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Adaptation and mutation dynamics in populations evolving under nutrient limiting conditions

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Abstract of the Dissertation

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To study adaptation to nutrient limitation and to understand the generation of adaptive diversity in evolving populations, we evolved *Escherichia coli* populations in twelve chemostats under three nutrient limiting regimes: four under nitrogen limitation, four under magnesium limitation and four in environments where the concentrations for both these nutrients are low. We traced the fitness trajectory of these populations over a period of ~400 generations. We also sequenced populations at two different time points to identify targets of selection and to investigate the mutation dynamics in these different nutrient-limiting environments.

Our results show that populations evolving under nutrient limiting conditions have high levels of adaptive heterogeneity. We found 39 and 35 potential targets of selection in environments limited by nitrogen and magnesium respectively. We also found 21 potential targets of selection in environments where the concentrations of both these nutrients are low. Global gene regulators NtrBC were primary target of selection under nitrogen limiting conditions while genes involved in regulating membrane physiology were important targets of selection under magnesium limiting conditions. Our experiments also showed that the evolutionary dynamics of populations evolving in single nutrient limited environment is different to that of

populations evolving in environments where concentrations of multiple nutrients are low; with different nutrients becoming potentially limiting for populations over their evolutionary trajectory in the latter. We also found a transient nature of adaptive mutations in these populations suggesting a complicated process of birth and death of lineages with adaptive mutations. We use this data to describe the fitness landscapes for populations evolving under nutrient limiting conditions.

Overall this thesis increases our understanding of how populations adapt to nutrient limitation and highlights the intricate nature of adaptive mutation dynamics in these evolving populations.

Dedicated in the memory of my late Father, Mr. Mahmud Warsi,
and my family, for the never-ending support

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Chapter One

Introduction

One of the central goals of the discipline of evolutionary biology is study of adaptive mutations, adaptive diversity and mutation dynamics (Orr, 2000; Orr, 2010; Good et al., 2012, Lang et al., 2013). An understanding of the distribution of adaptive mutations and their fitness effects gives one a better understanding of how natural selection works. Most studies that have attempted to investigate these characteristics in natural populations have run into problems of lack of controlled environment, unknown selective pressure, small sample size and no replicates. Alternatively, experimental evolution offers a unique way of getting around these issues by allowing us to use a tractable and a controlled system to study natural selection. My thesis thus makes use of an experimental evolution approach to study the dynamics of adaptive mutations in evolving populations.

Given the advantages of an experimental evolution approach, many evolution experiments have been performed to date, in a plethora of environments. Adaptation to sub-optimal temperature (Lenski and Bennett, 1993; Ketola et al. 2013), limiting carbon resources (Lenski et al., 1991; Dykhuizen and Dean, 2004; Maharajan et al. 2007) and UV light (Alcantara-Diaz, 2004) are just a few in a long list of studies. Most of these have increased our understanding of different aspects of natural selection, mutation dynamics, fitness landscapes, adaptive mutations and interactions between mutations. Three papers from the above-mentioned list of studies were instrumental in setting the objectives for this thesis. These were Luzner et al. (2002), Dykhuizen and Dean (2004) and Maharajan et al. (2007). Maharajan et al. (2007) showed the existence of high level of adaptive diversity in a population adapting to limiting glucose in chemostat. They showed six different adaptive mutations arising in separate clones and being maintained in the same population. Dykhuizen and Dean (2004) and Luzner (2002) demonstrated the mechanisms by which different resource specialists are maintained in the population. Thus two central themes summarize these papers: 1) There are different evolutionary pathways leading to

adaptation to the same selective pressure, and 2) Different adaptive mutations are maintained in the population by different mechanisms that can be tested in the lab.

To study the generation of adaptive diversity in a population, we made use of limiting nutrients as the selective pressure. Resource limitation and competition for nutrients are common selective pressures in nature (Chapin, 1987; Egli, 1992; Klausmeier, 2004; Elser et al., 2007). This has been studied extensively from a theoretical perspective (Tilman, 1980; Huston and DeAngelis, 1994) and many short-term experiments have also been performed in laboratories investigating ecological questions of species diversity and community structures (Tilman, 1981; Grover, 1988). And although nutrient limitation as a selective pressure has been studied before using experimental evolution approaches, there are two aspects of these selective pressures that have not been dealt with properly. These include study of limitation of non-carbon nutrient requirements and study of multiple selective pressures simultaneously. We have formed our understanding of how populations adapt to limiting nutrient conditions based on the many studies that have used carbon conditions as a selective pressure. However, given that different nutrient limitation poses different challenges to an organism, we wanted to investigate if these expectations are upheld when we limit the organism for nutrients that have different effects on the physiology than carbon limitation. ***The first chapter of this thesis thus deals with looking at adaptive responses, both genotypic and phenotypic, under conditions nitrogen limitation and magnesium limitation***.

We also wanted to address the question of adaptive responses in environments where multiple nutrients are in low concentrations. Use of nutrients with multiple selective pressures is difficult to study for two reasons: Firstly, one runs into the problem of Liebig's law of minimum. This law states that in an environment where multiple nutrients are in low concentration, the growth of the organism is only limited by the most limiting of these nutrients. Consequently, one might expect the evolutionary dynamics of the population also to be dictated by the most limiting nutrient. Secondly, it is quite possible that initial adaptation to the most limiting nutrient might result in the population experiencing limitation for the second most limiting nutrient. This results in changing selective pressure

and gives a noisy signal for adaptation. Our experimental set-up allowed us to ask two important questions: 1) Does the evolutionary dynamics in environments where multiple nutrient are in low concentrations mimic those observed in single nutrient limiting environment 2) Is there evidence of changing selective pressure in these environments. ***The second chapter of this thesis thus looks at evolutionary responses in populations evolving in environments where multiple nutrients are in low concentrations; and compares it to the evolutionary responses under single nutrient limitation.***

Finally, we wanted to understand the mutation dynamics under all these three different nutrient limiting conditions. Mutation dynamics can help us infer the distribution of beneficial mutations, fixation of mutations and selective sweep events. These characteristics help us better under the mechanistic nature of natural selection. ***The third chapter of this thesis thus looks at dynamics of adaptive mutations in all the evolving populations under the different nutrient limiting regimes***.

Thus, this thesis intends to study the dynamics of adaptive mutations in populations evolving under limiting nutrient conditions, and compare these dynamics with environments that have potentially multiple nutrient limitations. Controlled environment in chemostats, relatively fast growing microbial populations and use of Next Generation Sequencing (NGS) technology to identify adaptive mutations allows us to go in greater depth to understand the mechanistic nature of natural selection.

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Chapter One
Simplicity, complexity, and heterogeneity of adaptive responses to nutrient limitation

Abstract:

To study adaptation to nutrient limitation, we evolved *Escherichia coli* in nitrogen limited and in magnesium limited chemostats, comparing the evolutionary trajectories under these very different limitations. Population sequencing was performed at the end point of these experiments to identity potential targets of selection and to look at the repeatability of the evolutionary response. With limited nitrogen, the global gene regulators NtrBC are important targets of selection; cellular membrane proteins and nucleotide binding proteins are targets for selection with limiting magnesium. There are high-levels of genotypic heterogeneity in populations across both nutrient-limiting conditions. Multiple low frequency SNPs were found to be repetitive between at least two populations under each selective regime. We also found high disparity between the increase in fitness of these populations and increase in fitness of individual clones further confirming the scenario of a highly complex and heterogeneous evolving population. To understand the physiological mechanism of these adaptations, we measured fitnesses of evolved clones under limitation of alternate nitrogen sources and under energy limitation. We did not find any consistent pattern across different replicates. Together, our results suggest that evolution in the face of nutrient limitation is likely to be far more complex than previously thought.

Introduction:

Nutrient starvation is a common occurrence in many ecosystems (Harder and Dijkhuizen, 1983; Elser et al., 2007; Menge et al., 2012; Farrior et al., 2013; Moore et al., 2013). Adaptation to nutritional stress is thus an important part of the biology of various organisms. Consequently, understanding how organisms adapt to limiting nutrient conditions is an important question in ecology and evolution. To study these adaptations we have employed an experimental evolution approach. This approach allows us to replicate experiments under controlled environments to examine the repeatability in their evolutionary responses (Dykhuizen and Hartl, 1983b)

Most experimental evolution studies on nutrient starvation have been focused on carbon source limitation, finding selection for enhanced uptake of the limiting carbon source (Dykhuizen et al., 1987; Sonti and Roth, 1989; Wenger et al., 2011). Most of these studies have not explored the heterogeneity and complexity in these evolving populations, and have mostly studied individual clones instead of populations. Work done on glucose limitation by Maharajan et al. (2006) gave the first suggestion of high level of adaptive diversity in populations evolving to limiting glucose conditions. Adaptive mutations in global gene regulators, metabolic genes and membrane proteins were found in these populations.

Additionally, very few studies have looked at limitation of other essential nutrients like nitrogen (Jezequel et al., 2013; Hong and Gresham, 2014) or phosphorous (Wang et al., 2010) and only one study has looked at evolutionary adaptation to limitation of metal ions (cobalt metal ion limitation by Chou et al., 2009). And although the common adaptive response in all these studies was an increased uptake of the limiting nutrient these experimental evolution studies have not explored the adaptive diversity in these evolving populations. Using a population sequencing approach that we employ here, we look at the heterogeneity in adaptive responses within populations evolving under nitrogen and magnesium limiting conditions. To study these evolutionary responses in *Escherichia coli*

we carried eight evolution experiments in chemostats under nutrient limiting conditions (four each) for 400 generations

Nitrogen and magnesium perform different functional roles in the physiology of *E. coli*, and thus offer an opportunity for comparing evolutionary responses to different types of nutrient deprivation. Nitrogen limitation results in the induction of the NtrB-NtrC regulon, which induces large numbers of nitrogen-scavenging proteins (Reitzer, 2003, Gyaneshwar et al., 2005). There is also increased transcription of genes involved in murein metabolism and catabolic genes like *ast* (arginine degrative enzymes) and *gab* (GABA degradative enzymes) (Gyaneshwar et al., 2005) under nitrogen limiting conditions. Magnesium is an important cofactor for multiple enzymes and for ribosomes. It is important in stabilizing the outer cell membrane. Magnesium limitation induces phenotypic changes like biofilm formation and reduced cell motility in bacteria (Guina, 2003; Minagawa, 2003) and affects the turnover rate of ribosomes. Both nitrogen and magnesium limitation effects pathogenicity. Uropathogenic *E.coli* colonizing nitrogen deficient urinary tracts overexpress genes involved in nitrogen scavenging (Snyder et al., 2004). Magnesium ion limitation has been shown to induce antibiotic resistance in gram-negative bacteria (Guina et al., 2003),

Populations exposed to nutrient limiting conditions show adaptive responses in diverse sets of genes. Previous studies have shown transporter proteins, metabolic enzymes and regulatory proteins to be involved in adaptive responses to nutrient limitation (Dykhuizen et al., 1987;Sonti and Roth, 1989; Maharajan et al., 2006; Wang et al., 2010; Hong and Gresham, 2014). In our experiments, we demonstrate adaptation to limiting nutrient conditions by competing evolving populations (and clones) against the ancestral strain in the selective environment. Increase in relative fitness of the evolving populations is taken as an indication of adaptation. To identify targets of selection under our experimental conditions we use a population-sequencing approach. Because it reveals the frequencies of SNPs in the evolving populations, population sequencing can identify targets of selection better than clone sequencing. We analyzed the population sequence data to look for genes that showed high frequency SNPs, genes that showed SNPs and were

consistent between replicate experiments and genes that showed multiple independent SNPs within the same population. Genes that show at least two of these three characteristics have a high potential of being targets of selection. Also, given that these nutrients offer contrasting roles to the organism, comparing their evolutionary responses offers greater insight into specificity of natural selection. More specifically, our experimental design allows us to ask whether under nutrient limiting conditions genes involved in general stress response or genes specifically associated with nutrient metabolism are more likely the targets of selection?

Although population sequencing of evolving populations can identify potential targets of selection, it does not tell us anything about the physiological response of adaptive mutations. We attempted to understand the physiological affects of potentially adaptive mutations by measuring fitness of evolved clones on limitation of three sets of alternate nutrients.

1) Relative fitness of clones evolved under limitation of ammonium ion (nitrogen source) was measured on limitation of alternate nitrogen sources. Increased relative fitness on limitation of alternate forms of nitrogen indicates an adaptive response that involves common sets of non-specific transporters, metabolic enzymes or common global gene regulators. On the other hand fitness trade-offs on limitation of alternative nitrogen sources is suggestive that the adaptive response under limitation of ammonia results in loss of functions of unnecessary scavenging operons (for alternate nitrogen sources) or regulators that are induced under nitrogen limiting conditions.

2) Relative fitness of clones evolved under magnesium limitation was measured on nutrient replete conditions. Magnesium limitation results in cell membrane becoming rigid and induces starvation response in the cell. (Guina, 2003; Minagawa, 2003). The changes in cell membrane, which result in making the organism more resistant to antibiotic, might also results in effecting nutrient uptake. A trade-off on nutrient-rich conditions might thus indicate selection for traits involving increased starvation resistance and decreased uptake of nutrients through the cellular membrane. Previous studies have found clones evolved under limiting nutrient conditions to show trade-offs under nutrient rich conditions (Wenger et al., 2011).

3) Relative fitness of both sets of clones (clones evolved under limiting nitrogen conditions and clones evolved under limiting magnesium conditions) was also measured under energy (glucose) limiting conditions. Decreased fitness on energy-limitation might indicate a trade-off due to antagonistic pleiotropy. This might result from constitutive (unregulated) expression of transporters, metabolic enzymes or global regulators. Increased fitness on energy-limitation would suggest selection for a common nutrient stress signal in the cell.

Our study thus has four objectives. First, we highlight the diversity in populations evolving under nutrient limiting conditions. Second, we study the evolutionary responses under limiting nitrogen and limiting magnesium conditions, and look at repeatability of this response. Third, we compare the evolutionary response of populations evolving to limiting nitrogen and magnesium conditions. Fourth, we attempt to understand the physiological basis of adaptation by measuring fitness on alternate nutrient limiting environments.

Results:

Fitness of clones and of the populations under nutrient limitation:

To demonstrate adaptation to respective nutrient limiting conditions, we measured the increase in relative fitness of the populations (with respect to ancestor) at different time points during the course of the experiment (Fig. 1). Populations from four replicate experiments each showed different levels of increase in relative fitness, suggesting multiple possible evolutionary trajectories. The total increase in fitness for populations that were evolved under nitrogen limitation ranged from ~0.027 to 0.041 (Table 1), with most of the fitness increase being seen in the first ~168 generations. Under magnesium ion limitation, the fitness increase in populations showed even greater variation between replicate experiments (fitness increase ~0.011 to 0.0507; Fig. 1, Table 1).

We also isolated single clones from each of the four populations at the end-point of the experiment (400 generations) to measure their fitness (as opposed to fitness

measurements on the entire population, performed above). As shown in Fig. 1, the fitness of the individual clones from each population was statistically different from the population fitness measures (Table 1). To measure how much of the fitness increase observed in the clones was associated with adaptation to nutrient limitation, we measured relative fitness of these clones under non-limiting nutrient conditions. The fitness measure of most of these clones was statistically different between nutrient limiting and nutrient non-limiting conditions (Fig. 2 and Table 2). This demonstrates that most of the fitness increase seen in our clones can be attributed to nutrient specific adaptation. Although we did not investigate the cause of increased fitness to nutrient non-limiting conditions, we think that adaptation to chemostats or change in cell size is responsible for this observation.

Given that we found a difference between the fitness levels of single clones and their respective populations, we also measured the fitness of another single clone from four populations (two from each nutrient limitation). Not surprisingly, the clones had different levels of fitness increase as compared to the population and to other clones (Table 1).

Potential adaptive mutations and evolutionary responses under nitrogen and magnesium limitation

We used whole-genome next-generation population sequencing to identify potentially adaptive mutations under both of the nutrient limiting conditions (Table 2).

Under nitrogen limiting conditions we found a total of three genes containing high frequency SNPs, eighteen genes containing a SNP and being consistent between at least two replicates and sixteen genes containing two independent SNPs in the same population (Table 3). Three genes were found to show at least two of the three above-mentioned characteristic: *glnG (*NtrC*)*, *xdhA* and *paoC*. We describe these below. We also describe a high frequency mutation in gene *glnL* (NtrB), because this gene codes for a protein that interacts with the product of *glnG*.

Three populations showed non-synonymous SNPs (V14L) in gene *glnG* (protein NtrC) while the fourth population showed a 2 bp deletion in gene *glnL* (protein NtrB) (Fig. 3). Specifically, 1) Two populations showed a non-synonymous SNP leading to amino acid change of V14L in gene *glnG* (NtrC). We will refer to this as type1 change in the following discussions (Fig.3), 2) The third population, in addition to the non-synonymous SNP leading to the amino acid change of V14L in gene *glnG* (NtrC), also showed a SNP upstream of the start codon, in a putative regulatory region. We will refer to this as type2 change in the following discussions (Fig.3), 3) The fourth population showed a 2 bp deletion in gene *glnL* (NtrB), leading to premature stop codons. We will refer to this as type3 change in the following discussions (Fig.3). V14L mutation occurs in a conserved region of the protein NtrC, which has been shown to affect interactions between NtrB and NtrC proteins (Pioszak and Ninfa, 2004). The 2bp deletion in *glnL* (referred to as clones with allele type3 in Fig.3) results in a premature stop codon in the protein NtrB, suggesting a complete loss of the functional protein. Thus it appears that nitrogen limitation selects for mutations that affect the interaction between NtrB and NtrC, suggesting that the functionality of this gene complex is not retained in populations exposed to nitrogen limitation.

Two populations showed non-synonymous mutations in gene *xdhA*, while one of these populations also showed two independent SNPs in this gene. *xdhA* codes for the protein xanthine dehydrogenase. Mutations in this gene have been shown to increase the efficiency of utilizing aspartate as a nitrogen source by the organism (Xi et al., 2000). *paoC* codes for a protein which functions as an aldehyde dehydrogenase. It is induced as a response to DNA damage and is involved in purine metabolism. The physiological basis of *paoC* being a target of selection is not clear, and more work will be needed to understand the adaptive response associated with this gene.

Under magnesium limiting conditions we found a total of five genes containing high frequency SNPs, nineteen genes containing a SNP and being consistent between at least two replicates and eleven genes containing two independent SNPs in the same population (Table 3). Three genes were found to show at least two of the three above-mentioned characteristic: *yhaV, fabR,* and *sstT*.

Two out of four populations evolving under magnesium limitation showed non-synonymous SNPs in gene *fabR*, with one of these SNPs reaching a high frequency in the population. *fabR* codes for a protein that regulates fatty acid biosynthesis and is involved in maintaining cell membrane homeostasis. Two populations showed deletion in gene *yhaV*. In one of these populations the deletion reached close to fixation in the population. *yhaV* codes for the toxin in the toxin-antitoxin system in *E.coli*. Two populations showed non-synonymous SNPs in gene *sst,* with one population showing two independent mutations in this same gene. *sstT* codes for an sodium coupled amino acid transporter.

Fitness trade-off in alternate nutrient conditions:

Clones that showed mutations in *glnG* (type1 and type2 in fig. 3 and 4a) and in *glnL* (type3 in fig. 3 and 4a) were isolated from the populations to measure their relative fitness on limitation of alternate nitrogen sources. All the three types showed an increased relative fitness upon limitation of glutamate and arginine as nitrogen sources but these were less than the relative fitness for these clones under limitation of ammonia as nitrogen source. For all the clones, the relative fitness upon glutamate limitation was less than that upon arginine limitation. For two of the clone types, the relative fitness was either comparable or less than the increased fitness under non-limited (nutrient-rich) conditions (Fig. 4a).

Magnesium limitation increases starvation resistance in cells and alters the cell membrane making it rigid (Guina, 2003; Minagawa, 2003). We measured relative fitness of magnesium-adapted clones on nutrient rich conditions to see if continuous selection for starvation resistance and for membrane rigidity results in trade-offs under nutrient rich conditions. We did not find any trade-off under nutrient rich conditions (Fig.4b). The fitness increase of the evolved clone was not significantly different from that of the fitness increase under nutrient replete conditions.

All the clones adapted to magnesium and nitrogen limitation grew better than the ancestor when competing under glucose limitation. However, the three types of clones

adapted to limiting nitrogen conditions all behaved differently under energy (glucose) limiting conditions than under energy replete conditions. Clones harboring the type one and the type three change showed an increase in fitness under glucose limitation that was similar to the fitness increase seen under glucose replete conditions. In contrast, the clone with type two change showed a lower fitness increase in energy-limiting environment than in non-limited conditions (t-test; p=0.108). Clones adapted to magnesium limitation showed an increase in fitness under energy (glucose) limiting conditions (Fig.4c), suggesting selection for general nutrient stress response under limiting magnesium conditions.

Comparing evolutionary response under limiting nitrogen and magnesium conditions:

Magnesium and nitrogen perform drastically different function in the cell. To compare evolutionary response under these nutrient limiting conditions, we first isolated clones that had adapted to limiting nitrogen and limiting magnesium environment. We measured their fitness on limiting magnesium and limiting nitrogen conditions respectively (Fig.5 and Table 4). Clones evolved on nitrogen limitation do not show any significant increase in relative fitness under limiting magnesium conditions. However, clones that evolved under limiting magnesium conditions did show substantial increase in relative fitness under nitrogen limiting conditions.

Discussion:

Adaptation to nutrient limitation is heterogeneous within and between populations:

Time-course population fitness measures and relative fitness of the clones indicates that in our experiments we find adaptation to limitation of nitrogen and magnesium ions. Replicates under the same selective pressure showed different rates of fitness increase and different final fitness values, suggesting that there are many complex evolutionary trajectories for clones and populations in response to the same environmental stress.

Another pattern that repeats in our data is the difference between the fitness of the evolved population and that of clones from these populations. The different evolutionary trajectories and high variability in fitness of clones suggests not only an extensive heterogeneity in adaptive responses between populations under the same selective pressure, but also within populations. Recent studies (Lang et al., 2013; Blundell et al., 2014) looking at mutation dynamics in evolving populations suggest initial stochastic genetic variation and genetic drift in early stages of adaptation to affect the evolutionary trajectories and evolutionary outcomes. Both these factors might well play important roles in giving rise to heterogeneity in our populations. Although variability between evolutionary dynamics of replicate populations is expected, the high level of variability between relative fitness of different clones within a population and between clones and their respective populations is only recently being appreciated. This variability can also be an outcome of interactions between the clones in these populations. Different positive (cross-feeding) or negative (for e.g. release of toxins) interactions are possible in these populations that can result in these observations. One of our future goals is to investigate these interactions.

Dual global gene regulator NtrBC is potential target of selection under limiting nitrogen conditions:

Under nitrogen limiting conditions, we found non-synonymous mutations in global gene regulators NtrC and NtrB (Fig 3). The non-synonymous SNP in the gene coding for protein NtrC is observed in the region of the protein that interacts with protein NtrB, while the 2 bp deletion in the gene coding for NtrB protein results in a premature stop codon. NtrB protein responds to extracellular nitrogen levels, and modulates activity of NtrC through phosphorylation-dephosphorylation steps. NtrC induces the expression of downstream operons that are mainly involved in scavenging of nitrogen sources from the environment and in regulating amino acid synthesis. While mutations in protein NtrB suggest loss of function, mutations in protein NtrC suggest loss of interaction with NtrB protein leading to the unregulated expression of the former (Pioszak and Ninfa, 2004). This suggests that the adaptive response to nitrogen limiting conditions might occur through

constitutive expression of protein NtrC. Clones having mutation in the regulatory region of protein NtrC showed a lower increase in fitness under energy limiting conditions, also suggesting a cost associated with constitutive expression of NtrC (Stoebel et al., 2008). Our pleiotropic fitness experiments further strengthened this view of selection for unregulated expression of NtrC. Growth under both arginine and glutamate is NtrC dependent (Gyaneshwar et al., 2005). Predictably, clones adapted to ammonium limitation also showed increased fitness on limitation by these other nitrogen sources. If the mutations to NtrC involved loss of efficiency of the resulting protein, we would have found a decrease in fitness of clones as compared to the ancestor, under limitation of alternative nitrogen sources.

