

**EXPERIMENTAL EVOLUTION IN *PSEUDOMONAS*
FLUORESCENS CELL DIVISION AND PROTEIN
TRANSLATION**

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ABSTRACT

The evolution of organisms over time is caused by the interaction between selective pressure, genetic drift, gene flow, and available mutations. This interaction creates what is often referred to as fitness landscape: the fitness of an organism changes depending on new mutations, which in turn will change the effect on fitness caused by the other mutations that can arise next. When facing a process of natural selection caused by selective pressure, different organisms will have different options available to overcome it, depending on the different available adaptive mutations. What options and mutations will be adopted and selected depends on how easily and readily achievable they are. This is hugely dependent on the genome of the organism and which options require the minimum possible mutations to be realized. Selection upon these *de novo* mutations will in turn further drive the organism along the fitness landscape, opening up new options and losing others.

As presented in the first introductory chapter, the process resulting in adaptation by selective pressure on a sequence of mutations can be studied by experimental evolution. By forcing an organism to overcome an evolutionary challenge that decreases its fitness, it is possible to favor a process of natural selection that allows the observer to find the range of adaptations that are likely to be favored. Identification of the mutations that are selected in this way in turn allows further studies to analyze different things: what advantage they provide, how they alter the phenotype and genotype of the organism, and how useful they are to explain and predict adaption happening in the natural world. It can also reveal previously unknown connections between biological functions.

In this work I applied experimental evolution in the model bacterium *Pseudomonas fluorescens* SBW25 to study two processes that are both shared and essential to all cell-based life forms on Earth: cell growth, and protein synthesis. In both cases I proceeded by first deleting non-essential genes central to the process, then by measuring the phenotypic effect caused by the gene deletions, then by allowing natural selection to find *de novo* mutations that could recover a deleterious phenotype, and in the end by identification and analysis of the *de novo* mutations. This in turn allowed formulation of a testable hypothesis about how the roles of different genes are connected.

In the second chapter I study how the loss of the gene *rodA* affects cell wall synthesis. Disrupting the process by deletion of this gene enables understanding of how the protein RodA specifically produces a distinctive rod-shape phenotype in *P. fluorescens*. Such disruption also demonstrates how the decrease

in fitness directly caused by the loss of *rodA* is due to alterations of the spatial structure of the cell, on which evolution relied as a framework to regulate the activity of other genes involved in cell division, despite these genes not being directly connected to the *rodA* pathway.

In the third chapter I switch the focus from cell growth and division to ribosome biology. The deletion of five different ribosomal proteins, both universally shared or specific to bacteria, allowed quantification of the consequent decrease in translation rate. This decrease in translation comes with a reduced fitness, that is then partially or completely overcome by *de novo* mutations. As in the previous chapter, the compensatory mutations allowed construction of a picture that connects separate biological processes, in this case translation and transcription, at the level of the paths available to reach adaptation.

In the fourth chapter I dive deeper in the recovery of fitness after ribosome disruption to better explain how the compensatory mutations identified can provide a fitness advantage. The results, while still not completely understood in every detail, expands our knowledge of how the reciprocal regulation of translation and transcription is deeply affected by the environment and is tied to the metabolic requirements of the cell.

ABSTRAKT

Die Evolution von Organismen im Verlauf der Zeit beruht auf der Interaktion zwischen Selektionsdruck, genetischem Drift, Genfluss und verfügbaren Mutationen. Diese Interaktion bringt die sogenannte Fitnesslandschaft hervor: Die Fitness eines Organismus verändert sich abhängig vom Erwerb neuer Mutationen, wodurch wiederum die Fitness, durch weitere, nachfolgende Mutationen, beeinflusst wird. Wenn unterschiedliche Organismen mit dem, durch Selektionsdruck verursachten, Prozess der natürlichen Selektion konfrontiert werden, haben sie, abhängig der zur Verfügung stehenden adaptiven Mutationen, verschiedene Möglichkeiten, diesen zu überwinden. Welche Optionen und Mutationen übernommen und selektiert werden, hängt davon ab, wie leicht und schnell diese realisierbar sind. Diese sind sowohl maßgeblich abhängig vom Genom des Organismus, als auch davon, welche Optionen die geringstmöglichen Mutationen erfordern. Selektion auf Basis dieser De-Novo Mutationen treibt den Organismus wiederum weiter entlang der Fitnesslandschaft, und eröffnet neue Optionen oder sorgt dafür, dass andere verloren gehen.

Wie im Einführungskapitel dargestellt, kann der Prozess der Anpassung durch Selektionsdruck auf eine Abfolge von Mutationen mit Hilfe von experimenteller Evolution untersucht werden. Indem ein Organismus gezwungen wird, eine evolutionäre Herausforderung zu überwinden, die seine Fitness reduziert, kann ein Prozess der natürlichen Selektion gefördert werden, der es dem Beobachter erlaubt, eine Bandbreite an wahrscheinlich bevorzugten Anpassungen zu identifizieren. Die Identifizierung der auf diese Weise selektierten Mutationen ermöglicht wiederum die Analyse unterschiedlicher Aspekte: Welchen Vorteil bieten diese Mutationen, wie verändern sie Phänotyp und Genotyp des Organismus und wie nützlich sind sie für die Erklärung und Vorhersage von Anpassungen an die Natur? Auch bisher unbekannte Zusammenhänge zwischen biologischen Funktionen können so aufgedeckt werden.

In dieser Arbeit habe ich experimentelle Evolution im Modellbakterium *Pseudomonas fluorescens* SBW25 angewendet, um zwei Prozesse zu untersuchen, die für alle zellbasierten Lebensformen auf der Erde essenziell sind und die alle von ihnen gemeinsam haben: Zellwachstum und Proteinsynthese. Um beide Prozesse zu untersuchen, habe ich zunächst nicht-essentielle Gene, die für den jeweiligen Prozess von zentraler Bedeutung sind, deletiert und den Effekt der Gen-Deletion auf den Phänotypen gemessen, anschließend der natürlichen Selektion erlaubt, De-Novo Mutationen zu finden, die den beeinträchtigten Phänotypen wieder regenerieren konnten und schlussendlich die De-Novo Mutationen identifiziert und analysiert. Diese Vorgehensweise ermöglichte mir das Aufstellen einer überprüfbaren Hypothese darüber, wie die Rollen unterschiedlicher Gene miteinander verknüpft sein könnten.

Im zweiten Kapitel habe ich untersucht, wie sich der Verlust des Gens *rodA* auf die Zellwandsynthese auswirkt. Das Unterbrechen dieses Prozesses durch Deletion des Gens ermöglicht es zu verstehen, wie das Protein RodA den spezifischen und charakteristischen stäbchenförmigen Phänotyp in *P. fluorescens* erzeugt. Die Deletion von *rodA* zeigt auch, wie der daraus resultierende Fitnessverlust auf Veränderungen der räumlichen Struktur der Zelle zurückzuführen ist, auf die sich die Evolution als Rahmen stützt, um die Aktivität anderer an der Zellteilung beteiligter Gene zu regulieren, auch wenn diese Gene nicht direkt mit dem *rodA*-Signalweg verbunden sind.

Im dritten Kapitel habe ich den Fokus von Zellwachstum und -teilung auf die Ribosomen-Biologie verlegt. Die Deletion von fünf verschiedenen ribosomalen Proteinen, die entweder universell oder Bakterien-spezifisch sind, erlaubte mir die Quantifizierung der daraus resultierenden Abnahme der Translationsrate. Diese Abnahme der Translation ging mit einer reduzierten Fitness einher, die anschließend teilweise oder ganz durch De-Novo-Mutationen überwunden werden konnte. Wie im vorherigen Kapitel ermöglichten die gefundenen kompensatorischen Mutationen die Konstruktion eines Bildes, das getrennte biologische Prozesse, in diesem Fall Translation und Transkription, auf der Ebene der verfügbaren Wege zur Anpassung miteinander verknüpft.

Im vierten Kapitel gehe ich tiefer auf die Wiederherstellung der Fitness nach einer Ribosomen-Störung ein, um besser erklären zu können, wie die identifizierten kompensatorischen Mutationen zu einem Fitnessvorteil führen können. Die Resultate erweitern unser Wissen darüber, wie die wechselseitige Regulierung von Translation und Transkription stark von der Umwelt beeinflusst und an die metabolischen Anforderungen der Zelle gebunden ist.

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CHAPTER 1 - INTRODUCTION

1.1 Understanding biological systems via evolution

Evolutionary cell biology is the study of cellular mechanisms using experimental evolution as an investigation tool. It relies on observing how a population changes while adapting, revealing how evolution generates novel genotypes and phenotypes to increase population fitness. Since even before the discovery of the DNA molecule as source of the genetic information, experimental evolution helped researchers to understand both how adaptation happens and the mechanisms that propel evolution, for example, during *Escherichia coli* evolution of bacteriophage resistance (Avery et al 1944, Luria & Delbrück 1943).

Through evolutionary processes, populations can adapt and develop new phenotypes. Several factors influence which genetic novelties arise and are selected, including population size, horizontal gene transfer, genetic background, predation pressure, and the size and number of available ecological niches. Each of these factors shapes how populations evolve in response to specific challenges, ultimately affecting their overall fitness (Haldane 1927, Kimura 1992, Murray 2020). Notably, fitness is environment-specific; thus, de novo mutations that are beneficial in one context may not be advantageous in another. Conversely, mutations can also unexpectedly increase fitness in alternative environments. This complexity gives rise to the concept of a fitness landscape, where species tend to evolve toward local fitness optima. Genetic mutations that alter phenotypes move the population across this landscape, with the direction of beneficial mutations depending on both genotype and phenotype (Murray 2020, Wright 1932). In addition to the direct impact of mutations on fitness, other evolutionary forces such as epistasis, genetic hitchhiking, and genetic drift also play crucial roles (Van den Bergh et al 2018).

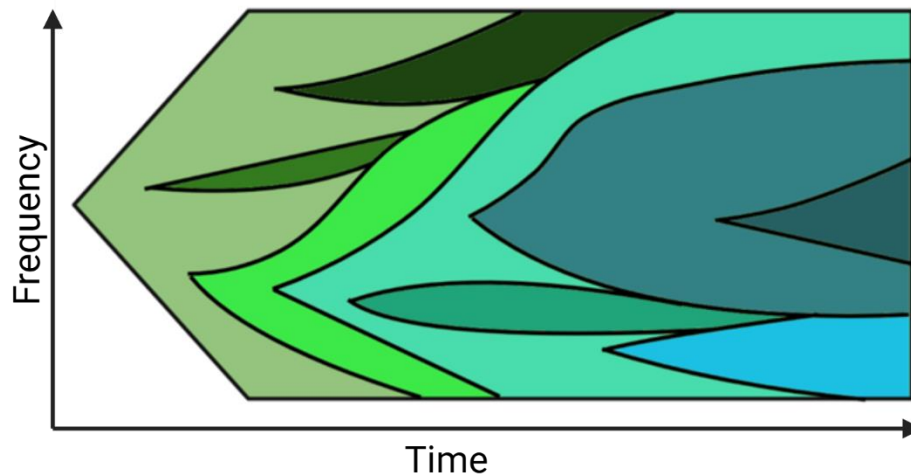


Figure 1: Evolution of multiple lineages from an ancestral clone in a large population. Over time, mutations arise and increase in frequency (depicted by different colors) with secondary mutations giving rise to additional changes in frequencies. Image produced using Biorender.

There are several approaches to studying evolution. One approach involves observing natural populations, both extant and extinct, to infer how they have changed over time (Grant & Grant, 2014; Green et al, 2016; Holmes et al, 2021). These studies, depending on the available data and timescale, can reveal which factors most strongly shape a population's evolutionary history. A second approach involves theoretical and mathematical modeling. Since the early days of the field, modeling has helped researchers understand and predict the outcomes of natural selection (Fisher, 1918). With the increasing availability of genomic data and detailed knowledge of genetic variation, these models are becoming increasingly accurate and informative (Cavalli-Sforza & Feldman, 2003).

A third method is experimental evolution, which involves monitoring populations over many generations under controlled conditions. This approach enables the direct observation of evolutionary dynamics and has gained prominence in the past two decades (Cohan & Hoffmann 1986, Dykhuizen & Hartl 1983, Lenski et al 1991, Levin et al 1977). Typically, a population of a chosen species is evolved under controlled environmental parameters, while changes in genotype and phenotype are tracked over time (**Figure 1**). Experimental variables such as genetic background, population structure, and environmental conditions can be systematically manipulated to investigate specific hypotheses (Van den Bergh et al, 2018; Remigi et al, 2019).

Controlling experimental parameters opens a wide range of research opportunities. Populations can be observed with minimal interference to assess the repeatability of evolution from identical starting conditions (Lenski et al, 1991). Experimental evolution can also be used to validate theoretical models, or conversely, to generate empirical data that inspire new models (Black et al, 2020; Jones et al, 2008). Researchers can artificially manipulate fitness to direct populations toward specific phenotypic outcomes, revealing the evolutionary pathways that lead to those outcomes (Barnett et al, 2025; Chen & Arnold, 1993). Another powerful approach, explored in this work, involves introducing a genetic perturbation and observing how the population can be rescued by further mutations. This can yield insights into the genotype-phenotype map and the preferential paths taken during adaptation. It can also uncover unexpected interactions between cellular pathways and regulatory networks (LaBar et al, 2020).

While conceptually similar to traditional genetic studies, such as identifying antibiotic- or phage-resistant mutants, experimental evolution allows for the observation of mutation sequences over time and how combinations of mutations contribute to fitness. It also highlights the different ways in which genetic variation can be produced in an organism, such as by SNPs, copy number variations, hypermutator genes, contingency loci (LaBar et al, 2020, Oliver et al 2000, Silverman et al 1979). It is also possible to learn about the historical evolutionary path of a species, although with some limitations: experimental evolutionary rescue studies rely on introducing a disadvantageous perturbation that would likely lead to extinction of the lineage in nature. As a consequence, not all the evolutionary paths observed in an experimental study can be expected to be directly found in natural environment. Despite this, genetic drift and naturally occurring population bottlenecks can still promote the survival of population with deleterious mutations analogous to those used in the experiments. Specific environment can also promote the survival of normally disadvantaged mutants (LaBar et al 2020, Rainey & Travisano 1998).

1.2 Evolutionary cell biology

Studies of evolutionary biology applied to questions about cellular mechanisms are in recent years coalescing into the field of evolutionary cell biology. Aim of it is recognizing that evolution is ultimately driven by modifications at the cellular level, and that cellular mechanisms are determined via an evolutionary process since the origin of cellular life (Doolittle 1980, Lynch et al 2014, Martin et al 2008).

A central aim of evolutionary cell biology is to understand the origins of cellular structures and functions, and the evolutionary forces that led to their presence in different species. Due to the stochastic and contingent nature of evolution, the features of extant organisms reflect both adaptive pressures and

historical events. By integrating cell biology with evolutionary theory, researchers can distinguish whether a particular cellular phenotype is an adaptive trait or a vestigial, inherited feature.

Comparative analysis of cellular structures and functions across species allows for hypotheses about the evolutionary pressures that shaped them. For instance, homologous genes can be mapped to phenotypes or environmental contexts across species, suggesting convergent evolution toward specific phenotypes in similar environments. The evolution of the cell wall provides one such example: cell shape is an adaptive trait that varies with environmental conditions such as nutrient availability and is often driven by parallel loss of the same genes across unrelated prokaryotic lineages. Similarly, ribosome complexity is influenced by the number of ribosomal protein genes, with more complex ribosomes typically found in species requiring greater regulatory control (Desmond et al, 2010; Lynch, 2024; Yulo & Hendrickson, 2019; Yutin et al, 2012).

Another possibility is to understand not only how and under which selective pressure an innovation arises, but also which are the required conditions for such innovation to provide a benefit to the individual. With the increasing availability of population-wide genomic data, it is now possible to evaluate the adaptive significance of traits in the context of broader population-level genetic and phenotypic variation, rather than in isolation or under the assumption of fixation (Lynch et al, 2014). This enables a more nuanced understanding of the interplay among fitness landscapes, gene frequencies, and ecological context.

The ongoing discovery of the natural biodiversity at the cellular and genetic level is also providing more resources to better understand the adaptation of different species and the processes shaping genetic variation. This is especially evident in studies of unicellular organisms. Broader, more systematic analysis of the organisms involved are leading to advances in the study of horizontal gene transfer, microbial ecology, and host-microbiome interactions (Rainey & Quistad, 2020; The Human Microbiome Project Consortium, 2012). In turn, deeper insights into these processes further elucidate how they affect evolution and the dynamics of adaptation.

Finally, because of their short generation time and simple genetic architecture, single cell organisms like bacteria and yeast makes for good model systems in experimental evolution studies of evolutionary cell biology, in which adaptation and genetic innovations can be easily identified and linked together (Van den Berg et al 2018). A common mechanism of adaptation is the loss of gene function. On average, most random mutations are either neutral or slightly deleterious (Eyre-Walker & Keightley 2007, Lang & Murray 2008, Murray 2020). Importantly, most beneficial mutations tend to result from gene inactivation, as the

number of possible mutations leading to loss of function far exceeds those that result in a specific gain-of-function SNP. Therefore, the probability of gene inactivation mutations emerging and being positively selected is higher, particularly when they confer a fitness advantage. Consequently, gene inactivation is often the first evolutionary response to adaptive challenges, a phenomenon that has been confirmed experimentally (Lind et al 2015).

Given the importance of loss of function as a mechanism of genetic innovation, it is useful to study how experimentally induced gene knockouts influence evolutionary trajectories and fitness changes across the fitness landscape. This approach has been widely used across diverse species and genes (LaBar et al 2020, McCloskey et al 2018, Remigi et al 2019, Tobin et al 2016, Yulo et al 2025). Analyzing the genetic evolution of organisms following targeted knockouts enables the identification of positively selected mutations and helps uncover the factors that make some mutations more likely to occur or be beneficial. Such analyses also illuminate how selected genes contribute to adaptive phenotypes.

Genomic studies of evolved organisms not only reveal new gene-function relationships but also aid in predicting how organisms might adapt to novel environments. For example, prior research has shown how ribosomal proteins from different species can be integrated and adapted within new hosts, illustrating the role of horizontal gene transfer in shaping the evolution of the protein translation machinery (Lind et al 2010). Other studies have used evolution to investigate how *E. coli* develops bacteriophage resistance and how this process is modulated by antibiotic exposure (Parab et al 2024). Further, *Bacillus subtilis* has been experimentally evolved to survive in a simulated Martian atmosphere, highlighting the potential for microbial contamination of extraterrestrial environments (Nicholson et al 2010). These experiments offer valuable foresight into ecological dynamics before similar conditions arise in natural settings.

