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Adaptive Landscapes in Evolving Populations of *Pseudomonas* Fluorescens in Simple Environments

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Adaptive landscapes in evolving populations of *Pseudomonas fluorescens* in simple environments

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ABSTRACT

The adaptive landscape heuristic can be used to answer the question "how predictable is evolution?" because its topology will impact the repeatability of evolution. In my Masters research I addressed this question in two ways: (1) I reviewed empirical adaptive landscape studies in the fields of directed protein evolution and microbial experimental evolution and (2) I performed a selection experiment to characterize adaptive landscape topology by measuring variance in fitness and metabolic phenotype within and among genetically distinct *Pseudomonas fluorescens* strains in two environments. Empirical studies have found that protein level landscapes are generally smooth, however, population level landscapes are rugged even in simple environments. Experimentally I found that the pattern of variance in fitness and metabolic phenotype was unique to the selection environment. The response to selection was highly repeatable at the level of fitness, but the underlying genetic routes taken were different for each environment and more variable in xylose than in glucose, suggesting a more rugged underlying landscape. More generally, my research suggests that making statements about the predictability of adaptive evolution at the population level may be challenging and will likely depend on the specifics of the environment in which selection occurs.

RÉSUMÉS

Le paysage adaptatif heuristique peut être utilisé pour répondre à la question « l'évolution est-elle prédictible ?» parce que sa topologie influencera la répétabilité de l'évolution. La recherche de ma Maîtrise adresse cette question de deux manières: (1) J'ai révisé les études empiriques sur les paysages adaptatifs dans les domaines de l'évolution dirigée des protéines et de l'évolution microbienne expérimentale et (2) j'ai effectué une expérience de sélection pour caractériser la topologie du paysage adaptatif en mesurant la variance de la valeur sélective ainsi que le phénotype métabolique au sein et entre des lignées génétiquement distinctes de *Pseudomonas fluorescens* dans deux environnements. Les études empiriques ont trouvé que les paysages au niveau des proteines sont généralement doux, cependant, au niveau de la population, les paysages sont rugueux même dans des environnements simples. Expérimentalement, j'ai trouvé que le patron de variance de la valeur sélective et du phénotype métabolique était spécifique à l'environnement de sélection. La réponse à la sélection était hautement répétable au niveau de la valeur sélective, mais les voies génétiques sous-jacentes prises étaient différentes pour chacun des environnements et étaient plus variables dans le xylose que dans le glucose, suggérant un paysage adaptatif plus rugueux. Plus généralement, ma recherche suggère que donner des affirmations sur la prédictibilité de l'évolution adaptative au niveau de la population peut être difficile et dépend surtout des spécificités de l'environnement dans lequel la sélection se produit.

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Chapter 1

An introduction to adaptive landscapes

Selection is the process whereby individuals with heritable traits that make an organism more likely to survive and successfully reproduce become more common in a population over successive generations. We know that selection results in adaptation to a particular environment, but many questions remain unanswered. How do populations adapt? Do multiple populations adapt in the same way, or in different ways? What processes are important in determining the adaptive course of a population through time? Answering these questions can be aided by utilizing the adaptive landscape heuristic. The adaptive landscape has been proposed as an abstract representation of the fitness of genotypes or phenotypes in a given environment. Genotypes or phenotypes are shown along the x- and y-axes, and fitness is shown along the z-axis with increasing height representing greater fitness.

The shape of the landscape plays a major role in evolutionary processes because natural selection generally drives populations up peaks by increasing fitness. Thus the topology of landscapes is of great interest to evolutionary biologists as it will be a major determinant of the course, and also the repeatability of evolution. The idea has inspired much theoretical work, but little is known about empirical landscapes, as they are difficult to study because constructing every possible genotypic or phenotypic combination is a daunting task. Landscapes can be "smooth" -having only a single fitness peak, or "rugged" - having multiple fitness peaks. In a smooth landscape multiple evolving populations will likely converge on the same final adaptive solution. Conversely rugged landscapes with many

peaks will greatly increase the chances of different populations finding divergent adaptive solutions.

THEORIES OF ADAPTIVE LANDSCAPES

In the literature " adaptive landscape" is typically, but not exclusively, used to refer to two different types of landscapes: mapping genotypes to fitness or mapping phenotypes to fitness. Biologists are not always specific with respect to what type of landscape they are referring to (individual genotypes, gene frequencies, or phenotypes) and this has added to some of the confusion and debate surrounding the adaptive landscape concept. Another source of confusion is that there are a number of formalized theories describing what a landscape is; these theories differ in the evolutionary mechanisms important in determining the topology of the landscape and thus ultimately what the topology would look like. I will briefly review several of the leading theories below.

Sewall Wright (1932) was the first to introduce the concept of the adaptive landscape in an evolutionary context. Prior to its introduction as a concept in evolutionary biology it was used extensively in studying energy potentials in thermodynamics and also aided in developing quantum mechanics theory (Ao 2008). Wright used the adaptive landscape as a heuristic to explain the process of adaptation in a given environment, and in particular to demonstrate his shifting balance theory. His landscape consisted of a two-dimensional space of genotypic frequencies with the height of the landscape representing population fitness. He stressed that representing the population genetics of a real evolutionary process requires thousands of dimensions because the number of possible gene combinations in the field of gene frequencies of a population is vast. Thus Wright used the two-dimensional adaptive

landscape as a simple model of what could in actuality be thousands of dimensions. He thought that even this simplified two-dimensional representation would have a rugged topology as a result of epistatic (non-additive) interactions between genes. He envisaged a landscape with adaptive valleys separated by many adaptive peaks, and he used his shifting balance theory to explain how populations move through this landscape by random genetic drift (Wright 1932). Wright's genetic landscape, and the idea of step-wise movement across it (see Figure 2; Wright 1932) were later formalized by Kauffman and Levin (1987) in their rugged *NK* genetic adaptive landscape theory.

The first to challenge Wright's interpretation of the landscape concept was R. A. Fisher (Fisher 1936). Fisher thought that as the dimensionality of the field of gene combinations in the field of gene frequencies increases, the number of stable peaks on the surface of the landscape must decrease. Movement on this type of landscape would not require the random drift processes of Wright's shifting balance theory, only selection and mutation. Fisher also thought the problem of peak shifts was relatively unimportant because he felt that genetic variation for fitness would always be available largely due to constantly changing environments. Thus Fisher viewed adaptive landscapes as constantly changing, using the analogy that adaptive peaks were like waves on an ever-changing ocean (Fisher 1930; Ridley 2004) whereas changes to the topology of Wright's landscape occurred over a much longer time scale (Wright 1932).

Simpson (1944) took Wright's idea of adaptive landscapes defined in genotypic space and translated it to phenotypic space; phenotypic characters are plotted against fitness. On Simpson's landscape as the number of phenotypic dimensions increases, the relative

elevation of all fitness peaks declines, but the number of peaks and the coarseness of the landscape increases. It should be noted, however, that these genetic and phenotypic landscapes could be difficult to relate to each other. The connection between the two can be formalized using a "genotype-phenotype mapping function" proposed by Alberch (1991), however, we rarely have any idea of how to construct such a map. One important exception comes from studies of directed protein evolution where such a function can be realized because all combinations of genotypes and phenotypes are known. Directed protein evolution studies will be explored in greater detail in a later section (see also Poelwijk *et al.* 2007). The genotype-phenotype map becomes much more difficult to study in higher-level phenotypes that are expressed by more complex organisms because there are a large number of unknown molecular steps or networks between different evolutionary states.

Kauffman and Levin (1987) built on Wright's idea of a genetic adaptive landscape by developing a theory to understand the fitness of different gene combinations given epistatic gene interactions. In Kauffman and Levin's *NK* model the fitness contribution of each N loci is dependent in a random way on K other loci. Thus K can be seen as describing the degree of epistasis; if $K=0$ then there is a single adaptive peak, but as K increases the number of peaks increases and the mean fitness of the nearest peak decrease toward that of an entirely random genotype. Typically K has a positive value and epistasis is present, resulting in a rugged adaptive landscape. This landscape theory has been applied successfully in biochemistry (Fontana *et al.* 1989; Fontana *et al.* 1993).

Gavrilets has used a genetic landscape framework to develop a theory of holey landscapes (Gavrilets 1997; Gavrilets and Gravner 1997; Gavrilets 1999). He fundamentally disagrees

with Wright's view of rugged adaptive landscapes, suggesting instead that as the number of gene combinations in a field of gene frequencies increases, the number of incompatible combinations in that field will also increase, resulting in a holey landscape. Thus Gavrilets's landscape contains holes representing locations of incompatible gene combinations that cause reproductive isolation within an environment. Gavrilets bases his theory on several, arguably unrealistic assumptions; (i) fitness of gene complexes are randomly generated, (ii) fitness's are generated independently, and (iii) fitness values are either 0, genotype unviable, or 1, genotype viable (Gavrilets 1997; Gavrilets and Gravner 1997; Gavrilets 1999). The presence of sterile hybrids in nature would appear to support the holey adaptive landscape theory, however, testing its applicability to asexual species proves more difficult.

As the concept of the adaptive landscape was developing within evolutionary biology it was also beginning to be utilized in different biological fields. It continues to have a progressively more important role in the development of systems biology and bionetwork dynamics modeling (for review see Ao 2009). As it becomes increasingly used in other fields it seems to be the time to discuss the progress of empirically determining adaptive landscapes as well as examining their utility as a predictive evolutionary tool.

EMPIRICAL ADAPTIVE LANDSCAPES

Our understanding of the ruggedness of empirical landscapes, and thus the repeatability of evolution, is limited because experimental studies investigating their topology are difficult to execute (for review see Whitlock *et al.* 1995; Arnold *et al.* 2001). However, with the development of novel experimental approaches empirical landscapes can now be studied in an increasingly thorough and systematic manner. Specifically two fields are shedding light

on landscape topology. Directed evolution reconstructs protein intermediate states and allows for the investigation of all possible evolutionary trajectories. Experimental evolution using microbes allows adaptation in large populations to be followed in real time for thousands of generations. The major conclusions from these two disciplines will be reviewed in chapter two of this thesis in the context of answering the question of how rugged empirical adaptive landscapes are? The second chapter is written as a stand-alone scientific review with the intention of submitting it to the Journal of Evolutionary Biology. This necessarily means that there is a small amount of overlap in the background material presented in this introductory chapter and the introductory section of the second chapter.

Microbial experimental evolution studies generally seem to infer rugged underlying landscapes and unpredictable evolutionary trajectories (for review see chapter two). However these past studies are limited in several methodological aspects. Few studies have utilized starting genotypes that vary in their characteristics. Typically all replicate lines are initiated from a single clone, and thus inferences about the underlying landscape are limited to a small area around the location of that one ancestor. Also, few studies have attempted to quantify evolutionary repeatability by measuring the variance in fitness (or any other phenotypic trait) through time among starting genotypes and within replicate selection lines. Previously, these variance measures, if calculated, were not used to explicitly infer the ruggedness of the empirical adaptive landscape underlying their experimental system. My research is unique in that it makes use of the adaptive landscape concept to construct an informative design that examines the predictability of evolution, specifically making use of the two under-explored aspects discussed above.

My Master's research followed the dynamics of the variance of fitness and metabolic phenotypes within and among six distinct *P. fluorescens* genotypes adapting to two different single carbon source environments. From these measures of variance I was able to make conclusions about the relative ruggedness of each selection environment. I used distinct initial genotypes to found multiple replicate populations so that at the start of the selection experiment populations were spread across the underlying landscape. Thus an extensive range of possible evolutionary trajectories was sampled. This experiment thus extends similar analyses on the variance among replicate selection lines (e.g. Lenski *et al.* 1991; Korona *et al.* 1994; Nakatsu *et al.* 1998; Barrett *et al.* 2005) to include information on how divergent genotypes respond to selection within the same environment. My experiment is thus a more robust means of inferring the ruggedness of adaptive landscapes in different environments. To my knowledge this is the first microbial experimental evolution study to investigate adaptive landscape topology in this manner.

The third chapter of this thesis presents this experiment. It is written as a stand-alone scientific manuscript that is in review at Evolution. Again, there is a small amount of overlap in the background material presented in this chapter and the introductory section of the third chapter, but I have endeavoured to keep this to a minimum.

