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Deciphering molecular mechanisms in the evolution of new functions

HIND ABDALAAL

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Abstract

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The evolution of new genes and functions is considered to be a major contributor to biological diversity in organisms. Through *de novo* origination, "duplication and divergence", and horizontal gene transfer, organisms can acquire new genetic material that can evolve to perform novel functions. In this thesis, we investigate how functional trade-offs, "gene duplication and amplification", and neutral divergence contribute to the emergence of a new function from a preexisting gene.

In Paper i, we investigated the ability of *Salmonella enterica* to compensate for the loss of peptide release factor 1 (RFI) and the potential of peptide release factor 2 (RF2) to gain a new function to replace RFI. The amplification of RF2 and accumulated mutations within RF2 were the main evolutionary routes by which the fitness cost was restored. However, further characterization of the evolved RF2 showed a toxic effect to the cell due to the termination on tryptophan codon (UGG). This evolutionary trade-off - which we named "collateral toxicity" - might present a serious barrier for evolving an efficient RF2 to replace RF1.

In Paper ii, we determined whether we could evolve a generalist enzyme with two functions (HisA + TrpF) from the specialist enzyme HisA, which can only synthesize histidine. In a previous study, we showed that HisA evolved a TrpF activity through strong trade-off trajectories. Here, we developed a selection scheme in which we constantly selected for keeping the original function (HisA), while intermittently selecting for the new function (TrpF). Our results showed that all evolved lineages shared the same "stepping stone" mutations in the *hisA* gene, which enabled them to grow well in the absence of both histidine and tryptophan. Additional accumulated mutations in the *hisA* gene gave the strains an increased ability to grow without both amino acids, indicating that the HisA enzyme evolved to be an efficient generalist.

In Paper iii, we explored how differences between diverged orthologs influence evolvability. We generated artificial orthologs using a random mutagenesis approach. First, we screened for orthologs with a lower HisA activity and then selected for orthologs with a higher HisA activity; these steps were repeated in alternating rounds. We then tested the ability of each ortholog to evolve TrpF activity. As expected, the orthologs showed varying abilities to evolve the new function. In particular, orthologs with higher HisA activity levels showed both a higher potential to evolve the new function and a higher TrpF activity when they acquired the new function.

Keywords: Evolvability, functional trade-offs, collateral toxicity, duplication and amplification

Hind Abdalaal, Department of Medical Biochemistry and Microbiology, Box 582, Uppsala University, SE-75123 Uppsala, Sweden.

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To my family

List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

I **Abdalaal, H***., Pundir, S., * Ge, X., Sanyal, S., & Näsvall, J. (2020). Collateral toxicity limits the evolution of bacterial Release Factor 2 towards total omnipotence. *lecular Biology and Evolution* (*.pub a head of print*)

II **Abdalaal, H** Näsvall, J. (2020). Intermittent selection directs the evolution of a specialist enzyme to become a generalist. *anuscript*

III **Abdalaal, H** Näsvall, J. (2020). Evolvability of orthologous genes. *anuscript*

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* These authors have contributed equally to the work.

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Abbreviations

.coli	*scherichia coli*
S.enterica	*Salmonella enterica*
P. aeruginosa	*Pseudomonas aeruginosa*
P.fluorescence	*Pseudomonas fluorescence*
SNP	Single nucleotide polymorphism
HGT	Horizontal gene transfer
N_e	Effective population size
DNA	Deoxyribonucleic acid
IAD	Innovation–amplification–divergence
PCR	Polymerase chain reaction
WGS	Whole genome sequencing
s	Selection coefficient
RNA	Ribonucleic acid
MutSHL	Methyl directed mismatch repair enzymes
rpoS	Alternative transcription sigma factor S

Introduction

In the early nineteenth century, Charles Darwin highlighted in his book natural selection as the primary mechanism by which evolutionary change occurs (Darwin, 1859). He defined natural selection as "the principle by which each slight variation [of a trait], if useful, is preserved". According to his definition for natural selection, individuals with traits that provide them a higher capacity to survive in their surroundings are more likely to survive and pass these traits to their offspring. Natural selections will occur when the following conditions are met: (i) there are traits variations in population; (ii) these variations are heritable; (iii) the fitness of these variants have different rates of reproduction (differential fitness) (Lewontin, 1970). Therefore, natural selection needs a reliable hereditary mechanism between generations, however Darwin's theory left a gap in explaining how inheritance works. During the same era, Gregor Mendel studied heredity mechanisms, and concluded that, traits can be passed down by heredity units or heredity factors (Laird and Lange, 2011) It is important to remember that Darwin and Mendel developed these theories only through sharp observations. With today's progress in molecular genetics, we know that heredity units are the genes that are passed down to the next generation, and natural selection alone does not account for all the evolutionary changes in a biological system. In the 1960s Kimura suggested that most of the genetic variations in the population could not be caused by natural selection, but was the result of genetic drift (mechanisms by which a genetic traits can be lost or become widespread in a population due to a random chance) on neutral mutations (genetic changes that does not affect organism ability to survive and reproduce) (Kimura, 1968). Later, Otha suggested the nearly neutral theory which included both processes – natural selection and drift – as the driving forces for the evolutionary changes (Ohta, 1973).

With the progress of molecular genetics, we are now better equipped to decipher the molecular mechanisms that lead to evolutionary changes. We can also answer most of the problematic questions in our time such as: how to tackle antibiotic resistance? How to overcome food shortages? How to provide clean energy?

Microbial experimental evolution as a research tool

Most of the early evolutionary theories were based on observational studies. These observations were recorded for events that happened billions of years ago. Experimental evolution provides the chance to test our hypotheses by replicating the same conditions in the lab. Experimental evolution studies have been conducted both on unicellular and multicellular eukaryotic model systems (Marden et al., 1997; Ratcliff et al., 2012), prokaryotes (Buckling et al., 2007; Heineman and Bull, 2007; Gallie et al., 2019; Lind et al., 2015), single enzymes (Bloom and Arnold, 2009; Moses and Davidson, 2011) and ribozymes (Salehi-Ashtiani and Szostak, 2001). The laboratory experiments conducted on microbial populations have yielded a robust understanding of various theories, and facilitated greater exploration of new ideas in evolutionary biology. Bacteria are useful as model system for studying evolution because they grow fast, allowing researchers to evolve several generations quickly, and compare them to their unevolved ancestors. In addition, several replicates from a common ancestor can be included which helps to determine repeatability of these evolutionary changes. With bacterial models, it is easy to control the environment and manipulate conditions. For example , the evolutionarily changes can be tested in fluctuating (Beaumont et al., 2009; **Paper II**) or stable environments (Näsvall et al., 2012; **Paper II**). Also, bacterial populations from different points in time can be easily saved by freezing them at low temperature (-80°C) to be studied later (Lenski et al., 1991; Elena and Lenski, 2003; Näsvall et al., 2012; **Paper I**; **Paper II**).

In general, most bacterial evolution experiments are conducted by inoculating cells into liquid media and letting them grow until the culture reaches its maximum density. A fraction of the culture is then transferred to fresh medium to continue the growth until the culture again reaches a high population density (Fig.1). This process is referred to as cycling or serial passaging, and as the cycling progresses, natural selection might drive the adaptation process. The genetic changes of the populations are often analyzed by WGS.

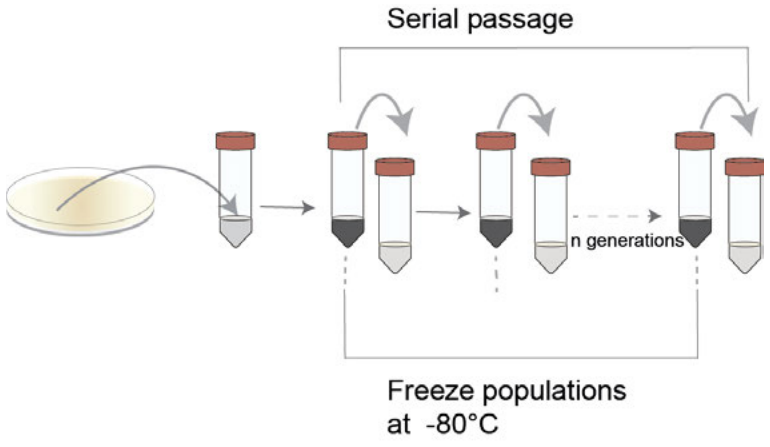

Fig 1. **Bacterial evolution experiments.** This is an example of a standard experimental evolution experiment for bacterial population which is done by many labs. Populations are passaged frequently in order to achieve several generations of growth.

The experimental setup of each evolution experiment is tailored to suit the question at hand, for example:

(i) The evolution of a single species (one strain), addresses the fundamental question of the adaptive response of this species to a certain environment. An example of this is the long-term and on going evolution experiment performed by Lenski's group since 1988, which has now reached approximately 70000 generations (Grant et al., 2020). The experiment started with 12 replicates of *E. coli* grown in media containing glucose and citrate; the initial strain did not utilize citrate. As this experiment has continued many concepts have been elucidated, such as the origin of a new function (citrate utilization) (Blount et al., 2008), evolution of mutation rate (Sniegowski et al., 1997), and trade- off (Travisano and Lenski, 1996).

