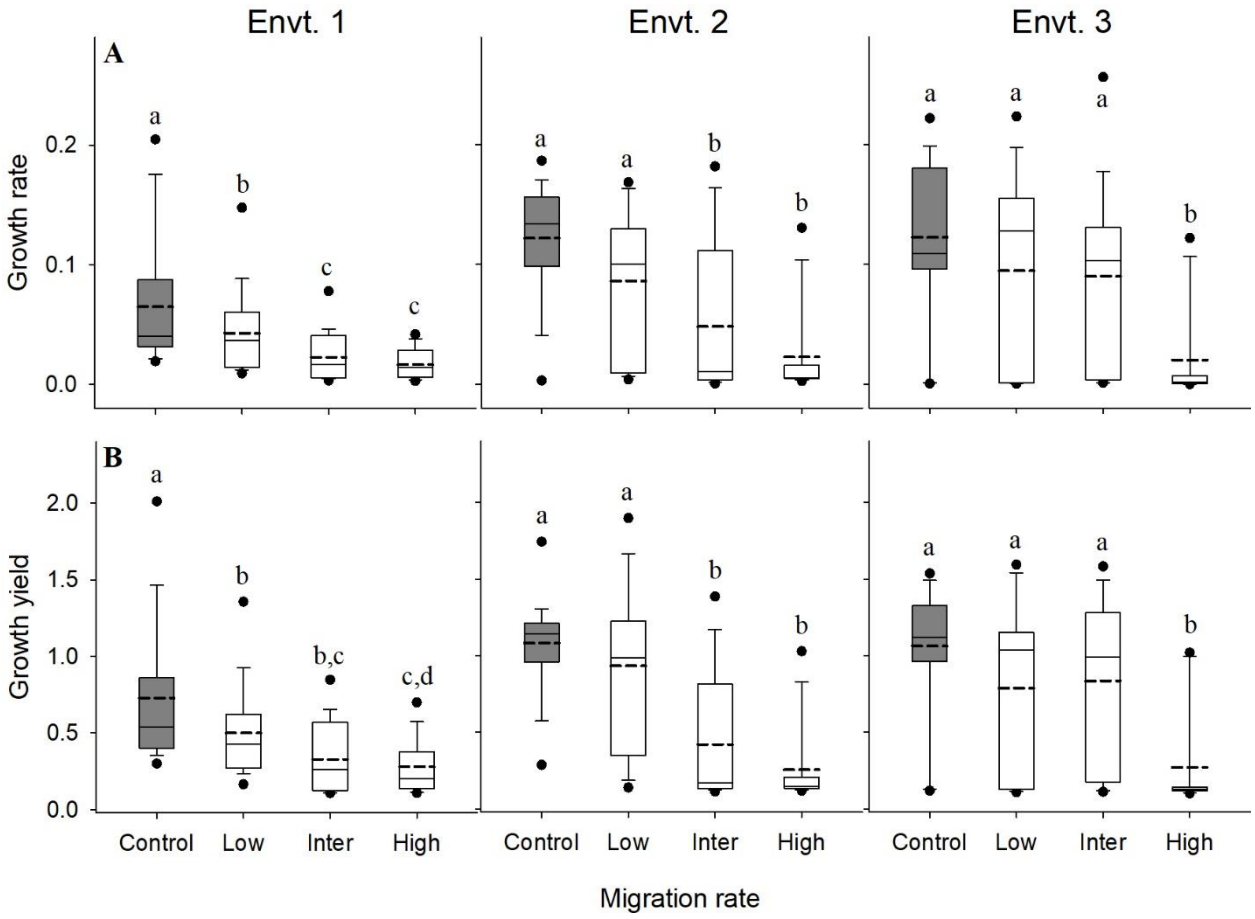

Figure 4.2. Effect of clonal migration on fitness, post selection. Two fitness proxies A) growth rate and B) growth yield were measured in three complex environments. See methods for composition of the three complex environments. Each box plot represents data of 24 values i.e., 12 replicate population, assayed twice. Solid lines represent median, dotted lines denote mean, whiskers denote 10th and 90th percentiles and dots denote 5th and 95th percentile. Box plots denoted by different letters are significantly different from each other ($P < 0.05$ in Tukey's posthoc analysis).

Extent of adaptation, post selection, was estimated as growth rate and growth yield in three complex environments. When the fitnesses across all three environments were analyzed together in a single ANOVA, we see that the interaction between migration treatments and assay environment is significant (Fig. 4.2; Growth rate: $F_{6,144} = 2.43$, $P = 0.03$; Growth yield: $F_{6,144} = 4.62$, $P = 4.0E-04$). Therefore, the effect of clonal migration on fitness in the three complex environments were analyzed separately.

The results of ANOVAs, P values and Cohen's d of all pairwise comparisons are summarized in Tables 4.3 and 4.4. Briefly, all migration treatment had significantly lower growth rate and yield than the no migration control populations in environment 1. Growth rate of low migration treatment was significantly higher than both intermediate and high migration treatments which were not significantly different from each other. Similarly, growth yield of low migration treatment was higher but it was significant w.r.t only high migration treatment. But again, both intermediate and high migration treatments were not significantly different from each other. In environment 2, only two migration treatments (intermediate and high) had significantly lower growth rate and yield w.r.t the control. However, the growth rate of the low migration populations was only marginally insignificant (Tukey's p = 0.054) w.r.t control populations in environment 2. Both growth rate and yield of the low migration treatment was significantly higher than the intermediate and high migration treatment. However, there was no significant difference between growth rates and yields of intermediate and high migration treatment. In environment 3, both growth rate and yield of only the high migration treatment was significantly lower than all other treatments. Thus, taken together, we see that when populations are faced with complex and unpredictable environments, migration can have an overall negative effect on the evolutionary outcomes. Additionally, the negative effects experienced by these populations were monotonic with the level of migration received.

Table 4.3. Summary of the P values for growth rate of clonal migration, post selection. P value of the main effect of migration is reported after Holm-Šidák correction for familywise error. Migration treatments were compared with each other using Tukey's post-hoc analysis. Pairwise effect sizes were computed as Cohen's d.

Growth rate					
	Holm Sidak Corrected *P* value	Pairwise comparison	*P* value (Tukey's post hoc)	Cohen's *d*	Interpretation
Envt. 1	0.001252	Cont. vs. Low	0.011	0.491	Small
		Cont. vs. Inter	1.67E-04	1.043	Large
		Cont. vs. High	1.67E-04	1.245	Large
		Low vs. Inter	0.023	0.702	Medium
		Low vs. High	0.002	0.997	Large
		Inter vs. High	0.814	0.348	Small
Envt. 2	5.68E-06	Cont. vs. Low	0.054	0.684	Medium
		Cont. vs. Inter	1.75E-04	1.321	Large
		Cont. vs. High	1.67E-04	2.330	Large
		Low vs. Inter	0.037	0.626	Medium
		Low vs. High	2.98E-04	1.302	Large
		Inter vs. High	0.255	0.486	Small
Envt. 3	5.07E-05	Cont. vs. Low	0.462	0.390	Small
		Cont. vs. Inter	0.330	0.473	Small
		Cont. vs. High	1.76E-04	1.915	Large
		Low vs. Inter	0.995	0.059	Small
		Low vs. High	0.002	1.184	Large
		Inter vs. High	0.003	1.177	Large

Table 4.4. Summary of the P values for growth yield of clonal migration, post selection. P value of the main effect of migration is reported after Holm-Šidák correction for familywise error. Migration treatments were compared with each other using Tukey's post-hoc analysis. Pairwise effect sizes were computed as Cohen's d.

Growth yield					
	Holm Sidak Corrected *P* value	Pairwise comparison	*P* value (Tukey's post hoc)	Cohen's *d*	Interpretation
Envt. 1	0.000116	Cont. vs. Low	0.171	0.598	Medium
		Cont. vs. Inter	0.321	1.113	Large
		Cont. vs. High	1.68E-04	1.304	Large
		Low vs. Inter	0.984	0.642	Medium
		Low vs. High	0.002	0.894	Medium
		Inter vs. High	0.001	0.227	Small
Envt. 2	1.11E-08	Cont. vs. Low	0.532	0.350	Small
		Cont. vs. Inter	1.68E-04	1.763	Large
		Cont. vs. High	1.67E-04	2.851	Large
		Low vs. Inter	2.85E-04	1.088	Large
		Low vs. High	1.67E-04	1.668	Large
		Inter vs. High	0.441	0.465	Small
Envt. 3	1.5E-05	Cont. vs. Low	0.034	0.567	Medium
		Cont. vs. Inter	2.13E-04	0.479	Small
		Cont. vs. High	1.72E-04	2.122	Large
		Low vs. Inter	0.159	0.088	Small
		Low vs. High	0.044	1.137	Large
		Inter vs. High	0.934	1.271	Large

4.3.3 Variant (V) population has higher variation in fitness than the clonal (C) population

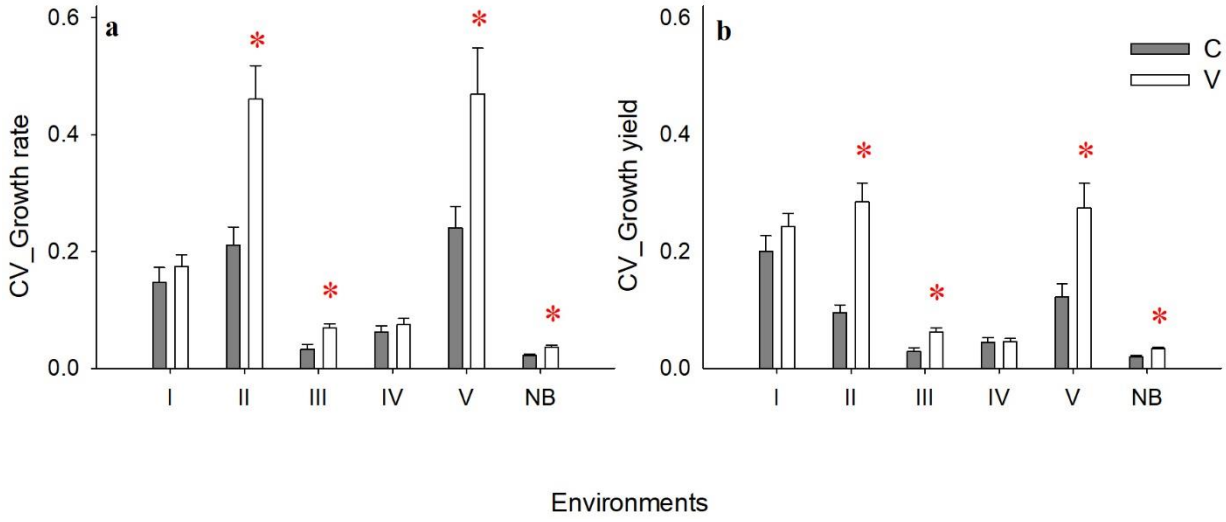

Figure 4.3. Average coefficient of variation of the clonal and variant population.

Variation of fitness (CV) was measured in growth rate and yield of the two populations assayed in 6 representative environments. Each bar represents the average CV over 72 individual colonies assayed over 12 days. Error bars are SE of mean. * denote p value < 0.05 in paired t-Test after Holm-Šidák correction.

Next, we investigate the effects of increased variation in the migrant population on the evolutionary outcome of the recipient populations. For this, we enriched the variation of the ancestral population (variation source, V). After ~100 generations of lenient bottlenecking ($1/10^{th}$), in benign environment, the V population showed significant increase in variation in fitness, measured as coefficient of variation, in 4 out of 6 tested environments (Fig. 4.3 and Table 4.5). The increase in variation was seen w.r.t both growth rate and yield in environments II, III, V and NB. The p values of all the paired t-Test after Holm-Šidák correction is listed in Table 4.5. Thus, V population had larger within-population fitness variance than C population and was used as the source of migrants in a second selection experiment.

Table 4.5. Summary of the P values of paired T-test of coefficient of variation in fitness between C and V ancestors, after Holm-Šidák correction

	p value after Holm-Šidák correction	
Environment	Growth rate	Yield
I	0.649	0.425
II	0.003	0.0001
III	0.018	0.005
IV	0.413	0.858
V	0.044	0.014
NB	0.006	0.009

4.3.4 Presence of variation in the immigrant pool counters the negative effect of migration.

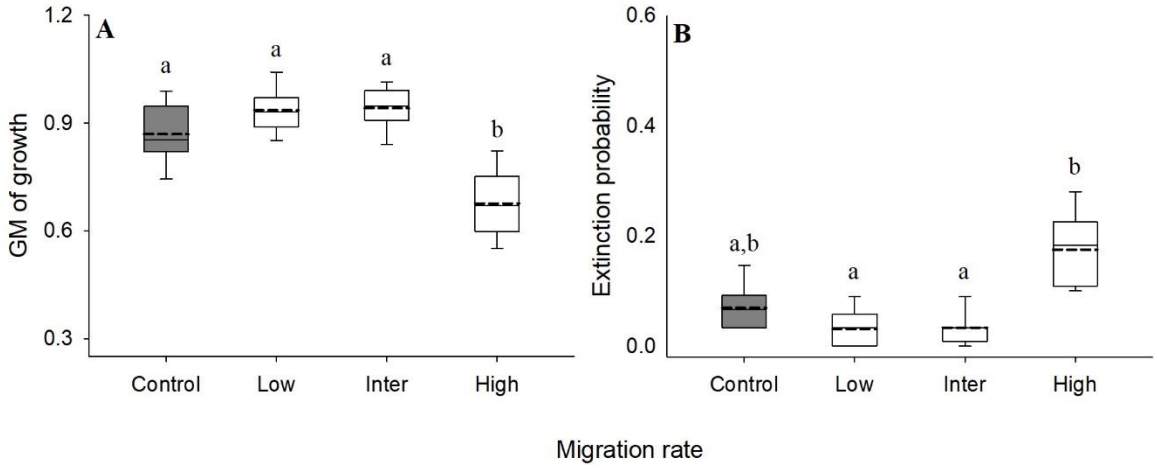

Figure 4.4. The effect of migration, when the immigrants carry variation. Fitness was measured as A) Geometric mean of growth, during selection. B) Probability of extinction in the selection environments, during selection. Each box plot represents data from 12 replicate populations. Solid lines represent median, dotted lines denote mean, whiskers denote 10th and 90th percentiles and dots denote 5th and 95th percentile. Box plots denoted by different letters are significantly different from each other ($P < 0.05$ in Tukey's posthoc analysis).

When the migrant populations carried variation (V population), migration had an effect only at high levels of migration. A significant main effect was observed w.r.t geometric mean of growth (Fig 4.4A; $F_{3,44}$ = 34.14, P = 1.48E-11). GM of growth was reduced at high levels of migration and this reduction was significantly different from all other treatments in Tukey's post-hoc analysis (control: p = 1.69E-04, Cohen's d = 2.25 (large); low: p = 1.69E-04, Cohen's d = 3.37 (large); intermediate: p = 1.69E-04, Cohen's d = 3.52 (large)). Subsequently, extinction probability also had a significant main effect of migration (Fig 4.4B; $F_{3,44}$ = 6.07, p= 0.0015) and high migration treatment had an elevated probability of extinction. However, this increase in extinction was significantly different from only low and intermediate migration treatment (low: p = 9.32E-04, Cohen's d = 2.02 (large); intermediate: p = 0.03, Cohen's d = 0.90 (large)). Thus in contrast to migration from a clonal source, the negative effect of migration, during selection, is diminished when the migrants carry variation.

Table 4.6. Summary of P values of the effect of migration with variation on fitness measured during selection.

	ANOVA *P* value	Pairwise comparison	*P* value (Tukey's post hoc)	Cohen's *d*	Interpretation
GM of growth	1.48E-11	Cont. vs. Low	0.133	0.932	Large
		Cont. vs. Inter	0.089	1.037	Large
		Cont. vs. High	0.0002	2.254	Large
		Low vs. Inter	0.998	0.097	Small
		Low vs. High	0.0002	3.371	Large
		Inter vs. High	0.0002	3.519	Large
Extinction probability	1.50E-03	Cont. vs. Low	0.218	1.494	Large
		Cont. vs. Inter	0.906	0.242	Small
		Cont. vs. High	0.138	1.192	Large
		Low vs. Inter	0.576	0.439	Medium
		Low vs. High	0.001	2.021	Large
		Inter vs. High	0.030	0.898	Large

4.3.5. Presence of variation in the immigrant pool counters the negative effect of migration.

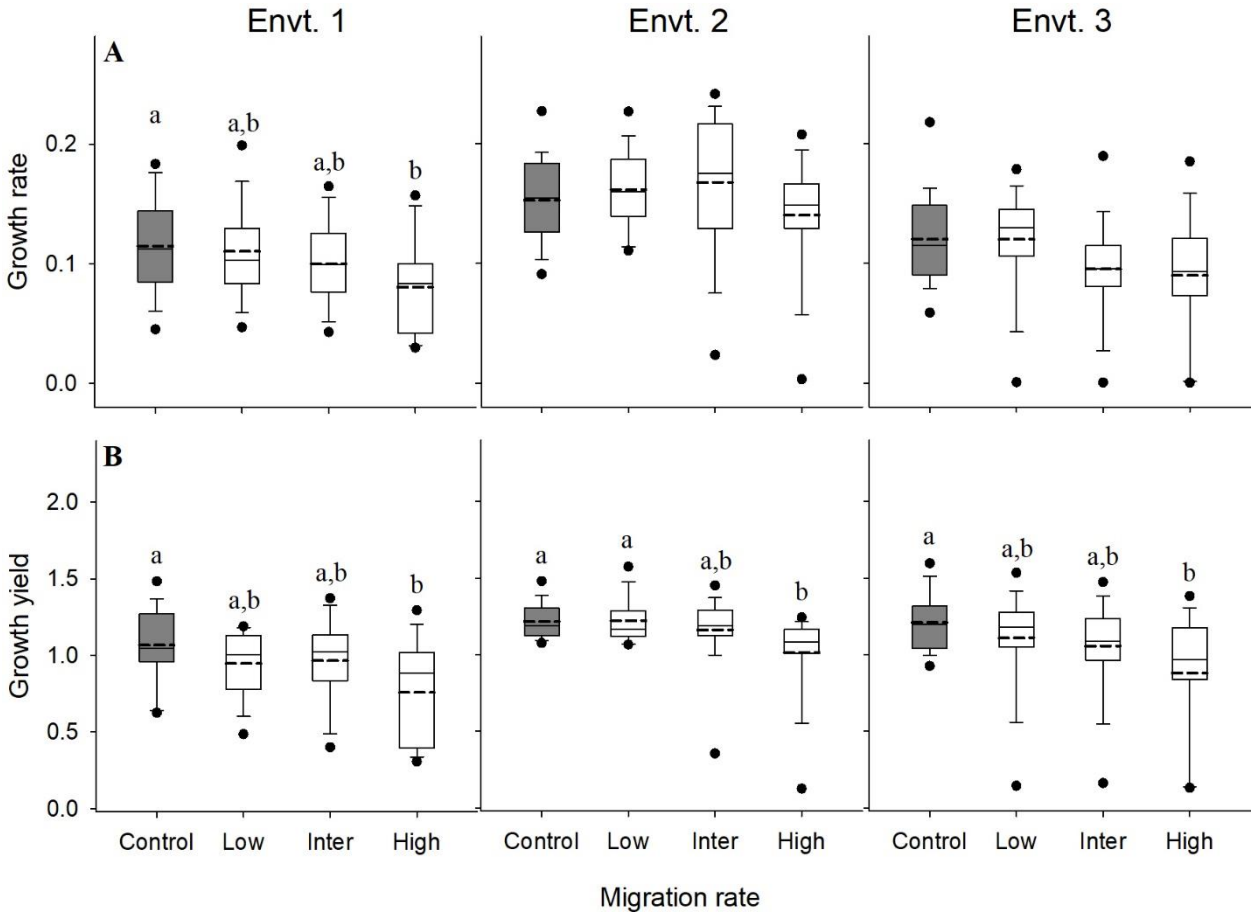

Figure 4.5. Effect of migrant, with variation, on fitness post selection. Fitness was estimated as A) growth rate and B) growth yield in three complex environments. Each box plot represents data of 24 values i.e., 12 replicate populations, assayed twice. Solid lines represent median, dotted lines denote mean, whiskers denote 10th and 90th percentiles and dots denote 5th and 95th percentile. Box plots denoted by different letters are significantly different from each other ($P < 0.05$ in Tukey's posthoc analysis).