Chapter 2

A review of the ruggedness of empirical adaptive landscapes

INTRODUCTION

The adaptive landscape has been proposed as an abstract representation of the fitness of genotypes or phenotypes in a given environment. Genotypes or phenotypes are shown along the x- and y-axes, and fitness is shown along the z-axis with increasing height representing greater fitness. The shape of the landscape plays a major role in evolutionary processes because selection increases fitness thereby moving populations up peaks. Thus the topology of landscapes is of great interest to evolutionary biologists as it is a major determinant of the course, and also the repeatability of evolution. The idea has inspired much theoretical work, but less is known about empirical landscapes as they are difficult to study because constructing every possible genotypic or phenotypic combination is a daunting task. Landscapes can be "smooth," having only a single fitness peak, or "rugged," having multiple fitness peaks. In a smooth landscape multiple evolving populations will likely converge on the same final adaptive solution. Conversely, rugged landscapes with many peaks will increase the chances of different populations finding divergent adaptive solutions.

In the literature, the term adaptive landscape is typically, but not exclusively, used to refer to two different types of landscapes: mapping genotypes to fitness or mapping phenotypes to fitness. Alternately individual selection surfaces plot an individual's fitness (Arnold 2005). Genes are the unit of inheritance, not phenotypes, and thus landscapes have to be rugged at the genotypic scale for genetic interactions to affect the course of evolution. However, empirically often little is known about the underlying genetic basis of phenotypes, while

more is known about the relationship between phenotype and fitness. Also phenotypic landscapes can be particularly informative if traits are determined by many loci that interact additively; complex phenotypic fitness functions will imply complex genotypic fitness functions. Biologists are not always specific with respect to what type of landscape they are referring to (individual genotypes, gene frequencies, or phenotypes) and this has added to some of the confusion and debate surrounding the adaptive landscape concept. There is also confusion because there are a number of formalized theories describing what a landscape is (Wright, 1932; Simpson 1944; Kauffman and Levin 1987; Gavrilets 1997); these theories differ in the evolutionary mechanisms important in determining the topology of the landscape and thus ultimately what the topology would look like (for review see Skipper Jr. 2004). For the purposes of interpreting empirical studies in this review the basic Wrightian interpretation (Wright, 1932) of a genotypic landscape, or alternately where appropriate Simpson's phenotypic interpretation (Simpson 1944) of an adaptive landscape will be utilized.

There are several other criticisms and areas of concern that should at least be mentioned with respect to adaptive landscapes. A practical concern is how to portray a genotypic landscape: how does one order genotypes along a landscape? Allelic frequencies and some phenotypic traits can be ordered along axes and thus the problem is eliminated, however, it is less than intuitive as to how one would actually construct genotypic axes. Another matter of debate is whether landscapes tend to be static or changing. In this review I generally treat landscapes as static, however changing landscapes need to be addressed in future studies of natural systems. Regardless of whether the adaptive landscape concept is tractable in either its genotypic or phenotypic interpretation, it still has value as a heuristic by generating modes of

inquiry. But do adaptive landscapes have predictive value, and therefore a role separate from being a heuristic? Will they allow us to enhance predictive value? Yes, if empirical topology can be quantified it would allow us to change evolutionary studies from a retrospective to a prospective science. However whether practically we are able to design experimental studies that can directly measure topological features of landscape remains to be seen. To date there are issues with the relative scales of landscape analysis and the ordering of axes that have yet to be fully overcome in the two research areas that are at the forefront of this type of endeavour.

Our understanding of the ruggedness of empirical landscapes is limited because experimental studies investigating landscape topology are difficult to execute (for review see Whitlock *et al.* 1995; Arnold *et al.* 2001) and in the past were generally limited to inferences about the process of evolution based on its end points. However, with the development of novel experimental approaches empirical landscapes are now being studied in an increasingly thorough and systematic manner. Specifically two fields are shedding light on landscape topology. Recent advancements in biochemical studies of directed protein evolution have allowed for all possible evolutionary trajectories between two different protein or enzyme states to be reconstructed and the likelihood of different adaptive trajectories to be measured. Populations adapt in real time for hundreds or thousands of generations in controlled laboratory environments in the field of experimental evolution.

The main aim of this review article is to determine how rugged empirical adaptive landscapes are, and in so doing demonstrate the utility of experimentally inferring the topology of adaptive landscapes. This is not intended to be a comprehensive review of either

directed protein evolution studies, or microbial experimental evolution studies. Rather, I hope to shed light on the major findings of these two new fields in the context of adaptive landscape topology, highlighting evolutionary implications with respect to how landscapes would constrain adapting proteins or populations.

LANDSCAPE INFERENCES FROM DIRECTED PROTEIN EVOLUTION STUDIES

Recent technological advancements have made it possible to examine all possible evolutionary trajectories between two protein states via the reconstruction of all intermediate protein forms. In these studies the fitness of the constructed intermediate forms are used to infer the course of evolution for the protein or enzyme in question, and from this one can then make conclusions about the underlying landscape topology (for review see Poelwijk *et al.* 2007). Generally these studies allow for the construction of both genotypic and phenotypic landscapes. Of these studies several have been performed with a more evolutionary, as opposed to biochemical, perspective and thus their major findings will be outlined below.

Weinreich and colleagues (2006) looked at changes in the β-lactamase protein in *Escherichia coli*. They used a penicillin-resistant strain known to achieve resistance to a newer antibiotic, cefotaxime, by the accumulation of five mutations. All possible mutational trajectories were explored by constructing 32 possible intermediates and 120 trajectories (they did not consider the possibility of reverse mutations); 102 trajectories were inaccessible to selection because one or more steps did not increase fitness. Only a very small number of trajectories are likely to be realized; 18 trajectories were accessible to selection because each of the 5 steps conferred a resistance, and thus fitness, increase. These 18 trajectories indicate that there are areas of the landscape that allow a continuous increase in fitness towards a single peak. Therefore the area of the β-lactamase landscape surveyed has a single peak representing cefoxtaxime resistance.

Another study examined the connection between the adaptive landscape and the underlying molecular properties of the protein isopropylmalate dehydrogenase (IMDH), which is involved in the biosynthesis of leucine (Lunzer *et al.* 2005). IMDH naturally uses the coenzyme nicotinamide adenine dinucleotide (NAD) as a hydride acceptor during oxidative decarboxylation. By changes in six amino acids IMDH can be engineered to use a different coenzyme, nicotinamide adenine dinucleotide phosphate (NADP) as a hydride acceptor (Lunzer *et al.* 2005). Intermediates from the evolutionary pathway between using NAD to NADP show that each amino acid change contributes additively to enzyme function *in vitro*. Fitness effects were measured in a second *in vivo* assay. Resultant fitness measurements were used to construct genotype-fitness, phenotype-fitness, and genotype-phenotype maps. The genotype-fitness map had a single peak because a fitter genotype was mutationally accessible to every genotype except for the NAD-dependent wild type that was the most fit (and represented the adaptive peak on the underlying adaptive landscape). Thus this study provided an evolutionary mechanism that explained why all IMDHs use NAD. Both the IMDH and the β-lactamase protein studies found smooth underlying genotypic landscapes, but whether these findings can be generalized to conclude that protein evolution is predictable, reproducible and proceeds deterministically towards a single adaptive peak remains to be determined.

Repressor-operator binding adaptation, specifically the epistatic interactions between different genetic components within a network, was investigated (Poelwijk *et al.* 2006) using published *E. coli lac* system mutation data (Lehming *et al.* 1990). In this study, no single evolutionarily superior repressor or operator was discovered. This means that there was no evolutionary path of constantly increasing fitness, specifically no path where transient loss in

affinity from one tight repressor-operator pair to another did not occur. Two repressor residues and four base pairs in the operator are central to altering the specificity of binding in this system, and a large set of mutants with substitutions at these loci were assayed for their repression value, defined as the ratio between repressed and unrepressed expression of the controlled gene (Lehming *et al.* 1990). The connecting paths between these alleles had reduced affinity, indicative of the presence of valleys of decreased fitness within the landscape, and epistasis (Poelwijk *et al.* 2006). These results infer the presence of a rugged landscape, different from the single-peaked landscapes of β-lactamase and IMDH. Molecular interactions, intuitively, should be subject to epistasis; mutating one binding partner will likely only benefit a new interaction if the other binding partner is mutated first, and vice versa. Not only does this mean that epistasis will introduce ruggedness into the adaptive landscape, it also suggests a role in the evolution of enzyme diversity because it enables the formation of multiple independent lock-key combinations. Thus interpreting the results of these type of biochemical directed evolution studies in an adaptive landscape perspective may yield a potential evolutionary mechanism explaining how complex biological systems composed of tightly integrated parts might evolve by stepwise process of natural selection.

In recent years the ability to perform analyses on evolutionary intermediate proteins and their putative pathways has resulted in a surge in the number of these types of directed protein evolution studies and provided a first look at small scale molecular adaptive landscapes. The methodology allows for the linkage of genetic architecture to fully functioning phenotypes and the construction of detailed genotype-phenotype-fitness maps. But, however promising, caution must be used when expanding the protein fitness landscapes that are the result of directed evolution to settings involving evolution in natural systems. It's unclear how protein

fitness assayed during directed evolution is related to organismal fitness. Differences could result due to interactions between the protein and the cellular environment, and may be constrained by metabolic limitations, regulation, and interactions with other cellular machinery as well as many other factors. Similarly it is known that protein function can be dramatically influenced by post-translational modification, for example epigenetic modification like DNA methylation. It is possible that by disconnecting proteins from their *in vivo* function the resultant proteins may be biologically irrelevant and thus not useful with respect to applying results to applying results to evolutionary landscape theory.

LANDSCAPE INFERENCES FROM MICROBIAL EXPERIMENTAL EVOLUTION

Microbial experimental evolution studies have rapidly increased in number over the last decade and their results can often be used to make inferences about empirical adaptive landscapes at the population level by following adaptation across this surface in real time. The most well-known long-term microbial evolution study was begun in 1988 with twelve isogenic populations of *E. coli* propagated in a glucose-limited environment, and to date this ongoing experiment is in excess of 50 000 generations of evolution (for example Lenski *et al.* 1991; Lenski and Travisano 1994). Initially all populations showed a rapid increase in fitness, but the rate of fitness increase slowed dramatically after generation 1000. The slowing of the rate of fitness increase is suggestive of populations reaching a fitness peak(s). Patterns of evolution between replicate populations are very similar, but small (although significant) differences in fitness (accompanied by larger variation in other phenotypic traits like cell size) continue to be maintained between these populations. This suggests that populations may have reached different fitness peaks and the maintained fitness variance implies they are on peaks of slightly different heights on a rugged landscape. A similar, but shorter experiment using the bacterium, *Alcaligenes eutrophus*, has produced qualitatively similar results, again supportive of a rugged underlying landscape (Korona 1996). Although these results are consistent with the hypothesis that populations have reached a peak in the adaptive landscape they are also consistent with an alternative interpretation; populations may have simply run out of genetic variation and become stalled at different locations within the landscape. However this is unlikely as bacterial population sizes are very large. Calculating within-lineage variation at different points during the course of the selection experiment would provide estimates of the amount of genetic variation present within

populations, allowing one to make stronger inferences with respect to the topology of the adaptive landscape.

Both of these studies, however, are only crude approximations of what the underlying landscape may be like because all populations are initially isogenic and thus start at the exact same location in the landscape. Another caveat is that only fitness is used as a measurement and thus it is possible similar fitness values are created by drastically different underlying genetic variation. When grown in novel environments, Lenski's long-term *E. coli* populations exhibited much greater variation in fitness suggesting the genetic basis of adaptation may be different in the replicate populations, despite similar fitness in the selection environment (Travisano *et al.* 1995). Fitness differences in novel environments could also be the result of variation that is neutral within the selection environment, and thus not indicative of a smooth underlying landscape. This alternative is unlikely because all populations are initially isogenic and maintained at a large size and thus it would take a very long time for neutral mutations to fix. Subsequent genetic analysis of these populations has shown that mutations in one gene, *spoT*, account for a significant amount of the changes in the DNA expression array, however the location of this mutation was different in all cases, further supporting the conclusion that these populations are adapting on a rugged landscape (Cooper *et al.* 2003). Divergent genetic changes occurring over the course of selection are the result of populations finding different ways to adapt to the same environment, implying the presence of multiple adaptive solutions, or peaks.

The pleiotropic effects of independently arising beneficial mutations from the long-term *E. coli* populations were measured in the selection environment, glucose, and in five novel

environments (Ostrowski *et al.* 2008). In glucose, widespread parallel adaptation was demonstrated because most of these beneficial mutations occurring in only a few genes. There was little or no variation among these beneficial mutations, even across several loci. However considerable heterogeneity in pleiotropic effects was revealed by correlated effects on fitness as determined in the five novel environments. The contrast between the similarity of the direct response to selection and the diversity in the correlated response to selection demonstrates that multiple genetic paths were taken by different lineages over the course of 20 000 generations of evolution. These results suggest that the underlying landscape is rugged with populations reaching different fitness peaks of similar heights. They also serve to illustrate the importance of secondary measures of fitness when analyzing data in an evolutionary context.