(ii) Comparative evolution, examines how different strains with different genetic backgrounds respond to the same selection pressure. For example, Vogwill et al, (2014) compared how different strains of *P.aeruginosa* develop resistance to rifampicin using a short-term selection experiment. They found that the growth rate of the evolved clones has varied significantly (around 30%) between different genetic backgrounds.

However, these experimental evolution setups are simple and do not necessarily reflect the real picture of microbial community complexity found in nature. Furthermore, bacteria often accumulate mutations that respond not only to the subjected stress but also to the laboratory conditions (Knöppel et al., 2018).

Directed experimental evolution

To investigate the evolution of a specific gene or protein, directed experimental evolution is usually a convenient approach. This process begins with choosing a protein with the properties of interest, such as promiscuous activity (see page 21), or functional and structural arrangements needed in evolving a new function. Then, researchers use random mutagenesis in this protein to generate a wide range of genetic diversity. Lastly, there is screening or selection for the viable variant that gives the desired new function; this step is a shortcut for the selection and accumulation of beneficial mutations that happen in nature. If one mutagenesis round does not yield the desired level of the new activity, more rounds can be performed to improve the function (Bershtein et al., 2008; Bloom and Arnold, 2009; Cirino et al., 2003; Lundin et al., 2020, **Paper III**) (Fig.2).

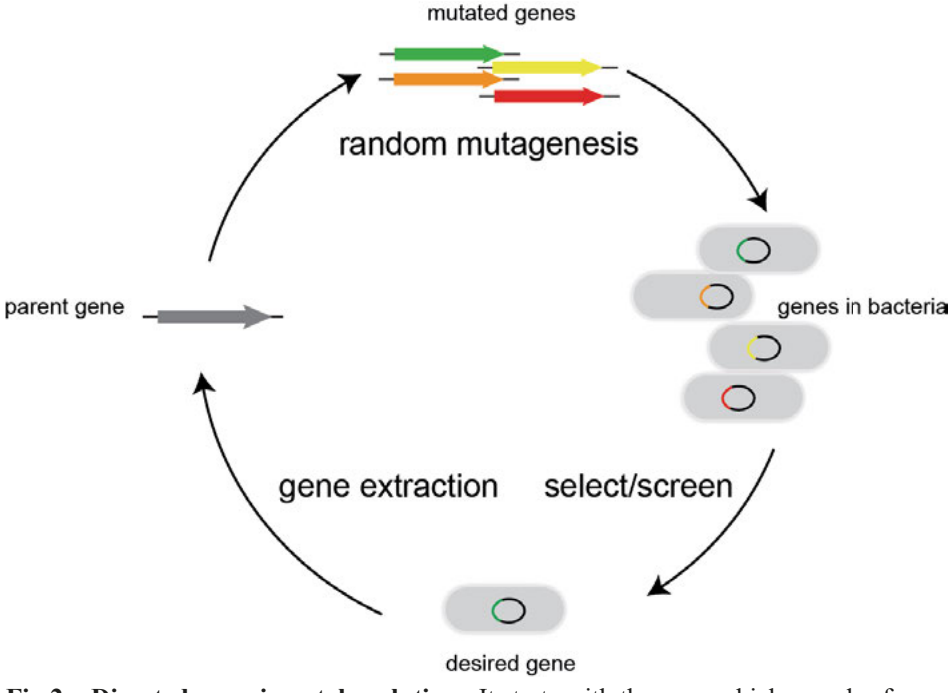

Fig 2. **Directed experimental evolution.** It starts with the gene which encodes for the parent protein. This parent gene is randomly mutagenized using error prone PCR, or similar techniques that generate sequence diversity. The library is introduced to the bacterial chromosome or plasmid. Then, it is screened or selected for the desired property. This process is repeated until the evolved protein shows the required level of activity.

Researchers in the protein engineering field sometimes use this approach to improve protein properties such as catalytic activity, binding affinity, or stability (Eijsink et al., 2005; Jäckel et al., 2008). Directed evolution is also used

for manipulating protein scaffolds to perform non-biological chemistries. For example, Carbon-Silicon bond (Kan et al., 2016) or C-H amination (Prier et al., 2017). Directed evolution has not only helped understanding the function of evolved biomacromolecules, but also allowed using it in different industrial applications (Cherry and Fidantsef, 2003), from improving laundry detergents (Vojcic et al., 2015) to synthesizing medicinal drugs (Ebo et al., 2020)

Fitness

Fitness is the ability of a microorganism to survive and reproduce (Orr, 2009). There are two ways by which a microorganism's fitness can be measured: (i) absolute fitness; (ii) relative fitness.

The absolute fitness ($|\omega|$) is the total number of genotypes that are successfully passed on to the next generation. An absolute fitness value of 0 is considered lethal, while 1 is beneficial. Relative fitness which is described by the concept selection coefficient s is usually normalized to the fittest (generally the WT) is given a value of 1, s is calculated by the equation $s = 1 - \omega$. A positive value indicates that the genotype has higher fitness, while a negative value indicates the opposite (Orr, 2009).

Fitness in experimental evolution is normally measured by comparing two genotypes. Therefore, relative fitness or selection coefficient s is more informative in this case. The genotype with higher fitness will reproduce and increase at a higher frequency over time than the less fit ancestor or competitor. Several experimental studies have shown that evolutionary changes in fitness can be reliably quantified (Wiser and Lenski, 2015)

Competitive fitness is one of the most robust assays that is used in the lab to determine selection coefficient. A typical example in measuring fitness in experimental evolution is including a mutant that has acquired a new function or phenotype, such as the ability to grow in the presence of a certain concentration of an antibiotic. The evolved strains or populations can be mixed with their ancestor; both can be phenotypically marked (with fluorescence markers or mutations with easily identifiable phenotypes) to differentiate between them while they grow under the same conditions (Gullberg et al., 2011; Wistrand-Yuen et al., 2018). Obviously, the evolved mutant will grow better than its parent in the presence of the antibiotic.

The second assay for measuring fitness are exponential growth rate assays. In this case, the mutant and the WT grow separately over a period of time The value of s is estimated from the maximum growth rate of both genotypes (Lundin et al., 2020, 2018)

Mutations

Mutation is the engine of evolution –the process that generates a novel function – that may be favored by natural selection. Mutations play a major role in generating sequence diversity by introducing changes at the nucleotide level, which result in heterogeneous organisms and enable evolutionary change within a population. In brief, mutations can have different sizes, which range from the substitution of a single base pair, to deletions and insertions of DNA fragments, inversions, translocations and duplications. If the mutation happens in genes that are essential for survival (e.g. resistance to antibiotic) or reproduction (e.g. growth rate), it might have a fitness effect on the microorganism. Therefore, based on the mutation fitness effect they are divided into three classes: deleterious (decreasing fitness), beneficial (increasing fitness), and neutral (no effect). The fitness effects of mutations might determine its fate as its mentioned by Darwin's "survival of the fittest" concept Therefore, a mutation that has the highest fitness in a population will be fixed, while the one with highest fitness cost might be purged from the population (Lenski et al. 2006; Soskine & Tawfik 2010). However, this is not the full picture of how mutations are fixed in a population. Mutations can be stochastically fixed by genetic drifts, or because they are coupled with other mutations in linked location.

At the protein level SNPs, in particular, are known to have different effects on activity (Khanal et al., 2015; Soskine and Tawfik, 2010; Wrenbeck et al., 2017). When SNPs occur in protein coding sequences they can be classified as either synonymous or nonsynonymous substitutions. Synonymous changes have long been assumed to be neutral for organisms, however, several studies have shown that synonymous mutations can have non-negligible impact on protein activity and organism fitness (Agashe et al., 2013; Kimchi-Sarfaty et al., 2007; Knöppel et al., 2016; Shabalina et al., 2013). Synonymous mutations can drive adaptive evolution by increasing fitness to a level comparable to that of a non-synomous mutation. Therefore, it can be positively selected and become fixed in population (Bailey et al., 2014)

Deleterious mutations

A deleterious mutation can have a detrimental effect on the organism's fitness and protein function. The deleterious effect can range from slightly deleterious to lethal. In . *coli,* RNA polymerase, encoded by *rpoB*, showed that a majority (363/465) of amino acid substitutions have a lethal effect on the organism's fitness (Nene and Glass, 1982). However, this finding might not be general ,for example several fitness effect studies have estimated that the occurrence of mutations with a lethal effect on an organism's fitness is only 3-13% (Firnberg et al., 2014; Jacquier et al., 2013; Lundin et al 2017).

At the protein level, the effect of a mutation “especially SNPs” varies based on its location. SNPs that result in changes to the active site have a severe effect on protein activity, particularly when they affect core amino acids and proline substitution which can disrupt the protein folding (Suckow et al., 1996). SNPs outside the active site can affect the protein activity in several ways: through changes in protein stability or changes in both expression and stability rather than actual function (Soskine and Tawfik, 2010; Tokuriki and Tawfik, 2009).

Mutations that confer a new function may also have negative effects on protein stability, which can affect the binding affinity to the original substrate (Bershtein et al., 2008; Tokuriki and Tawfik, 2009)

A deleterious mutation may become fixed in population with small population size via genetic drift Also, The fixation of a deleterious mutation may occur through coupling with another beneficial mutations (genetic hitchhicking event) (Buskirk et al., 2017), or when the fitness effect is ameliorated by other mutation (compensatory).