Our results are surprising at two levels. Firstly, given the futile usage of energy and resources by the scavenging proteins induced by NtrC, inactivation of NtrC had appeared to be a more likely outcome. However we see selection for continuous expression of this protein. It is interesting to note that unregulated expression of scavenging operons is also seen in diatoms in environments that are iron-limited. This results in an increased efficiency of reallocation of iron to needed proteins, which might alleviate the cost for loss of regulation. It is possible that clones adapted to nitrogen limitation show unregulated expression of NtrC for the same reason i.e. increased re-usage of elemental nitrogen for needed proteins. Secondly, most cases of adaptation to limitation of carbon resources, which are metabolized by genes in a single operon, also proceed by constitutive expression of the operon. Our results show the same type of response under a more complicated biochemical set-up, where the regulator is controlling multiple operons. It is interesting to contrast this result with other studies that have looked at adaptive responses to nitrogen limitation. Hong et al. (2014) showed that adaptive responses to nitrogen limitation in yeast results in copy number variation for the transporter protein. And Jezequel et al. (2013) have shown that one of the adaptive responses to nitrogen limitation for prokaryote *Acinetobacter baylyi* involve mutation in gene *glnK.* The protein coded by this gene interacts with NtrB to induce expression of NtrC, the latter two being targets of selection in our experiments. Thus, we find different proteins of the same regulatory protein complex to be targets of selection in the two prokaryotic species that have been studied, while we

find copy number variation for transporter proteins to be the adaptive response in eukaryotes. Further work is needed to understand the consistency and the significance of these different adaptive strategies in different groups of organisms.

Cell membrane proteins and deletion of a toxin gene are potential adaptive mutations under Magnesium ion limitation:

In contrast to starvation of nitrogen, starvation of magnesium ion is more diverse in its evolutionary response. Across the four replicates evolving under magnesium ion limitation,

we found genes involved in cell-membrane physiology to either consistently show SNPs between replicate populations (*fabR*) or show high-frequency SNPs (*fabR, lptG*) in a given population. This result is expected because magnesium ion plays an important role in stabilization of cellular membrane. As early as 1969, Fiil and Branton showed that cells growing in magnesium deficient environment show changes in cell membrane structuring. Importantly these authors also showed that these cells had the same the amount of magnesium ion present per cell as compared to cells grown in magnesium replete conditions. Thus the mutations we observe in genes regulating the cell membrane might result in it's restructuring, probably resulting in reallocating of magnesium ions within the cell for needed proteins. We also found two populations showing a deletion in gene *yhaV*, which codes for a toxin protein in a toxin-antitoxin system in *E.coli*. The antitoxin component of this complex is coded by the gene *prlF*, with which YhaV forms a non-toxic complex. In these toxin-antitoxin systems, the antitoxin protein is degraded under unfavorable conditions resulting in the toxin protein causing cell growth arrest and cell death. Thus it appears that magnesium-limiting conditions induce these events resulting in this kind of programmed cell death. It is possible that once the population adapts to magnesium limitation, deletion in gene *yhaV* is quickly selected for in order to bypass this programmed cell death. The presence of multiple potential targets of natural selection under magnesium ion limitation is not unexpected. Magnesium ion plays a role in stabilization of the cellular membrane, is a cofactor for enzymes and proteins involved in replication, transcription and translation, and is needed to make the energy molecule ATP

biologically active (Misra and Draper, 1998; Hartwig, 2001; Berg et al., 2002). Given the diversity and importance of these functions, it is not surprising that we do not see a repeatable adaptive signal in our experiments.

Physiological studies have shown that under magnesium ion limitation, bacterial cells show biofilm formation, reduced susceptibility to antibiotics and increased rigidity of the cellular membrane (Mulcahy and Lewenza, 2011; Monsieurs et al., 2005). The increased membrane rigidity makes the organism less susceptible to antibiotics, and in general to movement of molecules across the membrane. These studies have also suggested that the cellular survival rather than growth becomes the important strategy under magnesium ion starvation. To see if adaptation under magnesium limiting conditions resulted in trade-off due to antagonistic pleiotropy, we measured relative fitness of clones under nutrient rich conditions. Our experiments did not show any fitness trade-offs. Instead, we found evolved clones to have increased fitness under nutrient rich conditions. This is contrary to what has been shown before under adaptation to limiting nutrient conditions (Wenger et al., 2011). We also found the evolved clones to have an increased fitness, with respect to the ancestor, under energy limiting conditions. Magnesium ions are required to make the energy molecules in the cell, ATP, physiologically active. Thus it is not surprising that adaptation to magnesium limitation shows a pleiotropic increase in fitness under energy limiting conditions.

Adaptation to nitrogen-limitation proceeds by nutrient specific proteins, while adaptation to magnesium-limitation proceeds by more general sets of proteins:

Our results suggest two different patterns of evolutionary response under two different nutrient limiting conditions. Clones adapted to nitrogen limitation show nutrient specific evolutionary response. Our results did not generally show a pleiotropic increase in relative fitness of these clones on magnesium limitation (Fig.5 and Table 4). Given that we find proteins NtrBC to be the major targets of selection under nitrogen limiting conditions, it does add up to a nutrient specific evolutionary response for these sets of clones. On the other hand, adaptation to magnesium limitation might proceed via a more general stress

response route. Clones adapted to magnesium limitation showed an increase in relative fitness on nitrogen limiting conditions and on glucose limitation. Given that our results indicate that under magnesium limitation the potential targets of selection include genes involved in translation related functions, cell membrane biogenesis and flagellar operon induction, a pleiotropic increase in relative fitness in other nutrient limiting environments is not surprising. Thus although our population sequencing results do not show any general stress response genes to be targets of selection, the pleiotropic increase in fitness is seen by affecting general sets of phenotypes. Another important pattern observed in our data is that the increase in relative fitness of clones evolved under magnesium limitation is less than those evolved under nitrogen limitation. This suggests a potential trade-off between the ability to adapt under a selective pressure and the consequent evolutionary pleiotropic response. Magnesium ion has diverse functional roles and interacts with multiple proteins. It is possible that this results in the adaptive response for magnesium limitation to have a wider ranging pleiotropic response as compared to the adaptive response for nitrogen limitation; but this also results in it having a lower adaptive response under any single selective pressure.

Adaptive heterogeneity in populations evolving under nutrient limiting conditions

Our results highlight large levels of adaptive diversity in populations evolving under both nitrogen limitation and magnesium limitation. We found 38 and 35 potentially adaptive mutations in populations evolving under nitrogen limiting and magnesium limiting conditions respectively. Although we describe only a few mutations from this list, it is worthwhile to mention the nature of the remainder of these. Genes containing these mutations are involved in diverse sets of functions. Populations evolving under nitrogen limiting conditions show potentially adaptive mutations in genes involved in amino acid metabolism, DNA replication, oxygen sensing, multidrug efflux pumps and carbon metabolism. Populations evolving under magnesium limiting conditions show potentially adaptive mutations in genes involved in metabolism of cations (namely copper and iron), low pH resistance, multidrug efflux pumps and carbon metabolism. Interestingly, most of these mutations remain at low frequency in the population. This suggests that there are

many possible adaptive routes that result in only small increments in relative fitness of populations evolving under nutrient limiting conditions. Our work is the first to highlight these potentially small affect mutations in these evolving populations. Previous work investigating diversity in populations evolving under nutrient limiting condition either did not have population sequencing results to look at the existing adaptive diversity (Maharajan et al., 2006) or did not have replicates in the experiment, making it difficult to infer if the diversity was adaptive or not (Jezequel et al, 2013). More work will be needed to see if these mutations are maintained in the population or that the mutation dynamics involve a rapid turnover of these low frequency, potentially adaptive SNPs.

In conclusion, our results have highlighted the complexity associated with adaptation to nutrient limitation. Our results showed different patterns of evolutionary responses between adaptation to starvation of nitrogen and magnesium ion, and showed the presence of multiple potentially adaptive mutations in the populations evolving to nutrient limiting conditions. The results we find here are different from previously described nutrient limitation studies in that we do not find any transporter proteins being selected for in either of our treatments. Nitrogen limitation studies performed using yeast showed copy number variation in the transporter proteins as the adaptive response (Hong and Gresham, 2014), while similar adaptive outcomes were observed when *Methylobacterium extroquens* was allowed to evolve on metal-deficient nutrient media (Chou et al., 2009). Our study points at a novel evolutionary strategy as adaptation to nutrient limiting conditions. While NtrBC protein complex appeared to be the major target of selection under limiting nitrogen conditions, the evolutionary response was more diverse under magnesium starvation with proteins effecting cell membrane physiology being potential targets of selection. These results show that our understanding of how organisms adapt to nutrient starvation is still not complete, and points at further avenues that need to be investigated to understand the adaptive responses to this commonly occurring selective pressure.

Material and Methods:

Strain and media used:

The ancestor used in the study is a derivative of E.coli K-12 MG1655. It is cured of lambda phage and contains no plasmid. It also contains a deletion in a region of its lac operon making it *lac*- and a deletion in the rpoS gene making it *rpoS*-. Minimal media M9 with different concentrations of salts was used for the long-term evolution experiments. In general, minimal M9 was made by adding 1.75g potassium dibasic phosphate, 0.5g potassium monobasic phosphate, 1g ammonium sulphate, 0.5g sodium citrate and 0.1g Magnesium sulphate in one liter of water. The sugar used in all the experiments was glucose at a concentration of 1g/L. For nitrogen starvation experiments ammonium ion was used at a concentration of 0.05g/L (0.7mM). Sodium sulphate was used to compensate for sulphate concentrations (0.9g/L). For magnesium ion starvation experiments, no Magnesium chloride was added in the media. In order to demonstrate nutrient limitation, growth curves were plotted under the mentioned concentrations of ammonium ion and magnesium ions. For pleiotropic fitness experiments the nitrogen source used was glutamic acid and arginine at concentrations of 5 mM. No ammonium sulphate was added in these experiments. For energy limitation experiments, glucose was used at a concentration of 0.1g/L of media. For the long-term evolution experiments, chemostats were changed every 10 days to avoid wall effects. The flow rate was maintained to get a ~2 hr generation time. Samples were taken every 24 hours and were frozen as glycerol stocks at – 80 ^{o} C. Contamination checks were performed every 24 hours by plating the samples on citrate plates. The experiments were allowed to run for 34 days, that equaled ~400 generations.

Fitness assays for clones and populations:

Competition was carried out with the ancestral strain using lac as the neutral marker. *lac* operon was transduced into the parent strain by P1 transduction and was confirmed to be neutral under conditions of nitrogen and magnesium-ion limitation (Fig.6). All competitions were carried out in chemostats under appropriate nutrient conditions.

Each chemostat was inoculated with both the ancestral strain and the evolved population or evolved clone. Competitions were carried out typically for 48-72 hrs. Selection coefficient was calculated by plotting log of ratios of cell counts to time and calculating the slope of linearly regressed line. Each competition experiment was done in a duplicate. Error bars represent standard errors to the mean.

Next-gen sequencing analysis:

We constructed the library from DNA extracted from populations and individual clones. We extracted DNA using the DNeasy blood and tissue kit from Qiagen. Protocols were followed as mentioned in the manual, except for increasing the lysis time to one hour. Libraries were made using the NexteraXT sample preparation kit. Samples were dual-indexed and pooled together. Illumina's Miseq was used for sequencing using the Miseq reagent kit v2 (500 cycle). 9.9 Gb of data were obtained from the run with 76% of reads being above the Q30 score. For the populations and clones discussed in the paper, the average coverage obtained was 24X . Geneious was used to map the reads onto the reference genome and to find SNPs. Conservative values were used for trimming the raw reads, aligning these reads and for finding variants in the data. For SNP detection the cut-off values used were a minimum coverage of 15 and the SNP frequency of 15%.

Tables:

Table1: Relative fitness values for populations and clones. Samples were taken from the end point of the experiment i.e. after ~400 generations. p values indicate statistical significance for difference between fitness of population and clone1 (p1), population and clone2 (p2) and the two clones (p3). * indicates statistical significance.

Nutrient limiting environment analyzed	Fitness increase of population	Fitness increase of clone-1	Fitness increase of clone-2	Comparison between means
Limiting Nitrogen environment-Replicate 1	0.038 ± 0.003	0.1002± 0.0056	0.03085± 0.00077	p1=0.0459* ,p2=0.1892 p3=0.0367*
Limiting Nitrogen environment-Replicate 2	0.0413±0.00007	0.05655± 0.0001	0.04060± 0.0011	p1= 0.0036* ,p2=0.5341 p3=0.031*
Limiting Nitrogen environment-Replicate 3	0.0288±0.0007	0.0939± 0.00536	_	p1= 0.0367*
Limiting Nitrogen environment-Replicate 4	0.0324±0.0008	0.097785± 0.01	_	p1= 0.0018*
Limiting Magnesium environment-Replicate 1	0.0288±0.0004	0.04252± 0.0004	0.0012± 0.00007	p1=0.0186* ,p2=0.0066 p3=0.0044*
Limiting Magnesium environment-Replicate 2	0.05075±0.001	0.0354±0.0038	0.02395± 0.0007	p1=0.116 ,p2=0.0248* p3=0.1491
Limiting Magnesium environment-Replicate 3	0.033±0.001	0.0453± 0.005	-	p1= 0.4381
Limiting Magnesium environment-Replicate 4	0.01125±0.001	0.0245±0.0045	-	p1=0.016*

Table 2: Relative fitness of clones used in the study, in nutrient limiting environment and in nutrient non-limiting environment.

Clone used	**Environment in which relative fitness is measured**		**p values for comparison between the two environments**
	Nitrogen-limiting	**Non-nutrient limiting (control)**	
Nitrogen specialist from population 1	0.0565±0.0003	0.0125±0.0013	0.0684
Nitrogen specialist from population 2	0.1002±0.011	0.0342±0.002	0.0391
Nitrogen specialist from population 3	0.0897±0.008	0.0347±0.002	0.012
Nitrogen specialist from population 4	0.0939±0.007	0.0536±0.0017	0.0605
Magnesium specialist from population 1	0.0425±0.0008	0.0096±0.0119	0.083
Magnesium specialist from population 2	0.0354±0.0075	0.0215±0.0085	0.1833
Magnesium specialist from population 3	0.0453±0.0098	0.0001±0.0037	0.053
Magnesium specialist from population 4	0.0245±0.002	0.00001±0.002	0.0059

Table 3: Potential adaptive mutations and Evolutionary responses under Nitrogen and Magnesium limitation

Nitrogen-limitation:

Non-synonymous mutations repeating between replicates	High-frequency Non-synonymous mutations	Mutations showing clonal interference pattern
bglJ *paoC* *dosP* *qseB* *emrB* *topA* *empB* *wzyE* *yihM* *xdhA* *glnG* *prlF* *gntR* *nudK* *mdtM* *yhgE* *melA* *ytfR*	*glnG (NtrC)* *glnL* *paoC*	*ydbA* *add* *yjiR* *xdhA* *araG* *resC* *yjiR* *eco* *panD* *eptC* *yjhG* *fhuB* *atpF* *proX* *insM*

b) Magnesium ion-limitation:

Non-synonymous mutations repeating between replicates	*High-frequency Non-synonymous mutations*	*Mutations showing clonal interference pattern*
araG *proY* *avtA* *rhaS* *cusS* *sstT* *fabR* *ygfT* *fecD* *yhaV* *fimH* *yhgE* *inaA* *ypjA* *mdfA* *mdtM* *paoC* *yiaN* *yjaB*	*yhaV* *fabR* *lptG* *phoQ* *aldA*	*fabR* *ftsZ* *fimD* *selA* *insM* *sstT* *nfrA* *yfeD* *agaS* *cybB* *fimH*

Table4: Comparing evolutionary response under limiting nitrogen and magnesium conditions

Clone type	**Environment fitness measured in**	**Relative fitness measures**
Nitrogen specialist from population 1	Nitrogen-limiting	0.0565±0.0003
	Magnesium-limiting	0.0033±0.006
Nitrogen specialist from population 2	Nitrogen-limiting	0.1002±0.011
	Magnesium-limiting	0.0014±0.002
Nitrogen specialist from population 3	Nitrogen-limiting	0.0897±0.008
	Magnesium-limiting	0.00035±0.0002
Nitrogen specialist from population 4	Nitrogen-limiting	0.0939±0.007
	Magnesium-limiting	0.002±0.002
Magnesium specialist from population 1	Magnesium-limiting	0.0425±0.0008
	Nitrogen limiting	0.0244±0.004
Magnesium specialist from population 2	Magnesium-limiting	0.0354±0.0075
	Nitrogen limiting	0.0265±0.0048
Magnesium specialist from population 3	Magnesium-limiting	0.0453±0.0098
	Nitrogen limiting	0.0652±0.0001
Magnesium specialist from population 4	Magnesium-limiting	0.0245±0.002
	Nitrogen limiting	0.0162±0.002

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Fig.1 Fitness trajectories of populations adapting under nitrogen starvation and magnesium starvation is shown for four replicates. Relative fitness of clones isolated from each population after ~400 generations is also shown. Relative fitness is calculated by competing evolved populations against ancestral strain.

Fig 1a. Adaptation to Nitrogen limitation

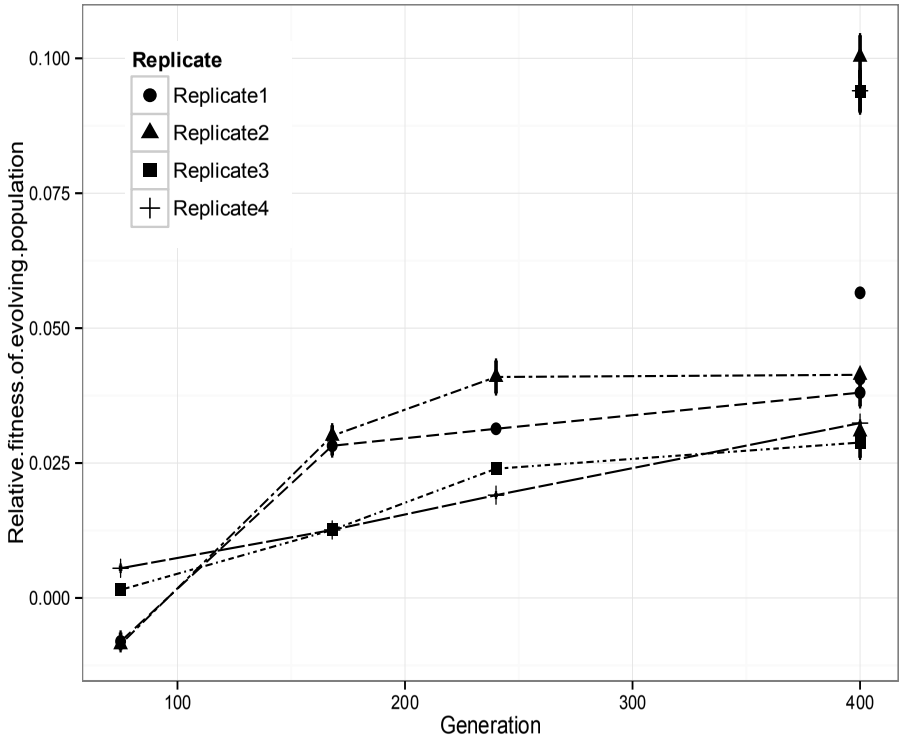


Fig1b. Adaptation to Magnesium ion limitation

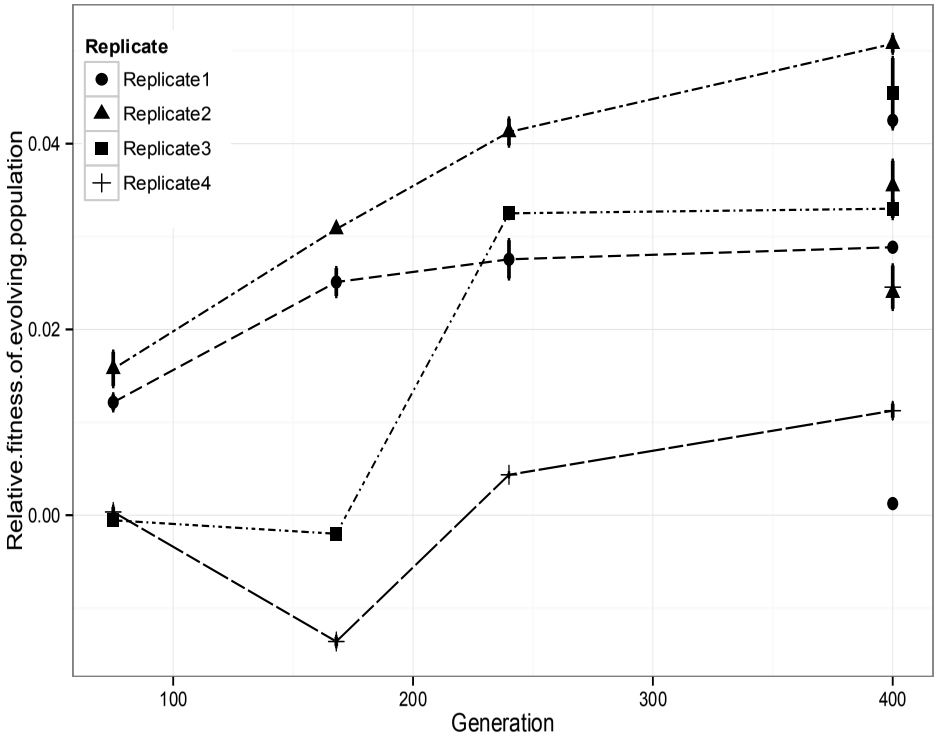


Fig.2 Relative fitness of individual clones on limiting nutrient and non-limiting nutrient conditions (control)

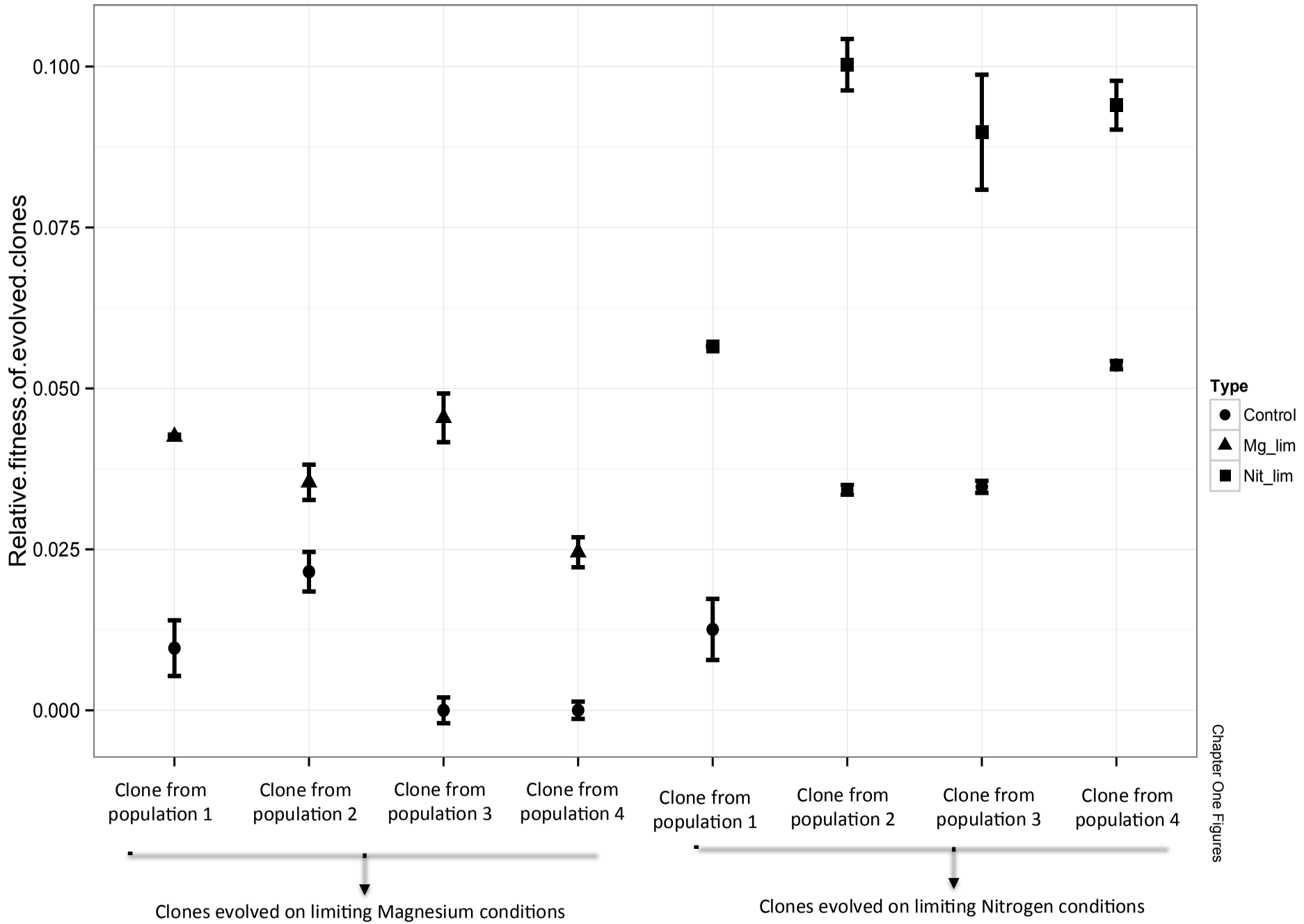


Fig.3 Mutations in the dual gene regulator NtrBC in populations evolving under Nitrogen limiting conditions