The majority of these studies indicate microbial adaptive landscapes are rugged. The prevalence of rugged landscapes is further supported by the results of several studies that found epistasis between mutations in *E. coli* (Remold and Lenski 2004; Elena and Lenski 2007) and between mutations in an RNA virus (Sanjuan *et al.* 2004). However, limited fitness differences often occur suggesting that the multiple peaks on the rugged landscapes may generally be of similar height. Populations appear to be evolving on rugged landscapes but do not appear to become trapped on local peaks of greatly reduced fitness.

Experimental reverse evolution

A clear alternative explanation exists for populations having limited fitness differences. Populations may be approaching the same adaptive peak along different paths and at different rates. It will be nearly impossible to conclude experimentally that populations have

reached a fitness peak; it is always possible, even if very unlikely, for example, that the *E. coli* populations will all converge on the same peak after 100 000 generations of selection. A different approach to studying adaptive landscapes that eliminates this matter of debate is to test whether evolution is reversible. One does this by seeing if a population pushed off its current fitness peak, returns to the same peak or a different one. On a smooth landscape a population will return to the same peak, whereas on a rugged landscape there is an increased likelihood that the population will ascend different peaks. A caveat to this approach is that populations are pushed off of fitness peaks via selection in a novel environment. This displaces populations from their original peak but it is unknown how far these populations have moved within the landscape. The displacement distance will depend on how correlated genotypic fitness's are in the two environments. If fitness is highly correlated across environments then populations will only be displaced a short distance. If fitness is poorly correlated across environments populations have likely been displaced a much greater distance. Thus by not knowing how great a distance populations have moved in genotype space, it is unknown whether subsequent fitness changes reflect landscape topology at a local or global scale.

Three studies of antibiotic resistance show that evolution is not reversible, implying a rugged underlying landscape (Schrag *et al.* 1997; Levin *et al.* 2000; Maisnier-Patin *et al.* 2002). Two of these studies utilize an *E. coli* strain sensitive to streptomycin and presumed to be approaching a fitness peak in the absence of streptomycin (hereafter termed the permissive environment). Populations are removed from the adaptive peak by changing the environment via the addition of streptomycin. Strains are then allowed to evolve in the presence of streptomycin and many become resistant. Evolved resistant bacteria typically have reduced

fitness in the original environment. When resistant bacteria are moved back to the permissive environment, do they return to their original fitness peaks? Generally, no they do not. Strains do not tend to lose their resistance by back mutation but instead adapt by compensatory mutations at other genetic sites that alleviate the cost of resistance. Compensatory mutations are typically only beneficial in the resistant background, and are often somewhat deleterious in the original background, thus providing even greater evidence that populations have varying genetic compositions and are climbing adaptive peaks different from the one on which they started. This was conclusively demonstrated in *E. coli* when resistance persisted even after selection in permissive conditions for over 10 000 generations (Schrag *et al.* 1997). Similar results were found using *Salmonella typhimurim* that becomes resistant to streptomycin by a single amino acid substitution. Eighty-one resistant lineages were evolved in the permissive environment, selecting for the amelioration of costs of resistance (Maisnier-Patin *et al.* 2002). Seventy-seven lineages retained resistance, and more interestingly, within these lineages there was a broad range of compensatory mutations that occurred. Mutations involving at least 35 different amino acid substitutions, affecting numerous ribosomal proteins, were found. The variety of mutations was unexpected, and illustrates that there are many different accessible pathways on this landscape that alleviate the cost of resistance.

Reverse evolution studies have also explored the adaptive radiation that occurs in *Pseudomonas fluorescens* grown in static microcosms (Rainey and Travisano 1998). Briefly, isogenic populations propagated in spatially heterogeneous (static) microcosms rapidly diversify into a number of distinct types. Each type is specialized for a particular microenvironment; wrinkly spreaders form mats on the surface where oxygen levels are

highest, smooths reside in the broth, and fuzzy spreaders occupy the bottom of the tube in oxygen-depleted conditions. Therefore it appears bacteria evolve to three different peaks on the underlying phenotypic landscape, corresponding to three different niches within the static microcosm. Reverse evolution occurs when the wrinkly spreader morphotype is placed within a homogeneous environment; full convergence back to the ancestral smooth morphotype is observed (Rainey and Travisano 1998). Differences between the relative fitness's of the evolved and reverse-evolved morphotypes indicate a number of different evolutionary paths were taken. Generally reverse evolution studies are in agreement with the other microbial studies and point to a prevalence of rugged underlying landscapes.

Microbial studies are beginning to provide key insights into the nature of population level adaptive landscapes. So far the results suggest that these types of adaptive landscapes are rugged even within simplified laboratory environments, and that there are many potential adaptive solutions to the same environmental constraints. This would suggest rugged landscapes are the rule rather than the exception because natural environments will likely be much more complex than laboratory environments. The inference that most landscapes are rugged is strengthened by the fact that microbial landscapes would be predicted to be more smooth because of their large population sizes within which many mutations can occur resulting in populations are that are constantly sampling large areas of the landscape. Thus even if multiple peaks existed it is likely an individual within these large populations will ascend the highest peak before the whole population becomes fixed on a suboptimal peak. The importance of the ruggedness of adaptive landscape topology in terms of constraining adapting populations may be different in asexual and sexual species. In asexual species all that is required is a single mutant genotype that occurs within the realm of attraction of the

highest peak within the landscape for the entire population to reach the optimal adaptive solution. In sexual species if this type of mutant were to arise it might be rapidly destroyed by recombination. Alternately, however, it may also arise by recombination. Thus whether or not asexual or sexual species are more likely to be constrained is dependent of the details of the landscape and the genetics of the adapting populations. Experimental evolution studies directly comparing sexual and asexual species, utilizing facultatively sexual organisms such as *Chlamydomonas* spp. and *Saccharomyces* spp. would provide insight into this issue.

CONCLUSIONS

Experimental studies of both proteins and microbes have provided insight into the ruggedness of empirical adaptive landscapes. Results suggest varying topologies at different levels of complexity. The experimental reconstruction of evolutionary intermediates and their putative trajectories suggest a prevalence of smooth landscapes at a molecular level, with the eventual evolution of a single optimal protein state. However at more complex levels of organization, such as those explored in microbial studies where entire populations are adapting, it appears that landscapes are rugged and there are many adaptive solutions to even a simplified laboratory environment. The scale of the landscape that is being surveyed may easily explain this discontinuity between protein and population level studies. Directed protein evolution studies characterize a very small subspace of the full genotype space, whereas population level studies increase the area of the landscape surveyed. Thus simply due to differences in scale the *a priori* likelihood of population level landscapes being smooth decreases dramatically.

Empirical investigations examining adaptive landscape topology –either in the form of constructing evolutionary intermediate proteins states or microbial selection experiments - are powerful tools to begin to explore natural adaptive landscapes. Both types of studies represent only starting points for the investigation of empirical landscapes, but existing results will hopefully spark further research. However, there are several caveats to empirical adaptive landscape literature that need to be addressed by the community in general.

Empirical studies rarely address the scale of the adaptive landscape studied. This has implications with respect to how results should be interpreted. Choosing a larger scale means

considering a wider variety of alternatives with respect to genotypes, phenotypes, or strategies, but does not permit the same depth of analysis that can be performed at smaller scales. The scale of the adaptive landscape studied will be dependent on what types of questions one hopes to answer. At smaller scales, where individuals and not populations are points on the landscape, questions about the distribution of individuals within a population, as well as the processes determining this distribution, can be studied. Larger scale analyses consider the patterns of population occupancy across large regions of the adaptive landscape. Populations are single points within the landscape and broad scale patterns can be examined. Questions that can be asked include how predictably does selection explore the landscape and to what extent are the occupied peaks the highest peaks?

Similarly within the literature there is no agreed upon method for ordering axes. This is problematic because the smoothness of the experimental landscape will depend on how genotypes are ordered along axes. Fitness is assigned to a particular point in genotype space, and depending on the ordering of genotypes, the topology of the landscape in the immediate vicinity of any point will change. Thus the inference about the ruggedness of the landscape depends on how genotypes are ordered, which in some studies may be arbitrary.

Even if the results of empirical adaptive landscape studies address the caveats mentioned above they cannot tell us directly about the topology of the landscape in natural systems. It is likely that within natural environments, where systems are more complicated and likely to change temporally, adaptive landscapes will be increasingly rugged. While intuitive, this inference has yet to be tested experimentally. Another unexplored caveat is whether landscape topology changes as natural environments change through time. Experimental tests

exploring the permanence of landscape through time in the face of environmental change are necessary to test whether landscapes are more like turbulent oceans or fixed alpine ridges. If adaptive landscapes are constantly changing they will not constrain the adaptive process. Microbial experiments in changing landscapes would provide insight into whether simplified landscape topology change through time.

Ultimately it appears that adaptive landscapes at the population level are rugged even in very simple environments, indicating that in nature it is likely there will be many adaptive solutions in a given environment. Implications from this are that anthropogenic changes to the environment, such as those seen currently in natural systems through climate change and in clinical settings with the evolution of antibiotic resistance, will result in unpredictable outcomes and make it difficult to predict the long-term consequences of our actions.

Chapter 3

Adaptive landscapes in evolving populations of *Pseudomonas fluorescens*

INTRODUCTION

Stephen Jay Gould often made the point that if life's tape were rewound and replayed, the outcome would be markedly different (Gould 1989). Adaptive evolution, in his view, is a contingent process dominated by historical accidents. The implication is that making substantive claims about the predictability or repeatability of the evolutionary process will be difficult if not impossible. Gould's view contrasts with a growing number of empirical examples of parallel evolution, the evolution of the same genes and phenotypes repeatedly and independently in response to similar selection. Hallmark examples of parallel evolution include morphological traits in lizards (Losos 1992) and fish (Colosimo *et al.* 2005). Parallel evolution also seems to be a common feature in the evolution of antibiotic resistance among many pathogenic bacteria where the genetic targets of resistance to specific classes of drug are well known. For example, resistance to quinolone antibiotics in Gram-negative bacteria is often rapid and stereotypical, with characteristic mutations in genes such as *gyrA* being almost invariably the first to emerge in both the clinic and laboratory (Piddock 1999; Jacoby 2005).

Whether or not adaptive evolution is predictable or repeatable in terms of the particular traits or genes that respond to selection depends to a large extent on the nature of the adaptive landscape (Wright 1932), an abstract graphical representation constructed by plotting fitness against genotypes or phenotypes in a given environment. Selection increases population

mean fitness, thus driving populations up peaks within a landscape. As such the shape of the landscape plays a major role in the evolutionary process. The ruggedness of the landscape is often taken to be a measure of the variety of genetic routes available, and thus the predictability of adaptive evolution. Smooth landscapes contain a single, Mt Fuji-like fitness peak and selection is expected to be highly repeatable such that independently evolving populations starting from arbitrary positions in the landscape will eventually end up at the same genetic and phenotypic 'solution', defined by the location of the fitness peak. More rugged landscapes, by contrast, contain multiple fitness peaks and valleys such that populations that occupy different locations in genotype or phenotype space are likely to explore only local fitness peaks, making the outcome of adaptation more difficult to predict.

Assessing the ruggedness of 'real' adaptive landscapes requires a method to quantify evolutionary repeatability. The most direct way is to follow the evolutionary process in many replicate selection lines adapting to a novel environment and then map the underlying genetic routes taken. The variance in fitness, or any other phenotypic measure, among evolving lines provides a measure of repeatability. Two sorts of inferences can be made. The first comes from the analysis of the variance among replicate selection lines derived from the same initial genotype. This variance necessarily increases initially as mutations occur and are substituted. Over longer time scales, the dynamics of this variance shed light on the ruggedness of the underlying landscape. If the variance among replicate lines is maintained, as it has been in the *Escherichia coli* long-term selection lines (Lenski *et al.* 1991; Lenski and Travisano 1994), the implication is that the underlying landscape is rugged. A smooth landscape, by contrast, can be inferred if the variance among replicate lines eventually decreases as fitness increases. The second inference comes from the analysis of

the variance in fitness among distinct starting genotypes. Now, the initial variance is expected to be large because each genotype starts from a different location in the landscape. If the underlying landscape is rugged, this large variance will be maintained or even increase through time. On a smooth landscape, however, the variance among genotypes will decline as all types converge on a single adaptive solution.