1.3 Aims of this thesis

In this dissertation, my goal is to study two essential cellular processes from an evolutionary perspective: cell wall synthesis and protein translation. In both cases I relied on the model bacterium *Pseudomonas fluorescens* SBW25. Cell wall synthesis involves the incorporation of peptidoglycan units into the cell wall, extending it along the growth axis of the cell, during cell growth, or between two daughter cells, during cell division. Thus, is necessary to allow proliferation. Meanwhile, protein translation is essential in providing all the components that cells need and it is performed by ribosomes. New ribosomes are synthesized starting from multiple ribosomal RNAs and ribosomal proteins assembled together in a

complex. Both cell wall synthesis and protein translation are ancestral processes already present in the last universal common ancestor and are performed by very conserved genes. Studying these processes through experimental evolution offers a unique opportunity to reveal novel interactions between essential cellular pathways. To date, neither of these processes has been extensively studied through experimental evolution, and existing studies have focused on different genes than those explored here. This work therefore provides a valuable opportunity to observe the evolution of essential, conserved traits.

In **Chapter 2**, I investigate cell wall synthesis during longitudinal cell growth by focusing on the gene *rodA*, which is part of a protein complex responsible for inserting peptidoglycan along the cell's axis. Building on previous research on the actin-like protein *mreB*, I assess the phenotypic consequences of a $\Delta rodA$ mutation on growth and morphology. I then examine whether and how evolution enables the recovery of fitness and morphology via secondary mutations. The analysis of compensatory mutations offers insights into how bacterial cells coordinate growth and division.

In **Chapter 3**, I apply a similar approach to study the ribosome. Here, I generate knockouts of ribosomal protein genes *rplI*, *rplX*, *rpmA*, *rpmG*, *rpmJ*, encoding respectively L9, L24, L27, L33, L36. Evolution again facilitates the recovery of fitness following the initial deletion. Analyzing the resulting compensatory mutations enhances our understanding of the regulatory mechanisms governing ribosome function. Notably, I observe that the knockout of different genes can be rescued by identical *de novo* mutations, suggesting convergence within the underlying functional network.

In **Chapter 4**, I dive deeper from the findings of Chapter 3 by further exploring the mechanistic basis of both fitness loss following ribosomal protein deletion and recovery through compensatory evolution. I focus on evolved mutants harboring *de novo* mutations in genes involved in transcription and transcription-translation coupling, including *nusG*, *rpoC* and *rpoD*. I measure translation rates across wild-type, deletion mutants, and evolved mutants, and evaluate how these correlate with fitness. Additionally, I investigate how the energetic balance between transcription and translation shifts during adaptation.

Finally, in **Chapter 5**, I summarize the key findings from the preceding chapters, discuss ongoing work in our laboratory, and highlight promising avenues for future research. I conclude by reflecting on how these findings contribute to our understanding of the evolutionary dynamics that shape essential cellular processes.

CHAPTER 2 – RodA directs cell morphology and affect fitness

2.1 INTRODUCTION

2.1.1 Peptidoglycan constitutes the cell wall

To grow, proliferate and divide, cells need to increase in size before division. In bacteria, cell expansion of cell volume is driven by an increase in the surface area of the cell wall. The bacterial cell wall is located outside the cell membrane and consists of a mesh of peptidoglycan. Peptidoglycan is a *N*-acetylglucosamine-*N*-acetylmuramyl-peptide, which is a disaccharide bound to a peptide chain of five amino acids. New peptidoglycan units are added to existing chains, elongating them via glycosidic bonds; separate chains are then linked orthogonally with peptide cross-links, providing structural strength to the cell wall (Schleifer & Kandler 1972, Vollmer et al 2008).

While the general nature of peptidoglycan is very conserved, details of cell wall structure vary slightly among different bacterial lineages. In different species, the exact composition of the disaccharide and peptide components of the peptidoglycan may also differ. The type and number of other components, such as proteins or sugars, integrated or anchored in the cell wall, also vary between species. One of the main distinctions between species is the difference between Gram-positive and Gram-negative bacteria. In Gram-positive species the peptidoglycan wall is up to 100 nm thick, whereas in Gram-negative it is approximately 2 nm thick, but is surrounded by a second layer of phospholipidic membrane (Kumar et al 2022, Vollmer et al 2008). Another defining feature is the process by which bacteria synthesize peptidoglycan and incorporate it to the cell wall. This process is intertwined with the growth pattern of the cell, and with the final shape it assumes. Therefore, bacteria of different shapes are expected to have distinct genes involved in cell wall synthesis and synthesis regulation.

2.1.2 The elongasome extends the cell wall longitudinally

The focus of this chapter is on the rod-shaped bacterium *Pseudomonas fluorescens* SBW25. In *Pseudomonas*, as in other rod-shaped species, like *Bacillus subtilis* and *Escherichia coli*, the cell wall is synthesized by two complexes: the divisome and the elongasome. As their names suggest, the divisome is mainly involved in synthesizing the peptidoglycan cell wall during septation, producing the separating wall between daughter cells leading to cell division. In contrast, the elongasome extends the cell wall along the growth axis of the cell (Begg & Donachie 1985). Consequently, it is not surprising that the genes constituting the elongasome are usually found only in rod-shaped bacteria. Just as the divisome, the

elongasome is a membrane protein complex that performs a series of tasks in order to incorporate new peptidoglycan molecules in the cell wall. It consists of membrane proteins such as MreC, MreD, RodA, RodZ and PBP2 along with the membrane bound actin homologue MreB (**Figure 2**) (Liu et al 2020, van den Ent et al 2001).

For a long time, before the elongasome and divisome components were properly identified and characterized, the insertion of peptidoglycan into the cell wall was thought to be carried out exclusively by penicillin-binding proteins of class A (aPBPs), such as PBP1a: these proteins can catalyze both transglycosylation and transpeptidation (Straume et al 2021). In recent years, other proteins such as RodA in the elongasome and FtsW in the divisome have been shown to not only be involved in the export of peptidoglycan outside the cell membrane but also capable of actively inserting it into the cell wall. As a result, these proteins have been classified as SEDS (shape, elongation, division, sporulation) proteins. SEDS proteins usually work in concert with penicillin-binding proteins of class B (bPBPs), which unlike aPBPs can only perform transpeptidation to cross-link peptidoglycan chains (Henriques 2002, Philippe 2008). Further studies have demonstrated that aPBPs operate independently of the elongasome or divisome (Kumar 2022).

In *E. coli*, the elongasome is first recruited by the cytoskeletal protein MreB (Van der Ploeg et al 2015). MreB forms filaments adherent to the cytosolic surface of the cell membrane. The filaments rotate around the circumference of the cell, driving the movement of the elongasome to sites where peptidoglycan synthesis will occur. Different models have been proposed to explain the pattern by which MreB filament assembly is achieved, how the filaments move around the cell membrane, and how they direct the elongasome positioning (Middlemiss et al 2024, Yulo & Hendrickson 2019). The rotation of MreB filaments around the cell ensures a uniform distribution of peptidoglycan insertion; disruption of MreB filaments prevents proper elongation of the cell (Van Teeffelen et al 2011, Yulo et al 2025). MreB centrality to the elongasome activity is evident from the fact that cells lacking elongasome proteins such as MreC, MreD, or RodA cannot assemble MreB filaments (Kruse et al 2004).

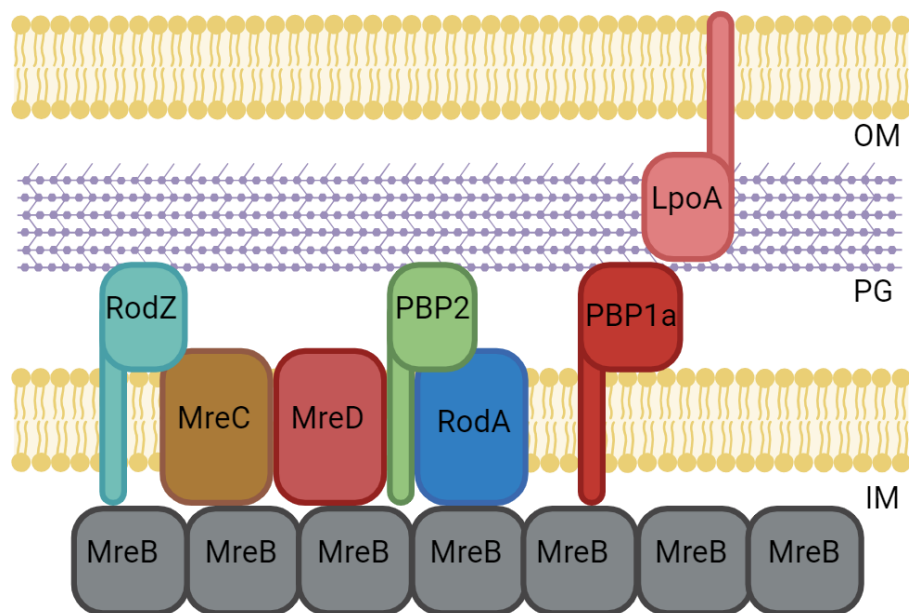


Figure 2: Elongasome components in *E. coli*. OM: outer membrane. PG: peptidoglycan layer. IM: inner membrane. MreC, MreD and RodA are mostly integrated in the cell membrane. RodZ, PBP2, and PBP1a are anchored in the membrane but their main body is in the periplasmic space. LpoA is anchored on the outer membrane. Image based on Kumar et al 2022 and produced using Biorender.

2.1.3 Multiple enzymes in the elongasome synthesize the peptidoglycan

For the cell wall synthesis to occur, first of all, peptidoglycan monomers have to be synthesized in the form of the membrane-anchored precursor lipid II. Lipid II is synthesized in the cytoplasm from nucleotides and amino acids via a series of enzymes, classified from MurA to MurG. Then, the precursor lipid II has to be exported outside the cell membrane by flippases such as MurJ. Its phospholipid component is removed during the subsequent steps. The peptidoglycan is then integrated in the cell wall. RodA first catalyzes the transglycosylation of the peptidoglycan to an existing chain. Finally, separate chains are linked together by transpeptidation of their peptide moieties by PBP2. MreC and MreD in turn regulate the interaction and activity between RodA and PBP2, while RodZ connects the elongasome to the MreB filaments. Many details of this process are still unknown (Cho et al 2016, Ishino et al 1986, Kumar et al 2022, Liu et al 2020, Sjødt et al 2018, Umbreit & Storminger 1972, van den Ent et al 2010, van der Ploeg et al 2015).

2.1.4 *rodA* and the elongasome share a common origin with the divisome

In the elongasome, RodA forms a stable complex with PBP2, a bBPB, thus conferring to the elongasome glycosyltransferase and transpeptidase activity. RodA and PBP2 are coded by, respectively, the genes *rodA* and *pbpA* (also called *mrdB* and *mrdA*), which are located together in an operon (Spratt et al 1980). The elongasome complex itself can navigate around the circumference of the cell similar to MreB (Stoker et al 1983, Van der Ploeg et al 2015). There is still much to know about RodA: despite the gene being identified in 1973 (Matsuzawa et al 1973), its structure is still unknown due to inability to crystalize the protein (Sjodt et al 2018, Stoker et al 1983). Our knowledge about its structure and function have been provided both by experimental analysis of knock out mutants and by evolutionary insights obtained from its amino acid sequence (Henriques et al 1998, Henriques 2002, Iwaya et al 1975, Sjodt et al 2018)

Many details are known about the evolution of *rodA* and the elongasome. Evidence suggests that the elongasome and divisome share a common evolutionary origin. This is supported by similarities in the functionality, structure, and composition of the complexes. For instance, the elongasome and divisome interact with the same enzymes of the lipid II synthetic pathway, such as MraY and MurG (Mohammadi et al 2007, Szwedziak & Löwe 2013). The localization of both the elongasome and divisome on the guiding filaments of MreB (for the elongasome), or FtsZ (for the divisome), is guided by RodZ (Ago & Shiomi 2019). RodA itself is homologous to FtsW, in the divisome: although sequence identity is poorly conserved, they share structural features, functional sites, and catalytic activity. Just as RodA forms a stable complex with PBP2, FtsW is in a complex with a bBPB, PBP3, to perform the final steps of peptidoglycan assembly (Ikeda et al 1989, Szwedziak & Löwe 2013).

Evolutionary modifications in the regulation of peptidoglycan synthesis have contributed to species differentiation at both macroscopic (e.g., cell shape) and microscopic level (e.g., the number of peptidoglycan units per chain), fitting the physical and chemical properties of the cell wall to the needs of the organism (Costa et al 1999, Vollmer et al 2008). To complicate matters further, these needs are not constant, and bacteria must be able to adjust cell wall structure depending on growth conditions. For example, the density, length and cross-linking of peptidoglycan chains in *E. coli* vary with growth rates (Pisabarro et al 1985). Long-term experimental evolution studies of *E. coli* have also shown how mutations in the *rodA-pbpA* operon can be positively selected to favor the organism. These studies revealed that changes in cell morphology, including the loss of rod shape, can spontaneously evolve to enhance ecological performance (Philippe et al 2008).

2.1.5 Goals of the chapter

In this chapter I aim to understand how the SEDS protein, RodA, affects the growth of *P. fluorescens*, what phenotype results from its loss, and what mechanisms the cell can eventually use to adapt to such loss. Previous studies in *P. fluorescens* SBW25 highlighted how the experimental deletion of *mreB* caused loss of fitness by impairing the regulatory mechanisms directing peptidoglycan synthesis, and how the organism could adapt through experimental evolution by *de novo* mutations of PBP1a, an aPBP that doesn't rely on MreB filaments to build the cell wall (Yulo et al 2025). Moreover, further experiments in our lab showed how alterations of the cell morphology are produced not only by manipulating genes involved in directly maintaining the cell shape phenotype such as *mreB*, but also by disrupting cell division, for example by deletion of the division inhibitor gene *minC*. These results together indicate how cell growth and morphology are strictly connected.

Extending these experiments to multiple genes involved in cell wall synthesis can help us understand how the activity of such genes is interconnected. This could provide insights into how the various functions within the cell interact together to solve its necessities, and show how evolution can explain the variability observed in different species.

2.2 RESULTS

2.2.1 Construction of *rodA* deletion mutants

In *P. fluorescens* SBW25, *rodA* is a gene of 1104 nucleotides, located in position 5955822 of the genome, preceded by *pbpA*. The two genes constitute an operon and are separated by 31 nucleotides. The reference genome predicts a signal peptide overlapping the last 19 nucleotides of *rodA*. The signal peptide is part of the gene PFLU_5421, which is homologous to lytic murein transglycosylase B in *E. coli*, involved in lipid II biosynthesis. As such, it was decided to produce two *rodA* deletion mutants, one with the gene completely deleted, and the other with the deletion leaving the last 19 nucleotides intact in order to preserve the possible signal peptide (and prevent alteration of the lipid II synthetic pathway while knocking out *rodA*).

The mutants were produced by two-step allelic exchange (Hmelo et al 2015), adapted to work in *P. fluorescens* SBW25 in-house by David Rogers and Ellen McConnell. The resulting mutant only carries the desired mutation, without any inclusion of reporter genes or other lesion or leftovers from the

mutagenesis process. The successful mutations were confirmed by gel electrophoresis and Sanger sequencing of the junction point to confirm deletion of *rodA* (**Table 1**).

After deletion of the gene *rodA*, both full and partial deletion mutants were separately tagged with GFP or mScarlet by Tn7 transposon insertion from a plasmid provided by conjugation from a donor *E. coli* culture (Choi & Schweizer 2006). The insertion was confirmed by Sanger sequencing of the tagged gene and visual identification of fluorescent colonies.

Table 1: $\Delta rodA$ mutants produced in this work

Mutant	Mutation	Tag
MPB23648	Partial <i>rodA</i> deletion	GFP
MPB23672	Partial <i>rodA</i> deletion	mScarlet
MPB23673	Full <i>rodA</i> deletion	GFP
MPB23652	Full <i>rodA</i> deletion	mScarlet

2.2.2 $\Delta rodA$ mutants have lower fitness and altered morphology

Two phenotypes were initially measured: fitness and cell shape. The fitness of $\Delta rodA$ mutants was measured by competitive fitness assay against SBW25. Fitness is measured as relative growth of mutant and SBW25 – in co-culture – over time (**Figure 3**).

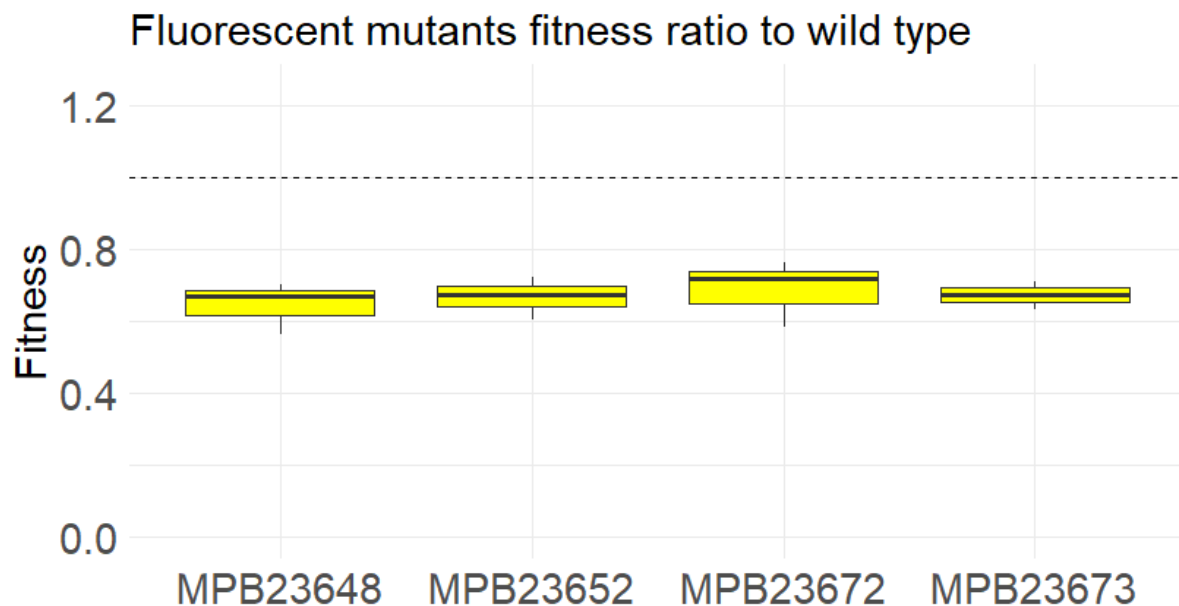


Figure 3: Fitness of *rodA* deletion mutants. Y-axis: competitive fitness against the ancestor. Data are means of three biological replicates. The boxes represent the mean within the 1st and 3rd quartile.