Beneficial mutations

In general, beneficial mutations are much less common than deleterious mutations; their proportion varies across species and enzymes, but does not exceed 10% (Firnberg et al., 2014; Lind et al., 2017).

Beneficial mutations are needed to adapt to and survive environmental stress such as exposure to a toxic compound such as antibiotics (Gullberg et al., 2011). Thus, the rate or percentage of beneficial mutations increases when organisms change environment, therefore beneficial mutations are fixed by natural selection. In cases with a high rate of beneficial mutations, there is a competition for fixation, so mutations with the strongest effect will be successfully fixed, a phenomenon called “clonal interference”(Conrad et al., 2011; Visser and Rozen, 2006) (Fig 3).

eutral mutations

Neutral mutations have negligible impact on biological systems. Therefore, these mutations are overlooked by the purifying selection and accumulate in the genome over time. These mutations become fixed in a population by two mechanisms: genetic drift or hitchhiking (Kishimoto et al., 2015) (Fig 3).

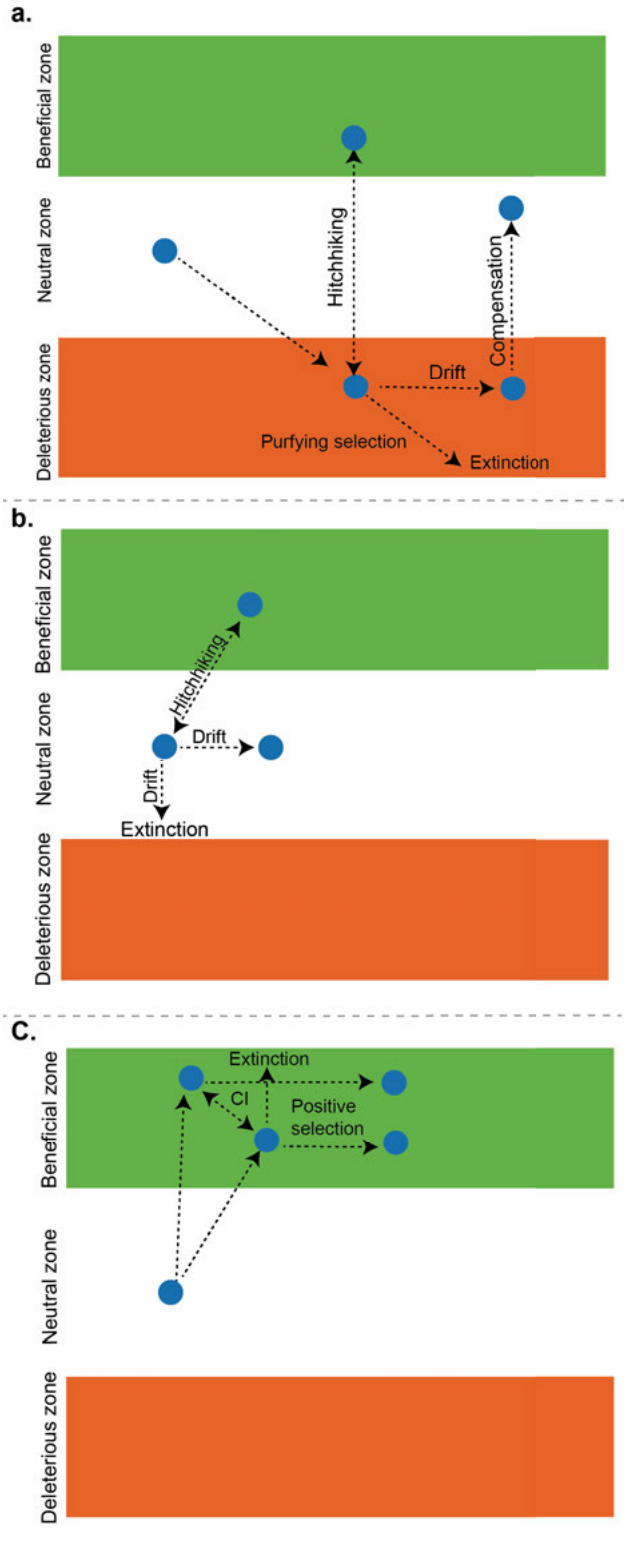

Fig 3: Fate of new mutations. **a.** Deleterious mutations can be eliminated by purifying selection. However, in the case of a small population size it can get fixed by genetic drift. Some times its effect might be restored by compensatory mutation(s), also it may become fixed if it is coupled with other beneficial mutations (hitchhiking) **b.** Neutral mutations can be lost or fixed by genetic drift. Also, genetic hitchhiking can contribute to their fixation. **c.** Beneficial mutations can be positively selected. However, in case of the presence of another beneficial mutation with higher fitness, the one with the lower fitness will be lost, a phenomenon called clonal interference (CI).

A neutral mutation is perceived as one that does not affect protein evolution because it has a negligible impact on protein activity.

However, it can affect the biophysical properties of encoded proteins (Soskine and Tawfik, 2010). Bloom et al., (2007) found that the accumulation of neutral mutations can generate a subtle change in the protein function that are not currently under selection, but that may be selected for under different environmental conditions.

Compensatory mutations

Compensatory mutations are a type of mutation that are positively selected for, due to their ability to completely or partially reduce the negative impacts of other mutations. The effect of a deleterious mutation can be suppressed by a compensatory mutation located somewhere else in the chromosome (intergenic mutations), or in the same gene (intragenic mutations).

The effect of such a mutation is conditional, and results from a particular form of epistatic interaction with the deleterious mutation. This form of epistasis is more frequent than back mutation (reverting to the wild type sequence) (Whitlock and Otto, 1999). Compensatory mutations can be either neutral or deleterious if appearing solely in the population. A deleterious mutation can be compensated by several different compensatory mutations; Poon and Chao (2005) showed that for every deleterious mutation there is an average of 11.8 compensatory mutations. These mutations play a critical role in the adaptive evolution of different pathogens, for example, antibiotic resistance mutations come with a fitness cost (Andersson and Levin, 1999; Andersson and Hughes, 2010). This phenotype could be a useful strategy to escape the antibiotic resistance problem, and reduce its spread by decreasing the use of antibiotics (Nissinen et al., 1996). However, since compensatory mutations are more common than mutations that revert to the wild-type sequence; this may lead to a restored fitness without losing the resistance phenotype. Thus, several studies have suggested that compensatory mutations may play a crucial role in the evolution of drug resistance (Nagaev et al., 2001; Brandis and Hughes, 2013; Knopp and Andersson, 2015).

Since the compensatory effect of intragenic suppressors are dependent on deleterious mutations, they have been assumed to be clustered close to the deleterious mutation (Davis et al., 2009). Nonetheless, some of the intragenic suppressors are located far away from the deleterious mutation they compensate for (Bershtein et al., 2008; Winkler and Bonomo, 2016). Additionally they are able to ameliorate the effect of a wide range of deleterious mutations, these mutations referred to as a global suppressor. Aramli and Teschke (1999) reported global suppressors in the P22 bacteriophage, where a single amino acid substitution in the coat protein was able to improve folding of the temperature sensitive protein by suppressing the effect of 18 different mutations that negatively affected folding. In bacteria, global suppressors

were identified in TEM-1 β-lactamase, and were found in combination with deleterious mutations that changed the substrate specificity of these enzymes towards third generation cephalosporins (Huang and Palzkill, 1997). The impact of global suppressors on protein evolvability and stability was extensively studied in TEM-1 β-lactamase. or example, although Bershtein et al. (2008) subjected TEM-1 β-lactamase to extensive mutagenesis (18 rounds), penicillin hydrolysis of the mutated enzymes was still as the same level as the WT enzyme. The authors identified several global suppressors that compensated for the effect of the destabilizing deleterious mutations and increased their activity toward cephalosporins. While some of the global suppressors did not restore the penicillin hydrolysis function to the wild type level, they still enhanced the evolvability toward hydrolyzing extended spectrum β-lactams (Brown et al., 2010). Thus, global suppressors may play more constructive roles in adaptive evolution than neutral mutations, generating new evolutionary pathways.

Evolution of new genes and functions

The evolution of new genes is considered a major contributor to adaptive evolutionary innovation. Geneticists have started to study the underlying mechanisms of this process long before the recent progresses in genome sequencing. Recent methodological advances have greatly improved our ability to investigate the molecular mechanisms of how evolution of new functions occurs.
Biological evolution may start with (i) a gene that does not have a function (termed *de novo* gene evolution), (ii) gene duplication and divergence, (iii) horizontal gene transfer (HGT) (Fig 4).

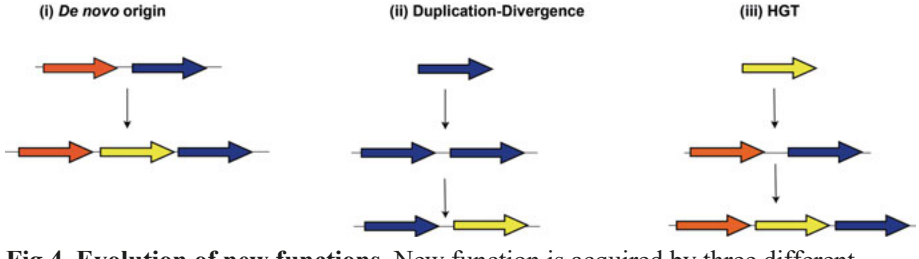

Fig 4. Evolution of new functions. New function is acquired by three different routes **(i)** *De novo* origin, **(ii)** duplication and divergence, **(iii)** HGT.