Following the analysis in section 4.3.1, fitnesses of the populations, receiving migrants with variation, in the three complex environments, were also analyzed as independent ANOVAs (Tables 4.7 and 4.8). In contrast to clonal migration, we found that the main effect of migration was either non-significant (growth rate in environments 2 and 3) or when significant, the effect was limited to only the populations receiving high level of migration. The growth rate and yield of the populations in environment 1 is significant only between the no-migration control and high migration treatments. The main effect of growth rate in environment 2 was not significant ($F_{3,44} = 1.9$, $P = 0.144$). However, growth yield is significant and Tukey's post hoc analysis show that high migration treatment has significantly lower yield than control and low migration treatments (Table 4.8). Alike environment 2, growth rate in environment 3 was also not significant ($F_{3,44} = 2.56$, $P = 0.13$). Similarly, growth yield is significant and Tukey's post hoc analysis show that high migration treatment has significantly lower yield than only no-migration control (Table 4.8). Taken together, these results illustrate that the presence of variation in the migrant pool can ameliorate the negative effects of migration.

Table 4.7. Summary of the P values for growth rate of migration with variation. P value of the main effect of migration is reported after Holm-Šidák correction for family-wise error. Migration treatments were compared with each other using Tukey's post-hoc analysis. Pairwise effect sizes were computed as Cohen's d.

Growth rate					
	Holm Sidak Corrected *P* value	Pairwise comparison	*P* value (Tukey's post hoc)	Cohen's *d*	Interpretation
Envt. 1	0.02406	Cont. vs. Low	0.984	0.109	Small
		Cont. vs. Inter	0.612	0.395	Small
		Cont. vs. High	0.032	0.847	Large
		Low vs. Inter	0.819	0.283	Small
		Low vs. High	0.074	0.747	Medium
		Inter vs. High	0.377	0.510	Medium
Envt. 2	0.1435	NA	NA	NA	NA
Envt. 3	0.1296	NA	NA	NA	NA

Table 4.8. Summary of the P values for growth yield of migration with variation. P value of the main effect of migration is reported after Holm-Šidák correction for familywise error. Migration treatments were compared with each other using Tukey's post-hoc analysis. Pairwise effect sizes were computed as Cohen's d.

Growth yield					
	Holm Sidak Corrected *P* value	Pairwise comparison	*P* value (Tukey's post hoc)	Cohen's *d*	Interpretation
Envt. 1	1.9E-05	Cont. vs. Low	0.581	0.528	Medium
		Cont. vs. Inter	0.703	0.397	Small
		Cont. vs. High	0.010	1.070	Large
		Low vs. Inter	0.997	0.077	Small
		Low vs. High	0.200	0.681	Medium
		Inter vs. High	0.136	0.690	Medium
Envt. 2	0.007	Cont. vs. Low	1.000	0.046	Small
		Cont. vs. Inter	0.808	0.284	Small
		Cont. vs. High	0.009	0.934	Large
		Low vs. Inter	0.755	0.298	Small
		Low vs. High	0.007	0.922	Large
		Inter vs. High	0.084	0.555	Medium
Envt. 3	0.010	Cont. vs. Low	0.680	0.371	Small
		Cont. vs. Inter	0.328	0.587	Medium
		Cont. vs. High	0.003	1.057	Large
		Low vs. Inter	0.933	0.164	Small
		Low vs. High	0.061	0.627	Medium
		Inter vs. High	0.210	0.491	Small

4.4. Discussion

In this study, we investigated the effect of immigration on adaptation of asexual populations in complex and unpredictably fluctuating environments. Immigration from clonal and non-evolving source (ancestor) population, resulted in reduction in fitness during selection (Fig. 4.1) as well as post-selection (Fig. 4.2). During selection, as the proportion of immigrants increased, the geometric mean (GM) of growth decreased (Fig. 4.1A) and the extinction probability increased (Fig: 4.1B). After ~200 generations, the populations that received immigrants during selection, had adapted less with reduced growth rate (Fig. 4.2A) and yield (Fig. 4.2B). The reduction in fitnesses increased with the fraction of immigrants in the evolving populations.

These results are in contradiction with a number of studies where the presence of migration promotes larger and/or faster adaptation in asexual organisms (Bell & Gonzalez 2011; Lagator *et al.* 2014). In particular, it disagrees with a previous study (Perron *et al.* 2007) that used a similar experimental setup of clonal source population and showed that increasing immigration rates leads to rapid evolution. In their study, although the rates of adaptation were faster in benign (single antibiotic) environments than in harsh (two antibiotic) environments, the effect of migration was positive in all cases. In contrast, we see increasing negative effects of migration with increasing rates of migration. One possible reason for the discrepancy in results could be the intensity of the stress used in the selection environments. While both experiments had multiple stress components, Perron *et. al.*, (Perron *et al.* 2007) used lethal concentrations of stress whereas we used sub-lethal concentrations. Lethal stresses create a sink environment where population size is expected to decline without sustained migration (as pointed out by the authors themselves) (Dias 1996; Holt 1997; Holt &

Gomulkiewicz 1997). As in the case of the two experiments ((Perron *et al.* 2007) and this study), immigration from a clonal source population can be expected to promote adaptation in terms of its demographic effect, i.e., changes in population sizes, which in turn can influence the adaptive dynamics of these populations. However, in non-lethal/ non-sink environments, like in our experiment, migration provided no demographic advantage as populations can persist here without immigration. Instead, the effect was largely negative as selection for locally fitter individuals can be diluted by increasing proportions of immigrants (Kawecki & Holt 2002; Lenormand 2002; Kawecki & Ebert 2004). The two studies taken together highlight that the nature of the environment faced by the evolving populations needs to be considered when studying the effect of migration on adaptation. The results can potentially be very different when the populations are evolving in sub-optimal environments compared to those evolving in lethal environments.

Since clonal immigrants in sub-optimal environments did not provide any significant advantage to adaptation, we next investigated how variation in the immigrants influences adaptation to complex and unpredictable environments. To this end, we conducted a second selection experiment using source population with larger variation in fitness (Fig. 4.3). Immigration from this variant population had little or no effect on the evolving population as seen from fitness measured during as well as post selection (Figs. 4.4 and 4.5). Populations receiving low and intermediate levels of migration did not show reduction w.r.t any aspects of fitness. However, reduction in fitness was observed when the populations were subjected to high migration.

It has been previously shown in bacteriophages that increased variation, due to migration, can promote adaptation (Dennehy *et al.* 2010). However in that study, the positive effect was limited to immigration from source populations grown in the same environment as the

selection environment. Migration between similar environments could have promoted the spread of beneficial variation between sub-populations (Kassen 2014). On the other hand, in our study the source population was grown in a benign environment, unrelated to the selection environment. Migration from such a source is not limited to only beneficial variants. Additionally, the distribution of fitness effects of new mutations is expected to greatly vary in a complex and fluctuating environment with a rugged and shifting fitness landscapes (Van Cleve & Weissman 2015). Thus, in non-sink but complex unpredictable environments, we see that the benefit of increased variation was only enough to counter the negative effects of migration. These results are in agreement with theoretical predictions that variation in the migrant pool can ameliorate the negative effects of migration (Barton 2001). However, if the amount of variation is too high, then one can expect negative effects on fitness, including extinctions (Barton 2001). Significant reduction in all aspects of fitness with high migration can be indicative of existence of such a limit on the positive effects of increased variation via migration.

Populations receiving high levels of migration, from both clonal and variant sources, relied on recurrent immigration and revival from previous time points for survival under complex unpredictable conditions. This is a clear demonstration of the creation of pseudo-sinks where viable environments appear to have become a sink environment as a result of high migration (Watkinson & Sutherland 1995). Repeated introduction of individuals into sub-optimal environments can limit adaptation and result in the populations being in a constant state of maladaptation, a phenomenon commonly observed at range margins (Kirkpatrick & Barton 1997).

4.5. Conclusions

Migrating individuals play multiple roles (demographic, variation) in the destination environment (Garant *et al.* 2007). The relative importance of these aspects of migration and their influence on adaptation is dependent on the quality of environment. Maintenance of a sustainable population might be more critical in a lethal sink environment but supply of variation is more important in sub-optimal environments, without which migration can result in the creation of pseudo-sink environments. Since sub-optimal environments are likely more prevalent in nature, it is important that studies be conducted under such conditions to fully understand the effects of migration. Additionally, it becomes important to consider the interactions between the role of migrating individuals and the environment they migrate into.

Chapter 5: Frequency and predictability of fluctuations have very little effect on adaptation in *Escherichia coli* populations

5.1. Introduction

Experimental evolution studies have played an important role in developing our understanding of the different aspects of evolution. However, until recently, most experimental evolution studies have typically considered static/constant environment (Lenski *et al.* 1991; Gresham *et al.* 2008; Goldman & Travisano 2011; Puentes-Téllez *et al.* 2013). But, the environment is rarely ever constant. Organisms are constantly challenged with heterogeneity and fluctuations in their environment. More specifically, the physical, chemical and biological components of the microscale environments surrounding microorganisms can be extremely heterogeneous.. For example, nutrients are not always uniformly distributed but maybe localized (Pett-Ridge & Firestone 2005; Billerbeck *et al.* 2006; Or *et al.* 2007; Stocker 2012). Such heterogeneities can in turn strongly influence the growth and distribution of microorganisms in the environment. However, it is only in the last two decades that we have started understanding evolution in fluctuating environments (reviewed in (Nguyen *et al.* 2021).

Evolution in temporally fluctuating environments differs in many ways from that in constant environments. Firstly, the speed of adaptation is expected to be slower when the environment fluctuates vs. when the environment is constant (Kassen 2002, 2014). Secondly, a relatively homogeneous environment and a variable one are known to promote the evolution of specialist and generalist phenotypes respectively (Gilchrist 1995; Condon *et al.* 2014; Haaland *et al.* 2020). However, predictability and the relative scale of fluctuation (fine-grained vs. coarse-grained) influence the finer details of these phenotypes. For example, predictable coarse-grained environmental fluctuations can support the evolution of phenotypic plasticity and/or sequential emergence of specialists (Crill *et al.* 2000; Simons 2011; Rezaie *et al.* 2021). Unpredictable fine-grained fluctuations, on the other hand, can

favour the evolution of broadly adapted generalists with increased fitness in all component environments (Tufto 2015). Coarse-grained and unpredictable fluctuations can in general favour the evolution of bet-hedging strategies (Kussell *et al.* 2005; Acar *et al.* 2008). Studies have shown that intermediate levels of fluctuations can result in higher levels of genetic variation by promoting stable coexistence of diverse communities where constant environments would result in the exclusion of one or more species (Rodríguez-Verdugo *et al.* 2019). Again, the exact outcomes are highly dependent on the timescales of fluctuation where, too fast or too slow fluctuations have been shown to result in extinction (Rodríguez-Verdugo & Ackermann 2021).

Thus, microorganisms have been known to employ diverse strategies when adapting to fluctuations in the environment, which in turn, are influenced by the nature of fluctuations itself. Intuitively, adaptation to predictable fluctuation is expected to be different from adaptation to unpredictable fluctuations. Similarly, the frequency of fluctuations is expected to influence the rate and extent of adaptation. While prior studies have demonstrated differences in evolutionary outcomes depending on the nature of fluctuation (discussed above), relatively few empirical studies have directly compared the effects of differences in the nature of fluctuations. Studies on the effects of predictable vs. unpredictable fluctuations have yielded conflicting results. Some studies (Hughes *et al.* 2007; Alto *et al.* 2013) provide evidence for the effect of predictability on adaptation while others (Turner & Elena 2000; Karve *et al.* 2018) observed no difference between predictable and unpredictable fluctuations. Empirical studies comparing the effects of frequency of fluctuations have resulted in non-significant results. In clonal populations of *Chlamydomonas reinhardtii*, there was no effect of the frequency of fluctuations (at intra- or inter-generational timescales) on the extent of adaptation (Kassen & Bell 1998). Similarly, *Pseudomonas fluorescens* adapting to fluctuations in nutrient concentration and a wide range of frequencies (between every day to

every 48 days) also did not show a significant effect of the rate of fluctuations (Buckling *et al.* 2007). However, it is to be noted that both these studies considered only predictable fluctuations. To the best of our knowledge, the interaction between the predictability and frequency of fluctuations has not received sufficient attention.

We subjected replicate populations of *Escherichia coli* fluctuations between stress (sub-lethal concentration of cobalt chloride) and no-stress (nutrient broth) environment. Our goal in this study was to investigate the effect of fluctuations, with more emphasis on fluctuation and less on the choice of environment. Therefore, to simplify our experimental system, we used fluctuations between stress and no-stress conditions, such that the populations had to adapt to only one stress and not two separate ones. *E. coli* populations were subjected to both predictable and unpredictable fluctuations at three different frequencies. The populations were also subjected to gradual increase in the concentrations of cobalt chloride, every 240 generations. After 240 generations, the populations subjected to rapid predictable fluctuations in cobalt chloride had evolved growth rate similar to populations constantly exposed to cobalt chloride. However, the populations experiencing the other combinations of fluctuation and predictability had a significant reduction in growth rate in the same environment. Interestingly, the pattern was completely reversed after 720 generations. At this point, the populations experiencing rapid predictable fluctuations had a significantly lower growth rate than the control populations, in cobalt chloride, whereas the fitness of all the other treatments were similar to that of the control.

5.2. Materials and Methods

5.2.1 Experimental evolution

This study was conducted on *Escherichia coli* MG1655 strain with kanamycin resistance. A single colony was inoculated in 10 ml nutrient broth with kanamycin (NB^{Kan}) and allowed to grow for 24 hours at 37^{o}C and 150 RPM. A total of 64 replicate populations were initiated, by inoculating 20 µl of this grown culture in 2ml NB^{Kan}. These populations were equally divided between 8 treatment regimes with 8 replicate populations per treatment. The replicate populations were subjected to either constant stress (sublethal concentration of cobalt chloride) or benign (NB^{Kan}) environment, or fluctuations between the two. The fluctuations were either predictable or unpredictable, fluctuating at one of the three different frequencies: change in environment every 1, 2 or 6 subcultures. Predictable fluctuations involved alternating between the two environments and a random sequence of fluctuations was used for the unpredictably fluctuating treatment (see supplementary material for sequence of unpredictable fluctuations). The number of times populations were exposed to each of the two environments was kept the same in all fluctuating treatments (54 exposures to each environment), which was half of the number of exposures in the constant regimes (108 exposures). Populations were subcultured every 12 hours by inoculating 20 µl of grown culture in 2ml fresh media, resulting in ~6.64 ($\log_2$ 100) generations per transfer. All populations were maintained at 37^{o}C and 150 RPM throughout the selection duration. At the beginning of the selection, populations were exposed to 10mg/ml cobalt chloride which resulted in ~60% reduction in growth, measured as both growth rate and yield, compared to growth in NB^{Kan}. With repeated exposures, populations showed an increase in fitness in the stress environment. Therefore, to ensure that the populations were continuously stressed, the concentration of cobalt was increased every 36 transfers (after ~240 generations). The

concentration was increased twice, from 10mg/ml to 20 mg/ml and then to 30 mg/ml. The selection lasted for a total of 108 transfers (54 days). After every ~240 generations, glycerol stocks were made for each population by adding 700 µl of culture to 300 µl of 50% glycerol.

5.2.2 Fitness assays

Fitness of the evolving populations was estimated after 240 and 720 generations of adaptation. All 64 populations, including the fluctuation and constant regimes, were assayed together in both stress and benign (cobalt and NB^{Kan}) environments. The populations after 240 generations were subjected to 10mg/ml cobalt chloride whereas populations after 720 generations were subjected to 30 mg/ml. 4µL glycerol stocks were revived in 2ml NB^{Kan}, overnight. The assays were performed in conditions similar to the selection. Briefly, 20µL of the revived culture was inoculated in 2ml assay environments, in 24-well tissue culture plates and incubated at 37^{o}C and 150 RPM. Every 2 hours, for 24 hours, OD_{600} of the cultures was measured using a plate reader (Synergy HT BioTek, Winooski, VT, USA). Following previous studies (Karve *et al.* 2015; Chavhan *et al.* 2019), we estimated fitness as the populations' growth rate and yield using a custom python script which computes the maximum slope (growth rate) of the growth curve data over a period of 6 hours and maximum OD_{600} in 24 hours (yield). The fitness of the treatment populations, scaled by the fitness of the corresponding control populations in the respective assay environments were compared. The entire assay was repeated twice to obtain two measurement replicate data.