The measurement confirmed that the deletion of *rodA*, while viable in *P. fluorescens*, leads to a decrease in fitness to around 70% of the ancestor. The mutants with full and partial deletion show similar decreases in fitness. The similarity between the mutants is consistent with the results of further experiments described below, suggesting that the predicted signal peptide, if actually expressed by the cell, does not contribute toward a measurable phenotype.

The next test consisted of observing cells by phase contrast microscopy to analyze their shape. Samples were imaged at the phase contrast microscope (**Figure 4**). To further explore how the shape of the cell is a product of the interaction of different genes involved in cell growth, *minC* deletion mutants previously produced in the lab by Malavika Venu and David Rogers were also observed and measured. The study of *minC* is not the focus of this work, but the analysis of the $\Delta minC$ mutant provides a comparison with $\Delta rodA$ mutants, since both genes are involved in cell wall synthesis, and their opposite effects on cell morphology is helpful in underscoring how both MinC and RodA alter the cell shape. As is the case of $\Delta mreB$ mutants, $\Delta rodA$ mutants lose the rod-shape phenotype, and appear instead spherical. Moreover, the cells are also of more variable size, with the largest cells being much bigger than the average wild-type cell. Deletion of *minC* leads to an opposite phenotype, namely, that of elongated cells of various length, caused in many cases by defective septation during cell division. Mutants with *minC* fused with a reporter *GFP* show a phenotype much closer to the wild-type (**Figure 5**).

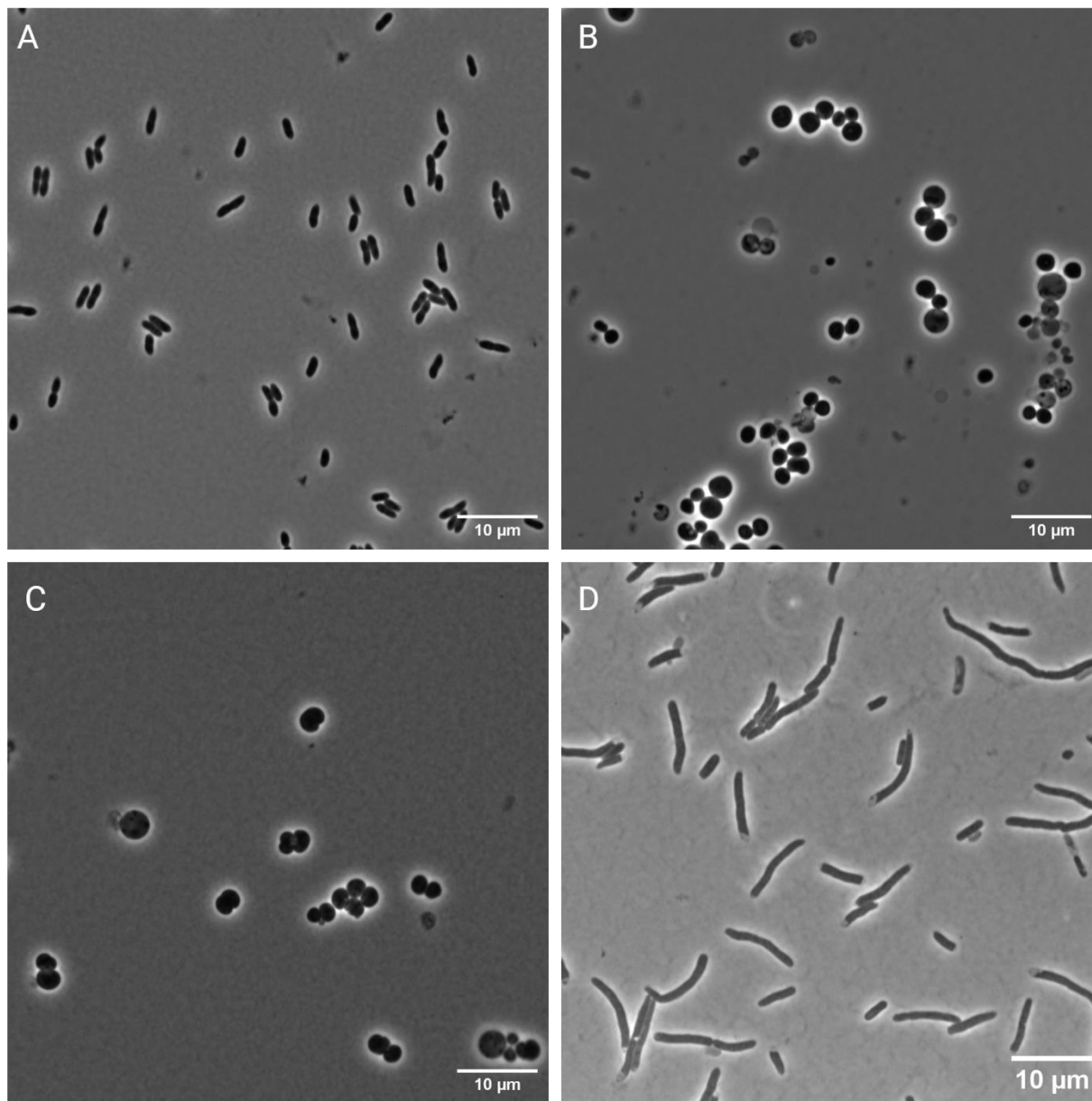


Figure 4: Phase contrast imaging of the *P. fluorescens* cells used in this work. A: wild-type. B: partial *rodA* deletion mutant. C: full *rodA* deletion mutant. D: *minC* deletion mutant.

Overall these results show that deletion of *rodA* leads to a loss of fitness coupled with an alteration in the cell shape phenotype. It was still not clear if the loss of fitness was directly caused by the loss of rod shape, or if the two effects are independent.

Area and roundness of *rodA* and *minC* mutants

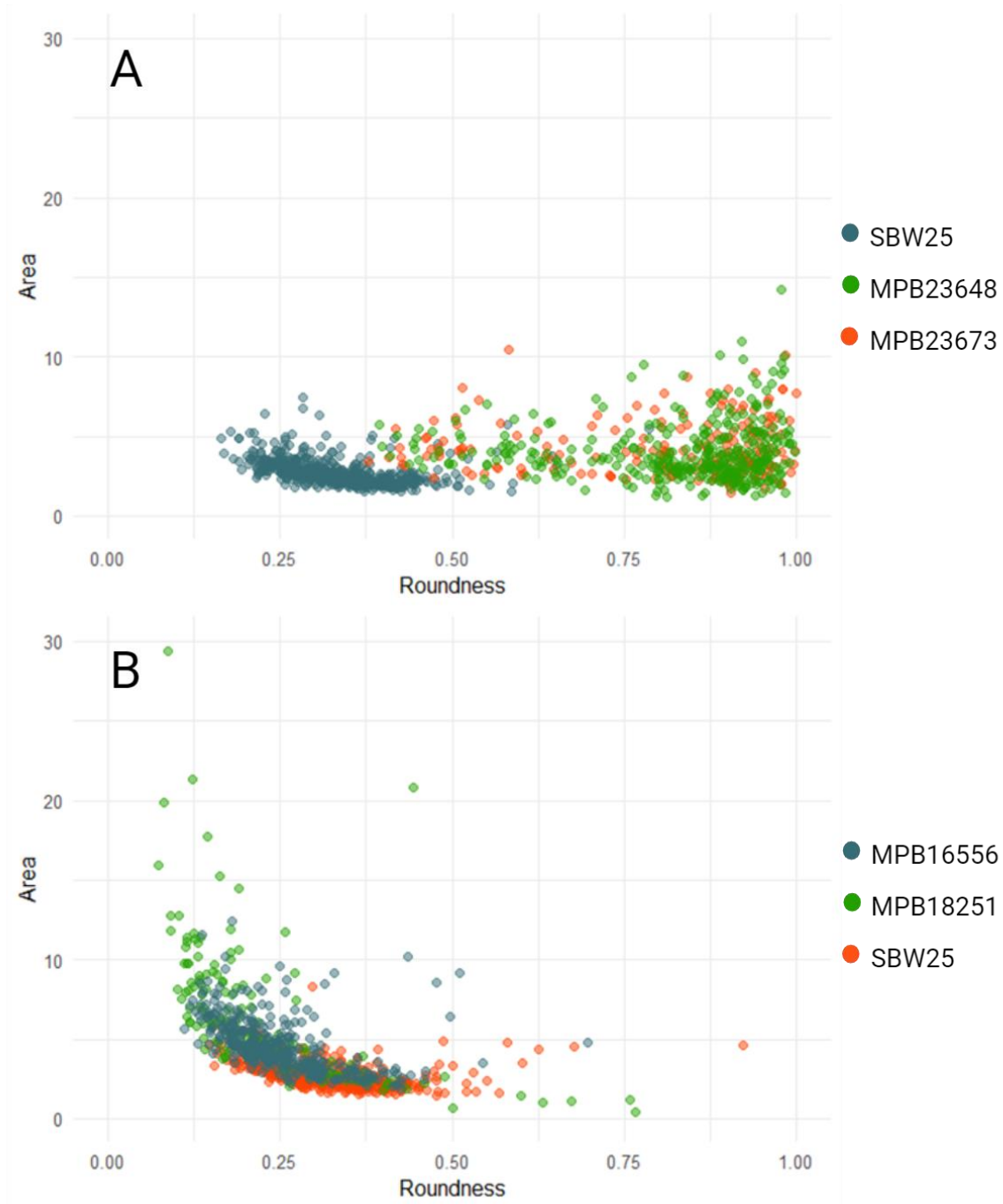


Figure 5: Measurement of area and roundness of $\Delta rodA$ mutants. X-axis: roundness. Y-axis: area. The area is calculated in μm^2 . Roundness is calculated as the ratio between observed area and predicted area calculated as a circle from the longest axis in the cells. Each dot represents the measurement of an individual cell. A:

measurement of $\Delta rodA$ mutants. Blue: wild-type. Orange: full *rodA* deletion mutant MPB23673. Green: partial *rodA* deletion mutant MPB23648. B: measurement of $\Delta minC$ mutants. Orange: wild-type. Green: $\Delta minC$ MPB18251. Blue: *minC-GFP* MPB16556.

The high number of minicells identified at the microscope in *rodA* deletion mutants suggested that a possible cause of fitness decrease is provided by an increased number of asymmetrical cell divisions producing daughter cells deprived of DNA and other essential components.

2.2.3 Experimental evolution allows fitness recovery by selection of compensatory mutations

Since it is known that *rodA*, and with it, the rod shape, has been repeatedly lost in different bacterial lineages, the next step was to understand if and how the loss of fitness could be recovered over time.

To do so, the cells were allowed to acquire compensatory mutations through experimental evolution. Each of the four different $\Delta rodA$ mutants previously produced was propagated in five independent (replicate) populations by serial daily 1:1000 dilution in fresh medium, allowing the population to go through ~10 cell division cycles between dilutions. Each population was propagated for a total of 69 days, equating around 690 generations. Every 7 days fitness was measured relative to SBW25.

Fluorescent mutants fitness ratio to wild type

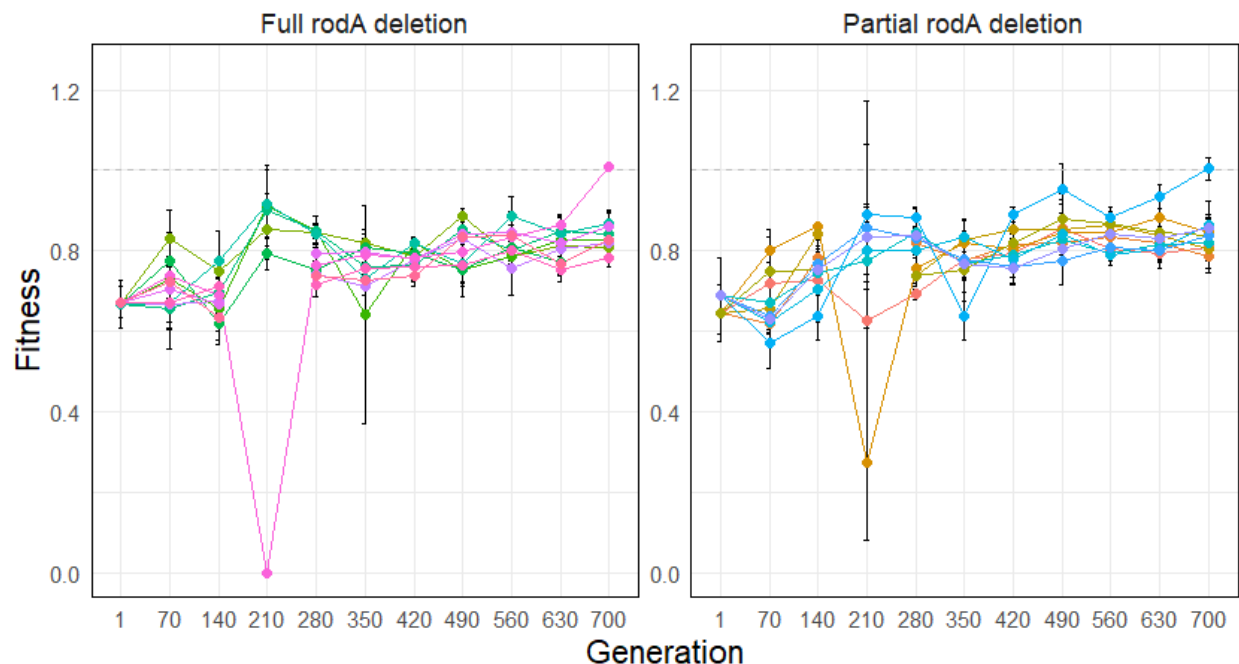


Figure 6: Fitness of the evolving $\Delta rodA$ mutant populations. X-axis: number of generations. Y-axis: competitive fitness against the ancestral type. Each color represents an independent population. Each fitness measurement was repeated three times from independent biological replicates, and is indicated with the mean plus and minus the standard deviation. Left: Full *rodA* deletion mutant populations. Right: partial *rodA* deletion mutant populations.

Most lines managed to recover fitness through the course of the experiment, in some cases up to the level of the wild-type (**Figure 6**). This shows how, even in short time, bacteria can compensate for the loss of *rodA*. Observation of evolved populations at the microscope similarly showed how, while the rod shape was not recovered, the evolved cells still showed a partially recovered capability of performing a correct symmetrical division. Evolved cells are consistently smaller than both the ancestral deletion mutants and SBW25, and there are lower amounts of minicells and giant cells (**Figure 7**).

Morphology of evolved $\Delta rodA$ mutants

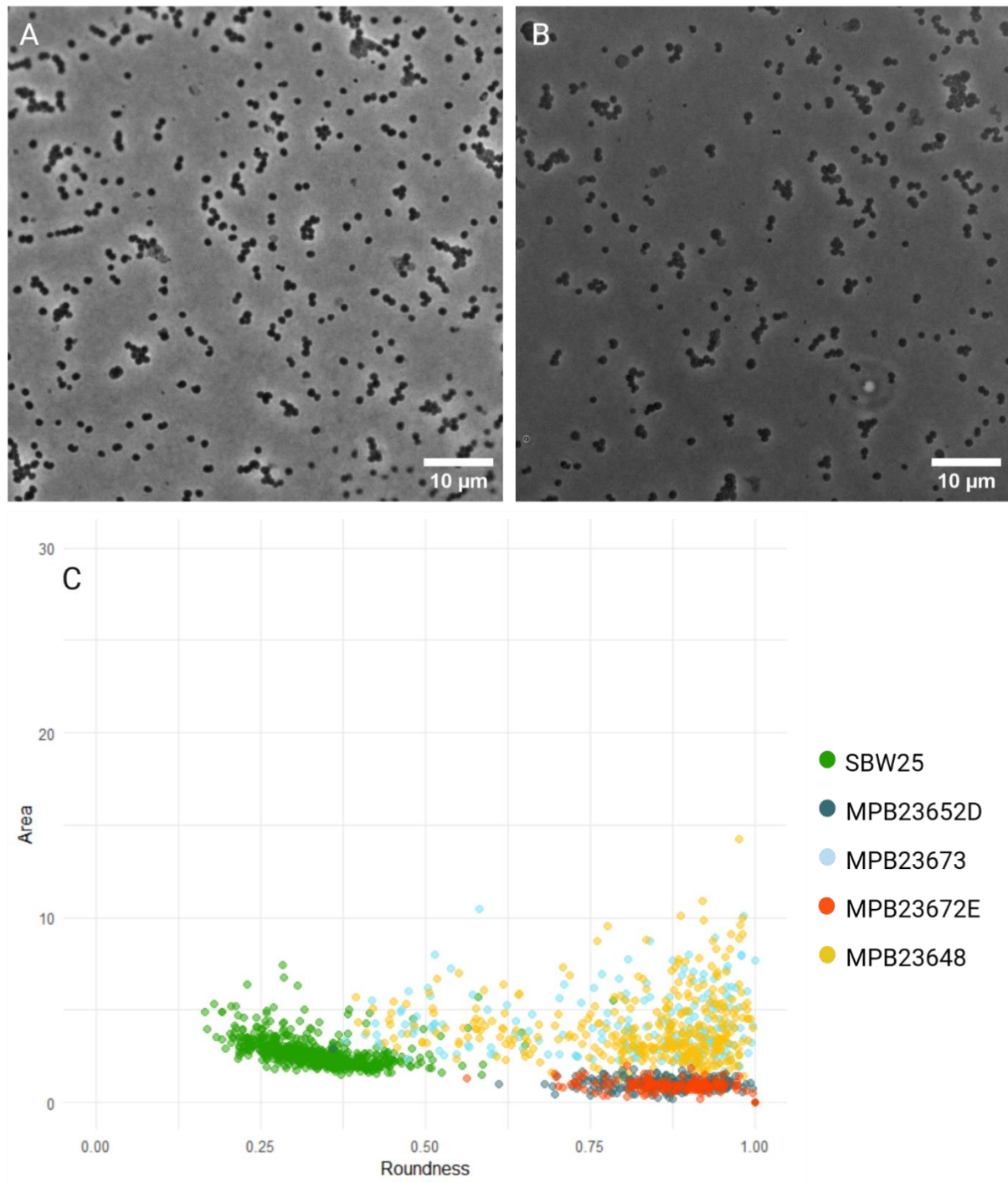


Figure 7: Derived *rodA* deletion mutant populations sampled at 690 generations of evolution. A: evolved full *rodA* deletion mutant population MPB23652D. B: derived partial *rodA* deletion mutant population MPB23672E. C: Measurement of area and roundness of evolved $\Delta rodA$ mutants. X-axis: roundness. Y-axis: area. The area is

calculated in μm^2 . Roundness is calculated as the ratio between observed area and predicted area calculated as a circle from the longest axis in the cells. Each dot represents the measurement of an individual cell. Green: ancestral SBW25. Blue: evolved full *rodA* deletion mutant MPB23652D. Cyan: ancestral full *rodA* deletion mutant MPB23673. Orange: evolved partial *rodA* deletion mutant MPB23672E. Yellow: ancestral partial *rodA* deletion mutant MPB23648.