Historically, the emergence of new functions from *de novo* sequences was less supported (Jacob, 1977). Recently, however, comparative genomics (Levine et al., 2006; Wu and Zhang, 2013) and experimental studies (Brown et al., 2010; Digianantonio and Hecht, 2016; isher et al., 2011; Knopp et al., 2019) have provided evidence for accepting this mechanism. In contrast, the role of gene duplication in evolution of new function has been widely studied for 80

years. Duplication provides a significant reservoir for the emergence of new function from preexisting genes, while HGT is known for transferring ready-made functional genes. Overall, there is no discrete line between these three mechanisms, indicating that the evolution of new functions may involve overlaps between them.

Because the primary focus of our study is to examine the evolution of new functions in pre-existing genes, we discuss the mechanistic explanations for this phenomenon in greater detail.

Functional Promiscuity

Many enzymes have been shown to be able to catalyze reactions other than their main (known) function, and these side-reactions are often referred to as promiscuous activities. Native reactions are catalyzed with high efficiency, while secondary reactions are catalyzed with lower efficiency (Copley, 2003; Khersonsky et al., 2006). This phenomenon has been linked to either flexibility of the active site loop (Zhang et al., 2015), or conformational diversity that allows binding of different substrates (Tokuriki and Tawfik, 2009). There is evidence from several experimental studies showing that overexpression of various genes in *E. coli* can complement the auxotrophic effect of deleting other genes. For example: the *ytjC , gph* or *hisB* genes can rescue the loss of the *serB* gene (Patrick et al., 2007; Matsumura, 2013); and four different *.coli* sugar kinase can rescue a glucokinase mutant (Larion et al., 2007; Miller and Raines, 2005, 2004). The role of promiscuous activity on the evolution of new genes was suggested by Jensen, (1976). Recently, several evolution studies have concluded that functional promiscuity can potentiate evolution of a new function by providing the substrate upon which natural selection can act, and ultimately lead to further functional divergence (Aharoni et al., 2005; Amitai et al., 2007; Näsvall et al., 2012; Wrenbeck et al., 2017) For these reasons, promiscuous enzymes have become the starting point in protein evolution studies, to promote acquisition of new functions from its pre-existing genes.

ene duplication and Divergence

Gene duplication is an event in which one or several genes can have two identical copies that cannot be distinguished from each other. Duplication can also occur at a larger scale by duplicating an entire chromosome or whole genome in eukaryotes (Lynch 2007).

Historically, Haldane (1932) and Muller (1935) suggested gene duplication as a key player on the origination of new functions. Later, Ohno (1970) viewed gene duplication as the primary source of the emergence of new gene

functions and species evolution. Over the last decades, the role of gene duplication in evolution of new function has been refined (Piatigorsky, 1991; Hughes, 1994; Bergthorsson et al., 2007; Näsvall et al., 2012).

Gene duplications have been observed in many different organisms (Dunham et al., 2002; Powles, 2010; Wong et al., 2007). In humans, it is a source of genetic variation, adaptation, and a major cause of diseases (Conrad and Antonarakis, 2007 ;Perry et al., 2007)

Anderson and Roth (1977) estimated that the duplication formation is 10% in S. enterica, which was observed to duplicate certain chromosome locus around 0.005 to 13% within an overnight batch culture without any selection.

The fate of duplicated genes

There are several different suggested models that describe maintenance of duplicated gene copies.

Ohno (1970) suggests that when a successful fixation of duplicated copies in a given population occurs, then the duplicated gene copy will acquire the new beneficial function that increases the organism's fitness, while the other gene will retain the original function. The problem with Ohno's model (known as "Ohno's dilemma") is the assumption that duplications are a neutral event. Therefore, the duplicates can be maintained in the population for long enough to accumulate beneficial mutations that could introduce a new beneficial function (Bergthorsson et al., 2007). However, the birth of duplication may be accompanied by several problems: (i) non-functionalization, in which the gene is inactivated by accumulation of deleterious mutations, subsequently, the duplicated genes will be lost from population as the result of no clear evolutionary advantage (Lynch et al., 2001); (ii) the duplication may become costly to the cell due to the replication of the extra copy(s) of the DNA, or the expression of RNA, and production of protein (Stoebel et al., 2008; Veitia et al., 2008; Wagner, 2005a); (iii) the duplication may also be lost due to a genetic drift which will be hard to overcome in small populations (the rate of loss of duplications in S. enterica is said to be 0.15% per pb per generation (Pettersson et al., 2009)); (iv) duplication could be unstable, especially the tandem repeats which can be lost by recombination (Kugelberg et al., 2006 Bergthorsson et al., 2007) (Fig 5).

Due to all of these above mentioned conditions, the duplication has to be positively selected for in order to be maintained in a given population (Adler et al., 2014; Andersson et al., 2015)

Another suggested model is called sub-functionalization, also known as duplication–degeneration–complementation (DDC). This model suggests that the duplicated genes are functionally redundant, which might allow them to accumulate neutral mutations. Subsequently, divergence is supported by two sequential events, the accumulation of beneficial mutations, and positive selection for the required function. However, the two gene copies tend to

complement each other and share the same function as the original gene (Braun and Liberles, 2003; Force et al 1999) (Fig 5).

A solution to Ohno's dilemma suggests that new function arises as a weak secondary (promiscuous) activity, while the original function of the gene is still maintained. The promiscuous activity is positively selected for due to an environmental change (innovation). In order to increase the level of the selected function, an increase in gene dosage (amplification) is more common than point mutations. The amplification process is then followed by an accumulation of beneficial mutations, which helps improving the selected functions (divergence) (Bergthorsson et al., 2007; Näsvall et al., 2012). Together, this is known as the innovation-amplification-divergence (IAD) model (Fig 6).

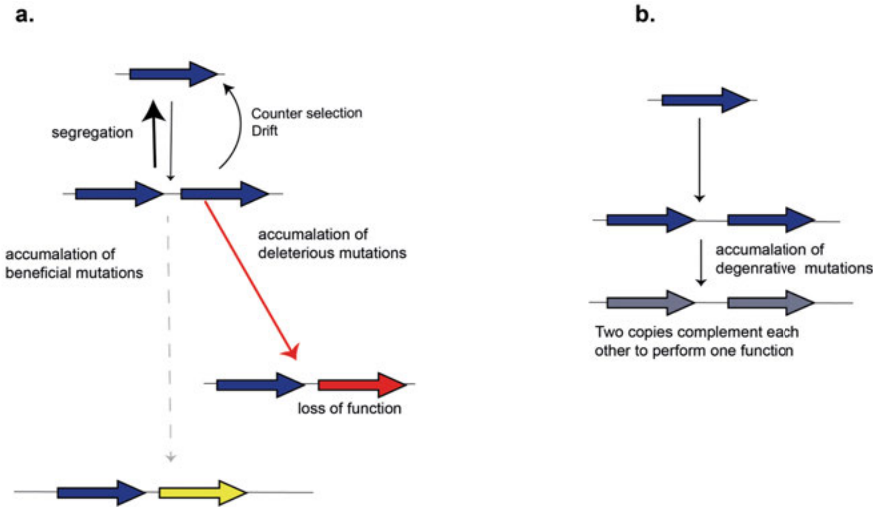

Figure 5: The possible outcome of duplicated genes. **a**. Ohno's model and associated problems. Ohno assumed that duplication is a neutral event, which is neither selected for nor against. Therefore, the duplicated copy is redundant, which accumulates beneficial mutations until it is positively selected for. However, there several factors that might make evolution unlikely, (i) duplications are more likely to accumulate deleterious mutations than a beneficial one, (ii) in a small population it can get lost by a genetic drift, (iii) it can be very costly to the cell, (iv) it can be unstable, which can segregate by recombination. Sup -functionalization model, it also assumes neutrality of duplication. The two copies will complement each other in performing one function.