5.2.5 Statistical analysis

The scaled fitnesses of the treatment populations, after evolution, were analyzed using two-way mixed model ANOVA with treatment (7 levels; 6 treatments and 1 control line) as fixed factor and replicate populations (8 populations) as random factor nested in treatment. Fitnesses, measured as growth rate or yield, in the two assay environment and at two time-

points were compared using independent ANOVA. Since a total of 8 ANOVAs were performed, to correct for the inflation of family-wise error rates, a Holm-Šídák correction was performed on p-values of the treatment main effect (Abdi 2010). When the main effect of treatment was significant, pairwise comparisons were performed using Tukey's post-hoc comparisons. We also computed the Cohen's *d* statistics (Cohen 2013) as a measure of the effect sizes (Sullivan & Feinn 2012). The biological significance of the differences between the treatments were interpreted as small, medium and large for $0.2 < d < 0.5$, $0.5 < d < 0.8$ and $d > 0.8$, respectively.

All the ANOVAs were performed on STATISTICA v7.0 (Statsoft Inc.). Cohen's *d* statistics were estimated using the freeware Effect Size Generator v2.3.0 (Devilly 2004).

5.3. Results

5.3.1 Fitness of the treatment populations measured as growth rate

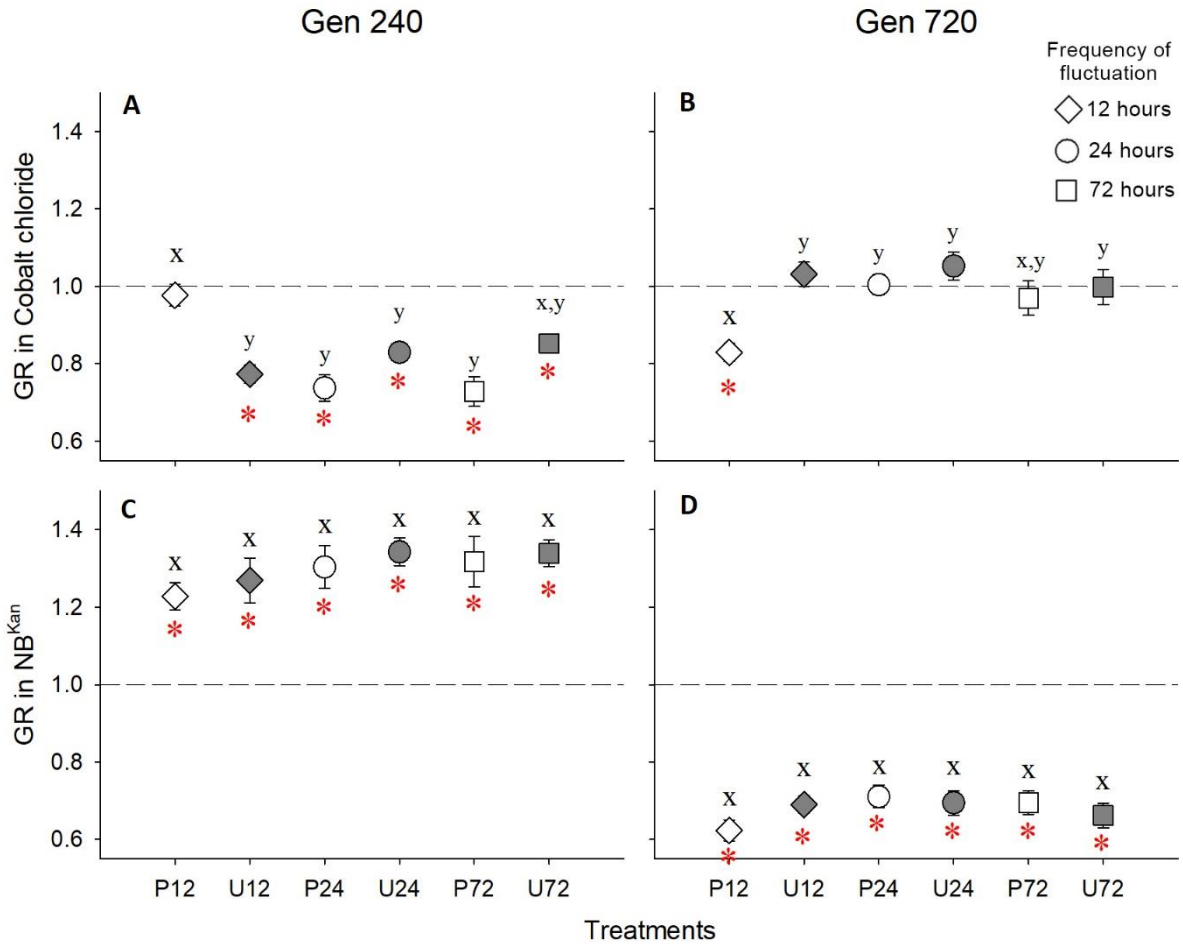

Figure 5.1. Mean scaled growth rate of the treatment populations w.r.t constant regime in the two assay environments. Fitness of the different treatment populations were measured as growth rate in the two environments i.e., 10mg/ml cobalt chloride after generation 240 (A) and 30mg/ml cobalt chloride after 720 generations (B) along with nutrient broth (NB) (C and D). Predictability of fluctuations are represented either as P (predictable, open symbols) or U (unpredictable, closed symbols). Error bars represent SEM. Horizontal reference lines represent growth rate of the constant populations in the corresponding environment. Letters above the mean represents the significance between the different treatments. Populations represented by different letters are significantly different from each other ($P < 0.05$, in Tukey's posthoc analysis). An * below the

mean denote that the treatment is significantly different ($P < 0.05$, in Tukey's posthoc analysis) from 1.0 (i.e., growth rate of the constant populations).

Growth rate of the fluctuating treatments, scaled by the growth rate of the control populations, showed significant main effect of treatment (after a Holm-Šídák correction) in both assay environments at both time-points: cobalt-240 generations (Fig. 5.1A; $F_{6,56}$ = 28.67 P = 1.57E-13), cobalt-720 generations(Fig. 5.1B; $F_{6,56}$ = 5.32 P = 5.46E-04), NB-240 generations (Fig. 5.1C; $F_{6,56}$ = 8.22 P = 1.77E-05), NB-720 generations (Fig. 5.1D; $F_{6,56}$ = 11.58 P =6.92E-10). Tukey's post-hoc analysis was performed to further compare the fluctuating treatment populations with each other as well as with the constant population. After 240 generations of adaptation with fluctuating exposures to sub-lethal concentrations of cobalt chloride and NB^{Kan}, treatment populations had significantly lower growth rate in cobalt chloride (Fig. 5.1A, table 5.1) with significantly higher growth rate in NB^{Kan} (Fig. 5.1C, table 5.3) than the corresponding constant populations. However, this pattern had completely changed by the time the populations evolved for 720 generations. Here, the growth rate of the treatment populations were not significantly different from the constant population in cobalt environment (Fig. 5.1B, table 5.2). But, they had a significantly reduced growth rate in NB^{Kan} compared to the NB^{Kan} constant populations (Fig. 5.1D, table 5.4). While all treatment populations had this pattern of trade-off in fitness, there was one exception. The growth rate of populations exposed to predictable fluctuation every 12 hours (P12) was similar to the constant population after 240 generations (Fig. 5.1A, table 5.1) but had a significantly lower growth in cobalt chloride w.r.t the constant populations after 720 generation (Fig. 5.1B, table 5.2). Furthermore, P12 was significantly different from all treatments except U72 after 240 generations (Fig. 5.1A, table 5.1) and P72 after 720 generations (Fig. 5.2B, table 5.2) of evolution. However, growth rate of P12 in NB^{Kan} environment was similar to the other treatment populations after 240 and 720 generations i.e., there was no significant difference between the six treatment populations.

It is interesting to note that rapid predictable fluctuations were significantly different from the other treatments. However, the growth rate of these populations were similar to or significantly lower than the growth rate of the control populations after 240 and 720 generations, respectively. Additionally, this effect was limited to the fitness in cobalt.

Table 5.1. Summary of Tukey's *P* value of the scaled growth rate in cobalt chloride after 240 generations

	Treatment	{1}	{2}	{3}	{4}	{5}	{6}	{7}
1	P 12		0.000384	0.000145	0.017410	0.000139	0.071447	0.998031
2	U 12	0.000384		0.981023	0.840128	0.938816	0.516171	0.000166
3	P 24	0.000145	0.981023		0.340522	0.999989	0.121126	0.000136
4	U 24	0.017410	0.840128	0.340522		0.229608	0.998176	0.003484
5	P 72	0.000139	0.938816	0.999989	0.229608		0.072028	0.000135
6	U 72	0.071447	0.516171	0.121126	0.998176	0.072028		0.017084
7	Co	0.998031	0.000166	0.000136	0.003484	0.000135	0.017084	

Table 5.2. Summary of Tukey's *P* value of the scaled growth rate in cobalt chloride after 720 generations

	Treatment	{1}	{2}	{3}	{4}	{5}	{6}	{7}
1	P 12		0.001953	0.010859	0.000559	0.073994	0.015333	0.013943
2	U 12	0.001953		0.997480	0.999511	0.854919	0.992770	0.994492
3	P 24	0.010859	0.997480		0.951941	0.990986	1.000000	1.000000
4	U 24	0.000559	0.999511	0.951941		0.607304	0.919415	0.929642
5	P 72	0.073994	0.854919	0.990986	0.607304		0.996668	0.995508
6	U 72	0.015333	0.992770	1.000000	0.919415	0.996668		1.000000
7	Co	0.013943	0.994492	1.000000	0.929642	0.995508	1.000000	

Table 5.3. Summary of Tukey's *P* value of the scaled growth rate in nutrient broth after 240 generations

	Treatment	{1}	{2}	{3}	{4}	{5}	{6}	{7}
1	P 12		0.996461	0.920336	0.622611	0.837920	0.655924	0.021944
2	U 12	0.996461		0.998631	0.928659	0.990895	0.942857	0.003744
3	P 24	0.920336	0.998631		0.997267	0.999993	0.998413	0.000812
4	U 24	0.622611	0.928659	0.997267		0.999795	1.000000	0.000228
5	P 72	0.837920	0.990895	0.999993	0.999795		0.999915	0.000462
6	U 72	0.655924	0.942857	0.998413	1.000000	0.999915		0.000245
7	Co	0.021944	0.003744	0.000812	0.000228	0.000462	0.000245	

Table 5.4. Summary of Tukey's *P* value of the scaled growth rate in nutrient broth after 720 generations

	Treatment	{1}	{2}	{3}	{4}	{5}	{6}	{7}
1	P 12		0.445739	0.150774	0.358838	0.354331	0.911457	0.000135
2	U 12	0.445739		0.996105	0.999999	0.999999	0.981434	0.000135
3	P 24	0.150774	0.996105		0.999172	0.999260	0.777959	0.000135
4	U 24	0.358838	0.999999	0.999172		1.000000	0.958344	0.000135
5	P 72	0.354331	0.999999	0.999260	1.000000		0.956704	0.000135
6	U 72	0.911457	0.981434	0.777959	0.958344	0.956704		0.000135
7	NB	0.000135	0.000135	0.000135	0.000135	0.000135	0.000135	

5.3.2 Fitness measured as yield

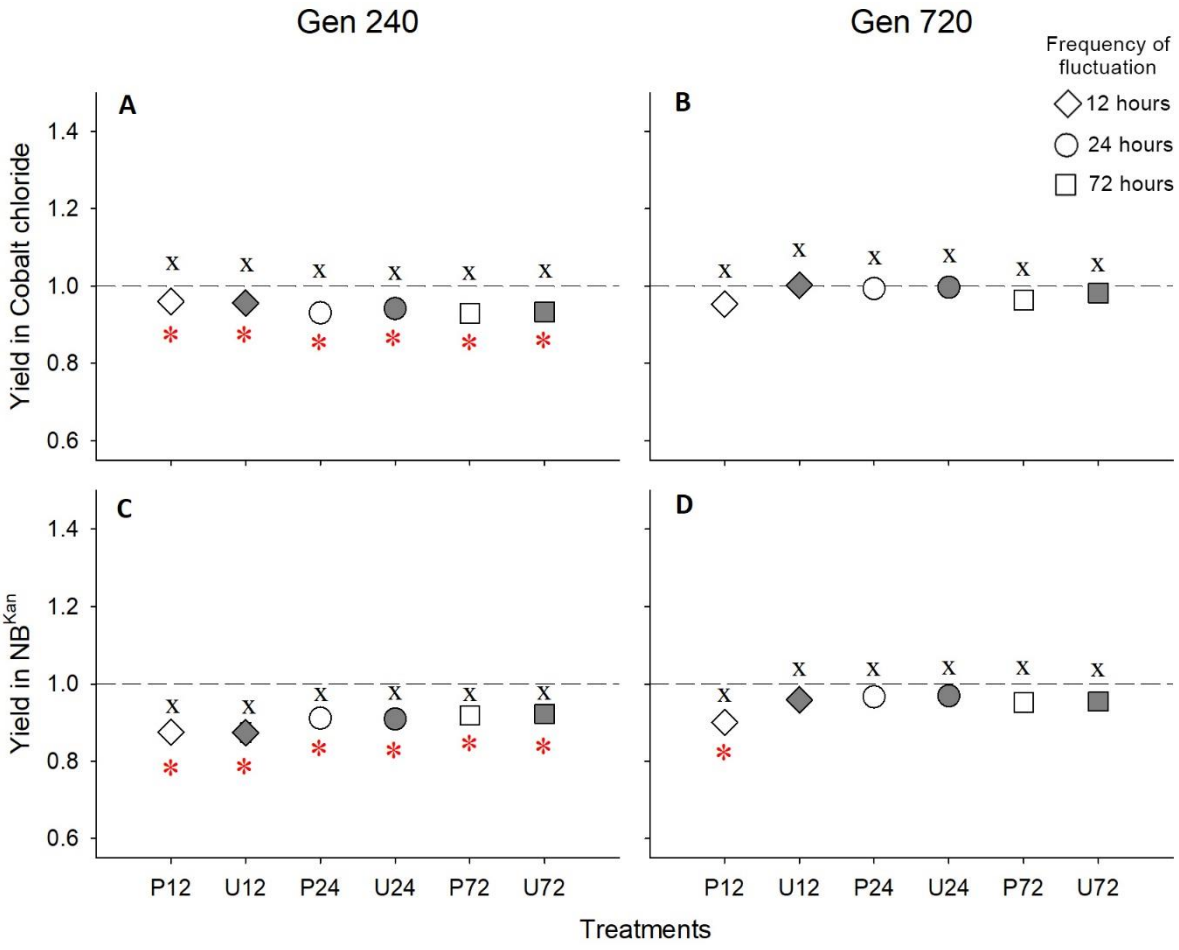

Figure 5.2. Mean scaled growth yield of the treatment populations w.r.t constant regime in the two assay environments. Fitness of the different treatment populations were measured as growth yield in the two environment i.e., 10mg/ml cobalt chloride after generation 240 (A) and 30mg/ml cobalt chloride after 720 generation (B) along with nutrient broth (NB) (C and D). Predictability of fluctuations are represented either as P (predictable, open symbols) or U (unpredictable, closed symbols). Error bars represent SEM. Horizontal reference lines represent growth yield of the constant populations in the corresponding environment. Letters above the mean represents the significance between the different treatments. Populations represented by different letters are significantly

different from each other ($P < 0.05$, in Tukey's posthoc analysis). An * below the mean denote that the treatment is significantly different ($P < 0.05$, in Tukey's posthoc analysis) from 1.0 (i.e., growth yield of the constant populations).

The scaled growth yield show significant main effect of treatments (after a Holm-Šídák correction) for all four comparisons; cobalt-240 generations (Fig. 5.2A; $F_{6,56}$ = 15.33 P = 5.1E-09), cobalt-720 generations (Fig. 5.2B; $F_{6,56}$ = 3.17 P = 0.01), NB-240 generations (Fig. 5.2C; $F_{6,56}$ = 6.52 P = 1.25E-04), NB-720 generations (Fig. 5.2D; $F_{6,56}$ = 7.59 P =3.47E-05). Tukey's post-hoc comparisons showed that there was no significant difference between the fluctuating treatments in all four comparisons. However, they significantly differed from the corresponding constant populations in terms of yield in both cobalt and NB, after 240 generations. The fluctuating populations had evolved significantly lower yield in both environments (Figs. 5.2A and 5.2C, tables 5.5 and 5.7). On the other hand, all treatment populations (except P12) had evolved growth yield similar to both constant populations, after 720 generations (Figs. 5.2B and 5.2D, tables 5.6 and 5.8). Although P12 treatment was not significantly different from the other treatment populations, it was the only treatment that had a lower fitness than the constant population in NB, after 720 generations (Fig. 5.2D, table 5.8).

Taken together, the fluctuating treatment populations did not show differences in yield across the two assay environments.