Previous microbial evolution experiments have provided examples of both parallel and divergent evolution in traits at different levels of organization (i.e. fitness, genes, or sequences). As previously mentioned, the long-term *E. coli* lines have small variance in fitness among replicate lines maintained over 50 000 generations (Lenski *et al.* 1991). However fitness measures in this experiment provide only a very approximate measurement for determining whether populations are following different evolutionary trajectories because very similar phenotypes may be achieved by considerably different underlying genetic sequences. This caveat was addressed in further studies. The same evolving populations showed greater variation in fitness when assayed in novel assay environments (Travisano *et al.* 1995), indicating populations had reached different adaptive solutions. Genetic analysis confirmed these results; while changes occur in the same 59 genes in evolving replicate populations, and in the gene *spoT* in 8 of 12 populations, the location of the mutations within the *spoT* gene was different in all cases (Cooper *et al.* 2003). Similar expression patterns and fitness values may seem to indicate similar adaptive solutions, however, these traits were the result of mutations at different sites within a gene and different genes, indicative of populations evolving along different trajectories within a rugged landscape. Studies examining the evolution of antibiotic resistance also seem to indicate a prevalence of rugged underlying landscapes (Schrag *et al.* 1997; Levin *et al.* 2000; Maisnier-Patin *et al.* 2002).

Generally, resistance is costly in the absence of the antibiotic but when resistant strains are placed back in a permissive environment they do not ascend the same peak on the landscape by losing resistance, instead they ascend a new peak via compensatory mutations.

Here we follow the dynamics of the variance of fitness and metabolic phenotypes within and among six distinct *P. fluorescens* genotypes to two different single carbon source environments. The use of distinct initial genotypes ensures that the starting populations are spread over a large area of the underlying landscape, capturing as much of the present variability as possible and sampling a more extensive range of possible evolutionary trajectories. Our experiment thus extends similar analyses on the variance among replicate selection lines (e.g. Lenski *et al.* 1991; Korona *et al.* 1994; Nakatsu *et al.* 1998; Barrett *et al.* 2005) to include information on how divergent genotypes respond to selection in the same environment. This analysis thus provides a more robust means of inferring the ruggedness of adaptive landscapes in different environments. To our knowledge this is the first microbial experimental evolution study investigate the topology of the underlying adaptive landscape in this way.

MATERIALS AND METHODS

Starting Strains and Growth Conditions

The original founding ancestor of all genotypes was the soil bacterium *P. fluorescens* strain SBW25. Strains used for the selection experiment were neutrally marked with the insertion of the *lacZ* gene (Zhang and Rainey 2007) and are isogenic to the wild type. Colonies with l*acZ* grow blue on agar plates supplemented with 40 mg L^{-1} of 5-bromo-4-cholor-3-indolyl-beta-D-galactopyranoside (X-Gal), and can be easily distinguished from pale yellow wild type colonies. Both *P. fluorescens* SBW25*lacZ* and SBW25 clones were frozen at -80°C in 16% (v/v) glycerol.

Six genotypes were used to found 24 selection lines, with 4 replicates per genotype, in two different environments. The selection experiment consisted of 48 selection lines: (6 genotypes X 4 replicates/starting genotype X 2 environments) diluted by serial transfer daily for 75 days. The six founding genotypes had all evolved from the ancestral SBW25*lacZ* strain in different environmental conditions ensuring they were in disparate starting locations within the landscape. Four of the six genotypes had previously been evolved in various carbon-limited environments for approximately 400 generations (M: mannose, G: glucose, X: xylose and MGX: a combination of all three). Two other genotypes were included; a wrinkly-spreader phenotype previously cultured in static experimental microcosms (Meyer *et al.* 2010), and finally SBW25*lacZ*, the ancestor of all other genotypes.

All populations were cultured in 24 well plates (Corning Incorporated, Corning, NY), with 2 ml of media in each well, in an orbital shaker (150 rpm) at 28°C. The culture media consisted of M9 minimal salts (1 g l^{-1} NH_4Cl, 3 g l^{-1} KH_2PO_4, 0.5 g l^{-1} NaCl and 6.8 g l^{-1} Na_2HPO_4)

supplemented with 15 mg l^{-1} $CaCl_2$ and 0.5 g $l^{-1}MgS0_4$, and a source of carbon. Two monosaccharides were used as sole carbon sources: glucose ($C_6H_{12}O_6$) and xylose ($C_5H_{10}O_5$). The chosen concentration of each sugar (glucose: 9.57mg/L, xylose: 255.22 mg/L) resulted in equal population densities of the ancestral strain after 24 hours (~$2x10^7$ colony forming units/ml) and is the same concentration used in the previous evolution experiment from which the M, G, X and MGX genotypes were derived.

The two selection environments differ only in their carbon source, glucose or xylose. Generally, Pseuodomonads metabolize glucose via the Entner-Doudoroff pathway and/or the pentose pathway, while xylose can only be metabolized via the pentose pathway (Rahkimova *et al.* 1998; Fuhrer *et al.* 2005). Thus two possible metabolic pathways exist to metabolize glucose and only one pathway to utilize xylose. Little is known, however, about the variety of uptake pathways. Previous work from our lab has shown that *P. fluorescens* SBW25 grows much better on glucose than on xylose, consistent with the observation that this strain lacks *xylB*, a key component of the xylose utilization operon in other Pseudomonads and Gram-negative bacteria (Song and Park 1997; Rainey 1999).

Selection Experiment and Competitive Fitness Assay

The selection experiment consisted of four replicates of each of the six genotypes in two single carbon source environments (glucose or xylose). A 20 ul sample of each culture was transferred to fresh media every 24 hours (~6.6 generations). This transfer regime was repeated for 75 transfers resulting in approximately 500 generations of selection. Every five days samples from each evolving population were routinely checked for contamination and frozen for further analysis.

The fitness of evolved populations in their selection environment was estimated approximately every 100 generations by competing the evolved populations against the original ancestral genotype, SBW25, lacking the *lacZ* marker. All competing strains were first acclimated from frozen cultures separately on the substrate of interest for two growth cycles (48 hours). Evolved and ancestral cultures were then mixed at a ratio (evolved: ancestor) of 1:1 in glucose and 1:100 in xylose for a transfer volume of 20 ul. The xylose-evolved lines required a greater proportion of ancestral culture at the start of competition in order to prevent evolved strains from entirely out competing the ancestor by the end of the assay period. Strains were grown together for three growth cycles, with estimates of the relative frequency of both types quantified after the first growth cycle (24 hours) and the third growth cycle (72 hours) by plating on agar plates containing M9 minimal salts and glucose, supplemented with X-Gal. We estimated relative fitness, ω , by calculating the rate at which the frequency of the evolved population changes relative to its ancestor, with the following equation:

$$\omega = 1 + \frac{\ln(f\ \text{final}) - \ln(f\ \text{initial})}{doublings} \qquad (1)$$

where f initial and f final are the relative ratios of the evolved population relative to the ancestral populations after the first growth cycle (initial) and after the third growth cycle (final) respectively. *Doublings* refers to the number of doublings or generations (calculated for the ancestral strain) that occur between the initial and final measurements (48 hours). Note that tracking the dynamics of mean fitness and the variance in fitness among lines over

time necessitates a large number of competition experiments so we elected to obtain just a single fitness estimate for each replicate population at each time point. This necessarily means that we sacrifice the ability to conduct significance tests on any given estimate of fitness for a given selection line.

BIOLOG Assays

To assay for changes in the metabolic physiology of the selected strains as a result of adaptation to different carbon sources, we assayed evolved and ancestral genotypes on commercially available BIOLOG GN2 microplates (BIOLOG, Hayward CA) to obtain a metabolic profile. BIOLOG plates are 96-well microtiter plates that contain simple agar medium and a tetrazolium dye that acts as an indicator of oxidative carbon metabolism. Ninety-five wells contain a unique single carbon compound that acts as the sole source of carbon available in the microcosm, and one well acts as a carbon-free control. Following previous work (MacLean *et al.* 2004) we assayed a given strain on a BIOLOG plate we first grew it overnight in 6 ml of M9KB media (28^0C at 150 rpm). A total of 20 ul of this culture was then diluted in 20 ml of M9 salts [Na_2HPO_4, $6g \bullet 1^{-1}$; KH_2PO_4, $3g \bullet 1^{-1}$; NH_4Cl, $1g \bullet 1^{-1}$; and NaCl, $0.5g \bullet 1^{-1}$] and incubated for approximately 2 h at 28^0C to starve the cells ensuring all cells were in the same physiological state. A total of 150 ul of starved culture was then added to each well of the BIOLOG plate. The absorbance of the microplate was read immediately at 630 nm by using an automated Bio-Tek El_x800 microplate reader, and again after 24 h of incubation +/- 15 min at 28^0C. We calculated a quantitative score for each well (OD_{660} corr) as the observed absorbance after 24 h minus the observed absorbance immediately after inoculation (+/- 15 min), corrected by the maximum OD reading of the control well within the biolog assay for the respective selection environment (OD_{660} corr=

(OD_{660} final- OD_{660} initial)-max OD_{660} control). Single assays of each strain were performed for each line following previous work demonstrating the assay technique to be highly reproducible (MacLean and Bell 2003). Only carbon substrates that sustained growth of at least one strain, either ancestral or evolved at any time point, were retained in the metabolic profiles for analysis.

Statistical Analysis of BIOLOG Profiles

The distance metric used to quantify the divergence of metabolic phenotypes in multivariate space was Mahalanobis distance because it takes into account correlation between the environmental variables that comprise the BIOLOG profile. At each time point within a selection environment, the Mahalanobis distance between each population and the centroid mean of all 24 evolving populations were calculated. Principal component analyses were performed on the covariance matrix of the metabolic profiles of all populations from a given selection environment at a given time point. Similar patterns of variation were found throughout the course of the selection experiment and thus analyses in this paper were performed with the principal component axes calculated from the start of the experiment. This was deemed appropriate because data varied in the same directions throughout the experiment in both environments. All statistical tests were performed in R version 2.9.2.

RESULTS

Relative Fitness

The fitness of all populations increased through time in both glucose (Fig. 1A) and xylose (Fig. 1B), with progress in xylose exceeding that in glucose (Tables 1 and 2). In order to assess whether lines were approaching evolutionary equilibrium the initial and final mean rates of change of fitness for each genotype in both environments were compared (Tables 1 and 2); in glucose there was no significant difference between rates of fitness change at the start and the end of the evolution experiment (Table 1). The mean rate of fitness change in xylose decreased for all six starting genotypes, and this was formally significant for four genotypes and marginally significant for the remaining two genotypes (Table 2). We also asked whether the magnitude of the rate of change for each line was less in the final 100 generations when compared to the first 100 generations using a sign test. 21/24 lines showed evidence of the expected decline in rate of change of fitness in xylose ($P < 0.0001$) whereas only 10/24 lines displayed this pattern in glucose ($P = 0.840$). Taken together, these results suggest that lines evolved in xylose, but not glucose, are approaching evolutionary equilibrium.

Linear and polynomial regressions were fit to the fitness trajectories of the 24 populations evolved in glucose (Table 3) and xylose (Table 4) in order to quantify the evolutionary course each population followed. An ANOVA was used to compare model fits. If neither the linear nor the polynomial regressions had a significant model fit the linear results are presented (Table 3 and 4). Model parameters b_1 (linear slope) and b_2 (curvature of the polynomial) are presented and can be taken as measures of overall rate of fitness increase or the decrease in rate of fitness increase towards the end of the experiment, respectively. Three

glucose selected populations and two xylose selected populations had fitness trajectories that were better fit by a polynomial regression, however for all the glucose trajectories b2 was positive, meaning the curvature of the fit was concave and not implying the populations were approaching a plateau by the end of the experiment. Thus 2 xylose populations no glucose populations appeared to have decreasing rates of fitness increase towards the end of the experiment.

Variance in Fitness Within and Among Starting Genotypes

The variance in fitness among evolving lines provides a measure of the repeatability of evolution. The variance in relative fitness among replicate selection lines of a given starting genotype differed in glucose (Fig. 2A) and xylose (Fig. 2B). In glucose this variance did not significantly increase when initial and final time points are compared (t_5=-0.262, P=0.402) whereas in xylose it did (t_5=-2.421, P=0.030). Figure 3 shows the variance in fitness among all starting genotypes. In generations 300 and 500 lines selected in xylose have significantly larger variance than glucose selected lines (Table 5). These results suggest that the underlying landscape is more rugged in xylose than in glucose.

Metabolic Divergence

We examined the metabolic divergence of all selection lines by calculating the Mahalanobis distance between the metabolic profile of each of the 24 replicate populations and the centroid mean profile of all populations for a given time point and a given environment. If evolving populations tend to converge on the same set of metabolic solutions within a given environment, the mean Mahalanobis distance in a given environment will decrease through time. The trajectory of the mean Mahalanobis distance in glucose and xylose is shown in

figure 4. The Mahalanobis distance among populations in glucose remains modest and approximately constant throughout the experiment. In xylose, however, populations diverge to a much greater extent towards the end of the experiment.

