The Genetics of Adaptation and Evolvability in Yeast

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Cover illustration: The adaptive walk, by Karl Persson

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The most important things are the hardest to say,
because words diminish them.

— Stephen King

Abstract

Evolution is the hereditary change in life forms that has shaped the divergence of all organisms that inhabit planet Earth. I used the yeast *Saccharomyces cerevisiae* to study how adaptive evolution increases the fitness and changes the properties of experimental and natural yeast populations. In **Paper I**, I screened for evolvability genes that control how fast *S. cerevisiae* adapts using experimental evolution and high-throughput growth phenotyping. I investigated the rate of adaptation of nearly all viable single gene deletion strains. I found that the dynamics of adaptation was decided by diminishing returns epistasis, i.e. the decreasing effect size of beneficial mutations in fitter backgrounds, with almost no impact of specific evolvability genes. In **Paper II**, my co-workers and I found that *S. cerevisiae* adaptation to high mitochondrial superoxide production paraquat was extraordinarily swift. We revealed a novel regulatory mechanism whereby this adaptation was achieved: a genetically controlled reduction in the copy numbers of mitochondrial ETC genes through induction of mitochondrial DNA deletions. Intact mitochondrial genomes were rapidly restored after release from short-term stress, while the mitochondrial genome deletions become irreversible during long-term exposure to high mitochondrial superoxide production. In **Paper III**, my co-workers and I evolved *S. cerevisiae* populations with different levels of pre-existing genetic variation under exposure to anticancer drugs. We found that a higher amount of pre-existing variation speeded up adaptation and that selection on pre-existing and new variation acted on the same proteins, albeit on different aspects of the functions of these proteins. In **Paper IV**, my co-workers and I studied how DNA introgressions from the wild yeast *Saccharomyces paradoxus* have appeared in its sister species *S. cerevisiae*, despite the reproductive isolation of these two species. We show that this can be explained by the hybrid going through a genome destabilization event that leads to scattered islands of homozygosity. These in turn provide sufficient base-pairing for meiosis to proceed, and thereby allow two reproductively isolated species to generate offspring, and in the process, also serve as origins of the *S. paradoxus* introgressions into *S. cerevisiae*. Finally, in **Paper V**, my co-workers and I studied how the domestication of *S. cerevisiae* affected its phenotypes, particularly its life cycle. We compared key properties of the life cycle across nearly 1000 wild and domesticated yeast isolates. We found that domestication recently had profoundly altered the life cycle of *S. cerevisiae*, raising questions on how suitable domesticated yeast isolates are as models. Together, these works shed light on the molecular mechanisms whereby one of our key model organism adapts, and have adapted, to changes in the environment and what the consequences of this adaptation are.

Sammanfattning

Evolutionen har förändrat livet på jorden och skapat dess enorma artrikedom. Jag använde bak- och bryggjästen *Saccharomyces cerevisiae* för att undersöka hur adaptiv evolution ökar livsdugligheten och förändrar egenskaperna hos experimentella och naturliga jästpopulationer. I min första artikel undersökte jag om enskilda gener kan påverka anpassningsförmågan hos jäst. Vi använde oss av en samling jäststammar där varje stam saknade en enskild gen. Denna samling anpassade vi i labbet och studerade hur tillväxten förändrades över tid, allt eftersom stammarna anpassade sig. Jag fann att anpassningsdynamiken bestämdes av den minskande effektstorleken av fördelaktiga mutationer i bättre bakgrunder, men påverkades nästan inte alls av specifika evolvabilitetsgener. I min andra artikel fann vi att jästens anpassning till giftet paraquat, som kraftigt ökar superoxidproduktionen i mitokondrien, gick otroligt snabbt. Vi avslöjade en ny regleringsmekanism till denna anpassning: en genetiskt kontrollerad minskning av antalet kopior av mitokondriella gener, via en induktion av mitokondriellta DNA deletioner. De mitokondriella generna återställs efter kortvarig stress, men blir irreversibla efter långvarig stress. I min tredje artikel undersökte vi hur jästpopulationer med olika nivåer av redan existerande variation anpassar sig till två olika cancermediciner. Vi upptäckte att en större existerande variation ökade anpassningen, och att selektion på redan existerande och ny variation verkade på samma proteiner, om än på olika aspekter av dessa proteiners funktioner. I min fjärde artikel studerade vi hur arvsmassa från andra arter kan förekomma i reproduktivt isolerade arter. Dessa så kallade genetiska introgressioner från bakjästens närmsta släkting förekommer i vissa grupper av bakjäst, trots att de är reproduktivt isolerade. Vi visar att instabilitet i hybridens arvsmassa skapar utspridda öar av homozygositet. Detta tillåter två reproduktivt isolerade arter att kringgå sterilitetsbarriären och utbyta gener med varandra. I min femte och sista artikel så undersöker vi hur bakjäst har påverkats av domesticering. Vi jämförde och karaktäriserade olika livscykelegenskaper för runt tusen vilda och domesticerade jäststammar och fann tydliga skillnader mellan domesticerade och vilda stammar. Vi fann att domesticering förändrat livscykeln för bakjäst, vilket väcker frågor om hur lämpliga domesticerade jästisolat är som modeller. Tillsammans belyser mina fem artiklar molekylära mekanismer med vilka en av de främsta modellorganismera anpassar sig, och har anpassat sig, till förändringar i miljön och vilka konsekvenserna av denna anpassning är.

List of included papers

I. **Persson, K.**, Stenberg, S., Tamás, M. J., & Warringer, J. Adaptation of the yeast gene knockout collection is near-perfectly predicted by fitness and diminishing return epistasis. *G3 Genes|Genomes|Genetics*, 1, 5–14. (2022).

II. Stenberg, S., Li, J., Gjuvsland, A. B., **Persson, K.**, Demitz-Helin, E., González Peña, C., Yue, J.-X., Gilchrist, C., Ärengård, T., Ghiaci, P., Larsson-Berghund, L., Zackrisson, M., Smits, S., Hallin, J., Höög, J. L., Molin, M., Liti, G., Omholt, S. W., & Warringer, J. Genetically controlled mtDNA deletions prevent ROS damage by arresting oxidative phosphorylation. *ELife*, 11. (2022).

III. Li, J., Vazquez-Garcıa, I., **Persson, K.**, Gonzalez, A., Yue, J-X., Barre, B., N. Hall, M., Long, A., Warringer, J., Mustonen, V. & Liti, G. Shared Molecular Targets Confer Resistance over Short and Long Evolutionary Timescales. *Mol. Biol. Evol.* 36, 691–708 (2019).

IV. Angiolo, M. D., Chiara, M., Yue, J-X., Irizar, A., Stenberg, S., **Persson, K.**, Llored, A., Barré, B., Schacherer, J., Marangoni, R., Gilson, E., Warringer, J. & Liti, G. A yeast living ancestor reveals the origin of genomic introgressions. *Nature* 587, 420–425 (2020)

V. De Chiara, M., Barré, B. P., **Persson, K.**, Irizar, A., Vischioni, C., Khaiwal, S., Stenberg, S., Amadi, O. C., Žun, G., Doberšek, K., Taccioli, C., Schacherer, J., Petrovič, U., Warringer, J., & Liti, G. Domestication reprogrammed the budding yeast life cycle. *Nature Ecology & Evolution*. (2022)

My contributions to the included papers

I. I designed and performed all the experiments, analysed, and visualised all the data, wrote the draft of the manuscript and co-wrote the final version of the paper.

II. I performed the de-adaptation experiments of sequentially stored adaptation lines. I performed multiple growth phenotyping experiments, analysed growth curves, and contributed with figures. I assisted in preparing the manuscript.

III. I performed all the growth phenotyping experiments, analysed data, and contributed with text and editing of the manuscript.

IV. I performed all growth phenotyping experiments in a diverse set of environments and stress conditions. I contributed with text and suggestions on manuscript and figures.

V. I performed all growth phenotyping experiments in a diverse set of environments and stress conditions. I contributed with text and suggestions on manuscript and figures.

My other papers, not included

VI. Mozzachiodi, S., Tattini, L., Llored, A., Irizar, A., Škofljanc, N., D'Angiolo, M., De Chiara, M., Barré, B. P., Yue, J.-X., Lutazi, A., Loeillet, S., Laureau, R., Marsit, S., Stenberg, S., Albaud, B., **Persson, K.**, Legras, J.-L., Dequin, S., Warringer, J., ... Liti, G. Aborting meiosis allows recombination in sterile diploid yeast hybrids. *Nature Communications*. (2021).

VII. Barre, B., Hallin, J., Yue, J-X., **Persson, K.**, Mikhalev, E., Thompson, D., Molin, M., Warringer, J. & Liti, G. Intragenic repeat expansions control yeast chronological aging. *Genome Res.* 30, 697–710 (2020)

VIII. Johansson, N., **Persson, K. O.**, Norbeck, J. & Larsson, C. Expression of NADH-oxidases enhances ethylene productivity in Saccharomyces cerevisiae expressing the bacterial EFE. Biotechnol. *Bioprocess Eng.* 22, 195–199 (2017).

IX. Yue, J-X., Li, J., Aigrain, L., Hallin, J., **Persson, K.**, Oliver, K., Bergström, A., Coupland, P., Warringer, J., Lagomarsino, M., Fischer, G., Durbin, R. & Liti, G. Contrasting evolutionary genome dynamics between domesticated and wild yeasts. *Nat. Genet.* (2017).

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Abbreviations

ALE, EE	Adaptive Laboratory Evolution, Experimental Evolution
CNV	Copy Number Variation
DS	Domestication Syndrome
ETC	Electron Transport Chain
GWAS	Genome-Wide Association Study
LOH	Loss Of Heterozygosity
mtDNA	mitochondrial DNA
QTL	Quantitative Trait Loci
ROS	Reactive Oxygen Species
SNP	Single Nucleotide Polymorphism

Terminology and glossary

Bottleneck - a drastic reduction in population size
Clonal interference - when two or more beneficial mutations emerging in different clones compete with their fitness in an asexual clonal population and thus interfere with each other's frequency change
Diminishing returns epistasis - a decrease in effect size of beneficial mutations in fitter genetic backgrounds
Drift - when the allele frequency changes in a population due to random sampling
Epistasis - the effect of one gene depends on the presence or absence of other genes/alleles
Evolvability - the capacity of an individual or a population for adaptive evolution
Fitness - the success of an organism in survival and reproduction relative to that of the population
Fixation - when an allele is present in all DNA copies in all individuals in a population of organisms or cells, it has reached fixation
Genetic hitchhiking - the capacity of neutral, deleterious or only slightly beneficial mutations to increase in frequency in a population due to linkage to a beneficial mutation
Mutator, mutator genotype - a genotype that has an elevated mutation rate
Phenotype - an observable characteristic of a cell or an organism other than the primary sequence of its DNA
Phenotypic plasticity - changes to the phenotypes of an organism in response to changes in its environment
Pleiotropy - a single mutation or genotype affecting multiple traits
Selective sweep - increase in the frequency of a beneficial allele, and variants linked to it, in a population under selection
Yeast - eukaryotic single-celled microorganisms that are members of the fungus kingdom; often used to mean the model yeast *Saccharomyces cerevisiae*

1. Introduction

Evolution is the process of hereditary change in living organisms that have shaped the divergence of the millions of species that inhabit earth. Understanding how evolutionary processes transform living organisms in ways that often make them better suited to survive and reproduce in the environment in which they exist is undeniably one of the most important fields of research in biology. And finding out how the genetics of an organism, often together with particular factors in its environment, affect evolution is, in my opinion, one of the most fundamental objectives in evolutionary biology.

This is not only because of the key role evolution has in shaping the world around us, but also because evolution has a direct impact on human health. This is most evident in terms of cancer and infection. Cancer is caused by mutated somatic cells that adapt evolutionarily to the tissue environments surrounding them. This allows them to break free from mechanisms that constrain their capacity to replicate and expand clonally and to eventually spread, invade, and colonise new tissues and organs in the host body. Additionally, the adaptive evolution of pathogenic viruses, bacteria, fungi, and parasites can cause both more virulent and more drug-resistant strains to emerge and threaten human health. Future severe pandemics of bacteria that have evolved resistance to all available antibiotics may be unavoidable. As a side effect, many medical procedures that carry a high risk of infection or leave the patient with a compromised immune defence might not be possible to perform in the future due to the risk of untreatable infections.

Additionally, a fundamental understanding of evolution has many applications in industrial sectors based on bioproduction, such as food, feed, and biotech production. For example, the production of chemicals, enzymes and medicines from industrial yeast and bacterial strains can be improved using adaptive laboratory evolution (ALE) to enhance the strain's resistance to inhibiting growth conditions. This will, in many cases, improve the yields of products and lower production costs. In conclusion: evolution can provide both improved quality of life and suffering - two sides of the same coin. And the recent development of new methodology to acquire and analyse genetic, phenotypic and fitness information from naturally or artificially evolved organisms, will help us achieve the former, and prevent the latter.

1.1. Main findings of included papers

In my PhD work, I used the model yeast *Saccharomyces cerevisiae* to study adaptation in both experimental and natural yeast populations. This thesis demonstrates how ALE, sequencing and high-throughput phenotyping of yeast can be used to understand evolution.

In **Paper I**, in which I am first and corresponding author and on which I have spent most of my work, we aimed to disclose evolvability genes, i.e. genes that control the rate at which organisms adapt to changes in their environment. To do this, we investigated the rate of adaptation of many isogenic yeast populations, all corresponding to single-gene deletion *S. cerevisiae* strains, to stressful environments. First, we set up an ALE experiment using a high throughput growth phenotyping platform where we tracked the adaptation of ~4600 unique deletion strains to the model stress compound arsenite. We found that the pre-adaptation fitness of a deletion strain in a given condition almost perfectly predicted its adaptation speed, i.e. accounted for nearly all variation in adaptation rates. In other words: no genes had evolvability functions with substantial effects on adaptation. Next, we selected a subset of ~330-350 deletion strains and repeated the ALE experiment, but with very high replication to better account for mutational chance and at other selection strengths and with other selection pressures. These all confirmed that the initial fitness near perfectly predicts adaptation, suggesting that this observation can be generalised. Based on previous reports, and as deletion strains with lower pre-adaptation fitness had larger fitness improvement, we suspected that our findings were due to a global diminishing return epistasis, such that beneficial mutations have a smaller effect in fitter backgrounds. We tested this assumption by reconstructing the three strong effect mutation types that drive arsenic resistance, in a large subset of gene deletion strains. This indeed confirmed that favourable mutations are less beneficial in fitter gene deletions, and this diminishing return epistasis explained most of the non-technical variation in doubling times. Our screen represents the first truly exhaustive exploration of the role of individual genes in evolvability, and our findings underscore the immense power and generality of diminishing returns epistasis. Moreover, the lack of evolvability genes with a meaningful effect on yeast adaptation dynamics reduces the prospects of developing drugs that slow down antimicrobial and chemotherapy resistance.

In **Paper II**, we adapted populations of a wild *S. cerevisiae* isolate to paraquat, to understand how cells adjust to mitochondrial production of superoxide via the energy transport chain. We found that adaptation to paraquat was extraordinarily swift; much faster than to other selection pressures. By modelling the adaptation rate using population genetics we

found that nuclear mutations could not realistically account for the rapid adaptation. We also discounted mitophagy, which in mammalian cells is a common response to mitochondrial superoxide production. Using sequencing and quantitative PCR, we next showed that the fast adaptation to superoxide coincided with deletions in the mtDNA. By mimicking these deletions using mutants lacking important mitochondrial proteins, such as the mitochondrial DNA polymerase Mip1, we showed that mtDNA deletions drive the fast adaptation to mitochondrial superoxide by reducing respiration, i.e. they are a cause rather than a consequence of the superoxide. We also showed that this adaptation depended on the presence of the mitochondrial superoxide dismutase Sod2 and on signalling through the retrograde (Rtg) pathway - and therefore was under genetic control. The early partial loss of mtDNA segments - and the reduced respiration - was always reversed a few generations after stress release despite selection not favouring such a reversion. This is consistent with the process being under genetic control. However, more prolonged chronic paraquat exposure causes irreversible mtDNA and respiratory loss when the copy number of intact mtDNA molecules approaches zero, i.e. when the heteroplasmic state becomes a homoplasmic state. One should treat this subject with caution, but our Paper provides some fresh perspectives on the axiom that mutations are independent of the needs of the organism. Moreover, they may have important implications for understanding diseases linked to mitochondrial ROS generation.

In **Paper III**, we aimed to understand how pre-existing and de novo genetic variation interplay during clonal adaptation to anticancer drugs, and whether they involve the same genes. To do this, we asexually evolved diploid *S. cerevisiae* populations with different levels of pre-existing variation under exposure to the model anticancer drugs hydroxyurea and rapamycin. We used initially isogenic populations derived from four widely diverged *S. cerevisiae* lineages and contrasted these against populations with substantial pre-existing variation that were obtained by random mating between two or four of these lineages. We then used time-resolved growth phenotyping and sequencing to observe how alleles and fitness changed in the populations over time. We found that the presence of pre-existing variation speeded up adaptation, with a doubling of pre-existing variation boosting adaptation by more than 50%. This is consistent with a higher standing genetic heterogeneity conferring a higher fitness variance, and faster adaptation. We also found that the causative pre-existing and de novo variants were selected on shared genetic targets, *RNR4* in hydroxyurea and *TOR1*, *TOR2* in rapamycin. However, the mechanisms through which standing and de novo variants caused drug resistance differed. This study deepens our understanding of how pre-existing and de novo variants interactively drive adaptation in clonally evolving populations.

In **paper IV**, we aimed to understand how genome introgressions from the wild yeast *Saccharomyces paradoxus* have appeared in its sister species *S. cerevisiae*, despite the two species being reproductively isolated. The two species form hybrids, but the hybrids can normally not pass through meiosis to generate viable progeny. It is therefore a puzzle how such progeny nevertheless emerge and can backcross to the *S. cerevisiae* parent to give rise to modern-day *S. cerevisiae* lineages with extensive *S. paradoxus* introgression. We shed light on this mystery by identifying a clonal descendant of the ancestral *S. cerevisiae* and *S. paradoxus* that founded the extant *S. cerevisiae* Alpechin lineage, whose members have 4-5% *S. paradoxus* introgressions. We found that this clonal descendant had retained its ancestral genome structure with separate *S. cerevisiae* and *S. paradoxus* sub-genomes, but with extensive homozygosity regions scattered across the chromosomes. We show that these local homozygosities allow the clonal descendent of the ancient hybrid to pass through meiosis, and recombine, i.e. to overcome the reproductive barrier between *S. cerevisiae* and *S. paradoxus*. We also show that the regions of homozygosity in the clonal descendent of the ancient hybrid match (in a way that cannot be explained by chance) with the introgressions in the Alpechin lineage, clearly identifying the ancient hybrid as the ancestor of the Alpechins and the homozygosity regions as the origins of the introgressions. Because the losses of heterozygosity in the ancient hybrid represent a form of genome instability, our findings show that genome instability allows two reproductively isolated species to bypass the hybrid sterility barrier and exchange genetic information. This is important from many perspectives, and not the least because genes transferred from one species to another can have positive and negative effects on the species, as emphasised by the Nobel prize committee in the motivation for the 2022 Nobel prize in Medicine to Svante Pääbo.