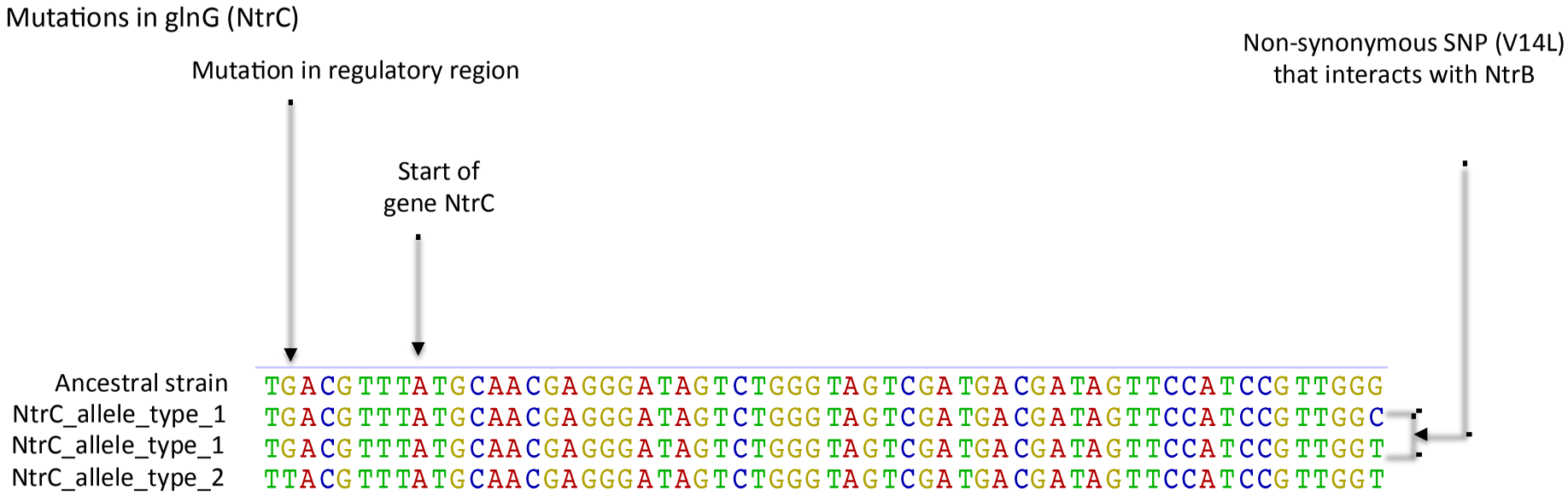
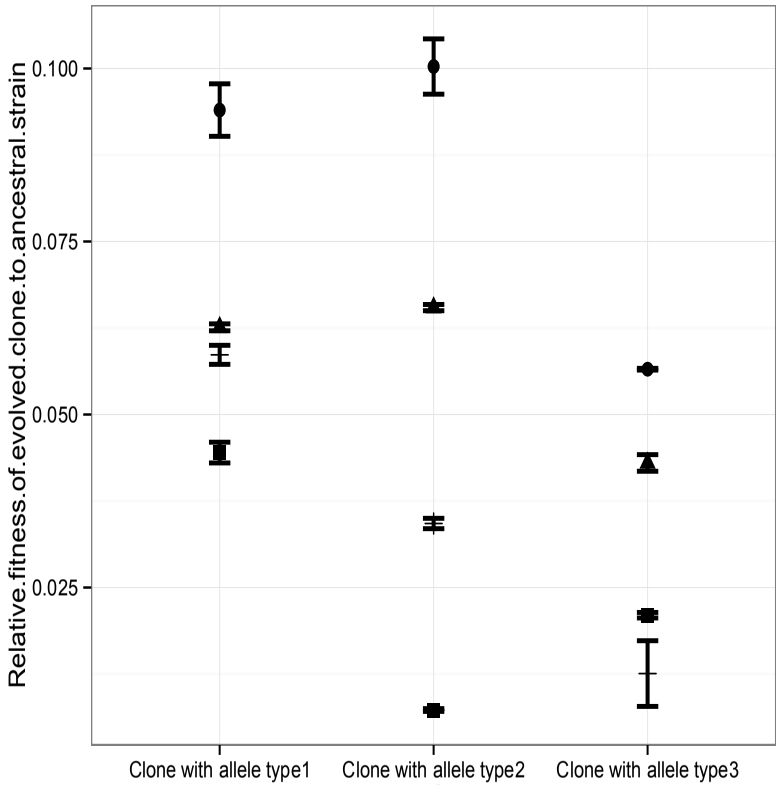


Fig.4 Relative fitness of individual clones on alternate nutrient limiting conditions

Fig.4a Relative fitness measurements for three clones containing the three different allele types on limitation of alternate nitrogen resources

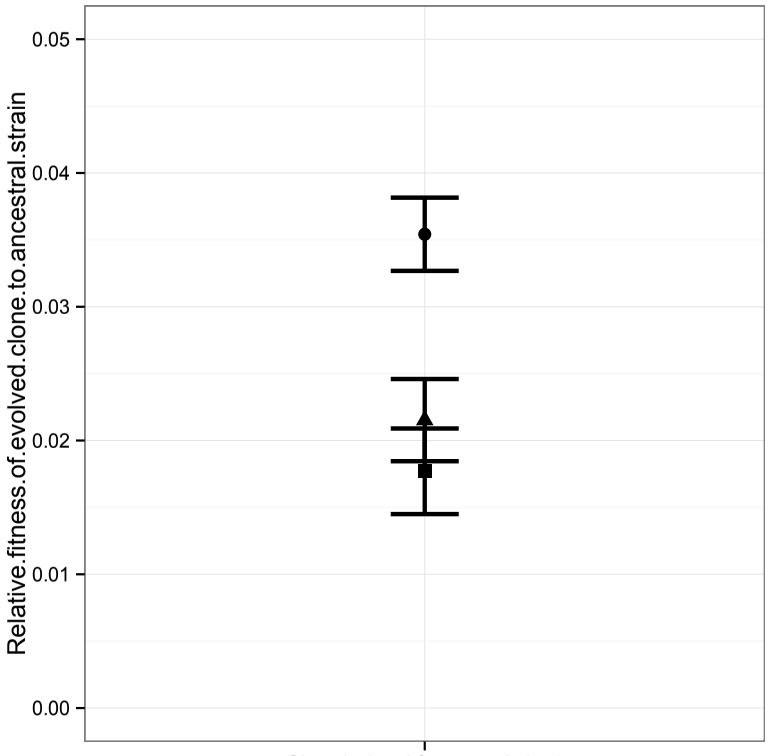


Clone with allele type1 — V14L mutation in NtrC

Clone with allele type2 — V14L mutation and mutation in putative regulatory in NtrC region in NtrC

Clone with allele type3 — 2 bp deletion in NtrB

Fig.4b. Relative fitness of clones evolved under magnesium ion limitation in nutrient rich environment



Clone isolated from populations evolving under limiting magnesium conditions

Fig.4c. Relative fitness of clones under energy-limiting environment

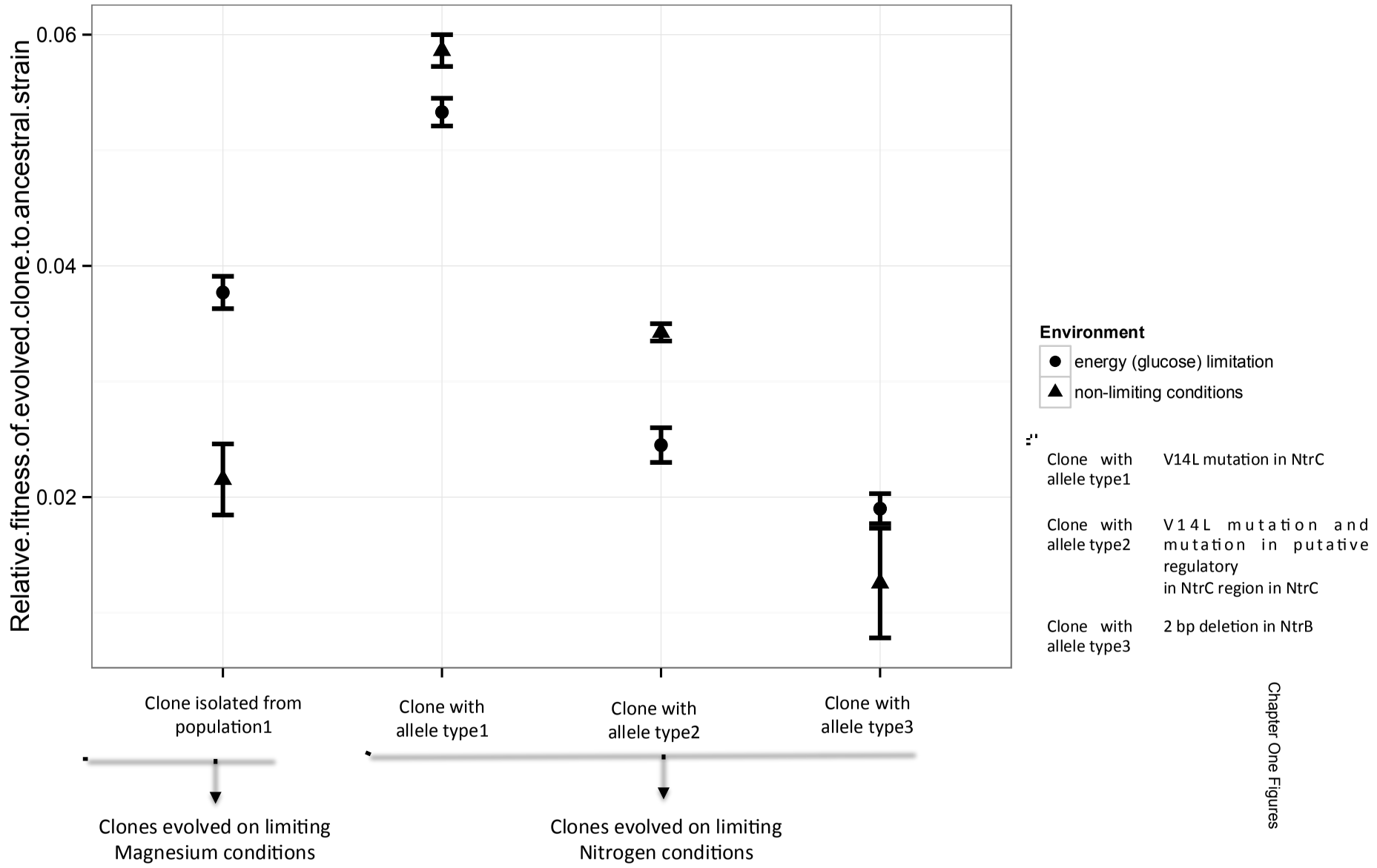
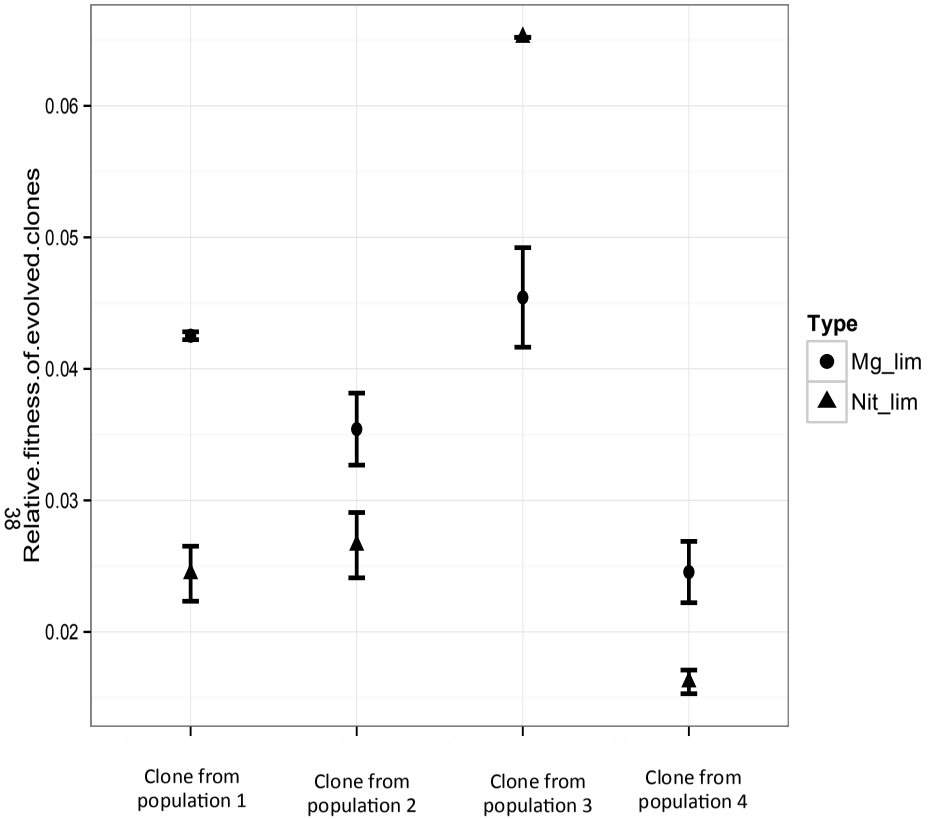


Fig.5. Comparing evolutionary response under limiting nitrogen and magnesium conditions

5a.Relative fitness of Magnesium-limitation adapted clones on limiting Nitrogen conditions



5b.Relative fitness of Nitrogen-limitation adapted clones on limiting magnesium conditions

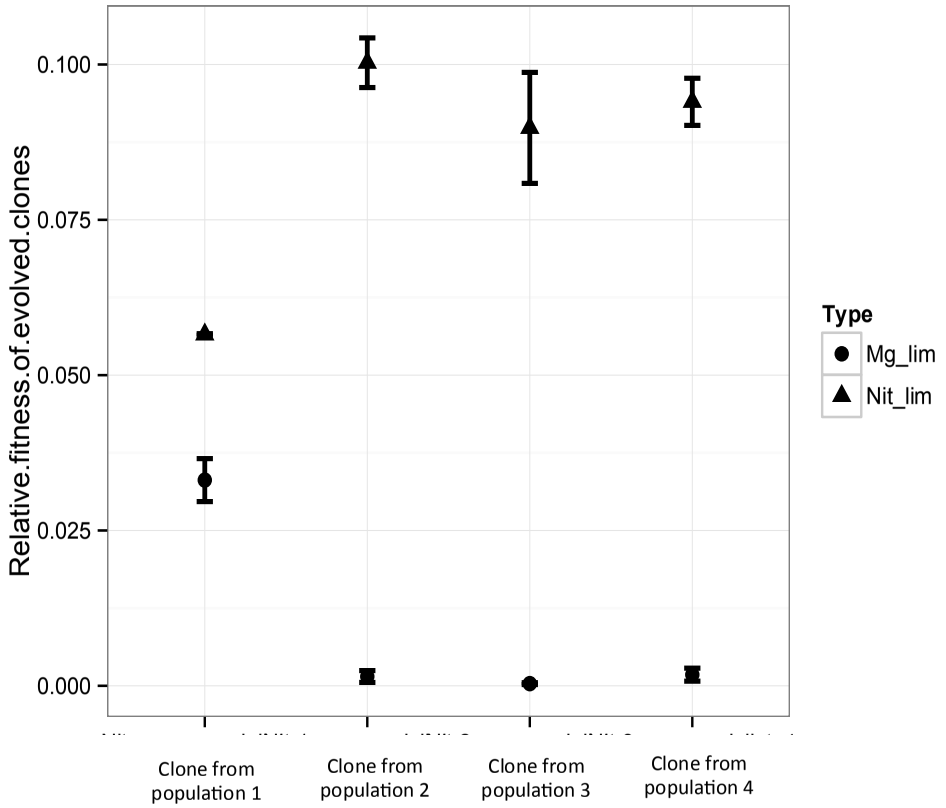
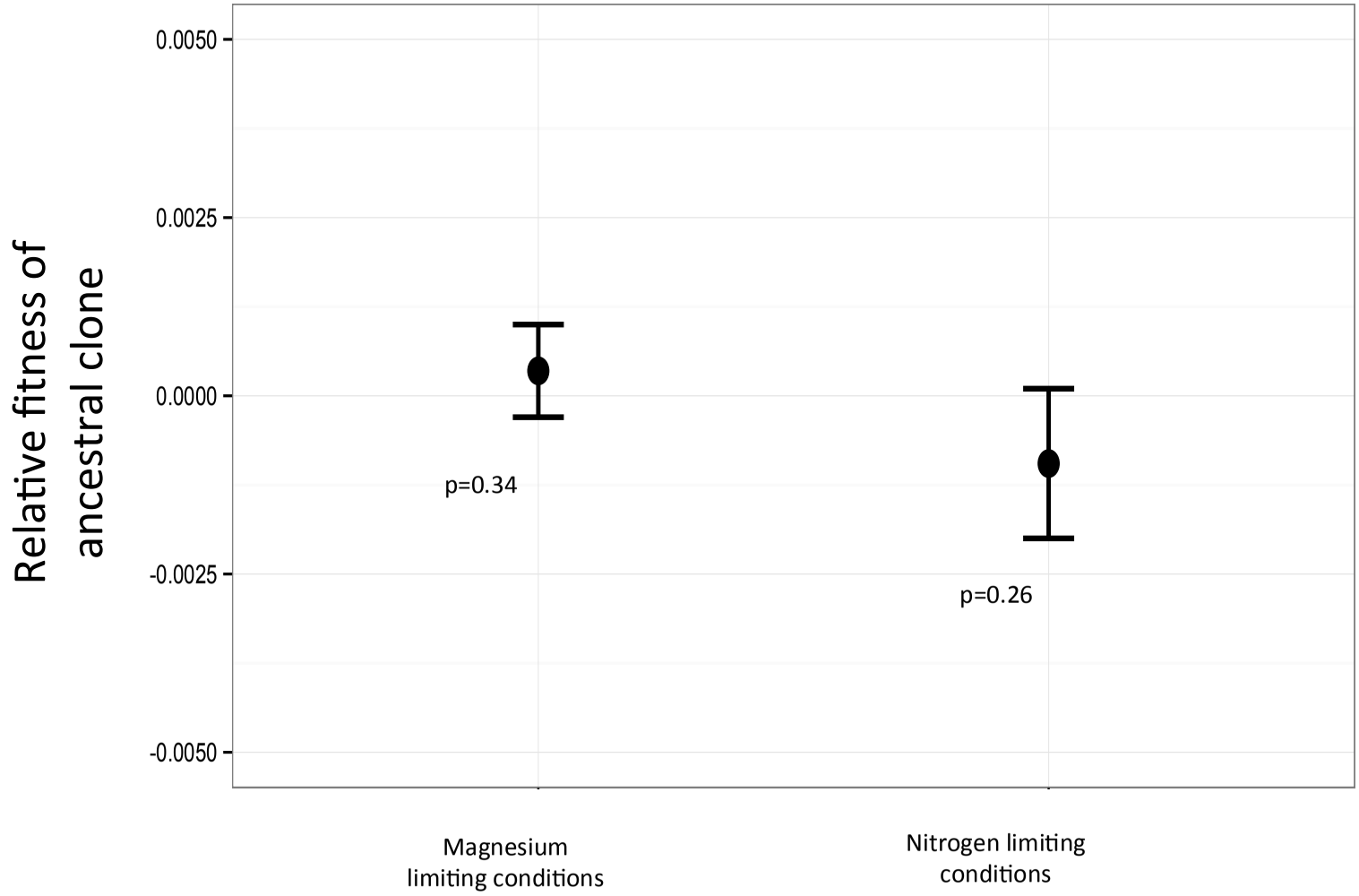


Fig.6. Testing for neutrality of genetic marker *lac* that is used in competition experiments. p values indicate no statistical difference between any of the values and zero.



Chapter Two
Evolutionary implications of Liebig's law of minimum

Abstract:

Different axes of an organism's niche have different selection intensities and the interactions between these axes determine the evolutionary trajectory of a population. An extreme case of these interactions is predicted from ecological theory in Liebig's law of minimum. This law states that in environments where multiple nutrients are in relatively low concentrations, only one nutrient will affect the biomass production of the organism. This would then imply that the evolutionary response of the population would be dictated by the more limiting of the two nutrients. We used experimental evolution approach to test this hypothesis. We used resource usage of nitrogen and magnesium as our two niche axes and identified zones along their concentration gradients where growth limitation is observed. We then conducted twelve evolution experiments in chemostats: four each on limiting nitrogen conditions, limiting magnesium conditions and in environments where both nitrogen and magnesium are in low concentration. We hypothesized, based on Liebig's law, that evolutionary outcomes in environments where both nitrogen and magnesium are in low concentrations will be similar to evolutionary outcomes under single nutrient limitations of the nutrient that is most limiting in the former environment. Evolutionary response was measured by measuring relative fitness of evolving populations and individual clones, and by population sequencing. Increase in relative fitness of the evolving populations and clones were observed under the different nutrient limiting conditions. Fitness measures show that clones isolated from population evolving in low nutrient zones, where populations are growth limited by magnesium (LNML zones), are more adapted to limiting magnesium conditions than to limiting nitrogen conditions. However clones and populations show different levels of fitness increases under single magnesium limiting environment and in LNML zones. Population sequencing results show only two genes to be common targets of selection under these environments. These results show that although LNMLzones is a magnesium-limited environment, the evolutionary dynamics observed in it are different from those in a single nutrient-limiting environment. We further demonstrate that this difference might arise because evolutionary adaptation causes fluctuations between nitrogen-limitation and magnesium limitation in environments where both these nutrients are present in low concentrations.

Introduction:

The evolutionary potential along different axes of an organism's niche varies and has been the focus of many questions in evolutionary biology. It is inherently linked to niche evolution and its importance can be attributed to its association to concepts of trade-offs (Mole, 1994; Dykhuizen et al., 2004), evolutionary constraints (Mole et al., 1994; Futuyma, 2010), evolutionary rates (Benett, 1992; Bolnick, 2001), causal explanations of biodiversity (Fryer, 1972; Kambysellis, 1997; Maharajan, 2006), stable community structures (Tilman, 2004; Polechová et al., 2008) and outcomes of ecological competition (Futuyma et al., 1988; Silvertown, 2004; Tilman, 2004;). It has also been a central concept in models of adaptive evolution like Fisher's geometrical model (Fisher, 1930; Orr, 2005) and Haldane's ellipsoidal model of adaptation (Waxman and Welch, 2005). Two aspects constitute this evolutionary process: adaptation along each axis of a niche and the interaction between different axes. The optimal evolutionary trajectory for a population is dependent on the nature of these interactions. Despite their importance, our understanding of how these interactions dictate evolutionary outcomes is very limited. In this study we combine ecological theory and an experimental evolution approach to predict and test the outcomes of these interactions. We use the niche axes of resource usage and use Liebig's law of minimum to predict evolutionary outcomes for populations evolving in environments where the concentration of multiple nutrients is low, such that, if taken individually, the concentration of each nutrient will limit growth in an otherwise nutrient replete environment.

Liebig's law of minimum, which was originally applied to plant growth, states that in environments where multiple nutrients are in low concentrations, only the more limiting nutrient will affect the biomass production of the organism (de Baar, 1994, Saito et al., 2008). On the same lines, the law of Blackman limitation states the more limiting nutrient will affect the growth rate of the organism (Blackman, 1905; Saito, 2008). In natural systems, these laws have been shown to be valid in some situations (Tilman 1987, Tilman 1990,

Elser et al. 2007, Harpole et al. 2011) while are not applicable under others (Harpole et al. 2011). These laws have been used in mathematical modeling of nutrient-limiting conditions, in quantitative ecological theories of bottom-up control in population dynamics and in understanding species co-existence (Droop, 1973; Legovic and Cruzado, 1997; Ballantyne IV et al., 2008; Hutchinson, 1961). However these laws have not been used to predict adaptation to multiple environmental factors.