2.2.4 Colony size measurement

To confirm the fitness recovery through multiple measurement method, the fitness was compared by measuring the size of colonies. Multiple measurements are useful ensure the results of the previous fitness assays are not skewed by experimental conditions, or by adaptation of the evolved mutants to the experimental evolution setup. Colonies of fit mutants are expected to grow faster in size than those of sick mutants. To measure it, fluorescent ancestral ΔrodA mutants were plated together with a sample from the evolved population of the different color (**Figure 8**). The results show how the adaptation of *P. fluorescens* to the loss of *rodA* helps to recover fitness both in liquid environment, by competitive fitness assay (**Figure 6**), and solid environment, by colony size measurement on plates (**Figure 9**).

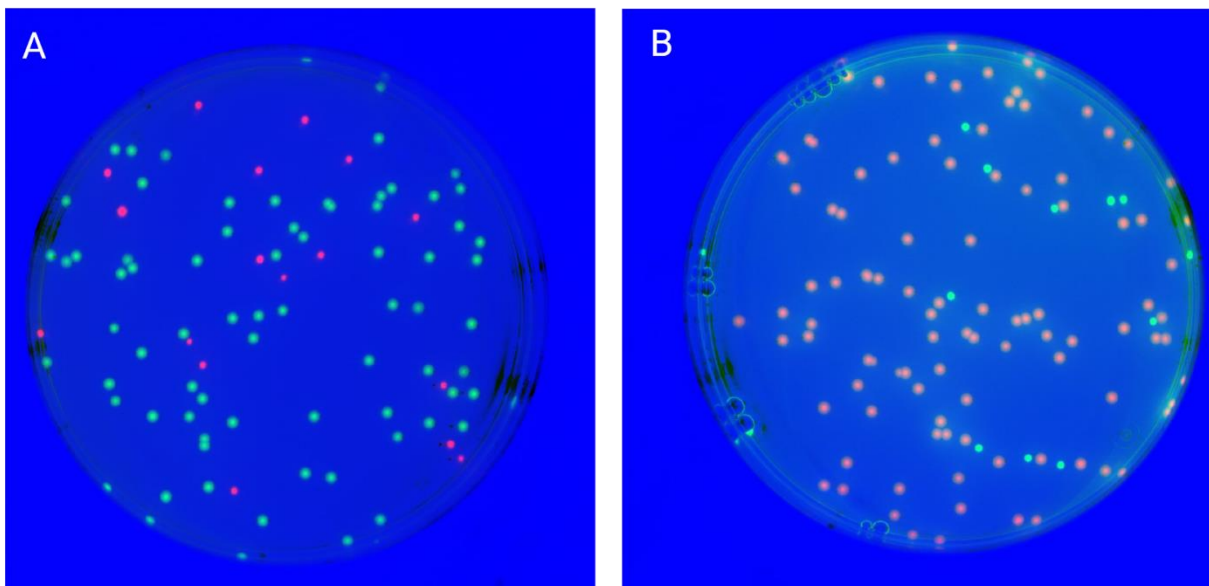


Figure 8: colonies of evolved and ancestral full *rodA* deletion mutants plated on LB. A: Red: colonies of the ancestral mutant MPB23652 tagged with mScarlet. Green: colonies from the population MPB24184, evolved from the ancestral mutant MPB23673 tagged with GFP. B: Green: colonies of the ancestral mutant MPB23673

tagged with GFP. Red: colonies from the population MPB24186, evolved from the ancestral mutant MPB23652 tagged with Scarlet.

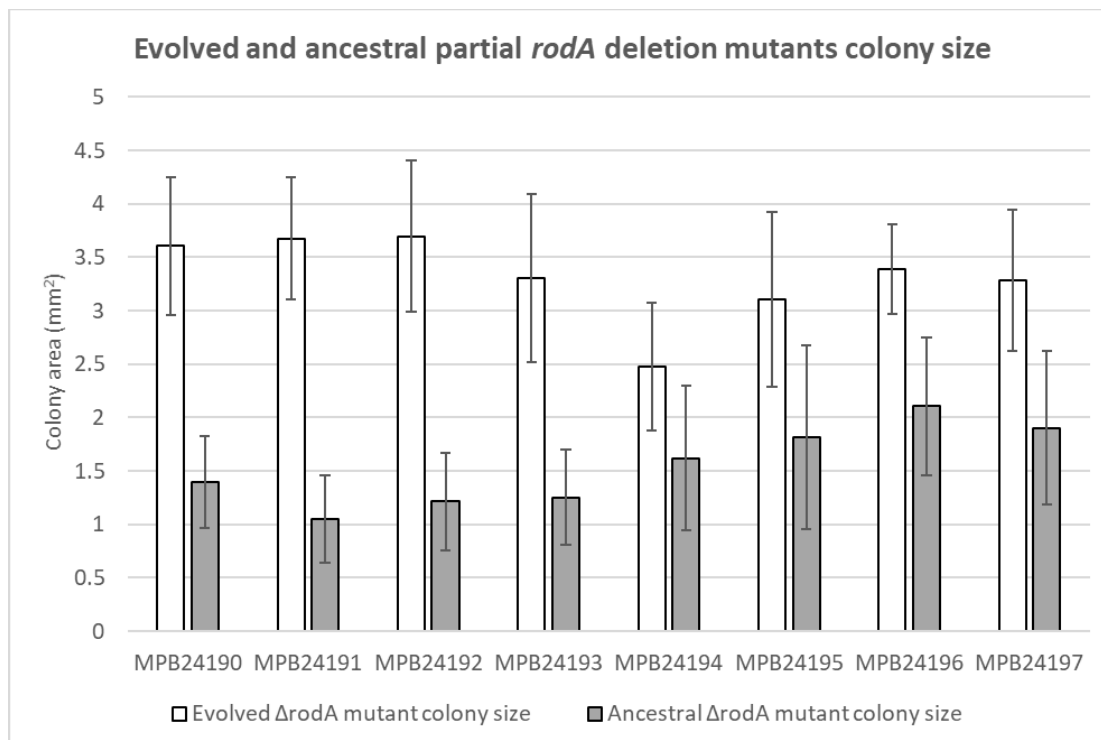
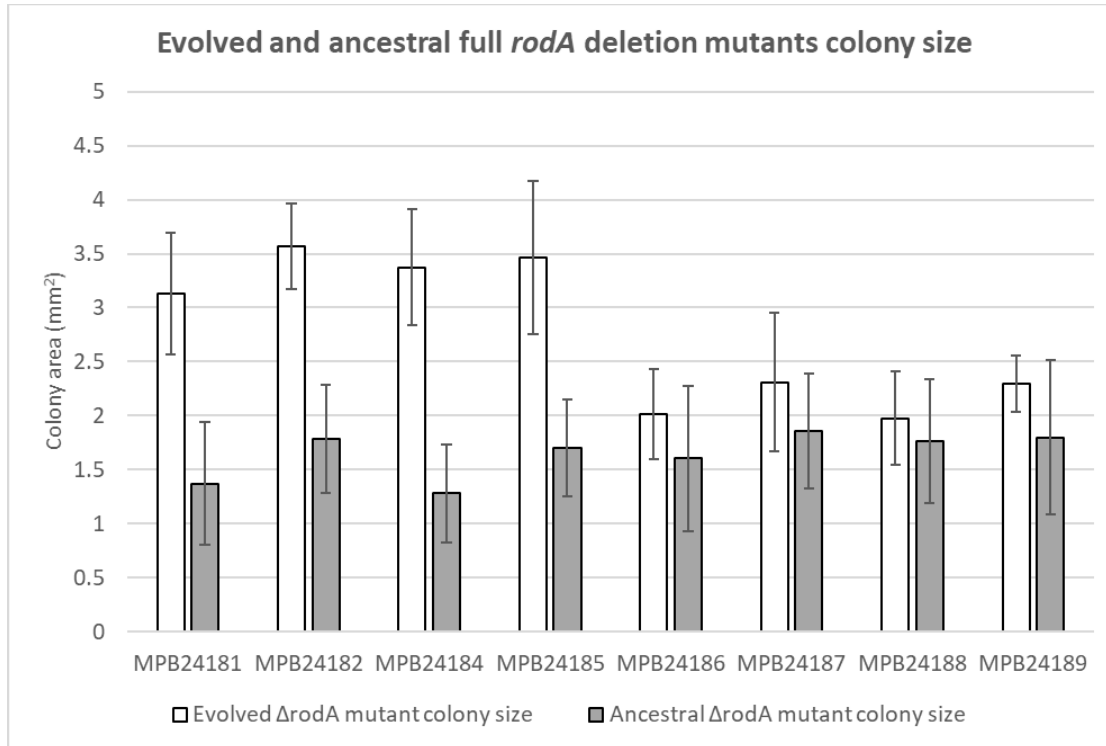


Figure 9: Colony size of evolved *rodA* deletion mutants and colony size of ancestral *rodA* deletion mutants. Each pair of columns corresponds to a separate evolved population sampled at 34 days of experimental evolution (white bar) plated together with the corresponding ancestral $\Delta rodA$ mutant of opposite color (grey bar). Top: full *rodA* deletion mutant populations. Bottom: partial *rodA* deletion mutant populations. X-axis: evolved mutant. Y-axis: average area of the colonies in mm². The data are means and standard deviations with data recorded 72 hours after plating. Between 44 and 319 colonies were observed for each mutant.

2.2.5 Sequencing of evolved mutants reveal *de novo* mutations

To better understand how *P. fluorescens* adapted to the loss of *rodA*, and to obtain insights into how this could have happened during evolution, 16 evolved populations were subject to genome sequencing. Genomic DNA was extracted from samples of each population from two timepoints, at 34 and 69 days of experimental evolution. The DNA was then sequenced and the reads mapped on the *P. fluorescens* SBW25 reference genome. The output was then analyzed using Geneious to identify mutations, including both SNPs and large-scale duplications (**Figure 10**).

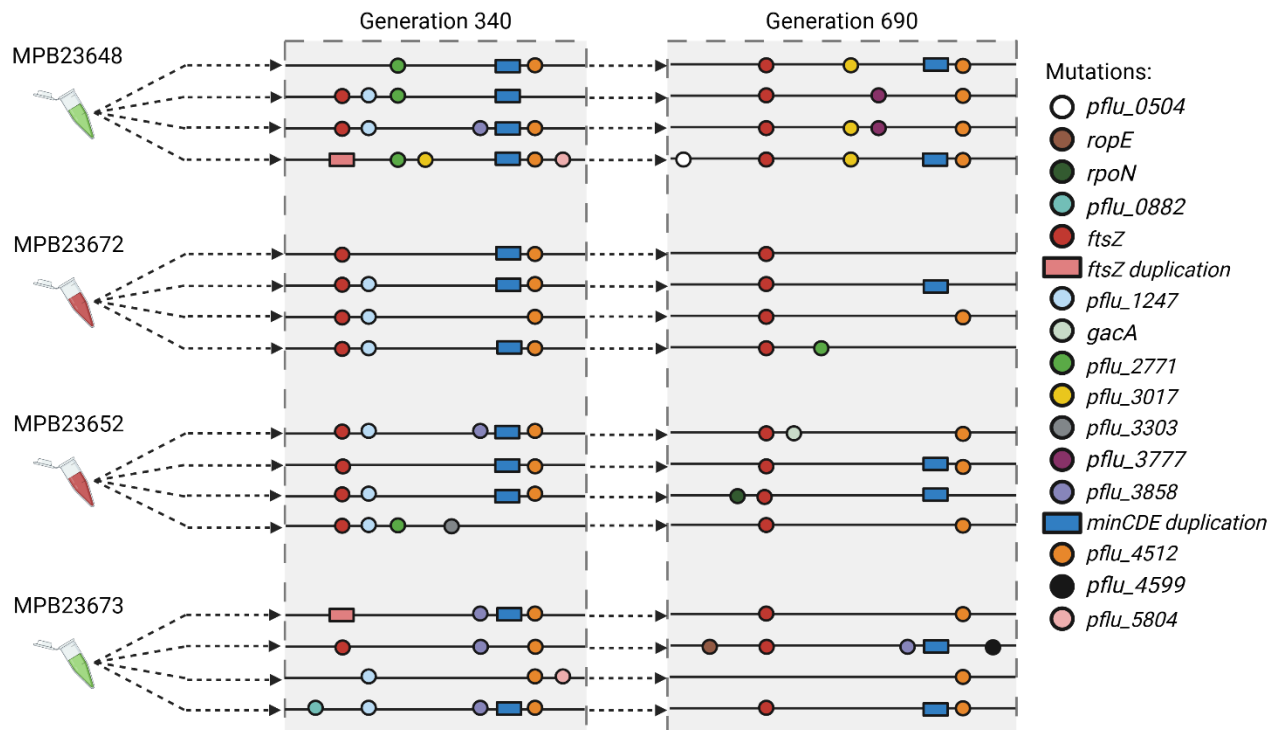


Figure 10: *De novo* mutations in evolved $\Delta rodA$ mutants. For each population, all genes with at least one mutation above 60% of frequency in at least one population are marked. In the left box are the mutations identified in populations evolved 340 generations; in the right box are the mutations at 690 generations. Colored circles represent SNPs, rectangles represent duplications. The order of the genes in the graph follows the order of the genes in *P. fluorescens* chromosome. The full list of SNPs and duplications and their chromosomal positions are in the supplementary materials (Supplementary Table 1, 2, 3).

The two most striking results are how most evolved populations show a high frequency of clones with SNPs in *ftsZ*, and the many large-scale duplications all between positions 4040000 and 4890000. The duplications almost always involve the *min* operon, comprising *minC*, *minD*, *minE*. The Min proteins regulate cell division. Similarly, FtsZ is an essential protein that polymerizes during cell division to create the division septum; MinC and MinD directly prevent this by antagonizing FtsZ. Some duplications also involve the gene *ftsZ*. Interestingly, populations with *ftsZ* duplications at 340 generations replace them with *ftsZ* SNPs at 690 generations. All the *ftsZ* mutations found in this experiment affect amino acids located at the interface between FtsZ and MinC (**Figure 11**). Notably, multiple populations evolved identical amino acidic substitutions in *ftsZ*, as for example S281R appearing independently in four populations. Other genes show a high degree of ongoing selection; the most recurring mutations appear in the genes *pflu_1247* and *pflu_4512*. The gene *pflu_1247* codes a 194 amino acids-long hypothetical cytoplasmatic protein, for which no homologous gene with known function could be found. The gene *pflu_4512* codes a 530 amino acids-long cytosine permease. All the SNPs in the genes *pflu_4512* and *pflu_3858* are located in palindromic R2 repetitive regions; such regions are more prone to both *de novo* mutations and sequencing errors. It is still possible that SNPs in such regions alter folding of secondary structure of the mRNA, thus altering the regulation of the mRNA processing, translation and degradation. Other common mutations are SNPs in the gene *pflu_2771*, a 200 amino acids NAD(P)H dehydrogenase, and a 42 nucleotides deletion in *pflu_3017*, a 1185 amino acids-long signal transduction protein.



Figure 11: Ribbon model of a FtsZ dimer. The two monomers are in different colors, respectively red-yellow and magenta-orange. The amino acids changed by the SNPs identified in this work are highlighted in spacefilling in white and cyan. Model based on the FtsZ dimer structure of *Methanocaldococcus jannaschii* (Oliva et al 2004). When polymerizing, further FtsZ monomers are included elongating the polymer from the top and the bottom of the structure.

The interplay between *ftsZ* and *minC* is further displayed by the fact that the *min* operon duplications appear at high frequency at 34 days and then disappear from many populations at 69 days, while the *ftsZ* SNPs increase in frequency in most populations. Out of 16 evolved and sequenced $\Delta rodA$ populations, 13 have duplications of the *min* operon at 34 days, down to 9 at 69 days; there are SNPs in *ftsZ* in 11 populations at 34 days, up to 15 populations at 69 days.

An interesting feature is that all the mutations in *ftsZ* appear to decrease the local amount of negative charge at the interface with *minC*, going in most cases from negatively charged to non-polar amino acids and from polar to positively charged amino acids (**Table 2**).

Table 2: *De novo* mutations in *ftsZ* at 34 and 69 days of experimental evolution.