Finally, in **paper V**, we aimed to understand how the historical domestication of many lineages of the model yeast *S. cerevisiae* affected its phenotypes, particularly its core life cycle phenotypes. We did this by comparing key life cycle phenotypes, such as growth, gamete formation, and cell survival, across nearly 1000 genome sequenced yeast wild and domesticated yeast isolates. We found a clear phenotypic separation between domesticated and wild isolates. Specifically, we found that wild yeasts enter meiosis and sporulate when nutrients are depleted. In contrast, domesticated isolates have typically lost or suffered grave impairments in the capacity to enter and pass through meiosis. Instead, they typically stay in the mitotic cell cycle, or its G_0 state, when resources are consumed. We also found that domesticated yeasts had generally enhanced fermentative growth traits, at the cost of a reduction in the capacity to grow in respiratory conditions, and had decreased tolerance to many stresses. We refer to the characteristic phenotypes of domesticated yeasts as the yeast

domestication syndrome, and we traced its genetic origins using GWAS and genetic engineering. We could thereby identify aneuploidies and many loss of function mutations in e.g. the key meiotic transcription factor Ime1 as drivers of the yeast domestication syndrome. The dramatic phenotypic consequences of domestication lead us to propose that domestication has been the most dramatic event in the evolution of *S. cerevisiae*. We also point out that many yeast phenotypes, potentially including many molecular and cellular traits that are not measured here but that may be strongly affected by the profound changes in the yeast life cycle, are recent consequences of domestication. Thus, they could not reasonably have been present in the shared ancestor of, e.g. yeast and humans. This raises some questions about the risks of using domesticated yeast, such as common lab strains, as models and argues for the use of strains that have only been exposed to natural selection and evolution.

1.2. An introduction to evolution

Five major forces in nature cause an evolutionary change in natural and experimental populations. **Natural selection** acts on the existing variation in the population, selecting the fittest individuals, i.e., those best suited to survive and reproduce, for future generations. The selective pressure will be determined by the composition of the current environment, which will favour the individuals with the highest current fitness, regardless of the trade-offs involved with these individuals potentially being less fit in other environments. Environments are rarely constant in nature, making it difficult to understand what, and how strong, the selective pressure is under natural evolution, but they can be kept reasonably constant in laboratory evolution experiments of the type that I have performed in **Paper I, II** and **III**, which often is a key advantage.

Random genetic drift arises when chance influences who survives and how many offspring they have - and tend to reduce the genetic variation in small populations over generations. The population size can be drastically reduced due to rare, dramatic environmental factors, or during the founding of a new population from a few emigrants. These so-called bottleneck events cause extensive genetic drift such that even negative genetic variants may become common, when a few random, rather than the fittest individuals, will parent the next generation. Bottlenecks are challenging to identify and correct for in naturally evolving populations but again – they can be avoided or controlled for in laboratory evolution experiments (**Paper I, II** and **III**), where the operator influences the population size.

Mutation is the source of all de novo (new) genetic variation. Most mutations result from errors in the copying of the heritable genetic information (DNA, except in some viruses) and failure to correct these errors, leading to a change being passed on to the next and future generation, either when they occur in single-celled organisms or in the germ-line cells of multicellular organisms.

Non-random mating will not change the frequency of alleles, but will change the frequency of genotypes, i.e., how alleles are combined with each other into genomes, in natural populations. This force will have limited influence on the results in this thesis because, except in a few cases, there is no mating involved. Finally, **migration** from one population to another can introduce new variants into the recipient population or change the frequencies of its alleles. Again, this force will have limited influence on the results presented in this thesis because, except for in the (rare and unwanted) case of contamination between colonies representing different populations, we have set migration to zero in our experiments.

All the five forces of evolution are typically acting in ways that can be hard to estimate on natural populations, while in ALE, researchers can control or remove some of these forces to study certain aspects of evolution. For example, the most common type of ALE experiments, including the ALE experiments presented in **Paper I, II** and **III**, use microbes maintained in controlled environments. Mating and migration, other than rare, unwanted cross-contamination between populations, are not part of the experimental design.

1.3. The yeast *Saccharomyces cerevisiae*

Research can be simplified and speeded up by using a small set of model organisms, hoping to find new insights that are relevant also in a broader set of organisms for which experiments are hard to, or cannot be, performed. The yeast *S. cerevisiae*, which I have used as model organism in all my five papers, has many properties that make it a good model organism. It is typically non-pathogenic, even if there are sporadic cases of *S. cerevisiae* infections in immunocompromised patients. Moreover, being single-celled, it has no nerve system: experiments that would be considered painful, or in other ways unethical, to higher organisms can be conducted in yeast with no moral implications.

The central core functions of especially eukaryotic cells are conserved over large evolutionary distances; thus, yeast and humans, the organism which is most important to model, share many of these core functions even after a billion years of diverged evolution. Thus, many essential yeast genes can be replaced with their human orthologs without a substantial loss of fitness, at least in standard laboratory conditions (Kachroo *et al.* 2015). Therefore, yeast can be used as a simple but highly useful model to study the mechanisms underlying some human diseases and serve as an initial step before moving to models of higher complexity, such as human cell lines or animal models. Yeast can survive a wide range of stresses and environmental challenges, including those encountered in standard laboratory and industrial environments, making it suitable for both scientific research and as a microbial cell factory.

Being the first eukaryote to be whole genome sequenced in 1996 (Goffeau *et al.* 1996), *S. cerevisiae* has served as the test organism in the development of many new, large-scale techniques. It is also unusually easy to manipulate genetically. This means that almost all yeast genes have now been studied individually, from many different perspectives, and extensive functional annotations of nearly all yeast genes are available in the Saccharomyces Genome Database (SGD). We consequently now understand better how a yeast cell works, more than any other eukaryotic cell. This leverages work on yeast compared to research in organisms with less studied and functionally annotated genomes, and means that more meaningful conclusions can be drawn with higher confidence.

The cost of propagating yeast at large population sizes in the lab is low, as it grows rapidly on cheap laboratory media. Yeast cell populations can also be stored indefinitely, with no change to their genomes occurring, in glycerol at only -80°C. Stored yeast cells can then be revived, and their properties studied again and again. This is particularly useful in adaptive laboratory evolution (ALE) experiments, as cells from different stages of evolution can

be stored and then revived and compared or competed against each other. Yeast can be propagated both as haploids and diploids and prevented from shifting from one ploidy state to the other, simplifying the work on gene characterisation. Compared to many other model species, genetic engineering is easy and efficient, exploiting the high rate of homologous recombination in yeast. This means that conclusions based on inference, or association, can be confirmed by direct intervention – i.e., we can manipulate the gene that is believed to underlie a particular trait and see if the genetic change really causes a phenotypic change. The introduction of new and more sophisticated techniques to edit genomes, such as the CRISPR/Cas9 system (Cai *et al.* 2019), has moved some of the spotlights from *S. cerevisiae* to other yeasts or other organisms. However, it remains a robust and popular model and, in my view, will continue to be so for the foreseeable future.

1.4. *S. cerevisiae* as a model organism in evolution studies

The genomes of species can be compared with those of their close species relatives to uncover the genetic and molecular underpinnings of their recent divergence. The evolution of *S. cerevisiae,* with its relatively small 12 Mb genome, has been studied in higher resolution than perhaps any other eukaryote (Duina *et al.* 2014). The *Saccharomyces* genus, encompassing *S. cerevisiae* and its closest relatives, originated approximately 10-20 million years ago (Hittinger 2013). But, due to its short generation time, the genome of *S. cerevisiae* has diverged from that of its closest known relative, *S. paradoxus,* to a degree that is roughly comparable to that of human and mouse genomes (Hittinger 2013).

S. cerevisiae has recently emerged as a model organism for population genomic studies. It can be found worldwide, both in domesticated forms, on a wide array of human-associated (fermented beverages), and wild (e.g., plants, insects and soil) biotopes (Peter *et al.* 2018). Because of its recent close association with humans, the population structure of *S. cerevisiae* does not follow geographic boundaries as strictly as that of many other species, but also reflects its hitchhiking with human migrations and the human breeding of yeasts, e.g., for wine or beer production (Liti 2015). Thus, in addition to the rather diverged and distinct wild lineages, which have been separated by geography, many *S. cerevisiae* strains have mosaic, recombinant genomes and mixed ancestries resulting from intentional or unintentional human crosses of genetically distinct lineages (Liti *et al.* 2009). A recent sequencing effort revealed extensive genome sequences for over 1000 *S. cerevisiae* isolates (Peter *et al.* 2018). The natural evolution of *S. cerevisiae* is further discussed in chapter 2 and **Paper IV** and **Paper V.** More recently, and thanks to its short generation time, ease of storing and handling in the lab, and small genome, the yeast *S. cerevisiae* has become a favourite model for ALE. In chapter 3 and 5, I will explain how evolution can be studied using ALE experiments. In **Paper I**, **II** and **III**, we use ALE to explore different aspects of yeast evolution.

1.4.1. The life cycle of yeast

One important advantage of using *S. cerevisiae* as a model has to do with its life cycle, and how we researchers can control it. Yeast cells divide by budding, and a budding mother cell produces a genetically identical daughter cell. Before the daughter cell is separated from its mother, a copy of each chromosome is generated and segregated into the daughter cell in a process called mitosis. A haploid cell (1N) carries one copy of each chromosome. Two

haploid cells, one of each mating type, can mate by fusing into a diploid cell (2N). The diploid cell can either grow clonally or undergo meiosis, a double round of cell division where an ascus (spore sack) is formed containing four haploid gametes, two of each mating type, which in yeast are referred to as spores (Figure 1).

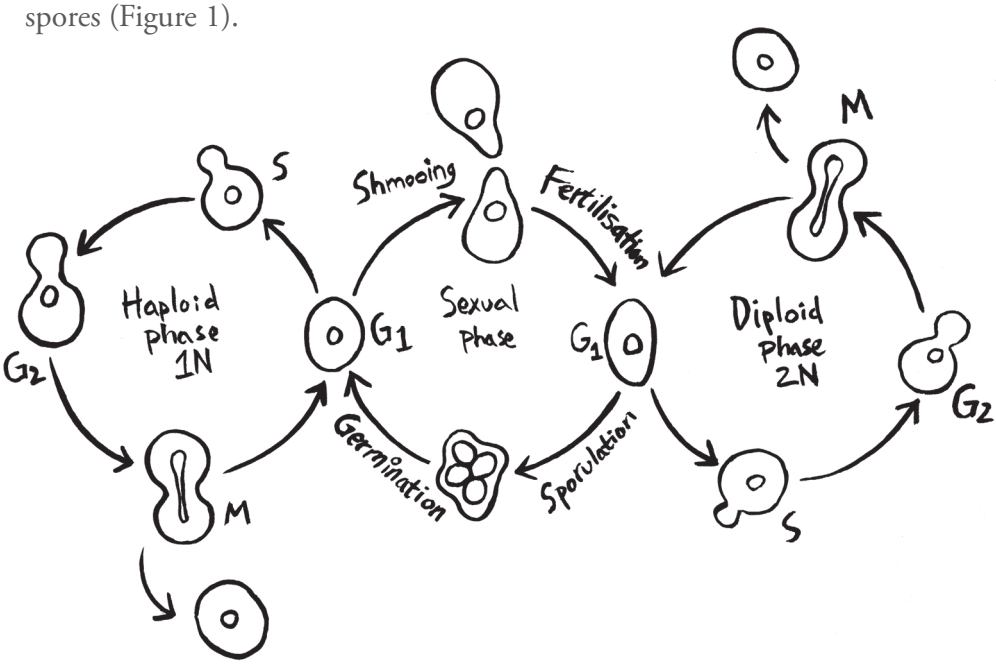

Figure 1. The life cycle of yeast. See text for details.

Meiotic spores have a high tolerance for various stresses, which presumably means they can persist in this state for long periods in the wild, until more favourable conditions allow them to germinate and become vegetative, haploid cells in their own right. Commonly, such haploid cells mate and form diploids with another germinated spore from the same meiotic event, or with their own mitotic daughter cells (after a switch in mating type) or, much more rarely, by outcrossing with an unrelated cell (Sun *et al.* 2019). The sexual cycle and meiosis are typically triggered by nutrient depletion, and can therefore be controlled in the lab, through regulation of access to nutrients. Population genomic studies have shown that yeast most commonly reproduces asexually, rarely mates and very rarely mates with an unrelated individual. It has been estimated that only one in a thousand divisions is sexual, and most of these are self-fertilisations (Tsai *et al.* 2008; Cubillos *et al.* 2009). Outbreeding and migration (dispersal) can be promoted by various insects, such as the fruit fly *Drosophila melanogaster*, which is attracted to yeast cells living on fruit. Fruit flies consume the yeast and act as a vector, flying between different geographical

locations and dispersing yeast stuck on their bodies (Madden *et al.* 2022). The most diverged strains, i.e. the strains with least outbreeding with other lineages, have been isolated in the Chinese primaeval forests, indicating that the species originated in East Asia and migrated globally from there (Wang *et al.* 2012). However, even if sexual reproduction and outcrossing are rare in wild strains, they nevertheless play important parts in the natural yeast life cycle and can greatly affect its evolution, which we show in **Paper V**.

Haploid yeast cells only mate with haploids with the opposite mating type, termed **a** and alpha. Each mating type secretes a pheromone that can be detected by the opposite mating type, stimulating directional growth towards a prospective mating partner. In a process called shmooing, two haploid cells reach each other and merge to form a diploid.

In the earliest days of yeast research, the strains studied were homothallic, i.e., haploid cells could change their mating type. Homothallic strains are self-fertile, allowing them to mate rapidly after budding with their clonal offspring and become diploid. To avoid mating type switching, uncontrolled mating and a transition to diploid cells, most modern lab strains are heterothallic, stable haploids, with the *HO* endonuclease that is responsible for the mating-type switch having been knocked out. The strains I use in **Paper I**, **II** and **III** are of this type, while most strains in **Paper IV** and **V** are natural isolates. Early in *S. cerevisiae* research, one reference isolate, s288c and derivates of it, was selected by the research community as a common model, because it was naturally heterothallic, having lost the HO function, and therefore remained a stabile haploid (Mortimer and Johnston 1986).

The yeast life cycle shown in Figure 1 depicts a strict alternation between haploid and diploid phases. However, it is worth keeping in mind that several yeast strains isolated from the wild are triploids or tetraploids (Peter *et al.* 2018; Fischer *et al.* 2021) From the global collection of 1011 *S. cerevisiae* isolates, 11.5% are polyploids (Peter *et al.* 2018), and I use these strains in **Paper V**.

A cell can exit the proliferating state and enter a resting state called quiescence**,** or G_0, typically by exiting the cell cycle in the G_1 phase and arresting as unbudded cells. They have a reduction in overall protein synthesis to approximately 0.3% relative to logarithmically growing cells (Fuge *et al.* 1994) and thickened cell walls that are often held to explain their better resistance to various hazardous environments (Plesset *et al.* 1987; de Nobel *et al.* 2000). However, it is believed, and we show data that support this in **Paper V**, that a quiescent cell is not as stress-tolerant as a spore. In contrast to a quiescent cell, a cell is considered senescent if it is metabolically active but cannot, or does not, re-enter the cell cycle.

Wild, natural yeast exists where conditions permissive for proliferation, such as access to sufficient nutrients, occur only sporadically. Oak bark, for example, which is believed to be one such natural habitat, has a seasonal cycle

of tree sap flow, that depends on climate, geography and temperature. Wild *S. cerevisiae*, e.g. on oak bark, is therefore likely to stay in a quiescent state, or enter into the spore state, for extended time periods to exit from these states only sporadically for short bursts of cell division.

During meiosis, the genetic material of the two parental haplotypes is shuffled and recombined. In that way, alleles that were previously in separate genomes can be combined. The physical distance of two loci on the same chromosome, together with the recombination rate per base pair in the intervening DNA, determines how likely they are to be separated during recombination. Two loci are linked if they segregate together more often than expected by chance. Genetic linkage to a beneficial mutation can allow neutral, deleterious or weakly beneficial mutations to increase in a population, in what is called genetic hitchhiking or genetic draft. In clonally dividing (heterothallic) populations, meiosis and recombination do not occur. Here, the linkage between loci is not dependent on physical distance. In this case, the entire genome acts as a single lineage group.

The yeast cultivated in the ALE experiments in **Paper I**, **Paper II** and **Paper III** are all clonally propagated, heterothallic lines; therefore, no recombination occurs. Since there is no sexual reproduction, beneficial alleles in separate cells cannot be recombined into the same haplotype. Thus, both genetic hitchhiking and clonal interference, which arise when clones carrying favourable mutations compete and interfere with each other's frequency increase, are common phenomena. In **Paper IV** and **V**, we work with yeasts that can go through sexual recombination but do so only when exposed to nutrient depletion. In these strains, there is still hitchhiking but only for variants that are closely genetically linked to a beneficial variant.

2. Natural yeast evolution and adaptation

2.1. Yeast speciation, hybridisation, and introgression

2.1.1. The Saccharomyces sensu stricto complex

S. cerevisiae is a member of the *Saccharomyces sensu stricto* complex comprising eight *Saccharomyces* yeast species: *S. cerevisiae*, *S. paradoxus*, *S. mikatae*, *S. kudriavzevii*, *S. arboricola*, *S. eubayanus*, *S. uvarum* and its newest member *S. jurei* joining the family in 2017 (Naseeb *et al.* 2017, 2018). The taxonomic groups of this genus have been changed and rearranged several times over the years (Boynton and Greig 2014). Recent phylogenic analysis of the genus excluded *S. bayanus* because it was found to be of hybrid origin (Hittinger 2013; Boynton and Greig 2014; Naseeb *et al.* 2017). *S. cariocanus* was earlier included as a separate species because of reproductive isolation but was, after genome sequence analysis, shown to belong to the American population of *S. paradoxus* (one of three defined populations) (Liti *et al.* 2006, 2009). All the species have a typical budding morphology, and their genomes are distributed on 16 chromosomes of approximately the same size (Duina *et al.* 2014).