Both these laws allow one to investigate how interactions between the different axes of a niche determine the evolutionary outcome in a constant environment where multiple nutrients are in low concentration. A possible extrapolation of these ecological laws is that in environments where multiple nutrients are in low concentrations, the evolutionary outcome is dependent only on the most limiting of these nutrients. In other words, the adaptive response of a population evolving in such environments will be dictated by only the most limiting nutrient, and will mirror the adaptive response in populations evolving on single nutrient limiting environments. Alternatively, it is possible that the cell senses the low concentrations of both these nutrients simultaneously, even if it is being growth limited by only one of the nutrients. In such situations, the cell will make physiological changes in different transporters, enzymes and metabolic pathways to cope up with low concentrations of both the nutrients. Mutations in these genes that can increase the efficiency of cell functioning at low concentrations of these nutrients will thus be selected for. Thus, even with growth limitation being seen due to a single nutrient, the evolutionary response will be dependent on low concentrations of both the nutrients. More specifically, Liebig's law of minimum will give a good approximation of outcomes over a relatively short time scale but not over an evolutionary time scale. Both these cases highlight the different ways in which different niche axes interact with one another to determine an evolutionary outcome. We test these competing hypothesis using nitrogen limiting and magnesium limiting conditions.

Nitrogen and magnesium ion fulfill different needs of the organism. Nitrogen is an essential nutrient for the organism and is a necessary elemental component of different biomolecules. Magnesium ion is functionally involved in stabilizing proteins, other

biomolecules and cell membranes and can be substituted for some of its functions (Pleshchitser, 1958; Hartwig, 2001; Misra and Draper, 1998). We identified the concentrations below which growth of *E. coli* was limited for these nutrients. These consisted either of single nutrient limited zones for magnesium and nitrogen or of low nutrient zones where the concentration of both the nutrients individually results in growth limitation, but when combined together, only the more limiting of the two nutrients affect the growth rate (Fig.1). We then conducted twelve evolution experiments in chemostats: four each under limiting nitrogen conditions, limiting magnesium conditions and in a low nutrient zone, where limitation of growth was seen due to low magnesium concentrations. These ***L***ow ***N***utrient zones where populations are ***M***agnesium ***L***imited are abbreviated as ***LNML*** zones for the rest of this paper***.*** Results from single nutrient limitation are described in detail in a separate paper and their results are mentioned here only for comparisons.

Use of an experimental evolution approach allowed us to measure the evolutionary response of these evolving populations. Relative fitness measurements for the evolving populations gives a phenotypic basis of the evolutionary response, while a population sequencing approach, using Next-Gen sequencing technology, allowed us to understand the genotypic underlying of the evolutionary response. We identified potential targets of selection using three criteria: 1) Mutations that repeat between replicate experiments; these are considered hallmarks of adaptive evolution, 2) a given gene showing two or more independent SNPs at the same time point in a single evolving population. This is synonymous to the concept of clonal interference and 3) high frequency non-synonymous SNPs.

This study thus allows us to investigate the evolutionary trajectories of populations evolving in zones of single nutrient limitation and in LNML zones. Evolutionary trajectories in the latter will allow us to study how different niche axes interact to determine evolutionary outcomes.

Results:

1) **Identification of zones of nutrient limitation:**

We used a combination of growth curve analysis and analytical chemistry methods to identify concentrations of magnesium and nitrogen at which *E.coli* populations are growth limited by these nutrients. We analyzed four different concentrations for ammonium ion (0 mM, 0.07 mM, 0.7 mM and 7 mM) and three different concentrations for magnesium ion (0, 0.04 mM and 0.08 mM), giving us a total of twelve environments with differing nutrient concentrations.

Analytical chemistry methods: In environments where a particular nutrient becomes limiting, it's concentration becomes negligible and in most cases undetectable. Using the same rationale, non-limitation of a particular nutrient in an environment will result in its accumulation. We first allowed *E.coli* to grow in each of the twelve different environments in side-arm flasks. After the populations had reached a stationary phase we used analytical chemistry (indophenol method of detection of ammonia) methods to detect the presence of ammonium ion (See methods for more explanation). Seven out of twelve nutrient combinations showed accumulation of ammonia suggesting that these were zones where either the population was magnesium limited or faced no nutrient limitation, while five nutrient combinations showed no ammonia being present in the environment suggesting that these environments were nitrogen-limited (Fig 1a). We did not perform analytical methods (like Eriochrome Black T method) for detection of magnesium ions because other salts present in our medium interfered with its detection. Thus these methods allowed us to conclusively identify nutrient combinations that resulted in nitrogen limiting conditions. We next performed growth curve analysis to identify nutrient combinations that would result in limiting magnesium conditions.

Growth curve analysis: Growth curves were made in each environment, in replicates of eight. Controls were performed under conditions where no nutrient was limiting. A difference in u_{max} or the population density at which stationary phase is seen, between the

different nutrient combinations and the control (nutrient replete conditions) would indicate nutrient limitation. In six out of seven environments where accumulation of ammonium ion was seen, the final population density was lower than that seen under nutrient non-limiting case, however we did not find any change in u_{max} in any of these environments. This suggests that these were zones where the population was limited for magnesium ion. Evolution experiments were performed on single nutrient limiting conditions and under LNML zones.

2) Evolved clones and populations show increase in relative fitness in all three nutrient -limiting conditions

Clones, isolated from the end-point of our evolution experiments, showed increase in relative fitness compared to the ancestral strain. The fitness increase varied in range from 0.056-0.10 for clones adapted in nitrogen limiting conditions, 0.0245-0.0454 for clones adapted in magnesium limiting conditions and 0.0245-0.03 for clones adapted in LNML zones (Fig 2, Table1). Clones isolated from populations evolving under LNML zones of nutrient limitation also showed an increase in relative fitness in both single nutrient-limiting environments (Fig.3, Table2). For all the clones tested, this increase was much more pronounced in limiting magnesium conditions than in limiting nitrogen conditions. We investigated whether this observation was an outcome of adaptation to both the nutrient limitations individually or was a correlated response to adaptation to only one of the nutrient limiting conditions To this effect, we measured the relative fitness of clones adapted to limiting nitrogen conditions on limiting magnesium conditions and vice versa (Fig.4, Table3). Our results showed that clones adapted to nitrogen limitation do not show any adaptation to magnesium limitation but clones that adapt to magnesium limitation show increased fitness on nitrogen limiting conditions. This pattern is identical to clones evolving under LNML zones of magnesium limitation. Thus clones evolving under single nutrient limitation of magnesium behave in the same manner as clones evolving under LNML zones. However, our results also show that clones evolved under LNML zones of nutrient limitation show a greater increase on magnesium limitation than do clones evolved under magnesium limiting conditions (Fig.3, Table2).

Increase in relative fitness was also observed for each of the evolving populations. Populations evolving under single nutrient limiting conditions showed different trajectories of fitness increase. These ranged from ~0.027 to 0.041 for populations evolving in nitrogen limiting conditions to ~0.011 to 0.0507 for populations evolving in magnesium limiting conditions (Table1 and Fig 5). Populations evolving in LNML zones had an increase in fitness ranging from ~ 0.0148 – 0.0374 and showed a different pattern of fitness change as compared to populations evolving under single nutrient limitation. In three of these populations the fitness increased for the first ~168 generations, only to then decrease over the next ~100 generations, to then increase again (Fig.5c). The fourth population showed an initial increase in fitness for the first ~72 generations, then showed a decrease in fitness for the next ~100 generations and then increased in fitness again (Fig.5c). Overall, all the populations evolving in LNML zones show fluctuating changes in fitness. These fitness dynamics are suggestive of changing selective dynamics along the evolutionary trajectories for these populations.

3) **Different population dynamics observed under single nutrient limiting environment and in LNML zones:**

To test the hypothesis of changing selective pressure in LNML zones, we first measured the change in relative fitness of three populations that had adapted in LNML zones, under single nutrient limited environments (both magnesium and nitrogen). An important pattern emerges from these fitness measurements. The fitness trajectory of the population evolving in LNML zones is different from the fitness trajectory observed in either of the single nutrient limiting conditions (Fig.6 and Table 4). In four out of twelve cases (four time points per population), the fitness measurement of the population under LNML zones of magnesium limitation condition is statistically different from single nutrient limitations for magnesium and in eight cases it is statistically different from fitness measurements on limiting nitrogen conditions. These results indicate that even if an evolutionary response of a population under LNML zones is qualitatively similar to single

nutrient limitation for magnesium, there are quantitative differences observed in the evolutionary trajectories for these two environments.

We further measured the change in ammonium ion concentration over the course of our experiment. Fluctuations in ammonium concentration over the course of ~400 generations was observed (Fig.6 and Fig.7). All the populations start in limiting nitrogen conditions. However, since at this early stage the populations only experience nutrient limitation of magnesium ion, nitrogen (ammonium ion) starts increasing in concentration in these environments. As the population starts increasing in fitness, the concentration of ammonium ion starts decreasing in concentration. This observation suggests that the environment moves toward being nitrogen limited. After this point two patterns are seen. In two environments (those in which population 2 and population 4 are evolving) the concentration of ammonium ion continues to decrease. While in the remaining two cases (those in which population 1 and population 3 are evolving), the concentration of ammonium ion starts increasing. Thus for all the populations, these fluctuations resulted in environments changing between nitrogen limiting to nitrogen replete. In most cases these changes in ammonium concentration was correlated with changes in fitness of the population under limiting nitrogen condition.

Overall our results show that population dynamics in LNML zones and single nutrient limiting conditions are quantitatively different; and that in LNML zones, adaptation to the more limiting of the two nutrients (magnesium ion in our experimental set up) increases evolutionary potential for the initially non-limiting nutrient (ammonium ion in our experimental set up).

4) **Evolutionary response in populations evolving in** LNML zones**:**

We sequenced all the evolving populations at the end of the experiment to identify targets of selection. As mentioned before we use three criteria to identify targets of selection:

1) Non-synonymous mutations in the same gene across replicates

2) High frequency non-synonymous SNPs

3) A given gene showing two or more independent SNPs at the same time point in a single evolving population

Table 5 lists genes that fall in the above-mentioned criteria for all the three nutrient limiting conditions. Genes that show more than one type of signature of selection are highly probable of being targets of selection. Since we have previously described evolutionary responses under single nutrient limiting conditions, we will describe here the results only for populations evolving in LNML zones. We find only two genes to show at least two types of signature of selection: *rho* and *yhaV.* While *rho* shows non-synonymous mutations, which in one population reaches to fixation, gene *yhaV* shows a deletion, which reaches to fixation in another population. Functionally Rho is involved in translation termination while YhaV is a toxin of a toxin-antitoxin system.

A comparative analysis of evolutionary responses between populations evolving in single nutrient limiting environment and under LNML zones further highlights dissimilarity between nutrient limiting conditions. We found two genes to show mutations that repeat between all three nutrient limiting environments: *mdtM* (multidrug efflux pump) and *paoC* (DNA damage repair). These might have role in adaptation to the chemostats or might be a part of general stress response in the cell. Only one gene, a potential target of selection, is found selected in both populations evolving under magnesium limitation and populations evolving in LNML zones: deletion in toxin producing gene *yhaV*. Besides this, we find two genes that are part of the same operon, to be targets of selection under these nutrient-limiting regimes. These include *lptG* and *lptA* respectively, which are both involved in cell membrane biogenesis. No gene targets were found to be consistent only between populations evolving under nitrogen limiting conditions and populations evolving in LNML zones. Overall our results show that although deletion in toxin gene *yhaV* and genes involved in cell membrane physiology are potential targets of selection under both LNML zones and single nutrient limiting conditions for magnesium, the overlap in the targets of selection for these two environments is minimal. We further carried a Mantel test for SNPs shared between populations that evolved under magnesium limitation and under

environments where multiple nutrients are in low concentrations that further suggested that the evolutionary dynamics are different between these environments (Mantel's r= 0.1895, p= 0.1388).

Discussion:

Liebig's law of minimum suggests that in environments where multiple nutrients are in low concentrations, the most limiting of these will determine the growth rate of the organism. This study was designed to investigate if Liebig's law of minimum can be used for making predictions for evolutionary outcomes in environments where multiple nutrients are in low concentrations. Our results show that although this law can be extrapolated for predicting evolutionary outcomes, and that we can reject our alternative hypothesis of limitation of both nutrients having an affect on evolutionary outcomes, this law does not give a complete picture of the evolutionary dynamics in these environments. In other words, populations evolving in environments with multiple nutrients in low concentrations are growth limited because of low concentrations of the most limiting nutrient. Consequently, these adapt to this single nutrient limitation. However, this initial adaptation results in other nutrients that are present in the environment in low concentration, to start affecting, and possibly limiting the growth rate of the population. These varying nutrient limiting conditions results in changing selective pressures and selection intensities, leading to complicated population dynamics.

Clones evolved in LNML zones are more adapted to magnesium limiting conditions than clones evolved under single magnesium limiting conditions

Clones isolated from populations evolving in LNML zones were similar to clones adapted to single nutrient limitation for magnesium. However, these clones show a peculiar characteristic: these are more adapted to limiting magnesium conditions than the clones that evolved under magnesium limiting conditions. This is unexpected because if LNML zones are similar to magnesium limiting conditions then the relative fitness of these clones should not show such a unidirectional difference. This implies that the intensity of selective

pressures is different between these two environments, even if both are magnesium limiting. Our results suggest that an LNML zone consists of temporally changing selective pressures. This fluctuating environment might result in this pattern of high adaptation to limiting magnesium conditions. Ketola et al., (2013) observed similar results in that populations adapting to fluctuating thermal environment had a higher increase in fitness at the mean temperature of fluctuation as compared to the populations that had adapted to the constant mean temperature. We do think that evolutionary dynamics in fluctuating environment can lead to the pattern we observe for the following reasons:

1) This can take place due to inefficient removal of deleterious mutations in populations evolving under constant environment. Populations evolving under single nutrient limiting conditions show a concave pattern of increase in fitness. This has been observed in some of our populations evolving in single nutrient limiting conditions, as well as in other studies (Wiser et al., 2013). This has been shown to result in fitness epistasis for incoming mutations where the affect of both detrimental and beneficial mutations is reduced, especially when the population is close to fitness maxima (Dykhuizen, 1987; Chou et al., 2011; Khan, 2011). This fitness epistasis results in purifying selection not being efficient, resulting in accumulation of mildly deleterious mutations in the population. On the other hand, fitness trajectories of populations evolving in LNML zones show fluctuating patterns of selective pressure, which might be responsible for purifying selection being more efficient.

2) Our population sequencing results show populations evolving under single nutrient limiting conditions and Liebig's law of nutrient limitation only have two genes in common. Thus, it is likely that this difference in targets of selection results in a unidirectional difference in fitness between these evolved clones.

3) This could also be an outcome of correlated response of beneficial mutations under the alternating environment. In our case this comprises limiting nitrogen conditions. Mutations that increase fitness under limiting nitrogen conditions can have a correlated response to limiting magnesium conditions. Although possible, we think this is unlikely. This is because populations evolving under limiting nitrogen conditions show a nutrient specific evolutionary response, and do not show any correlated response under limiting magnesium conditions (Fig.4).

Evolutionary trajectories in LNML zones show temporal changes in selective pressures

Our results show that populations evolving under zones of low nutrient concentrations show signatures of changing selective pressure. We confirmed this hypothesis by measuring the concentration of the nutrient that was non-limiting initially (nitrogen in our experiments, (Fig.7). The concentration of nitrogen in these environments fluctuates between nitrogen limiting and nitrogen-replete conditions, with different environments showing different levels of fluctuation. It is likely that as populations adapt to the more limiting of the two nutrients, they start experiencing limitation of the second nutrient. i.e. adaptation to limiting magnesium conditions results in population being more nitrogen limited, and vice versa. Gorban et al. (2010), using empirical data and theoretical models, pointed out similar outcomes as a result of evolutionary adaptation. Their models demonstrated that as the organism can start meeting the demands of the limiting nutrient, through adaptation, it starts coming closer to the co-limiting zones for nutrient limitation.

Changing niche: adaptation environments with multiple low nutrient concentrations:

Our study also highlights a more general aspect of adaptation to multiple environmental factors. Different factors affect an organism's physiology in different ways: some might have a larger impact on the organism's physiology while others might have a lower or no impact at all. These factors define the niche space for the organism. However, our results demonstrates that as an organism evolves to a given niche axis, the evolutionary potential along the other axes changes as well. In our experimental system, we start with two axes where only one determines the evolutionary dynamics of the population. This would imply that only this first axis is important in characterizing the niche space of the organism. However adaptation along this first axis then increases the evolutionary potential along the second axis, increasing its role in defining the niche space of the organism. This idea of a changing niche space is slightly different from what is mostly

found in literature i.e. involving a spatial component or the one involving the concept of niche construction (Odling-Smee et al., 1996; Pearman, 2008). The former would imply adaptation to previously inhabitable areas outside the normal range of the organism, while the latter would imply the organism changing its environment. In our case, adaptation in a given environment makes initially less important factors contribute more to evolutionary dynamics, however these factors were always present in the environment. And our experimental set up has no spatial component to it.

The decrease in fitness in the evolutionary trajectories of the populations evolving in LNML zones also implies that the environment fluctuates between single nutrient limitations for magnesium and nitrogen, instead of being co-limited for these nutrients. Such dynamics might be more specific to the limiting factors in question. It is quite likely that the zone of co-limitation for the two nutrients we used in our study is very narrow, resulting in the environment shifting between the single nutrient limiting zones and not easily stabilizing on the co-limiting zones. In other cases, where these co-limiting zones are broad, the population might have a higher probability to equally being affected by both the factors simultaneously. This latter case is analogous to predictions from metabolic control theory on adaptive changes in one enzyme affecting the evolutionary potential of other enzymes in a given metabolic pathway (Kascher and Burns, 1973; Dykhuizen, 1987). In this situation, beneficial mutations in the enzyme that has a higher control coefficient (measured as change in end flux per unit change in protein activity) than other enzymes in the pathway results in a more equal and stable distribution of control coefficients along the pathway.

In conclusion, our results highlight a concept of changing niche space as a result of natural selection i.e. adaptation along a given axes result in an increase of complexity of the niche. This changing niche space highlights why evolutionary dynamics in environments where multiple nutrients are in low concentrations is different from that seen in single nutrient limiting conditions.

Material and Methods:

Strain and media used:

The ancestor used in the study is a derivative of E.coli K-12 MG1655 (strain designation DD1953). This strain is *rpoS-, lac-* and has no plasmids present. Minimal media M9 with different concentrations of salts was used for the long-term evolution experiments and has been explained in detail in Chapter 1. Additionally, for media used in experiments involving multiple nutrients with low concentrations, the concentration of ammonium sulphate used was 0.05g/L and no Magnesium sulphate was added. For the long-term evolution experiments, chemostats were changed every 10 days to avoid wall effects. The flow rate was maintained to get a ~2 hr generation time. Samples were taken every 24 hours and were frozen as glycerol stocks at – 80 ^{o} C. Contamination checks were performed every 24 hours by plating the samples on citrate plates.

Indophenol assay for detection of ammonia:

Different time point population samples, from different evolution experiments, that were stored at -80 C were thawed and used for detection of ammonium ions. These were centrifuged for 15 mins at 13,000 rpm. 750 ul of the supernatant was taken for further analysis. 5ul of the remaining solution was streaked on an LB plate to make sure that the supernatant was clear of bacteria. To each sample, 30ul of phenol and 30ul of sodium nitroprusside was added. This was followed by addition of 120 ul of oxidizing solution (Oxidizing solution consists of 4 parts of Alkaline solution and 1 part of bleach; alkaline solution consists of a mixture of Sodium citrate (0.2g/ml) and Sodium hydroxide (0.01g/ml) in water). These were vortexed and were then allowed to mix on a shaker for an hour at room temperature. Ammonium ion was quantified by measuring optical density at 640 nm. For each sample this analysis was done in replicates. For all the evolution experiments, ammonium concentration in the medium was measured at generation 0, ~72, ~168, ~240, and ~400.

Fitness assays for clones and populations:

Competition was carried out with the ancestral strain using lac as the neutral marker. The *lac* operon was transduced into the parent strain by P1 transduction and was confirmed to be neutral under conditions of nitrogen limitation, magnesium-ion limitation and under conditions of where both nitrogen and magnesium are in low concentrations (Fig.8). Competitions were carried out typically for 48-72 hrs. Selection coefficient was calculated by plotting log of ratios of cell counts to time and calculating the slope of linearly regressed line. Each competition experiment was done in a duplicate. Error bars represent standard errors to the mean.

Next-gen sequencing and analysis of SNPs:

This is same as described in Chapter One. Briefly, Illumina's Miseq was used to sequence bar coded populations. The average coverage obtained was 24X. SNPs were identified using the software Geneious and only SNPs reaching 15% or more were analyzed in the study. For comparison of evolutionary response for populations evolving in magnesium limited conditions and those evolving in LNML zones, we also carried a mantel's test for pairwise SNPs obtained between all eight of the populations, carrying 9999 permutations of rows.

Tables:

Table 1. Relative fitness increases for populations and clones evolving under different nutrient limiting conditions. Fitness was also measured for clones evolving in nutrient non-limiting conditions (controls). p values in each cell indicates if the difference between the relative fitness value and zero is statistically significant.

Nutrient limitation analyzed	Relative fitness of Population on nutrient limiting condition	Relative fitness of Clone on nutrient limiting condition	Relative fitness of clone on nutrient non-limiting conditions
Limiting nitrogen conditions – Replicate1	0.038 ± 0.003 p=0.021*	0.1002± 0.0056 p=0.0005*	0.0125±0.0013 p value=0.114
Limiting nitrogen conditions – Replicate2	0.0413±0.00007 p value=0.0003*	0.05655± 0.0001 p value=0.0126*	0.0342±0.002 p value=0.006
Limiting nitrogen conditions – Replicate3	0.0288±0.0007 p value=0.031*	0.0939± 0.00536 p value=0.013*	0.0347±0.002 p value=0.008
Limiting nitrogen conditions – Replicate4	0.0324±0.0008 p value=0.003*	0.097785± 0.01 p value=0.0128*	0.0536±0.0017 p value=0.003
Limiting magnesium conditions – Replicate1	0.0288±0.0004 p value=0.003*	0.04252± 0.0004 p value=0.012*	0.0096±0.0119 p value=0.133
Limiting magnesium conditions – Replicate2	0.05075±0.001 p value=0.005*	0.0354±0.0038 p value=0.024*	0.0215±0.0085 p value=0.045
Limiting magnesium conditions – Replicate3	0.033±0.001 p value=0.009*	0.0453± 0.005 p value=0.026*	0.0001±0.0037 p value=0.5
Limiting magnesium conditions – Replicate4	0.01125±0.001 p value=0.021*	0.0245±0.0045 p value=0.030*	0.00001±0.002 p value=0.49
LNML zones Replicate1	0.02165±0.003 p value=0.05	0.02455±0.00044 p value=0.005	0.002±0.00003 p value=0.5
LNML zones	0.0148±0.0002	0.0245±0.0015	0.0365±0.0035

Replicate2	p value=0.004	p value=0.01	p value=0.03
LNML zones Replicate3	0.0374±0.0018 p value=0.015	0.03±0.001 p value=0.01	0.016±0.003 p value=0.05
LNML zones Replicate4	0.0271±0.001 p value=0.012	0.0252±0.0007 p value=0.009	0.0145±0.0005 p value=0.01

Table 2: Relative fitness of clones that evolved on LNML zones on single nutrient limiting conditions. p values in each cell indicates if the difference between the relative fitness value and zero is statistically significant.

Clone analyzed	Relative fitness of clones in		
	LNML zones	Limiting nitrogen condition	Limiting magnesium condition
Clone isolated from Population1	0.02455±0.00044 p value=0.005	0.0150±0.0009 p value=0.02	0.0589±0.009 p value=0.05
Clone isolated from Population2	0.0245±0.0015 p value=0.01	0.024±0.002 p value=0.03	0.0532±0.002 p value=0.01
Clone isolated from Population3	0.03±0.001 p value=0.01	0.0126±0.0005 p value=0.013	0.0545±0.008 p value=0.046
Clone isolated from Population4	0.0252±0.0007 p value=0.009	0.027±0.004 p value=0.04	0.0353±0.004 p value=0.04

Table 3. Relative fitness of clones evolved on limiting nitrogen conditions on magnesium limiting conditions and vice versa. p values in each cell indicates if the difference between the relative fitness value and zero is statistically significant.