Table 5.5. Summary of Tukey's *P* value of the scaled growth yield in cobalt chloride after 240 generations

	Treatment	{1}	{2}	{3}	{4}	{5}	{6}	{7}
1	P 12		0.999873	0.168308	0.704846	0.135890	0.243853	0.017420
2	U 12	0.999873		0.317632	0.882150	0.266378	0.426943	0.006448
3	P 24	0.168308	0.317632		0.959200	1.000000	0.999996	0.000137
4	U 24	0.704846	0.882150	0.959200		0.934794	0.986450	0.000243
5	P 72	0.135890	0.266378	1.000000	0.934794		0.999948	0.000136
6	U 72	0.243853	0.426943	0.999996	0.986450	0.999948		0.000139
7	Co	0.017420	0.006448	0.000137	0.000243	0.000136	0.000139	

Table 5.6. Summary of Tukey's *P* value of the scaled growth yield in cobalt chloride after 720 generations

	Treatment	{1}	{2}	{3}	{4}	{5}	{6}	{7}
1	P 12		0.098240	0.264271	0.186707	0.996857	0.668732	0.129099
2	U 12	0.098240		0.999027	0.999941	0.318933	0.907189	1.000000
3	P 24	0.264271	0.999027		0.999997	0.616302	0.993607	0.999825
4	U 24	0.186707	0.999941	0.999997		0.496806	0.977377	0.999997
5	P 72	0.996857	0.318933	0.616302	0.496806		0.944681	0.387546
6	U 72	0.668732	0.907189	0.993607	0.977377	0.944681		0.943830
7	Co	0.129099	1.000000	0.999825	0.999997	0.387546	0.943830	

Table 5.7. Summary of Tukey's *P* value of the scaled growth yield in nutrient broth after 240 generations

	Treatment	{1}	{2}	{3}	{4}	{5}	{6}	{7}
1	P 12		1.000000	0.728767	0.783073	0.537267	0.441519	0.000173
2	U 12	1.000000		0.689834	0.746986	0.496042	0.402723	0.000164
3	P 24	0.728767	0.689834		1.000000	0.999940	0.999370	0.007597
4	U 24	0.783073	0.746986	1.000000		0.999704	0.998041	0.005727
5	P 72	0.537267	0.496042	0.999940	0.999704		0.999999	0.018115
6	U 72	0.441519	0.402723	0.999370	0.998041	0.999999		0.027365
7	Co	0.000173	0.000164	0.007597	0.005727	0.018115	0.027365	

Table 5.8. Summary of Tukey's *P* value of the scaled growth yield in nutrient broth after 720 generations

	Treatment	{1}	{2}	{3}	{4}	{5}	{6}	{7}
1	P 12		0.315633	0.183670	0.154211	0.464283	0.406966	0.007852
2	U 12	0.315633		0.999947	0.999763	0.999977	0.999998	0.723226
3	P 24	0.183670	0.999947		1.000000	0.997948	0.999312	0.875335
4	U 24	0.154211	0.999763	1.000000		0.995406	0.998123	0.907843
5	P 72	0.464283	0.999977	0.997948	0.995406		1.000000	0.561007
6	U 72	0.406966	0.999998	0.999312	0.998123	1.000000		0.621685
7	NB	0.007852	0.723226	0.875335	0.907843	0.561007	0.621685	

5.4. Discussion

When fitness was estimated as yield, the populations subjected to the different kinds of fluctuations had either a significantly lower yield (after 240 generations; Fig. 5.2A and 5.2C) or comparable yield (after 720 generations; Fig. 5.2B and 5.2D) to the corresponding constant populations. However, when fitness was estimated as growth rate, the fluctuating treatments show tradeoffs, where they evolved fitness in only one of the environments at the expense of fitness in the other. After 240 generations of adaptation, all treatments, except P12, had significantly lower growth rate in cobalt and higher growth rate in NB than the corresponding constant populations (Fig. 5.1A and 5.1C). These results are similar to previous studies showing that the rate and extent of adaptation of populations exposed to fluctuating environment is lower than populations evolved in constant environments (Kassen 2014). However, it has also been shown that the reduction in fitness need not be uniform w.r.t all constant environments (Hughes *et al.* 2007; Jasmin & Kassen 2007; Cooper & Lenski 2010). This supports our result where populations evolve reduced growth rate in cobalt with higher growth rate in NB, even higher than constant NB populations. We observed contrasting results at the end of 720 generations. Here, the growth rate of the fluctuating populations was comparable to the constant cobalt populations in cobalt (Fig. 5.1B) but lower than constant NB populations in NB (Fig. 5.1D). Thus, although the fitness patterns after 240 and 720 generations were similar i.e., populations were able to evolve increased fitness in only one component environment. It is not clear at this point why the identity of this component environment changes between the time-points.

Additionally, contrary to the expectation that predictable and unpredictable fluctuations result in divergent evolutionary outcomes (Turner & Elena 2000; Hughes *et al.* 2007), we see little to no difference in fitness between the treatments. Furthermore, despite the intuitive

expectation that the frequency of environmental fluctuation could influence the evolutionary outcome, we see that it had little to no effect on fitness outcomes of our populations. The only difference is seen w.r.t short predictable fluctuations (P12). P12 was the only treatment with growth rate in cobalt equivalent to the constant populations, after 240 generations. Additionally, this was also significantly greater than all other treatment populations, except U72 (unpredictable fluctuations every 72 hours). Interestingly, the pattern was completely reversed after 720 generation of adaptation. Here, P12 was also the only treatment with significantly lower growth rate in cobalt than the constant populations and other fluctuating treatments, except P72 (fluctuation every 72 hours, but predictable fluctuations). P12 also showed a significant reduction in yield in NB compared to NB selected populations, although none of the fluctuating treatments showed a significant different w.r.t each other. Despite the observed differences, the frequency of fluctuations cannot be said to have had a strong effect on adaptation. This is because the effects were observed in only one treatment (P12) and only under limited conditions (fitness measured as growth rate in cobalt chloride).

Overall, our results are in agreement with other empirical studies on the effect of frequency of fluctuation (Kassen & Bell 1998; Scheiner & Yampolsky 1998; Buckling *et al.* 2007). Like these earlier studies, we detected no significant effect of the frequency of environmental fluctuations on fitness. In particular, Buckling *et al*. (Buckling *et al.* 2007), covered fluctuation ranging from every day to every 48 days, and still failed to detect any difference. Interestingly, short exposures to minute-scale fluctuations, that match the timescales of protein expressions, have been shown to initiate differential response in microorganisms (Lambert & Kussell 2014; Graham *et al.* 2017). Although our fluctuation were not of the same scale, we found that our rapidly fluctuating treatment i.e., 12 hour fluctuation, either had a higher or lower growth rate than all treatments at the end of 240 and 720 generations of adaptation, respectively. However, this is limited to the predictable fluctuation and also to

fitness measured as growth rate in cobalt environments. Nevertheless, to the best of our knowledge, this is the first empirical study to report some significant effect of frequency of fluctuation on an evolutionary outcomes. It is possible that greater resolution of fluctuations is needed to observe the effect of frequency of fluctuation on adaptation.

Chapter 6: Conclusions and future directions

Conclusion

In my thesis, I studied the effects of genetic variation and environmental fluctuations on microbial adaptation. In the first half, I focused on Ultraviolet radiation, an environmentally important mutagen, as a source of genetic variation. Laboratory populations of *Escherichia coli*, selected for increased resistance to UV, revealed multiple possible mechanisms of UV resistance. The mutated genes and pathways revealed notable overlaps with that UV resistance mechanisms in the naturally radio-resistant *Deinococcus sp*. Resistance in this genus has been attributed to multiple direct and indirect prevention, protection, and repair mechanisms such as effective removal of damaged cellular components and ROS, increased proteome protection, and cell cycle checkpoints to ensure complete repair before cell division (reviewed in (Makarova *et al.* 2001; Blasius *et al.* 2008; Jin *et al.* 2019). More interestingly, there were major differences between the accumulated mutations, influenced by the growth phase during UV exposures. For example, UV exposure during lag phase resulted in mutations in genes involved in cellular transport whereas, exponential phase exposures resulted in mutation in signal transduction and cell adhesion. Since there was no difference in the intensity and duration of UV exposure, the observed genomic differences can only be attributed to the physiological differences between lag and exponential phase cultures. However, the way by which UV induced selection and mutagenesis is shaped by the physiological differences between the growth phases is as yet unknown, and can be a fruitful topic of investigation.

Bacterial cultures in the different growth phases are known to differ in multiple physiological factors such as cell size, protein content or metabolite composition (Kolter *et al.* 1993; Rolfe *et al.* 2012; Jun *et al.* 2018). A key effector of such differences between growth phases is differential gene expression. During transcription, highly expressed genes may be at a greater

risk of UV induced damages resulting from lesions that block RNA polymerase combined with complex transcription-coupled repair (Tornaletti & Hanawalt 1999). Consequently, differences in the gene expression patterns between growth phases can constrain or potentiate biases in UV induced mutagenesis. Alternatively, differences in the proteins and metabolites subjected to UV induced damages, in the different growth phases, can result in differential selection pressures on cellular components and functions. UV radiation has been extensively studied as an agent of selection as well as a mutagen (Weigand & Sundin 2009; Wassmann *et al.* 2010; Goldman & Travisano 2011; Shibai *et al.* 2017; Tom *et al.* 2018). However, UV selection and mutagenesis in the context of bacterial physiology has remained relatively unexplored. I showed that these interactions are extensive and can have significant effects on the organisms' response to UV radiation. Further experiments can be designed to understand UV induced selection and mutagenesis and the nature of their interaction with cell physiology. For example, overexpression of proteins in the different growth phases can be used to study the effects of UV selection and the significance of these proteins in the specific growth phases. Alternatively, single exposures to UV radiation in the different growth phase, combined with deep sequencing, can help understand constraints and biases of UV induced mutagenesis. Such experiments can further our knowledge regarding genes and pathways that are fundamental to or highly expressed in the different growth phases. Extending the study of growth phase specific effects on mutagenesis to other physical and chemical mutagens can have interesting implications for applied microbiology. Random mutagenesis using UV radiation and other chemical mutagens is routine in strain improvement studies (Meireles *et al.* 2003; Lotfy *et al.* 2007; Fang *et al.* 2009; Joshi *et al.* 2010; Zhu *et al.* 2018). However, by understanding the physiology-induced biases in mutagenesis, experiments can be designed to subject cultures to the mutagens during specific growth phase. Such experiments may have a greater chance of obtaining the targeted mutations specific for the desired traits.

An important observation in the UV resistant populations is that they had evolved collateral resistance to different classes of antibiotics. In chapter 3, I further explored the effects of UV radiation on the evolution of antibiotic resistance. UV radiation had no effect on the resistance phenotype i.e., populations evolved similar levels of resistance irrespective of whether they were exposure to UV or not. However, UV radiation had strongly influenced the genomics of resistance. Resistance in the un-exposed control populations was accompanied by mutation that altered antibiotic target site and efflux pump protein (*rplD* and *acrB*). In contrast, resistance in UV exposed populations was associated with mutations in efflux pump protein as well as its transcriptional regulator (*acrB* and *acrR*). It is expected that mutations altering drug targets site confer high resistance at the expense of slower growth rate (Andersson & Hughes 2010; Hughes & Andersson 2017). On the other hand, mutations in regulators of efflux pumps have been shown to result in lower fitness however, without any costs in antibiotic-free medium (Santos-Lopez *et al.* 2019). Although the UV exposed populations were fixed for regulator mutations instead of target altering mutations, they had evolved equal resistance to erythromycin as control populations. Furthermore, populations subjected to fluctuating exposures of UV and erythromycin had evolved the highest growth rate in the presence and absence of erythromycin. It is possible that the increased mutational supply and/or mutational biases of UV radiation favoured the selection of cost-free and high-fitness mutations. This can have serious implications for public health since UV radiation is a popular disinfection technology to control microbial growth on surfaces, air, and water (Das 2001; Lim & Blatchley III 2009; Kowalski 2010; Bolton & Cotton 2011). In addition, UV radiation is a popular treatment for dermatological infections and diseases such as psoriasis, eczema, mycosis fungoides and sézary syndrome (Dai *et al.* 2008; Olsen *et al.* 2016; Wiznia *et al.* 2017). More recently, endotracheal application of UV radiation has shown promising results in reducing viral load and severity of COVID-19 (Rezaie *et al.* 2021). UV radiation

has also been suggested as alternate / adjunctive treatment for controlling antibiotic resistant infections (Thai *et al.* 2002; Thai *et al.* 2005) despite UV radiation having been shown to have conflicting effects on antibiotic resistance evolution. Some studies show that UV resistance is accompanied with reduced susceptibility to antibiotics (Zhang *et al.* 2017; Li *et al.* 2021) while others show that antibiotic resistant and susceptible populations are equally susceptible to UV radiation (Meckes 1982; Conner-Kerr *et al.* 1998). My results showed that exposure to UV can result in correlated antibiotic resistance and more importantly, the evolution of cost-free resistance. Without a systematic understand of the effect of UV on antibiotic resistance evolution, its extent and mechanisms, overuse of UV radiation may contribute to the already overwhelming problem of antibiotic resistance evolution.

Another key result from chapter 3 is that fluctuations in exposure to UV and erythromycin resulted in increased mean and variation in fitness in novel antibiotic environments. Although mutation rates of these populations were not directly measured, alternating exposures to UV is expected to produce an inherent fluctuation in mutation rate. While mutation rates and fluctuating environment have been extensively studied in the context of antibiotic resistance evolution (Oliver 2005; Woodford & Ellington 2007; Roemhild *et al.* 2015; Lin & Kussell 2016; Windels *et al.* 2019), the effect of fluctuating mutation rate on adaptation can be an interesting avenue for future studies. Fluctuations in antibiotic exposures can constrain and slowdown the evolution of resistance (Kim *et al.* 2014; Roemhild *et al.* 2015). On the other hand, elevated mutation rates may promote resistance evolution (Eliopoulos & Blázquez 2003; Blázquez & Gómez-Gómez 2007; Jolivet-Gougeon *et al.* 2011; Mehta *et al.* 2019) but the cost of increase in deleterious mutations results in the evolution of reduced mutation rates (Giraud *et al.* 2001; de Visser 2002; Turrientes *et al.* 2013). The dynamics of mutator strains in fluctuating environments has been studied using theoretical models (Travis & Travis 2002) and *in silico* replicator populations (Stich *et al.* 2010). However, simultaneous exposures to

elevated mutation rates and fluctuating environments can have serious implications for antibiotic resistance evolution in nature: a possibility that has not yet been fully investigated. While my results are limited to *E. coli* populations, future studies need to be expanded to other pathogenic organisms, to include organisms such as *Pseudomonas sp.* which have higher prevalence of hypermutable strains. Given that combination and fluctuating therapy is routinely suggested as a way to control/slow down antibiotic resistance evolution (Mouton 1999; Baym *et al.* 2016; Davis *et al.* 2021), future studies can be extended to understand the effects of interaction between fluctuating mutation rates and environments in complex antibiotic environment.

In chapter 4, I studied the effects of migration when populations are faced with complex and unpredictably fluctuating environments. Under these conditions, I showed that clonal migration has a negative effect on adaptation. My results are in contrast with Perron *et al.*, (Perron *et al.* 2007), who showed that clonal migration can promote adaptation in predictably fluctuating environment. Adaptation in lethal environments, of the kind that was used in Perron *et al.*, (Perron *et al.* 2007), can be dependent on recurrent migration to maintain stable population sizes. In contrast, my selection environment was sub-lethal, that could support growth and adaptation even in the absence of migration. The differences in these results point towards the importance of the nature of the environment and selection pressure, in determining the effect of migration. Organisms at the edge of an expanding range may be subjected to both complex selection pressures as well as migration from the core population (Bridle & Vines 2007). Future studies that directly test and compare migration under different strengths of selection pressure can help make better predictions about the effects of migration under varying environmental conditions, as experienced in range margins. I also showed that the presence of variation in the migrating individuals can alleviate some of the negative effects of migration. However, at high migration rate, variation resulted in reduced fitness.

This observation warrants further investigations into whether continuous increase in genetic variation leads to proportional increase in fitness or if there is a limit to the benefit of genetic variation in migrating individuals. Additionally, it is also known that the effect of genetic variation on adaptation can be influenced by other factors such as the strength of selection pressure and fitness of the organisms. Therefore, additional experiments designed to study the interaction between genetic and environmental variation and its effect on migration may help understand the contrasting effects of migration on adaptation.

My experiments to study the effect of frequency and predictability of fluctuations on adaptation, in chapter 5 showed that the extent of adaptation was not influenced by either of the two factors. The only exception was the predictable, 12 hour fluctuation treatment. Although this treatment significantly differed from the other treatments, the effect was not consistent in all selection environment. My results are congruent with prior studies that have shown that adaptation is not influenced by the frequency of fluctuation (Kassen & Bell 1998; Scheiner & Yampolsky 1998; Buckling *et al.* 2007). However, the lack of empirical evidence to support the literature surrounding the effects of frequency and predictability of temporal fluctuations leaves us with outstanding questions. Do we need more resolution in the frequency of fluctuations to observe any effects on adaptation? Will the predicted effects of frequency of fluctuations be observable at all evolutionary timescales? i.e., short-term evolution vs. long-term evolution. Experiments designed to answer these questions can help understand the discrepancies between theory and experimental studies.

Future directions

One of the key observation from my thesis was that strong and continuous selection pressures resulted in uniform fitness increase (chapter 2 and 3). At first glance, such results may have suggested that the different treatments i.e., physiological differences between growth phases or constant vs. fluctuation exposures to UV, had no effect on the evolutionary dynamics. However, whole genome sequencing (WGS) of the evolved populations revealed crucial and interesting differences in genomic evolution. Given the complex and degenerate interactions between genotype and phenotype, WGS in combination with experimental evolution can provide a much richer understanding of the evolutionary processes.

Recent advancements have facilitated the extension of sequencing technologies to include analysis of gene expression, epigenetic factors, and microbiome as well as in-situ sequencing of fixed cells and tissues (Gupta & Verma 2019). While epigenetic modifications do not alter the genetic material, they can have profound effect on gene expression, contributing to phenotypic plasticity and phenotypic variance. Such epigenetic variation can be adaptive as it can increase rates of phenotypic change in the face of environmental change (Bonduriansky *et al.* 2012; Burggren 2016). This allows the organisms to detect and survive environmental change by altering gene expression to match the environmental condition (Angers *et al.* 2010; Bollati & Baccarelli 2010). While I have not looked at epigenetic changes in my thesis, I observed that mutations in global regulator genes were unique to populations subjected to fluctuating environment (chapter 2). This hints at the importance of variation in gene expression for survival in fluctuating environments. Future studies that combine sequencing technologies with the ability to detect genetic, epigenetic, and expression level changes can be crucial to furthering our understand evolution in fluctuating environment.