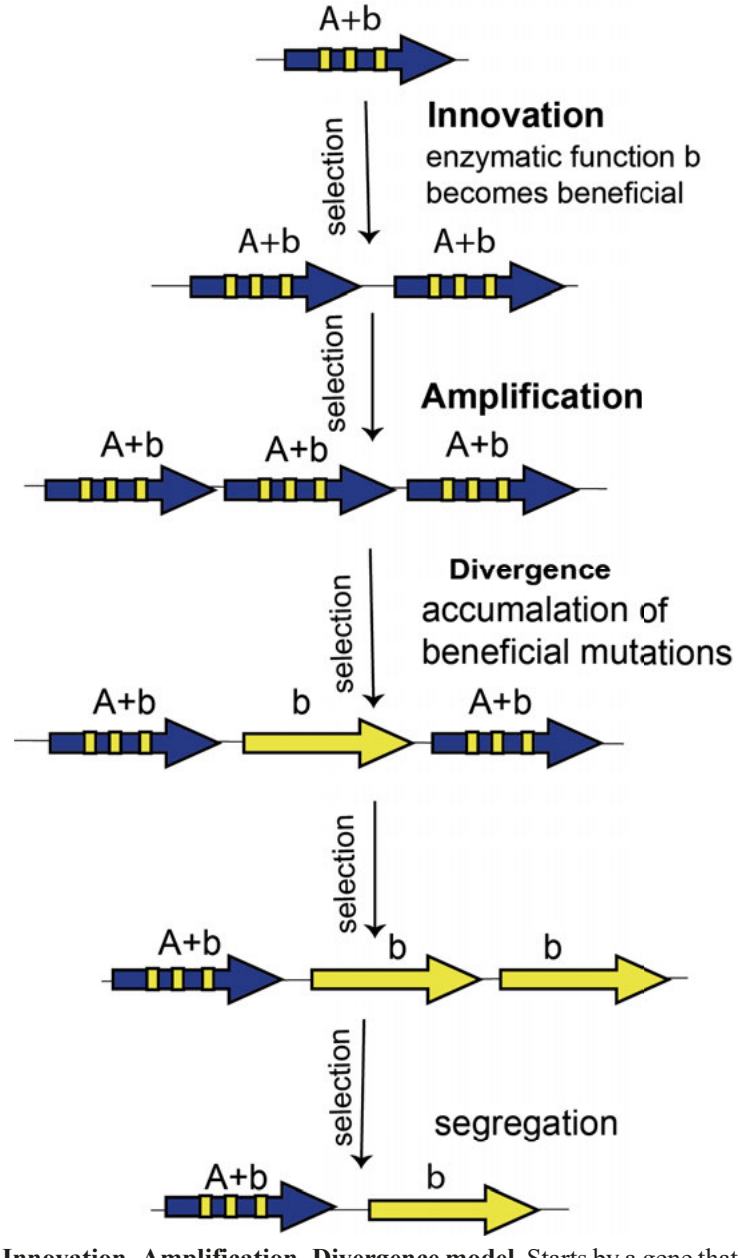

Fig. : Innovation- Amplification -Divergence model. Starts by a gene that has side activities which are not selected for (innovation). Amplification to have more of function b might become a necessity in case of environmental change. This selection can favor further improvement of the same function. Accumulation of beneficial mutations will lead to the divergence of new functions (divergence).

Horizontal gene transfer (HGT)

HGT is the lateral transfer of genetic material between two organisms. In the absence of sexual reproduction in prokaryotes, HGT is considered a major driving force in bacterial genome evolution. Its occurrence is frequent; an estimated 32% of bacterial genes are thought to have been acquired through ancestral HGT events (Koonin et al., 2001)

Bacterial HGT can be mediated *via* three well-studied mechanisms: (i) conjugation, (ii) transformation (the uptake of naked DNA from the surrounding environment), and (iii) transduction (mediated by phage). Successful HGT events are highly dependent on the survival of the transferred gene throughout bacterial generations, in addition to overcoming several immunity constraints in organisms – such as restriction-modification systems and CRISPR (Thomas and Nielsen, 2005)

HGT is considered as one of the most effective ways of diversifying bacterial gene pools, this promotes adaptability to new environments (Baltrus et al., 2008). Nakamura et al. (2004) showed that informational genes involved in the central dogma (replication, transcription, and translation) are less likely to be transferred through HGT events compared to operational genes involved in cell maintenance.

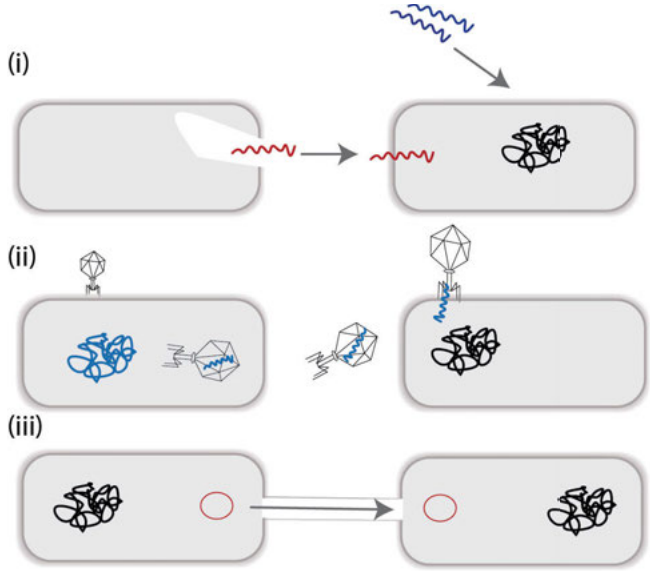

Fig : Mechanisms of horizontal gene transfer: (i)**Transformation**: uptake of a short fragment of naked DNA from the surrounding environment or from a lysed cell; (ii) **Transduction**: transfer of DNA from one bacterium into another *via* bacterio- phages; (iii) **Conjugation**: requires cell to cell contact to transfer DNA *via* a pilus.

Functional trade-offs

To survive in a new environment, it is sometimes necessary to evolve a new function. However, in the process of evolving a new function from a preexisting gene, the original function is often traded-off by the new one (Ferenci, 2016; Soskine and Tawfik, 2010).

As summarized by Ferenci (2016), functional trade-offs are prevalent between many microbial traits that affect important characteristics. Acquiring resistance to phages (Bohannan et al., 2002; Quance and Travisano, 2009) antibiotics (Kurabayashi et al., 2014; Phan and Ferenci, 2013) and several environmental stresses (Maharjan et al., 2013; Zakrzewska et al., 2011) can result in a fitness cost. For example, resistance to rifamycin is usually due to mutations in the β subunit of RNA polymerase (RpoB) (Nitta et al., 1968). These mutations confer resistance to this antibiotic but can severely affect the fitness and other phenotypes due to the importance of RNA polymerase in transcription. These mutations impair metabolism, germination, and sporulation control in B. subtilis (Perkins and Nicholson, 2008; Sonenshein et al., 1974), affect the proper termination of transcription in the attenuator of the tryptophan operon in E.coli (Yanofsky and Horn, 1981), and change the concentrations of siderophores and sulfoglycolipids in M. tuberculosis (Lahiri et al., 2016).

The evolution of new enzymatic functions *via* mutations that change substrate specificity can also be accompanied by trade-offs acting on the original substrates versus the new substrates (Ferenci, 2016; Soskine and Tawfik, 2010) This process is widely discussed in studies in directed protein evolution (Khersonsky et al., 2006; McLoughlin and Copley, 2008). These studies demonstrate that the effects of mutations are context dependent *i.e*. the mutations that improve the new function may have varying impacts on the original function (Fig.8). Some mutations may exhibit a strong trade-off between the new and the original function, *i.e.* emergence of the new function is accompanied by either a significant drop, or complete loss of the original function. or example, a mutation that introduces a new function in Glutamylphosphate reductase (ProA) increased its promiscuous activity by 12-fold, while reduced the original function approximately 2,800-fold (McLoughlin and Copley, 2008). Enzymes that evolve a new function *via* mutational changes that alter the substrate specificity, typically increasing the affinity for alternative substrates or the level of weak promiscuous activities. These changes involve mutational adjustments at the active site, which might change the active site of the protein, making it difficult for the original substrate or ligand to bind (McLoughlin and Copley, 2008; O'Loughlin et al., 2006; Vick et al., 2005; Wei et al., 2005). In these cases, the original function is suggested to be retained by duplication and amplification (Soskine and Tawfik, 2010). However, the sharp reduction of the original function does not

always occur. Several directed evolution experiments have shown that mutations that create new functions improve the promiscuous activity by several folds, while the original functions fall slightly or remain at the same level (Aharoni et al., 2005; Roodveldt and Tawfik, 2005; Rothman and Kirsch, 2003). However, a weak trade-off is not always sustained when improving a new enzymatic function. Tokuriki et al., (2012) showed that the rate of the new function improvements diminishes when further mutations accumulate

Sometimes, mutations that introduce a new enzymatic function in a protein do not all have the same trade-off trajectories. or example, when Lundin et al. (2020) evolved the HisA enzyme towards a new activity (using random mutagenesis), they observed a strong trade-off in most of the trajectories, while fewer mutations showed a weak trade-off. Occasionally, trade-offs are not only between the old and the new functions. This is illustrated in **paper 1** where we evolved a generalist bacterial release factor 2 (RF2) gene, which can terminate at three stop codons. However, we discovered a new trade-off (between termination on stop codons, and mis-termination on sense codon) which has a toxic effect to the cell.

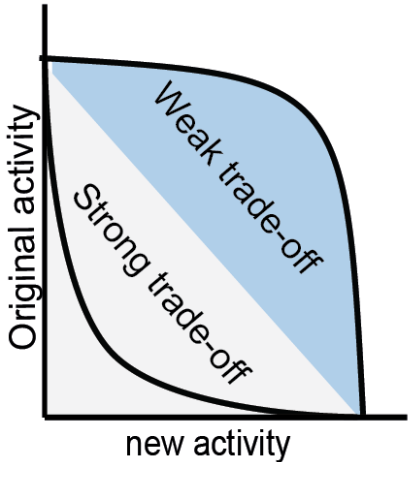

Fig 8: Trade-offs in evolution of new enzymatic functions. A mutation that introduces or improves new enzymatic function may lead to (i) a large loss of the new function, phenomenon known as strong trade-off, or (ii) a small loss in the original function, phenomenon known as weak trade-off.

Evolution of generalists

Generalist organisms have the ability to use a wide range of resources enabling them to survive multiple environments. Kassen (2002) summarized that a fluc tuating environment gives rise to generalists.