Clone type	**Environment fitness measured in**	**Relative fitness measures**	**p value for comparison of the two means**
Nitrogen specialist from population 1	Nitrogen-limiting	0.0565±0.0003 p value=0.0007	0.06
	Magnesium-limiting	0.0033±0.006 p value=0.033	
Nitrogen specialist from population 2	Nitrogen-limiting	0.1002±0.011 p value=0.0126	0.04
	Magnesium-limiting	0.0014±0.002 p value=0.18	
Nitrogen specialist from population 3	Nitrogen-limiting	0.0897±0.008 p value=0.031	0.06
	Magnesium-limiting	0.00035±0.0002 p value=0.12	
Nitrogen specialist from population 4	Nitrogen-limiting	0.0939±0.007 p value=0.012	0.06
	Magnesium-limiting	0.002±0.002 p value=0.168	
Magnesium specialist from population 1	Magnesium-limiting	0.0425±0.0008 p value=0.002	0.0835
	Nitrogen limiting	0.0244±0.004 p value=0.027	
Magnesium specialist from population 2	Magnesium-limiting	0.0354±0.0075 p value=0.024	0.1833
	Nitrogen limiting	0.0265±0.0048 p value=0.029	
Magnesium specialist from population 3	Magnesium-limiting	0.0453±0.0098 p value=0.026	0.017
	Nitrogen limiting	0.0652±0.0001 p value=0.0004	
Magnesium specialist from population 4	Magnesium-limiting	0.0245±0.002 p value=0.030	0.053
	Nitrogen limiting	0.0162±0.002 p value=0.017	

Table 4. Relative fitness of populations evolved on LNML zones on limiting nitrogen and limiting magnesium conditions for four different time points :~72 generation, ~168 generation, ~240 generation and ~400 generation. '*' Indicates statistically different values as compared to LNML zones at p=0.1, while '**' indicates statistically different values as compared to LNML zones at p=0.05. p values in each cell indicates if the difference between the relative fitness value and zero is statistically significant.

Population analyzed	**Generation**	**Relative fitness measured on**		
		LNML zones	Limiting-nitrogen conditions	Limiting-magnesium condition
Population evolving in LNML zones-Population1	72	0.00825±0.00055 p value=0.02	0.03055±0.00065** p value=0.006	0.0295±0.0049** p value=0.05
	168	0.03965±0.00775 p value=0.06	0.0434±0.002 p value=0.01	0.037±0.0043 p value=0.03
	240	0.0178±0.0007 p value=0.12	0.0243±0.0012** p value=0.01	0.04375±0.00445** p value=0.03
	400	0.02165±0.00375 p value=0.05	0.01885±0.00005 p value=0.0008	0.0326±0.0009* p value=0.008
Population evolving in LNML zones-Population3	72	0.0184±0.0023 p value=0.04	0.0343±0.0011** p value=0.01	0.01165±0.00105 p value=0.028
	168	0.05805±0.00375 p value=0.02	0.0648±0.0016 p value=0.007	0.06495±0.00335 p value=0.016
	240	0.0221±0.0045 p value=0.06	0.02455±0.00095 p value=0.01	0.0408±0.0004* p value=0.003
	400	0.0374±0.0018 p value=0.01	0.0493±0.002** p value=0.01	0.06815±0.00705* p value=0.032
Population evolving in LNML zones-Population4	72	0.0184±0.0023 p value=0.04	-0.0363±0.0015** p value=0.01	0.01165±0.00105* p value=0.03
	168	0.01595±0.00415 p value=0.08	-0.0114±0.0029** p value=0.08	0.0282±0.0018* p value=0.02
	240	0.0198±0.0026 p value=0.04	-0.0218±0.0001** p value=0.000001	0.0371±0.0023** p value=0.02
	400	0.0271±0.0011 p value=0.01	-0.00635±0.00185** p value=0.09	0.05585±0.00345** p value=0.02

Table 5. Evolutionary response under the different nutrient-limiting conditions.

Table 5a. Population sequencing results for populations evolving on LNML zones. Potential targets of selection were identified using three different criteria.

Non-synonymous mutations repeating between replicates	**High-frequency Non-synonymous mutations**	**Mutations showing clonal interference pattern**
ade *thiB*	*rho*	*ccmB*
basS *yddB*	*lptA*	*dptG*
fabR *ydeP*	*yhaV*	*ggt*
insM	*paoC*	*infC*
mdtM		*insM*
paoC		*pgaB*
rho		*phoE*
yhaV		

Table 5b: Population sequencing results for populations evolving on limiting nitrogen conditions. Potential targets of selection were identified using three different criteria.

Non-synonymous mutations repeating between replicates	**High-frequency Non-synonymous mutations**	**Mutations showing clonal interference pattern**
bglJ ***paoC***	*glnG*	*ydbA* *add*
dosP *qseB*	*pnp*	*yjiR* *nudK*
emrB *topA*	*glnL*	*araG* *resC*
empB *wzyE*	*paoC*	*yjiR* *eco*
xdhA *prlF*		*panD* *eptC*
glnG *nudK*		*yjhG* *fhuB*
gntR *yhgE*		*atpF* *proX*
mdtM *yihM*		*insM* ***xdhA***
melA		
ytfR		

Table 5c: Population sequencing results for populations evolving on limiting magnesium conditions. Potential targets of selection were identified using three different criteria.

Non-synonymous mutations repeating between replicates	**High-frequency Non-synonymous mutations**	**Mutations showing clonal interference pattern**
araG *proY*	*yhaV*	*fabR* *ftsZ*
avtA *rhaS*	*fabR*	*fimD* *selA*
cusS *sstT*	*lptG*	*insM* ***sstT***
fabR *ygfT*	*phoQ*	*nfrA* *yfeD*
fecD *yhaV*	*aldA*	*agaS*
fimH *yhgE*		*cybB*
inaA		*fimH*
mdfA		
mdtM		
paoC		
yiaN		
yjaB		
ypjA		

Table 6: p values for pairwise t test comparisons of u_{max} for populations growing in environments with different nutrient combinations. Environment descriptions are shown in Fig 1a. Environment 3 (multiple nutrients in low concentrations), 4 (magnesium limitation) and 11 (nitrogen limitation) are used in evolution experiments in our study.

	Environment1	Environment2	Environment3	Environment4	Environment5	Environment6	Environment7	Environment8	Environment9	Environment 10	Environment 11
Environment2	0.02	-	-	-	-	-	-	-	-	-	-
Environment3	0.048	0.652	-	-	-	-	-	-	-	-	-
Environment4	0.616	0.024	0.07*	-	-	-	-	-	-	-	-
Environment5	0.475	0.046	0.119	0.794	-	-	-	-	-	-	-
Environment6	0.663	0.02	0.058	0.936	0.733	-	-	-	-	-	-
Environment7	0.728	0.015	0.046	0.851	0.653	0.914	-	-	-	-	-
Environment8	0.555	0.032	0.088	0.913	0.879	0.85	0.766	-	-	-	-
Environment9	0.479	0.045	0.117	0.799	0.994	0.738	0.658	0.885	-	-	-
Environment10	0.804	0.011	0.035	0.756	0.568	0.818	0.903	0.675	0.573	-	-
Environment11	0.723	0.016	*0.057	0.858	0.659	0.921	0.993	0.772	0.664	0.896	-
Environment12	0.628	0.038	0.094	0.992	0.816	0.933	0.854	0.927	0.821	0.766	0.86

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Fig 1. Identification of growth limiting nutrient in environments with low concentrations of multiple nutrients

Indophenol method for detection of ammonium ions and growth curve analysis were used to identify nutrient limiting zones. Environments that are limited for a ammonium ion will have undetectable concentrations of these ions. Comparisons of stationary phase of growth curves in different environments highlights the nature of nutrient limitation.

Fig 1a. Different combinations of nutrient concentrations analyzed. Cells marked grey represent environments where no ammonia was detected after the population had reached a stationary phase of growth.

Mg^{2+} (mM) / NH_4^+ (mM)	0	0.04	0.08
0	Environment1	Environment5	Environment9
0.07	Environment2	Environment6	Environment10
0.7	Environment3	Environment7	Environment11
7	Environment4	Environment8	Environment12

Fig 1b. Growth curve analysis for all the different environments mentioned in Fig1a. Statistical analysis on u_{max} is shown in table 6

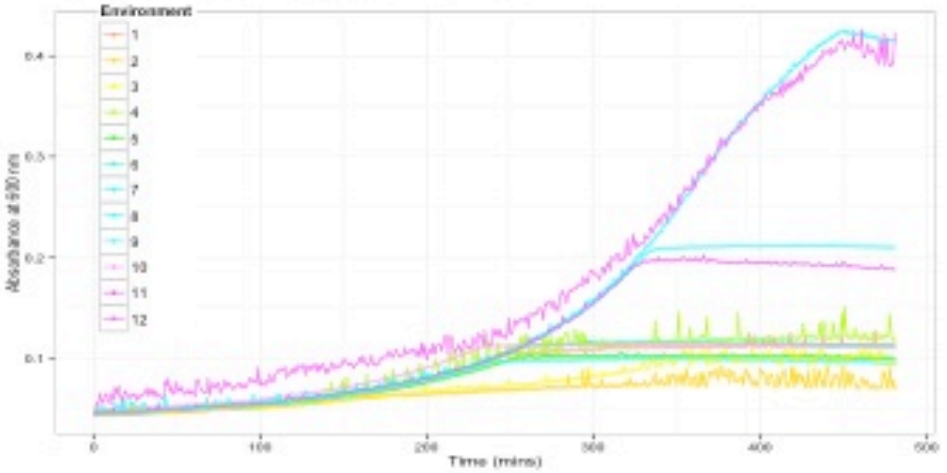


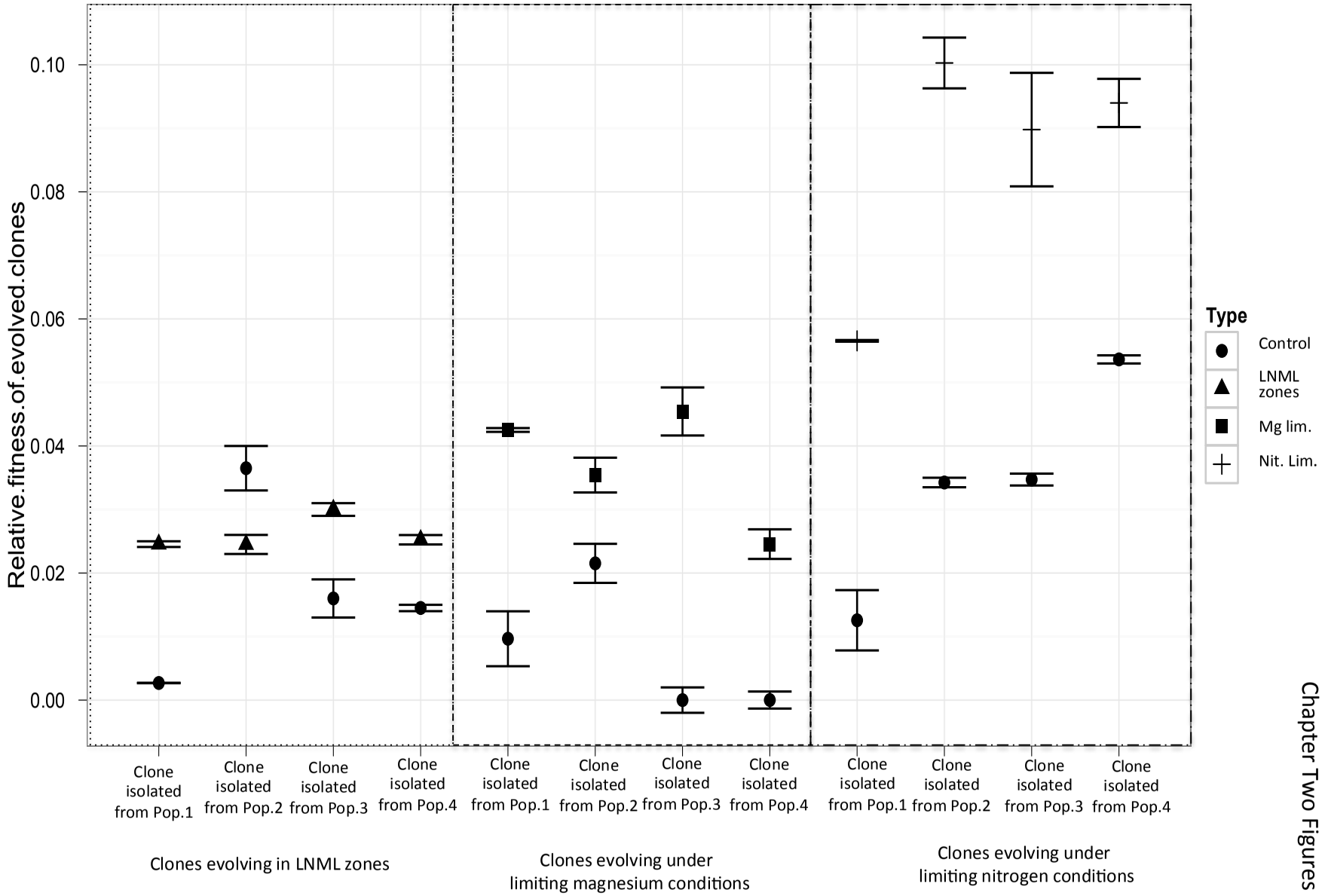
Fig 1c. Identification of growth limiting nutrient in environments with low concentrations of multiple nutrients

Mg^{2+} (mM) / NH_4^+ (mM)	0	0.04	0.08
0	N-limited	N-limited	N-limited
0.07	LNML* zone	LNML zone	N-limited
0.7	LNML zone	LNML zone	N-limited
7	Mg-limited	Mg-limited	Non-limiting Conditions

*refers to **L**ow **N**utrient zones with **M**agnesium **L**imiting Growth of the population

Fig.2: Relative fitness of clones evolving under the three nutrient limiting regimes

Clones isolated from end point of the evolution experiment were competed with ancestral strain in appropriate nutrient limiting environments. Controls were carried out in environments with excess of all the nutrients.



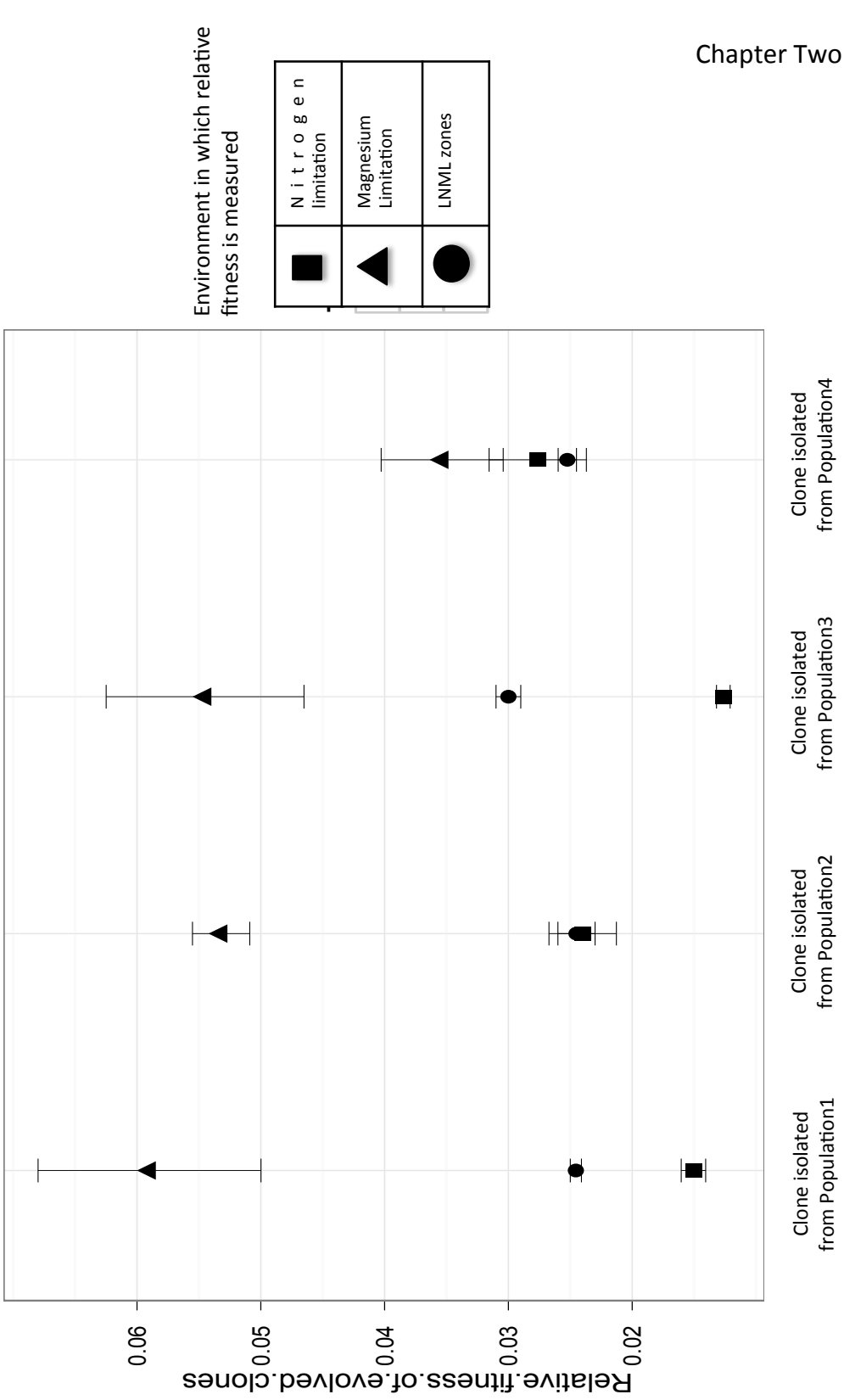


Fig.3: Relative fitness of clones evolved in LNML zones in limiting nitrogen and limiting magnesium conditions. Evolved clones show a higher level of fitness increase on limiting magnesium condition as compared to limiting nitrogen conditions.

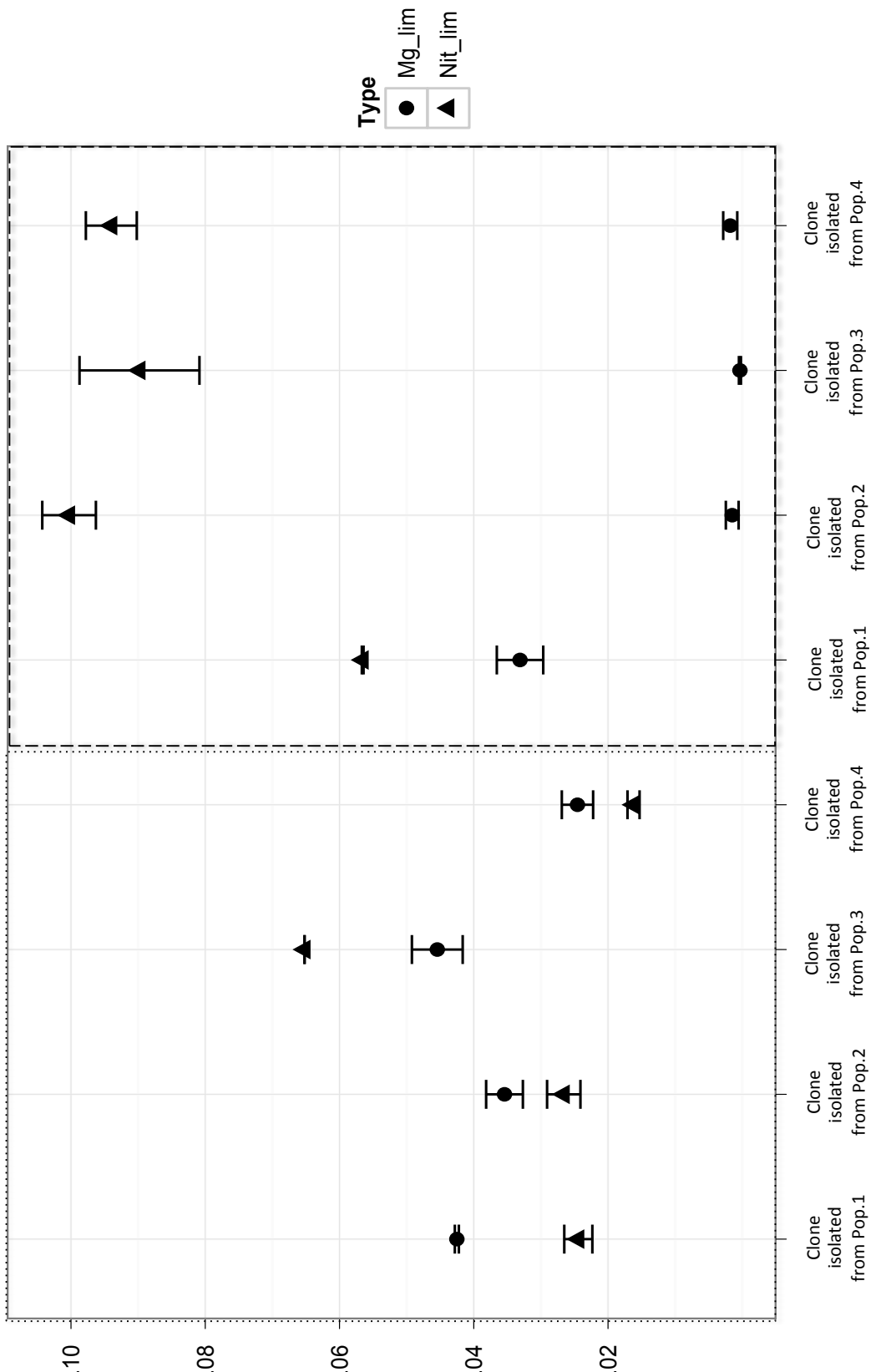


Fig.4 Relative fitness of clones evolved on limiting nitrogen conditions on magnesium limiting conditions and vice versa. Clones adapted on limiting nitrogen conditions do not show any fitness increase on limiting magnesium conditions, while clones that evolved on limiting magnesium conditions do show fitness increase on limiting nitrogen conditions.

Fig. 5 Relative fitness of populations evolving under limiting nutrient conditions for four time points: ~72 generation, ~168 generation, ~240 generation and ~400 generation. Relative fitness was measured by performing competition experiments against the ancestor, using growth on lactose as neutral marker. Change in ratios of the two types were regressed on time, and slope was calculated to measure the relative fitness. Two replicates were done for each measurement, and the average was taken for the slope obtained in each case. Error bars represent variation obtained in the replicates.