Next generation sequencing technologies, although a powerful tool, is largely used to identify beneficial variations that rose to high frequencies as a result of selection. However, recent studies have shown that by introducing unique sequences or neutral DNA barcodes in clonal populations, it is possible to track the distribution of variants even when they are rare and independent of the effects of selection (Levy *et al.* 2015; Jasinska *et al.* 2020). Lineage tracking via random barcodes coupled with WGS can be an exciting tool to study the generation, maintenance and distribution of variation in fluctuating environments. It can also be used to study the effects of migration since, migration is an important factor that influences variation. Furthermore, factors such as selection and drift strongly influence the probability of invasion of alleles introduced by migration (Blanquart *et al.* 2012).

One common shortcoming of existing experimental evolution studies is the generalizability of the results since, in most cases, the evolutionary outcomes are highly restricted to the choice of environments. This points to an interesting issue regarding the robustness of evolutionary predictions across different environmental conditions. For example, it has been shown that the most important conditions that determine whether populations of *E.coli* evolved to become specialist or generalist, in a fluctuating environment, is the choice of environmental pair (Sandberg *et al.* 2017). Trait correlations such as those between glucose/glycerol and glucose/xylose fluctuation supported the evolution of generalists. Whereas, glucose/acetate fluctuation, that lack such correlations resulted in the evolution of specialist (Sandberg *et al.* 2017). The effect of migration that I report in my thesis, are in support with some of the prior studies while also contrasting with other studies (chapter 4). This also holds true for the effect of predictability and frequency of fluctuations. Given the contrasting and environmental specific effects on adaptive outcomes, future studies that make use of high-throughput technologies to study the evolutionary effect of the different factors in multiple environments may led to a unified robust understanding of evolution. Recent high-

throughput experiments (Dragosits *et al.* 2013; Horinouchi *et al.* 2017; Maeda *et al.* 2020) with multiple environments have been successful in addressing questions such as the evolution of trade-offs and cross resistance across multiple stress/antibiotic environments. Such high-throughput experiments can be expanded to better predict the effects of mutagenesis, migration and fluctuating environment.

References

Abdi, H. (2010). Holm's sequential Bonferroni procedure. *Encyclopedia of research design*, 1, 1-8.

Acar, M., Mettetal, J.T. & Van Oudenaarden, A. (2008). Stochastic switching as a survival strategy in fluctuating environments. *Nature genetics*, 40, 471-475.

Ackermann, M. (2015). A functional perspective on phenotypic heterogeneity in microorganisms. *Nature Reviews Microbiology*, 13, 497-508.

Aertsen, A. & Michiels, C.W. (2004). Stress and how bacteria cope with death and survival. *Critical reviews in microbiology*, 30, 263-273.

Albright, M.B. & Martiny, J.B. (2018). Dispersal alters bacterial diversity and composition in a natural community. *The ISME journal*, 12, 296-299.

Alcantara-Diaz, D., Brena-Valle, M. & Serment-Guerrero, J. (2004). Divergent adaptation of Escherichia coli to cyclic ultraviolet light exposures. *Mutagenesis*, 19, 349-354.

Alper, T. (1977). The role of membrane damage in radiation-induced cell death. In: *Membrane toxicity*. Springer New York City, pp. 139-165.

Alto, B.W., Wasik, B.R., Morales, N.M. & Turner, P.E. (2013). Stochastic temperatures impede RNA virus adaptation. *Evolution*, 67, 969-979.

Andersson, D.I. & Hughes, D. (2010). Antibiotic resistance and its cost: is it possible to reverse resistance? *Nature Reviews Microbiology*, 8, 260-271.

Andersson, D.I., Nicoloff, H. & Hjort, K. (2019). Mechanisms and clinical relevance of bacterial heteroresistance. *Nature Reviews Microbiology*, 17, 479-496.

Angers, B., Castonguay, E. & Massicotte, R. (2010). Environmentally induced phenotypes and DNA methylation: how to deal with unpredictable conditions until the next generation and after. *Molecular Ecology*, 19, 1283-1295.

Ansari, I. & Datta, A. (2003). An overview of sterilization methods for packaging materials used in aseptic packaging systems. *Food and Bioproducts Processing*, 81, 57-65.

Arevalo, M.A., Tejedor, F., Polo, F. & Ballesta, J. (1988). Protein components of the erythromycin binding site in bacterial ribosomes. *Journal of Biological Chemistry*, 263, 58-63.

Arroyo, C., Gayán, E., Pagán, R. & Condón, S. (2012). UV-C inactivation of Cronobacter sakazakii. *Foodborne pathogens and disease*, 9, 907-914.

Baharoglu, Z., Lestini, R., Duigou, S. & Michel, B. (2010). RNA polymerase mutations that facilitate replication progression in the rep uvrD recF mutant lacking two accessory replicative helicases. *Molecular microbiology*, 77, 324-336.

Bailey, S.F., Rodrigue, N. & Kassen, R. (2015). The effect of selection environment on the probability of parallel evolution. *Molecular biology and evolution*, 32, 1436-1448.

Band, V.I. & Weiss, D.S. (2019). Heteroresistance: a cause of unexplained antibiotic treatment failure? *PLoS pathogens*, 15, e1007726.

Barrett, T.C., Mok, W.W., Murawski, A.M. & Brynildsen, M.P. (2019). Enhanced antibiotic resistance development from fluoroquinolone persisters after a single exposure to antibiotic. *Nature communications*, 10, 1-11.

Barton, N. (2001). Adaptation at the edge of a species' range. In: *Integrating ecology and evolution in a spatial context* Cambridge, UK.

Battista, J.R. (1997). Against all odds: the survival strategies of Deinococcus radiodurans. *Annual review of Microbiology*, 51, 203-224.

Baym, M., Stone, L.K. & Kishony, R. (2016). Multidrug evolutionary strategies to reverse antibiotic resistance. *Science*, 351.

Bearden, D.T. & Danziger, L.H. (2001). Mechanism of action of and resistance to quinolones. *Pharmacotherapy: The Journal of Human Pharmacology and Drug Therapy*, 21, 224S-232S.

Beaumont, H.J., Gallie, J., Kost, C., Ferguson, G.C. & Rainey, P.B. (2009). Experimental evolution of bet hedging. *Nature*, 462, 90-93.

Bell, G. & Gonzalez, A. (2011). Adaptation and evolutionary rescue in metapopulations experiencing environmental deterioration. *Science*, 332, 1327-1330.

Berney, M., Weilenmann, H.-U., Ihssen, J., Bassin, C. & Egli, T. (2006). Specific growth rate determines the sensitivity of Escherichia coli to thermal, UVA, and solar disinfection. *Applied and environmental microbiology*, 72, 2586-2593.

Bertrand, R.L. (2019). Lag phase is a dynamic, organized, adaptive, and evolvable period that prepares bacteria for cell division. *Journal of bacteriology*, 201, e00697-00618.

Billerbeck, M., Werner, U., Polerecky, L., Walpersdorf, E., DeBeer, D. & Huettel, M. (2006). Surficial and deep pore water circulation governs spatial and temporal scales of nutrient recycling in intertidal sand flat sediment. *Marine Ecology Progress Series*, 326, 61-76.

Blanquart, F., Gandon, S. & Nuismer, S. (2012). The effects of migration and drift on local adaptation to a heterogeneous environment. *Journal of evolutionary biology*, 25, 1351-1363.

Blasius, M., Sommer, S. & Hubscher, U. (2008). Deinococcus radiodurans: what belongs to the survival kit? *Critical reviews in biochemistry and molecular biology*, 43, 221-238.

Blázquez, J. & Gómez-Gómez, J.M. (2007). Evolution of antibiotic resistance by hypermutation. *Evolutionary Biology of Bacterial and Fungal Pathogens*, 319-331.

Bódi, Z., Farkas, Z., Nevozhay, D., Kalapis, D., Lázár, V., Csörgő, B. *et al.* (2017). Phenotypic heterogeneity promotes adaptive evolution. *PLoS biology*, 15, e2000644.

Bolger, A.M., Lohse, M. & Usadel, B. (2014). Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics*, 30, 2114-2120.

Bollati, V. & Baccarelli, A. (2010). Environmental epigenetics. *Heredity*, 105, 105-112.

Bolton, J.R. & Cotton, C.A. (2011). *The ultraviolet disinfection handbook*. American Water Works Association, Denver, CO.

Bonduriansky, R., Crean, A.J. & Day, T. (2012). The implications of nongenetic inheritance for evolution in changing environments. *Evolutionary applications*, 5, 192-201.

Boots, M. & Sasaki, A. (1999). 'Small worlds' and the evolution of virulence: infection occurs locally and at a distance. *Proceedings of the Royal Society of London. Series B: Biological Sciences*, 266, 1933-1938.

Brash, D.E. (2015). UV signature mutations. *Photochemistry and photobiology*, 91, 15-26.

Bridle, J.R. & Vines, T.H. (2007). Limits to evolution at range margins: when and why does adaptation fail? *Trends in ecology & evolution*, 22, 140-147.

Bruckbauer, S.T., Trimarco, J.D., Martin, J., Bushnell, B., Senn, K.A., Schackwitz, W. *et al.* (2019). Experimental evolution of extreme resistance to ionizing radiation in Escherichia coli after 50 cycles of selection. *Journal of bacteriology*, 201, e00784-00718.

Bucheli-Witschel, M., Bassin, C. & Egli, T. (2010). UV-C inactivation in Escherichia coli is affected by growth conditions preceding irradiation, in particular by the specific growth rate. *Journal of applied microbiology*, 109, 1733-1744.

Buckling, A., Brockhurst, M.A., Travisano, M. & Rainey, P.B. (2007). Experimental adaptation to high and low quality environments under different scales of temporal variation. *Journal of evolutionary biology*, 20, 296-300.

Bürger, R. & Gimelfarb, A. (2002). Fluctuating environments and the role of mutation in maintaining quantitative genetic variation. *Genetics Research*, 80, 31-46.

Burggren, W. (2016). Epigenetic inheritance and its role in evolutionary biology: re-evaluation and new perspectives. *Biology*, 5, 24.

Byers, D.L. (2005). Evolution in heterogeneous environments and the potential of maintenance of genetic variation in traits of adaptive significance. *Genetica*, 123, 107-124.

Cadet, J., Sage, E. & Douki, T. (2005). Ultraviolet radiation-mediated damage to cellular DNA. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis*, 571, 3-17.

Canino-Koning, R., Wiser, M.J. & Ofria, C. (2019). Fluctuating environments select for short-term phenotypic variation leading to long-term exploration. *PLoS computational biology*, 15, e1006445.

Cano, A., Rozhonova, H., Stoltzfus, A., McCandlish, D. & Payne, J. (2021). The mutation spectrum shapes the spectrum of adaptive substitutions. *bioRxiv*

Cano, A.V. & Payne, J.L. (2020). Mutation bias interacts with composition bias to influence adaptive evolution. *PLoS computational biology*, 16, e1008296.

Carja, O., Liberman, U. & Feldman, M.W. (2014). Evolution in changing environments: Modifiers of mutation, recombination, and migration. *Proceedings of the National Academy of Sciences*, 111, 17935-17940.

Carja, O. & Plotkin, J.B. (2017). The evolutionary advantage of heritable phenotypic heterogeneity. *Scientific reports*, 7, 1-12.

Carson, M.J., Barondess, J. & Beckwith, J. (1991). The FtsQ protein of Escherichia coli: membrane topology, abundance, and cell division phenotypes due to overproduction and insertion mutations. *Journal of Bacteriology*, 173, 2187-2195.

Charpentier, X., Kay, E., Schneider, D. & Shuman, H.A. (2011). Antibiotics and UV radiation induce competence for natural transformation in Legionella pneumophila. *Journal of bacteriology*, 193, 1114-1121.

Chavhan, Y.D., Ali, S.I. & Dey, S. (2019). Larger numbers can impede adaptation in asexual populations despite entailing greater genetic variation. *Evolutionary Biology*, 46, 1-13.

Chen, P. & Zhang, J. (2020). Antagonistic pleiotropy conceals molecular adaptations in changing environments. *Nature ecology & evolution*, 4, 461-469.

Child, M., Strike, P., Pickup, R. & Edwards, C. (2002). Salmonella typhimurium displays cyclical patterns of sensitivity to UV-C killing during prolonged incubation in the stationary phase of growth. *FEMS microbiology letters*, 213, 81-85.

Ching, J., Musheyev, S.A., Chowdhury, D., Kim, J.A., Choi, Y. & Dennehy, J.J. (2013). Migration enhances adaptation in bacteriophage populations evolving in ecological sinks. *Evolution*, 67, 10-17.

Chollet, R., Chevalier, J., Bryskier, A. & Pagès, J.-M. (2004). The AcrAB-TolC pump is involved in macrolide resistance but not in telithromycin efflux in Enterobacter aerogenes and Escherichia coli. *Antimicrobial agents and chemotherapy*, 48, 3621-3624.

Clark, C., Bozdogan, B.l., Peric, M., Dewasse, B., Jacobs, M.R. & Appelbaum, P.C. (2002). In vitro selection of resistance in Haemophilus influenzae by amoxicillin-clavulanate, cefpodoxime, cefprozil, azithromycin, and clarithromycin. *Antimicrobial agents and chemotherapy*, 46, 2956-2962.

Claverys, J.-P., Prudhomme, M. & Martin, B. (2006). Induction of competence regulons as a general response to stress in gram-positive bacteria. *Annual Review of Microbiology*, 60, 451-475.

Cohen, J. (2013). *Statistical power analysis for the behavioral sciences*. Academic press, New York.

Condon, C., Cooper, B.S., Yeaman, S. & Angilletta Jr, M.J. (2014). Temporal variation favors the evolution of generalists in experimental populations of Drosophila melanogaster. *Evolution*, 68, 720-728.

Conner-Kerr, T., Sullivan, P., Gaillard, J., Franklin, M. & Jones, R. (1998). The effects of ultraviolet radiation on antibiotic-resistant bacteria in vitro. *Ostomy/wound management*, 44, 50-56.

Cooper, T.F. & Lenski, R.E. (2010). Experimental evolution with E. coli in diverse resource environments. I. Fluctuating environments promote divergence of replicate populations. *BMC evolutionary biology*, 10, 1-10.

Cooper, V.S. (2018). Experimental evolution as a high-throughput screen for genetic adaptations. *MSphere*, 3, e00121-00118.

Couce, A., Rodríguez-Rojas, A. & Blázquez, J. (2015). Bypass of genetic constraints during mutator evolution to antibiotic resistance. *Proceedings of the Royal Society B: Biological Sciences*, 282, 20142698.

Crill, W.D., Wichman, H.A. & Bull, J. (2000). Evolutionary reversals during viral adaptation to alternating hosts. *Genetics*, 154, 27-37.

Cuevas, J., Moya, A. & Elena, S. (2003). Evolution of RNA virus in spatially structured heterogeneous environments. *Journal of evolutionary biology*, 16, 456-466.

Cunningham, C., Jeng, K., Husti, J., Badgett, M., Molineux, I., Hillis, D. *et al.* (1997). Parallel molecular evolution of deletions and nonsense mutations in bacteriophage T7. *Molecular Biology and Evolution*, 14, 113-116.

Dai, T., Tegos, G., Rolz-Cruz, G., Cumbie, W. & Hamblin, M. (2008). Ultraviolet C inactivation of dermatophytes: implications for treatment of onychomycosis. *British Journal of Dermatology*, 158, 1239-1246.

Dantur, K.I. & Pizarro, R.A. (2004). Effect of growth phase on the Escherichia coli response to ultraviolet-A radiation: influence of conditioned media, hydrogen peroxide and acetate. *Journal of Photochemistry and Photobiology B: Biology*, 75, 33-39.

Das, T.K. (2001). Ultraviolet disinfection application to a wastewater treatment plant. *Clean Products and Processes*, 3, 69-80.

Davies, R. & Sinskey, A.J. (1973). Radiation-resistant mutants of Salmonella typhimurium LT2: development and characterization. *Journal of bacteriology*, 113, 133-144.

Davis, K., Greenstein, T., Colindres, R.V. & Aldridge, B.B. (2021). Leveraging laboratory and clinical studies to design effective antibiotic combination therapy. *Current Opinion in Microbiology*, 64, 68-75.

de Visser, J.A.G. (2002). The fate of microbial mutators. *Microbiology*, 148, 1247-1252.

Deatherage, D.E. & Barrick, J.E. (2014). Identification of mutations in laboratory-evolved microbes from next-generation sequencing data using breseq. In: *Engineering and analyzing multicellular systems*. Springer, pp. 165-188.

Delcour, A.H. (2009). Outer membrane permeability and antibiotic resistance. *Biochimica et Biophysica Acta (BBA)-Proteins and Proteomics*, 1794, 808-816.

Dennehy, J.J., Friedenberg, N.A., McBride, R.C., Holt, R.D. & Turner, P.E. (2010). Experimental evidence that source genetic variation drives pathogen emergence. *Proceedings of the Royal Society B: Biological Sciences*, 277, 3113-3121.

Descours, G., Ginevra, C., Jacotin, N., Forey, F., Chastang, J., Kay, E. *et al.* (2017). Ribosomal mutations conferring macrolide resistance in Legionella pneumophila. *Antimicrobial agents and chemotherapy*, 61, e02188-02116.

Dettman, J.R., Rodrigue, N., Melnyk, A.H., Wong, A., Bailey, S.F. & Kassen, R. (2012). Evolutionary insight from whole-genome sequencing of experimentally evolved microbes. *Molecular ecology*, 21, 2058-2077.

DeVeaux, L.C., Müller, J.A., Smith, J., Petrisko, J., Wells, D.P. & DasSarma, S. (2007). Extremely radiation-resistant mutants of a halophilic archaeon with increased single-stranded DNA-binding protein (RPA) gene expression. *Radiation research*, 168, 507-514.

Devilly, G. (2004). The effect size generator for windows: Version 2.3 (computer programme). *Centre for Neuropsychology, Swinburne University, Australia*.

Dias, P.C. (1996). Sources and sinks in population biology. *Trends in Ecology & Evolution*, 11, 326-330.

Dragosits, M., Mozhayskiy, V., Quinones-Soto, S., Park, J. & Tagkopoulos, I. (2013). Evolutionary potential, cross-stress behavior and the genetic basis of acquired stress resistance in Escherichia coli. *Molecular systems biology*, 9, 643.