<i>ftsZ</i> mutations at 34 days of evolution					<i>ftsZ</i> mutations at 69 days of evolution				
Line	AA position	Frequency	Original AA	Mutation	AA position	Frequency	Original AA	Mutation	Frequency change
MPB23648A	-				176	96.00%	ASP	GLY	96.00%
MPB23648B	242	96.50%	GLU	ALA	242	100.00%	GLU	GLY	3.50%
MPB23648D	256	89.00%	GLN	PRO	87	98.60%	GLU	LYS	9.60%
MPB23648E	-				252	98.40%	ASP	ALA	98.40%
MPB23652A	306	99.30%	GLU	ALA	306	100.00%	GLU	ALA	0.70%
MPB23652B	234	99.10%	PRO	LEU	234	100.00%	PRO	LEU	0.90%
MPB23652D	176	98.90%	ASP	GLY	176	100.00%	ASP	GLY	1.10%
MPB23652E	281	98.80%	SER	ARG	281	100.00%	SER	ARG	1.20%
MPB23672A	305	100.00%	ASP	ALA	305	100.00%	ASP	ALA	0.00%
MPB23672B	235	100.00%	ASN	LYS	235	100.00%	ASN	LYS	0.00%
MPB23672D	281	98.10%	SER	ARG	281	100.00%	SER	ARG	1.90%
MPB23672E	176	100.00%	ASP	GLY	176	86.40%	ASP	GLY	-13.60%
MPB23673A	-				270	76.00%	ASP	GLY	76.00%
MPB23673B	281	99.20%	SER	ARG	281	97.20%	SER	ARG	-2.00%
MPB23673C	234	99.10%	PRO	LEU	-				-99.10%
MPB23673D	-				-				0.00%
MPB23673E	-				281	99.30%	SER	ARG	99.30%

Column 1: evolving population. Columns 2-6: amino acid position. Columns 3-7: SNP frequency. Columns 4-8: wild-type amino acid. Columns 5-9: mutant amino acid. Column 10: change in frequency of mutant *ftsZ* allele between 34 and 69 days of experimental evolution. The amino acids are color coded by their side chain. Red: negatively charged amino acids. Blue: positively charged amino acids. Grey: polar amino acids. Yellow: non-polar amino acids.

2.3 DISCUSSION

2.3.1 Fitness decrease in $\Delta rodA$ mutants could have multiple causes

In this chapter I showed that *rodA* could be deleted in *P. fluorescens* without the mutation being lethal. The deletion led to a decrease in fitness, to a switch from rod to round cell shape, and to a high number of asymmetrical divisions. Mutants carrying a knock-out of *rodA* have successfully been constructed and characterized in previous studies, although not in *P. fluorescens* (Begg & Donachie 1985, Henriques et al 1998, Matsuzawa et al 1973, Sieger et al 2013). Such studies demonstrated the role of RodA on cell shape phenotype and fitness, and its interactions with other components of the elongasome and divisome. In the present work, *rodA* deletion mutants were also allowed to recover fitness through evolution, highlighting the different genes which activity can be altered by *de novo* mutations in order to recover

fitness after the experimental deletion of *rodA*. This work helps to better understand the role of RodA and lateral cell wall extension in the physiology of the cell; to understand how the rod-shape phenotype provided by *rodA* is integrated in complex cellular functions; and how evolution can modify an organism following the perturbation of such functions via the loss of *rodA*.

RodA, as part of the elongasome, integrates new peptidoglycan molecules in the cell wall in a helical pattern around the sides of the cell, elongating it, starting from the middle of the cell length. As such, the round shape phenotype, as observed here in *P. fluorescens*, is a recurring phenotype of the *rodA* deletion in different species. A similar phenotype can arise when disrupting other genes involved in the elongasome activity (Begg & Donachie 1985, Sieger et al 2013, Yulo et al 2025). Notably, the details of the phenotype produced by the loss of *rodA* are different in different species. So, for example, in the apically-growing *Corynebacterium glutamicum* a *rodA* deletion doesn't lead to a loss of rod shape, but in a decrease of elongation rate, while in *B. subtilis* and *E. coli* the same deletion produces a round shape and a strong decrease in cell viability (Henriques et al 1998, Matsuzawa et al 1973, Sieger et al 2013). These differences reflect the different ways in which bacterial species can regulate and localize the activity of both elongasome and divisome. Consistently with this picture, the results of this work suggest that also in *P. fluorescens* the deletion of *rodA* causes a malfunctioning elongasome, leading to the loss of the rod shape. The fact that the deletion of a gene involved in cell wall synthesis such as *rodA* leads to a decrease in fitness, as observed in the experiments here presented, requires a more nuanced explanation; various are possible, as several possibilities exist.

First of all, losing one of the proteins that are responsible for actively integrating peptidoglycan into the cell wall may impede the ability of the cell to grow in size fast enough to keep up with all the other processes required for cell division, such as DNA replication and ribosome synthesis. As a consequence, the cell wall synthesis could become the bottleneck of the division process.

Another consequence of the loss of *rodA* is that the now round cell may be less capable of properly integrating the flagellum into the cell wall. Since the flagella in *P. fluorescens* are assembled and located at the cell poles, a round cell without a pole could struggle to properly assemble them. This could decrease the control that the cell has in its own motility, and as such being less capable of reaching nutrients in the growth medium. Additionally, decreased motility could also force a decreased capability of separating sister cells after division, increasing the chances for growing cells to cluster together, in turn diminishing their access to the nutrients and free space necessary to divide, thus hampering cell division (Capdevila et al 2004, Kojima et al 2020, Siwach et al 2021). That said, the experimental setup used in this chapter

relies on liquid, shaking cultures in all stages of growth and evolution: such environment, by mixing the culture, decrease the need of a flagella. Previous studies showed how the production of flagella is negatively selected in shaking cultures (Wang et al 2025).

Lastly, previous work on *E. coli* and *B. subtilis* demonstrated how bacterial cells employ their own shape as a tool to direct cell division (Blasios et al 2013, Raskin & de Boer 1999). It is known that the rod shape generates a continuous gradient within the cells of the Min and FtsZ proteins that dictate where and when the division septum is assembled. Thus, altering cell shape can disrupt this process. The compensatory mutations identified in this work support the hypothesis that this is the mechanism that evolution is adapting to recover the fitness loss.

Moreover, in this work the *rodA* deletion mutants show an increase in unviable minicells and cell lysis. Both phenomena could be caused by the frequent asymmetrical cell divisions observed, since asymmetrical division can often produce daughter cells lacking some of the essential components required to viability (Yulo et al 2025). Thus, this also suggests a direct link running between *rodA*, cell shape, cell division, and fitness.

2.3.2 *De novo* mutations in *ftsZ* and *min* operon recover fitness

Experimental evolution of *rodA* deletion mutants saw compensatory mutations favored by selection reaching high frequency. Compensatory mutations that provided a significant positive effect on fitness spread in each evolving population. By the end of the experiment, all the evolving lines recovered part of the fitness lost with the deletion, in some cases up to the level of the wild-type. Most evolving lines showed SNPs in the gene *ftsZ*, and duplications surrounding and including the operon *minCDE*.

FtsZ is the essential protein required to perform septation, dividing the daughter cells during cell division. It has been studied extensively in both *E. coli* and *B. subtilis*. FtsZ functions by polymerizing, through its GTPase activity, in a bundle of filaments around the circumference of the cell, around the middle of its length; the proteins ZipA and FtsA act as an anchor of FtsZ on the cell membrane (Bi & Lutkenhaus 1991, Lutkenhaus 2007, Moore et al 2016). The formation of this Z-ring in turn drives the localization and activity of the enzymes of the divisome, such as FtsW and PBP3. This allows for the cell wall synthesis to be directed inward, progressively separating the two halves of the mother cell (Lutkenhaus 2007).

The activity of FtsZ has to be guided to ensure the Z-ring is formed in the middle of the cell, leading to symmetrical division (**Figure 12**). This guidance is provided by a complex system, in which the proteins MinC, MinD and MinE prevents the polymerization of FtsZ outside the proper location. This works by the

antagonistic interaction of MinD and MinE. MinD localizes to the cell membrane at the cell poles, while MinE prevents it. This disperses MinD in the cell, promoting its migration from one pole to the other. When bound to the membrane, MinD acts as a carrier of MinC (Raskin & de Boer 1999). The localized high concentration of MinC at the cell pole prevents FtsZ polymerization (de Boer et al 1990). This happens by direct interaction of MinC dimers with FtsZ, leading both to sequestering of FtsZ and to favoring the disassembly of FtsZ polymers. The result is that the intracellular gradient of free FtsZ is highest in the middle of the cell, where the Z-ring can be assembled.

Deletion of *minC*, while not lethal, completely breaks this system (Adler et al 1967, de Boer et al 1989, Lutkenhaus 2007). Interestingly, while the MinCDE system was apparently already present in the last universal common ancestor (LUCA), some lineages lost either *minE* (as in *B. subtilis*) or *minC* (as in the chloroplasts), in some cases replacing their function with other genes while maintaining the rod shape. This demonstrate the evolutionary flexibility available to cells in maintaining fitness and shape, and the robustness and adaptability of the FtsZ-MinC system. Another level of regulation is provided by the balance of the anchors ZipA and FtsA, since an increase in their concentration prevents FtsZ monomers from interacting with each other and polymerize (Lutkenhaus 2007).

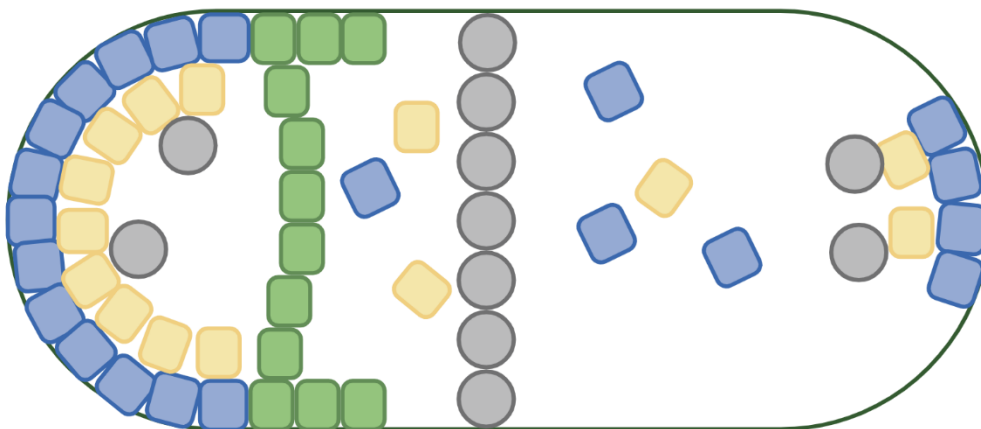


Figure 12: Schematic assembly of the Z-ring in *E. coli*. Blue: MinD. Yellow: MinC. Green: MinE. Grey: FtsZ. MinD localizes at the cell pole, together with MinC. MinE follows MinD along the cell membrane and promotes its separation from the membrane, leading to a migration of MinD and MinC across the cell to the other pole. The resulting local low concentration of MinC in the middle of the cell prevents it from disassembling FtsZ filaments. Image produced using Biorender.

2.3.3 *De novo* mutations in FtsZ affect its interaction with MinC

The structure of FtsZ is known and well characterized. This showed how FtsZ is homologous to eukaryotic tubulin; both can perform GTP-dependent assembly in filaments (Löwe & Amos 1998, Lutkenhaus 2007). Some of the compensatory SNPs in *ftsZ* identified in this work affect the homologous amino acids that previous work showed to be involved in the interaction with MinC. Specifically, the residues N280 in *E. coli* (homologous to S281 in *P. fluorescens*) and D255 in *B. subtilis* (homologous to Q256 in *P. fluorescens*) have been shown to be necessary to allow proper interaction between MinC and FtsZ (Blasios et al 2013, Shen & Lutkenhaus 2010). Therefore, the SNPs S281R and Q256P identified in evolved $\Delta rodA$ mutants suggest a change in affinity between MinC and FtsZ. Moreover, most of the *ftsZ* SNPs identified in this work are located in the region of FtsZ between amino acids 240 and 310, corresponding to helix 10 and the two loops between helix 9 and sheet 8 and between helix 10 and sheet 9; many mutations to this region are known to alter the strength of interaction between FtsZ and MinC (Machado et al 2024).

The other functional sites of FtsZ, which are the GTP binding site, the FtsZ-FtsZ interaction sites, the interaction sites with divisome proteins such as FtsA and ZipA, and the interaction sites between separate FtsZ filaments, looks to be mostly unaffected by the SNPs (Cameron et al 2021, Guan et al 2018, Pichoff & Lutkenhaus 2002, Wang et al 1997). GTPase activity of *P. fluorescens* FtsZ is mainly performed by the residues N208, D210, D213, situated at the interface between two FtsZ subunits when they form a polymer; there are no SNPs altering those amino acids. S281, while part of the interaction surface with MinC, also appear close to the FtsZ-FtsZ interface, potentially being involved in it. The interaction of FtsZ with FtsA and ZipA is mediated by the conserved C-terminal peptide of FtsZ, especially through the residues I392, F395, L396. No SNPs were identified in this region. No SNPs are identified in the residues involved in filament to filament interaction, such as those between the residues R79 and E156 and the residues R232 and D302.

While no SNPs were found in *min* genes, it has been shown that mutation of the MinC amino acids at the interaction site with FtsZ leads to decrease in MinC functionality if the mutant amino acids acquire a negative charge (LaBreck et al 2019, Machado et al 2024). Conversely, most of the SNPs in *ftsZ* here identified show a consistent change in the electric charge of the interaction site surface between FtsZ and MinC, with an overall increase of local positive charge and decrease of negative charge, suggesting that the FtsZ-MinC interaction is under selection by regulation of the electric affinity between the two proteins. Since no SNPs appeared in *minC*, it is possible that mutations of MinC, while potentially providing positive

regulation in the interaction with FtsZ, could also interfere with other protein-protein interactions, processes, and protein folding.

2.3.4 Conclusions

The compensatory mutations in *ftsZ*, together with the widespread duplications encompassing the *min* operon, suggest a scenario in which one of the driving causes of fitness loss experienced by the cell after experimental deletion of *rodA* is the misregulation of the septation process. This misregulation is caused by the loss of the rod shape phenotype which the cells use as a physical reference for localizing the proteins involved in cell division in the correct location.

The experimental evolution showed how this issue can be overcome by two main solutions: increasing the dosage of the Min proteins by *min* operon duplication, and changing the affinity between FtsZ and MinC by SNPs in *ftsZ*. Another, although rarer, mutation is the duplication of *ftsZ*. These three parallel solutions suggest that the preferred compensatory strategy adopted by evolved $\Delta rodA$ cells consists in tuning the likelihood of interaction between MinC and FtsZ. It is interesting to note that the *min* operon duplication can produce an increase of expression of all MinC, MinD and MinE. A possible explanation is that the resulting outcome of such duplication is for the probability of MinC to interact with FtsZ not to be the double as in the wild-type, due to the increase of both MinC and of the inhibitors MinD and MinE. But the higher concentration of proteins in the cell could lead to a higher frequency of interactions between the different components, which is maybe required for proper Z-ring assembly in the round-shaped cell, by slightly altering the balance of the reaction between FtsZ and MinC. Another potential explanation is that the mutant $\Delta rodA$ cell, being not only round but also enlarged and with a longer circumference than the wild-type, requires a higher expression of *ftsZ* and *min* genes to divide at the correct moment of the cell cycle.

Moreover, since the duplications of the *min* operon and *ftsZ* appear to decrease in frequency as the experiment progressed, while the frequency of *ftsZ* SNPs increased, it is possible that the increase in *min* genes expression caused by large-scale duplication is a first, easily accessible solution provided by a relatively frequent mutation. The duplication is then reverted when a clone further evolves by a rarer but more efficient mutation to a specific and more efficient SNP in *ftsZ*, as illustrated by the changes in frequency of the two categories of mutations between day 34 and 69 of evolution.

The adaptation of the cell to the loss of rod shape by changing the regulation of the Z-ring assembly is not without precedent. Different bacterial lineages are known to regulate the gradient and concentration-

dependent polymerization of FtsZ in different ways. For example, both *B. subtilis* and *Caulobacter crescentus* retain at least part of the *min* operon, but show no oscillation of MinC; other molecules and mechanism direct the septation to the middle of the cell (Lutkenhaus 2007).

Finally, the link between the loss of rod shape and regulation of cell division regulation is rooted in the shared evolutionary history of elongasome and divisome. This is evidenced by the homology between the effectors RodA in the elongasome and FtsW in the divisome, as well as their interaction with shared penicillin-binding proteins, and by the reliance on the same enzymes MraY and MurG to synthesize the peptidoglycan precursor lipid II (Szwedziak & Löwe 2013). In a previous experimental evolution study, the loss of *mreB* was compensated by *de novo* mutations to *pbp1A*, an aPBP (Yulo et al 2025). While MreB and RodA work together in the elongasome, it is interesting to note that MreB functionality goes beyond the elongasome, and is involved in other processes such as interacting with the divisome and in providing structural strength to the cell. The different compensatory mutations arising after the loss of either *rodA* or *mreB* could help elucidate how the phenotypic effect produced by the deleted genes overlap, and how they do not. As in the case of the present work, $\Delta mreB$ mutants showed round cell phenotype, coupled with asymmetric division and low fitness. Similarly to the evolved $\Delta rodA$ mutants, the evolved $\Delta mreB$ mutants managed to recover both fitness and symmetry, but not the rod shape.

An interesting detail is how, in the case of the *mreB* deletion, evolution selects for compensatory mutations altering proteins directly interacting with MreB such as Pbp1A, as opposed to the *ftsZ/min* operon mutations observed in evolved $\Delta rodA$ mutants. A possible cause for this difference is that RodA is the final effector of its own pathway, inevitably compromising it after deletion. This would force the cell to adapt to the loss of the pathway, instead of fixing it. On the contrary the loss of MreB, given its role as a structural support, could be overcome by mutations to other genes originally depending on it, such as *pbp1A*, in order to balance again the functionality of the elongasome. Pointedly, one of the evolved $\Delta mreB$ populations showed compensatory mutations in *ftsZ*, with the same D176A SNP observed in this work. This suggests that altering the regulation of the elongasome or of the divisome are both viable adaptive strategies to $\Delta mreB$ mutants, while only the second one is available to $\Delta rodA$ mutants. Another possibility is dictated by the fact that the loss-of-function *pbp1A* SNPs identified in evolved $\Delta mreB$ mutants are easier to access than gain-of-function mutations in *ftsZ*, increasing the probability for a cell to acquire them. It is possible that the deletion of *rodA* cannot be fixed by other, easily accessible loss-of-function mutations.

Overall, the results in this chapter show how the regulation of the elongasome and divisome remains interconnected, providing insights into the evolution of bacterial lineages that over time altered their regulation of cell wall synthesis, shape determination, and cell division.