Inspection of the distribution of Mahalanobis distances in each environment illustrates the same conclusions. The differences in scale of the Mahalanobis distribution through time (Figure 5), specifically the decreasing variance in glucose and drastically increasing variance in xylose, as well as the difference in the shape of the distribution, increasingly unimodal in glucose and bimodal in xylose, also indicate glucose populations appear to be clustered around a single metabolic profile throughout the experiment (Fig. 5A) whereas in xylose two distinct metabolic profiles evolve by the end of the experiment (Fig. 5B). This result is consistent with the results from the analysis of variance in fitness through time, and lends further support to the notion that the metabolic landscape in xylose is more rugged than that in glucose.

Principal Component Analysis of Metabolic Profiles

We performed a principal component analysis on the metabolic profiles to investigate how the location of all populations in metabolic space changed through time. These results are shown in figure 6. We first conducted a principal components analysis using the founding populations. As expected, the analysis distinguishes clearly among the different founding genotypes and replicate populations within each genotype cluster close together using just the first two principal components, which together explain 93% of the variation. We then examined how the evolved populations had changed along these same two principal components at the end of the experiment. At generation 500 the first two principal

components from the start of the experiment explained 68% of the variation in glucose and 87% of the variation in xylose. In glucose, all populations appear to group together within metabolic space whereas in xylose there are at least two groupings at generation 500. Figure 7 shows this contrast more directly by illustrating the consensus metabolic profiles that one would see when visually inspecting a BIOLOG plate for all populations in glucose and for each of the two population groupings in xylose. The consensus metabolic profile in glucose is clearly distinct from both profiles in xylose. Moreover, the six populations that represent the most divergent group in xylose have, intriguingly, lost the ability to metabolize most substrates present on a BIOLOG plate. Taken together, these results are consistent with the underlying landscape being more rugged for xylose than it is for glucose.

DISCUSSION

The ability to predict the outcome of adaptive evolution is often thought to depend on the ruggedness of the underlying adaptive landscape. However the topology of 'real' adaptive landscapes remains difficult to assess. Here we have presented an empirical approach to exploring the relative ruggedness of adaptive landscapes that makes use of information on the dynamics of the variance in fitness and metabolic phenotype coming from replicate evolving populations of microbes in the laboratory. Our results reveal that both types of variance displayed a tendency to increase through time in xylose, as mean fitness appeared to plateau. In glucose, by contrast, the magnitude of variance in fitness and metabolic profile remained relatively modest throughout the experiment, while mean fitness continually increased. Taken together, these results imply that the underlying adaptive landscape is more rugged for xylose than for glucose.

Further support for this interpretation comes from recent theory on the genetics of adaptation (Orr 2006). Analysis of mutational landscape models that view adaptation as a sequence of moves in DNA sequence space suggest that adaptive walks – the number of sequential substitutions of beneficial mutations by selection required to reach the fittest genotype – tend to be longer on smooth than rugged landscapes because in rugged landscapes local optima are more common and therefore usually closer to the adapting population (Orr 2006). Although we have not explicitly tracked the number of beneficial mutations fixed in our experiment, our observations on the dynamics of mean fitness are at least consistent with the interpretation of mutational landscape models: the majority of lines in xylose display a decline in the rate of change of mean fitness by the end of the experiment, consistent with them reaching or approaching evolutionary equilibrium. This is not true in glucose, where

mean fitness shows no signs of reaching a plateau at any point in the experiment. Thus the lines in glucose are still adapting whereas the bulk of adaptation in xylose has been accomplished by generation 500. The interpretation of this result with respect to adaptive walks is that more substitutions, perhaps of smaller effect, remain to be fixed in glucose evolving populations before evolutionary equilibrium is reached. This is consistent with the predictions of DNA sequence-based models of adaptation and this result can be used to infer that glucose has a landscape that is smoother when compared to that of xylose.

Two alternative interpretations of our results seem possible. First, all populations in both selection environments might share the same rugged underlying landscape but populations adapting to xylose are near a valley, while populations adapting to glucose are close to a local peak. This interpretation can readily explain the result that the variance in fitness and metabolic profile remains low over the entire experiment in glucose but increases dramatically in xylose. However, it is difficult to explain why the rate of change of mean fitness shows no evidence of decreasing in glucose but does in xylose; if this interpretation were true it would be expected that glucose populations would have decreased rates of fitness change as they approach the adaptive peak, which is not the case.

Second, all populations in both selection environments might share the same smooth underlying landscape, with xylose populations starting farther away from the optimum than glucose populations. Previous work has shown that there are a greater number of available mutations that have a wider range of beneficial fitness effects as populations move further from the optimum (Kassen and Bataillon 2006; Martin and Lenormand 2008; Schoustra *et al.* 2009). Thus, glucose populations would have few beneficial mutations available and, among

these, most would have relatively small fitness effects. Xylose populations, by contrast, would have more beneficial mutations with a range of effect sizes from small to large. Although genetic drift and clonal interference may narrow the range of fitness effects ultimately fixed, the variance in fitness effects among mutations fixed is still expected to be larger for populations starting farther from an optimum. Thus in this second alternative explanation of our results the variance in fitness among replicate selection lines sharing a common ancestor is expected to be higher for populations adapting to xylose than for glucose in the early stages of adaptation, as was observed. Although this pattern was present at the beginning of the experiment we also note that the variance remains high in xylose throughout the 500 generations of selection, whereas it declines again in glucose by generation 500. Such a result is indicative of a more rugged underlying landscape underlying xylose compared to glucose.

Mechanistic Explanations for Differences in Landscape Ruggedness

We have provided experimental evidence that populations adapting to glucose all appear to be converging on the same metabolic phenotype, suggestive of a smooth underlying landscape. Similar patterns of phenotypic convergence have been found in other microbial studies. Evolutionary repeatability was investigated using adaptation of *E. coli* to two different single carbon source environments. Convergent growth phenotypes occurred after 600 generations (Fong *et al.* 2005) but interestingly, the transcriptional states underlying these phenotypes were different and showed patterns of divergence. The extent of convergence in populations from Lenski's long-term *E. coli* lines was assessed after 20 000 generations of selection in glucose limited environments (Cooper *et al.* 2003; Woods *et al.* 2006); extensive parallelism was present at the genic level but this pattern did not hold at the

sequence level where substitutions were rarely identical. Both of these studies found patterns of parallel convergence in phenotype, similar to the pattern of convergence in metabolic phenotype seen in glucose evolving populations in this study. Evidently this phenotypic convergence belies underlying genetic divergence at the sequence level and so suggests that we might expect to observe similar patterns in our experiment. Whole-genome sequencing would obviously resolve this issue directly, however such an effort is beyond the scope of the present paper. We will address this issue in a future contribution.

It is notable that metabolic network studies often lead to the prediction that a single, optimal global phenotype exists which is achieved through equal usage of the metabolic network (Ibarra *et al.* 2002; Fong *et al.* 2003), a result that implies most metabolic networks exist on a rather smooth landscape. Our glucose results are consistent with this expectation but our xylose results are not. Why, then, are there multiple peaks within the adaptive landscape underlying xylose? Several possible explanations seem plausible. The simplest is that there are multiple ways in which fitness on xylose can be improved through modifications to different metabolic pathways or transport mechanisms. Our current knowledge of metabolic pathways in *P. fluorescens* is limited, but we do know that strain SBW25 shows limited growth on xylose, probably because it lacks the xylulose kinase gene *xylB*, a central component of the *xylAB* operon in other Gram-negative bacteria (Rainey 1999). Our experiment clearly demonstrates that this inability to easily use xylose can be readily overcome through mutation, although the genetic changes responsible are not known and will have to await the results of whole genome sequencing.

A second possibility is that the adaptive landscape in xylose is not static but, instead, changes through time. There is good evidence from microbial selection experiments that adaptive landscapes can change as a result of adaptation. For example, Spencer and colleagues (2008) documented the emergence of niche specialists from a common ancestor in *E. coli* populations evolving on a mixture of glucose and acetate, and a host of experiments have observed either frequency-dependent selection maintaining diversity (Elena and Lenski 1997; Rainey and Travisano 1998) or the emergence of diversity through cross feeding (Treves *et al.* 1998, Rozen *et al.* 2000). It is notable that in our experiment adaptation to xylose is associated with an increase in density of nearly 10-fold, suggesting that density-dependent interactions, and thus the strength of resource competition, may become more important in xylose as adaptation proceeds. Increased resource competition could lead to the emergence of diversity within populations. Although an investigation of the extent of diversification within populations is beyond the scope of the present work, we note that finding such within population diversity would not change our leading result that the landscape underlying xylose is more rugged than the landscape underlying glucose.

The Utility of Adaptive Landscapes

The notion of an adaptive landscape, although pervasive in the evolutionary literature since its introduction by Wright (1932), remains controversial largely because it is so difficult to study empirically. Ideally, one would reconstruct all possible combinations of genotypes or phenotypes in a given environment and assess their fitness. This approach is typically not feasible for whole organisms, although it may be for specific molecules (see Lunzer *et al.* 2005; Weinreich *et al.* 2006; Poelwijk *et al.* 2006; Hayashi *et al.* 2006). Our aim here has been to present an alternative methodological approach that makes use of the range of

realized pathways that adaptive evolution takes to make inferences about the underlying landscape. Although we have not investigated these pathways at the DNA sequence level, our use of the variance in fitness and metabolic phenotype through time provides useful information about the *relative* ruggedness perceived by evolving populations in different environments. This approach is not new (see for example Lenski *et al.* 1991) but our study is unique in that it examines the dynamics of variance among both replicate lines derived from a single genotype as well as among different genotypes that occupy distinct locations in fitness and metabolic space. The strength of this approach is that our inferences are more robust than if we restricted attention to just the variance among replicate selection lines derived from a common ancestor because they explicitly incorporate information from genotypes with widely different genetic backgrounds. The underlying landscape is thus more fully explored than if we had started from just a single, isogenic genotype as most selection experiments in microbes do.

One might be tempted to argue, however, that our study still tells us little about real landscapes, perhaps because although they remain a useful heuristic, they do not, in fact, exist. Our feeling, however, is that the pervasiveness of the adaptive landscape idea in the literature justifies its empirical study. The utility of this idea may be limited in practice, but nevertheless it seems worthwhile to at least attempt to test the logical implications of viewing the natural world through an adaptive landscape lens. In this vein, we have attempted only to assess the relative ruggedness of landscapes imposed by two different environments on the same initial set of genotypes. Stronger inferences about the actual topology of the landscape – the steepness of the peaks and the depth of the valleys, whether it includes ridges, or is flat with a series of holes – are not possible with our approach. What we can say is that, from the

perspective of the populations occupying these two landscapes, one appears to be more rugged than the other. Our approach provides a useful starting point that can inform the design of future experiments. We are not alone in the belief that it is possible to study empirical landscapes; our study joins an increasing number of studies that attempt to experimentally investigate the topology of adaptive landscapes at a range of scales, from within genes to within a protein (e.g. Lunzer *et al.* 2005; Hayashi *et al.* 2006; Polewijk *et al.* 2006; Weinreich *et al.* 2006; Lozovsky *et al.* 2009). Previous microbial experimental evolution studies can also be interpreted in terms of adaptive landscapes (see Colegrave and Buckling 2005).

The Predictability of Adaptive Evolution

The ruggedness of the adaptive landscape is often used to make inferences about the predictability of adaptive evolution. Our results lend some support to this idea. Populations evolved in glucose tended to converge on a single metabolic profile, irrespective of the position in metabolic space from which they started. This result is consistent with the underlying landscape being rather smooth and containing a single optimal metabolic peak. In xylose, however, the results were substantially different. Populations starting from different metabolic positions did not necessarily converge on the same adaptive solution. Although the majority of populations (18/24) did converge on a similar metabolic profile by the end of the experiment, the remaining 6 did not. It is notable that not all of these 6 populations were derived from the same common ancestor, suggesting that this result is not a unique feature of a given genetic background or starting position in metabolic space. Rather, these results imply that making *a priori* statements about the predictability of adaptive

evolution of the traits underlying fitness may be challenging, and is likely to depend on the specifics of the environment in which selection occurs.

Chapter 4

Conclusions

Empirical adaptive landscapes are only beginning to be explored in laboratory settings via directed protein evolution and microbial experimental evolution (reviewed in chapter 2). There is a disconnect between the topologies found when studying proteins versus those found studying entire populations; protein landscapes are smooth with a single optimal adaptive solution whereas populations appear to be adapting to multiple adaptive solutions along a rugged landscape even in very simple laboratory environments. The general ruggedness of population level adaptive landscapes seems to indicate evolution will be difficult to predict in most environments.