S. paradoxus, *S. mikatae*, *S. jurei*, *S. kudriavzevii*, *S. arboricola* and *S. eubayanus* are wild species, while *S. cerevisiae* and *S. uvarum* exist both as domesticated and wild isolates. The species that are found in the wild are often associated with trees, such as soil, bark and leaves. Frequent isolation of *Saccharomyces* species from oak trees (*Quercus spp.*) and surrounding soil suggest that this could be one of the natural habitats of this group of species. Strains have also been isolated from tree sap, fruits and insects (Wang and Bai 2008; Naseeb *et al.* 2017). Since *S. cerevisiae* has been isolated from multiple niches, it has been suggested to be nomadic, having no niche (Goddard and Greig 2015).

Most strains isolated within the genus are *S. cerevisiae* and *S. paradoxus*, and the rest are mostly restricted to only a handful of isolates (Boynton and Greig 2014). *S. paradoxus* is the closest known relative of *S. cerevisiae*. It is commonly isolated from the environment in Northern Europe and North America, but is rarely found to be associated with fermentation. It is therefore considered a thoroughly wild yeast. *S. paradoxus* shows a lower phenotypic variation than its closest relative, *S. cerevisiae*, despite having a much higher nucleotide variation. One explanation for this could be the fact that *S. cerevisiae* occupies a wider range of ecological niches and has higher levels of structural variation (Liti *et al.* 2009; Yue *et al.* 2017).

The relationship between the two sister species, *S. cerevisiae* and *S. paradoxus,* is the focus, or part of the focus, of **Paper IV** and **V**. I believe that it is likely that additional *Saccharomyces sensu stricto* complex members exist and will be identified in the near future. Currently, I am involved in an ongoing international collaborative project to isolate *Saccharomyces* species worldwide from oak bark and soil samples. The Swedish strain isolates we identified so far have been *S. paradoxus* and *S. uvarum* (unpublished data).

2.1.2. Reproductive isolation and hybridisation of *Saccharomyces* species

The biological species definition states that for a new species to emerge, reproductive barriers that limit gene flow between populations must be established. This can happen before fertilization (pre-zygotically), through differences in geography, mating behaviour or physiology. However, the *Saccharomyces* species easily hybridise with each other, and there are no significant prezygotic barriers (Chou and Leu 2010). Reproductive isolation instead occurs post-zygotically, though genetic incompatibilities between the two genomes combined into the hybrid zygote. Three such mechanisms have been shown to exist in yeast. First, populations evolving independently accumulate mutations that may be adaptive or neutral in the background in which they appear, but that display deleterious effects when combined with other variants with which they have not co-evolved in hybrids. The observed lower viability or fertility of offspring from species-species hybrids of some yeast crosses is due to such accumulation of incompatible mutations, in what is called the Bateson–Dobzhansky–Muller model (Hunter *et al.* 1996; Naumov *et al.* 2006; Baker and Bradley 2006; Lowry *et al.* 2008). Other yeast lineages are postzygotically reproductively isolated from each other because of differences in chromosome number or structure, the latter resulting because of chromosomal rearrangements in one or both of the lineages (Chou and Leu 2010; Charron *et al.* 2019). The Malaysian *S. cerevisiae* lineage, e.g. cannot produce viable offspring with other *S. cerevisiae* lineages because of recent structural mutations on chromosomes VII, VIII, X, XI and XIII (Yue *et al.* 2017), which prevents sufficient base-pairing and correct separation of homologous chromosomes during meiosis.

However, the major species barrier in the *Saccharomyces* genus is believed to be the anti-recombination effect of extensive sequences divergence between species. When sister species of the genus *Saccharomyces* hybridise, meiotic recombination is inhibited by the high sequence divergence, which results in incorrect or insufficient base-pairing of homologous chromosomes, in a process that is believed to involve the DNA mismatch-repair system. Because of the abortion of meiosis, few viable gametes are formed and those that

are formed are entirely or predominantly non-recombined, reproductively isolated from their parental backgrounds and have reduced fitness (Greig *et al.* 2002).

Saccharomyces hybrids are commonly used in industrial fermentation but are seldom found in wild and clinical samples (Alsammar and Delneri 2020). The best-known industrial hybrid is *S. pastorianus*, a hybrid cross between *S. cerevisiae* and *S. eubayanus* (Gallone *et al.* 2018). This hybrid (shown in red, Figure 2) has been used industrially for centuries in lager production, which is brewed at lower temperatures compared to other beer styles that use *S. cerevisiae*.

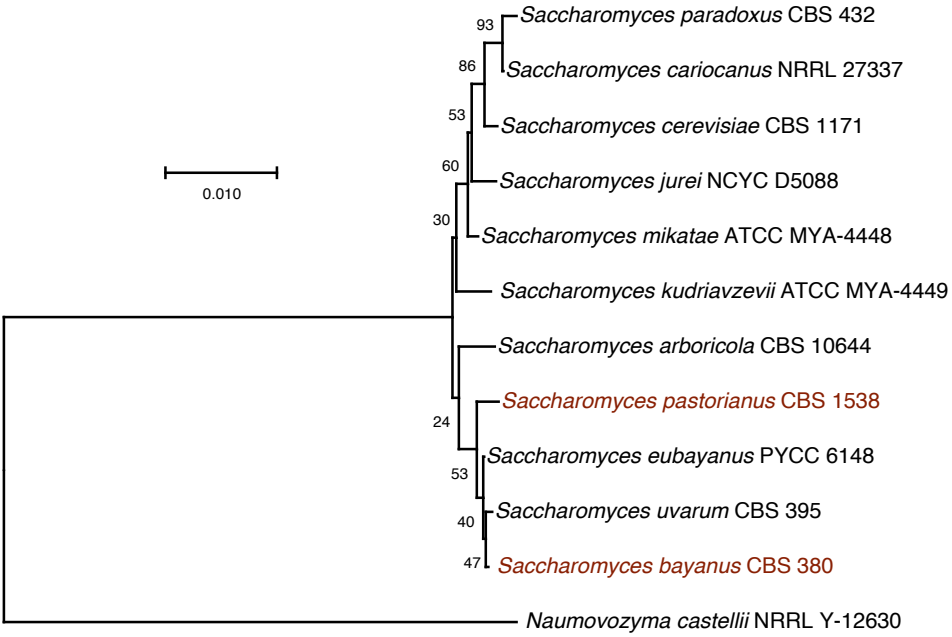

Figure 2. The phylogeny of the Saccharomyces genus. A Neighbour Joining dendrogram based on the ITS regions of the family. Hybrids are indicated in red. Bootstrap values were determined from 1000 replicates shown at branch nodes. Bar, 1 base substitution per 100 nt. Made using MEGA11.

2.1.3. Genomic introgressions

Movement and insertion of a gene or a chromosomal region from one species into the gene pool of another occur in a process called introgressive hybridisation, or introgression. The hybrid offspring of two species repeatedly backcross with one of its parental species, retaining genetic regions from the other parental species. The introgression blocks are scattered throughout the genome, indicating extensive recombination in the past.

The recent global *S. cerevisiae* strain collection is divided into 26 defined lineages representing ecological niches and geographical areas (Peter *et al.* 2018). Abundant (give number) introgressions from the sister species *S. paradoxus* are observed in four of these defined lineages. One of these is the Alpechin lineage, associated with olives or the wastewater from olive oil production in Spain.

How is it possible that reproductively isolated species still harbour introgressions? We discovered, by chance, an *S. cerevisiae - S. paradoxus* hybrid (**Paper IV**) that proved to be the direct clonal descendant from an ancestral hybridisation event which founded the Alpechin lineage. That is, this strain, which we call "the living ancestor", retained the genome structure, with two completely separated *S. cerevisiae* and *S. paradoxus* subgenomes, of the ancestor of the Alpechins. That is - the lineage had not passed through a single meiotic recombination event. However, we found that it had passed through a genome destabilization process, which had caused it to lose heterzygosity and become homozygotic for primarily *S. paradoxus* DNA over a hundred scattered homozygous blocks. The Alpechins had formed through repeated backcrossing of the ancestral hybrid to the *S. cerevisiae* lineage and therefore only retained introgressions of *S. paradoxus* DNA, had access to all the supposed benefits of sexual recombination. However, what is even more remarkable is that the regions of *S. paradoxus* homozygosity in the living ancestor coincided in a way that could not be explained by chance, with the *S. paradoxus* introgressions in the Alpechins. This clearly implicated the regions of loss-of-heterozygosity, as the origins of the introgressions. It also showed that the explanation for the introgressions, and for the crossing of the reproductive barrier between *S. cerevisiae* and *S. paradoxus*, was the genome instability event that gave rise to the *S. paradoxus* homozygosity blocks in the ancestral hybrid. It is unclear how the living ancestor managed to compete with its modern descendants in the same ecological niche. Wild yeasts go through meiosis every thousand generations (Tsai *et al.* 2008). Still, the living ancestor has passed through mitosis for more than a million generations without sexual reshuffling of its genome and formation of spores, even though it is capable of this (**Paper IV**). This could reflect a relaxation of what has been believed to be strong selection for sex and spore formation when living in a human-made environment (**Paper V**). The origin of introgressions we discovered in yeast may help us understand how introgressions arise also in other species.

2.2. Domestication of yeast

Domestication is a form of evolution, where the selection pressure of one organism is controlled by another, typically (but not exclusively) humans. Artificial selection and breeding are done to domesticate wild organisms, generating cultivated variants that have improved and desirable properties in the intended (human) controlled environments, often at the expense of decreased fitness in other niches, including in its native habitat. Specific genotypic patterns of domestication are frequently observed in agricultural crops, pets, and livestock, including genome decay, abundant chromosomal rearrangements, deletions and amplifications of chromosomes, chromosome segments and individual genes, and an overall loss of genetic diversity. Domestication, or quasi-domestication, which is the unintended domestication of a species due to its close association with humans or human activities, often also results in the emergence of sets of specific traits, collectively referred to as domestication syndromes (DS). Examples of DS in mammals include increased docility and tameness, changes to coat colour, reduced teeth size, alterations in cranial morphology, ear and tail form, changes in hormone levels, prolongation in juvenile behaviour and reduction in brain size (Wilkins *et al.* 2014). Plants' DS are e.g., grain retention by loss of shattering of seeds when ripe, reduced lateral branching and altered flowering times (Kantar *et al.* 2017).

Domestication is shaped by three evolutionary forces: drift from the bottlenecks imposed by humans non-random mating (i.e. breeding), and artificially imposed selection. This means that DS can emerge from the fixation of traits associated with the selected alleles, with alleles that are genetically linked to the selected alleles and with alleles that have drifted to fixation during the bottlenecks.

Humans have used the fermentable properties of yeast for millennia. Ancient organics absorbed in pottery have identified human alcoholic fermentation as early as nine millennia ago (McGovern *et al.* 2004). This raises the possibility that yeast genomes and phenotypes have been extensively exposed to domestication long before understanding the underlying process for producing bread and alcoholic beverages. In the 1930s, Øjvind Winge performed breeding experiments on yeast, crossing different isolates at the Carlsberg laboratory to improve desirable traits for brewing (Barnett 2007).

Besides selection for niche-specific traits, domestication also causes relaxation of selection for traits that are no longer advantageous or disadvantageous in the particular artificial environment. Extended relaxed selection can cause gene loss or pseudogenisation of genes that are no longer selectively advantageous to maintain, leading to genome decay (Gallone *et al.* 2018).

It has long been known that some of the domesticated yeast lineages possess specific traits and specific genotypic characteristics. For example, about half of all wine yeasts carry a mutation in the promotor of the plasma membrane sulfite pump *SSU1* (Sicard and Legras 2011). The mutation causes an induction of the *SSU1*, allowing the expulsion of sulfite from the cytoplasm and higher resistance to sulfite, an antiseptic used since the middle ages to clean wine canisters.

Similarly, beer yeasts can ferment maltotriose; the sugar is uncommon in high concentrations of natural yeast environments, but the second most common carbohydrate in beer wort, after maltose. Brewing yeasts with an efficient maltotriose metabolism have a selective advantage in this industrially specialised niche. Maltotriose metabolism has evolved independently through different pathways in the two main beer lineages (Gallone *et al.* 2016). And many ale isolates lack a functional sexual cycle (Gallone *et al.* 2018).

However, which of these properties in individual domesticated clades that are consequences of domestication, and should be seen as parts of DS that is shared across domesticated yeasts, and which that are due to clade-specific selection events or bottlenecks that pre- or postdate domestication, have not been easy to establish.

In **paper V**, we study the effects of domestication broadly, across many domesticated and non-domesticated lineages. This allows us to exclude other lineage-specific influences, that manifested before or after domestication, as causes of the observed traits. We find that these independent lineages, with separate domestication histories, often share the same signatures of domestication. We compared asexual growth, sexual gamete formation, and cell survival across nearly 1000 genome sequenced wild and domesticated yeast isolates. We found a clear phenotypic separation between domestic and wild isolates; wild yeasts could enter meiosis and sporulate when facing starvation, while the domesticated strains had typically lost this trait or had impaired capacity to do so. Additionally, domesticated yeast showed a reduced stress tolerance and higher fermentative over respiratory ability.

We describe these shared phenotypes as a yeast specific domestication syndrome, and trace their genetic origins using GWAS and genetic engineering. We identified aneuploidies and multiple loss of function mutations in *IME1*, the key meiotic transcription factor, as the drivers of this DS. We conclude that the loss of sexual recombination caused by domestication impaired the capacity of yeast strains to create new genetic diversity by combining beneficial genetic variants from different genomes into one, and prevented the removal of the genetic load caused by deleterious variants hitchhiking with linked beneficial variants.

3. Experimental evolution

3.1. The principles and applications of experimental evolution

The classical way to study evolution is to look at patterns of variation and co-variation in nature and try to infer a time scale, e.g., on genetic data, radioactive decay or the age of sediments, to when that variation and co-variation emerge. Based on such patterns, e.g., in terms of co-variation between the emergence of a trait and a particular environmental factor, researchers can also draw loose conclusions on how organisms have adapted to their environment. However, studying evolution through natural variation and co-variation only allow tentative conclusions on causality because we cannot rewind time and replay evolution. We study associations and infer causality from these associations, but we can rarely disprove alternative explanations for these associations. For example, we can see that the emergence of defence mechanisms against reactive oxygen species (ROS) coincides in time with the oxygenation of the atmosphere, and we can infer that ROS gene emergence was caused by oxygenation. Still, we cannot easily test and confirm that inference.

Another problem with studying natural evolution, in addition to the difficulty of assigning causality, is that we can only view a few snapshots from a continuous process. The picture that we get therefore becomes relatively crude, and we typically miss all the details of the underlying evolutionary dynamics.

To address these shortcomings of studying natural evolution, scientists in the 1980s started doing evolutionary experiments in the lab, following the evolutionary process as it happened. This branch of evolutionary biology become known as experimental evolution (EE), artificial laboratory evolution (ALE) or selection (ALS) and has more recently branched again to given rise to subfields characterised by particular subtypes of evolution experiments, such as directed evolution (DE), evolutionary engineering, and mutation accumulation lines (MAL). Common model organisms in experimental evolution are viruses, such as the bacteriophage lambda, the bacteria *E. coli,* the fruit fly *Drosophila melanogaster,* and the yeast *S. cerevisiae*. Due to their relatively small size, large populations can be maintained in small volumes in parallel, allowing multiple replicate lineages to be evolved simultaneously and thereby, to some degree, account for mutational chance and random genetic drift. Moreover, since these model organisms divide fairly rapidly, many generations can be generated in the lab in reasonable timescales. This allows selection to drive not only pre-existing, standing genetic variants to high frequencies, but also new mutations to manifest and be selected.

As an example, the famous long-running evolution experiment on *E. coli* was initiated in 1988 with 12 isogenic cultures, and is still running after

over 75,000 generations, continuing to reveal important insights into the evolutionary process – all due to the emergence of novel genetic variation (Callaway 2022). Storing frozen records from ALE experiments lets researchers replay evolution from any given point in the past, and compare, or even compete, different evolutionary stages against each other, in experimental designs that account for almost all confounding factors and that can be extensively replicated to account for chance effects. This often allows firm conclusions on what causes what, i.e., on causality.

A typical ALE experiment follows the same simple principle: populations of cells or multicellular organisms are maintained under a specific selective pressure, while keeping all other environmental variables constant and keeping the population size within fixed limits, for a set period of time. During this time, they are allowed to pass through the mitotic, and potentially meiotic, cell cycle, and the number of such cell cycles are estimated. Selection acts on the pre-existing variants and, given a sufficient number of mitotic cell cycles, also on new mutations arising mostly in association with the copying of the genomes, such that genotypes with higher fitness outcompete other genotypes. Beneficial variants, and sets of variants, become more common than deleterious variants, and sets of variants decrease in frequency over time, and as a consequence, the mean fitness in populations increases.

3.2. Genetic basis of yeast adaptation in the lab

3.2.1. Fitness and adaptation in experimental yeast populations

Selection acts on the fitness of an organism, which in turn is decided by its capacity to survive and produce copies of itself. Chances of survival and the number of offspring are in turn decided by the progression of the organism through its life cycle, i.e. its life history traits, which sometimes are referred to as its fitness components. Phenotypes, at the level from molecules to whole organisms, can be selected only by affecting fitness components, and genotypes are exposed to selection solely if they alter such phenotypes. Several parameters have been used as fitness components in microbial evolution experiments, the main ones being (i) growth rate (typically taken as a reflection of the mitotic cell division rate but in reality affected not only by birth rates, but also by death rates), (ii) growth yield (which is often considered to be a reflection of how efficiently a cell population can convert growth limiting nutrients into new cells, and is sometimes equated with the carrying capacity of the experimental environment) and (iii) lag time (which is equivalent to the time it takes before a cell population shows net growth after a period of no net growth). Figure 3 shows an example growth curve and these three main fitness components. Given that experiments are typically performed in conditions where nutrients are available in constant access, or are replenished soon after being depleted there is a general agreement that these parameters together are good proxies for fitness in experimental conditions. In my experimental evolution experiments, I use growth rate (**Paper I**, **II** and **III**) as proxies for fitness and measure adaptation as change in these parameters over generations. While the approximation of fitness to growth rate in my experimental populations is reasonable based on how I designed experiments, it contrasts somewhat against how we treat fitness in **Paper IV** and **V**, where we look at yeast evolved in nature. In natural environments, where nutrients are often depleted for long periods, and cells starve and die and employ other life history strategies to cope with this (such as shifting into the meiotic cell cycles), other life cycle parameters become important to fitness. To give a better account of fitness in natural environments, we therefore, in **Paper IV** and **V** also report the chronological life span of starving cell populations, the rate at which diploid cells pass through the meiotic life cycle and generates spores (as reflected in its spore efficiency) and how long the generated spores survive (i.e. spore viability). In my experimentally evolving populations,

these fitness components are unlikely to have a substantial role, because the cell populations do not starve long enough for the chronological life span to become relevant, and because cells are either genetically incapable of engaging in mating and recombination or do not encounter the stimuli (nitrogen starvation) for doing so. An alternative approach to measuring fitness, instead of using life cycle parameters as proxies, is to compete different genotypes against each other and to measure their frequency change, which is a direct reflection of their fitness relative to the mean fitness in the cell population. This gives a more accurate view of fitness per se, but is hard to do at the scale required for my experiments and fails to connect the fitness measure to the underlying life cycle, i.e. the biological levels between the genotype and fitness are treated as a black box.