Buckling et al., (2007) provided a clear evidence for how generalist organisms emerge. They evolved *P. fluorescence* in alternating environments (poor and rich media) compared with control that evolved in one media. Control populations only improved local fitness, and had up to 20% lower fitness in the other media, while the populations that were cycled between alternating media had better fitness in all conditions.

In directed experimental evolution for proteins, Khersonsky et al., (2006) reviewed several cases in which a generalist intermediate is possible when the weak trade-off trajectory is followed. However, with a strong trade-off it is difficult to maintain the generalist phenotype. This is demonstrated by Lundin et al., (2020), when they tried to maintain bifunctionality of HisA enzyme with random mutagenesis, while using a constant selection pressure. However, in **Paper II** we used constant selection to keep histidine biosynthesis enzyme A (HisA) activity, and fluctuating selection for gaining the new activ ity tryptophan biosynthesis enzyme (TrpF) By using this setup, we evolved enzymes that maintained both functions.

Evolvability

Evolvability is a relatively recent concept that has emerged in evolutionary biology (Brown, 2014). It has carried various definitions in the last 2 years (Houle, 1992; Wagner, 2008, 2005b; Wagner and Altenberg, 1996). The main definition that we use this thesis is "the ability of generating heritable variation in traits, that can shape the outcome of evolution".

hat causes evolvability?

Many human microbial pathogens have evolved to evade the immune system (Reddick and Alto, 2014).With the increasing problem of antibiotic resistance, it is possible that we are facing the end of the efficacy of most available antibiotics (Alanis, 2005)

Therefore, understanding the causes of evolvability is important for predicting these problems before they occur, and for developing strategies to mitigate them.

Phenotypic heterogeneity

Bacterial communities are diverse, and this diversity plays a crucial role in shaping bacterial properties and functions. Communities with isogenic individuals (cells with identical genotypes), that live in the same environment can have subpopulations with phenotypic variation; this is referred to as phenotypic heterogeneity (Avery, 2006).

Phenotypic heterogeneity might provide individuals in the group with ben eficial traits that might not be available in a community with homogenous phenotypes. These variations may rise as small subpopulations, giving them a competitive advantage in fluctuating environments, for example by conferring tolerance to antibiotics (Allison et al., 2011), or stress (Chastanet et al., 2010)

Phenotypic heterogeneity is a phenomenon of "persister cells", where subpopulations of bacteria show a slow or no growth phenotype that allows them to persist in the presence of bactericidal antibiotics. One reason of this variation can be the above-average level of *hipA* gene expression (Intracellular toxin) (Rotem et al., 2010). The antibiotic tolerance subpopulation also showed a more prolonged lag phase, enabling it to stay dormant during antibiotic exposure and resume growth when there is no antibiotic in the media. Adopting this behavior has facilitated the rise of antibiotic resistance. The rise of antibiotic tolerance precedes the antibiotic resistance phenotype; this has been reported in different settings either as the result of intermittent exposure to antibiotics (Fridman et al., 2014) or during a drug combination treatment (Haaber et al., 2015). Levin-Reisman et al. (2017) found that 80% of the sequenced clones of the *ampC* mutation – that confer resistance to ampicillin – is coupled with various mutations that belong to the "tolerome"of *.coli* . These findings indicate that a population with phenotypic heterogeneity has higher evolutionary potential compared to a homogenous population (Fig. 9).

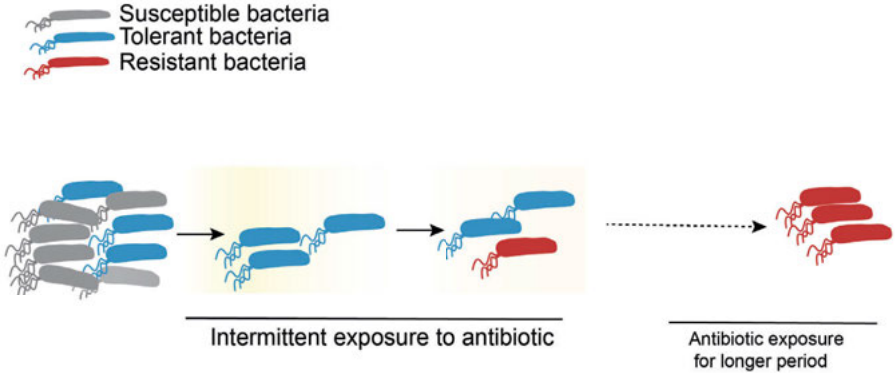

Fig. 9: Phenotypic heterogeneity can increase evolvability. When a certain population change environment, phenotypic heterogeneity may generate a subpopulation of cells that shows a new phenotype, such as tolerant bacteria (blue cell) during the intermittent exposure of antibiotic. This phenotype can be adaptive because it allows a subpopulation to survive this environmental challenge, and can facilitate the accumulation of beneficial mutations that gives rise to a new function (antibiotic resistance, red cell)

utational robustness and cryptic genetic variations

Mutational robustness is the ability of biological systems to accommodate mutations without showing a phenotypic change (de Visser et al., 2003; Wagner, 2008, 2005b).

The difference between genotypes' robustness can influence their evolvability by facilitating the accumulation of genetic variations, which can

eventually lead to a genotype that expresses a novel function (de Visser et al., 2003; McBride et al., 2008; Wagner, 2005b). Robustness also allows for the accumulation of deleterious mutations by masking their effects so they appear as “neutral”. Since the effects of these genetic changes are not pronounced in the system they are known as cryptic genetic variations (Gibson and Dworkin, 2004)

The ways that cryptic genetic variations accumulate in biological systems were revealed by investigating mutations buffering mechanisms. These mechanisms include the expression of protein chaperones like Hsp90, which can prevent the accumulation of misfolding of proteins. These chaperones can also alleviate the effect of deleterious mutations which sometimes introduces new function (Rutherford and Lindquist, 1998) .

When biological systems are exposed to a new selective pressure, cryptic variations do not always remain hidden, they can manifest in the form of a new phenotype (Félix and Wagner, 2008; Hayden et al., 2011). Cryptic genetic variations have a major impact on human health. Several genetic variants can maintain neutral effects but can increase the health risks under certain conditions (Gibson and Dworkin, 2004). These genetic variations can also play a key role in evolvability, by providing a distinct genetic background that permits the accessibility of adaptive mutations (Payne and Wagner, 2014; Zheng et al., 2019). Promiscuous functions in different orthologs can respond in varying ways to mutations that introduce a new function. or example Khanal et al. (2015) showed that a single amino acid change significantly improved the promiscuous activity of the enzyme ProA, but that the size of the improvement varied substantially between orthologs, independently of the magnitude of their inherent promiscuous activity (Fig.10). The main reason for this is considered to be the cryptic variations that accumulate during the divergence of orthologs (Baier et al., 2019.)

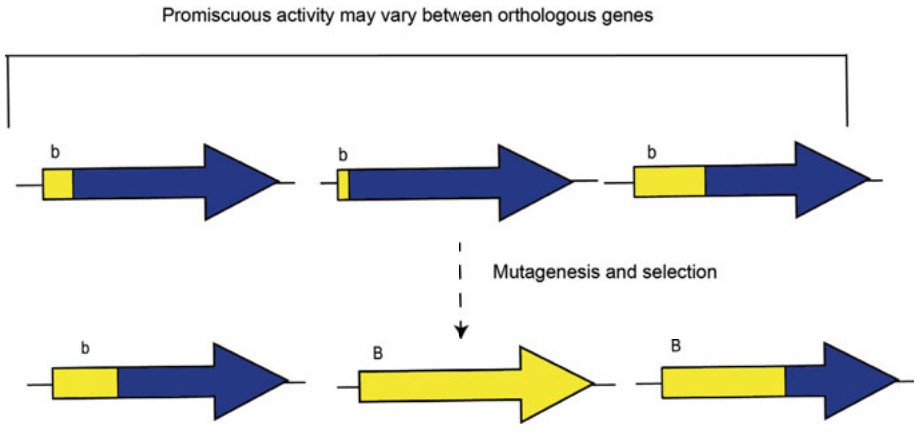

Fig. 10: Evolvability of promiscuous activity in orthologous genes. Orthologs with the lowest level of promiscuous activity may evolve a significantly higher fitness plateau than its counterparts. By contrast, the ortholog with the highest initial promiscuous activity may evolve to a less-optimal fitness level. The level of promiscuous activity is indicated by either lower case "b" for lower level, or upper case "B" for higher level.

rotein evolvability

Recent adaptive evolution experiments at the molecular level have focused on the features that facilitate the ability of proteins to acquire new functions

The focus has been placed on proteins because there are correlations between functional changes in proteins and acquiring certain phenotypes in a population (Bloom et al., 2007; Soskine and Tawfik, 2010; Takano et al., 2013).

Proteins are considered the ideal systems for studying evolvability because of their biophysical properties, and biochemical activities that can be easily changed by a few mutations (Bloom et al., 2006; Salverda et al., 2011; Soskine and Tawfik, 2010). Protein evolvability is defined as the ability of the protein to acquire sequence diversity and conformational flexibility, which can enable it to evolve toward a new function. For example, cytochrome P450 enzymes have been engineered to acquire new substrate specificity and showed catalytic activity towards new substrates with native-like (Fasan et al., 2007), or non-native-like structure (Appel et al., 2001; Sulistyaningdyah et al., 2005)

Understanding protein evolvability will help us understand (i) the long-term evolution of protein's superfamily, (ii) the factors that determine how much a protein function can be altered. The later can be beneficial for the protein engineering field.