In my thesis I contributed to this growing body of research by investigating the empirical adaptive landscapes that underlie two different simple environments using several strains of *Pseudomonas fluorescens*. First, I explored evolutionary trajectories of all populations as inferred from fitness data. Measures of variance in fitness within and between genotypic populations indicated that the xylose environment had a more rugged underlying landscape when compared with the glucose environment. Secondly, I measured metabolic phenotypes of each population throughout the selection experiment. Metabolic phenotypes diverged over the course of 500 generations of selection; in glucose populations all appeared to be reaching a similar adaptive solution on a smooth underlying landscape whereas xylose populations appear to be clustering into at least two different metabolic solutions on a more rugged landscape.

I found that even simple environments vary in adaptive landscape topology and that the repeatability of evolution is likely to be dependent upon the environment in which selection is occurring. More broadly I advocate the use of multiple starting strains within a selection experiment in order to explore broader areas of the adaptive landscape and also because it allows for contrasts in both within and between starting genotype variance. This is the first study to use such methodology and hopefully in the future others will utilize this approach

Table 1. Mean initial and final relative fitness measures of the four replicate lines initiated from a single starting genotype, evolved in glucose. The differences between initial and final rates of fitness change for each genotype were evaluated using a paired-sample, one-tailed *t*-test (*df*= 3 in all cases).

Selection line	Initial mean fitness (+/- standard deviation)	Final mean fitness (+/- standard deviation)	Mean initial rate of change in relative fitness	Mean final rate of change in relative fitness	*t*	*p*
M	1.03 (0.0673)	1.11 (0.0260)	$6.22*10^{-4}$	$2.48*10^{-4}$	1.76	0.0878
G	1.07 (0.00865)	1.16 (0.0346)	$3.17*10^{-4}$	$3.84*10^{-4}$	-0.128	0.547
X	0.954 (0.0727)	1.13 (0.0335)	$9.90*10^{-4}$	$5.44*10^{-4}$	0.657	0.279
MGX	1.06 (0.0141)	1.12 (0.0826)	$-4.18*10^{-4}$	$2.35*10^{-4}$	-2.11	0.937
SBW25	0.990 (0.0229)	1.10 (0.0435)	$3.26*10^{-5}$	$3.23*10^{-4}$	-0.956	0.795
WS	1.01 (0.00871)	1.10 (0.0395)	$-8.75*10^{-5}$	$2.70*10^{-4}$	-1.65	0.901

*Significant at $P < 0.05$

Table 2. Mean initial and final relative fitness measures of the four replicate lines initiated from a single starting genotype, evolved in xylose. The differences between initial and final rates of fitness change for each genotype were evaluated using a paired-sample, one-tailed *t*-test (*df*= 3 in all cases).

Selection line	Initial mean fitness (+/- standard deviation)	Final mean fitness (+/- standard deviation)	Mean initial rate of change in relative fitness	Mean final rate of change in relative fitness	*t*	*p*
M	1.07 (0.0695)	1.24 (0.0708)	$9.76*10^{-4}$	$-4.03*10^{-4}$	2.10	0.0631
G	1.01 (0.0342)	1.31 (0.119)	$2.27*10^{-3}$	$2.20*10^{-4}$	1.93	0.0750
X	1.07 (0.0208)	1.30 (0.0477)	$2.03*10^{-3}$	$5.42*10^{-4}$	3.05	0.0463 *
MGX	1.11 (0.0660)	1.19 (0.0867)	$1.35*10^{-3}$	$-1.49*10^{-3}$	3.65	0.0178 *
SBW25	1.04 (0.0647)	1.21 (0.102)	$1.99*10^{-3}$	$-6.41*10^{-4}$	4.13	0.0128 *
WS	0.915 (0.0293)	1.27 (0.0607)	$3.86*10^{-3}$	$4.38*10^{-4}$	4.68	0.00924 *

*Significant at $P < 0.05$

Table 3. Regression fits of relative fitness trajectories of all populations evolving in glucose. Linear and polynomial models were compared using an ANOVA. If neither model was significant the linear model fits are presented.). Model parameters b_1 (linear slope) and b_2 (curvature of the polynomial) are presented and can be taken as measures of overall rate of fitness increase or alternately the decrease in rate of fitness increase towards the end of the experiment.

Starting Genotype	Rep	*p* (ANOVA)	Regression Type	*df*	$b_{1/2}$	*Multiple* R^2	*F*	*p*
G	1	0.556	linear	1,4	$1.440*10^{-4}$	0.377	2.42	0.195
G	2	0.491	linear	1,4	$1.777*10^{-4}$	0.793	15.4	0.0173*
G	3	0.464	linear	1,4	$2.734*10^{-4}$	0.674	8.25	0.0454*
G	4	0.628	linear	1,4	$9.810*10^{-4}$	0.0615	0.262	0.636
M	1	0.732	linear	1,4	$1.594*10^{-4}$	0.270	1.48	0.291
M	2	0.0316 *	polynomial	2,3	$1.190*10^{-6}$	0.907	14.6	0.0284*
M	3	0.586	linear	1,4	$1.433*10^{-5}$	0.00325	0.0140	0.912
M	4	0.332	linear	1,4	$9.994*10^{-5}$	0.0604	0.257	0.639
MGX	1	0.199	linear	1,4	$1.579*10^{-4}$	0.150	0.708	0.447

MGX	2	0.00106 *	polynomial	2,3	$1.75*10^{-6}$	0.991	165	$8.58*10^{-4}$*
MGX	3	0.0257 *	polynomial	2,3	$1.36*10^{-6}$	0.935	21.6	0.0166*
MGX	4	0.861	linear	1,4	$-6.06*10^{-5}$	0.121	0.554	0.498
WS	1	0.123	linear	1,4	$2.90*10^{-4}$	0.331	1.98	0.232
WS	2	0.299	linear	1,4	$4.31*10^{-5}$	0.0103	0.0416	0.848
WS	3	0.116	linear	1,4	$2.50*10^{-4}$	0.759	12.6	0.0237*
WS	4	0.360	linear	1,4	$2.21*10^{-4}$	0.598	5.96	0.0711*
WT	1	0.454	linear	1,4	$2.24*10^{-4}$	0.713	9.94	0.0309*
WT	2	0.850	linear	1,4	$2.74*10^{-4}$	0.784	14.5	0.0190*
WT	3	0.935	linear	1,4	$9.41*10^{-5}$	0.162	0.773	0.429
WT	4	0.950	linear	1,4	$2.70*10^{-4}$	0.813	17.4	0.0140*
X	1	0.563	linear	1,4	$2.94*10^{-4}$	0.436	3.09	0.154
X	2	0.931	linear	1,4	$3.07*10^{-4}$	0.583	5.60	0.0772*
X	3	0.823	linear	1,4	$3.86*10^{-4}$	0.740	11.4	0.0279*
X	4	0.570	linear	1,4	$1.59*10^{-4}$	0.152	0.781	0.445

*Significant at $P < 0.05$

Table 4. Regression fits of relative fitness trajectories of all populations evolving in xylose. Linear and polynomial models were compared using an ANOVA. If neither model was significant the linear model fits are presented.). Model parameters b_1 (linear slope) and b_2 (curvature of the polynomial) are presented and can be taken as measures of overall rate of fitness increase or alternately the decrease in rate of fitness increase towards the end of the experiment.

Starting Genotype	Rep	*p* (ANOVA)	Regression Type	*df*	$b_{1/2}$	*Multiple* R^2	*F*	*p*
G	1	0.627	linear	1,4	$6.89*10^{-4}$	0.942	65.7	0.00126*
G	2	0.163	linear	1,4	$1.88*10^{-4}$	0.063	0.270	0.631
G	3	0.557	linear	1,4	$5.02*10^{-4}$	0.665	7.94	0.0480*
G	4	0.742	linear	1,4	$4.99*10^{-4}$	0.777	14.0	0.0202*
M	1	0.756	linear	1,4	$3.14*10^{-4}$	0.559	5.06	0.0876
M	2	0.757	linear	1,4	$4.87*10^{-4}$	0.784	14.5	0.0190*
M	3	0.773	linear	1,4	$4.02*10^{-4}$	0.269	1.47	0.292
M	4	0.374	linear	1,4	$2.11*10^{-4}$	0.214	1.09	0.356
MGX	1	0.941	linear	1,4	$3.50*10^{-4}$	0.385	2.50	0.189

MGX	2	0.130	linear	1,4	$1.55*10^{-4}$	0.212	1.08	0.358
MGX	3	0.0112*	polynomial	2,3	$-2.42*10^{-6}$	0.939	22.9	0.0153*
MGX	4	0.179	linear	1,4	$1.18*10^{-4}$	0.0312	0.129	0.738
WS	1	0.213	linear	1,4	$2.53*10^{-4}$	0.172	0.832	0.413
WS	2	0.301	linear	1,4	$4.61*10^{-4}$	0.293	1.66	0.268
WS	3	0.331	linear	1,4	$3.71*10^{-4}$	0.188	0.925	0.391
WS	4	0.500	linear	1,4	$6.79*10^{-4}$	0.574	4.05	0.138
WT	1	0.390	linear	1,4	$9.24*10^{-5}$	0.107	0.481	0.526
WT	2	0.0658*	polynomial	2,3	$-3.07*10^{-6}$	0.838	7.74	0.0654
WT	3	0.187	linear	1,4	$1.23*10^{-4}$	0.0728	0.314	0.605
WT	4	0.917	linear	1,4	$4.67*10^{-4}$	0.675	8.32	0.0448*
X	1	0.270	linear	1,4	$2.95*10^{-4}$	0.447	3.24	0.146
X	2	0.180	linear	1,4	$4.14*10^{-4}$	0.380	2.45	0.193
X	3	0.771	linear	1,4	$2.98*10^{-4}$	0.201	1.01	0.372
X	4	0.683	linear	1,4	$-6.55*10^{-6}$	$1.41*10^{-4}$	$4.22*10^{-4}$	0.985

*Significant at $P < 0.05$

Table 5. Variance in relative fitness in glucose versus xylose evolving lines throughout the course of the selection experiment. F-tests were performed on the variance in fitness of each of the 6 genotype groups and compared between selection environments at 100-generation intervals.

Generation	*F*	*df*	*p*
0	1.99	22,23	0.108
100	1.49	23,23	0.347
200	2.49	23,23	0.034 *
300	1.05	22,23	0.910
400	1.86	23,23	0.146
500	3.31	23,23	0.00581 *

*Significant at $P < 0.05$

Figure 1. Mean relative fitness trajectories +/- standard error of evolving populations in glucose (A) and xylose (B) selection environments.