Dupont, C.L., Grass, G. & Rensing, C. (2011). Copper toxicity and the origin of bacterial resistance—new insights and applications. *Metallomics*, 3, 1109-1118.

Dykhuizen, D.E. & Dean, A.M. (2004). Evolution of specialists in an experimental microcosm. *Genetics*, 167, 2015-2026.

Ebbensgaard, A.E., Løbner-Olesen, A. & Frimodt-Møller, J. (2020). The Role of Efflux Pumps in the Transition from Low-Level to Clinical Antibiotic Resistance. *Antibiotics*, 9, 855.

El-Halfawy, O.M. & Valvano, M.A. (2015). Antimicrobial heteroresistance: an emerging field in need of clarity. *Clinical microbiology reviews*, 28, 191-207.

Eliopoulos, G.M. & Blázquez, J. (2003). Hypermutation as a factor contributing to the acquisition of antimicrobial resistance. *Clinical Infectious Diseases*, 37, 1201-1209.

Ettayebi, M., Prasad, S.M. & Morgan, E.A. (1985). Chloramphenicol-erythromycin resistance mutations in a 23S rRNA gene of Escherichia coli. *Journal of Bacteriology*, 162, 551-557.

Ewing, D. (1995). The directed evolution of radiation resistance in E. coli. *Biochemical and biophysical research communications*, 216, 549-553.

Eyre-Walker, A. & Keightley, P.D. (2007). The distribution of fitness effects of new mutations. *Nature Reviews Genetics*, 8, 610-618.

Fabre, F. (1970). UV-sensitivity of the wild-type and different UVS mutants of schizosaccharomyces pombe Influence of growth stages and DNA content of the cells. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis*, 10, 415-426.

Fang, X., Yano, S., Inoue, H. & Sawayama, S. (2009). Strain improvement of Acremonium cellulolyticus for cellulase production by mutation. *Journal of Bioscience and Bioengineering*, 107, 256-261.

Fisher, R.A. (1930). *The genetical theory of natural selection*. Oxford University Press, Oxford.

Foster, P.L., Lee, H., Popodi, E., Townes, J.P. & Tang, H. (2015). Determinants of spontaneous mutation in the bacterium Escherichia coli as revealed by whole-genome sequencing. *Proceedings of the National Academy of Sciences*, 112, E5990-E5999.

Fowler, R. & Schaaper, R. (1997). The role of the mutT gene of Escherichia coli in maintaining replication fidelity. *FEMS microbiology reviews*, 21, 43-54.

Freddolino, P.L., Yang, J., Momen-Roknabadi, A. & Tavazoie, S. (2018). Stochastic tuning of gene expression enables cellular adaptation in the absence of pre-existing regulatory circuitry. *Elife*, 7, e31867.

Friesen, M.L., Saxer, G., Travisano, M. & Doebeli, M. (2004). Experimental evidence for sympatric ecological diversification due to frequency-dependent competition in Escherichia coli. *Evolution*, 58, 245-260.

Frimodt-Møller, N., Sebbesen, O. & Thomsen, V.F. (1983). The pneumococcus and the mouse protection test: importance of the lag phase in vivo. *Chemotherapy*, 29, 128-134.

Galhardo, R.S., Hastings, P.J. & Rosenberg, S.M. (2007). Mutation as a stress response and the regulation of evolvability. *Critical reviews in biochemistry and molecular biology*, 42, 399-435.

Galperin, M.Y., Moroz, O.V., Wilson, K.S. & Murzin, A.G. (2006). House cleaning, a part of good housekeeping. *Molecular microbiology*, 59, 5-19.

Garant, D., Forde, S.E. & Hendry, A.P. (2007). The multifarious effects of dispersal and gene flow on contemporary adaptation. *Functional Ecology*, 21, 434-443.

Garcia-Bernardo, J. & Dunlop, M.J. (2015). Noise and low-level dynamics can coordinate multicomponent bet hedging mechanisms. *Biophysical journal*, 108, 184-193.

Garibyan, L., Huang, T., Kim, M., Wolff, E., Nguyen, A., Nguyen, T. *et al.* (2003). Use of the rpoB gene to determine the specificity of base substitution mutations on the Escherichia coli chromosome. *DNA repair*, 2, 593-608.

Gayán, E., Condón, S. & Álvarez, I. (2014). Biological aspects in food preservation by ultraviolet light: a review. *Food and Bioprocess Technology*, 7, 1-20.

Gefrides, L.A., Powell, M.C., Donley, M.A. & Kahn, R. (2010). UV irradiation and autoclave treatment for elimination of contaminating DNA from laboratory consumables. *Forensic science international: Genetics*, 4, 89-94.

Gerken, H. & Misra, R. (2004). Genetic evidence for functional interactions between TolC and AcrA proteins of a major antibiotic efflux pump of Escherichia coli. *Molecular microbiology*, 54, 620-631.

Ghai, I. & Ghai, S. (2018). Understanding antibiotic resistance via outer membrane permeability. *Infection and drug resistance*, 11, 523.

Gifford, D.R., Berríos-Caro, E., Joerres, C., Galla, T. & Knight, C.G. (2019). Mutators drive evolution of multi-resistance to antibiotics. *bioRxiv*, 643585.

Gilbert, P., Collier, P.J. & Brown, M. (1990). Influence of growth rate on susceptibility to antimicrobial agents: biofilms, cell cycle, dormancy, and stringent response. *Antimicrobial agents and chemotherapy*, 34, 1865-1868.

Gilchrist, G.W. (1995). Specialists and generalists in changing environments. I. Fitness landscapes of thermal sensitivity. *The American Naturalist*, 146, 252-270.

Giraud, A., Matic, I., Tenaillon, O., Clara, A., Radman, M., Fons, M. *et al.* (2001). Costs and benefits of high mutation rates: adaptive evolution of bacteria in the mouse gut. *science*, 291, 2606-2608.

Goldman, R.P. & Travisano, M. (2011). Experimental evolution of ultraviolet radiation resistance in Escherichia coli. *Evolution*, 65, 3486-3498.

Goldstein, B.P. (2014). Resistance to rifampicin: a review. *The Journal of antibiotics*, 67, 625-630.

Gomes, C., Martinez-Puchol, S., Palma, N., Horna, G., Ruiz-Roldan, L., Pons, M.J. *et al.* (2017). Macrolide resistance mechanisms in Enterobacteriaceae: Focus on azithromycin. *Critical reviews in microbiology*, 43, 1-30.

Gomes, M.C., Silva, S., Faustino, M.A., Neves, M.G., Almeida, A., Cavaleiro, J.A. *et al.* (2013). Cationic galactoporphyrin photosensitisers against UV-B resistant bacteria: oxidation of lipids and proteins by 1 O 2. *Photochemical & Photobiological Sciences*, 12, 262-271.

Gomez, K., Bertram, J. & Masel, J. (2020). Mutation bias can shape adaptation in large asexual populations experiencing clonal interference. *Proceedings of the Royal Society B*, 287, 20201503.

Goosen, N. & Moolenaar, G.F. (2008). Repair of UV damage in bacteria. *DNA repair*, 7, 353-379.

Graham, P.J., Nguyen, B., Burdyny, T. & Sinton, D. (2017). A penalty on photosynthetic growth in fluctuating light. *Scientific reports*, 7, 1-11.

Gregory, S.T. & Dahlberg, A.E. (1999). Erythromycin resistance mutations in ribosomal proteins L22 and L4 perturb the higher order structure of 23 S ribosomal RNA. *Journal of molecular biology*, 289, 827-834.

Gresham, D., Desai, M.M., Tucker, C.M., Jenq, H.T., Pai, D.A., Ward, A. *et al.* (2008). The repertoire and dynamics of evolutionary adaptations to controlled nutrient-limited environments in yeast. *PLoS genetics*, 4, e1000303.

Griffiths, J.F., Griffiths, A.J., Wessler, S.R., Lewontin, R.C., Gelbart, W.M., Suzuki, D.T. *et al.* (2005). *An introduction to genetic analysis*. Macmillan, New York.

Guerrero-Beltr· n, J. & Barbosa-C· novas, G. (2004). Advantages and limitations on processing foods by UV light. *Food science and technology international*, 10, 137-147.

Gupta, N. & Verma, V.K. (2019). Next-Generation Sequencing and Its Application: Empowering in Public Health Beyond Reality. In: *Microbial Technology for the Welfare of Society*. Springer, pp. 313-341.

Haaland, T.R., Wright, J. & Ratikainen, I.I. (2020). Generalists versus specialists in fluctuating environments: a bet-hedging perspective. *Oikos*, 129, 879-890.

Hallsson, L.R. & Björklund, M. (2012). Selection in a fluctuating environment leads to decreased genetic variation and facilitates the evolution of phenotypic plasticity. *Journal of evolutionary biology*, 25, 1275-1290.

Hao, Y., Qu, Y., Song, G. & Lei, F. (2019). Genomic insights into the adaptive convergent evolution. *Current genomics*, 20, 81-89.

Hashimoto, S., Ogura, M., Aritomi, K., Hoshida, H., Nishizawa, Y. & Akada, R. (2005). Isolation of auxotrophic mutants of diploid industrial yeast strains after UV mutagenesis. *Applied and environmental microbiology*, 71, 312-319.

Hastings, J., Holzapfel, W. & Niemand, J. (1986). Radiation resistance of lactobacilli isolated from radurized meat relative to growth and environment. *Applied and environmental microbiology*, 52, 898-901.

Hermsen, R. & Hwa, T. (2010). Sources and sinks: a stochastic model of evolution in heterogeneous environments. *Physical review letters*, 105, 248104.

Holland, S.L., Reader, T., Dyer, P.S. & Avery, S.V. (2014). Phenotypic heterogeneity is a selected trait in natural yeast populations subject to environmental stress. *Environmental microbiology*, 16, 1729-1740.

Holt, R.D. (1997). On the evolutionary stability of sink populations. *Evolutionary Ecology*, 11, 723-731.

Holt, R.D. (2003). On the evolutionary ecology of species' ranges. *Evolutionary ecology research*, 5, 159-178.

Holt, R.D. & Gomulkiewicz, R. (1997). How does immigration influence local adaptation? A reexamination of a familiar paradigm. *The American Naturalist*, 149, 563-572.

Hopkins, K.L., Davies, R.H. & Threlfall, E.J. (2005). Mechanisms of quinolone resistance in Escherichia coli and Salmonella: recent developments. *International journal of antimicrobial agents*, 25, 358-373.

Horinouchi, T., Suzuki, S., Kotani, H., Tanabe, K., Sakata, N., Shimizu, H. *et al.* (2017). Prediction of cross-resistance and collateral sensitivity by gene expression profiles and genomic mutations. *Scientific reports*, 7, 1-11.

Hornbæk, T., Jakobsen, M., Dynesen, J. & Nielsen, A.K. (2004). Global transcription profiles and intracellular pH regulation measured in Bacillus licheniformis upon external pH upshifts. *Archives of microbiology*, 182, 467-474.

Huang, D.W., Sherman, B.T. & Lempicki, R.A. (2009a). Bioinformatics enrichment tools: paths toward the comprehensive functional analysis of large gene lists. *Nucleic acids research*, 37, 1-13.

Huang, D.W., Sherman, B.T. & Lempicki, R.A. (2009b). Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nature protocols*, 4, 44-57.

Hughes, B.S., Cullum, A.J. & Bennett, A.F. (2007). An experimental evolutionary study on adaptation to temporally fluctuating pH in Escherichia coli. *Physiological and Biochemical Zoology*, 80, 406-421.

Hughes, D. & Andersson, D.I. (2017). Evolutionary trajectories to antibiotic resistance. *Annual Review of Microbiology*, 71, 579-596.

Ishii, K., Matsuda, H., Iwasa, Y. & Sasaki, A. (1989). Evolutionarily stable mutation rate in a periodically changing environment. *Genetics*, 121, 163-174.

Jasinska, W., Manhart, M., Lerner, J., Gauthier, L., Serohijos, A.W. & Bershtein, S. (2020). Chromosomal barcoding of E. coli populations reveals lineage diversity dynamics at high resolution. *Nature ecology & evolution*, 4, 437-452.

Jasmin, J.N. & Kassen, R. (2007). On the experimental evolution of specialization and diversity in heterogeneous environments. *Ecology Letters*, 10, 272-281.

Jin, M., Xiao, A., Zhu, L., Zhang, Z., Huang, H. & Jiang, L. (2019). The diversity and commonalities of the radiation-resistance mechanisms of Deinococcus and its up-to-date applications. *AMB Express*, 9, 138.

Jolivet-Gougeon, A., Kovacs, B., Le Gall-David, S., Le Bars, H., Bousarghin, L., Bonnaure-Mallet, M. *et al.* (2011). Bacterial hypermutation: clinical implications. *Journal of medical microbiology*, 60, 563-573.

Joshi, D., Singhvi, M., Khire, J. & Gokhale, D. (2010). Strain improvement of Lactobacillus lactis for D-lactic acid production. *Biotechnology letters*, 32, 517-520.

Jun, S., Si, F., Pugatch, R. & Scott, M. (2018). Fundamental principles in bacterial physiology—history, recent progress, and the future with focus on cell size control: a review. *Reports on Progress in Physics*, 81, 056601.

Karve, S.M., Bhave, D. & Dey, S. (2018). Extent of adaptation is not limited by unpredictability of the environment in laboratory populations of Escherichia coli. *Journal of evolutionary biology*, 31, 1420-1426.

Karve, S.M., Bhave, D., Nevgi, D. & Dey, S. (2016). Escherichia coli populations adapt to complex, unpredictable fluctuations by minimizing trade-offs across environments. *Journal of evolutionary biology*, 29, 2545-2555.

Karve, S.M., Daniel, S., Chavhan, Y., Anand, A., Kharola, S. & Dey, S. (2015). Escherichia coli populations in unpredictably fluctuating environments evolve to face novel stresses through enhanced efflux activity. *Journal of evolutionary biology*, 28, 1131-1143.

Kassen, R. (2002). The experimental evolution of specialists, generalists, and the maintenance of diversity. *Journal of evolutionary biology*, 15, 173-190.

Kassen, R. (2014). *Experimental evolution and the nature of biodiversity*. Roberts, Colorado, United States.

Kassen, R. & Bell, G. (1998). Experimental evolution in Chlamydomonas. IV. Selection in environments that vary through time at different scales. *Heredity*, 80, 732-741.

Katz, S. & Hershberg, R. (2013). Elevated mutagenesis does not explain the increased frequency of antibiotic resistant mutants in starved aging colonies. *PLoS genetics*, 9, e1003968.

Kawecki, T.J. & Ebert, D. (2004). Conceptual issues in local adaptation. *Ecology letters*, 7, 1225-1241.

Kawecki, T.J. & Holt, R.D. (2002). Evolutionary consequences of asymmetric dispersal rates. *The American Naturalist*, 160, 333-347.

Kawecki, T.J., Lenski, R.E., Ebert, D., Hollis, B., Olivieri, I. & Whitlock, M.C. (2012). Experimental evolution. *Trends in ecology & evolution*, 27, 547-560.

Keller, L.C. & Maxcy, R.B. (1984). Effect of physiological age on radiation resistance of some bacteria that are highly radiation resistant. *Applied and environmental microbiology*, 47, 915-918.

Keseler, I.M., Gama-Castro, S., Mackie, A., Billington, R., Bonavides-Martínez, C., Caspi, R. *et al.* (2021). The EcoCyc database in 2021. *Frontiers in Microbiology*, 2098.

Kim, S., Lieberman, T.D. & Kishony, R. (2014). Alternating antibiotic treatments constrain evolutionary paths to multidrug resistance. *Proceedings of the National Academy of Sciences*, 111, 14494-14499.

Kirkpatrick, M. & Barton, N.H. (1997). Evolution of a species' range. *The American Naturalist*, 150, 1-23.

Knöppel, A., Näsvall, J. & Andersson, D.I. (2017). Evolution of antibiotic resistance without antibiotic exposure. *Antimicrobial agents and chemotherapy*, 61, e01495-01417.

Kohanski, M.A., Dwyer, D.J. & Collins, J.J. (2010). How antibiotics kill bacteria: from targets to networks. *Nature Reviews Microbiology*, 8, 423-435.

Koivunen, J. & Heinonen-Tanski, H. (2005). Inactivation of enteric microorganisms with chemical disinfectants, UV irradiation and combined chemical/UV treatments. *Water research*, 39, 1519-1526.

Kolter, R., Siegele, D.A. & Tormo, A. (1993). The stationary phase of the bacterial life cycle. *Annual review of Microbiology*, 47, 855-874.

Kottemann, M., Kish, A., Iloanusi, C., Bjork, S. & DiRuggiero, J. (2005). Physiological responses of the halophilic archaeon Halobacterium sp. strain NRC1 to desiccation and gamma irradiation. *Extremophiles*, 9, 219-227.

Kowalski, W. (2010). *Ultraviolet germicidal irradiation handbook: UVGI for air and surface disinfection*. Springer science & business media, New York.

Kumar, J., Babele, P.K., Singh, D. & Kumar, A. (2016). UV-B Radiation Stress Causes Alterations in Whole Cell Protein Profile and Expression of Certain Genes in the Rice Phyllospheric Bacterium Enterobacter cloacae. *Frontiers in Microbiology*, 7, 1440.

Kussell, E., Kishony, R., Balaban, N.Q. & Leibler, S. (2005). Bacterial persistence: a model of survival in changing environments. *Genetics*, 169, 1807-1814.

Kussell, E. & Leibler, S. (2005). Phenotypic diversity, population growth, and information in fluctuating environments. *Science*, 309, 2075-2078.

Lagator, M., Morgan, A., Neve, P. & Colegrave, N. (2014). Role of sex and migration in adaptation to sink environments. *Evolution*, 68, 2296-2305.

Lambert, G. & Kussell, E. (2014). Memory and fitness optimization of bacteria under fluctuating environments. *PLoS genetics*, 10, e1004556.

Larsen, N., Boye, M., Siegumfeldt, H. & Jakobsen, M. (2006). Differential expression of proteins and genes in the lag phase of Lactococcus lactis subsp. lactis grown in synthetic medium and reconstituted skim milk. *Applied and environmental microbiology*, 72, 1173-1179.