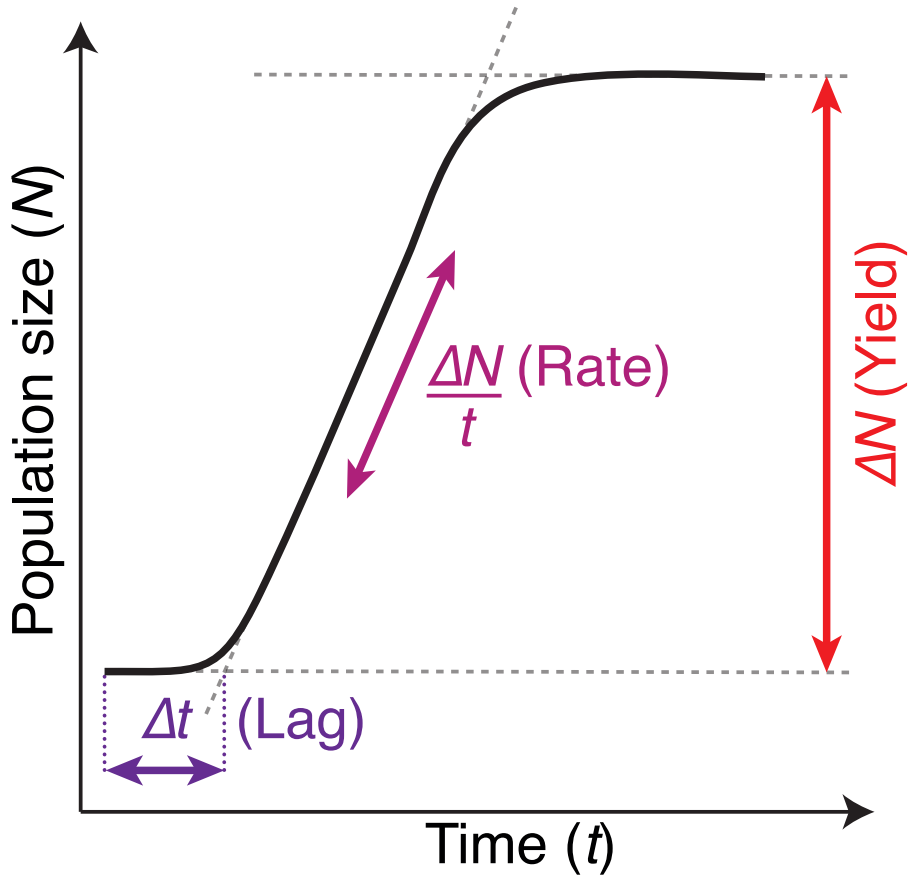

Figure 3. Fitness components in yeast are extracted from a growth curve.

3.3. Adaptation kinetics in lab evolution

The classical view of adaptation is that heritable change in fitness follows from the selection on variations in the primary sequence of the DNA that originally emerged independently of the needs of the organism. Selection is the only force that gives evolution a direction towards higher fitness. Positive selection favours advantageous genetic variants, leading to their increase in frequency, and negative selection removes variants with lower fitness. The first sections of this chapter will fit firmly within this classical, neo-Darwinistic view of evolution.

3.3.1. Mutation rates, mutation effect sizes and the strength of selection

Early mutation-accumulation (MAL) studies suggested that 2% (Wloch *et al.* 2001) or even as high as 5.75% (Joseph and Hall 2004) of all mutations in yeast are beneficial. Although later studies have shown that beneficial mutations may be more common than previously thought (Sniegowski and Gerrish 2010), it is still generally agreed that a majority of new mutations emerging in evolving experimental populations such as mine will have deleterious or neutral effects, i.e. beneficial mutations are rare. Exactly how many beneficial mutations that emerge in an experimental yeast population depend on how the fitness effects (DFE) of new mutations are distributed, the ploidy and the size of the population. Depending on these population genetics parameters, cell populations in experimental evolutions such as mine will follow one of three regimes (Sniegowski and Gerrish 2010). (i) In a strong selection, weak mutation (SSWM) regime, beneficial mutations are rare, but the selection on those is strong, i.e. their fitness effect size is large; (ii) in a weak selection, strong mutation (WSSM) regime, beneficial mutations, on the other hand, are common, but the selection on them is weak; (iii) and in a strong selection, strong mutation (SSSM) regime, beneficial mutations are both common and strong and will often co-exist in the same population. Which of these regimes that an experimental population experiences is relevant because it decides the adaptation dynamics, and how similar the adaptation dynamics of replicate, initially identical cell populations will be.

Under the SSWM regime, populations adapt due to scarce but strongly beneficial mutations. This occurs, e.g. if the population is small and there are a few highly beneficial mutations, e.g. resulting in the rare loss of the function of a single, small gene whose absence gives resistance to a particular environmental factor. When such a beneficial mutation occurs, it fixates rapidly

in the population. After that, the population will have to wait long before the next strongly beneficial mutation occurs, giving rise to a phenomenon that is called simple periodic selection. Because mutations are random, rare, and strong, different replicate populations will behave very differently, with a few likely to find the beneficial mutations early and adapt fast, whereas the majority may have to wait longer for a mutation that results in adaptation. Adaptation dynamics will differ - but on the other hand adapted populations will often finally hit the same or similar targets.

Under the WSSM regime, beneficial mutations are common, and the population size is so large that many beneficial mutations will be present in the population simultaneously – but they are all of small effect. The adaptation will then be slow and continuous, rather than fast and periodic, because it is limited by the rate that selection can incorporate these beneficial mutations into the population rather than by how often they emerge. Replicate populations will tend to follow very similar adaptation dynamics. Still, the evolutionary paths underlying the adaptation trajectories, i.e. the sequence of beneficial mutations accumulated, will tend to be very different, which we will see when sequencing populations. As an example of this, Desai and colleagues (Desai *et al.* 2007) evolved populations of diploid *S. cerevisiae* for 500 generations using six different combinations of population sizes and genomic mutation rates. They observed a smooth fitness increase in all their evolving populations, indicating a WSSM environment. In a WSSM regime, many beneficial mutations that impact the evolutionary dynamics will never, or only in rare populations, rise above extremely low frequencies and will not be detected through conventional genome sequencing methods (Levy *et al.* 2015). Weakly beneficial mutations may also often drift to extinction when they are still present at only low frequencies, i.e. before establishing. Using a sophisticated barcoding method, (Levy *et al.* 2015) could track these usually hidden evolutionary dynamics in WSSM regimes. Initial mutations that confer minor fitness improvements were soon outcompeted by rarer but somewhat large-effect mutations. They thus showed that adaptation (at least in their experimental environment) is driven by a large number of independent beneficial mutations and that mutations, in a WSSM regime, may occur stochastically but collectively have a predictable impact on the population's adaptation dynamics

Finally, the SSSM regime, is characterised by that strongly beneficial mutations are sufficiently common to manifest simultaneously in different genomes which will thus compete. This will occur, e.g. if there are several large genes whose loss gives resistance against a particular environmental factor. Loss-of-function mutations in these genes will then occur regularly and drive a reproducible fast adaptation that will be very similar in all replicate populations. The adaptation to arsenic in **Paper I**, occurring due to loss of either the arsenite importer Fps1 or its regulator Ask10, or to duplication of

the exporter Arr3, is of this type. So is the early, fast adaptation to paraquat in **Paper II**, driven by common, large effect size mtDNA deletions.

3.3.2. Drift, clonal interference, and hitchhiking

Census population sizes during experimental evolutions are typically set such sufficiently large that genetic drift is negligible. This holds true also when considering the population bottlenecks that occur when subsampling a part of the population and transferring it to a fresh medium in batch-to-batch experimental evolutions. Typically, the number of transferred cells is in the order of 20,000-100,000, at which drift should be negligible, assuming that transferred cells are randomly drawn from the population. Admittedly, this may not always be the case, in particular not in terms of the colony evolution experiments I perform in **Paper I** and **II**. Here, sampled cells are taken by robotics from one specific part of the colony: the centre. Yeast cells do not move substantially within a colony, as they are not exposed to any other moving forces than that coming from new buds emerging. Thus, colonies are genetically structured in the sense that neighbouring cells are related, often as mothers and daughters. This means that cells sampled for transfer in my colony evolution experiments may be more closely related than if each cell had been drawn randomly from the population. The census size of the sample, i.e. the number of cells, does therefore not necessarily reflect its effective size, i.e. its genetic variation, and the bottleneck may be more narrow, and the drift stronger, than the census sampling size implies. We have no firm data on how much drift occur in association with such bottlenecks, but I believe that it is still minimal. In addition to the drift that arises from random samplings of cells during bottlenecks, a certain amount of sampling error occurs for new mutations when they are still rare, i.e. present in only a few cells. This type of sampling error can lead, e.g. to some weakly positive mutations going extinct early on in some replicate populations, and contribute to variation between replicate populations.

Two additional population genetic phenomena complicate the understanding of adaptation dynamics in asexual experimental populations such as mine; clonal interference and genetic hitchhiking (Figure 4). Clonal interference occurs because of the lack of sex, which means that two beneficial mutations emerging in different genomes, or clones, can never be recombined into the same genome and therefore doomed to compete. The emergence of another competing, slightly fitter mutation in a population will thus first slow the frequency increase of a particular mutation, cause it to culminate and begin to decrease and ultimately drive it to extinction. In asexual populations, evolution in terms of the genotypes present, will therefore tend to take the form of waves, where clones carrying beneficial mutations replace each other

in sequential events (Figure 4). Genetic hitchhiking also connects to the lack of sexual recombination and means that neutral or slightly deleterious mutations emerging in an asexual population will be carried to higher frequencies by virtue of occurring in the same genome as a beneficial mutation. Most mutations rising to high frequencies in experimental asexual populations will often tend to be neutral or slightly deleterious hitchhikers. A high frequency, therefore, does not necessarily mean that a detected mutation is beneficial.

Moreover, the slightly deleterious hitchhikers will impose a genetic load on the cell population, i.e. its mean fitness will tend to be lower, and its adaptation dynamics will be somewhat slower, than they would have been in the absence of the hitchhiker mutations. Strongly deleterious, e.g. lethal mutations are prevalent, because the yeast genome is packed with genes whose functions have been optimised by many millions of years of adaptation and whose disruption will be catastrophic for cells. About a third of all mutations with fitness effects are believed to be lethal (Wloch *et al.* 2001). However, because of their adverse effects, strongly deleterious mutations will typically be removed by selection already at the single cell level. They will never reach frequencies that affect adaptation dynamics, or the variation between replicate populations. Their adverse effects also mean strongly deleterious mutations will not hitchhike to measurable frequencies.

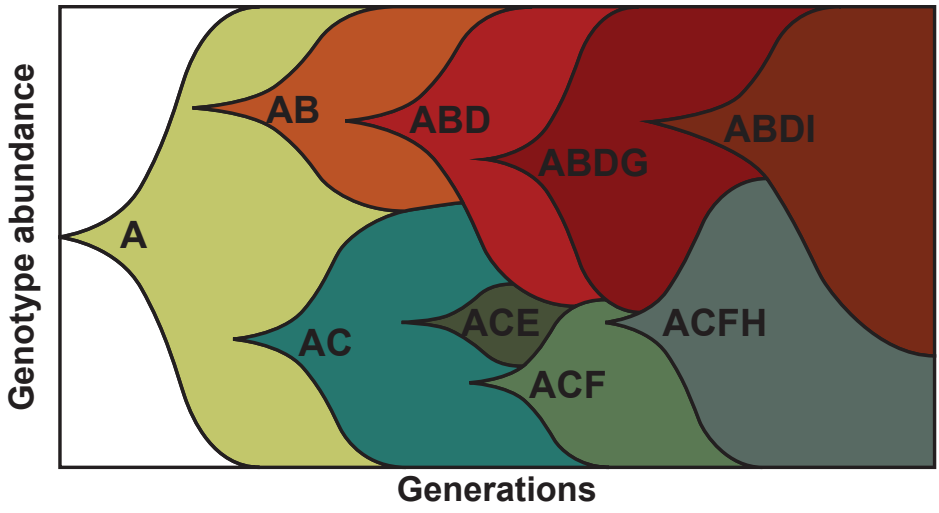

Figure 4. Muller diagram that shows how beneficial mutations arise and allow sublineages change in frequency (y-axis) when they compete for fixation in an asexual population over generations (x-axis).

3.3.3. Sexual recombination increases adaptation rate

One way in which cells can combat clonal interference is by mating followed by recombination. This can help alleviate the cells from linked deleterious mutations and combine multiple beneficial alleles within the same genome, resolving the competition between adapting lineages within the population. Several studies have shown that sex can increase the rate of adaptation (Zeyl and Bell 1997; Becks and Agrawal 2010, 2012; Gray and Goddard 2012; McDonald *et al.* 2016). McDonald et al. investigated the evolutionary dynamics of this observation at the genomic level (McDonald *et al.* 2016). Asexual reproduction avoids mating costs and allows an individual to pass a complete copy of its genome to its offspring. However, recombination relieves clonal interference and enables beneficial mutations to be brought together from different genetic backgrounds, which would otherwise compete with each other.

Beneficial mutations that occur in deleterious backgrounds can be rescued from extinction by recombination into genetic backgrounds of higher fitness. Using budding yeast to compare the sequence-level dynamics of adaptation in sexual and asexual populations, (McDonald *et al.* 2016) show that the adaptation rate is significantly improved in sexual populations. It allows beneficial mutations to be combined within the background and removes deleterious mutations from advantageous backgrounds. Sex enhances the efficiency of natural selection by allowing sorting of beneficial mutations from deleterious ones. Clonal interference and genetic hitchhiking are diminished when mating is allowed in evolving populations. In my experimental populations evolving in **Paper I** and **II**, cells start out as haploids and are genetically incapable of mating and forming **a**/alpha diploids, because they are all of the same sex, and the mating type switch has been genetically inactivated (HO deletion). Theoretically, they could form **a/a,** or alpha/alpha diploids through a genome duplication event, but such diploids would typically not enter meiosis and recombine sexually. In my experimental populations evolving in **Paper III**, cells are initially **a**/alpha diploids. However, they do not enter meiosis because they do not encounter a stimulus (nitrogen starvation, while energy is still available in a respiratory form to support spore formation) for meiosis. My experiments can, in terms of the absence of sex, be regarded as a simplification of natural yeast evolution. However, given that yeast engage so rarely in sex in nature (Tsai *et al.* 2008), I consider it to be a justifiable simplification. But it does limit the extent to which conclusions can be extrapolated from my experiments to organisms such as humans, which only reproduce through sexual recombination.

3.3.4. Epistasis

If an allele's fitness at one locus depends on what alleles are present at other loci, a phenomenon known as epistasis, it can have a huge impact on adaptation dynamics and evolution. In this situation, the order in which adaptive mutations appear in the genome, e.g. if a positive mutation emerges in a wildtype genome or a genome that already has acquired other mutations that can alter its mutation effect size, it has an enormous impact on what future evolutionary path the population will follow.

The long-term experimental evolution experiment (LTEE) of *E. coli* by the Lenski group is an excellent example of this. This multi-study experiment started with 12 initially identical, very large clonal populations of *E. coli*, evolving in the same, novel environment. The researchers expected that the same beneficial variations would appear in each lineage and that the lineages would then converge on the same fitness levels (Lenski *et al.* 1991).

Using the lines from the 2000th generation, each of the 12 lineages was grown separately on a second alternative growth medium(Travisano *et al.* 1995). These lines' initial fitness differed considerably; the observed differences were much greater than between the donor lineages in the first environment. These initial differences in the second environment suggest that the genetic differences at the end of 2000 generations in the first environment were far greater than they initially assumed. The various lines had evolved alternative means to reach similar fitness levels in the first environment. The genetic differences between the lineages had arisen because of the different timing and order of mutations. The initial differences had epistatic effects on other loci; strong selection in identical environments would shape different evolutionary outcomes. Some populations had incorporated mutations that were beneficial in the short-term but eventually led the populations to evolutionary dead ends.

The novel environment used in the LTEE experiment included citrate, which *E. coli* could not metabolise under aerobic conditions because the presence of oxygen completely repressed the citrate importer. It was, however, theoretically possible that *E. coli* could evolve the ability to use citrate as a carbon source. After 30,000 generations, none of the 12 populations had evolved this trait. After 31,500 generations, one lineage had succeeded in metabolising citrate (Pigliucci and Müller 2010; Leon *et al.* 2018).

How did this one population evolve the ability to utilise citrate as a carbon source? Was it an extremely rare mutation that could also occur in the other populations over time? Or did this population evolve through a series of contingencies, uniquely capable of going through the final evolutionary steps to citrate metabolism? To test this, they picked a frozen population from before the evolutionary event that caused citrate metabolism. Using this culture, they showed that the ability to metabolise citrate repeatedly arose in

that population. The population had become uniquely capable of making this breakthrough. The genetic background of this population had increased the mutability of citrate metabolism.

Beneficial alleles tend to provide a lower fitness gain when introduced in a lineage with high fitness compared to one with a lower fitness (Moore *et al.* 2000; Kryazhimskiy *et al.* 2009, 2014; MacLean *et al.* 2010; Chou *et al.* 2011; Khan *et al.* 2011; Wünsche *et al.* 2017). This is termed diminishing-returns epistasis (**Paper I**) and can be described as negative epistasis between beneficial mutations (the combined effect of mutations is lower than what would be predicted by the sum of individual mutations' effects). The timing and order of beneficial mutations matter. If a beneficial mutation doubles the growth speed early on during adaptation, the same mutation might only have a marginal effect on fitness if it occurs at a later point. This is because the lineage has already gained other beneficial mutations, and negative epistasis will result in a smaller effect size. Mutations are more likely to fixate when they occur during early adaptation than later.