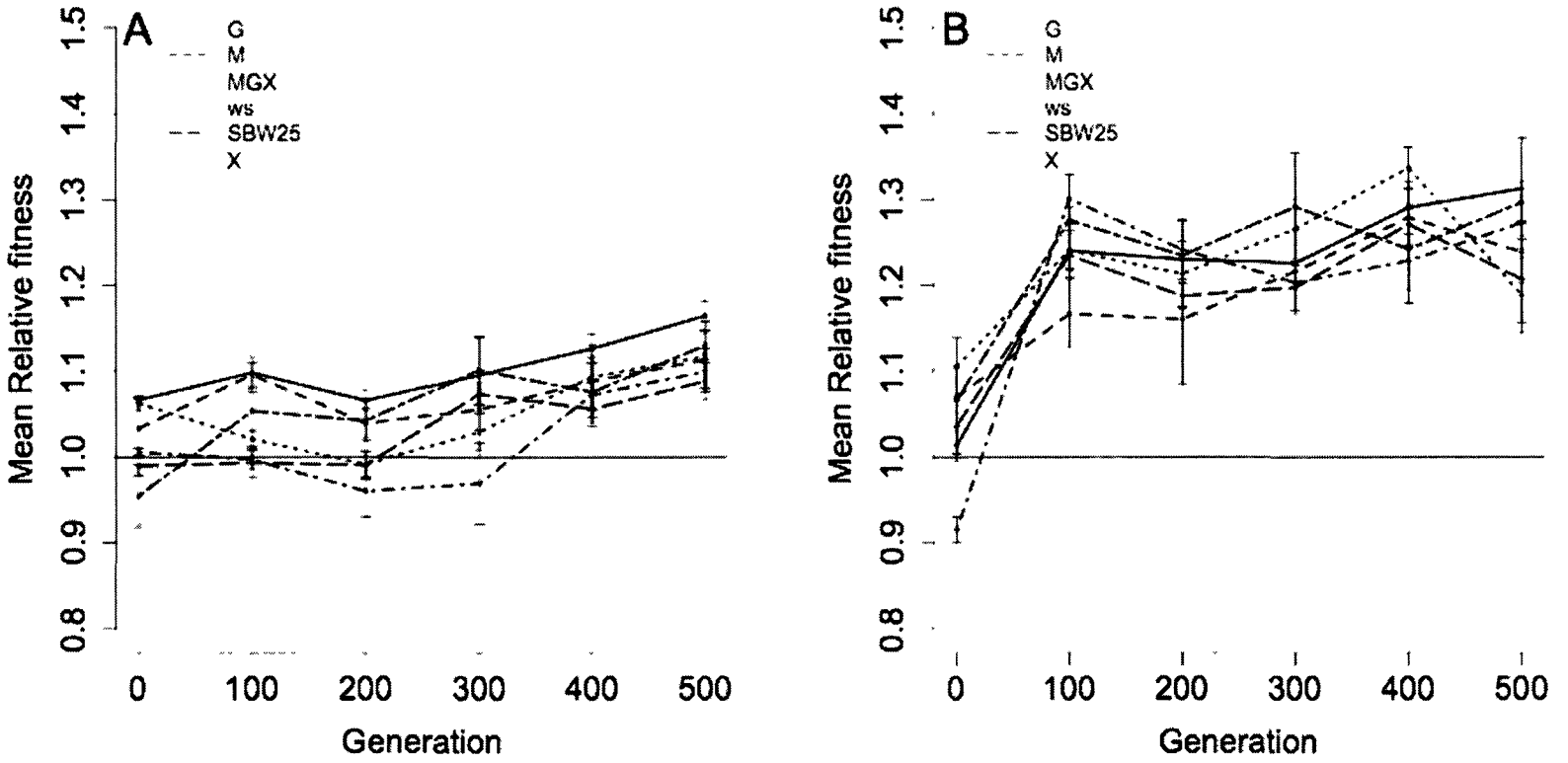


Figure 2. Variance in relative fitness within starting genotypes evolved in glucose (A) and xylose (B). Each line represents the variance in relative fitness among the four replicate populations initiated from the same genotype.

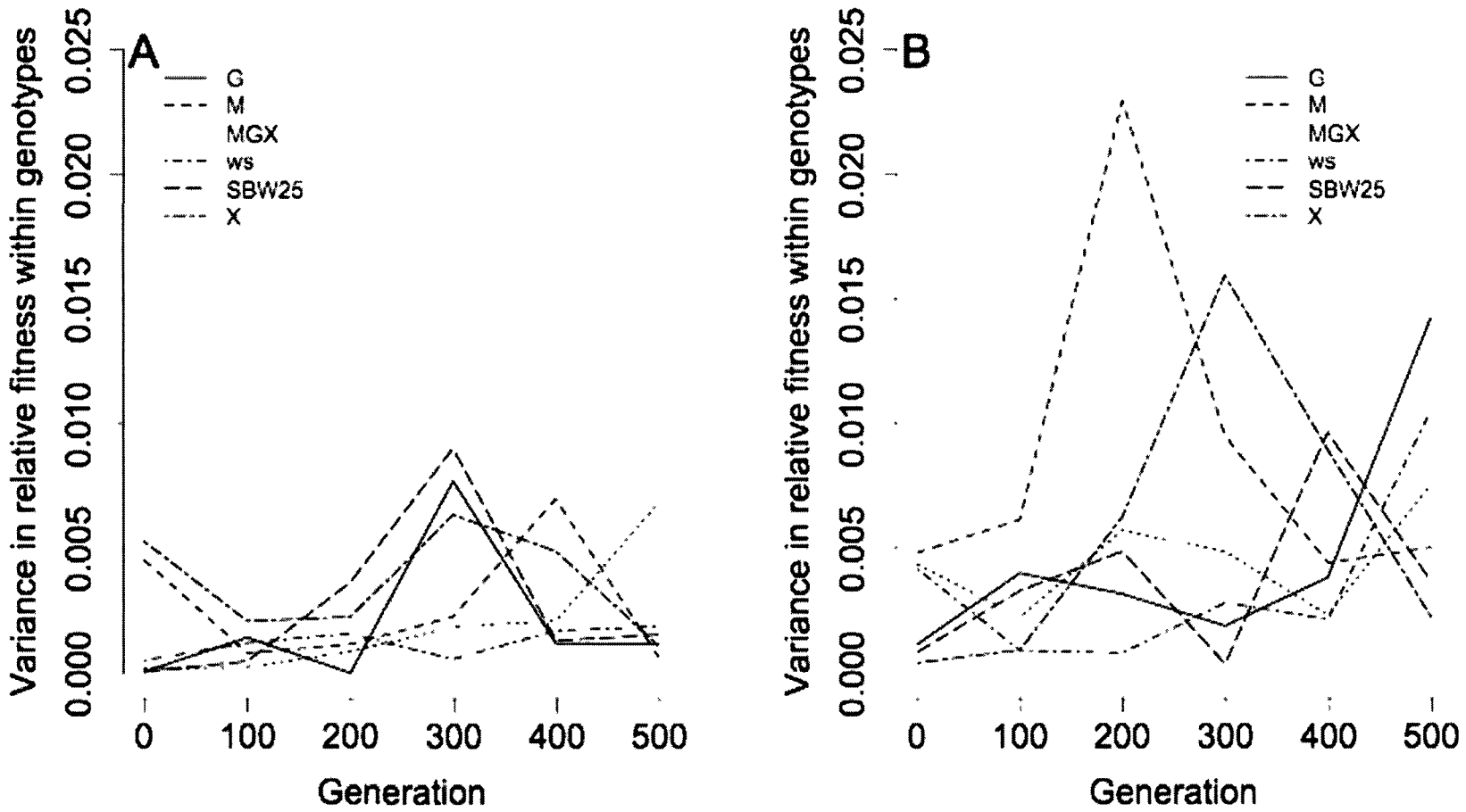


Figure 3. Variance in relative fitness among genotypes in glucose and xylose selection environments. Each line represents the variance in mean fitness among the six evolving genotypes.

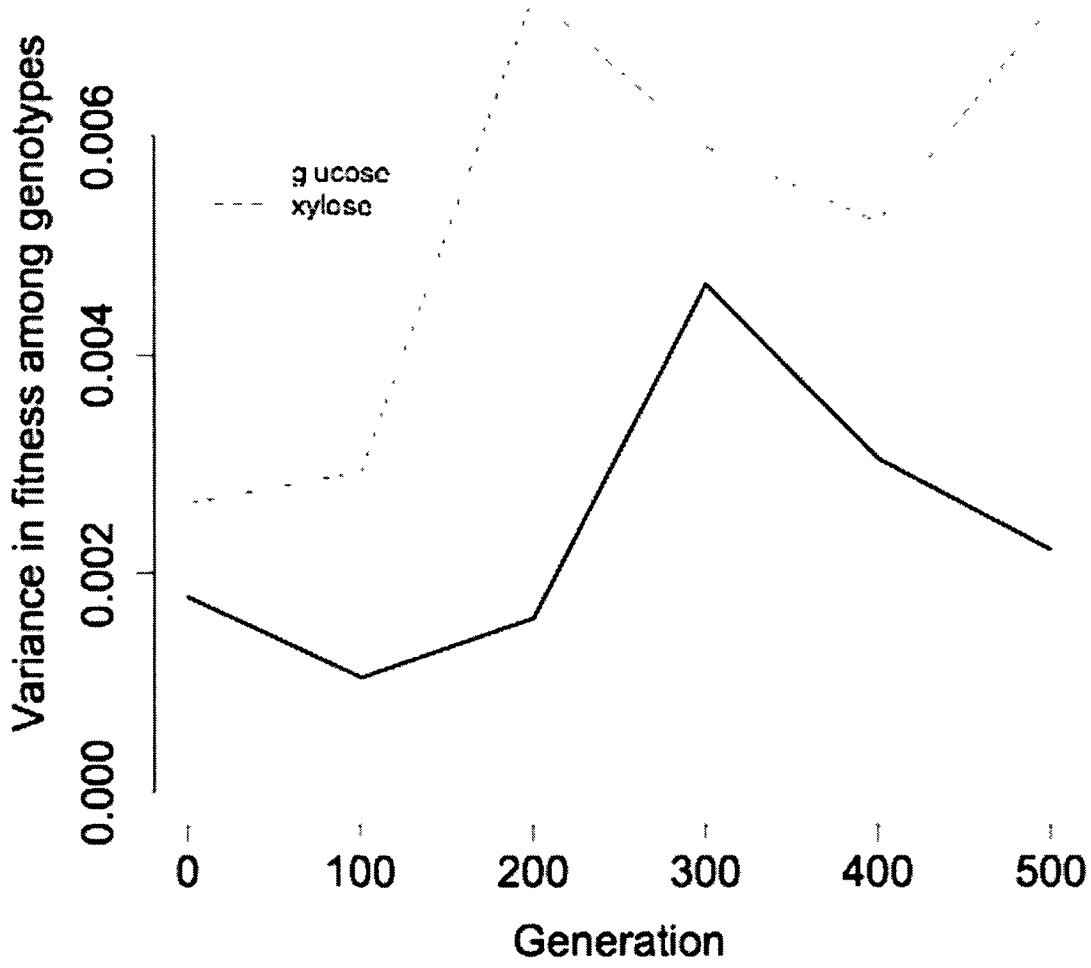


Figure 4. Mean interpopulation Mahalanobis distance of all 24 evolving population throughout the course of the experiment in glucose and xylose selection environments. Each point is the mean of the 24 population Mahalanobis distance measures.

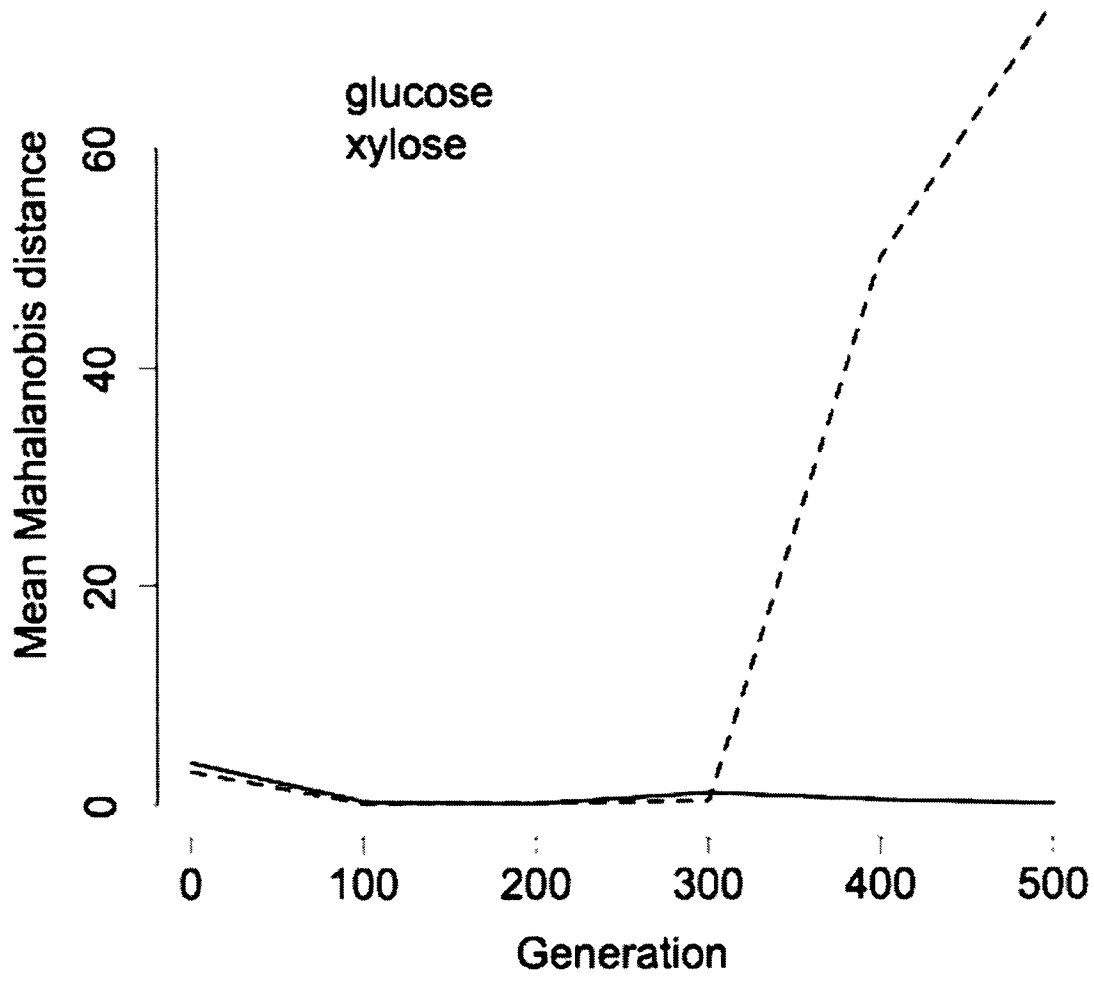


Figure 5. The distribution of interpopulation Mahalanobis distances in glucose (A) and xylose (B) over the course of the selection experiment.