Fig1a. Populations evolving under limiting nitrogen conditions

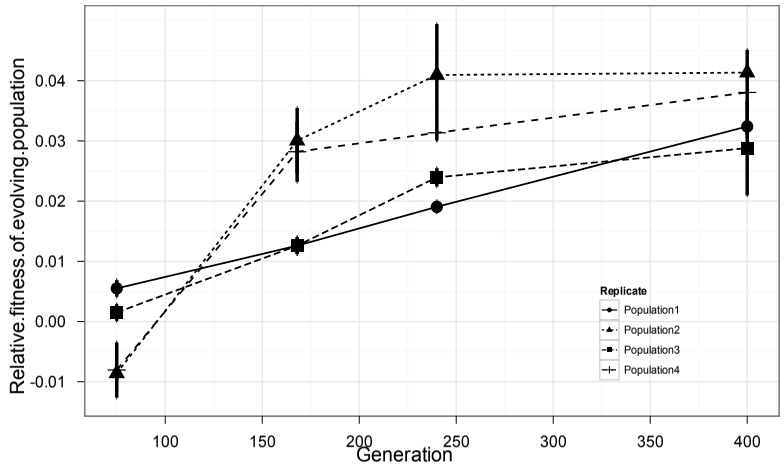


Fig1b. Populations evolving under limiting magnesium conditions

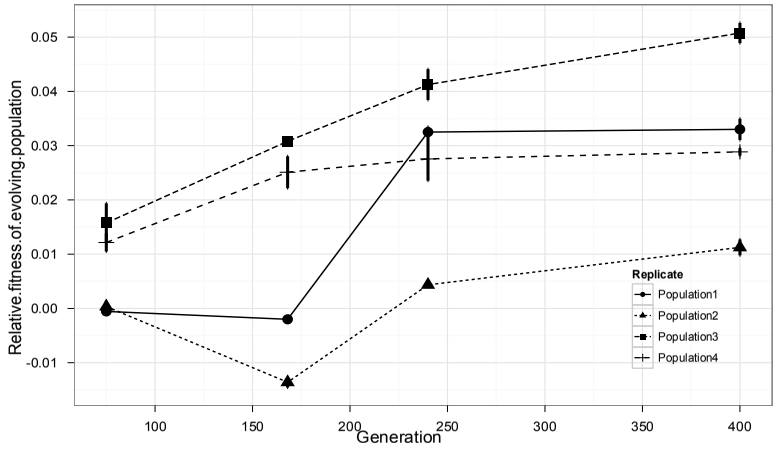


Fig1c. Populations evolving under dual nutrient limitations (Limited for both magnesium and nitrogen)

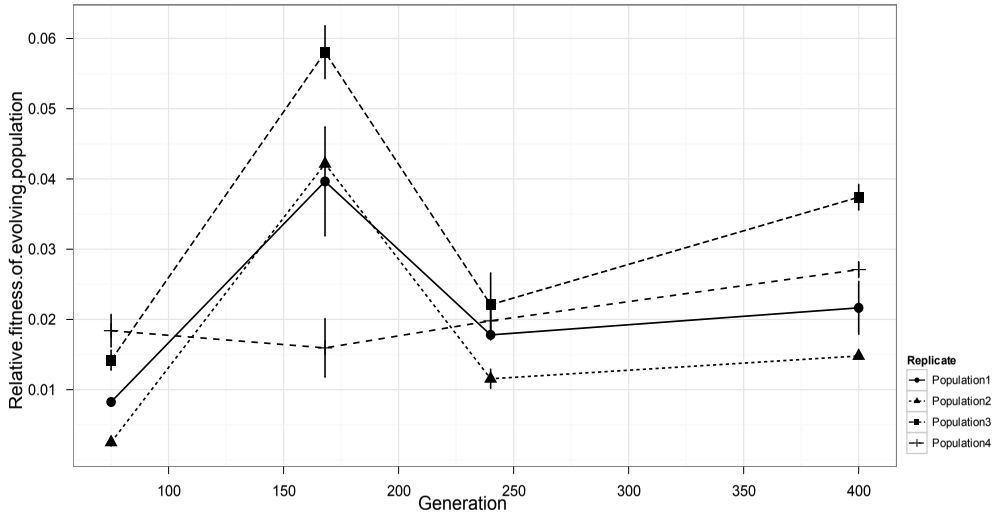
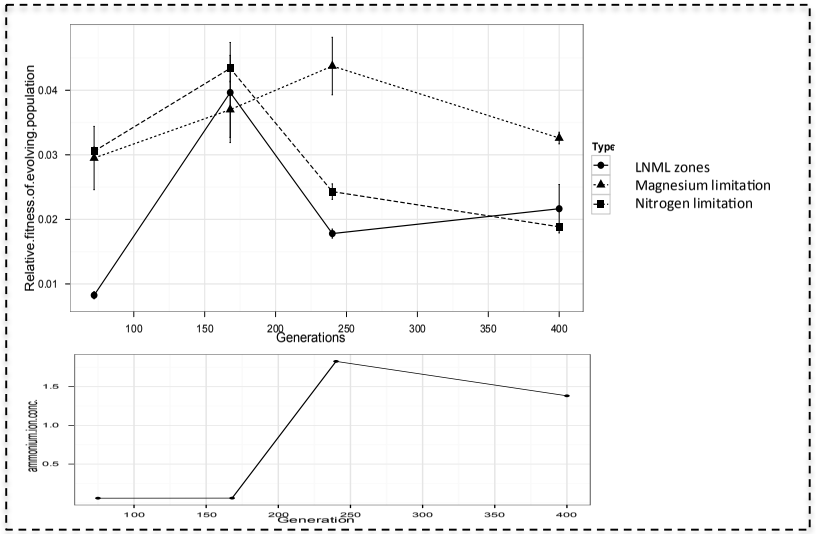
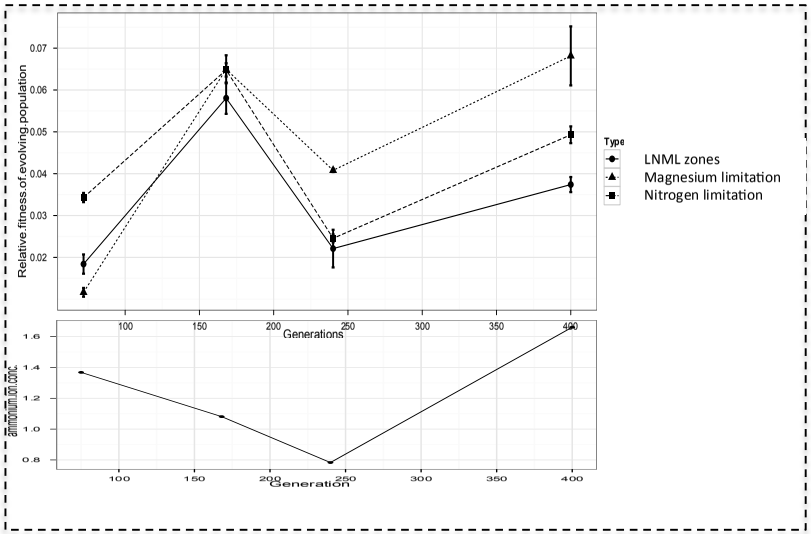


Fig.6: Relative fitness of populations evolved in LNML zone in environments that are nitrogen limited, magnesium limited and in LNML zones. Also shown in the lower panel for each figure is changing ammonium concentration during the experiment. These environments change from nitrogen limiting to nitrogen replete through the course of the experiment.

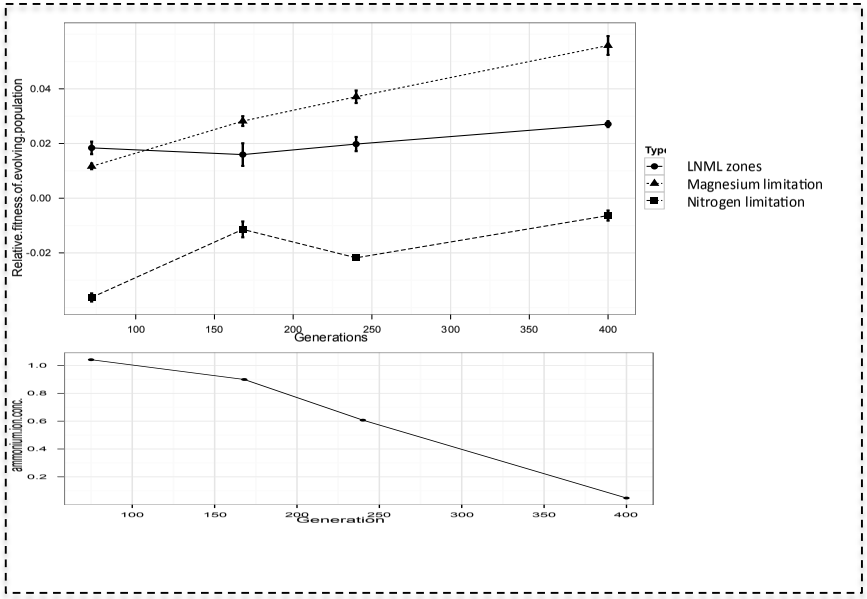
Population evolved in LNML zone: Population1



Population evolved in in LNML zone: Population3



Population evolved in LNML zone: Population4



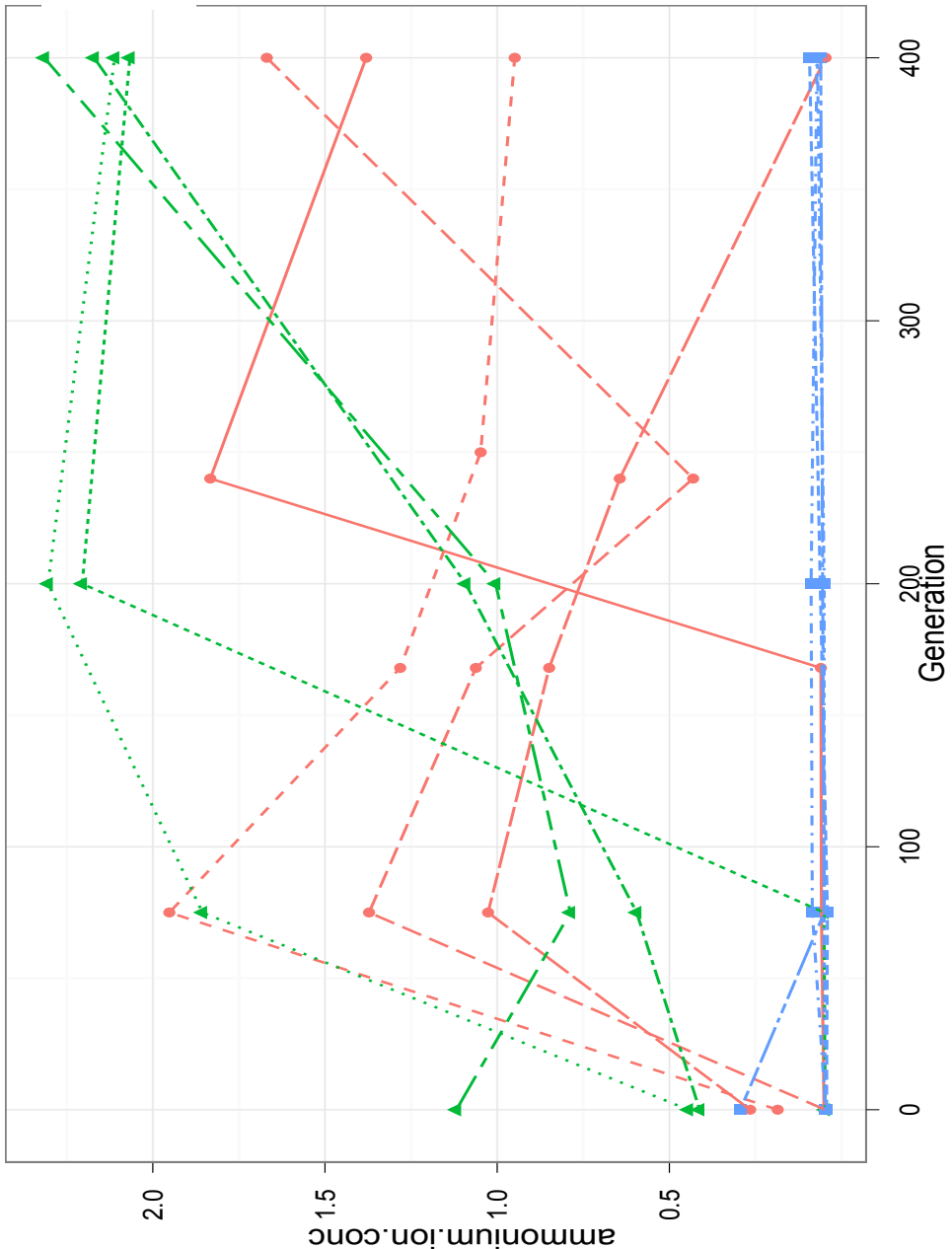
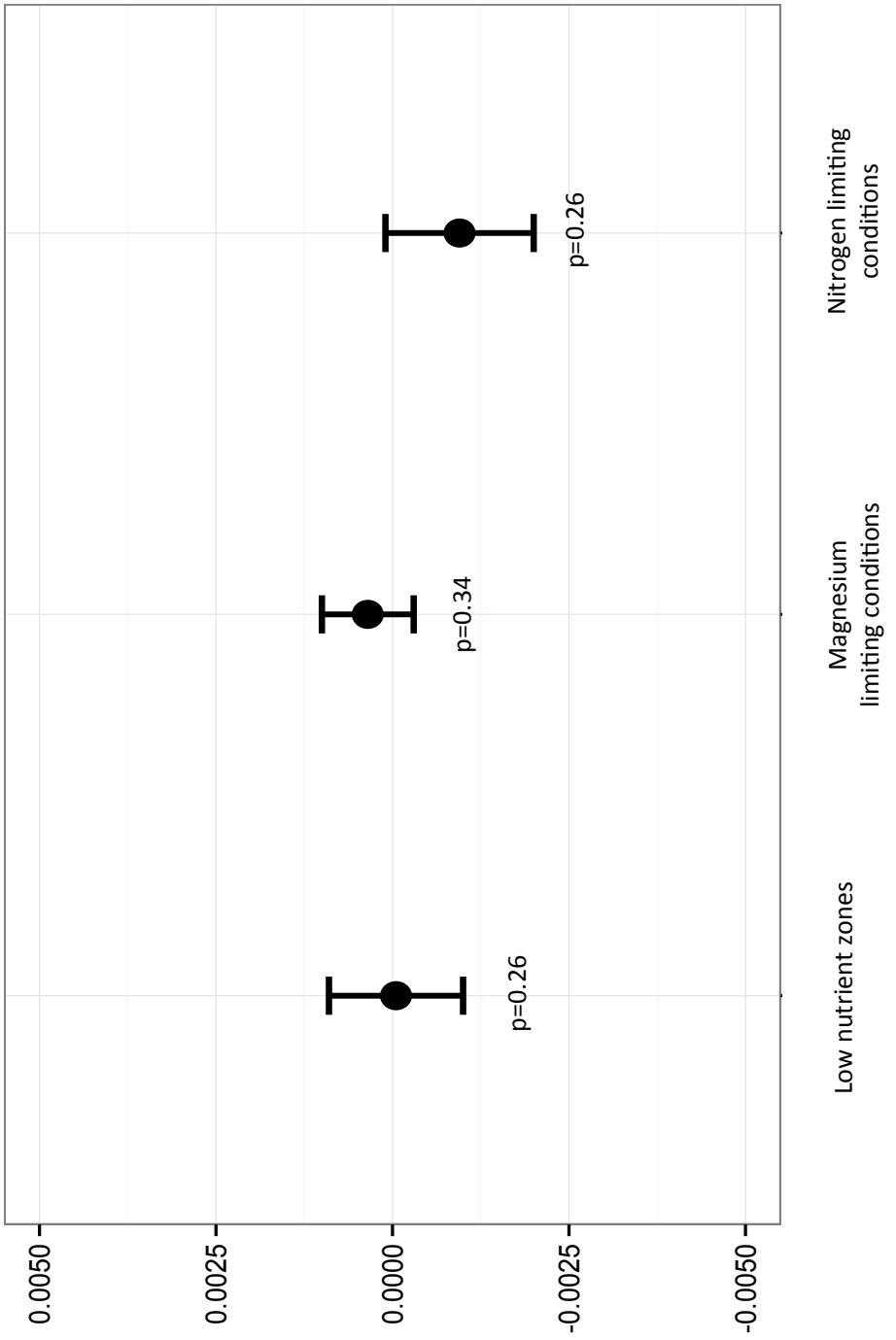


Fig.7: Fluctuating nitrogen concentration during the course of the experiment. Concentrations of ammonia in single nutrient limited magnesium and single nutrient limited nitrogen conditions are also shown for comparison.

Fig.8. Testing for neutrality of genetic marker *lac* that is used in competition experiments. p values indicate no statistical difference between any of the values below and zero.



Chapter Three
Multiple Fitness peaks characterize evolution under nutrient limitation

Abstract:

Fitness landscapes define the relationship between genotypes and fitness for a given selective pressure. Characterizing these landscapes is difficult, however, because of the large number of potential evolutionary trajectories and intermediates involved. We present an assessment of these fitness landscapes using an experimental evolution approach. We have evolved 12 *E. coli* populations under different nutrient limiting conditions (4 each) for 400 generations in chemostats. To compare fitness landscapes across different and ecologically relevant selective pressures, we evolve populations under limiting nitrogen, limiting magnesium and in environments where both nitrogen and magnesium are in low concentrations. We sequenced these populations after generation ~168 and generation ~400 to infer mutation dynamics and identify targets of selections. Our results show that initial fitness increases in the populations are either a result of selective sweeps (due to a single mutation with a large fitness effect) or multiple small effect mutations, and that these mutations (selective sweep or multiple mutations) are eventually replaced by other mutations over the course of the experiment. This discontinuity and non-linearity in adaptive mutations over the course of the evolutionary trajectory suggests fitness landscapes with many small peaks. Our results show that the fitness landscapes of single nutrient limiting conditions have more adaptive peaks of small fitness than landscapes for population adapting in environments where both nitrogen and magnesium are in low concentrations. On the other hand, the number of large fitness peaks varies between the different single-nutrient limiting environments. Overall our results show a complicated process of transient nature of adaptive mutations and highlight specific characteristics of fitness landscapes for different nutrient limiting conditions.

Introduction

Understanding the fitness landscapes of organisms in their environments is one of the major goals of evolutionary biology (Wright, 1932; Lewontin, 1974; Orr, 2002). This concept of association of different genotypes with multiple fitness 'peaks' (Whitlock et al., 1995) and the correlation between these 'peaks' (Kauffman and Levin, 1987) has helped in understanding the complex levels at which nature operates. Many theoretical models have speculated about different properties of these landscapes (Gavrilets, 2004; Orr, 2005). However, they are rarely characterized empirically (Weinreich, 2005; Poelwijk et al., 2007; Jiménez et al, 2013) because of the difficulty in tracing different evolutionary trajectories on a landscape and in identifying the different fitness intermediates along a given trajectory. There has also been criticism on the usage of fitness landscapes because of the ambiguity involved in defining it and some authors have also suggested disregarding their use to explain evolutionary models (Kaplan, 2008; McCandlish, 2011). However we still think that fitness landscapes provide great insights in understanding particular features of evolutionary trajectories. For example these landscapes allows one to visualize how the initial stochastic genetic variation in an evolving populations result in making some adaptive peaks more accessible than others. These also help in visualizing how some evolutionary trajectories on adaptive peaks result in other adaptive peaks becoming inaccessible for the population (Weinreich, 2005). Given these reasons, we present here the use of population sequencing data for populations evolving under three carefully designed selective regimes to assess, understand and compare these fitness landscapes.

Populations evolving under defined selective pressures in laboratories have been useful in increasing our understanding of mutation dynamics (Blundell, 2013; Wiegloss, 2013;) and in outlining characteristics of fitness trajectories (Wiser et al., 2013). We evolved populations under nitrogen-limitation, magnesium-limitation and in environments where both nitrogen and magnesium are present in low concentrations (Figure 1). Nitrogen is an important constituent of all proteins and many other biomolecules and can be rare in the environment. Consequently, various organisms across different ecosystems experience nitrogen limitation (LeBauer and Treseder, 2008). Magnesium is needed in replication,

transcription and translation. Magnesium limitation acts as a signal for transcription of virulent factors and can therefore increase pathogenicity (Guina et al., 2003)). Our study thus allows us to compare fitness landscapes for two nutrients that fulfill very different requirements for the organism. We also specifically analyzed evolutionary dynamics in environments where the concentrations of multiple nutrients are low. Both these kind of environments are observed in nature (Chapin, 1987; Egli, 1992; Klausmeier, 2004; Elser et al., 2007), and have been of major interests to ecologists for studies of ecological stoichiometry (Elser, 2000) and community ecology (Moe et al., 2005). And although single nutrient limitation has received some attention from an evolutionary perspective (Wang et al., 2010; Jezequel et al., 2013; Hong and Gresham, 2014), the same cannot be said about environments where multiple nutrients are in low concentrations. Evolutionary dynamics of populations evolving under multiple nutrient limitations are difficult to study for two reasons. First, an organism's biomass production and growth dynamics in environments with low concentrations of multiple nutrients are governed by the ecological theory of Liebig's law of minimum and Blackman limitation. (de Barr, 1994; Blackman, 1905; Saito, 2008). The first of these, i.e. Liebig's law of minimum, states that the growth of the organism is limited by the low availability of a nutrient relative to what is needed by the organism for its biomass production. The second law, i.e. Blackman limitation, states that the rate of growth of the organism is limited by the concentrations of the nutrient. By extension, one might expect that the evolutionary dynamics in environments where multiple nutrients are in low concentrations should be similar, if not identical, to those seen under limitation of single nutrients. In fact, work in our laboratory (unpublished data) has shown this to be true, where clones adapted in these environments show a stronger degree of adaptation to limiting magnesium conditions than to limiting nitrogen conditions. Secondly, initial adaptation of the population to the most limiting of the two nutrients might result in it experiencing limitation of other nutrients that are also present in low concentrations in the environment. This might result in change of the nature of selective pressure over time, giving difficult to interpret signals of selection. Our results here show that this might indeed be one of the characteristics of populations evolving in these environments. We refer to these as ***L***ow ***N***utrient zones where populations are ***M***agnesium

Limited and abbreviate these as ***LNML*** zones for the rest of this paper***.*** (See Chapter two for more explanation).

To characterize the fitness landscapes in these three nutrient-limiting environments, we make use of a population sequencing approach. Whole genome studies that allow assessment of mutation dynamics and empirical identification of adaptive targets have become achievable with Next generation sequencing (NGS) technologies. Understanding the mutation dynamics under different selective pressures helps in inferring the nature of fitness landscapes in these environments. We sequenced the populations at two different time points in our evolution experiments to identify different targets of selection and to investigate the genetic similarity/dissimilarity in these evolving populations. We identified targets of selection based on five primary criteria.

1) A given gene consistently showing a SNP across different replicates for the same experimental condition (Liao et al., 1986; Bull et al., 1997; Elard et al., 1996).
2) Increasing frequency of a mutation within a gene through time under experimental settings.
3) High frequency non-synonymous single nucleotide polymorphisms (SNPs) that are unique to the populations under experimental conditions. Although we realize that these might also be outcomes of hitchhiking or genetic drift events, we are inclined to think of these as adaptive responses for two reasons. First, the population size of each of our evolving population is large enough ($\sim 10^9$ cells) for genetic drift to be a weak force. Second, most of our evolving populations show only a single mutation reaching high frequencies, thus making a hitchhiking event unlikely.
4) A given gene showing two or more independent SNPs at the same time point in a single evolving population. This is synonymous to the concept of clonal interference. (Miralles et al., 1999; de Visser, 2006; Bollback and Huelsenbeck, 2007). Clonal interference is a common observation in evolving populations and has been described and formalized by Gerrish and Lenski (1998).
5) The last signature involves a given gene showing two or more independent SNPs in the same evolving population but at different time points. We think that these are adaptive

mutations because these genes appear to be targeted at both time points in our experiment. These adaptive mutations are either lost initially due to accumulation of detrimental mutations in the background or due to being outcompeted by more fit variants. We call these mutations Discontinuous Repeatable Targets (DRTs) to separate them from cases of clonal interference. (*In our experiment, this includes SNPs arising in a gene after ~168 generations, only to be lost eventually, followed by a different SNP arising at ~400 generations in the same gene*).

Our study thus investigates genetic variation and mutation dynamics in evolving populations under single nutrient limited environments and in environments where the concentration of multiple nutrients is low. We use population sequencing to identify targets of selection in these populations and compare mutation dynamics across the different environments. We further provide a qualitative assessment of the fitness landscapes under these nutrient-limiting conditions.

Results:

Variations in fitness trajectories of populations evolving under nutrient limiting conditions:

The fitness of the evolving populations was measured through competition experiments against the ancestral strain in different nutrient limiting conditions. Measurements were made for four time points in the evolution experiment i.e. ~72 generations, ~168 generations, ~240 generations and ~400 generations (Table 1). Our results show that different populations evolving under the same nutrient limiting conditions increased their fitness to varying degrees. This increase in fitness differed in terms of evolutionary trajectories with respect to one another.

Nitrogen limitation: Populations evolving under limiting nitrogen conditions showed an increase in relative fitness from ~0.027 to 0.041 (Refer to chapter two Fig. 5, Table 1).

Three of these populations (Population2, Population3 and Population4 in Fig 2a) showed most of the increase in fitness during the first ~168 generations, reaching an asymptote between 168 and 400 generations. The remaining population (Population1 and Fig 2a) increased in fitness at a relatively constant rate throughout the experiment.

Magnesium limitation: Populations evolving under limiting magnesium conditions increased in relative fitness ~0.011 to 0.0507 (Refer to chapter two Fig. 5, Table 1). The initial increase in fitness showed a more diverse response in these populations compared to populations evolving under nitrogen-limited conditions. Two populations (Population3 and Population4 in Fig 2b) had the largest increase in fitness during the first ~168 generations, with later increases becoming progressively smaller as if approaching an asymptote. The third population (Population1 in Fig 2b) did not have any change in relative fitness for the first ~168 generations, followed by a rapid increase between ~168 and ~ 240 generations, and almost no difference in fitness between ~240 and ~400 generations. The fourth population (Population2 in Fig 2b) showed a decrease in relative fitness through the first ~ 168 generations, followed by a rapid increase between ~168 and ~240 generations, and a much smaller increase in fitness between ~240 and ~400 generations.

Populations evolving in LNML zones: Populations evolving in environments where the concentrations of both nitrogen and magnesium were low showed an increase in relative fitness of ~ 0.0148 – 0.0374 (Refer to chapter two Fig. 5, Table 1). Three of these populations (Population1, Population2 and Population3) show an initial increase in relative fitness of the population till generation ~168, which is then followed by a distinct decrease in relative fitness between ~168 and ~240 generations. The relative fitness then gradually increased in these populations, but never equaled the initial rise (Fig. 2c). Population4 showed a large increase in relative fitness in the first ~72 generations but then did not show much change over the next ~328 generations.