Another example of epistatic interactions is sign-epistasis. This occurs when one mutation changes the effect of another beneficial mutation, i.e. making a beneficial mutation deleterious, or the other way around. For example, a large-scale study (Szamecz *et al.* 2014) found that the two mutations *MDM34* and *MGA2* resulted in higher fitness when they were both present. Both mutations are deleterious when they occur independently, an example of reciprocal positive sign epistasis. The *MGA2* mutations were observed during the adaptation of *mdm34Δ* strains (Szamecz *et al.* 2014).

3.3.5. Diminishing return epistasis and the rule of declining adaptability

The fitness change in experimental evolution experiments has been observed to follow similar dynamics of adaptation. Low fitness genotypes are repeatedly observed to adapt faster than higher fit genotypes. This phenomenon, termed "the rule of declining adaptability", seems to be universal, observed in diverse systems such as viruses, bacteria and yeast (Couce and Tenaillon 2015).

Using several published datasets from experimental evolution experiments of microbes, Couse and Tenaillon (Couce and Tenaillon 2015) show that a pattern of declining adaptability in higher fit genotypes is universal. Despite differences in the origin of the data, they all share the universal property: dependence of the rate of adaptation and initial fitness. Five studies consisting of different species, including eukaryotes, prokaryotes, and viruses, share the same principal experimental design: strains with different starting fitness were evolved in a controlled laboratory environment, measuring the fitness gains

after hundreds of generations of lab evolution.

It would make sense that lower fit genotypes would experience higher fitness gains simply because they have more possible mutations at their disposal. In relation to fitter genotypes, they can also substitute mutations that neutralise the causes for their initial fitness defect (Couce and Tenaillon 2015). This implies that low-fitness genotypes must fix both compensatory and adaptive mutations simultaneously, as a higher fit genotype only needs to fix adaptive mutations. How could the difference in fixation rate be explained? One explanation could be a shorter waiting time between selective sweeps in low-fitness genotypes because of the higher availability of beneficial mutations. Since the beneficial mutations have a more substantial effect on the low-fitness genotypes, selective sweeps should run faster to fixation. The universal patterns of early adaptation suggest that it might be predictable, at least at the macroscopic level (Kryazhimskiy *et al.* 2014).

High-fitness genotypes show lower adaptability since they already possess all or most of the possible strong-effect beneficial mutations and are therefore running out of beneficial mutations. The low-fitness genotypes have a higher adaptation rate because they have not yet gained these beneficial mutations. Some mutations may have redundant effects on functionality; any one gene in a pathway may be lost, resulting in the same adaptive gain. This hypothesis is defined as the modular epistasis model by (Kryazhimskiy *et al.* 2014): every beneficial mutation will improve a module, mutations within each module are redundant, and higher fit genotypes have a slower adaptation because they have fewer modules to improve, in particular modules that confer the largest fitness gains (Kryazhimskiy *et al.* 2014).

Alternatively, mutations occurring in higher-fitness backgrounds could provide less benefit than if they happened in a lower-fit background. Diminishing returns epistasis could be universal among adaptive mutations (Chou *et al.* 2009, 2011; Khan *et al.* 2011; Kryazhimskiy *et al.* 2014). Mutations can have widely different effects depending on the genetic background, but the average effect of a beneficial mutation is lower in higher fit backgrounds. If epistasis is global, every beneficial mutation will provide a lower adaptive gain in a fitter genetic background. This implies that each de novo mutation's effect depends on all other mutations present in the genome at the time and their combined effect on fitness. Kryazhimskiy *et al.*, selected three genes whose loss of function mutations were repeatedly found in their evolution experiment (Kryazhimskiy *et al.* 2014). By targeting deletions of these genes in the founder populations, they could observe that the deletions did negatively correlate with the fitness of the background of the strain.

I show that fitter yeast gene deletion mutants adapt slower to arsenite stress by a global diminishing return epistasis (**Paper I**). The adaptive benefit of excluding arsenite from the cell by loss of the arsenite influx (Fps1 or Ask10 loss of function mutations) or improved efflux (Arr3 duplication)

continuously decreases with the increasing fitness of the genetic background. Interpreted within this context, global epistasis makes sense. Since arsenite toxicity is entirely intracellular (Wysocki and Tamás 2010), it is irrelevant what other variants are affecting arsenite homeostasis if arsenite is effectively excluded from the cell.

3.3.6. Compensatory evolution

Adaptive evolution is often seen as a process where beneficial mutations are accumulated to improve fitness in natural populations. The contribution of deleterious mutations in the evolution of novel phenotypes is generally disregarded (Covert *et al.* 2013). Slightly deleterious mutations are more common than adaptive mutations, and can reach high frequencies in populations by hitchhiking with adaptive mutations and through genetic drift. While population bottlenecks and genetic drift may promote the accumulation of deleterious mutations, larger populations of microbes may be less affected. It is likely that loss-of-function mutations are positively selected when they are beneficial in one environment but detrimental in another (Szamecz *et al.* 2014; LaBar *et al.* 2020; Farkas *et al.* 2022). However, the accumulation of deleterious loss-of-function mutations would, over time, become devastating for an organism's survival unless their detrimental effects are weakened by conditionally beneficial mutations at other genetic locations. This process called compensatory evolution can help explain how core cellular functions can be conserved while the underlying genetic network changes during evolution (Szamecz *et al.* 2014; LaBar *et al.* 2020; Farkas *et al.* 2022).

Microorganisms can compensate for the loss of a gene in several ways. Cells can adapt to the loss of a gene by taking advantage of pre-existing genetic redundancy, replacing the lost gene by accumulating adaptive mutations or duplications of a paralogous gene (ancestral gene duplicate). Compensatory evolution to functional loss of genes regularly results in the acquisition of mutations in functionally related genes, but not always homologous genes (Vernon *et al.* 2008; Bergmiller *et al.* 2012; Szamecz *et al.* 2014; Liu and Rancati 2016). If a disrupted gene needed for a cellular function gets disrupted, downstream positive regulators may be amplified, or inhibitors of the pathway could be lost to restore the cellular function (Liu and Rancati 2016).

The compensatory process might change the direction of adaptive evolution, opening up new evolutionary paths that would otherwise be unreachable. Reversion of a lost gene's function is less likely when the target size of the compensatory mutations is much larger than the loss of function mutation. The compensatory mutations can improve fitness either by restoring the lost molecular function or reducing the dependence of the function altogether. Gene loss may even promote genetic changes that drive a population to a new

adaptive peak.

Nearly half of the non-essential genes have been suggested to be involved in at least one cell morphology trait (Ohya *et al.* 2005). In a recent study, Farkas *et al.* explored how compensatory evolution following gene loss can promote morphological novelties in yeast (Farkas *et al.* 2022). They used high-dimensional image analysis to quantitatively measure the single-cell morphology of gene deletion strains before and after compensatory adaptation. They found that morphological diversity emerges rapidly due to gene loss and subsequent compensatory evolution (under ~400 generations). Additionally, they discovered that multicellular phenotypes, like invasive growth, biofilm formation and aggregation spontaneously evolved in response to gene loss.

Interestingly, the researchers found that when reconstructing some of the compensatory mutations in the wildtype background, it had no major morphological impact, indicating a synergistic epistasis on the compensatory mutation and observed morphological trait. Compensatory evolution may change cellular morphology in three different ways. First, it may revert back to wildtype cell morphology. Second, it may retain the new morphological change that was initiated by gene loss without accumulating any further morphological changes, and third, novel morphological alterations may be generated (Farkas *et al.* 2022).

About every 5-6th gene in budding yeast has been reported to be essential for growth (Li *et al.* 2011), meaning that disrupting these genes results in an inviable cell. However, the status of essentiality depends on the genetic background and under what condition the mutant grows. Altering the composition of nutrients in the cultivation may change a cells dependence on a gene. This suggests that both genetic and environmental contexts influence essentiality. Many genes essential for growth in *E. coli* are the most conserved throughout the bacterial kingdom, indicating that these genes have a slower evolution than non-essential genes (Gerdes *et al.* 2003; Baba *et al.* 2006; Liu *et al.* 2015). The essential yeast genes have more homologs in other organisms (Giaever *et al.* 2002) and have higher protein-protein interactions than non-essential genes have (Hwang *et al.* 2009). Several strains in the systematic non-essential yeast gene deletion collection have shown to carry additional mutations, likely resulting from compensatory mutations. The secondary mutations include aneuploidy, polyploidy and point mutations (Giaever and Nislow 2014; Liu *et al.* 2015).

Liu *et al.* performed a stringent screen to investigate how many of the genes reported to be essential are, in fact, possible to overcome by adaptive evolution (Liu *et al.* 2015). They found that approximately 9% of all essential genes are evolvable by adaptive evolution. Deleting any of the 88 genes disrupted essential cellular functions, but this could be overcome by the adaptation of the mutant cells. The authors designated these 88 essential genes as "evolvable essential" to distinguish them from the remaining ~91% of the essential genes

that are not evolvable by compensatory mutations. Gene essentiality depends on available alternative mechanisms that can bypass the disrupted cellular function, how many mutational events are needed to achieve the alternative mechanism and the ability to generate a selectable phenotypic variation. The redundancy and interconnectivity of cellular pathways are important factors for an essential gene to be evolvable essential. The ability to generate selectable phenotypic variation will greatly depend on the mutation rate within the first few cell divisions following the removal of the essential gene. Both bacteria and yeast exhibit stress-induced genome instability during high cellular stress levels (Ponder *et al.* 2005; Forche *et al.* 2011; Chen *et al.* 2012; Liu *et al.* 2015). Once the cells have generated a mechanism that substitutes the lost evolvable essential gene and survived the initially lethal genetic insult, the additional fitness costs from aneuploidy could be reduced by replacing the aneuploidy with more specific genetic changes (Yona *et al.* 2012; Liu *et al.* 2015). Liu *et al.* found that some cellular functions may be more evolvable than others (Liu *et al.* 2015). Particularly essential genes that are only found in eukaryotes and therefore evolutionary younger could be more evolvable.

3.3.7. Outside the neo-Darwinistic paradigm

The coming sections will deal with phenomena that fall slightly outside the neo-Darwinistic box: history-dependent behaviour, heritable phenotypic plasticity, epigenetics and genetic control over adaptation.

3.3.8. Acclimatisation, history-dependent behaviour, epigenetics and genetic assimilation

Organisms often face short-term shifts in their environment, such as nutrient depletion, shifts in temperature and osmotic shock. Cells respond to these changes by turning on and off specific genes and proteins, through different mechanisms. These plastic molecular changes then cascade through the phenotypic layers, changing tissues, organs, and ultimately the form and behaviour of organisms, populations and ecosystems, in a process that is often referred to as phenotypic plasticity and results in acclimatisation. Acclimatisation takes time to manifest fully, ranging from minutes to weeks or even years in terms of higher organisms, but manifests at time scales that are shorter than a generation, or a few generations for microbes. Thus, both time scale, and the fact that it is not due to changes in the primary sequence of the DNA, distinguish it from adaptation. The time between an organism experiencing a change in environment and the time it takes for its phenotypic plasticity to manifest as acclimatisation is referred to as a lag time, or lag phase.

Populations of microbial cells that have recently been exposed to a specific environment can have a shorter lag time than if they had not been exposed to it recently. This history-dependent behaviour (HDB) is observed even if several generations have passed since the population last experienced this environment. The rapid acclimatization when the environment re-emerges, and the disappearance of the phenotype when the environment disappears, is deemed too fast to be explained by a classical neo-Darwinistic variation-selection model. The HDB, therefore, is often taken to represent an acquired, transgenerational cell memory that is based on hereditary changes to cells other than those that affect the primary sequence of its DNA.

In budding yeast, the most pronounced HDB has been observed when cells are repeatedly shifted between different carbon sources. Stockwell *et al.* e.g. looked at how a sudden switch from glucose to galactose as energy source results in a slow induction of the GAL genes that are responsible for galactose uptake and catabolism, and a long lag phase before acclimatisation manifests as growth on the galactose medium(Stockwell *et al.* 2015). Repeatedly shifting the same population between glucose and galactose resulted in HDB in terms of much faster induction of the GAL genes when galactose was encountered and a much shorter lag phase before galactose growth manifested. A similar phenomenon is observed in (New *et al.* 2014), where budding yeast is switched between glucose and maltose and in *E. coli* cells changed between glucose and lactose (Lambert and Kussell 2014). Cerulus *et al.* found that the lag phase duration when cells are switched from glucose to maltose depends on the time the cells were grown in glucose (Cerulus *et al.* 2018). Longer duration in glucose resulted in longer lag times. They found that repression of the respiratory pathways during glucose growth and subsequent reinduction when shifted to maltose is essential for HDB. They discovered that HDB in maltose-glucose-maltose shifts is linked to previous exposure to any carbon source that requires some degree of respiration and consequently gradual repression of respiration during growth on glucose. Cells grown for an extended period on glucose will gradually repress respiration in favour of fermentation; this will cause the cells to have difficulties in re-activating respiratory metabolism, accounting for the lag-phase before acclimatization to respiratory carbon sources becomes detectable. The HDB, therefore, may be seen as faster release from glucose repression of genes required for the use of carbon sources whose catabolism involve respiration.

Several explanations have been proposed to account for HDB, one of them being epigenetics. Epigenetic inheritance is a heritable cellular state that is not encoded in the primary DNA sequence but often relates to how the DNA is accessed by transcription factors that control gene expression. First, the nucleotides adenine and cytosine can be methylated, by the addition of a methyl group. Second, histones that bind DNA to form chromatin can be modified by both methylation and acetylation. DNA and histone

modifications of this type lead to changes in how the DNA is packaged, in the density of the chromatin, and how transcription factors can access it, leading to altered gene expression. DNA and histone modifications can both be induced as a plastic response to environmental factors and be passed on to daughter cells, as a transgenerational memory, after the inducing environmental stimuli have been removed. Prionic proteins are an alternative, non-canonical form of epigenetics, that can be environmentally induced and then stably propagated across generations after the stimuli have been removed. Their stable inheritance and complex phenotypes come about because prions are misfolded proteins that can transmit their misfolded shape onto normal variants of the same protein.

Guan *et al.* found that yeast cells can acquire tolerance to H_2O_2 after being exposed to a mild NaCl pretreatment (Guan *et al.* 2012). They could show that the H_2O_2 tolerance persisted for over three generations after being removed from the primary NaCl stress. The resistance decayed over generations in a manner that was too fast to be genetic and too slow to be phenotypic plasticity, which they interpreted as if it was due to epigenetics. Yeast has also been reported to maintain a similar epigenetic memory of DNA damage (Burrill and Silver 2011).

Another mechanism, which may be regarded as a form of heritable phenotypic plasticity, is protein inheritance, where some long-lived proteins needed in one environment do not get degraded when cells are exposed to the new environment. Some of the lingering proteins can be transmitted to daughter cells during cell division, and conceivably this can be propagated across several consecutive cell divisions.

This section is relevant to the thesis in context of results in **paper II**. Here we show a very rapid adaptation to paraquat that cannot be explained by classical neo-Darwinian selection for the fittest nuclear variants, and a loss of this adaptation over a few generations once the inducing stimuli are removed – despite the adapted cells having no selection against them. This directly leads, or lead, our thoughts to the phenomena above. However, as shown in **paper II** and discussed in the next section, the reality proved to be quite different.

3.3.9. Genetic control over adaptation

A fundamental axiom of evolutionary biology is that mutations emerge independently of the needs of the organism, i.e. a cell does not control whether the mutations are good or bad in the environment where it exists, and it is only selection that gives adaptation a direction. This is still a generally accepted assumption, although examples are now emerging of individual genes and mechanisms whereby cells can control the primary sequence of their

DNA. The previously mentioned yeast mating type switch is one of the best-known examples of this. The HO mating type gene encodes an endonuclease that acts specifically on the DNA encoded in the mating type locus. When activated, expressed and acting on its locus, an elaborate mechanism ensures that the deleted mating type information is replaced by that of the opposite mating type (copied from a neighbouring locus) (Hanson and Wolfe 2017). The mutation associated with mating type switching is clearly genetically controlled. It ensures that a haploid yeast mother can switch mating type after dividing to immediately mate with its daughter and become diploid (haplo-selfing). The diploid state is often beneficial, both in the sense that it confers faster mitotic growth in specific environments (Zeyl 2004; Zörgö *et al.* 2013), and because it better prepares cells for sudden starvation - by allowing passage through the sexual cycle and into the protective spore state when nutrients run out. In addition to this example, there is also accumulating data suggesting that mutation patterns genome-wide are not really independent of the organism's needs but shaped by selection (Monroe *et al.* 2022).

In **Paper II**, we show that the rapid adaptation of yeast to mitochondrial production of superoxide is due to genetic control over its mitochondrial mtDNA deletion rates. To understand how and why, a bit of expansion is needed. The primary role of mitochondria (although it also has many secondary roles) is to efficiently generate energy in the form of adenosine triphosphate (ATP) through the electron transport chain (ETC) by pumping electrons into the mitochondrial lumen and letting them leak out again through the energy-generating ATP synthase.

For reasons still a bit unclear, the mitochondria carry their own genomes (mtDNA), which are present in many copies, ranging from a few to many thousands depending on cell type (Rooney *et al.* 2015). The mtDNA organisation and size vary in different species, resulting from segmental loss or relocation to the nuclear genome (Gray *et al.* 2004). Despite the high variability in mtDNA content, a set of genes involved in oxidative phosphorylation and respiration are conserved in eukaryotes (Wallace 2007). The reason for the conservation of this set of genes is unknown. A reasonable explanation could be that the ETC core components are under rapid transcriptional feedback control by their oxidised protein products, resulting from the high generation of reactive oxygen species (ROS) when encoded inside the mitochondrion (Wallace 2007) – the CoRR hypothesis.

The electron transport occurs in the mitochondrial inner membrane; seven subunits of the protein complexes in the ETC are encoded in the mtDNA. ROS are produced when electrons leak prematurely from sites associated with substrate catabolism and ETC (Brand 2016). The main ROS formed in the mitochondrial matrix is the superoxide radical anion (O_2^-) by the complex I and III, and leakage of electrons from carriers connected to these complexes (Zou *et al.* 2017).

The ~75 kb mitochondrial genome of *S. cerevisiae* carries eight protein-encoding genes, compared to the human mitochondrial genome, which has 13 protein-encoding genes and a genome size of approximately 16 kb (Penta *et al.* 2001; Malina *et al.* 2018). A complete loss of the mtDNA is not fatal (but loss of the mitochondria is), but results in a petite phenotype, characterised by small colonies when grown on fermentable carbon sources, such as glucose (Chen and Clark-Walker 1999). The petite yeast cannot grow on non-fermentable carbon sources, such as glycerol or ethanol (Lipinski *et al.* 2010). *S. cerevisiae* prefers fermentation over respiration in the presence of oxygen (Diaz-Ruiz *et al.* 2011).