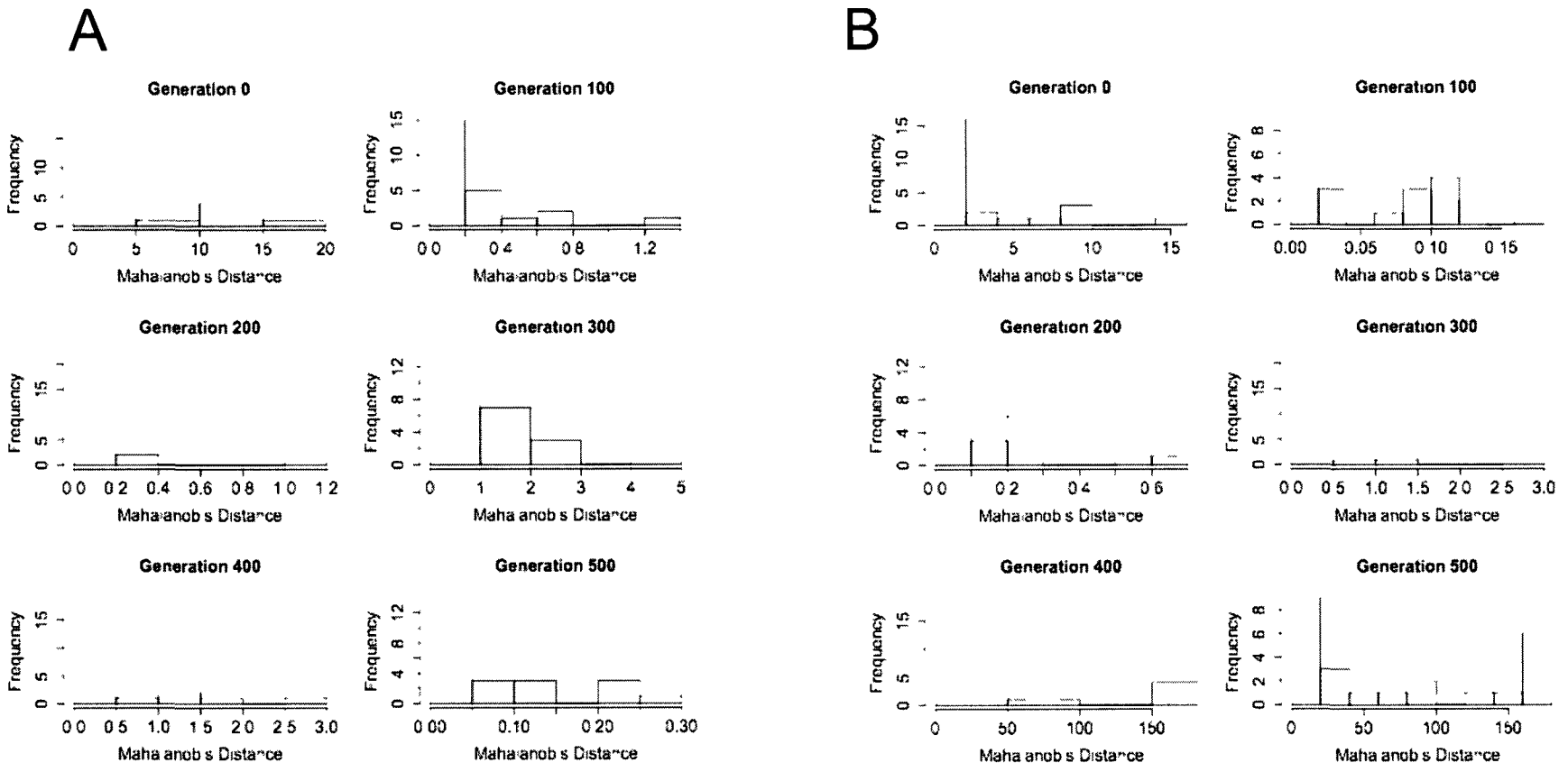


Figure 6. Populations in metabolic trait space along principal component axes one and two at the start of the experiment (A) and at the end of the experiment in glucose (B) and xylose (C) selection environments. Axes from the principal component analysis at the start of the experiment were used throughout to allow for direct visual comparison.

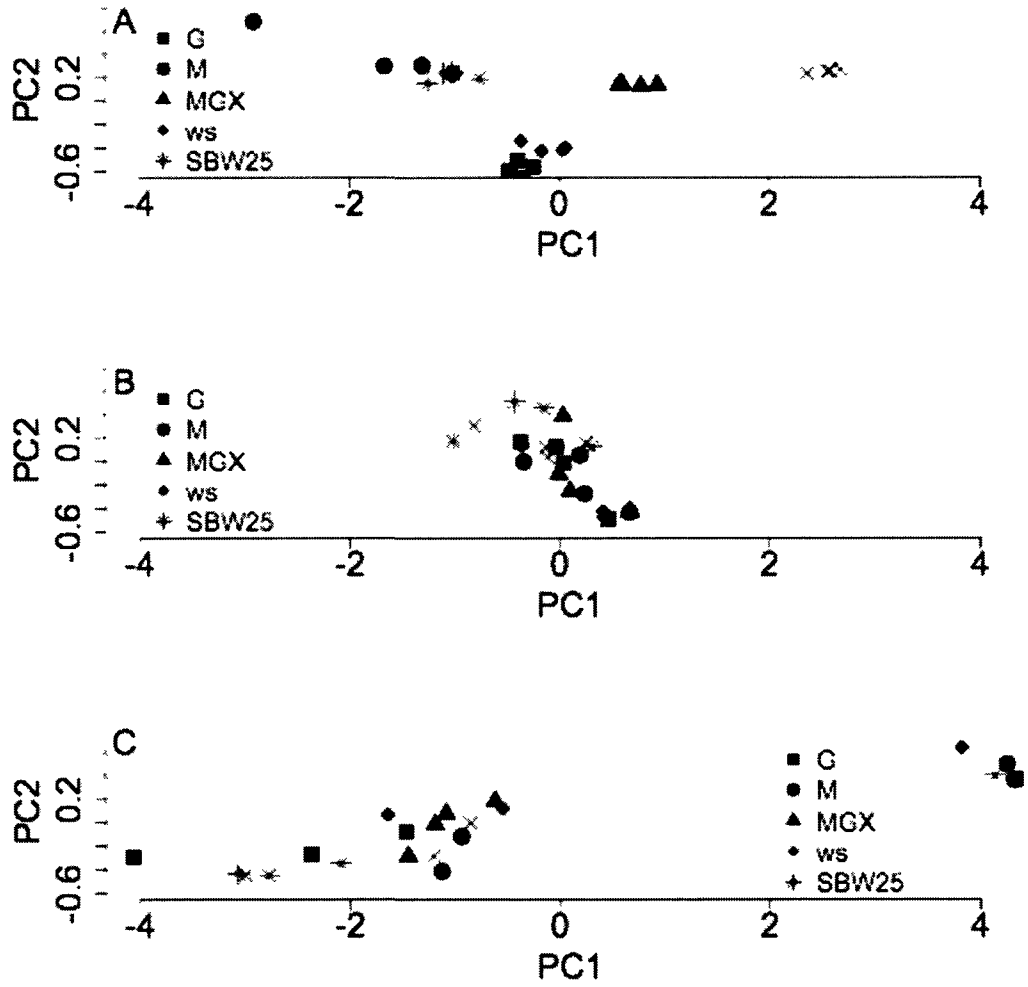
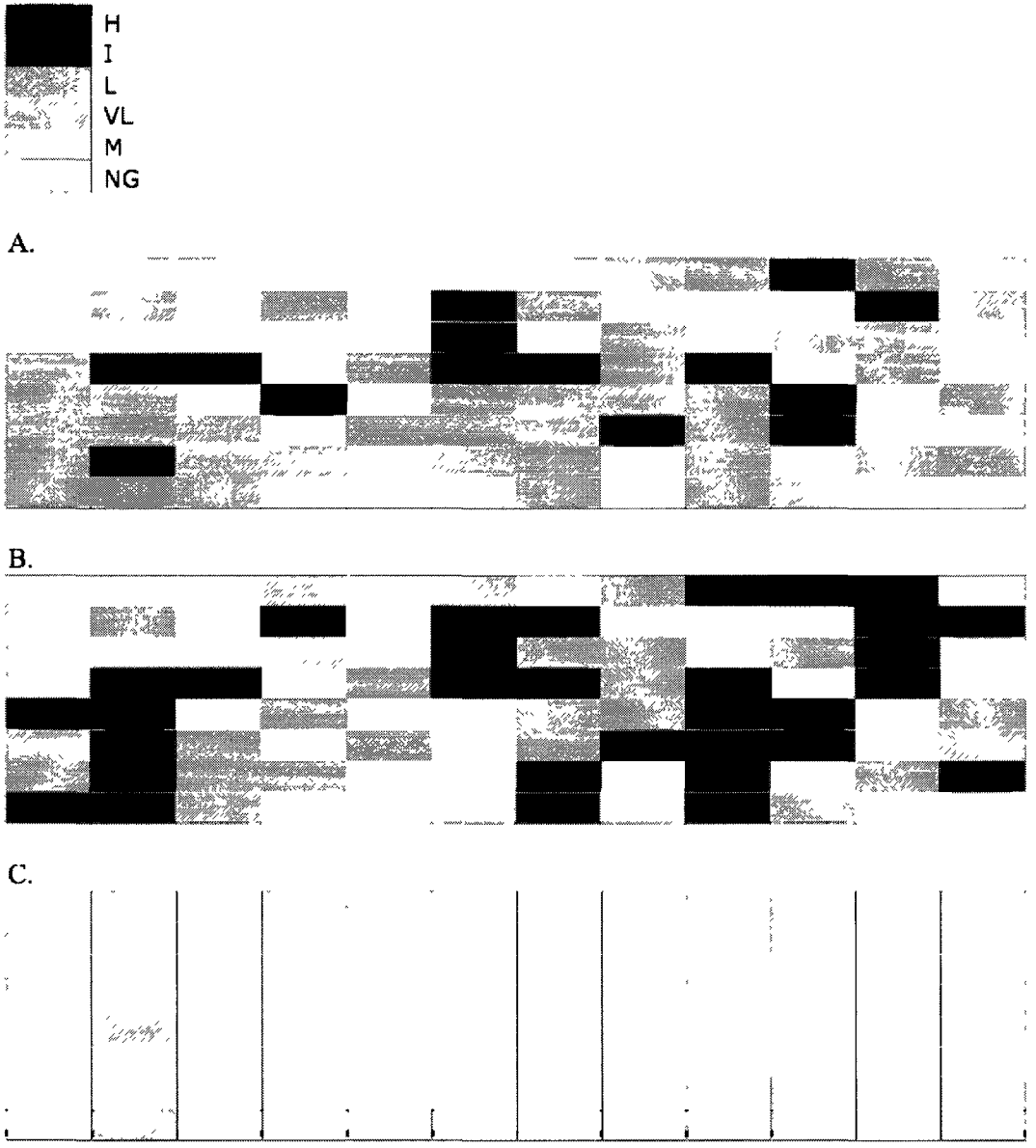


Figure 7. Mean metabolic profiles of glucose (A) and xylose (B, C) generation 500 populations as they would be viewed on a BIOLOG plate. Xylose populations were analyzed in two groups based on clustering from principal component analysis. Most xylose replicate lines had similar expression patterns (B); however, six lines (mannose replicate 1, glucose replicates 1 and 2, mannose-glucose-xylose replicate 2, SBW25 replicate2 and wrinkly spreader replicate 2) were clustered apart and have a different mean BIOLOG profile (C). From darkest to lightest; high (H) metabolism ($OD_{corrected}>1$), intermediate (I) metabolism ($1<OD_{corrected}>0.66$), low (L) metabolism ($0.65<OD_{corrected}>0.33$), very low (VL) metabolism ($0.32<OD_{corrected}>0.11$), minimal (M) metabolism ($0.10<OD_{corrected}>0.01$) or no growth (NG) ($OD_{corrected}<0$).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