Protein robustness

Protein robustness is the one of the main features that can enhance its evolvability. It enables proteins to tolerate the effect of a range of detrimental mutations providing the capacity for wider sequence diversity. Robustness might be due to thermal stability (Bloom et al., 2006; Takano et al., 2013) or as the result of a global stabilizing mutation (Baroni et al., 2004; Mitraki et al., 1991).

To have enough of a protein in its functional folded state, its stability needs to be above a certain threshold. In globular proteins, the residues that are located on the surface are more able to tolerate amino acid substitutions, than those located in the core, or the active site (Sikosek and Chan, 2014). What affects a protein's active site are mostly destabilizing mutations, which can reduce the stability below the required threshold. These mutations negatively affect protein function and organism fitness (Bloom et al., 2006). It has been shown that the trade-off between evolvability and protein stability is inevitable (Tokuriki et al., 2008). Therefore, modulating protein stability to allow fixation of new function destabilizing mutations is crucial for enhancing its evolvability, which in return can open new evolutionary trajectories or pathways (Brown et al., 2010; Winkler and Bonomo, 2016). For example, some archaeal proteins have higher thermal stability compared to bacterial proteins (Takano et al., 2013). Bloom et al., (2006) improved the function of the P450 enzyme and evolved it toward acting on five new substrates. They found that the improved variants have better ability to evolve toward catalyzing the new reactions, and they reasoned that to the higher stability which was acquired by improving its native function.

The threshold model for protein stability was proposed by Tokuriki and Tawfik, (2009) and describes two main mechanisms by which protein stability can be modulated to allow fixation of the mutation that introduces the new function. irst, compensatory mutations that increase protein stability allow the protein to tolerate a larger number of destabilizing mutations that introduce new function (Bershtein et al., 2008). Second, chaperone overexpression normally buffers the destabilizing effect by lowering the stability threshold. In PTE, GroEL/GroES overexpression has allowed a doubling of the number of mutations that accumulated and still have a neutral effect. Therefore, PTE variants with accumulated mutations have shown improved new activity under GroEL/GroES overexpression more so than the wild type, or less expressed GroEL/GroES (Tokuriki and Tawfik, 2009).

odel organisms in our studies

Salmonella enterica subsp. *enterica* serovar Typhimurium, is used as model organism in all of the projects included in this thesis. In **paper I** an *in vitro* translation system derived from *E. coli* K- 12 was used for

in *vitro* translation termination assays. *S. enterica* is a rod-shaped gram-negative aerobic enterobacterium that causes gastroenteritis in humans. The strains used in these studies are derived from the avirulent 2 strain, which has a defective *rpoS* (Swords et al., 1997)

It has 4600 genes, and the complete genome sequence was published in 2001(McClelland et al., 2001).We used it as the model system because it is a well-studied organism that has been used extensively in studies of bacterial genetics and experimental evolution due to: (i) being easy to culture in a wide range of laboratory media (ii) having short generation times, (iii) the availability of a wide range of molecular genetics tools, such as λ Red recombineering for homologous recombination and very efficient generalized transduction using bacteriophage.

Present investigations

per I

Collateral toxicity limits the evolution of bacterial release factor 2 toward total omnipotence

The hydrolysis of the nascent polypeptide-tRNA complex in the ribosomal A site is mediated by class 1 peptide release factors, which exist in all three domains of life. In bacteria, RF1 (encoded by the *prfA* gene) recognizes UAA and UAG stop codons, while RF2 (encoded by the *prfB* gene) recognizes UAA and UGA. Recently, it has been reported that the *prfA* gene is not essential in *E. coli*; and that previous reports that indicated the contrary were based on using *E. coli* K12, which has a defective RF2. Furthermore, even in *E. coli* K12, replacing UAG stop codons in six essential genes, or introducing the wild-type *prfB* (by reverting a single amino acid substitution) makes RF1 non-essential (Johnson et al., 2012; Mukai et al., 2010; Uno et al., 1996)

In this project, we investigated how the loss of RF1 in *S. enterica* is compensated. We found that the deletion of *prfA* in *S. enterica* is lethal unless *prfB* is duplicated. We deleted the *prfA* gene in two *S. enterica* strains, that contained duplications of the wild-type *prfB* (RF2[wt]), or a (E167K) mutant (RF2[E167K]) with a single amino acid substitution known to allow termination on UAG. We evolved these RF1 deficient *S. enterica* strains RF2[wt]) for 500 generations, and 1000 generations for (RF2[E167K]) in order to study how the bacteria could compensate for such an important function. Using several in vivo and in vitro assays we investigated how the evolved RF2 replaced the missing RF1.

While the ancestors had reduced growth rates (80% reduction for RF2[wt] and 40% reduction for RF2[E167K]), the growth rates of the evolved populations were significantly improved, in some cases close to the wild type *S. enterica* strain. We performed whole-genome sequencing (WGS) to compare between the ancestral and evolved lineages. The copy number of the ancestral clones in RF2[wt] was between 17-44 copies, while the RF2[E167K] did not exceed two copies. The number of copies of *prfB* gene at the end point has decreased in RF2[wt]. Several mutations were also accumulated in either RF1 or RF2 and other translational machinery.

We reconstructed the evolved RF2 variants in different genetic backgrounds in order to determine how they compensated for the effect of *prfA*

deletion. To effectively express RF2 at higher levels, the different evolved RF2 with FsFix mutation (which removes the regulatory frameshift site) variants were placed under P_{araBAD}, while RF2[wt] in its native locus, then we tried to introduce a *prfA* deletion. Some *prfB* variants (with two SNPs) in *prfA* deleted strains showed a higher growth rate compared with the variants with one SNP. We also measured the ability of the evolved RF2 variants to read through UAG stop codons using an in vivo reporter gene assay, and an in vitro translation termination assay.

Using in vitro assays, we measured the ability of the evolved variants to perform their original function (termination on UAA and UGA). The assays did not show any large loss (80% of the WT level for the poorest variants) and most of them released the peptide with the same efficiency as RF2[wt].

A noticeable toxicity was observed when some of the evolved RF2 variants were fully induced from ParaBAD promoter by 0.05% L-arabinose (in strains with intact prfA). The overexpression of these RF2 variants either caused poor growth (RF2 with one-point mutation), or were lethal (RF2 with two point mutations). We speculated that the toxicity could stem from mis termination on near-cognate UGG (tryptophan) codons. We developed an in vivo reporter gene assay to test this hypothesis. Our results indicate that the toxic RF2 variants indeed did terminate on UGG codons, and that there was a correlation between the level of toxicity and the level of mis-termination on UGG.

The evolution of release factor 2 has adopted the Innovation -amplification-divergence model. However, there were no significant trade-off between the original and the new function, which normally poses a barrier toward functional novelty which can be solved by gene amplification. The primary barrier is the toxicity that resulted from termination on sense codon and this phenomenon is difficult to be overcome by the amplification.

per II

Intermit ent selection directs evolution of a specialist enzyme to become a generalist

Several experimental studies have shown that the evolution of the new function often comes at the cost of the loss of the old function.

This project is based on the well-studied enzyme HisA in *S. enterica.* The HisA enzyme (ProFAR isomerase), plays a major role in synthesizing histidine (original activity; Henn-Sax et al., 2002). This enzyme can gain the ability to contribute in tryptophan biosynthesis (new activity, usually catalyzed by TrpF enzyme (PRA isomerase) (Jürgens et al., 2000; Näsvall et al., 2012; Newton et al., 2017). In our previous studies, which investigated the evolutionary routes of evolving *hisA* toward TrpF activity, most of the trajectories followed strong trade-off paths, where the gain in TrpF activity resulted in

rapid decreases in HisA activity. These results indicated that the evolution of a generalist enzyme with high efficiency in both activities may be unlikely. However, there are HisA orthologs that have evolved in nature to catalyze both reactions.

In this study, we investigate fluctuating selection pressures that could force evolution along rare "weak trade-off paths" and result in evolution of a generalist enzyme. We deleted the trpF gene in an S. enterica strain (with wild-type hisA in its native locus) and allowed it to evolve in the presence of a very low concentration of tryptophan but with no added histidine. This experimental setup allowed the bacterial populations to grow for several generations before tryptophan was depleted from the environment after a few hours. In this first phase of each cycle, there was no selection for TrpF activity but strong selection against the loss of HisA activity. By incubating the cultures for seven days (more than six days after tryptophan was depleted) before passaging into fresh media, each cycle had a prolonged phase with selection for gaining TrpF activity (while maintaining HisA activity). This process was conducted in every cycle. We then did WGS to the end point populations after 500 generations.

Five out of eight populations showed loss of function in one of the tryptophan biosynthesis genes trpD or trpE. The possible explanation of a beneficial effect from disrupting the function of these genes is that it prevents the toxic accumulation of phosporibosylanthranilate (the substrate of TrpF), or to avoid the metabolic cost of unnecessary flux through the first two steps in the broken tryptophan biosynthetic pathway. One of the populations had five mutations in the hisA gene. This population also had many other mutations, including a frameshift mutation in mutH, that probably caused a high general mutation rate.