Lawrence, D., Bell, T. & Barraclough, T.G. (2016). The effect of immigration on the adaptation of microbial communities to warming. *The American Naturalist*, 187, 236-248.

Lee, H., Popodi, E., Tang, H. & Foster, P.L. (2012). Rate and molecular spectrum of spontaneous mutations in the bacterium Escherichia coli as determined by whole-genome sequencing. *Proceedings of the National Academy of Sciences*, 109, E2774-E2783.

Lenormand, T. (2002). Gene flow and the limits to natural selection. *Trends in Ecology & Evolution*, 17, 183-189.

Lenski, R.E., Rose, M.R., Simpson, S.C. & Tadler, S.C. (1991). Long-term experimental evolution in Escherichia coli. I. Adaptation and divergence during 2,000 generations. *The American Naturalist*, 138, 1315-1341.

Levene, H. (1953). Genetic equilibrium when more than one ecological niche is available. *The American Naturalist*, 87, 331-333.

Levin-Reisman, I., Ronin, I., Gefen, O., Braniss, I., Shoresh, N. & Balaban, N.Q. (2017). Antibiotic tolerance facilitates the evolution of resistance. *Science*, 355, 826-830.

Levins, R. (1968). *Evolution in changing environments*. Princeton University Press, New Jersey.

Levy, S.F., Blundell, J.R., Venkataram, S., Petrov, D.A., Fisher, D.S. & Sherlock, G. (2015). Quantitative evolutionary dynamics using high-resolution lineage tracking. *Nature*, 519, 181-186.

Li, H.-b., Hou, A.-m., Chen, T.-j., Yang, D., Chen, Z.-s., Shen, Z.-q. *et al.* (2021). Decreased antibiotic susceptibility in Pseudomonas aeruginosa surviving UV irradition. *Frontiers in Microbiology*, 12.

Li, X.-Z., Plésiat, P. & Nikaido, H. (2015). The challenge of efflux-mediated antibiotic resistance in Gram-negative bacteria. *Clinical microbiology reviews*, 28, 337-418.

Lim, S. & Blatchley III, E.R. (2009). UV disinfection system for cabin air. *Advances in space research*, 44, 942-948.

Lin, W.-H. & Kussell, E. (2016). Complex interplay of physiology and selection in the emergence of antibiotic resistance. *Current Biology*, 26, 1486-1493.

Lindqvist, R. & Barmark, G. (2014). Specific growth rate determines the sensitivity of Escherichia coli to lactic acid stress: implications for predictive microbiology. *BioMed research international*, 2014.

Liu, H. & Zhang, J. (2019). Yeast spontaneous mutation rate and spectrum vary with environment. *Current Biology*, 29, 1584-1591. e1583.

Liu, S., Wu, N., Zhang, S., Yuan, Y., Zhang, W. & Zhang, Y. (2017). Variable persister gene interactions with (p) ppGpp for persister formation in Escherichia coli. *Frontiers in microbiology*, 8, 1795.

Lively, C. (1999). Migration, virulence, and the geographic mosaic of adaptation by parasites. *The American Naturalist*, 153, S34-S47.

Long, A., Liti, G., Luptak, A. & Tenaillon, O. (2015). Elucidating the molecular architecture of adaptation via evolve and resequence experiments. *Nature Reviews Genetics*, 16, 567-582.

Long, H., Miller, S.F., Strauss, C., Zhao, C., Cheng, L., Ye, Z. *et al.* (2016). Antibiotic treatment enhances the genome-wide mutation rate of target cells. *Proceedings of the National Academy of Sciences*, 113, E2498-E2505.

Lotfy, W.A., Ghanem, K.M. & El-Helow, E.R. (2007). Citric acid production by a novel Aspergillus niger isolate: I. Mutagenesis and cost reduction studies. *Bioresource Technology*, 98, 3464-3469.

Ma, D., Alberti, M., Lynch, C., Nikaido, H. & Hearst, J.E. (1996). The local repressor AcrR plays a modulating role in the regulation of acrAB genes of Escherichia coli by global stress signals. *Molecular microbiology*, 19, 101-112.

MacLean, R.C., Dickson, A. & Bell, G. (2005). Resource competition and adaptive radiation in a microbial microcosm. *Ecology Letters*, 8, 38-46.

Macomber, L. & Hausinger, R.P. (2011). Mechanisms of nickel toxicity in microorganisms. *Metallomics*, 3, 1153-1162.

Maeda, T., Iwasawa, J., Kotani, H., Sakata, N., Kawada, M., Horinouchi, T. *et al.* (2020). High-throughput laboratory evolution reveals evolutionary constraints in Escherichia coli. *Nature communications*, 11, 1-13.

Maharjan, R.P. & Ferenci, T. (2017). A shifting mutational landscape in 6 nutritional states: Stress-induced mutagenesis as a series of distinct stress input–mutation output relationships. *PLoS biology*, 15, e2001477.

Majtan, T., Frerman, F.E. & Kraus, J.P. (2011). Effect of cobalt on Escherichia coli metabolism and metalloporphyrin formation. *Biometals*, 24, 335-347.

Makarova, K.S., Aravind, L., Wolf, Y.I., Tatusov, R.L., Minton, K.W., Koonin, E.V. *et al.* (2001). Genome of the extremely radiation-resistant bacterium Deinococcus radiodurans viewed from the perspective of comparative genomics. *Microbiology and molecular biology reviews*, 65, 44-79.

Markham, B.E., Harper, J.E. & Mount, D.W. (1985). Physiology of the SOS response: kinetics of lexA and recA transcriptional activity following induction. *Molecular and General Genetics MGG*, 198, 207-212.

Maslowska, K.H., Makiela-Dzbenska, K. & Fijalkowska, I.J. (2019). The SOS system: a complex and tightly regulated response to DNA damage. *Environmental and molecular mutagenesis*, 60, 368-384.

May, K.L. & Grabowicz, M. (2018). The bacterial outer membrane is an evolving antibiotic barrier. *Proceedings of the National Academy of Sciences*, 115, 8852-8854.

McDonald, M.J. (2019). Microbial Experimental Evolution–a proving ground for evolutionary theory and a tool for discovery. *EMBO reports*, 20, e46992.

McDonald, M.J., Hsieh, Y.-Y., Yu, Y.-H., Chang, S.-L. & Leu, J.-Y. (2012). The evolution of low mutation rates in experimental mutator populations of Saccharomyces cerevisiae. *Current Biology*, 22, 1235-1240.

Meckes, M.C. (1982). Effect of UV light disinfection on antibiotic-resistant coliforms in wastewater effluents. *Applied and environmental microbiology*, 43, 371-377.

Mehta, H.H., Prater, A.G., Beabout, K., Elworth, R.A., Karavis, M., Gibbons, H.S. *et al.* (2019). The essential role of hypermutation in rapid adaptation to antibiotic stress. *Antimicrobial agents and chemotherapy*, 63, e00744-00719.

Meireles, L.A., Guedes, A.C. & Malcata, F.X. (2003). Increase of the yields of eicosapentaenoic and docosahexaenoic acids by the microalga Pavlova lutheri following random mutagenesis. *Biotechnology and bioengineering*, 81, 50-55.

Mok, W.W., Orman, M.A. & Brynildsen, M.P. (2015). Impacts of global transcriptional regulators on persister metabolism. *Antimicrobial agents and chemotherapy*, 59, 2713-2719.

Molina-Quiroz, R.C., Silva-Valenzuela, C., Brewster, J., Castro-Nallar, E., Levy, S.B. & Camilli, A. (2018). Cyclic AMP regulates bacterial persistence through repression of the oxidative stress response and SOS-dependent DNA repair in uropathogenic Escherichia coli. *MBio*, 9, e02144-02117.

Monod, J. (1949). The growth of bacterial cultures. *Annual review of Microbiology*, 3, 371-394.

Morgan, A.D., Gandon, S. & Buckling, A. (2005). The effect of migration on local adaptation in a coevolving host–parasite system. *Nature*, 437, 253-256.

Morimatsu, K. & Kowalczykowski, S.C. (2014). RecQ helicase and RecJ nuclease provide complementary functions to resect DNA for homologous recombination. *Proceedings of the National Academy of Sciences*, 111, E5133-E5142.

Morton, R. & Haynes, R. (1969). Changes in the ultraviolet sensitivity of Escherichia coli during growth in batch cultures. *Journal of bacteriology*, 97, 1379-1385.

Mouton, J. (1999). Combination therapy as a tool to prevent emergence of bacterial resistance. *Infection*, 27, S24-S28.

Murren, C.J., Auld, J.R., Callahan, H., Ghalambor, C.K., Handelsman, C.A., Heskel, M.A. *et al.* (2015). Constraints on the evolution of phenotypic plasticity: limits and costs of phenotype and plasticity. *Heredity*, 115, 293-301.

Nakashima, R., Sakurai, K., Yamasaki, S., Nishino, K. & Yamaguchi, A. (2011). Structures of the multidrug exporter AcrB reveal a proximal multisite drug-binding pocket. *Nature*, 480, 565-569.

Natali, F. & Rancati, G. (2019). The mutator phenotype: Adapting microbial evolution to cancer biology. *Frontiers in genetics*, 10, 713.

Nguyen, J., Lara-Gutiérrez, J. & Stocker, R. (2021). Environmental fluctuations and their effects on microbial communities, populations and individuals. *FEMS microbiology reviews*, 45, fuaa068.

Oliver, A. (2005). Hypermutation in natural bacterial populations: consequences for medical microbiology. *Reviews in Medical Microbiology*, 16, 25-32.

Olsen, E.A., Hodak, E., Anderson, T., Carter, J.B., Henderson, M., Cooper, K. *et al.* (2016). Guidelines for phototherapy of mycosis fungoides and Sézary syndrome: a consensus statement of the United States Cutaneous Lymphoma Consortium. *Journal of the American Academy of Dermatology*, 74, 27-58.

Or, D., Smets, B.F., Wraith, J., Dechesne, A. & Friedman, S. (2007). Physical constraints affecting bacterial habitats and activity in unsaturated porous media–a review. *Advances in Water Resources*, 30, 1505-1527.

Orr, H.A. (2009). Fitness and its role in evolutionary genetics. *Nature Reviews Genetics*, 10, 531-539.

Papkou, A., Hedge, J., Kapel, N., Young, B. & MacLean, R.C. (2020). Efflux pump activity potentiates the evolution of antibiotic resistance across S. aureus isolates. *Nature communications*, 11, 1-15.

Pastan, I. & Perlman, R. (1970). Cyclic Adenosine Monophosphate in Bacteria: In many bacteria the synthesis of inducible enzymes requires this cyclic nucleotide. *Science*, 169, 339-344.

Patra, P. & Klumpp, S. (2014). Phenotypically heterogeneous populations in spatially heterogeneous environments. *Physical Review E*, 89, 030702.

Payne, J.L., Menardo, F., Trauner, A., Borrell, S., Gygli, S.M., Loiseau, C. *et al.* (2019). Transition bias influences the evolution of antibiotic resistance in Mycobacterium tuberculosis. *PLoS biology*, 17, e3000265.

Perez, V., Hengst, M., Kurte, L., Dorador, C., Jeffrey, W.H., Wattiez, R. *et al.* (2017). Bacterial Survival under Extreme UV Radiation: A Comparative Proteomics Study of Rhodobacter sp., Isolated from High Altitude Wetlands in Chile. *Frontiers in Microbiology*, 8, 1173.

Peric, M., Bozdogan, B.l., Jacobs, M.R. & Appelbaum, P.C. (2003). Effects of an efflux mechanism and ribosomal mutations on macrolide susceptibility of Haemophilus influenzae clinical isolates. *Antimicrobial agents and chemotherapy*, 47, 1017-1022.

Perron, G.G., Gonzalez, A. & Buckling, A. (2007). Source–sink dynamics shape the evolution of antibiotic resistance and its pleiotropic fitness cost. *Proceedings of the Royal Society B: Biological Sciences*, 274, 2351-2356.

Perron, G.G., Hall, A.R. & Buckling, A. (2010). Hypermutability and compensatory adaptation in antibiotic-resistant bacteria. *The American Naturalist*, 176, 303-311.

Pett-Ridge, J. & Firestone, M. (2005). Redox fluctuation structures microbial communities in a wet tropical soil. *Applied and environmental microbiology*, 71, 6998-7007.

Pietsch, F., Bergman, J.M., Brandis, G., Marcusson, L.L., Zorzet, A., Huseby, D.L. *et al.* (2016). Ciprofloxacin selects for RNA polymerase mutations with pleiotropic antibiotic resistance effects. *Journal of Antimicrobial Chemotherapy*, 72, 75-84.

Prudhomme, M., Attaiech, L., Sanchez, G., Martin, B. & Claverys, J.-P. (2006). Antibiotic stress induces genetic transformability in the human pathogen Streptococcus pneumoniae. *Science*, 313, 89-92.

Puentes-Téllez, P.E., Hansen, M.A., Sørensen, S.J. & van Elsas, J.D. (2013). Adaptation and heterogeneity of Escherichia coli MC1000 growing in complex environments. *Applied and environmental microbiology*, 79, 1008-1017.

Ram, Y. & Hadany, L. (2012). The evolution of stress-induced hypermutation in asexual populations. *Evolution*, 66, 2315-2328.

Ramos, C.C.R., Roque, J.L.A., Sarmiento, D.B., Suarez, L.E.G., Sunio, J.T.P., Tabungar, K.I.B. *et al.* (2020). Use of ultraviolet-C in environmental sterilization in hospitals: A

systematic review on efficacy and safety. *International Journal of Health Sciences*, 14, 52.

Raynes, Y. & Sniegowski, P. (2014). Experimental evolution and the dynamics of genomic mutation rate modifiers. *Heredity*, 113, 375-380.

Reboud, X. & Bell, G. (1997). Experimental evolution in Chlamydomonas. III. Evolution of specialist and generalist types in environments that vary in space and time. *Heredity*, 78, 507-514.

Rechinger, K.B., Siegumfeldt, H., Svendsen, I. & Jakobsen, M. (2000). „Early” protein synthesis of Lactobacillus delbrueckii ssp. bulgaricus in milk revealed by [35S] methionine labeling and two-dimensional gel electrophoresis. *ELECTROPHORESIS: An International Journal*, 21, 2660-2669.

Reizer, J., Michotey, V., Reizer, A. & Saier Jr, M.H. (1994). Novel phosphotransferase system genes revealed by bacterial genome analysis: Unique, putative fructose-and glucoside-specific systems. *Protein Science*, 3, 440-450.

Rezaie, A., Melmed, G.Y., Leite, G., Mathur, R., Takakura, W., Pedraza, I. *et al.* (2021). Endotracheal Application of Ultraviolet A Light in Critically Ill Patients with Severe Acute Respiratory Syndrome Coronavirus 2: A First-in-Human Study. *Advances in therapy*, 38, 4556-4568.

Rodríguez-Rojas, A., Kim, J.J., Johnston, P.R., Makarova, O., Eravci, M., Weise, C. *et al.* (2020). Non-lethal exposure to H2O2 boosts bacterial survival and evolvability against oxidative stress. *PLoS genetics*, 16, e1008649.

Rodríguez-Verdugo, A. & Ackermann, M. (2021). Rapid evolution destabilizes species interactions in a fluctuating environment. *The ISME Journal*, 15, 450-460.

Rodríguez-Verdugo, A., Vulin, C. & Ackermann, M. (2019). The rate of environmental fluctuations shapes ecological dynamics in a two-species microbial system. *Ecology letters*, 22, 838-846.

Roemhild, R., Barbosa, C., Beardmore, R.E., Jansen, G. & Schulenburg, H. (2015). Temporal variation in antibiotic environments slows down resistance evolution in pathogenic Pseudomonas aeruginosa. *Evolutionary applications*, 8, 945-955.

Rogozin, I.B., Makarova, K.S., Natale, D.A., Spiridonov, A.N., Tatusov, R.L., Wolf, Y.I. *et al.* (2002). Congruent evolution of different classes of non-coding DNA in prokaryotic genomes. *Nucleic acids research*, 30, 4264-4271.

Rohlf, J. & Sokal, R.R. (1995). *The principles and practice of statistics in biological research*. WH Freeman and company, New York.

Rolfe, M.D., Rice, C.J., Lucchini, S., Pin, C., Thompson, A., Cameron, A.D. *et al.* (2012). Lag phase is a distinct growth phase that prepares bacteria for exponential growth and involves transient metal accumulation. *Journal of bacteriology*, 194, 686-701.

Rosenblum, E.B., Parent, C.E. & Brandt, E.E. (2014). The molecular basis of phenotypic convergence. *Annual Review of Ecology, Evolution, and Systematics*, 45, 203-226.

Ruiz, J. (2003). Mechanisms of resistance to quinolones: target alterations, decreased accumulation and DNA gyrase protection. *Journal of Antimicrobial Chemotherapy*, 51, 1109-1117.

Rutala, W.A., Gergen, M.F. & Weber, D.J. (2010). Room decontamination with UV radiation. *Infection Control & Hospital Epidemiology*, 31, 1025-1029.

Saier Jr, M.H., Reddy, V.S., Tamang, D.G. & Västermark, Å. (2014). The transporter classification database. *Nucleic acids research*, 42, D251-D258.

Sandberg, T.E., Lloyd, C.J., Palsson, B.O. & Feist, A.M. (2017). Laboratory evolution to alternating substrate environments yields distinct phenotypic and genetic adaptive strategies. *Applied and environmental microbiology*, 83, e00410-00417.

Santos-Lopez, A., Marshall, C.W., Scribner, M.R., Snyder, D.J. & Cooper, V.S. (2019). Evolutionary pathways to antibiotic resistance are dependent upon environmental structure and bacterial lifestyle. *Elife*, 8, e47612.

Santos, A.L., Moreirinha, C., Lopes, D., Esteves, A.C., Henriques, I., Almeida, A. *et al.* (2013). Effects of UV radiation on the lipids and proteins of bacteria studied by mid-infrared spectroscopy. *Environmental science & technology*, 47, 6306-6315.

Sanyal, R., Vimala, A. & Harinarayanan, R. (2020). Studies on the Regulation of (p) ppGpp Metabolism and Its Perturbation Through the Over-Expression of Nudix Hydrolases in Escherichia coli. *Frontiers in microbiology*, 11, 2561.