Each yeast cell contains approximately 50 copies of the mtDNA genome per haploid nuclear genome (Westermann 2010). During segregation for cell division, all mitochondrial genomes tend to be genetically identical (a state called homoplasmy). The mtDNA is maintained by nuclear-encoded proteins, such as the sole mitochondrial DNA polymerase *MIP1* (Lipinski *et al.* 2010).

In **Paper II**, we adapted populations of yeast cells to paraquat, which causes an elevated intramitochondrial superoxide anion (O_2^-) production (Cochemé and Murphy 2008). The cells responded by a swift reduction of the copy numbers of the mitochondrial ETC genes. We then returned yeast populations to unstressed conditions after short-term superoxide stress and found that this allowed the cells to rapidly restore the ETC genes to pre-stress levels. Long-term stress does, however, cause irreversible loss of the capacity to restore ETC genes (**Paper II**, Figure 5). Since chronic oxidative stress is a sign of ageing (Sun *et al.* 2016), this maladaptive mtDNA deletion could be an important driver in age-related mtDNA impairment.

We show that the swift paraquat adaptation is genetically controlled and depends on the presence of the mitochondrial superoxide dismutase Sod2 and signalling through the retrograde (Rtg) pathway. Even though it is not selectively beneficial in our environment, the mtDNA levels are reversed after stress release, indicating that the process is under genetic control. Our findings in **Paper II** provides perspectives on the axiom that mutations are independent of the needs of the organism.

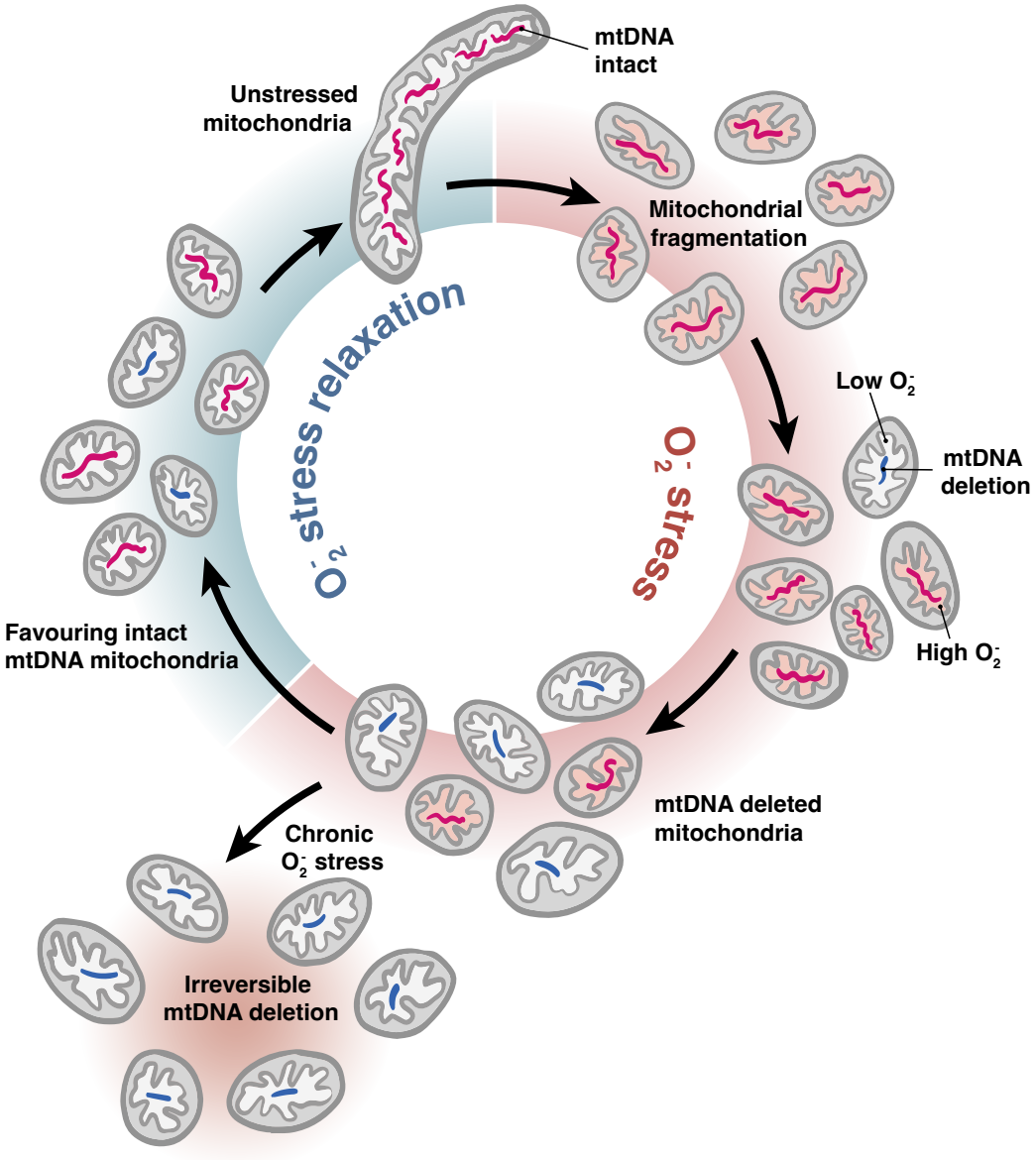

Figure 5. Schematic model of superoxide stress adaptation. Paraquat induces the production of superoxide through redox cycling. Cells stressed by the elevated superoxide levels induce fragmentation of mitochondria and deleted mtDNA encoding ETC components. This reduces mitochondrial superoxide production and a rapid adaptation. If the paraquat-induced oxidative stress is removed before all mtDNA is removed, cells with intact mtDNA will be selectively favoured, and the mitochondria will return their morphology to tubular structures. However, during prolonged stress, the mtDNA deletions will be irreversible.

4. The causes of evolvability

4.1. Evolvability and evolvability genes

With many concepts in biology, the same word can be used to describe a phenomenon but have a slightly different meaning. While there is a confusing pluralism also for the term evolvability, evolvability can be thought of as a refinement of Fisher's fundamental theorem of natural selection (Fisher 1930), which states that the genetic variance in fitness within a population is a measure of the populations potential to respond to natural selection. From this follows that the rate of adaptation of a population reflects its genetic variance in fitness, and its future rate of adaptation reflects its capacity to produce and maintain genetic variance in fitness. To this we need to add the qualifier that only genotypes that are fitter than the mean will contribute to adaptation, and we end up with the classical definition:

> *Evolvability is the ability of a population of organisms to not merely generate genetic diversity, but to generate adaptive genetic diversity, and thereby evolve through natural selection.*

However, it has become increasingly clear that heritable variation need not be based on genetic variants, that genetic variance in fitness may be cryptic and needs to be unmasked before contributing to adaptation. Also keeping a population alive by generating non-heritable variation in fitness may be seen as a form of evolvability in the sense that it is pre-requisite for future adaptation through genetic assimilation. Because there is no consensus around how common phenomena such as these are, there is also no consensus around whether a modern definition of evolvability needs to cover these phenomena. I will use the word evolvability to cover also these cases, but without making any assumptions on to what extent they actually contribute to the measured adaptation.

One might be tempted to use the word adaptability to complement and replace evolvability. However, we refrain from doing so in **Paper I** because of the many broad uses of this term in the literature, in many cases including phenomena such as acclimatisation, and ecological resilience (which is based on species richness), which falls distinctly outside the definition of evolvability. It should also be emphasised that a population can adapt immediately, purely based on its standing heritable variation in fitness. It can also adapt in the near or longer term, by generating, maintaining or unmasking heritable variation in fitness. The term evolvability covers all time perspectives, and should be seen as the potential for both immediate and future adaptation. For example, a currently isogenic population of cells lacking a key DNA repair enzyme may

have limited potential for immediate adaptation, because all existing cells are identical clones, but have a high potential for future adaptation because the mutation rate is high – and may therefore be said to have high evolvability. This is particularly relevant for this thesis, because in my **Paper I** and **II**, where I study the evolvability of close to isogenic populations, the evolvability estimated is determined almost exclusively by the capacity to generate, maintain, and unmask heritable fitness variation.

It should also be pointed out that evolvability is often used in the context of change in phenotypes over evolutionary time, with no explicit connection to fitness. When used in this sense, the concept is clearly phenotype specific and different phenotypes for a particular genotype or population may have different evolvability due to exposure to different degrees and types of evolutionary constraints. For example, a species of mammals, i.e. a population in the broadest sense, may have high evolvability for body size, but be less evolvable for the number of limbs because of developmental constraints: an embryo with diverging limb number is aborted before birth. The genetic variation for body size that is realised in adults is far greater than the corresponding variation in limb number. In my **Paper I**, I will use evolvability exclusively in terms of the life history phenotype cell doubling time, which, as discussed earlier, can be seen as a good proxy for fitness, at least under the conditions studied.

4.2. Standing variation, mutation, and recombination rates

A population will adapt, using its genetic variation in fitness, based on its current (standing) genetic variation in fitness. The level of standing genetic variation in fitness will be a product of all evolutionary forces, including selection, genetic drift, gene flow (migration) and the frequency and types of sexual recombination during mating. Thus, it is entirely plausible for genotypes that, e.g. increase the amount of immigration into a population to be seen as evolvability factors. However, two evolutionary forces are usually singled out when it comes to evolvability: mutation, which generates new genetic variants, and recombination, which is mainly associated with mating and sex and recombines these variants into new constellations.

The mutation rate, for microbes such as yeast, is defined by the number of de novo mutations per mitotic cell division. This is because most mutations occur as errors during DNA replication that are not repaired before the subsequent replication of mother and daughter cell genomes. However, a small number of mutations arise also during meiosis, and tick as a function of meiotic cell division, and a small number of mutations, which is larger for certain types of mutations, follows from environmentally induced DNA damage or spontaneous chemical reactions that tick as a function of chronological time. The point mutation rate, which is the most common and best understood type of mutation, for microbes tends to be ~10^{-9} - 10^{-10} mutations per base pair for each cell division (Gou *et al.* 2019).

Mutation rates have been suggested to be fine-tuned by natural selection to optimal long-term survival and evolvability (Lynch *et al.* 2016). But this is widely disputed, mostly because mutations tend to be negative much more often than positive. Some argue that mutation rates are not fine-tuned to be as low as possible, given the fitness cost of further improving the accuracy of the DNA replication and repair machinery (Ram and Hadany 2012). The DNA repair enzymes that prevent mutations can themselves experience mutations that lead to increased mutation rates. This type of evolution can be adaptive when facing new environments (Sniegowski *et al.* 1997; Healey *et al.* 2016; Payne and Wagner 2019). Likewise, stress-induced mutagenesis in microbes, through direct DNA damage by an external factor or through a more indirect strain, e.g. in terms of resource allocation that makes DNA replication more error prone, can generate beneficial variation that allows adaptation in extreme environments (Bjedov *et al.* 2003; Ram and Hadany 2012). The stress-induced mutagenesis can be seen, and has been seen, as a naturally selected cellular "intention" to increase the mutation rate, and thereby generate more variation upon which natural selection can act, when adaptation is required. But this remains controversial and is generally held

to simply reflect that cells are incapable of maintaining a low mutation rate when challenged.

The genome encodes many important functions that are essential for survival. Maintaining a low mutation rate ensures that these essential functions are intact and lethal mutations are selected against. Strains with highly elevated mutation rates, due to defective or error-prone DNA repair systems, are known as mutators. Mutator strains have been observed to have a mutation rate 10-1000 fold higher than wild-type, and can still be cultivated for thousands of generations without evident loss in viability (McDonald *et al.* 2012; Wiser *et al.* 2013). Strains with a high mutation rate have a higher chance of being linked to deleterious mutations with a fitness cost. This is particularly true when talking about asexual strains reproducing clonally. With an absence of beneficial mutations, natural selection will act to lower the mutation rate and balance it to genetic drift and physiological costs.

If beneficial mutations are available, lineages with a higher mutation rate have a higher chance of producing the beneficial genetic variation and simultaneously hitchhike on the higher fitness the new variant generates. ALE experiments with mutator *S. cerevisiae* lines were shown to adapt decreased mutation rates (McDonald *et al.* 2012).

When faced with near-lethal stress, rapid adaptation can save the population from extinction through evolutionary rescue. Swings *et al.* (Swings *et al.* 2017) found that hypermutation drove the adaptation of *E. coli* exposed to near-lethal ethanol stress. The near-lethal environments can trigger a change in the optimal balance of a constant genetic load and mutational supply, favouring a higher supply rate. Despite the increase in genetic load, the elevated mutational supply enables rapid adaptation of some individuals that later rescue the population from extinction. By measuring the mutation rate at different adaptation steps, (Swings *et al.* 2017) found that mutation rate recurrently increases in response to stress and decreases once the cells have adapted to the stress. Mortality could also act as a modulator of the population mutation rate. An elevated mutation rate is linked to higher mortality, with a buildup of genetic load and a higher chance of lethal mutations. The mortality differences give a selective advantage that favours strains with lower mutation rates once the population has adapted to a stressful environment.

The observation that minor elevation of mutation rate can confer a competitive advantage under near-lethal stress (Swings *et al.* 2017) gives support for the mutator genotypes also are evolvability genotypes. However, in my experiments in **Paper I**, we find that all gene deletion strains adapt according to their initial fitness, even gene deletions that are previously known to have an elevated mutation rate.

An elevated recombination rate can improve the population's adaptation rate by making more combinations of beneficial alleles and eliminating deleterious mutations. Sexually reproducing yeast populations have a faster

adaptation due to recombining and joining beneficial alleles from different genomes into one, while also releasing the load of deleterious variants (McDonald *et al.* 2016). The experimental populations adapted in **Paper I** and **II** are haploid and asexual. This means we will not capture the effects of evolvability on mating and inbreeding in diploid populations. Recessive variants, such as loss-of-function mutations, can drive adaptation in clonal diploid populations only if they are first converted into homo- or hemizygotic states (Vázquez-García *et al.* 2017). We may also miss genes causing increased meiotic recombination rates and loss-of-heterozygosity when screening for evolvability genes in **Paper I**.

4.3. Cryptic genetic variance

Genetic variation does not always contribute to heritable phenotypic variation in a population. This hidden variation, or cryptic variation, can bring forth phenotypic variation after an environmental change (Rutherford and Lindquist 1998; True and Lindquist 2000; True *et al.* 2004; Jarosz *et al.* 2010). Cryptic genetic variation can stay hidden, because it causes little to no phenotypic variation, but has the potential to cause phenotypic variation in a different environment or if recombined into a new genetic background (Payne and Wagner 2019).

Cryptic variation can allow populations to move with otherwise inaccessible trajectories to high fitness genotypes. If a population without variation adapts to an unfamiliar environment, it may reach a local fitness peak but will not climb other higher peaks if it would need to traverse in-accessible low-fitness genotypes. However, populations that carry cryptic genetic variation may reach these higher peaks if the necessary steps have arisen prior to the environmental change.

Similar to how electrical capacitors store and release charge, do biological systems allow evolutionary capacitance to store and release genetic variation. A living system can accumulate and store cryptic genetic variation that does not change the phenotype, due to the phenotypic robustness of the organism. When the conditions change, robustness breaks down by an increased amount of stress, and the previously hidden genetic variation will be unmasked and generate phenotypic variability (Payne and Wagner 2019). This newly uncovered variability will be subject to natural selection. An evolutionary capacitor works as a molecular switch, capable of masking and unmasking cryptic genetic variation.

The capacitance of a living system would allow a more rapid adaptation to an unfamiliar environment. The rate of the molecular switch is most likely determined by stress, affecting the phenotype when it is beneficial for adaptation.

Enzymes may, besides their primary function, perform side reactions. These side reactions performed by so-called promiscuous enzymes might confer an adaptive advantage if the side reaction would be beneficial in altered conditions (Copley 2003; Aharoni *et al.* 2005).

Chaperones are a group of proteins specialising in assisting protein folding and relieving the cell of cytotoxic protein aggregates. It has been hypothesised that chaperones provide additional robustness to mutations by assisting in folding the more unstable protein products. During elevated stress, chaperone-assisted folding will falter, and this previously cryptic variation can be unmasked (Jarosz *et al.* 2010). The heat shock protein Hsp90 is one of these chaperones believed to act as a critical player in evolutionary capacitance

(Rutherford and Lindquist 1998; Jarosz and Lindquist 2010). When using Hsp90 inhibitory drugs, several model organisms have displayed a broad range of phenotypes hidden by cryptic variation and uncovered once Hsp90 is inactivated (Rohner *et al.* 2013; Zabinsky *et al.* 2019).

The yeast protein Sup35 is involved in ensuring that translation stops correctly at one of the three stop codons (Halfmann *et al.* 2010; Halfmann and Lindquist 2010). Sup35 has a prion form, [*PSI*+]. When present in a yeast cell, it will convert the natural form of Sup35 into the prion form (Tyedmers *et al.* 2008). This results in an elevated stop codon readthrough rate, causing a higher number of erroneous proteins present in the cell. In some stressful environments, [*PSI*+] will be beneficial to the cell's ability to adapt (Lancaster *et al.* 2010).

Since genes involved in protein folding are suspected to influence evolvability, we expected to find gene deletions of the yeast Hsp90 chaperones Hsp82 and Hsc82 to have impaired evolvability. This is not the case in **Paper I**, we find that the two gene deletion mutants adapt as predicted by their fitness. However, the function of the two genes is redundant, and a double deletion results in synthetic lethality, and we might miss detecting a complete Hsp90 functional impairment in our screen.

4.4. Phenotypic heterogeneity

The best-known mechanisms that create heritable phenotypic variation is DNA mutation and recombination. In addition to this, several non-genetic mechanisms can create phenotypic heterogeneity by affecting gene expression. Some of these mechanisms are stochastic gene expression, protein synthesis errors, epigenetic modifications and protein promiscuity. The variation can benefit some subpopulations and give them a competitive advantage (Tawfik 2010).

Noise in gene expression can be caused by variations in transcription and translation efficiency (Ozbudak *et al.* 2002; Blake *et al.* 2003; Payne and Wagner 2019) and when gene expression is regulated by low-abundance molecules that fluctuate randomly in a cell (Elowitz *et al.* 2002). The evolution of increased gene expression noise has been observed in budding yeast adapting to an antifungal compound (Bódi *et al.* 2017), where increased expression noise in synthetic regulatory circuits of a resistance gene enhanced the effect of beneficial mutations.