To see if there were other possible mutational paths to generalist enzymes, we set up a larger evolution experiment with 24 populations using the same experimental set-up but with the mutS gene inactivated to increase the mutation rate. After 130 generations we screened the populations for cells able to grow without added tryptophan or histidine. We sequenced the hisA gene to identify mutations that introduced a generalist phenotype. Most of the lineages (22) had accumulated mutations in hisA. All of these lineages shared the same mutation (Q18R), and some had acquired additional mutations. This suggests that Q18R is the first mutation that is necessary to have TrpF activity without showing a dramatic loss in the original function of HisA. We confirmed this by measuring the strain ability to grow in the absence of histidine when only tryptophan is added to the media. As expected, the hisA(Q18R) growth rate was 20% less than the hisA (WT) growth rate. When additional mutations accumulated, the level of the original activity remained at the same level of the hisA (Q18R) variants, while the new function (TrpF) increased by 10% to 20%. This pattern was also observed when we cycled the isolated clones from the previous evolution experiment for improving the generalists HisA

enzyme. These clones gained one to two additional mutations on the hisA gene. These mutations increased activity, and the clones grew at nearly 50% of the WT's growth rate without added histidine or tryptophan.

Our results indicate that a fluctuating environment can direct evolution through rare mutational paths that show different trade-offs between the original and new functions compared with evolution in stable environments.

per III

Evolvability of orthologous genes

Orthologous genes are homologous genes in different species that diverged from a common ancestor. Thus, they retain the same function and other vital properties. The divergence of orthologs is assumed to be driven by the steady and slow accumulation of neutral mutations. Contemporary natural orthologs differ in their sequence and physical properties, such as stability, which influences their ability to accommodate mutations and evolve a new function. However, the understanding of how these differences affect evolvability is limited.

In this study we generated a set of artificial laboratory orthologs from a common ancestor (Salmonella enterica histidine biosynthesis enzyme HisA). We generated these orthologs by applying the same concept that transpires in nature when the natural orthologs diverge. We accelerated the process by using random mutagenesis to generate mutations (single nucleotide polymorphisms in each step). The wild-type (WT) HisA from S. enterica was subjected to alternating rounds of relaxed purifying selection by mutagenesis, and screens for partial loss of activity, followed by mutagenesis and selection for restored activity. By doing this the effect of each mutation will be known. We chose orthologs with non-synonymous SNPs for testing their ability to evolve towards a new function The level of HisA activity in the orthologs with compensatory mutations is 75-100%, while the one with the deleterious mutations is 40-85%. Most of the generated orthologs had unique mutational changes, however, some mutations appeared in two different lineages but exerted opposite effects.

We performed fluctuation tests to investigate the ability of the orthologs to acquire a new function (TrpF). We compared their evolutionary potential by determining mutation frequencies, characterizing the mutational changes that gave rise to TrpF activity, and measuring the level of the TrpF activity. The ability of hisA (WT) in S.enterica to gain spontaneous mutations that give a TrpF activity is very low. Therefore, we inactivated mutS gene in each background, by doing this we increased mutation rate by 100-fold.

Some orthologs with compensatory mutations had 2-fold higher mutation frequency compared to the hisA (WT), while the majority of the orthologs

with higher hisA activity did not show a significant difference in their mutation's frequencies compared to the hisA (WT). Fewer orthologs (2) with compensatory mutations have a significantly lower mutation frequency.

Most of the orthologs with deleterious mutations showed a significant decrease in the mutation frequencies (approximately 10-100-fold lower compared to the hisA (WT).

To investigate the mutations that caused TrpF activity in every tested ortholog, we sequenced hisA in the clones that had TrpF activity. Most of the sequenced clones shared the same mutations, while some variants had unique mutations that were not found in experiments with other orthologs including the common ancestor hisA (WT).

We also measured TrpF activity in all orthologs that have same shared mutations that give TrpF activity. When the mutations occurred in an ortholog with good HisA activity (i.e. the last mutation was a compensating one) they showed TrpF function that was similar to, or better than if the same mutation occurred in the WT. When they occurred in orthologs with poor HisA activity (i.e. the last mutation was a deleterious one) they always showed poorer TrpF function than if the same mutation occurred in the hisA (WT).

We re-constructed ten of the compensating mutations by themselves (without any deleterious mutations) – to observe the effect they have by themselves, both on HisA activity and on mutation frequency. While some mutations did not reduce the HisA activity (stayed at the same level of HisA (WT), others have reduced the growth rate between (3-20%).

Future perspectives

per II

In this project, we selected for a generalist HisA enzyme that could participate in both biosynthetic pathways. The resulting *hisA* variants from various evolution experiments acquired TrpF activity without showing a severe reduction in the original function. We tested the conditions for selecting generalist *hisA* enzymes in a small pilot experiment, which showed the accumulation of 5 mutations in the *hisA* gene, but we do not know the order in which these mutations appeared. Therefore, we must trace back this evolution experiment and determine the order of the *hisA* mutations. We will do this by plating on minimal media in the absence of both amino acids to detect the variant that has a generalist phenotype; then sequence the *hisA* gene from these variants. After that, every single mutation can be reconstructed to test the level of activity of both functions.

For the larger evolution experiment, we completed local sequencing of *hisA* in the isolated clones to determine the stepping-stone mutation that provided the initial TrpF activity, and to discover which additional mutations were selected for to improve TrpF activity. We determined the level of original activity loss in the strain where *hisA* exists in its native operon. In this context, it is difficult to measure TrpF activity because histidine must be added to the media, which contributes to reducing the expression of the *hisA* gene; this may affect the accuracy of the measured TrpF activity provided by this gene. Therefore, these variants had to be moved from the native location, for example by placing them under P_{araBAD} control (as in paper I and paper III). This allowed for measuring growth in the absence of tryptophan and the presence of histidine.

per III

In this project we examined the evolutionary potential of different *hisA* orthologs that we generated in the lab from a common ancestor (*hisA* of *S. enterica*).

We performed fluctuation assays to compare their ability to acquire a new function; our comparison was focused between the generated orthologs and the WT which was the common ancestor for the all diverged lineages.

However, in several experiments we did not include all orthologs within the same lineage, and in other experiments the WT was omitted. Therefore, the experiments did not always allow direct comparison between an ortholog and its immediate ancestor, or between an ortholog and the WT. Therefore, it will be necessary to repeat some of the mutation frequency experiments. By doing this, we will be able to determine the difference in evolvability between every ortholog, and compare them with both their immediate ancestor and the common ancestor (WT HisA).

We also noted that the evolutionary potential of some orthologs is higher than that of the common ancestor. It would be useful to investigate whether this stems from the effect of single compensating mutations, or from the specific mutation combinations in these orthologs, particularly in the higher activity orthologs that resulted from compensatory and deleterious mutations. To this effect, we re-constructed ten of the compensating mutations by themselves (without any deleterious mutations). We will perform fluctuation assays to test their evolvability, and compare it to the relevant orthologs with a combination of deleterious and compensatory mutations.

Concluding remarks

This work addresses fundamental questions in evolutionary biology, primarily regarding how new functions emerge from an existing function and which molecular mechanisms are involved in this process. We have addressed specific questions in the three projects included in this thesis:

1. What are the constraints in evolving omnipotent Bacterial Release Factor 2?
2. Can intermittent selection mitigate the strong trade-off constraints to evolving a generalist enzyme?
3. How does neutral sequence divergence affect evolvability? *i.e.*, which factors influence the difference in evolutionary potential between different orthologs?

To answer these questions, we have used a combination of experimental evolution and bacterial genetic tools.

In Paper I, we showed that RF2 duplication and amplification is a direct response for rescuing the loss of RF1, and that the accumulation of beneficial mutations on RF2 increased the efficiency of terminating on UAG stop codon. However, the ability of evolved RF2 to terminate on sense codon UGG also increased, which had a toxic impact on the cell. Our results indicate that evolving omnipotent RF2 might be blocked by the unwanted toxic new activity that seems difficult to avoid. This type of trade-off might be overlooked when the evolution of a new function is discussed; we suggest that it may be an evolutionary constraint that prevents the evolution of efficient generalists.

In our previous experiments on the HisA/TrpF model system, we tried to elucidate the mutational paths toward a new activity. We found that the ma jority of the evolutionary routes follow a strong trade-off trajectory. These results suggest that the evolution of generalist enzymes from a specialist may be quite rare. **In paper II**, we tried to perform evolution experiments by maintaining constant selection for the original activity, while exposing the new activity to intermittent selection. This selection scheme enabled us to evolve a generalist enzyme. Our results demonstrate that fluctuating selection may lead to evolution along a path of rare mutations that result in a generalist enzyme. A fluctuating environment is more likely to occur in nature; therefore, it is more likely to evolve generalist enzymes that can survive such conditions.

In paper III, we compared the evolvability of deleterious and compensating mutations in laboratory orthologs. We used a forward approach to

generate these orthologs by introducing single mutations; the effect of every deleterious mutation was restored by a compensatory mutation. This approach is likely to happen during the neutral sequence divergence, because when deleterious mutations become fixed in a population due to genetic drift, the effect of the deleterious mutation can be restored quickly by a compensatory mutation. Our preliminary results indicate that compensatory mutations are more likely to restore evolvability, while deleterious mutations reduce it.

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