CHAPTER 3 – Loss and recovery of fitness after ribosome disruption

3.1 INTRODUCTION

3.1.1 The ribosome is complex ribozyme

The ribosome is the cellular component that translates mRNA to synthesize new proteins. It reads the transcribed mRNA by matching the correct aminoacyl-tRNAs to their corresponding codons along the coding sequence of the mRNA. The ribosome then catalyzes the transpeptidation between the amino acid carried by the tRNA and the last amino acid of the growing peptide being synthesized. Besides its catalytic activity, the ribosome allows for interactions between multiple factors involved in the initiation, regulation and end of translation; it assists the folding of the growing peptide; it facilitates the translocation of new peptides towards their specific destination, such as the cell membrane or organelles.

The ribosome is a ribozyme composed of both rRNA and ribosomal proteins (r-proteins). It is divided in two components: a large subunit (LSU), also indicated as 50S due to its sedimentation gradient, and a small subunit (SSU), called 30S. In the SBW25 ribosome LSU there are 2 rRNA strands of respectively 23S and 5S and up to 34 r-proteins, while in the SSU there is one rRNA strand of 16S and up to 20 r-proteins. The whole ribosome is referred to as 70S (Garrett & Grisham 2009, Halfon 2019).

The rRNA is both the largest fraction of the ribosome mass and the main catalytic effector: the peptidyl-transfer center (PTC), where transpeptidation occurs to integrate new amino acids in the growing peptide chain, is entirely composed of rRNA. The same goes for the decoding center, where the codon-anticodon pairing takes place between the aminoacyl-tRNA and the mRNA. During its synthesis, the rRNA follows a complex folding process, resulting in a high number of loops and double stranded sections. Different domains of a rRNA tend to have different number of interactions with other each other, and with different numbers of r-proteins (**Figure 13, Figure 14**) (Noller & Chaires 1972).

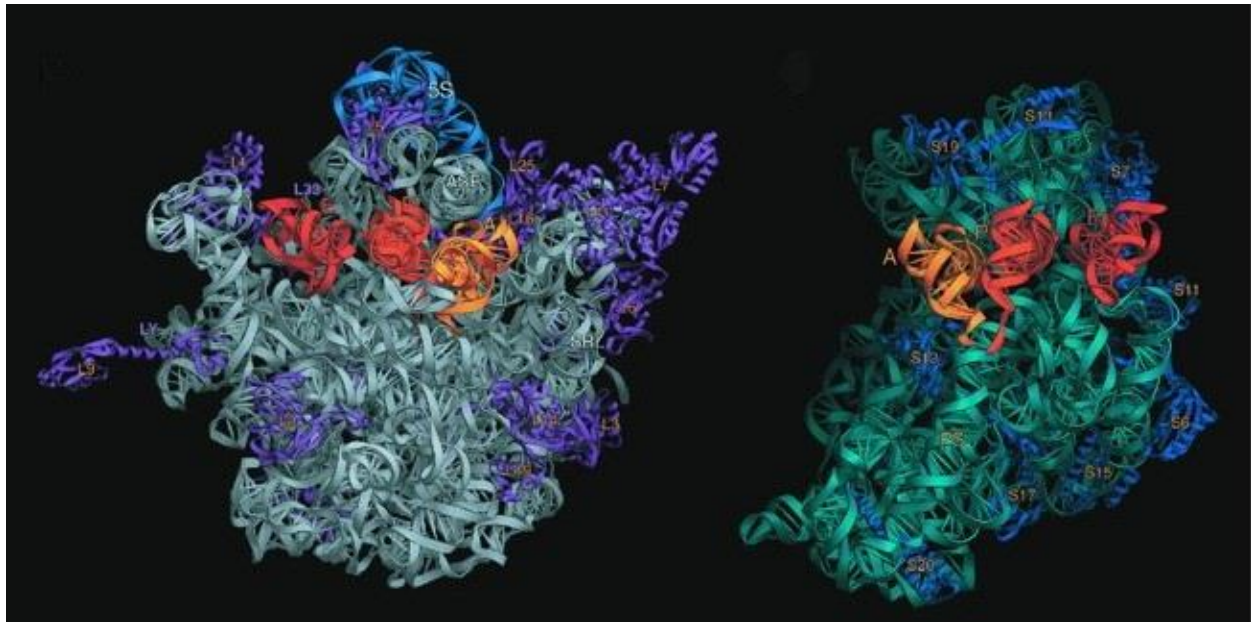


Figure 13: Structure of the ribosome subunits of *T. thermophilus*, the first organism for which the complete 70S ribosome structure was characterized. The two subunits are visualized as seen from their interface. Left: LSU. Right: SSU. Grey: 23S rRNA. Cyan: 5S rRNA. Green: 16S rRNA. Purple: LSU r-proteins. Blue: SSU r-proteins. Gold: A-tRNA. Orange: P-tRNA. Red: E-tRNA. From Yusupov et al 2001.

3.1.2 Ribosome components are highly conserved

The ribosome is highly conserved across all life forms, with some structural peculiarities specific to each domain of Bacteria, Archaea, and Eukarya. The rRNA sequence is extremely conserved, with the 16S sequence often used as the basis for phylogenetic trees between even evolutionary distant species. This is consistent with the fact that the most conserved part of the ribosome is the small subunit, at the intersection between tRNA and mRNA, together with the PTC and the interface between the two subunits (Hsiao et al 2009). These are the same structures of the ribosome where are located the 30-40 genes that constitute the minimal components (both RNA and proteins) of the ribosome, essential to enable the translational activity of the ribosome, as observed in studies of synthetic biology (Mears et al 2002, Mushegian 2005).

At the same time, r-proteins are also very conserved. It is possible to distinguish a set of universal r-proteins, common to every organism on Earth, and domain-specific r-proteins, which are unique to Bacteria, Archaea, or Eukarya (in this work, eukaryotic-specific r-proteins are indicated with the prefix e-, for example eL37). Within each domain there is also variability between different species, with events of

duplication, loss, and acquisition of r-proteins that can be used together with sequence-specific details to track evolutionary relationships. For example, all archaeal r-proteins are also found in the expanded eukaryotic ribosome, with the result that the archaeal ribosome is a smaller scale eukaryotic ribosome (Hartman et al 2005, Lecompte et al 2002, Londei & Ferreira-Cerca 2021).

One difference, possibly related to differences in regulatory mechanisms due to the higher plasticity and complexity of eukaryotic ribosome, is that bacterial r-proteins tend to be found in operons, with very rare duplication events; the opposite is true in eukaryotes. The arrangement of bacterial gene clusters also provides information on the evolution of the ribosome, with genes of similar ancestry usually being physically closer on the chromosome (even when not in an operon), in four different clusters: L2, L3, L4; L22, L23, L24; L5, L6, L10, L11, L13, L18, L29; L9, L31, L32-L34 (Lecompte et al 2002, Fox 2010).

It is known that the last universal common ancestor (LUCA) already had a functioning ribosome. Some parts of the rRNA tertiary structure can be traced back to LUCA, as is true for r-proteins (together with some essential elongation factors and tRNAs). Other rRNA structures and r-proteins were likely acquired later in different lineages. But it is fascinating to note how in the complete ribosome the most ancestral of both the rRNA sections and r-proteins are located deeper in the ribosome structure, where peptide synthesis occurs. As a consequence, the oldest proteins interact with the oldest domain of rRNA, and vice versa for the most recent ones, which interact with more recently evolved rRNA domains (Fox & Naik 2004, Hsiao et al 2009).

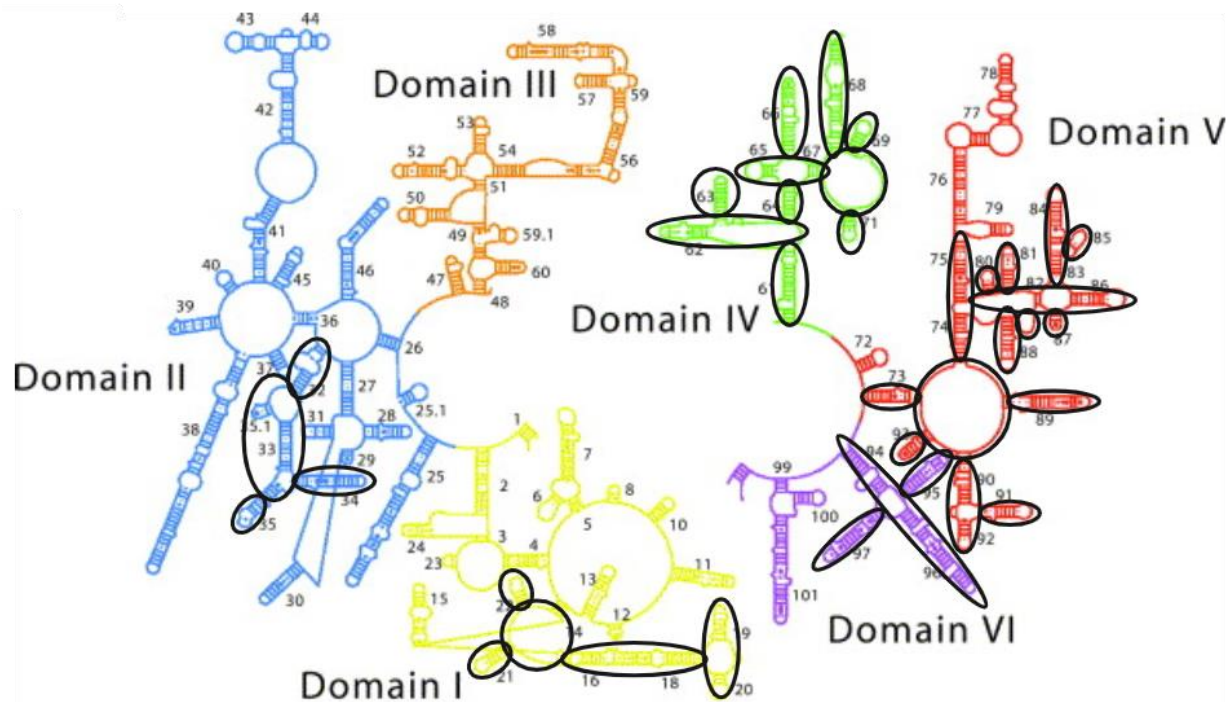


Figure 14: Interaction map of 23S rRNA in the ribosome of *Haloarcula marismortui*, one of the first and better characterized ribosomes. The six domains are indicated with different colors. The helices are indicated by number. The black circles indicate the regions more interconnected with other rRNA domains and r-proteins. Adapted from Klein et al 2004 and Hury et al 2006.

3.1.3 The ancestry of ribosome components informs as to function

Broadly, it can be said that rRNA structures and r-proteins that were acquired during evolution ended up being added as new layers, forming a structure of growing size and complexity (as in, higher number of components) (Hsiao 2009). This hierarchy is not only followed by the phylogenetic origin of the ribosome components, but also by their assembly: the less ancestral rRNA sections fold around more ancient ones, and r-proteins get into position in order, with more ancient r-proteins usually being the first to join the rRNA and newer r-proteins binding later in the process of ribosome assembly. (Fox & Naik 2004, Fox 2010, Hsiao et al 2009). This is consistent with the hypothesis that the ribosome originated as a purely RNA-based machine that performed non-specific catalysis, with proteins originating later, since r-proteins themselves are usually found more to the periphery of the full ribosome structure (Orgel 1968, Lecompte et al 2022). The ancient origin of the ribosome and its conserved features also provide information about when some structural motifs, like zinc fingers and leucine zippers, first appeared. This can be established

thanks to the high conservation of the ribosome, showing homologous genes between very distantly related species that both carry such motifs (Fox & Naik 2004).

Just as interesting are studies of r-proteins that have been lost or acquired following events of endosymbiosis. On average, the tendency for endosymbiotic organelles is to decrease in complexity, regarding both the number of components and of their gene expression regulation; most of the genes required by the endosymbiont are then moved to the nuclear chromosome of the host. This is consequence of both the consistent environment for the endosymbiont provided by the host cell, and of the need for the host to obtain control of the endosymbiont activity and reproduction (Gray 2012).

The complex process of adaptation of the ribosomes of mitochondria appear to have initially involved a series of acquisitions of r-proteins from the host, leading to an increased size and complexity of the mitochondrial ribosomes. This was followed by a dynamic evolution of the mitochondrial ribosomes across different eukaryotic lineages (Desmond et al 2010).

According to the differences between eukaryotic and bacterial ribosomes, the ribosomes of mitochondria and chloroplasts are smaller than their cytosolic counterpart, with fewer r-proteins than both the host and their free-living relatives, although in turn some r-proteins were acquired from the host. It has also been noted that r-proteins appear to be some of the last genes lost from the endosymbiont chromosome, while cytosolic r-proteins are more stable (Desmond et al 2010).

Opposite scenarios then emerge: some proteins, such as L25, are missing in chloroplasts but present in cyanobacteria, suggesting that the chloroplast lost them in the process of streamlining its genome; others, like eS31, are present only in chloroplasts but not cyanobacteria. These events raise questions about how selective pressure differed between endosymbiotic and free-living bacteria when it comes to the ribosome, and on which factors promoted the acquisition and loss of different r-proteins between different lineages (Desmond et al 2012, Yutin et al 2012).

3.1.4 Insights on ribosome structure

The ribosome plays a central role in the central dogma of biology. Despite this, it took a long time to produce a clear picture of its inner workings. It started from its discovery as a particle in the endoplasmic reticulum and its association with protein translation (Palade 1955), followed by the confirmation – at that time unexpected – that catalytic activity is performed not by r-proteins but by rRNA (Noller & Chaires 1972). This discovery helped to propel models for the origin of life known as the RNA world (Orgel 1968, Gilbert 1986).

The technical difficulties that had to be overcome for the study of such a complex molecular machine, composed of dozens of components, meant that it wasn't until the 21st century that it became possible to finally confirm the structure of the complete ribosome by X-ray crystallography, first in heat-resistant Archaea (Ban et al 2000, Schlünzen et al 2000, Wimberly et al 2000), then in *E. coli* (Noeske et al 2015), and finally in human cells (Khatter et al 2018).

Knowing the structure of the ribosome brought many benefits. Localization of the components allowed a better understanding of how they work together; other known important molecules involved in translation, such as tRNA and elongation factors, could be more easily mapped into the structure and function of the machine; new parts were identified. However, while the role of rRNA as the key component of translation has been elucidated, the functions of many r-proteins remain poorly understood.

3.1.5 Structure and function of ribosomal proteins

In parallel, thanks to the proteins for which a role has been identified, it is possible to determine which parts of the translation process are actively performed by proteins, instead of RNA. Over time it has become clear that the whole ribosome is required to ensure proper translation. This is evidenced by the massive r-protein reorganization and movement going on during tRNA translocation between sites in the LSU, with many proteins directly in contact with the tRNA. Many other SSU r-proteins interact with the mRNA, regulating both its entry and exit from the ribosome (Fox & Naik 2004, Korobeinikova et al 2012). Others, such as S4, S5, S12, are in direct contact with the decoding center; altering these proteins leads to a decrease in accuracy. Proteins like L5 and L18 provide a contact bridge between 23S and 5S rRNA in the LSU. Another group of r-proteins provide interactions that keep the two ribosome subunits together (Hsiao et al 2009, Klein et al 2004, Korobeinikova et al 2012).

The protein-specific functions that have been identified, and where these functions occur, align with what is known about how different regions of the ribosome have a different composition when it comes to the ratio of RNA to proteins. The LSU rRNA is evenly covered by proteins, except the PTC and the LSU-SSU interface, where the rRNA is more exposed (**Figure 13**) (Hsiao et al 2009, Klein et al 2004, Korobeinikova et al 2012). The regions binding translation factors are primarily composed of r-proteins, as is the exit tunnel of new peptides. This has direct functional role, since the r-proteins L7 and L12 are required to respond to elongation inhibition factors (Klein et al 2004, Korobeinikova et al 2012).

Despite the wealth of information now available, the study of r-proteins remains challenging. Many of these proteins are essential, and their genes are mostly packed together in few operons, together with other essential genes involved in the genetic information processing. These factors make genetic engineering of knock-out mutants and synthesis of in-vitro systems required for their study harder to obtain, for example by increasing the sensitivity of the cell to genetic manipulation in the region surrounding the r-protein gene of interest. For many r-proteins, all the information available now are only those that can be inferred by phylogenetic studies and localization in the ribosome structure (Kåhrström et al 2012).

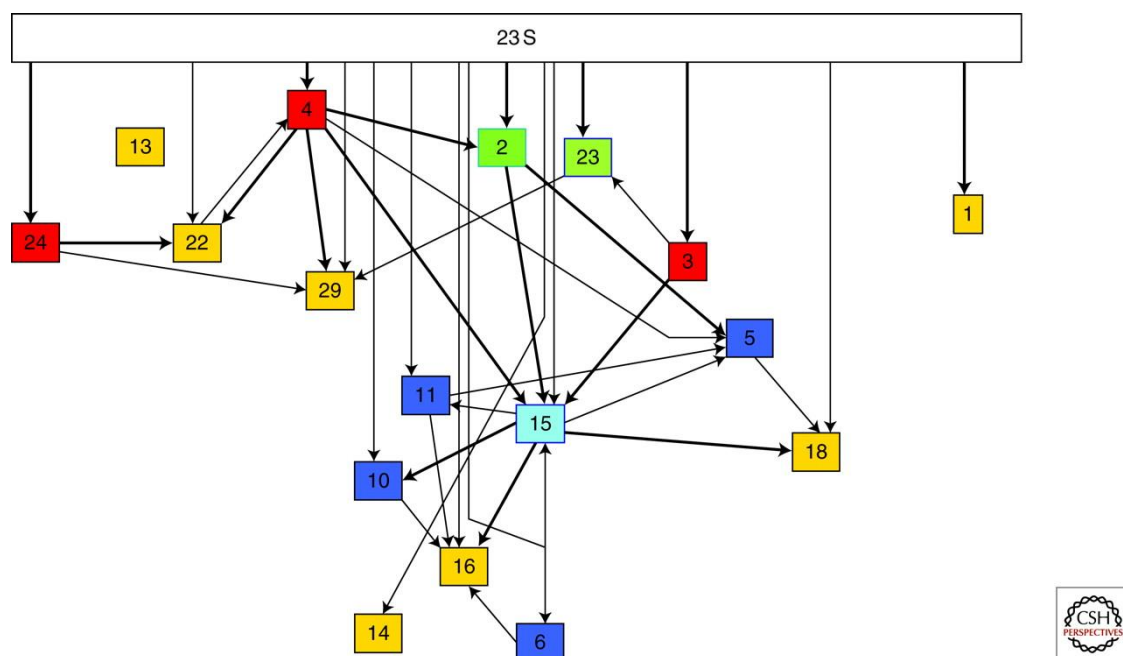


Figure 15: Order of assembly of r-proteins on the 23S rRNA in the LSU, from Fox 2010. Different colors represent the different order of assembly of each group of proteins.