The persistence phenotype, where some cells within an isogenic population stay in a physically dormant phenotype, is one example where stochastic gene expression is adaptive (Harms *et al.* 2016). The dormant subpopulation will survive exposure to drugs which require active growth to kill, causing the persisting subpopulation to bide time to adapt resistance to the drug. Recently, *E. coli* was observed to adapt resistance by persistence when exposed to periodic treatment of the antibiotic ampicillin (Levin-Reisman *et al.* 2017). The persistence can facilitate evolvability by allowing a small subpopulation to survive long enough to acquire adaptive mutations. Stochastic gene expression can also allow a subpopulation to express a beneficial phenotype to evade drug treatment (Spencer *et al.* 2009; Sharma *et al.* 2010). These cells do not need to be dormant; instead, they exhibit a brief transcriptional state that confers resistance. Stochastic gene expression may enable evolvability by changing the effect of mutations on fitness, enhancing the positive effects of beneficial mutations (Bódi *et al.* 2017).

Several types of errors are possible during protein synthesis and can produce non-genetic phenotypic heterogeneity. Wrong nucleotides can be added to the RNA sequence during transcription, splicing errors, translation mistakes causing incorrect polypeptide sequence and errors in the folding and post-translational modifications of the protein (Drummond and Wilke 2009). Stop-codon readthrough is a type of translation error that generates protein variation.

Promiscuous proteins have one primary function and other secondary, latent functions. Promiscuity can facilitate evolvability by providing a reservoir of potentially adaptive protein activities that can be further strengthened if the gene is duplicated, allowing one copy to be refined for the secondary

function.

In **Paper I** and **II**, we study the evolvability of close to isogenic populations, and in **Paper III**, we use isogenic populations derived from four widely diverged *S. cerevisiae* lineages and contrast these against populations with substantial pre-existing variation. We do not determine the degree of phenotypic heterogeneity in these populations, but I believe it is likely to have affected the evolvability and adaptive trajectories of my yeast populations. Additionally, gene deletions that increase phenotypic heterogeneity are part of the genome-wide evolvability screen in **Paper I**.

4.4.1 Robustness and networks

Non-genetic phenotypic heterogeneity generates phenotypic variation in the absence of genetic variation, while genetic robustness removes phenotypic variation in the presence of genetic variation. Many phenotypes are robust to mutations to some extent. DNA mutations that improve protein stability will increase the robustness by allowing the protein to fold correctly even when additional successive mutations occur (Bloom *et al.* 2006). Mutational robustness can evolve by increasing protein stability and duplication of genes (Bloom *et al.* 2006; Keane *et al.* 2014). Mutational robustness has likely evolved because it is adaptive and provides protection in scenarios when the mutation rate is higher (Sanjuán *et al.* 2007). Gene duplications can increase robustness by creating redundancy and allowing a higher tolerance to mutations in either duplicate. Robustness allows DNA mutations to cause genetic diversity without changing a phenotype.

Living organisms are organic units built up by modules; these functional units work together to build networks, such as metabolic pathways, gene regulation and protein interactions. Networks are considered modular if they consist of highly connected clusters of nodes and isolated or sparsely connected to the nodes of neighbouring clusters. Modular systems are more adaptable since rewiring a modular network of functional subunits requires fewer steps than a network where everything is entangled. Modularity provides robustness against pleiotropic effects from mutations and limits the effect within the concerned module, and adaptation will be less constrained. Modularity reduces the costs of connections in a network (Clune *et al.* 2013).

Protein functions are often mediated by physical interactions with other proteins. Large-scale protein-protein interaction studies have helped shed light on the modularity of these networks. A protein interaction network consists of central nodes, called hubs, with a larger than the average number of interactions. Han *et al.* identified two types of hubs in the protein-protein interaction network of yeast: party hubs interact simultaneously with many partners within their module in the same time and space, and date

hubs interact with their partners at a different time and space and integrate between modules (Han *et al.* 2004; Fraser 2005). The mutational effects at party hubs confer limited pleiotropy, indicating that their effect is mainly limited within the module. The date hubs have more extensive pleiotropy, with more widespread effects due to their higher interaction across many modules (Fraser 2005, 2006).

4.5. Fitness landscape topography

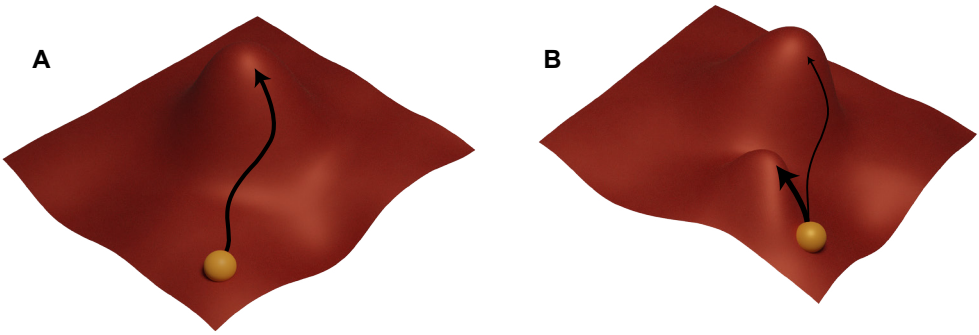

Figure 6. A conceptual fitness landscape. A) A single peak fitness landscape. B) A two peaked fitness landscape.

A fitness landscape comprises peaks and valleys, a peak represents a higher fitness, and a valley is detrimental to growth in the current environment. Different genetic backgrounds have diverse fitness landscapes. Depending on where the genotype is positioned on the fitness landscape, different adaptation routes will take place in an evolutionary experiment. When a genotype accumulates beneficial mutations and move across a fitness landscape, it is referred to as an adaptive walk (Payne and Wagner 2019).

A population of cells can climb fitness peaks to improve fitness (Figure 6). Most commonly, the most accessible fitness peak will be climbed since it will require fewer steps. A population that has reached a fitness peak will likely remain there and not climb down to reach another higher peak. That way, a population with a lower fitness will have a higher chance of crossing fitness valleys and reach new peaks. The shape of the fitness landscapes and the position of different genetic backgrounds in the landscape varies for the different populations in my experiments in **Paper I**, **II** and **III**. Beneficial mutations allow the populations to climb peaks in the fitness landscape. Populations that start with a low fitness tend to adapt more during the initial phase of the ALE experiments (Couce and Tenaillon 2015), which we further underscore in **Paper I**. Additionally, populations that have adapted a higher fitness in one environment may have lost fitness in another environment. These so-called trade-off effects result from adaptive or neutral mutations in the first environment being deleterious in the second environment. We show that adaptation of yeast deletion strains to various stresses incurs a cost in the absence of stress in **Paper I**.

5. Measuring yeast evolution - main methods

5.1. DNA Sequencing

In **Paper II**, **III**, and **IV**, we use DNA sequencing to understand existing and emerging genetic variation. In **Paper I** and **V**, we use sequence data produced in earlier studies. Several types of sequencing techniques are available; which techniques we use is determined by what kind of information we want to detect and also how much we are prepared to pay for it.

In **Paper II**, we first use long read (PacBio) sequencing of the YPS128 founder strain. We also incorporated the older assembly from our sequencing of the same YPS128 genotype (Yue *et al.* 2017) to make a de novo assembly and a reference genome to which we map genetic changes from our short-read sequences in adapting populations. The long-read sequencing has a 205x genome coverage. Our adapted populations and populations released from selection are resequenced with short-read sequencing (Illumina) to observe the change in copy numbers of the lost mtDNA segments, and genomic changes throughout the ALE experiments.

Short read sequencing, such as Illumina, produce high-quality data, based on mapping the short sequences onto a reference genome. The reference genome is preferably a high-quality assembly of the founder strain, and this is the case in **Paper II**. In **Paper III**, we use time-resolved short-read sequencing to determine how genetic variation changes in the populations as they adapt. Using only short-read sequencing means we may miss capturing some of the de novo structural rearrangements in the experimental populations. In **Paper IV**, we genome sequence the living ancestor. It contains large blocks of LOH, with near to identical sequence in the highly diverged parental subgenomes. To overcome these challenges, we use deep-sequencing using both short (200×, Illumina) and long (146×, PacBio) read technology.

Sequencing can be performed either on singe cells, on individuals drawn from a population and then clonally expanded to obtain sufficient DNA or on a large, random sample of a population, often after clonally expanding this large sample. The latter allows us to estimate the relative abundance of genetic variants within the evolving population, and the assumption is then that the clonal expansion step will not affect allele frequencies. In **Paper II** and **III**, we this last technique to detect de novo mutations (if they are at a high enough frequency in the population) and estimate their frequencies.

SNPs and smaller indels are called based on their differences to the reference genome, given that positions receive sufficient coverage (e.g. 10-fold) and have a sufficient quality score. The mean sequencing depth, i.e. how many reads we sequence for a sample, and the nature of the DNA in a particular region, with, e.g. centromeric, telomeric and sub-telomeric DNA being particularly hard to sequence, assemble and align, influence how much and what we miss to detect and the error in the estimated allele frequencies.

This then becomes a slight caveat when it comes to our conclusions. Some larger genetic variants, such as copy number variations and aneuploidies, will still map to the reference genome at their original position. They can still be called since the relative abundance of reads for these sequences will be higher. For example, if a chromosome is duplicated in a whole population sample, the relative abundance of reads mapping to that chromosome will be, on average, twice as high. However, large structural rearrangements, such as translocations, will be missed since the sequence abundance will remain the same. A de novo assembly will be necessary to detect such balanced structural variations. In addition to the difficulty of mapping short reads to repetitive regions, errors from non-random genome fragmentation, PCR amplification bias and variation in GC content across the genome are known sources of sequencing errors. Linking the genetic variants with phenotypes (chapter 5.3) will help determine if the gene variant is adaptive or not.

5.2. Phenotyping

Phenomics is the large-scale study of the phenotypes of organisms. Gathering robust and reliable genotypic data is generally simpler than for phenotypic data. The genotypic data may vary depending on the technical quality of the data. Still, there can only be one valid called genomic sequence for the individual carrying the genome, if the sequencing and assembly generate a complete error-free genome sequence. However, one individual expresses multiple phenotypes and these depend not only its genetics, which changes little across cells and times, but also on the exact composition of its environment, which often varies in ways that are hard to control. The limitations in phenotypic knowledge narrow our understanding of the relationship between phenotypes and genotypes.

Moreover, the phenotypic space is endless and cannot be exhaustively measured. A genetic variation may also affect several other phenotypes that are not measured, and these may affect fitness, leading to misinterpretations of why a genetic variant is selected. Phenomics is thus at the same time a more difficult and larger undertaking than genomics, and it is believed to be the next big step after the recent advances in genomics (Houle *et al.* 2010).

5.2.1 Measuring fitness

Fitness can be seen as the most important phenotypic parameter to measure in evolutionary biology. Fitness is a complex meta-phenotype comprising many partial phenotypes. While it can be measured directly in competition experiments, these are not high-throughput and are hard to parallelise across many populations. Instead, fitness is measured in high throughput experiments using different proxies, or fitness components which are then seen as approximating fitness. For microbes expanding clonally as asexual populations and where nutrients are rapidly refreshed once depleted, fitness will be determined almost exclusively by the properties of the mitotic cell division cycle; essentially the fraction of cells that pass through each step in the mitotic cell cycle and the time it takes for them to do that. Because cells in a population divide asynchronously, i.e. not at the same time, and because there is no easy readout for each cell cycle step, we cannot easily measure these individually. Instead, we measure meta-parameters that reflects the population level progression through the cell cycle and use these as fitness components that approximate fitness, individually or together.

The three main such fitness components that can reasonably reliably be measured at a large scale in yeast, and other microbial populations, are the lag, the rate and the yield of growth (Figure 3). The growth lag estimates

the time it takes for a population of yeast cells to acclimatise to the growth conditions they are provided and to start growing. A somewhat simplistic view is that it reflects the time it takes for a cell to exit the G_0 resting state, which they enter when resources in the preculture run out, i.e. the quiescent state, and re-enter the cell cycle in the G_1 step of the cell cycle. The conditions of the pre-cultivation, and the time spent in the preculture after resources are consumed, will impact the lag phase, which will be most affected. Populations that have remained long in a G_0 state will have a more prolonged lag phase than if they would have been freshly grown in a preculture and transferred to the experimental condition. This may be because a larger fraction of cells has entered quiescence and a larger fraction of cells have died. The latter reflects a particular problem with the lag phase; it does not only reflect the time to re-enter the cell cycle but is also influenced by how long it takes for detectable net growth to occur. If the inoculation contains few cells, or if many cells die after the transfer, the lag phase will be longer even if surviving cells re-enter the cell cycle rapidly. In all my experiments, I typically preculture yeast populations for three days before transferring them robotically to experimental plates for incubation and phenotyping. This ensures that the entire cell population has entered stationary phase, but that no cells have experienced an extended time in the stationary phase. Thus, all cells need to acclimatise when transferred to fresh medium, but the resulting lag-phases will not be so long as to substantially affect the extraction of the growth rate – or the fitness.

The growth rate is the time it takes for the population of cells to double during the logarithmic growth phase, where growth is the fastest. This is evidently influenced not only by the cell division time but also the death rate, but the latter is often assumed to be zero in this growth phase given that resources at this time are present in abundance, and the growth rate is taken to reflect the cell division rate. The strict definition of growth rate as the cell division rate means that its effect on adaptation can be directly modelled in evolutionary models, which we also do in **Paper II**. Empirically, the growth rate is estimated in two different ways: the maximal specific growth rate, which corresponds to the growth rate when it is at its maximum, and the mean specific growth rate, which corresponds to its mean along the entire growth phase. The latter typically requires modelling the growth, by fitting a function such as the Chapman-Richards or Gompertz function to the data. This imposes an exponential phase onto the growth data. However, in small cultivations with limited nutrients available, such as the ones explored in my papers, resources often become limited soon after all cells have left the lag-phase. This means that a clear exponential phase is not observed, and the fitting of a model is not a good choice. Therefore, we estimate the maximal specific growth rate in all my populations and use that as a fitness proxy. We use the maximal specific growth rate to determine fitness of yeast populations in **Paper I**, **II**, **III**, **IV** and **V.** Additionally, we estimated the phenotypes

growth yield, i.e. the total amount of cells generated during the experimental growth cycle, sporulation efficiency, and chronological life span in **Paper V**.

5.2.2. Growth phenotyping in high-throughput

Growth phenotyping of yeast cultures, at high throughput, has generally been conducted using liquid media micro-cultivations where measurements of the amount of light reflected or absorbed by, or transmitted through, each culture is used as proxies for population size. Liquid micro-cultivation stations can be used to track the growth of hundreds of cell populations at a time in this way. However, they tend to require considerable amounts of manual labour, the liquid cultivation step makes them prone to contamination and they cannot easily be made to handle tens of thousands of populations (Warringer and Blomberg 2003). In preparation for my PhD project, an alternative phenotyping platform was developed in the research group. Instead of liquid cultivations, this method relies on solid media and enables a high throughput (Zackrisson *et al.* 2016).

Scan-o-matic is a solid media growth phenotyping platform, and the primary method of phenotyping (**Paper I**, **II**, **III**, **IV** and **V**) for this thesis. The method provides many advantages compared to other phenotyping platforms, the main one is that it is highly scalable, allowing more than 100,000 populations to be phenotyped in parallel if run at maximum capacity. Figure 7 describes the pipeline from start to finish.

The core part of Scan-o-matic is a software that analyses flatbed images of agar plates with clonally growing microorganisms. Robot-assisted pinning enables replication and transfer of colonies from a source plate to a target plate. The density of colonies on the plates is up to 1536, where every fourth position is reserved for spatial controls. The plates are then incubated in scanners placed inside large incubators that control temperature and keep a constant humidity. Images of the plates are automatically scanned at intervals of 20 minutes. Each scanner takes four plates, bringing the total number of colonies that can be phenotyped to up to 6144 for each. The program analyses the image files and extracts the amount and intensity of the pixels, converting the values to cell counts using an internal standard based on flow cytometry measurements (Zackrisson *et al.* 2016). Single numeric phenotypic parameters (growth rate and yield) are extracted from each growth curve.

For the experimental evolution experiments (**Papers I, II**), the plates intended for serial transferral (evolution plates) are never placed in scanners; instead, copies from the evolution lines are pinned to plates that are phenotyped in scanners (Figure 7). The individual growth curves have a high quality, but it is still necessary for the user to quality-check the curves individually to remove technical errors. The curves are indexed by the quality and automatically sorted by the program, making the quality control

manageable. Future software updates might allow more sophisticated curve analysis, requiring only computational quality control to remove erroneous curves.

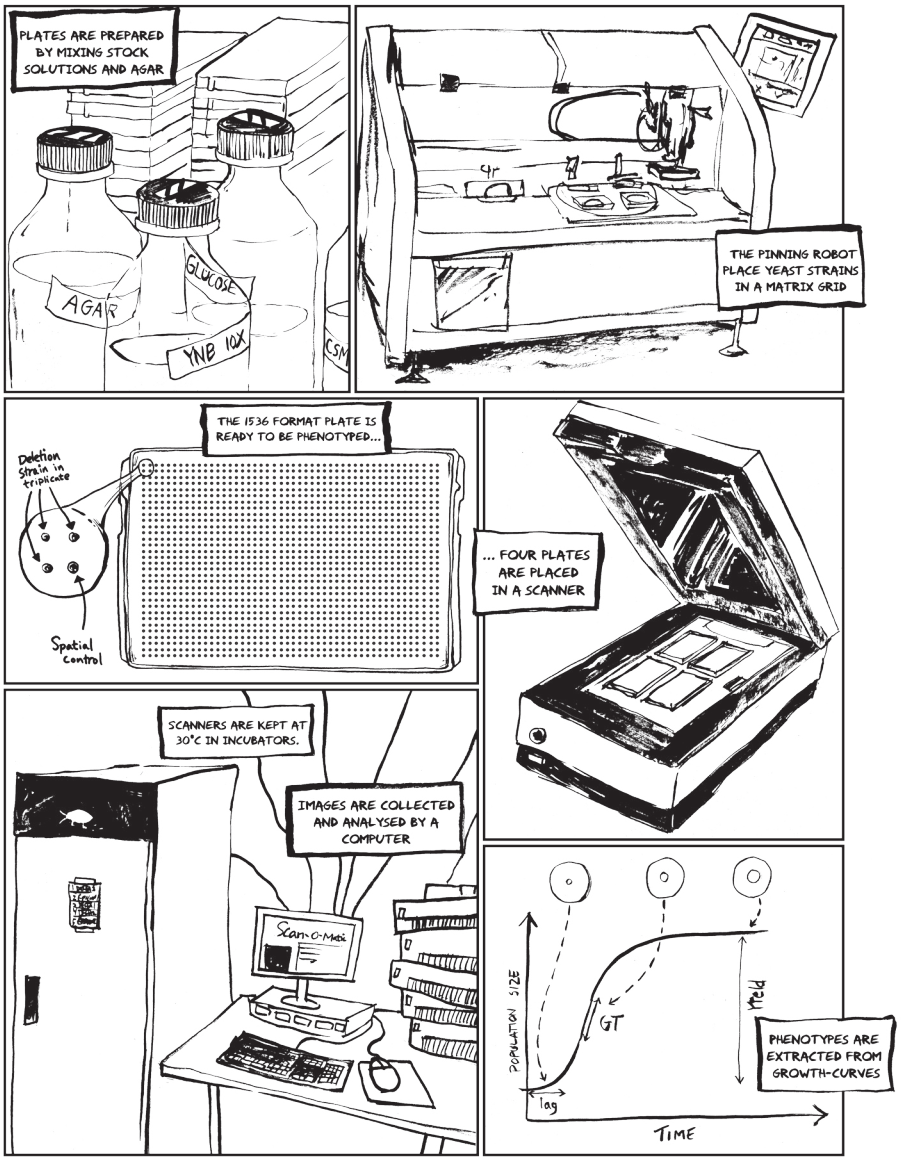

Figure 7. Graphical representation of the Scan-o-matic method for yeast phenotyping in high throughput. 1) Agar plates are prepared on a levelled surface. 2) Yeast strains are placed in a grid using robotics. 3) Every fourth position has a spatial control. 4) Plates are placed in the scanner, held in place by an acrylic fixture. 5) The scanners are kept inside incubators at 30°C, connected to a computer that saves scanned images at intervals of 20 minutes. 6) That way, each colony is tracked over time. The colony pixel values are converted into cell numbers. Phenotypes are extracted from each growth curve.