Distribution of SNPs across genomes in evolving populations:

Table 2 gives a summary of SNPs that reached 15% or higher in each of the evolving populations, across two time points during the course of the experiment. Three different trends are seen. First, we observe statistically fewer numbers of SNPs in populations evolving in environments where multiple nutrients are in low concentration, as compared to environments where single nutrients are in low concentrations (p=0.007). Second, given that 10% of the *E.coli* genome is intergenic and 90% is protein coding (Neidhart, 1996), only six out of twenty-four comparisons show a greater number of mutations in the intergenic regions than what is expected by chance. Thus, in most situations, we observe that the distribution of SNPs between intergenic regions and protein coding regions is similar to what is expected by chance. Lastly, dN/dS values show that in seven cases purifying selection is the dominant selective force across the genome and in the remaining seventeen cases dN/dS gives no signature of selection i.e. the genomes are evolving in a selectively neutral manner. dN/dS calculations are based on a null model of a random protein having ~74% of non-synonymous sites and ~26% synonymous sites (WenHsiung, 1997). On average, dN/dS for populations evolving under nitrogen limitation, under magnesium limitation and in low nutrient zones was 0.6319, 0.7618 and 0.8573 respectively; none of which are statistically different from each other.

The table also shows the proportion of mutations that we have identified as being potentially adaptive, based on the criteria mentioned in the introduction. In most cases this proportion is less than 5%, highlighting our conservative approach in identifying adaptive mutations. We described these adaptive mutations in the next section. Such a low proportion of adaptive mutations also suggests that most of the non-synonymous mutations that we see are either neutral or are deleterious in nature.

Identification of targets of selection after ~168 generations and ~400 generations:

We sequenced populations from two time points and characterized the genetic variation that accumulated during the experiment (~168 generation and ~400 generation).

NtrBC and genes involved in metabolism of alternate nitrogen sources are targets of selection under limiting nitrogen conditions:

Populations evolving under nitrogen limitation show non-synonymous SNPs in gene *glnG* in three out of the four populations. The fourth population consists of a two bp deletion in gene glnL. *glnG* and *glnL* code for proteins NtrC and NtrB respectively. Limiting nitrogen conditions acts as the environmental signal for induction of protein NtrB. NtrB in turn is responsible for activation of protein NtrC, which further induces the expression of multiple downstream operons that function to increase the efficiency of the cell under limiting nitrogen conditions and in scavenging for alternate nitrogen sources (Gyaneshwar et al., 2005, Reitzer, 2003). In our evolving populations, mutations in NtrB and NtrC occur in regions through which the two proteins interact. Previous studies on these proteins have shown that mutations in this region make NtrC insensitive to NtrB and result in constitutive (unregulated) expression of NtrC (Pioszak and Ninfa, 2004). SNPs in *glnG* (NtrC) reach a high frequency (~52% and ~93%) in two out of four of our populations while the two bp deletion in *glnL* (NtrB) reaches fixation in the population in which it arose. Both these observations point at proteins NtrBC being crucial targets of selection under limiting nitrogen conditions. Besides mutations in *glnG*, we found nineteen genes to contain a non-synonymous mutation in two out of four replicates (Table 3a), but these were all present at low frequencies in the population. These included genes with diverse set of functions that include oxygen sensing, multidrug efflux pumps and carbon metabolism.

One unexpected finding of our evolution experiments is that we did not find any mutation that was present at both ~ 168 generation and at ~400 generation in these evolving populations. This discontinuity in mutation dynamics highlights a complicated pattern of new adaptive lineages arising and being lost during the course of the experiment. We did find several instances of clonal interference both at generation ~168 and at generation ~400 (Table 3a). Across all the populations genes involved in metabolism of triethylamine (*torZ*), alanine (*ycfS*) and ethanolamine (*eutA*) show multiple

independent non-synonymous SNPs occurring at generation ~168. Genes involved in metabolism of aspartate (*xdhA*), in oxygen sensing (*dosP*), multidrug efflux pump (*mdtM*) and carbon metabolism show multiple independent SNPs at generation ~400. Surprisingly, none of the genes that show evidence of being selected are consistent between generations ~168 and ~400. This observation adds up nicely to our previous observations (lack of continuous mutations across different time points) further suggesting that multiple adaptive lineages arise and die over the course of evolution experiments. We find seven mutations to be DRTs (Table 3a) across populations evolving under limiting nitrogen conditions. These include *brnQ* (branched chain amino acid transporter), *malP* (maltodextrin phosphorylase), *ydbA* (autotranspoters), *yeeJ* (adhesion protein), *basS* (involved in response to excess iron), *pta* (phosphate acetyltransferase) and *mdtM* (multidrug efflux protein). These targets appear to be involved not only in transport of nitrogen sources but also in metabolism of alternate nutrients like iron and acetate. This highlights that the connectedness among different metabolic pathways in the cell might allow small affect mutations to be distributed across diverse metabolic pathways.

Overall our results show that under nitrogen limiting conditions, NtrBC dual regulator system is the major target of selection, while genes involved in metabolism of alternate sources of nitrogen and other nutrients might offer smaller fitness advantages. Our results also suggest that towards the end of the experiment these early mutations with small fitness effects are all replaced by the large affect mutation in NtrBC.

Deletion of toxin producing gene yhaV and cell-membrane physiology are major targets of selection under limiting magnesium conditions:

Populations evolving under limiting magnesium conditions show different mutation dynamics as compared to populations evolving under limiting nitrogen conditions. Unlike the cases described under limiting nitrogen conditions, three out of four populations evolving under magnesium limitation possessed similar mutations at generations ~168 and ~400, with the frequency of these mutations rising over the course of the experiment (Table 3b). These included a deletion in gene *yhaV* (an toxin system in E.coli), and non-

synonymous mutations in gene *fabR* (involved in regulating membrane homeostasis), *aldA* (non-specific aldehyde dehydrogenase) and *srlA* (involved in purine metabolism). Genome coverage data for the fourth population was too low for our analysis and hence was not considered. We found thirty-six genes, showing low frequency SNPs, to be repetitive between at least two out of four replicates (Table 3b). Like in the case of populations evolving under limiting nitrogen conditions, these genes performed diverse set of functions. These included genes involved in carbon metabolism, amino acid transporters and cell membrane physiology.

Two out of four populations showed evidence of clonal interference patterns after generations ~168 but did not show any instances of clonal interference after generation ~400 (Table 3b). These included genes involved in sensing extracellular iron concentrations (*basS*), genes involved in motility (*crl, bglX)* and those involved in cell membrane physiology (*fixA*). One population showed a single case of clonal interference at generation ~400, but this population did not show any at ~168 generations. Coverage data from the last population for generation 168 was not enough to call for SNPs, hence we did not analyze this population for this time point. At the 400-generation mark we did find two genes showing evidence of clonal interference. These include a psuedogene (*insM)* and gene involved in bacteriophage N4 adsorption (*nfrA)*. Thus, across all the four populations, genes involved in cell motility, cell division and in membrane biogenesis appear to be selected for under limiting magnesium conditions. This is not surprising because Mg^{2+} places a critical role in membrane stability, cell division and in flagellar assembly.

High frequency SNPs in these populations included the deletion in gene *yhaV*, and non-synonymous SNPs in genes *phoQ and lptG*. *phoQ* is the global regulator expressed under magnesium limitation and it induces genes involved in biofilm formation, cell division and motility. *lptG* is involved in cell membrane synthesis. These populations also show four cases of DRTs (Table 3b): *ydbA* (autotranspoters), *paoC* (involved in purine metabolism), *mrdA* (Proteins involved in maintaining cell shape), *potG* (puterscine transpoter) and *prfC* (release factor in translation). As was seen in populations evolving under nitrogen limiting

conditions, DRTs fall into diverse functional classes like translation and nitrogen metabolism.

Overall our results suggest that modification of cell membrane and cellular motility, and loss of toxin *yhaV* are selected for under limiting magnesium conditions. Similar to populations evolving under nitrogen limitation, we find discontinuity between potentially adaptive mutations that are found at ~168 generations and those that are found at ~400 generations. This again emphasizes the complex dynamics of multiple lineages in populations evolving under limiting nutrient conditions.

Populations evolving in LNML zones are only qualitatively similar to populations evolving under single nutrient limited environment:

Studies in our lab (unpublished) have shown that populations evolving under LNML zones have characteristics that are qualitatively similar to those evolving under magnesium limitation; i.e., these populations are adapted for limiting magnesium conditions. However, we find the nature of mutation dynamics to vary between these seemingly similar nutrient limiting conditions.

Two populations evolving in LNML zones harbored similar mutations at generations ~168 and ~400 (Table 3c). These genes included *ade* and *guaD* which are involved in nucleotide metabolism, *yhaV* which is a toxin producing gene and *mutM*, which is involved in DNA damage repair, for one of the populations and *lptA* (involved in membrane biogenesis) for the other population. Only one population out of four showed any clonal interference pattern at generation ~168 while two populations showed clonal interference pattern after generation ~400 (Table 3c). This included genes involved in synthesis of capsular polysaccharide, transporters and peptidases. Thus, clonal interference is less obvious in these populations as compared to populations evolving under limiting magnesium conditions. However, similar to populations evolving under limiting magnesium conditions, we did not find any mutations that were consistent between replicates. We did find high-frequency SNPs in genes *lptB, yhaV, rho* and *lptA* across three

replicates. It is interesting to note that one of the potential targets of selection under limiting magnesium conditions was gene *lptG*, which like *lptB* and *lptA*, are involvd in synthesis of cell-membrane. We found only one case of DRTs in populations evolving in LNML zones, which is deletion in toxin producing gene *yhaV* (Table 3c).

Taken as a whole, these results suggest that mutations in the genes in the *lpt* operon, which affects cell membrane synthesis, are major targets of selection in LNML zones. Similar mutations were observed under magnesium- limiting conditions, except that we found limited cases of clonal interferences and no cases of DRTs in populations evolving in LNML zones. This suggests that although the large effect mutations are similar between these two environments, the mutation dynamics of potentially small fitness affect mutations varies. As was done in the previous in Chapter 2, we also performed a Mantel test to further highlight the evolutionary dynamics between these populations (Mantel's r= 0.1999, p= 0.1211).

Discussion:

Mutation dynamics under single nutrient limitation suggest the fitness landscape to have multiple small peaks:

The increase in relative fitness in the populations evolving in single nutrient limiting environment most commonly followed an expected trajectory for populations evolving in a novel stressful environment. Most of these populations have a large initial increase in relative fitness, which then gradually slows as fitness reaches an asymptote (Lenski et al., 1991; Orr, 2002). Six out of eight populations show this trajectory. In four of these six populations, the initial increase in fitness is accompanied by a single mutation reaching a high frequency in the population, suggesting a selective sweep event. The other two populations, among these six, show multiple mutations accompanying this initial increase

in fitness. The latter of these patterns indicate multiple clones increasing in frequency, not allowing any single clone to reach high frequency in the population; resembling to the pattern of clonal interference. These results suggest that besides the expected selective sweep events, multiple small affect adaptive mutations in the population also lead to an increase in the fitness of the population, resembling a selective sweep event. Surprisingly, in all these six populations, both these kinds of mutations are lost over the course of our evolution experiment. The remaining two populations in these set of eight, which evolve under single nutrient limiting conditions, either show a continuous increase in fitness or show a decrease in fitness followed by an increase.

Further, discontinuity arises between mutations over the course of evolution and different SNPs evolve in the same gene, arising at two different time points (DRTs). Fitness trajectories of populations evolving in environments where multiple nutrients are in low concentration show unique features of increasing and decreasing fitnesses. These are discussed later.

Our observations paint a picture of the dynamics of adaptive mutations in populations evolving in novel environments that is different from the expectation of infrequent selective sweep events (Fig 2). First, besides the expected selective sweep events, we also find potentially small affect adaptive mutations dominating the initial increase in fitness (Fig 2c). Second, in most cases, the initial large affect mutation (Fig 2b) or the initial set of multiple small affect mutations (Fig 2c) are then replaced by higher fitness affect mutations resulting in a complicated dynamics of birth and death of these lineages. In fact, the most expected case of an initial selective sweep event that then persisted in the population at high frequencies (Fig. 2a) was seen in only one of our evolving population. Finally, this turn-over of adaptive mutations is not accompanied by large changes in fitness.

In our assessment of fitness landscapes we consider mutations that reach high frequencies in populations or those that are consistently found across all the replicates for a given experimental condition to represent large fitness peaks. Although large fitness peaks can be outcomes of both single mutations with large fitness effect or can be an

additive outcome of multiple small effect mutations, our experimental design does not allow us to distinguish between these two scenarios. Adaptive mutations that are identified by other forms of signature of selection, i.e. representing pattern of clonal interference or DRTs, and remain in low frequencies in the population are thought to represent small fitness peaks. With this distinction, we try categorizing the adaptive mutations in our experiments to either represent small fitness peaks or large fitness peaks. Given that we find many low frequency adaptive mutations with most of them being lost over the course of our experiment, it is quite probable that these represent small fitness peaks on a fitness landscape. These lineages are then generally stuck on these peaks to be gradually outcompeted by mutations of larger fitness affects. We also find adaptive mutations that potentially represent large fitness peaks. We describe these in the next section.

Our observation is similar to those seen in a recent study by Lang et al. (2013), which consisted of populations evolving in batch cultures. They found cohorts of mutations rising in the population together and being lost together. Unlike their analysis, however, we have specifically identified genes that are potentially being selected for in the populations. Thus, our results mainly concern the behavior of adaptive mutations in these populations. We show here that transient nature of adaptive mutations should be the expectation under nutrient-limiting conditions rather than the exception. Only a few studies have seen such dynamics (Papadopoulos et al. 1999; Wichman et al. 1999; Holder & Bull 2001; Bollback & Huelsenbeck 2007; Kao & Sherlock 2008; Pepin & Wichman 2008), with none investigating it at the resolution of population genomic data using next generation sequencing technology.

Effect of initial stochastic variation on large affect mutations is based on the environmental conditions:

Another unexpected observation in our evolution experiments is the different degrees of repeatability of targets of selection. Populations evolving under nitrogen-limited conditions show mutations in *glnG* in three out of four populations, with the fourth population

showing a deletion in gene *glnL*. *glnG* and *glnL* interact with each other under nitrogen limited conditions, suggesting that the phenotype resulting from this interaction is an important target of selection (Pioszak and Ninfa, 2004). On the other hand, populations evolving under magnesium limited conditions show minimal repeatability in mutations observed at the end point in our experiments. Deletion of toxin gene *yhaV* and mutations in gene *fabR* is seen in two out of four populations. Besides these two instances of repeatability between replicates, we do find many other genes that show signatures of being selected under magnesium limited conditions, but none of these targets are consistent in two or more populations. These include genes that play important roles in cell membrane synthesis and act as global gene regulators under magnesium limited conditions.

We try addressing this variation in repeatability based on our understanding of how microbial populations behave under stressful conditions. Microbial populations evolving in a stressful environment usually have an increased mutation rate by the action of SOS response and error prone polymerases (Tang et al., 2000). With population sizes in our experiments ($\sim 3 \times 10^9$), this leads to large amounts of initial genetic variation in the population. Consequently one expects to find large number of beneficial mutations in these populations. Recent studies have pointed out that the interplay between the initial stochastic genetic variation and the deterministic nature of adaptation decides the evolutionary response of evolving populations (Weigloss et al., 2013; Lang et al., 2013). Our results show that under nitrogen limitation the deterministic nature of evolution is the more dominating force resulting in repeatable targets of selection. While the diverse nature of evolutionary responses in populations evolving under magnesium limited conditions suggest that the initial stochastic variation has a greater impact on final evolutionary outcomes in these populations. Interestingly, populations evolving in LNML zones, which have been shown to be qualitatively similar to the populations evolving under magnesium limited conditions, also show a similar pattern of diverse evolutionary response. Repeatability due to the deterministic nature of natural selection can also be inferred in terms of fitness landscapes, which were discussed previously. Repeatable response under nitrogen-limited conditions suggests that the fitness landscape consists of limited number

of large affect fitness peaks. Using the same rational, the diverse response under magnesium-limited conditions might imply multiple large fitness peaks on the fitness landscape.

Overall this suggests that fitness landscapes under nitrogen limited conditions have multiple small peaks and limited large peaks, while fitness landscapes under magnesium-limited conditions have multiple small and large peaks.

Different mutation dynamics are observed under single-nutrient limited environment and in environments where multiple nutrients are in low concentrations:

In environments where multiple nutrients are in low concentration, ecological theory suggests that only the more limiting nutrient will affect the growth of the organism, and hence the evolutionary outcome. Indeed, in our experiments, evolution in LNML zones appears at first to be similar to evolution under magnesium limitation alone (unpublished results). Also the phenotype under selection in both these cases includes the cell membrane and deletion of toxin coding gene *yhaV*. In these environments, we have also seen the accumulation of nitrogen source in the media, further suggesting that the populations are only being limited by magnesium ion.

However, analysis of genetic variation and adaptive mutations in populations evolving in LNML zones show a pattern distinct from its single nutrient limited counterpart. Importantly, we find half the number of clonal interference instances in populations that evolved in LNML zones as compared to populations that evolved under single nutrient limitation. This suggests that even if these populations are evolving towards becoming adapted to magnesium limited conditions, the quantitative nature of small affect adaptive mutations varies between these environments. In terms of fitness landscapes, this implies that in LNML zones, fitness landscapes comprise of limited small and multiple large fitness peaks.

The fitness trajectories of populations evolving in LNML zones also show a decrease in relative fitness around generations ~168 for two of the populations. This suggests a complicated picture of potentially changing selective pressures in LNML zones. After initial adaptation on the more limiting of the two nutrients (which is magnesium ion in our case), it is possible that the population experiences some degree of nitrogen limitation. Although this hypothesis explains both our observation of decrease in relative fitness of these populations and of different mutation dynamics under these two environments, more work has to be done to prove this hypothesis.

Models at extreme ends of adaptive mutation dynamics: Clonal intereference model and multiple mutation model

Population sequencing results show that populations evolving under nutrient limiting conditions show genome-wide signature of purifying selection and of neutral evolution. However, we also observe a large number of potentially adaptive mutations under the three different nutrient limiting regimes. These observations might be a result of a high mutation rate under stressful conditions and of a large proportion of non-synonymous mutations being detrimental. A high mutation rate can help explain the large number of beneficial mutations giving a signature of positive selection; while both a high mutation rate and large proportion of detrimental non-synonymous mutations might result in an excess of observed synonymous SNPs from what is expected by chance, and a lower number of non-synonymous mutations than what is expected by chance.

Our study also highlights the birth and death process of multiple lineages that arise in populations under nutrient limited conditions. Such processes are captured in two adaptive mutation models, which are placed at extreme ends of the mutation dynamics spectrum in asexual populations: Clonal interference model and a multiple mutation model (Sniegowski and Gerrish, 2010). The former suggest single adaptive mutations in individual clones characterize evolutionary trajectories while the latter suggests clones with multiple adaptive mutations determining the outcome of evolutionary trajectories. In reality, and as has been acknowledged, nature works in between these models. Our data does not consist

of linkage data between mutations to identify which model is more relevant in our case. However the high level of non-linearity in adaptive mutations over the evolutionary time and the fact that only single mutations reached high frequencies in our populations suggests that our results fall more in line with the clonal interference model than with the multiple mutation model. This is in contrast to Lang et al. (2013), who demonstrated in their evolution experiments with yeast that a second adaptive mutation was usually needed to push a clone to high frequency in the population.

In conclusion, our data shows a complicated dynamics of birth and death of adaptive mutations. We demonstrate that adaptive mutations have a transient nature, more so than what was previously expected. Besides these general results on dynamics of adaptive mutation, our results show that fitness landscapes for single nutrient limitations and conditions where multiple nutrients are in low concentrations differ in the distribution of fitness peaks. Together these results highlight areas that need to be further explored at the functional level in such evolutionary experiments.

Material and Methods:

Strain and media used:

Ancestral strain used in all these experiments is a derivative of *E.coli* K-12 MG1655. that is cured of lambda phage and contains no plasmid. It is *lac*- (due to a deletion of lac operon) and *rpoS*-(due to deletion in a region of *rpoS* gene). All long-term evolution experiments were carried out for ~400 generations in chemostats in glucose minimal media. Description of the media is the same as in Chapter 1 and Chapter 2. Nutrient limitation was demonstrated by plotting growth curves at the mentioned concentrations of the nutrients. Long-term evolution experiments generally lasted for ~34 days, during which chemostats were changed every 10 days to avoid wall affects. The flow rate was maintained to get a ~2 hr generation time. Samples were taken every 24 hours and were frozen as glycerol stocks at – 80 ^{o}C. Contamination checks were performed every 24 hours by plating the samples on citrate plates.

Fitness assays for clones and populations:

A *lac+* derivative of the ancestor strain used in this study was derived by P1 transduction. This marker was shown to be neutral under the nutrient-limited conditions used in this study (See Chapter 2 Fig 8). All competitions were carried out in chemostats under appropriate nutrient conditions. Both, ancestral strain and evolved populations were grown in appropriate nutrient limited conditions in shaker flasks and were allowed to reach an optical density of 0.6. 1 ml of these strains was then inoculated in chemostats with the desired media. Competitions were carried out typically for 48-72 hrs. Selection coefficient was calculated by plotting log of ratios of cell counts to time and calculating the slope of linearly regressed line. For each chemostat we carried out two replicates. Values shown here represent averages of the slopes obtained in each replicate and the associated standard errors for the mean.

Next-gen sequencing:

We constructed genomic libraries from DNA extracted from these populations. Populations from two time points (~168 generations and ~400 generations) were re-grown using their glycerol stocks in appropriate nutrient limited conditions for 24 hours. DNA was extracted from 5 ml of this media using the DNeasy blood and tissue kit from Qiagen. Protocols were followed as mentioned in the manual, except for increasing the lysis time to one hour. Libraries were made using the NexteraXT sample preparation kit. Samples were dual-indexed and pooled together. Two separate runs on Illumina's Miseq was used for sequencing using the Miseq reagent kit v2 (500 cycle). 9.9 Gb of data was obtained in the first run with 76% of reads being above the Q30 score while 2.5 Gb of data was obtained in the second run with 74% reads being above the Q30 score. The average coverage for each of the populations is shown in the table 4. Geneious was used to map the reads onto the reference genome and to find SNPs. Conservative values were used for trimming the raw reads (40 bp from either end), aligning these reads and for finding variants in the data. The reads were aligned to E.coli K-12 MG1655 reference genome, which was downloaded from Genbank (NC_000913.3). For SNP detection the cut-off values

used were a minimum coverage of 15 and the SNP frequency of 15%. The ancestral strain was also sequenced using the same protocol to identify SNPs present in the ancestral strain in comparison to the reference genome. These were excluded from the analysis.

Tables:

Table1. Relative fitness measures for the populations used in this study. p values in each cell indicates if the difference between the relative fitness value and zero is statistically significant.

Population studied	Relative fitness of the population after			
	~72 Generations	~168 Generations	~240 Generations	~400 Generations
Population1-Nitrogen limiting conditions	0.0055±0.001 p value=0.03	0.0126±0.0013 p value=0.01	0.0190±0.0009 p value=0.005	0.0324±0.0008 p value=0.002
Population2-Nitrogen limiting conditions	-.0086±0.001 p value=0.1	0.030±0.005 p value=0.02	0.040±0.008 p value=0.023	0.0413±0.0001 p value=0.0003
Population3-Nitrogen limiting conditions	0.0015±0.001 p value=0.1	0.0126±0.001 p value=0.012	0.024±0.001 p value=0.007	0.0288±0.007 p value=0.031
Population4-Nitrogen limiting conditions	-0.008±0.004 p value=0.06	0.0282±0.005 p value=0.02	0.03135±0.001 p value=0.004	0.0381±0.007 p value=0.02
Population1-Magnesium limiting condition	-.0005±0.0002 p value=0.08	-0.002±0.0002 p value=0.06	0.0325±0.00098 p value=0.004	0.033±0.0019 p value=0.009
Population2-Magnesium limiting condition	0.0003±0.001 p value=0.05	-0.0136±0.0009 p value=0.011	0.00435±0.0003 p value=0.01	0.01125±0.00147 p value=0.021
Population3-Magnesium limiting condition	0.01575±0.0036 p value=0.03	0.031±0.0004 p value=0.002	0.0413±0.003 p value=0.011	0.05075±0.0018 p value=0.005
Population4-Magnesium limiting condition	0.01215±0.0016 p value=0.06	0.0251±0.00294 p value=0.02	0.02755±0.004 p value=0.02	0.02885±0.0006 p value=0.003
Population1-LNML zones	0.00825±0.0011 p value=0.021	0.0396±0.015 p value=0.06	0.0178±0.0013 p value=0.01	0.02165±0.0073 p value=0.05
Population2-LNML zones	0.0025±0.0005 p value=0.3	0.04215±0.00049 p value=0.01	0.01155±0.0013 p value=0.01	0.0148±0.0002 p value=0.02
Population3-LNML zones	0.05805±0.00735 p value=0.03	0.0221±0.008 p value=0.02	0.0221±0.0088 p value=0.06	0.0374±0.0035 p value=0.01
Population4-LNML zones	0.0184±0.0045 p value=0.04	0.01595±0.008 p value=0.08	0.0198±0.0051 p value=0.04	0.0271±0.0021 p value=0.01

Table 2: Summary of SNPs that reach 15% or higher across all the populations. p1 indicates if dN/dS is statistically different from 1. p2 indicates if SNPs seen in the intergenic regions are different than what is expected by chance.