3.1.6 Assembly and evolution of the ribosome go hand in hand

The study of ribosome structure is strictly linked with the study of phylogeny, and this is clear when the assembly process and the interactions of various components are taken into consideration. Given the complexity of the ribosomal structure, it is no surprise that the assembly process evolved both to be highly regulated and very efficient. Directing the assembly itself and to act as a scaffold to ensure correct folding of rRNA is one of the main functions of r-proteins (Klein et al 2004). Various studies exploited all these

characteristics to infer the ancestry of r-proteins: universality, order of assembly, number of interactions with other r-proteins, essentiality in promoting the assembly of other r-proteins. A common feature is how more recently evolved proteins are less critical for the assembly itself (**Figure 15**) (Fox & Naik 2004, Fox 2010).

The evolution of ribosome components is constrained by the already existing network of interactions that makes the whole machine possible in the first place. This constraint is clearly visible in cases of convergent evolution. This usually happens to maintain a certain number of interactions around a rRNA domain, ensuring its folding and providing structural support. This shows how evolution can maintain rRNA positioning. For example, the eukaryotic r-protein eL44 originated from a duplication of the universal r-protein L33, but then ended up occupying the same physical space in the structure that in bacteria is taken by L28. In other cases, proteins of different origin and even different structure evolve to occupy the same homologous spot and interact with the same components. Such examples are L27-eL21, L33-eL44 (Kovacs et al 2018, Korobeinikova et al 2012, Fox 2010).

More intriguing are examples of r-proteins like L16, whose folding creates a structure that simulates an rRNA helix. This structure can interact with the tRNA in a way similar to RNA-RNA interaction, suggesting that maybe the r-protein actually evolved to replace an ancestral, and now lost, rRNA domain. (Smith et al 2008). Besides these interesting exceptions, different proteins have different proportions of their surface in direct contact with rRNA, with some, such as L15 and L37e, having protrusions mostly buried into the rRNA. While many r-proteins are elongated or with protrusions, some are more globular. The protein-RNA interactions are often mediated by recognition of functional groups exposed in the RNA minor or major groove; others are based on recognition of bulged/flipped bases or insertion of nonpolar amino acids in hydrophobic binding pockets in the folded RNA (Klein et al 2004). During the assembly process, the proteins typically first bind to the growing ribosome with their more globular domains; this is then followed by folding of protein extensions into position, increasing the contact surface. This tail often extends deeper into the ribosome, in some cases directly contacting functional rRNA centers. (Harms et al 2001, Klein et al 2004, Korobeinikova et al 2012).

3.1.7 Assembly of the complete ribosome is a complex, regulated process

The assembly of the ribosome begins as soon as rRNA transcription starts. From *in vitro* experiments, it is known that several r-proteins, in particular, L3, L4 and L24, quickly bind to the growing rRNA 23S. This first interaction promotes both the correct rRNA folding, and it facilitates further integration of the other r-proteins in the structure. The proteins L3, L4, L13, L20 and L24 are essential for the formation of the 50S

subunit (Fox 2010, Klein et al 2004, Spillmann et al 1977, Spillmann & Nierhaus 1978). Both L3 and L4 are, with other r-proteins, in contact with the PTC. It has been suggested that the PTC in the LSU is the more ancestral part of the ribosome, because the rRNA self-folds there, with the structure stabilized by Mg^{2+} ions rather than by amino acid interactions provided by proteins in the other parts of the ribosome. This does not happen for example in the other candidate for the older component of the ribosome as suggested by just sequence phylogeny, which is the decoding center in the SSU. The proteins surrounding these two sites reflect this distinction: those around the PTC are universal, those around the decoding center are not (Hsiao et al 2009, Smith et al 2008).

Overall, the 50S subunit is assembled through four steps. In each step, different r-proteins join the growing ribosome. Some parallel assembly pathways evolved after LUCA, with all of the r-proteins of such pathways present only in some lineages of a domain from the tree of life. Besides using the analysis of sequence distance, it is possible to infer the relative age of the proteins both by their relative incorporation in the ribosome, and by how many downstream integrations they facilitate, as more ancestral r-proteins tend to be nested deeper in the ribosome and to provide more interactions with other r-proteins and rRNA sections. In evolutionary instances when one of these facilitator r-proteins are lost, usually the downstream proteins are lost as well (Fox & Naik 2004). This entire process is sequence-specific. Mutations in the rRNA can prevent the correct assembly of the ribosome, even in cases where the mutation is still permissive of translation *in vitro* (Briones & Amils 2004).

3.1.8 Ribosomal gene evolution is affected by multiple constraints

Given the constant need to translate new genes, every cell requires a high number of ribosomes. Their abundance and high complexity mean that the synthesis of new ribosomes consumes a significant portion of the metabolic reserves of a cell, so much so that ribosome biogenesis is the limiting process in cell division. Overall, around 50% of cellular energy expenditure in *E. coli* is invested in producing new ribosomes; this is part of the total 40% to 90% needed for protein synthesis. This means that in an exponentially growing bacterium, rRNA is 86% of the total RNA, and ribosomes make 30% of the biomass. The number of ribosomes in a cell is directly correlated to growth rate, but there are big differences between different growth conditions (Bowman et al 2020, Briones & Amils 2004, Hidalgo et al 2022, Kafri et al 2016, Maaløe et al 1966, Maaløe 1979, Scott et al 2014). Additionally, the rate of protein synthesis for unit of ribosomes is not constant, but decreases at lower growth rates; the same is true for tRNA and elongation factors (Churchward et al 1982, Kjeldgaard & Kurland 1963). All of these factors put a great

selective pressure in the optimization of ribosome components (both rRNA and r-proteins) to maintain efficiency, output, and regulation.

In this context, it can be surprising that some r-proteins, while highly conserved, are simultaneously non-essential. Many, as for example L9, S21, L30, eL7, have been repeatedly lost in multiple lineages. In some cases, it is possible to observe a drastic change in the number of r-proteins, like the set of 15 that separates the last eukaryotic common ancestor (LECA) from Eubacteria. Other r-proteins have been repeatedly acquired, probably by horizontal gene transfer, like eL7, S31. In the opposite case, some r-proteins are not only essential, but their structure and folding are always very similar, without acquiring or losing any additional domain, such as L1 or L29 (Desmond et al 2010, Yutin et al 2012). Knowing mutations that can follow the loss of r-proteins can provide cues concerning their roles in the ribosome. All of these changes can affect how the r-protein network can optimize the interconnection of the functional centers of the ribosome. This network itself is conserved enough to be traced back to LUCA (Timsit et al 2021).

An open question is the influence of the ribosome dual composition as a ribozyme composed of both proteins and RNA on ribosome evolution (Yao et al 2019). To keep the correct stoichiometric balance of rRNA and r-proteins, cells must coordinate its biosynthesis. This is shown by the fact that different r-proteins, despite their high number and different localization on the chromosome, are all expressed at around the same level (Shoji et al 2011). A first regulation is provided by the fact that many r-proteins self-regulate their own expression: free r-proteins, not part of a ribosome, can bind their own mRNA and suppress its translation (Ni & Buszczak 2024). Another layer of regulation is the amount of translated protein obtained from each transcript: r-protein genes are peculiar because represent some of the few genes that, to achieve the required expression level, are highly transcribed, but with relatively lower level of translation produced from each transcript (Frumkin et al 2007, Hausser et al 2019). Consistently, also the gene copy number of different r-proteins is usually even, with only one copy of each r-protein gene per genome, and few paralogs (Yutin et al 2012). While most organisms have a single copy of each r-protein gene, that's not the case for rRNA: in bacteria, the three rRNA genes are usually found all together in one operon, of which there are multiple copies in the DNA; different species have different amount of copies. It's been shown that in bacteria the copy number of rRNA genes is correlated with a higher optimization for growth in high-nutrient situations, while a low copy number comes with more efficient use of the resources. Similar constraints have been shown to affect evolution of tRNAs (Hidalgo et al 2022, Raval et al 2023).

The discrepancy in copy number between genes of rRNA and r-proteins might depend on the fact that, while every r-protein gene can be translated multiple times by each transcribed copy of the corresponding mRNA - resulting in an exponential output of gene expression to provide one copy of r-protein for each ribosome - there is need to transcribe a new copy of each rRNA gene for every new ribosome. Cells address this issue by putting the transcription of rRNA under control of some of the strongest promoters available, that are usually conserved. (Hidalgo et al 2022). Despite this, the differences in structure, copy number and synthetic pathway of the ribosome components could still affect the evolutionary paths available. Especially the loss of rRNA operons, while optimal in some circumstances, limits the possible routes of gene expression alteration by for example SNPs to weaken the operon promoter. Moreover, differences in chromosome localization can still alter the transcriptional activity of the remaining copies (Hidalgo et al 2022). In fact, the danger for the cell caused by stoichiometric unbalances of proteins that are part of a complex is in general proportional to the size, complexity and number of required balancing interactions of the complex (Liang et al 2008).

This becomes biologically relevant when ribosome-targeting antibiotics are considered. In almost all cases, the mechanism of action of the antibiotic involves binding to the domain V of rRNA 23S, in the PTC. In the same way, the mechanisms of resistance often involve mutations to the rRNA, not to r-proteins. Consequently, for bacteria the available mutational space of ribosome components is of extreme importance (Schlünzen et al 2001). Studying adaptation to alteration in the r-protein set in different species and strains with different rRNA genes copy number could help understand these evolutionary constraints.

3.1.9 Deleted r-proteins

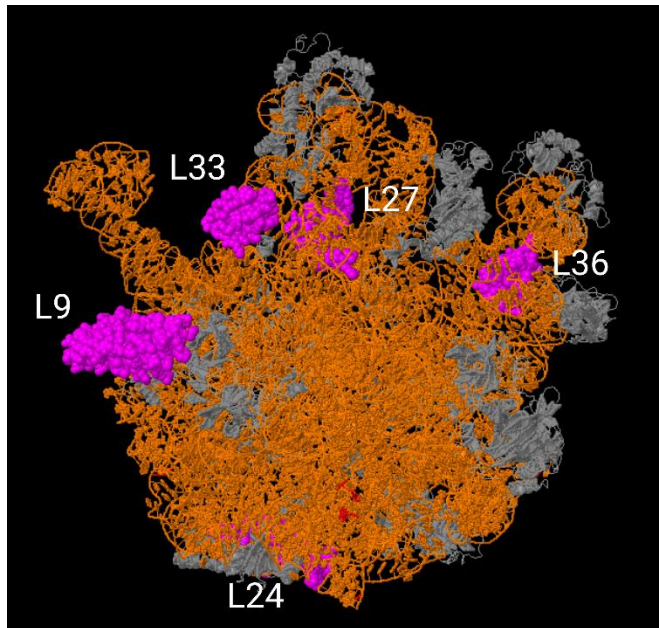


Figure 16: The r-proteins deleted in this work, visualized in the 50S subunit. Orange: 23S and 5S rRNA. Grey: r-proteins. Pink: deleted r-proteins. Image produced in Geneious from the crystallographic structure of the ribosome of *Pseudomonas aeruginosa* in Halfon et al, 2019.

In this work, I followed an evolutionary cell biology approach to study the evolution of the ribosome in the context of r-protein loss. This approach has already proved useful in previous studies (Tobin et al 2010), but it hasn't been applied in the model organism *P. fluorescens*, nor has it involved the deletion of the r-proteins considered in this work.

The first goal was to confirm whether it is possible to produce viable mutants of *P. fluorescens* SBW25 with deletion of r-proteins. To do so, I first investigated which r-protein genes would be good candidates for deletion. The choice was informed by different approaches:

- 1- Genes that have already been successfully deleted in other organisms. In particular, libraries of viable knock-outs that have been produced in *E. coli* and *B. subtilis* (Akanuma et al 2010, Shoji et al 2011). In other experiments, single r-protein genes have been deleted or inactivated by directed mutagenesis (Kåhrström et al 2012, Shirakawa 2023, Tobin et al 2010).
- 2- Genes that have been repeatedly lost through evolution. A number of r-proteins have been lost in different lineages, suggesting that at least in some circumstances they become non-essential.

Especially species with very small genomes, show a huge loss in their r-protein set, like in *Mycobacterium tuberculosis*, and in the mitochondrial ribosome.

- 3- Genes that can be inactivated by transposon (Tn) insertion in *P. fluorescens*. A previous experiment in our lab by Dave Rogers and Joanna Summers produced a library of mutants with random insertions of the transposon Tn5. Since gene expression can't occur at the insertion site, this provides a map of genes that are putatively non-essential.
- 4- Genes of different ancestry. While much is unknown about r-proteins, I was interested to delete genes that originated at different times, so to study how the cell reacts after the loss of r-proteins that are more or less universally shared between living organisms.
- 5- Genes that are not shared between separate lineages. As a future potential research venue, I remain interested in studying r-proteins that can be deleted both in a bacterium and an archaeon. This could show how very distantly related organisms adapt to the same gene lesion. Moreover, some non-related r-proteins appear to have evolved in parallel to fulfill the same structural role in both Bacteria and Archaea.

The work presented in this thesis is based on the successful deletion of the genes *rplI*, *rplX*, *rpmA*, *rpmG*, *rpmJ*, which code for, respectively, L9, L24, L27, L33, L36 (**Figure 16**).

L9

L9 is a primary rRNA-binding protein. It binds at domain V of 23S rRNA, at two separate sites separated by helix 76, close to L1, L2, L16, L19, L28. It appears to be involved in mRNA hopping, during which the ribosome bypass around 50 nucleotides in phage genes (Lillemoen et al 1997, Nag et al 1991). It is not required for peptidyl transfer activity, and it's non-essential in *E. coli* and *B. subtilis* (Akanuma et al 2012, Hampl et al 1981, Shoji et al 2011).

The non-essentiality of L9 is highlighted by the fact that it's been lost many times in mitochondria, endosymbionts like *Mycoplasma tuberculosis*, and other lineages such as *Aspergillus*, *Schizosaccharomyces*, *Chlamidomonas*, *Cyanidioschyzon*. This is consistent with the fact that it is part of the last stage of ribosome assembly. It was also successfully tagged with GFP without negative effects on growth (Desmond et al 2010, Fox & Naik 2004, Meteleev et al 2022).

L24

L24 is part of the peptide exit tunnel. It is nested in the domain I of 23S rRNA, binding in the first 480 transcribed nucleotides. There, L24 first tightly interacts with its own globular domain and is one of the

first r-proteins that gets assembled on the rRNA in the first ribosome assembly step. After folding of the rRNA, the tail of L24 also gets in position around the rRNA. It is essential for the assembly of 50S subunit, especially for the integration of L29 and L22, but it is dispensable later on and it does not appear to be directly involved in translation (Fox & Naik 2004, Klein et al 2004, Spillman & Nierhaus 1978).

When not integrate in a ribosome, the free L24 protein localizes at cell poles during stationary phase, together with naked 23S rRNA and complete ribosomes. It is hypothesized that this is caused by the localization of both r-protein and rRNA operons close to the *oriC* DNA replication starting site. Overexpression of L24 disrupts nucleoid segregation during cell division. It can also bind non-specifically to DNA, preferring supercoiled double helices (Exley et al 2000, Zouine et al 1996).

L24 is non-essential in *E. coli* but the deletion increases temperature sensitivity and fitness. The fitness decrease can be partially recovered by compensatory mutations to S11; mutations to 23S rRNA can also partially recover the heat-sensitive phenotype caused by the deletion (Nishi et al 1985, Nishi & Schnier 1986, Shoji et al 2011). Nonetheless, it was lost both in minimal endosymbionts like *M. tuberculosis* and in mitochondrion of LECA, being coded for in the nucleus (Desmond et al 2010).

L27

L27 is very close to different functional centers: it is one of the few r-proteins on the LSU-SSU interface, and its tail is in contact with the peptidyl-transfer center. Its structural homologue eL21 does not have this contact to the PTC, suggesting that the role is still non-essential. Still, it regulates ribosome catalytic activity by placing the 3' end of aminoacyl-tRNAs close to the PTC (Bischof et al 1995, Korobeinikova et al 2012, Harms et al 2001). The non-essentiality in *E. coli* was proved, and expression of only a truncated L27 partially compensate for the loss of fitness after deletion (Shoji et al 2011). The mitochondrial L27 is usually coded in the mitochondrial chromosome, although in some eukaryotic lines has been moved to the nuclear genome (Desmond et al 2010).

L33

L33 is in a small operon with L28. Its deletion in *E. coli* leads to no reduction in growth rate, while deletion of L28 prevents ribosome assembly (Maguire & Wild 1997). In eukaryotes L33 was duplicated, producing eL44 (Kovacs et al 2018). Already at the time of LECA, the gene of L33 was already integrated in the nuclear genome. It was then lost in some eukaryotic lineages, like the molds *Neurospora* and the parasites *Plasmodium*. In other lineages has been acquired by horizontal gene transfer. In general, it appears its assembly line in the ribosome evolved post-LUCA (Desmond et al 2010, Fox & Naik 2004).

L36

L36 is specific and universal to bacteria. After the endosymbiosis of the mitochondrion, already at the time of LECA its gene ended up in the nuclear genome. It then got lost in *Plasmodium*, *Thalassiosira* and *Leishmania*, while other lineages acquired it by horizontal gene transfer. The archaeal ribosome has a hole in its place. Its basic residues are conserved, as is a CCCH zinc finger motif. It's non-essential in *E. coli*, but the deletion leads to 50% decrease in growth rate. The deletion leads to structural perturbation of the inner ribosome, including the PTC (Desmond et al 2010, Fox & Naik 2004, Maeder & Draper 2005). While deletion doesn't affect the expression of adjacent genes, it alters those of many others. This differential gene expression leads to increased levels of proteins involved in translation and ribosome assembly, while decreasing those involved in anaerobic metabolism, stress response and nitrogen metabolism. The deletion also increases sensitivity to ribosome-targeting antibiotics, such as tetracycline and to a decrease in translational accuracy (Shirakawa 2023).