5.3. Connecting phenotypes to genetic variants across extant yeast lineages

There are several approaches to link traits to genotypes. However, when considering many genotypes for which the genealogy is not exactly known, only genome-wide association studies (GWAS) offer a realistic alternative. It is a method where genetic data from a population is statistically tested for genetic variants in different individuals that are associated with a genetic trait. It has been used to identify SNPs of many human diseases (Tam *et al.* 2019). In **Paper V**, we use GWAS to trace the genetic origins of the yeast DS and disclose causative effects of aneuploidy, gene presence/absence variations, CNVs and SNPs.

Despite the many successes with the method, there are some drawbacks. The SNVs identified with GWAS can typically only explain a smaller fraction of the heritability of complex traits, i.e. we have a high rate of false negatives that are not detected, typically because the variants are too weak or too rare to have a statistically significant effect. It may also identify false positives, yield too many loci and therefore not be informative enough, or it can fail to pinpoint the causal genetic variants (instead pointing to variants linked to these) (Tam *et al.* 2019).

Quantitative trait loci (QTL) mapping is another method for linking genotypes to phenotypic traits. This method relies on crossing two parental genomes. Genetic variants of the two parental genomes will recombine during meiosis. The combination of genetic alleles and phenotypes is determined for each of many progenies. By repeatedly crossing the progeny several rounds, which can be done in bulk, the resolution and accuracy of QTL mapping can be improved significantly (Märtens *et al.* 2016). We use QTL mapping on four-parent yeast populations in **Paper III**. We show that in contrast to two-parent populations (Vázquez-García *et al.* 2017), the higher level of pre-existing variation that is generated by the four-parent cross quantitatively alters the evolution dynamics. Only one pre-existing causative variant was mapped, in the two-parent population, while the four-parent populations revealed more than 20 quantitative trait loci (QTLs) contributing to drug resistance.

5.3.1. Validating candidate variants

Several approaches can be taken to test if an allele associated to a trait, or to change in a trait, is actually causing the observed phenotype or change

in phenotype. An allele suspected of causing a phenotype can be genetically reconstructed in the ancestral parental strain. If this mutant reproduces the same fitness as the evolved strain, the adaptation can be attributed to this mutation. If patterns of parallel evolution exist where several lineages have acquired mutations in the same gene, it will be more likely to be adaptive.

As stated, yeast can with ease be genetically engineered, relying on the process of homologous recombination to incorporate foreign DNA. Genes can be completely replaced by auxotrophic or resistance markers, allowing selection for knockout strains. In my papers, single deletion and duplication mutants are constructed using the LiAc/SS Carrier DNA/PEG Method (Gietz 2014). Double deletion mutants in **Paper I** was constructed using the Synthetic Genetic Array method (Tong and Boone 2006; Kuzmin *et al.* 2014), creating diploid crosses of two deletion strains, generating haploid spores and selecting for selectable markers to generate haploid double deletion strains. I introduced an extra *ARR3* gene on a single-copy plasmid, to hundreds of single gene deletion strains in **Paper I**. In **Paper II**, we generated strains lacking all mtDNA by deleting the mitochondrial DNA polymerase *MIP1*, strains with and without one extra chromosome II, III or V and various gene deletion strains to determine if they are adaptive. In **Paper III**, we constructed gene deletions, reciprocal hemizygotes, and crosses with various copy numbers of chromosome IX. In **Paper IV**, we deleted the *HO* gene, mated and sporulated yeast strains. In **Paper V**, we used the standard lithium acetate/PEG procedure and CRISPR-Cas9 technology to engineer deletion strains and swap alleles (Gorter de Vries *et al.* 2017; Barré *et al.* 2020).

5.4. Experimental evolution

The simplest ALE batch-to-batch experiment relies on manual serial dilution of the population after a certain time has passed (Figure 8A). However, when many replicate lines are maintained for a long time, it will be too labour intensive unless more sophisticated automation solutions handle the serial transfer of evolving populations. However, the batch-to-batch ALE experiments we use in **Paper I** and **II** are highly scalable, since we use robotics to handle all the transfers to fresh medium (Figure 7). The serial transfer causes cyclical alterations between low and high cell densities, an initial rapid growth rate that slows down once easily accessible nutrients are used up, and waste products accumulate.

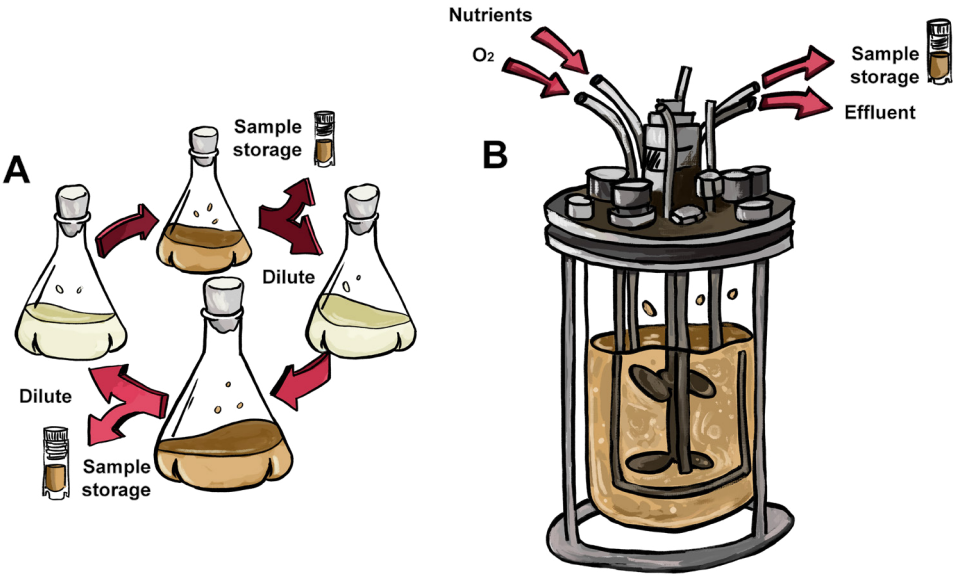

Figure 8. The classical experimental evolution setups.

The alternative to serial transfer is maintaining evolving microbial populations in continuous cultures (Figure 8B). Chemostats are closed culturing vessels, where a fresh medium is added at a fixed dilution rate, while the same volume of microbial culture is simultaneously removed from the vessel. The volume is held constant, and the culture is mixed by stirring. Commercial systems are expensive, which causes limitations in the propagation of multiple lines in parallel. Another side effect of ALE setups in chemostats is that there will be a selective pressure to remain in the vessel (van den Bergh *et al.* 2018). Microbes can evolve ways to adhere to the vessel wall by biofilm formation, or sediment faster to the bottom and avoid exit through the outlet. However, there are ways to reduce selection of biofilm and sedimentation phenotypes in continuous cultures by elevated stirring or use

of surfactants (Miller *et al.* 2013), usage of a twin-chemostat setup (de Crécy-Lagard *et al.* 2001), and stepwise movement of the growth chamber inner wall (de Crécy *et al.* 2007).

In ALE chemostat experiments, nutrients are available in excess throughout the experimental period and the cells remain continuously in the mitotic cell cycle. In ALE batch-to-batch growth experiments, one nutrient, typically a carbon/energy source (in most of our experiments: 2% glucose), is consumed and often completely depleted before being replenished. In contrast, other required nutrients are available throughout the experiment in substantial excess of the needs of the population. All other environmental conditions, often except for the imposition of one selection factor (e.g. a drug) in addition to competition for carbon/energy, are kept as constant as possible. The additional selection factor can be held constant throughout the experiment, or varied over time as the population adapts, to e.g. prevent the selection pressure from dropping over time, or to emulate variable environmental conditions. My ALE experiments in **Paper I, II** and **III** have all been batch-to-batch experimental evolutions on solid media, and all involve one extra selection factor, in addition to periodic carbon/energy limitation, at a fixed level. Both types of experimenters are great simplifications of natural environments, where nutrients are less or more sporadically available, and many other environmental factors vary over time. Organisms may pass through multiple other stages of their life cycle, and they might also compete for the resources with other species.

Yeast cells grown in agitated liquid cultures will all experience the same homogeneous environment. Since the cells cannot move by their own means, cultures grown on solid surfaces will develop distinct micro-environments within a colony. Inside a colony, many subpopulations can coexist. One might be tempted to use the mixing setting on the pinning-robot to sample cells from the entire colony. However, this increases the risk of cross-contamination between neighbouring colonies and is therefore not done in my evolution experiments. The ALE populations in this thesis are grown on solid media, in **Paper I** and **II**; we use robotic pinning to transfer each cycle to a fresh medium. Our setup has higher scalability and parallelisation than any liquid handling method I am aware of. The risk of cross contaminations from droplets between wells in a liquid microculture plate is inherently larger compared to colonies on agar. There is still a little risk of contamination and cross contamination in-between populations also in my solid agar evolution lines. Contamination of mould or bacteria is easily recognised visually, since they generally spread rapidly throughout a plate. Luckily, this did not happen to any evolution lines, but happened on rare occasions to plates placed in scanners where the risk of particles and microbes may land on the plate during phenotyping, and they had to be repeated. When transferring cells to a new plate at the end of the cycle, cells may also drop off from the pins

elsewhere. I believe that this is very rare. My evolution lines in **Paper I** and **II** are adapted with every fourth position empty; these positions are later filled with an unadapted spatial control. Contaminations on empty positions are easy to spot, which I do not recall observing during my experiments.

6. Future perspectives

6.1. Experimental evolution

Experimental evolution is an established and powerful tool to answer fundamental questions in evolutionary biology and evolutionary medicine and to refine strains for biotechnological applications. I am sure future ALE will benefit from more complex experimental setups, e.g. encompassing longer timespans and more complex populations and environments, as well as from increasing automation that lowers the labour intensity and reduces the risk of human errors. Developing sophisticated microfluidic systems that could be employed in ALE may help in this. The selective pressure can then be set to vary according to pre-defined parameters for individual populations, e.g. to keep the selective pressure constant as populations adapt, to change in strength or nature, or to be combinations of different selection agents. Additionally, the rapid expansion and improvements in machine learning and artificial intelligence will significantly impact what type of ALE experiments can be done, what questions can be answered and the resolution of the answers. With the technical advances in experimental and computational science, it may be possible to construct complete fitness landscapes in quite complex environments that better resemble those encountered in nature or medically relevant contexts.

6.2. Yeast in evolutionary biology studies

With its history and robust and efficient genetic engineering tools, *S. cerevisiae* will undoubtedly continue to play a major role in the future. Some of the basic biology of *Saccharomyces* species is still poorly understood. How do they interact with other microbes in their natural environment? Are they actively growing or dormant on bark and soil? How, when and where do they have sex? In what ways do they disperse in nature? Answering these questions would help us understand how ecology shaped their evolution. It may also help make yeast a model organism in ecology, a role it currently does not have. I also foresee that other yeast species will become more popular to study in the near future. What is often called non-conventional yeasts often have different properties that make them interesting for evolutionary studies. These other yeast species have also been shaped less by human activities, i.e. they will likely not have a pronounced domestication syndrome. In some cases, they have evolved symbiotic relationships with other species that we can explore to better understand species-species co-evolution. One example of this is the yeast *Wickerhamomyces anomalus* that is a mutualistic symbiont of

different insects (Cappelli *et al.* 2021). Additionally, industrial biotechnology applications would benefit from evolving or breeding non-conventional yeasts to efficiently produce enzymes and biochemicals from cheap resources such as plant biomass or industrial sidestreams, for which *S. cerevisiae* is not well suited.

6.3. The evolve and sequence approach

Sequencing technologies will continue to improve in the future. Already existing technologies will become more widely available and affordable, making the technology accessible also for high throughput purposes for research institutions. Higher accuracy in combination with longer reads will ensure higher quality genome assemblies. This means that the impact of structural variations will become easier to determine. The evolutionary dynamics can be studied in greater detail and at high precision, with large sample sizes. Massive lineage tracking with randomised DNA barcodes could reveal the distribution of fitness effects in many species, in high replication, under different selective pressures to study how it changes throughout evolution. Future sequencing technologies with a resolution at single genome levels will further improve the understanding of genetic change during evolution in experimental populations. Additionally, detecting DNA modifications allows a better understanding of the roles of epigenetic interactions during evolution.

6.4. Phenotyping

Enormous amounts of genomes are sequenced today, and the genetic databases that store these genomes are growing. However, these genomes tell us very little unless the containing genes are studied and their functions annotated. Linking the phenotypes to genes is far from a trivial task. Therefore, future high throughput phenotyping of microbes will be crucial to understanding these sequenced organisms' underlying genetics. Additionally, the different types of phenotypes that can be studied are enormous. In this thesis, I have phenotyped the growth rate of yeast in various kinds of stresses in solid agar. An organism may behave differently depending on the environment it is exposed to, and the number of different traits that can be measured is enormous. What other life history traits impact the fitness of an organism, depending on the environment? And how do morphological traits, such as cell shape, size, thickness etc., affect life cycle progression and

fitness? I believe that microscopical techniques to study the morphological characteristics of single cells will become even more advanced in the future; this will help to understand how morphological novelties allow cells to adapt in experimental populations.

6.5. The impact of natural variation on yeast evolvability

In my research, I have studied the contribution of non-essential genes to evolvability. The yeast gene knockout collection is derived from a lab strain, a cross between several wild isolates, with alleles that have never jointly been exposed to natural selection outside the lab. With this in mind, it would be interesting to apply our ALE methodology to adapt the yeast collection consisting of more than 1000 unique isolates (Peter *et al.* 2018). Future yeast sampling expeditions can further expand this collection of natural isolates with strains from more uncommon habitats and geographical locations. The effect of natural genome content variation on evolvability would be interesting to study using an extensive yeast collection like (Peter *et al.* 2018), which we worked with in **Paper V**. How does evolvability differ between domesticated and wild isolates? The variation in alleles, genome structure, gene copy numbers, gene presence/absence and ploidy number could affect evolvability. Another way to study evolution in natural yeast would be first to identify and separate strains that adapt slowly and quickly. These isolates could then be crossed in several rounds to create many crossover events. The evolvability of these highly chimeric stains can be measured in a second round of ALE. This way, genetic variation that promotes or prevents adaptation in the second round can be identified.

Additionally, expanding on the natural yeast collection could uncover the differences in evolvability between other yeasts in the *Saccharomyces* sensu stricto complex, or even more unrelated yeasts with ALE experiments. This could answer questions on how different yeast species adapt compared to others. How have hybridisation and genomic introgressions shaped evolution in yeasts? Could the speedy adaptive mtDNA deletions in response to superoxide stress in **Paper II** also happen in other yeasts or higher eucaryotes?

6.6. The role of essential genes in evolvability

So far, I have studied how non-essential genes impact evolvability in yeast populations, but what about essential genes? The essential status of a gene is determined if cells are non-viable without this gene. There are exceptions; essentiality can depend on the genetic background through epistatic effects on the background genome. Mutations can be conditionally essential, where the growth medium conditions determine if the gene is necessary for viability. Finally, several genes have been termed "evolvable essential" (Liu *et al.* 2015), since cell growth can be restored after disruption of these genes by compensatory mutations in other genes. However, some genes are critical for viability. These are essential for core cellular processes and cannot be compensated with altered growth conditions, genetic background or adaptation. Could any of these essential genes be linked to evolvability, and how could this be tested experimentally? One way to try this could be to use a library of temperature-sensitive essential gene mutants and adapt them to near-lethal temperatures. However, this could be experimentally challenging since the temperature sensitivity varies with each mutant, and temperatures need to be individually adjusted for each tested mutant. Another option could be to adapt a CRISPR interference library (Smith *et al.* 2017), in which the expression of essential genes can be systematically downregulated. This CRISPRi library has recently been phenotyped using the Scan-o-matic platform to investigate acetic acid tolerance (Mukherjee *et al.* 2021), suggesting that adaptation of this strain collection could be tested using the ALE method of **Paper I** and **Paper II**.

6.7. Evolvability research

Similar to smaller-scale studies, the results from the ALE experiments in **Paper I**, show that fitness determines adaptation almost deterministically. We could not find any strong links to genes with evolvability functions in our experimental setting. Does this mean that evolvability genes do not exist? Is the effect of diminishing returns epistasis so strong that the potential evolvability genes cannot be detected? Or do individual gene deletions have too weak an effect on evolvability to be measured? Maybe evolvability functions are redundant, and the impact of any single gene deletion on evolvability would be impossible to detect. Could there be a way to experimentally block diminishing returns epistasis to identify potential evolvability genes? How predictable is evolution, and can future evolutionary trajectories be theoretically modelled? Could the evolvability of cancer or pathogenic

infections be predicted and potentially prevented before it happens? How vital are epigenetics and plasticity for evolvability? Could this be tested by simply comparing the evolvability of strains with variations in plasticity or by chemically reprogramming the epigenetic coding of strains to see how they adapt in relation to the parental strain that retained the original epigenetic coding?

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