Population analyzed	**Time-point analyzed (Generation)**	**Total number of SNPS that reach 15% or higher**	**Type of mutations**					**Adaptive mutation**	
			Non-Synonymous	**Synonymous**	**Genome wide dN/dS**	**Coding**	**Intergenic**	**Coding**	**Intergenic**
Nitrogen limited population 1	168	292	165	79	0.4574 p1=0.0001	240	52 p2=0.007	**9**	**1**
	400	215	95	56	0.8309 p1=0.3618	203	12 p2=0.09	**4**	**0**
Nitrogen limited population 2	168	129	77	39	0.6951 p1=0.0682	116	13 p2=1	**2**	**0**
	400	43	19	24	0.2787 p1=.0001	38	5 p2=0.7359	**5**	**1**
Nitrogen limited population 3	168	292	177	39	0.4574 p1=0.0001	249	43 p2=0.09	**5**	**0**
	400	215	162	143	0.8309 p1=0.3618	183	32 p2=0.09	**8**	**1**
Nitrogen limited population 4	168	135	201	137	0.7383 p1=0.1713	106	29 p2=0.0001	**1**	**0**
	400	274	158	127	0.7663 p1=0.1713	246	28 p2=1	**5**	**2**
Magnesium limited population 1	168	171	115	94	0.7473 p1=0.1713	155	16 p2=1	**6**	**0**
	400	331	188	143	0.8826 p1=0.494	291	40 p2=0.505	**4**	**2**
Magnesium limited population 2	168	173	107	82	0.6377 p1=0.0402	160	13 p2=0.3173	**11**	**0**
	400	250	125	127	0.6930 p1=0.0682	209	41 p2=0.0455	**9**	**1**
Magnesium limited population 3	168	314	198	134	0.8802 p1=0.494	281	33 p2=1	**3**	**0**
	400	214	133	90	0.7446 p1=0.1713	193	21 p2=1	**7**	**2**
Magnesium limited population 4	168		-	-				**-**	
	400	253	142	116	0.7473 p1=0.494	214	39 p2=0.09	**1**	**1**
LNML population 1	168	139	71	68	0.8132 p1=0.3618	125	14 p2=1	**2**	**0**
	400	32	20	22	0.8215 p1=0.3618	21	11 p2=0.0001	**1**	**2**
LNML population 2	168	75	33	42	0.4481 p1=0.0001	64	11 p2=0.09	**5**	**0**
	400	115	86	53	1.0257 p1=1	98	17 p2=0.09	**10**	**1**
LNML population 3	168	221	119	102	0.6651 p1=0.0402	198	23 p2=1	**2**	**1**
	400	67	66	38	1.4788 p1=0.1712	53	14 p2=0.0002	**1**	**0**
LNML population 4	168	183	89	94	0.5497 p1=0.0014	157	26 p2=0.09	**2**	**1**
	400	43	57	36	1.0563 p1=0.8917	36	7 p2=0.0455	**2**	**0**

Table3: Genes showing signatures of selection in the evolving population. Five criteria were used to identify these (has been described in the text).

Table 3a: Signatures of selection in Populations evolving under limiting nitrogen conditions

Genes showing Non-synonymous mutations repeating between replicates	Genes showing Non-synonymous SNPs showing continuity during the course of the experiment	High-frequency Non-synonymous mutations	Genes showing patterns of clonal interference	Discontinuous repeatable Genes DRT
bglJ *paoC* *dosP* *qseB* *emrB* *topA* *empB* *wzyE* *fabR* *xdhA* *glnG* *prlF* *gntR* *nudK* *mdtM* *yhgE* *melA* *yihM* *ytfR*	-	*glnG (NtrC)* *pnp* *glnL* *paoC*	*ydbA* *add* *yjiR* *nudK* *araG* *resC* *yjiR* *eco* *panD* *eptC* *yjhG* *fhuB* *atpF* *proX* *insM* *xdhA*	basS pta mdtM

Table 3b: Signatures of selection in Populations evolving under limiting magnesium conditions

Genes showing Non-synonymous mutations repeating between replicates	Genes showing Non-synonymous SNPs showing continuity during the course of the experiment	High-frequency Non-synonymous mutations	Genes showing patterns of clonal interference	Discontinuous repeatable Genes DRT
araG *proY* *avtA* *rhaS* *cusS* ***sstT*** ***fabR*** *ygfT* *fecD* ***yhaV*** *fimH* *yhgE* *inaA* *mdfA* *mdtM* *paoC* *yiaN* *yjaB* *ypjA*	*yhaV* *fabR* *aldA* *srlA*	***yhaV*** ***fabR*** *lptG* *phoQ* *aldA*	*fabR* *ftsZ* *fimD* *selA* *insM* ***sstT*** *nfrA* *yfeD* *agaS* *cybB* *fimH*	*mrdA* *potG* *prfC* *ydbA*

Table 3c: Signatures of selection in Populations evolving in LNML zones

Genes showing Non-synonymous mutations repeating between replicates	Genes showing Non-synonymous SNPs showing continuity during the course of the experiment	High-frequency Non-synonymous mutations	Genes showing patterns of clonal interference	Discontinuous repeatable Genes DRT
ade *thiB* *basS* *yddB* *fabR* *ydeP* ***insM*** *mdtM* *paoC* ***rho*** ***yhaV***	rho bass eutA lptB yhaV fabR	***rho*** ***lptA*** ***yhaV*** ***paoC***	*ccmB* *dptG* *ggt* *pgaB* *phoE* *fimH* *cpsB*	-

Table4. Genome sequencing coverage results for all the populations used in this study

Population sequenced	**After time point**	**Coverage obtained (Miseq illumina)**
Population1-Nitrogen-limiting condition	Generation~168	13.3±5.6X
	Generation~400	7.3±3.8X
Population2-Nitrogen-limiting condition	Generation~168	5.8±3.4X
	Generation~400	18.2±7.8X
Population3-Nitrogen-limiting condition	Generation~168	6.3±3.1X
	Generation~400	12.2±5.6X
Population4-Nitrogen-limiting condition	Generation~168	8.9±4.1X
	Generation~400	15.3±7.0X
Population1-Magnesium limiting condition	Generation~168	24.4±9.5X
	Generation~400	11.7±5.6X
Population2-Magnesium limiting condition	Generation~168	14.3±6.4X
	Generation~400	19.6±8.2X
Population3-Magnesium limiting condition	Generation~168	9.7±5.3X
	Generation~400	19.2±7.7X
Population4-Magnesium limiting condition	Generation~168	3.3±2.5X
	Generation~400	18.1±7.8X
Population1-LNML zones	Generation~168	15.7±6.9X
	Generation~400	11.2±5.3X
Population2-LNML zones	Generation~168	31.2±11.9X
	Generation~400	18.8±7.7X
Population3-LNML zones	Generation~168	10.6±4.7X
	Generation~400	22.2±8.8X
Population4-LNML zones	Generation~168	12±5.5X
	Generation~400	15.4±6.7X

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Fig. 1. Experimental Design

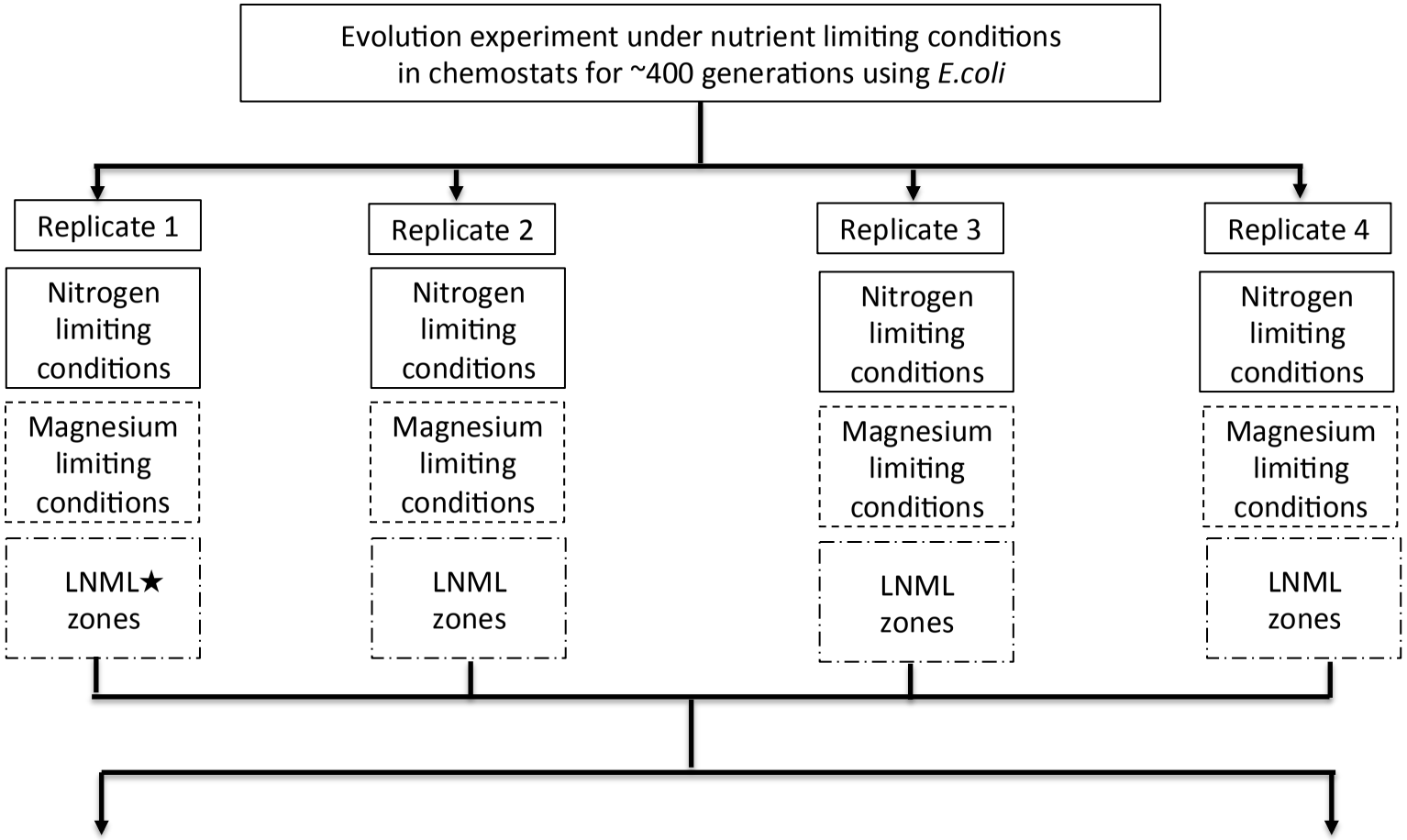


Fig. 2. Different models of the dynamics of adaptive mutations in novel environments. Fig 2b and 2c show a complicated model of birth and death of lineages. In our experiments we find one instance of model 2a,three instances of model 2b and three instances of model 2c.

Fig 2a. A single large affect mutation increases in frequency in the population and is fixed.

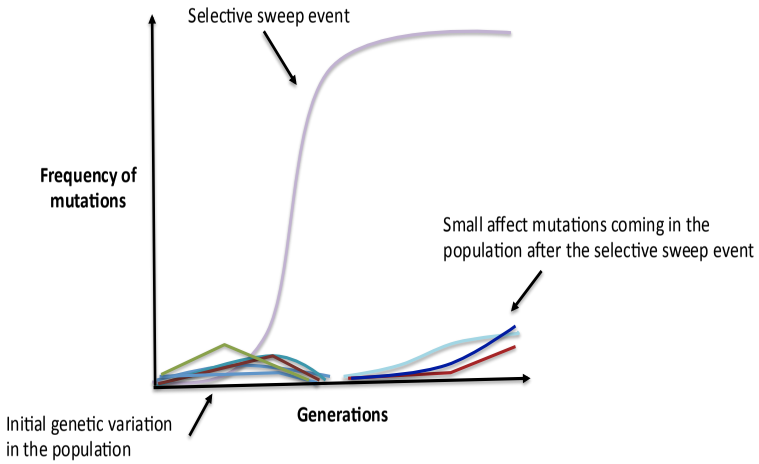


Fig 2b. A large fitness affect mutation increases in the population initially, but is outcompeted by another large fitness affect mutation in the population

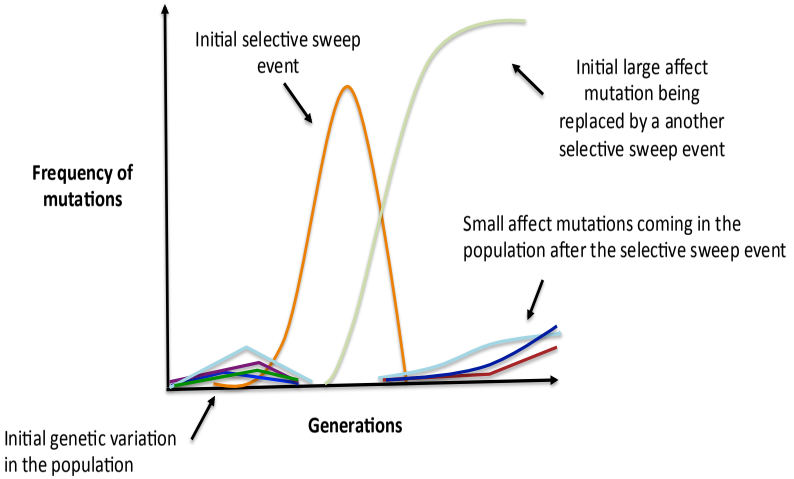
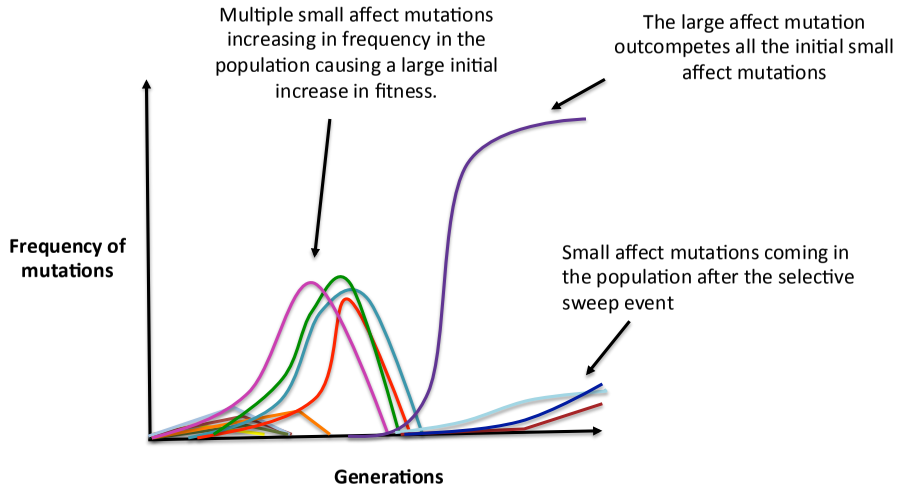


Fig 2c. Multiple small affect mutations increase in the population simultaneously. These result in an increase of the fitness of the population that is similar to a selective sweep..



Conclusion and Speculations

Competition for resources is a common selective pressure in nature (Chapin, 1987; Egli, 1992; Klausmeier, 2004; Elser et al., 2007). Ecologists and evolutionary ecologists have both investigated this concept, with the aim of explaining species diversity and community dynamics (Hutchison, 1961; Tilman, 1981; Grover, 1988). Many authors have written on the theoretical and empirical analysis for competition for resources (Tilman, 1980; Huston and DeAngelis, 1994). These have usually circled around the concept of competition of single and double resources, the equilibrium points around these competitions and the ability to predict different species composition under different resource levels. This thesis asks a similar question of resource competition but in an evolutionary context. The experiments in this thesis investigate adaptive strategies, dynamics of adaptive mutations and the generation of adaptive diversity in different nutrient limiting conditions.

Adaptation to micro and macronutrients: the specificity of resource competition

To understand how adaptation to resource limitation effects population diversity, we first wanted to identify the adaptive strategies that result in adaptation to resource limitation. To address these questions we conducted evolution experiments under limitation of a macronutrient i.e. nitrogen limitation and under limitation of a micronutrient i.e. magnesium limitation. This experimental design allowed us to compare evolutionary responses under different nutrient limitations, and hence look at the specific and general aspects of adaptation to nutrient starvation. It also allowed us to look at the repeatability of the evolutionary responses under nutrient limiting conditions. Our results show that microbial populations can adapt to limitations of both macro and micronutrients. We found variation in relative fitness of evolved clones within a population as well as between different populations, indicating phenotypic heterogeneity in these populations. This heterogeneity was common under both resource-limiting conditions suggesting that

this is a general characteristic of populations evolving under nutrient limiting conditions, irrespective of whether the nutrient is a macronutrient or micronutrient.

We also found specific patterns to adaptation under these two different selective regimes. Clones adapted to nitrogen limitation showed a higher degree of adaptation as compared to clones adapted to magnesium limiting conditions. Clones adapted to nitrogen limitation also showed a lower degree of pleiotropic response in fitness under limitation of alternate nutrients as compared to clones adapted to magnesium limiting conditions. We also identified the potential genetic basis of adaptation under both these nutrient limiting conditions. Global gene regulators were primary targets of selection under nitrogen limiting conditions while proteins involved in cell-membrane synthesis were primary targets of selection under magnesium limiting conditions. Understanding these general and specific responses to nutrient limitation is a necessary first step to understand how these effect changes in population diversity.

Resource competition in a 'more complex' environment: The environment and adaptive niche

To study the effect of resource competition in a more complex setting, we carried out evolution experiments in environment where both nitrogen and magnesium were in low concentrations. Although this does not in any way match the complexity of a natural environment, comparative analysis of evolutionary responses between this environment and the single nutrient limited environments can aid in forming expectations for adaptation under more complex settings. Our results from these experiments show that initial adaptation of populations evolving in these environments is a result of adaptation to the most limiting nutrient. This outcome is also predicted from ecological theory, more specifically from Liebig's law of minimum (de Baar, 1994, Saito et al., 2008). However after this initial phase of adaptation, we observed changes in concentrations of other nutrients in the environment, which although initially were also in low concentrations, were not growth limiting. In some cases we found that the concentration of these nutrients reach negligible

levels, indicating that these had now become growth limiting. It is quite possible that at this stage these 'other' nutrients also affect the evolutionary trajectories of these populations.

This change in environment as a result of adaptation highlights the complexity of natural selection. The environment has always been recognized as a fundamental part of natural selection; it forms a part of the ecology that results in the evolution of natural populations. However, unless defined by the experimenter, understanding the factors of an environment that result in adaptive evolution are difficult to decipher. We performed evolution experiments in controlled environments, where we increased the complexity of the environment by having multiple nutrients in low concentrations. This controlled set up not only allowed us to follow evolutionary trajectories of evolving populations, but also allowed us to detect the changes in nutrient concentrations in these environments, indicating how the environment changed over the evolutionary time course. Thus this work is an important example of how evolution affects the ecology of the system.

These experiments also lay out a more general understanding of adaptation of natural populations. In more complex environments, some factors are more important than the others. Our results show that as the populations start adapting to these more important factors, other environmental factors start playing a more significant role in their evolution. These evolutionary characteristics can be best envisioned as one of an evolving niche space, where the evolutionary potential along the niche axis changes as a result of the population adapting to the optimum.

Adaptive genotypic heterogeneity: the dynamic nature of physiology of the organism and potential community dynamics

Adaptation to a given environment depends upon the rate of incoming beneficial mutations and the rate of fixation of these mutations. In cases of microbial populations adapting to novel environments, the rate of incoming mutations is large, generally due to their large population sizes. However selective sweeps, both hard and soft, result in decrease in genetic variation in these populations, resulting in a more homogenous genetic

population. For a long period of time this was the expectation in evolving microbial populations, especially those evolving in laboratories (Lenski, 1991). Our work depicts a completely different picture of how homogenous these evolving populations are. We find high levels of adaptive genetic variation in populations evolving under nutrient limiting conditions. In total we found 39, 35 and 21 potential targets of selection in our three nutrient environments of nitrogen limitation, magnesium limitation and in environments where both these nutrients are in low concentrations respectively. Importantly, adaptation to all the three different nutrient limiting conditions and the large levels of adaptive heterogeneity is reflective of the dynamic nature of the physiology of an organism. Besides genes that are involved in metabolism of the nutrient in question (i.e. nitrogen and magnesium), we also found large number of genes that are involved in metabolism of other nutrients that show a signature of selection. In populations evolving under nitrogen limitation, genes involved in carbon and phosphorous metabolism are potential targets of selection. Likewise, in populations evolving under magnesium limitation, genes involved in nitrogen and carbon metabolism are potential targets of selection. These results highlight how the connectedness between different metabolic modules can impact adaptive evolution. While mutations with a large effect on fitness might be limited to the metabolic pathways for the limiting nutrient, mutations with small effects on fitness might be more evenly distributed throughout the different metabolic pathways.

These highly heterogeneous populations can also result from complicated interactions between clones as well as between clones and the environment. The former might include positive interaction (cross-feeding) or negative interactions (mortality by toxin production). The latter can include the clones releasing metabolic products in the environment. For example, microbes stressed under glucose limitation are know to release acetate and glycerol in the surrounding environment (Rosenzweig et al., 1994). In this case, this secretion of these by products results in generation and maintenance of population diversity. It is quite possible that the high levels of adaptive heterogeneity that is observed in our evolving populations might be results of these community dynamics. Irrespective of whether these interactions exist or not in our experimental populations, this work shows that these populations are highly heterogeneous both at the levels of genotypes and fitness.

The dynamics of adaptive mutations: A qualitative framework to infer fitness landscapes

The understanding of natural selection and adaptation not only comes from an understanding of the distribution of beneficial mutations but also from how frequently these mutations fix in populations. Our work, and work of others (Wielgoss, 2013; Lang et al., 2013) has shown that evolving microbial populations are generally not mutation limited. An understanding of the rate of fixation of these beneficial mutations will thus increase our understanding of the processes that give rise to the high levels of adaptive heterogeneity. Understanding the dynamics of these adaptive mutations will also help us understand the nature of selective sweeps in the populations. Our work highlights the shortsightedness of natural selection, which gives rise to the complexity in mutation dynamics. We see several adaptive mutations coming in the population that are eventually replaced by other adaptive mutations. Populations evolving in different nutrient limiting environments showed an initial increase in fitness, which was accompanied by either an increase in the frequency of a single mutation (possibly of large fitness affect) or multiple mutations rising in frequency simultaneously (possibly of small affects). However in most of the populations these adaptive mutations were completely outcompeted by other, possibly large effect, beneficial mutations. This same dynamics of birth and death of mutations was consistently observed in different selective regimes of limiting nitrogen, limiting magnesium and in environments where both these nutrients are in low concentrations. This complete turnover of genetic signature of selections over a period of ~200 generations, the large number of adaptive mutations, and the non-linearity in trajectories of these adaptive mutations can be best understood when depicted as adaptive landscapes. Our results show that landscapes for populations evolving under nutrient limiting conditions have multiple peaks of small fitness. As the population starts evolving under these nutrient limiting conditions, many small affect mutations are selected for and the population starts increasing in fitness. However as larger affect beneficial mutations start coming into the

populations, the small affect mutations are replaced. This is what gives rise to the non-linearity in trajectories of adaptive mutations.

Concluding remark:

Each of the points mentioned above are interpretations of complicated observations and thus are by themselves hypothesis generating. Although this thesis has answered several questions about the evolutionary response under nutrient limitation, it has also opened up several other questions for future research. Theoretical knowledge of resource competition and adaptive mutation dynamics can be combined with empirical results from this thesis to design experiments to disentangle the complexity of the adaptive responses in these populations.

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