Scheiner, S.M. & Yampolsky, L.Y. (1998). The evolution of Daphnia pulex in a temporally varying environment. *Genetics Research*, 72, 25-37.

Schimel, J., Balser, T.C. & Wallenstein, M. (2007). Microbial stress-response physiology and its implications for ecosystem function. *Ecology*, 88, 1386-1394.

Schlesinger, D.J. (2007). Role of RecA in DNA damage repair in Deinococcus radiodurans. *FEMS microbiology letters*, 274, 342-347.

Schlötterer, C., Kofler, R., Versace, E., Tobler, R. & Franssen, S. (2015). Combining experimental evolution with next-generation sequencing: a powerful tool to study adaptation from standing genetic variation. *Heredity*, 114, 431-440.

Schroeder, M.R. & Stephens, D.S. (2016). Macrolide resistance in Streptococcus pneumoniae. *Frontiers in cellular and infection microbiology*, 6, 98.

Schuster, S., Vavra, M. & Kern, W.V. (2016). Evidence of a substrate-discriminating entrance channel in the lower porter domain of the multidrug resistance efflux pump AcrB. *Antimicrobial agents and chemotherapy*, 60, 4315-4323.

Schwarz, T. (1998). UV light affects cell membrane and cytoplasmic targets. *Journal of Photochemistry and Photobiology B: Biology*, 44, 91-96.

Sekiguchi, M. (1996). MutT-related error avoidance mechanism for DNA synthesis. *Genes to Cells*, 1, 139-145.

Selveshwari, S., Lele, K. & Dey, S. (2021). Genomic signatures of UV resistance evolution in Escherichia coli depend on the growth phase during exposure. *Journal of evolutionary Biology*, 34, 953-967.

Sexton, J.P., McIntyre, P.J., Angert, A.L. & Rice, K.J. (2009). Evolution and ecology of species range limits. *Annual Review of Ecology, Evolution, and Systematics*, 40, 415-436.

Sherman, E.X., Wozniak, J.E. & Weiss, D.S. (2019). Methods to evaluate colistin heteroresistance in Acinetobacter baumannii. *Methods in molecular biology (Clifton, NJ)*, 1946, 39.

Shibai, A., Takahashi, Y., Ishizawa, Y., Motooka, D., Nakamura, S., Ying, B.W. *et al.* (2017). Mutation accumulation under UV radiation in Escherichia coli. *Scientific reports*, 7, 14531.

Shinohara, T., Ikawa, S., Iwasaki, W., Hiraki, T., Hikima, T., Mikawa, T. *et al.* (2015). Loop L1 governs the DNA-binding specificity and order for RecA-catalyzed reactions in homologous recombination and DNA repair. *Nucleic acids research*, 43, 973-986.

Simons, A.M. (2011). Modes of response to environmental change and the elusive empirical evidence for bet hedging. *Proceedings of the Royal Society B: Biological Sciences*, 278, 1601-1609.

Singh, S.K., SaiSree, L., Amrutha, R.N. & Reddy, M. (2012). Three redundant murein endopeptidases catalyse an essential cleavage step in peptidoglycan synthesis of E scherichia coli K 12. *Molecular microbiology*, 86, 1036-1051.

Sinha, R.P. & Häder, D.-P. (2002). UV-induced DNA damage and repair: a review. *Photochemical & Photobiological Sciences*, 1, 225-236.

Smith, A., Kaczmar, A., Bamford, R.A., Smith, C., Frustaci, S., Kovacs-Simon, A. *et al.* (2018). The culture environment influences both gene regulation and phenotypic heterogeneity in Escherichia coli. *Frontiers in microbiology*, 9, 1739.

Smith, K.C., Wang, T.-C.V. & Sharma, R.C. (1987). recA-dependent DNA repair in UV-irradiated Escherichia coli. *Journal of Photochemistry and Photobiology B: Biology*, 1, 1-11.

Smits, W.K., Kuipers, O.P. & Veening, J.-W. (2006). Phenotypic variation in bacteria: the role of feedback regulation. *Nature Reviews Microbiology*, 4, 259-271.

Sniegowski, P.D., Gerrish, P.J. & Lenski, R.E. (1997). Evolution of high mutation rates in experimental populations of E. coli. *Nature*, 387, 703-705.

Soberón-Chávez, G., Alcaraz, L.D., Morales, E., Ponce-Soto, G.Y. & Servín-González, L. (2017). The transcriptional regulators of the CRP family regulate different essential bacterial functions and can be inherited vertically and horizontally. *Frontiers in microbiology*, 8, 959.

Sprouffske, K., Aguilar-Rodríguez, J., Sniegowski, P. & Wagner, A. (2018). High mutation rates limit evolutionary adaptation in Escherichia coli. *PLoS genetics*, 14, e1007324.

Stich, M., Manrubia, S.C. & Lázaro, E. (2010). Variable mutation rates as an adaptive strategy in replicator populations. *PLoS One*, 5, e11186.

Stocker, R. (2012). Marine microbes see a sea of gradients. *Science*, 338, 628-633.

Stoltzfus, A. & McCandlish, D.M. (2017). Mutational biases influence parallel adaptation. *Molecular biology and evolution*, 34, 2163-2172.

Storz, J.F., Natarajan, C., Signore, A.V., Witt, C.C., McCandlish, D.M. & Stoltzfus, A. (2019). The role of mutation bias in adaptive molecular evolution: insights from convergent changes in protein function. *Philosophical Transactions of the Royal Society B*, 374, 20180238.

Streisfeld, M.A. & Rausher, M.D. (2011). Population genetics, pleiotropy, and the preferential fixation of mutations during adaptive evolution. *Evolution*, 65, 629-642.

Sukhi, S.S., Shashidhar, R., Kumar, S.A. & Bandekar, J.R. (2009). Radiation resistance of Deinococcus radiodurans R1 with respect to growth phase. *FEMS microbiology letters*, 297, 49-53.

Sullivan, G.M. & Feinn, R. (2012). Using effect size—or why the P value is not enough. *Journal of graduate medical education*, 4, 279-282.

Sun, D.-W. (2011). *Handbook of food safety engineering*. John Wiley & Sons, New Jersey.

Szostkova, M., Horakova, D. & Němec, M. (1999). The influence of the growth phase of enteric bacteria on electrotransformation with plasmid DNA. *Folia microbiologica*, 44, 177-180.

Taddei, F., Radman, M., Maynard-Smith, J., Toupance, B., Gouyon, P.-H. & Godelle, B. (1997). Role of mutator alleles in adaptive evolution. *Nature*, 387, 700-702.

Tait-Kamradt, A., Davies, T., Cronan, M., Jacobs, M., Appelbaum, P. & Sutcliffe, J. (2000). Mutations in 23S rRNA and ribosomal protein L4 account for resistance in pneumococcal strains selected in vitro by macrolide passage. *Antimicrobial agents and chemotherapy*, 44, 2118-2125.

Tenaillon, O., Denamur, E. & Matic, I. (2004). Evolutionary significance of stress-induced mutagenesis in bacteria. *Trends in microbiology*, 12, 264-270.

Tenaillon, O., Rodríguez-Verdugo, A., Gaut, R.L., McDonald, P., Bennett, A.F., Long, A.D. *et al.* (2012). The molecular diversity of adaptive convergence. *Science*, 335, 457-461.

Thai, T.P., Houghton, P.E., Keast, D.H., Campbell, K.E. & Woodbury, M.G. (2002). ULTRAVIOLET LIGHT C in THE TREATMENT OF CHRONIC WOUNDS WITH MRSA: A Case STUDY. *Ostomy/wound management*, 48, 52-60.

Thai, T.P., Keast, D.H., Campbell, K.E., Woodbury, M.G. & Houghton, P.E. (2005). Effect of ultraviolet light C on bacterial colonization in chronic wounds. *Ostomy/wound management*, 51, 32-45.

Thoma, F. (1999). Light and dark in chromatin repair: repair of UV-induced DNA lesions by photolyase and nucleotide excision repair. *The EMBO journal*, 18, 6585-6598.

Tillich, U.M., Lehmann, S., Schulze, K., Dühring, U. & Frohme, M. (2012). The optimal mutagen dosage to induce point-mutations in Synechocystis sp. PCC6803 and its application to promote temperature tolerance. *PLoS One*, 7, e49467.

Tom, E.F., Molineux, I.J., Paff, M.L. & Bull, J.J. (2018). Experimental evolution of UV resistance in a phage. *PeerJ*, 6, e5190.

Tornaletti, S. & Hanawalt, P.C. (1999). Effect of DNA lesions on transcription elongation. *Biochimie*, 81, 139-146.

Trautinger, B.W., Jaktaji, R.P., Rusakova, E. & Lloyd, R.G. (2005). RNA polymerase modulators and DNA repair activities resolve conflicts between DNA replication and transcription. *Molecular cell*, 19, 247-258.

Travis, J. & Travis, E. (2002). Mutator dynamics in fluctuating environments. *Proceedings of the Royal Society of London. Series B: Biological Sciences*, 269, 591-597.

Tritsch, D., Reinbolt, J. & Wittmann-Liebold, B. (1977). The primary structure of ribosomal proteins S7 from Escherichia coli strains K and B. *FEBS letters*, 77, 8-9.

Tufto, J. (2015). Genetic evolution, plasticity, and bet-hedging as adaptive responses to temporally autocorrelated fluctuating selection: A quantitative genetic model. *Evolution*, 69, 2034-2049.

Tupin, A., Gualtieri, M., Roquet-Banères, F., Morichaud, Z., Brodolin, K. & Leonetti, J.-P. (2010). Resistance to rifampicin: at the crossroads between ecological, genomic and medical concerns. *International journal of antimicrobial agents*, 35, 519-523.

Turner, P.E. & Elena, S.F. (2000). Cost of host radiation in an RNA virus. *Genetics*, 156, 1465-1470.

Turrientes, M.-C., Baquero, F., Levin, B.R., Martínez, J.-L., Ripoll, A., González-Alba, J.-M. *et al.* (2013). Normal mutation rate variants arise in a Mutator (Mut S) Escherichia coli population. *PloS one*, 8, e72963.

Uppal, S. & Jawali, N. (2016). The cyclic AMP receptor protein (CRP) regulates mqsRA, coding for the bacterial toxin-antitoxin gene pair, in Escherichia coli. *Research in microbiology*, 167, 58-62.

van Boxtel, C., van Heerden, J.H., Nordholt, N., Schmidt, P. & Bruggeman, F.J. (2017). Taking chances and making mistakes: non-genetic phenotypic heterogeneity and its consequences for surviving in dynamic environments. *Journal of The Royal Society Interface*, 14, 20170141.

Van Cleve, J. & Weissman, D.B. (2015). Measuring ruggedness in fitness landscapes. *Proceedings of the National Academy of Sciences*, 112, 7345-7346.

Van den Bergh, B., Swings, T., Fauvart, M. & Michiels, J. (2018). Experimental design, population dynamics, and diversity in microbial experimental evolution. *Microbiology and Molecular Biology Reviews*, 82, e00008-00018.

van der Putten, B.C., Remondini, D., Pasquini, G., Janes, V.A., Matamoros, S. & Schultsz, C. (2019). Quantifying the contribution of four resistance mechanisms to ciprofloxacin MIC in Escherichia coli: a systematic review. *Journal of Antimicrobial Chemotherapy*, 74, 298-310.

Van Tienderen, P.H. (1991). Evolution of generalists and specialists in spatially heterogeneous environments. *Evolution*, 45, 1317-1331.

Venail, P.A., MacLean, R.C., Bouvier, T., Brockhurst, M.A., Hochberg, M.E. & Mouquet, N. (2008). Diversity and productivity peak at intermediate dispersal rate in evolving metacommunities. *Nature*, 452, 210-214.

Venkateswaran, A., McFarlan, S.C., Ghosal, D., Minton, K.W., Vasilenko, A., Makarova, K. *et al.* (2000). Physiologic determinants of radiation resistance in Deinococcus radiodurans. *Applied and environmental microbiology*, 66, 2620-2626.

Via, S. & Lande, R. (1987). Evolution of genetic variability in a spatially heterogeneous environment: effects of genotype–environment interaction. *Genetics Research*, 49, 147-156.

Voet, D. & Voet, J.G. (2011). Biochemistry, 4-th Edition. *NewYork: John Wiley& SonsInc*, 492.

Vogwill, T., Fenton, A. & Brockhurst, M.A. (2011). Coevolving parasites enhance the diversity-decreasing effect of dispersal. *Biology letters*, 7, 578-580.

Volkova, N.V., Meier, B., González-Huici, V., Bertolini, S., Gonzalez, S., Vöhringer, H. *et al.* (2020). Mutational signatures are jointly shaped by DNA damage and repair. *Nature communications*, 11, 1-15.

Wagner, A. (2005). Robustness, evolvability, and neutrality. *FEBS letters*, 579, 1772-1778.

Wang, D., Kreutzer, D.A. & Essigmann, J.M. (1998). Mutagenicity and repair of oxidative DNA damage: insights from studies using defined lesions. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis*, 400, 99-115.

Wang, H., Dzink-Fox, J.L., Chen, M. & Levy, S.B. (2001). Genetic characterization of highly fluoroquinolone-resistant clinical Escherichia coli strains from China: role of acrR mutations. *Antimicrobial agents and chemotherapy*, 45, 1515-1521.

Wang, S. & Dai, L. (2019). Evolving generalists in switching rugged landscapes. *PLoS computational biology*, 15, e1007320.

Wassmann, M., Moeller, R., Reitz, G. & Rettberg, P. (2010). Adaptation of Bacillus subtilis cells to Archean-like UV climate: relevant hints of microbial evolution to remarkably increased radiation resistance. *Astrobiology*, 10, 605-615.

Wassmann, M., Moeller, R., Reitz, G. & Rettberg, P. (2011). Growth phase-dependent UV-C resistance of Bacillus subtilis: data from a short-term evolution experiment. *Archives of microbiology*, 193, 823-832.

Watkinson, A.R. & Sutherland, W.J. (1995). Sources, sinks and pseudo-sinks. *Journal of Animal Ecology*, 126-130.

Wedum, A.G., Hanel Jr, E. & Phillips, G.B. (1956). Ultraviolet sterilization in microbiological laboratories. *Public Health Reports*, 71, 331.

Weigand, M.R. & Sundin, G.W. (2009). Long-term effects of inducible mutagenic DNA repair on relative fitness and phenotypic diversification in Pseudomonas cichorii 302959. *Genetics*, 181, 199-208.

Weisblum, B. (1995). Erythromycin resistance by ribosome modification. *Antimicrobial agents and chemotherapy*, 39, 577-585.

West-Eberhard, M.J. (1989). Phenotypic plasticity and the origins of diversity. *Annual review of Ecology and Systematics*, 20, 249-278.

White, O., Eisen, J.A., Heidelberg, J.F., Hickey, E.K., Peterson, J.D., Dodson, R.J. *et al.* (1999). Genome sequence of the radioresistant bacterium Deinococcus radiodurans R1. *Science*, 286, 1571-1577.

Wielgoss, S., Barrick, J.E., Tenaillon, O., Wiser, M.J., Dittmar, W.J., Cruveiller, S. *et al.* (2013). Mutation rate dynamics in a bacterial population reflect tension between adaptation and genetic load. *Proceedings of the National Academy of Sciences*, 110, 222-227.

Williams, A.B. (2014). Spontaneous mutation rates come into focus in Escherichia coli. *DNA repair*, 24, 73-79.

Windels, E.M., Michiels, J.E., Fauvart, M., Wenseleers, T., Van den Bergh, B. & Michiels, J. (2019). Bacterial persistence promotes the evolution of antibiotic resistance by increasing survival and mutation rates. *The ISME journal*, 13, 1239-1251.

Wiznia, L.E., Quatrano, N.A., Mu, E.W. & Rieder, E.A. (2017). A clinical review of laser and light therapy for nail psoriasis and onychomycosis. *Dermatologic Surgery*, 43, 161-172.

Woodford, N. & Ellington, M.J. (2007). The emergence of antibiotic resistance by mutation. *Clinical Microbiology and Infection*, 13, 5-18.

Woods, R., Schneider, D., Winkworth, C.L., Riley, M.A. & Lenski, R.E. (2006). Tests of parallel molecular evolution in a long-term experiment with Escherichia coli. *Proceedings of the National Academy of Sciences*, 103, 9107-9112.

Wu, X., Zhang, J., Chao, L., Liang, J., Lu, Y., Li, H. *et al.* (2009). Identification of rifampin-resistant genotypes in Mycobacterium tuberculosis by PCR-reverse dot blot hybridization. *Molecular biotechnology*, 41, 1-7.

Yampolsky, L.Y. & Stoltzfus, A. (2001). Bias in the introduction of variation as an orienting factor in evolution. *Evolution & development*, 3, 73-83.

Yang, Z. & Bielawski, J.P. (2000). Statistical methods for detecting molecular adaptation. *Trends in ecology & evolution*, 15, 496-503.

Yeaman, S. & Guillaume, F. (2009). Predicting adaptation under migration load: the role of genetic skew. *Evolution*, 63, 2926-2938.

Zenteno-Cuevas, R., Zenteno, J., Cuellar, A., Cuevas, B., Sampieri, C., Riviera, J. *et al.* (2009). Mutations in rpoB and katG genes in Mycobacterium isolates from the Southeast of Mexico. *Memórias do Instituto Oswaldo Cruz*, 104, 468-472.

Zgurskaya, H.I. & Nikaido, H. (1999). Bypassing the periplasm: reconstitution of the AcrAB multidrug efflux pump of Escherichia coli. *Proceedings of the National Academy of Sciences*, 96, 7190-7195.

Zhang, C., Xu, L., Wang, X., Zhuang, K. & Liu, Q. (2017). Effects of ultraviolet disinfection on antibiotic-resistant Escherichia coli from wastewater: inactivation, antibiotic resistance profiles and antibiotic resistance genes. *Journal of applied microbiology*, 123, 295-306.

Zhu, X., Kong, J., Yang, H., Huang, R., Huang, Y., Yang, D. *et al.* (2018). Strain improvement by combined UV mutagenesis and ribosome engineering and subsequent fermentation optimization for enhanced 6′-deoxy-bleomycin Z production. *Applied microbiology and biotechnology*, 102, 1651-1661.