3.2 RESULTS

3.2.1 Deletion of r-protein genes

All deletions were performed separately on SBW25 MPB28225 by two-step allelic exchange, replacing the wild-type allele with the desired mutant allele carrying the full knock-out provided from a plasmid donor. This process makes it possible to engineer a strain that carries only the desired mutation, without leaving in the genome any selection gene or transposon unless desired. All genes were completely deleted, to ensure the cells couldn't recover their function just by reactivating them. The knock-out were confirmed by gel electrophoresis and Sanger sequencing of the junction point to confirm deletion of the desired genes. Importantly, all the successful deletions resulted in viable mutants, proving that it is possible to completely delete r-proteins in *P. fluorescens* by targeted mutagenesis, even for genes that are part of a cluster of r-proteins genes. The mutants were then tagged by Tn7 transposon insertion from a plasmid provided by conjugation from a donor *E. coli* culture, with alternatively GFP and kanamycin resistance or mScarlet and gentamycin resistance (**Table 3**).

Table 3: Mutants in which r-proteins have been deleted.

MUTANT	ID	Tag
Δ L9 GFP	MPB29185	GFP, Km
Δ L9 RFP	MPB29186	mScarlet, Gm

Δ L24 GFP	MPB30782	GFP, Km
Δ L24 RFP	MPB30781	mScarlet, Gm
Δ L27 GFP	MPB30867	GFP, Km
Δ L27 RFP	MPB30556	mScarlet, Gm
Δ L33 GFP	MPB29183	GFP, Km
Δ L33 RFP	MPB29184	mScarlet, Gm
Δ L36 GFP	MPB30841	GFP, Km
Δ L36 RFP	MPB30852	mScarlet, Gm

Column 1: Mutant name. Column 2: Mutant Id. Column 3: Fluorescence protein and antibiotic resistance gene tag.

I then proceeded to measure fitness of the resulting ribosomal mutant strains. In this work, fitness is measured as competitive fitness of the mutant against the ancestral type. It is measured by comparing the relative growth of mutant and SBW25 over a period of time.

I found that all r-protein deletion mutants showed decreased fitness compared to SBW25 (**Figure 17**). Interestingly, different deletions led to different degrees of fitness impairment. The fitness decrease correlated with the ancestry and localization of the gene deleted, with deletion of r-proteins more ancestral and localized deeper in the ribosome producing a stronger effect.

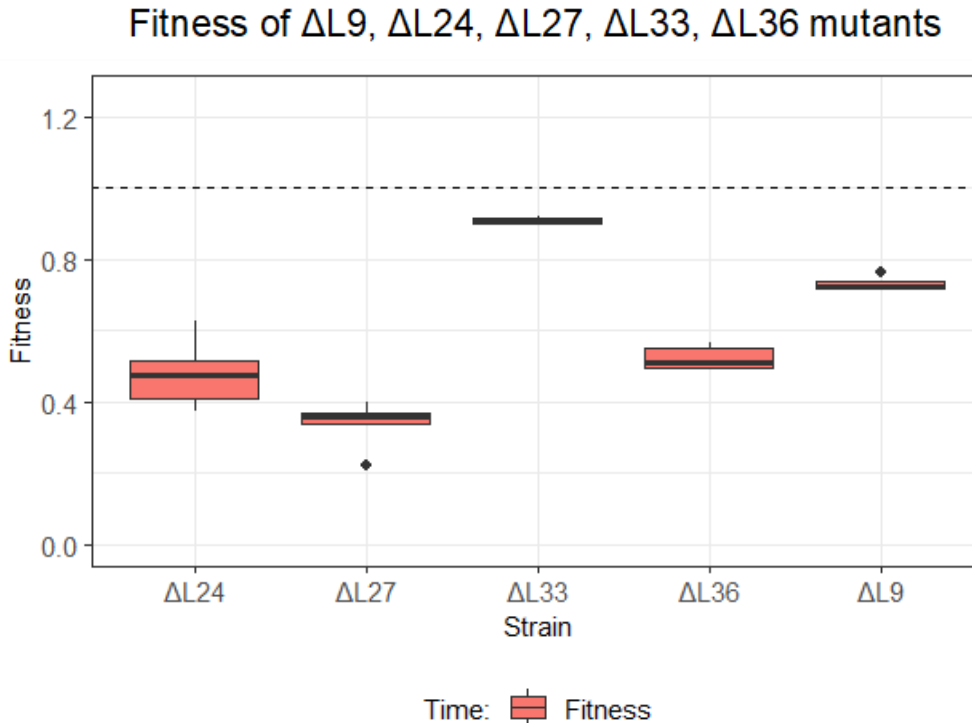


Figure 17: Fitness of r-protein deletion mutants tagged with mScarlet. X-axis: strain. Y-axis: competitive fitness against SBW25. Data are means from 5 biological replicates. The colored boxes indicate the values between the first to third quartile. Error bars indicate the maximum and minimum values. Outliers are indicated as dots.

3.2.3 Experimental evolution allows fitness recovery

Having confirmed that our r-proteins deletions are viable and that the mutations have phenotypic effects on fitness, I performed experimental evolution to see whether selection might favor mutations that compensate for deleterious fitness effects. This was done by propagating each mutant line by serial transfer and asking whether fitness was restored.

The setup consisted in 10 parallel evolving populations for each single r-protein deletion mutants (5 tagged with GFP, 5 with mScarlet). Each population went through a daily cycle of inoculation in new medium by 1:1000 dilution and overnight growth. Once a week, the populations were sampled to repeat the fitness measurement. The experiment proceeded for 56 days, which is approximately 560 generations. Populations derived from the $\Delta L27$ GFP mutant went through only 28 days of evolution due to contamination in later phases.

As measured by the repeated fitness assays, most evolving lines recovered fitness through the experiment, in some cases almost or at the level of SBW25, even in cases where the deletion carried by ancestral mutant caused a fitness decline down to around 40% of SBW25 (**Figure 18**). This result proved that, after the loss of non-essential r-proteins, bacteria can quickly recover fitness.

Fitness recovery over time of evolved r-protein deletion mutants

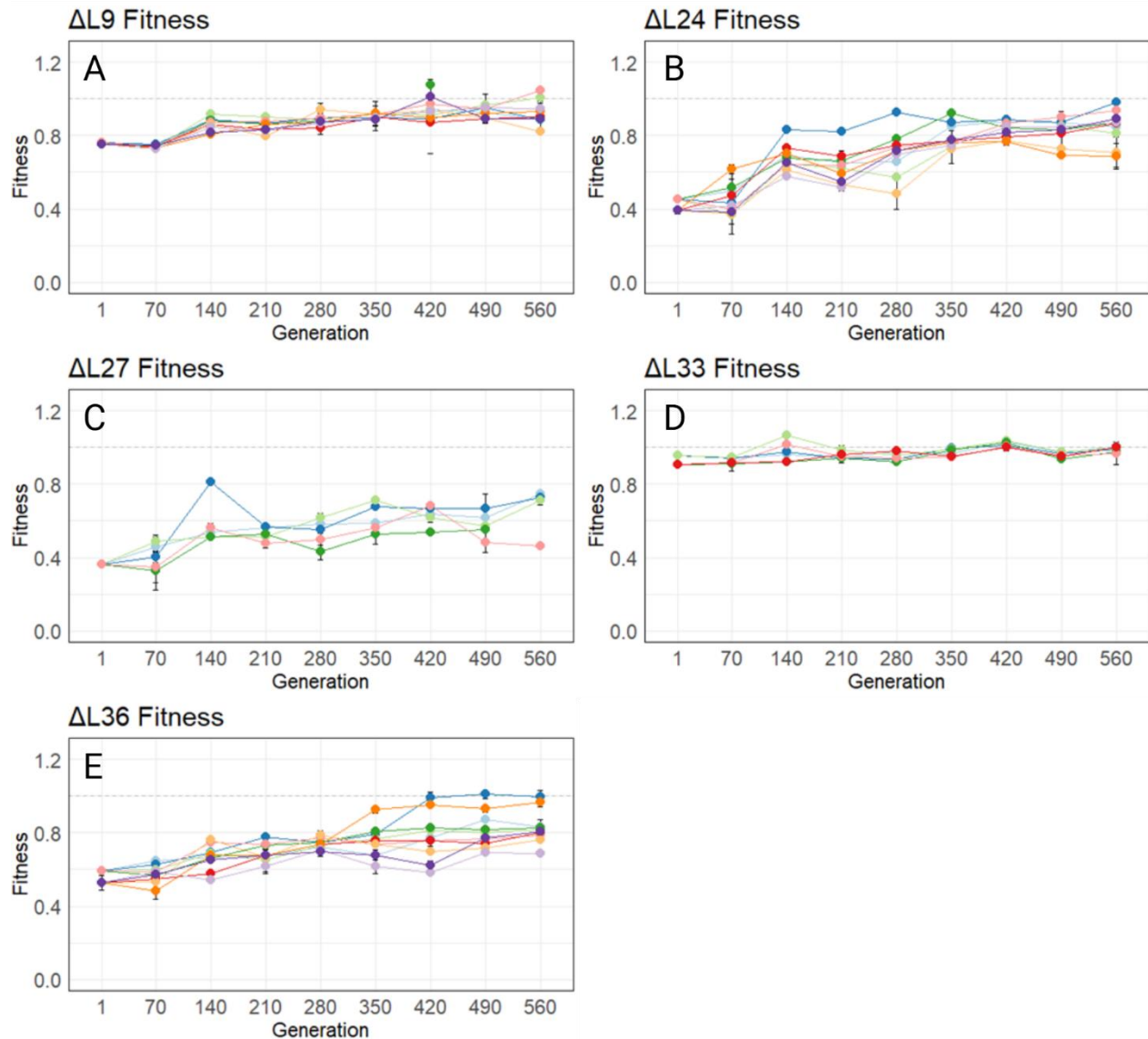


Figure 18: Fitness compensation during evolution of r-protein deletion mutant populations. Each line corresponds to a different population. X-axis: cell divisions since the beginning of the experiment (10 generations/day). Y-axis: Competitive fitness against SBW25. A: $\Delta L9$ 10 populations. B: $\Delta L24$ 10 populations. C:

ΔL27 5 populations. D: ΔL33 10 populations. E: ΔL36 10 populations. Each measurement was repeated two times from independent biological replicates, and is indicated with the mean plus and minus the standard deviation.

3.2.4 Genome sequencing of evolved mutants identifies *de novo* mutations

I wanted now to uncover how the evolved populations recovered fitness. Following literature and previous experiments in our lab (Yulo et al 2025), the hypothesis was that this was due to the appearance and spread in the populations of *de novo* mutations that compensated for the originally deleterious fitness effects.

To investigate this, the genomic DNA of evolved populations was extracted at two timepoints, day 28 and day 56. The DNA was then sequenced using Illumina technology, and the resulting reads mapped to the ancestral SBW25 genome in order to find mutations that distinguished derived from ancestral mutant types. The DNA was extracted by the whole populations. Clones carrying adaptive mutations will be more successful and their frequency in a population will increase. This leads to the fact that the mutations present in a population have their frequency proportional to how adaptive they are.

The analysis focused on the mutations that appeared at higher frequency, and especially those that fixed during the course of the selection experiment. Interestingly, some genes appeared to be target by the same compensatory mutation across different evolving lines, in some cases even in lines descending from different ancestral r-protein deletions.

Many populations acquired mutations in genes that are known to be under selection in *P. fluorescens* in this kind of experimental regime, such as *sucA*, *purA*, *aspA*, *mgtE*, and the operons *wss* and *wsp*. Many of these are involved in nutrient acquisition, aerobic metabolism, and cellulose synthesis. Many mutations can increase fitness of *P. fluorescens* by providing adaptation to this experimental regime, independently of their interaction with the r-protein deletions, and spread in the population. In this work such mutations were excluded from further analysis in order to focus on genes more directly involved in the ribosome function.

The hypothesis that some *de novo* mutations provide an advantage by specifically compensating for r-protein gene deletions is based on the function of the genes target of mutation. Additionally, such mutations don't appear in other experimental evolution tests performed in the lab on different *P. fluorescens* mutants. Finally, no compensatory mutations are present at high frequency in evolved ΔL33

populations, which are still growing at ~95% fitness after the gene deletion, consistent with a ribosomal activity not requiring adjustments. No duplications or large-scale genome rearrangements were detected. The attempt to genetically reconstruct *de novo* mutations of interest in the corresponding ancestral mutant background to confirm their effect on fitness failed. Evolved clones carrying such mutations could still be isolated, and their fitness was measured in Chapter 4.

3.2.5 *De novo* mutations in evolved Δ L9 mutants

De novo mutations in evolved Δ L9 mutants

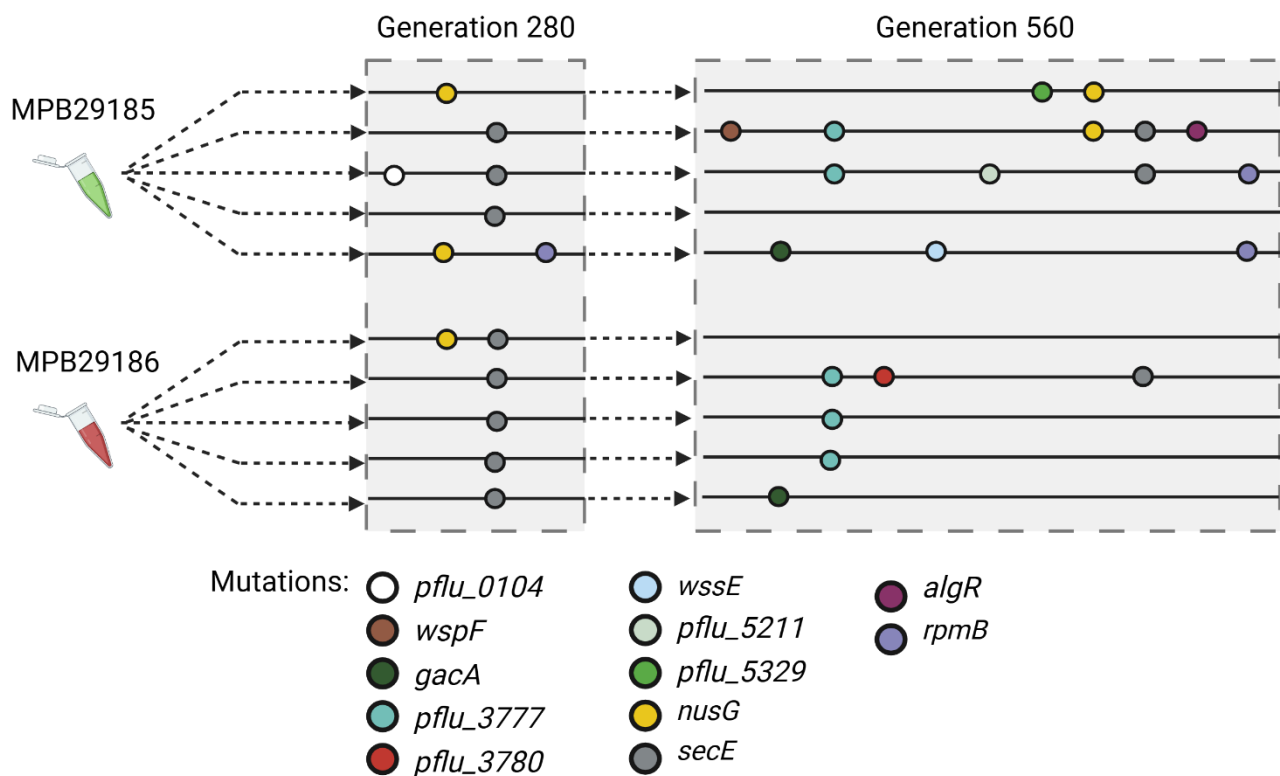


Figure 19: *De novo* mutations in evolved Δ L9 mutants. For each population, all genes with at least one mutation above 30% of frequency in at least one population are marked. In the left box are the mutations identified in populations evolved 280 generations; in the right box are the mutations at 560 generations. Colored circles represent SNPs to different genes. The order of the genes in the graph follow the order of the genes in *P. fluorescens* chromosome. The full list of SNPs and their chromosome positions are in supplementary materials (Supplementary Table 4).

The most common *de novo* mutations appeared in the *secE-nusG* operon (**Figure 19**). SecE is a protein of 123 amino acids and is part of the SecYEG translocon complex (van den Berg 2004). The translocon is a protein complex that helps to bind the translating ribosome to the cell membrane in order to allow secretion of new translated peptides. The role of SecE is to regulate the opening and closing of the membrane channel, but it doesn't appear to directly bind to the ribosome (Nijeholt et al 2013). The *de novo* mutations we found (I116V, I120V) are located at the C-terminal domain of the protein. The amino acids I116 and I120 are not known to perform specific functions, nor to be necessary to bind to other proteins in the complex (Nijeholt et al 2013). The structural model of the wild-type and mutant proteins was predicted on SwissModel (Waterhouse et al 2018) using the wild-type *E. coli* protein structure (from van den Berg et al 2004) as a template. This analysis shows that the mutations don't appear to alter the secondary or tertiary structure of the protein.

nusG is an essential gene ubiquitous in prokaryotes. It codes a 178 amino acids-long translation regulator. Its activity is performed by acting as a bridge between the ribosome and the RNA polymerase. By binding to both the ribosome on one side and to the RNA polymerase, the growing RNA, or other transcription factors on the other, it can affect the speed of both transcription and translation. Its function varies between different species (Eckart et al 2024, Ingham & Furneaux 2000, Werner 2012); moreover, it is known to affect differently the transcription of mRNA and rRNA (Wang & Artsimovich 2021). As in the case of *secE*, the mutations we found (R20P, I38N, W5C) do not appear to affect amino acidic residues with a known function, nor to alter the folding of the mutant protein compared to the wild-type models predicted on SwissModel (based on *Aquifex aeolicus* NusG crystal from Steiner et al 2002) (Waterhouse et al 2018).

The *nusG* and *secE* are in an operon of 912 bp and separated by 9 bp. 4 different mutations were found in the coding regions of the two genes closer to their spacer. From analysis on the software SAPPHIRE.CNN, each of the mutation decrease the probability of the region to act as a promoter for *nusG* (Coppens et al 2022).

Some evolving lines showed multiple mutations in this operon at different frequency. To check if they were evolving sequentially in a single clone or independently one from each other in separate clones, the single reads from the Illumina sequencing were analyzed. Since all the mutations are located at a very short distance from each other, some of the 150 bp-long reads were covering multiple compensatory mutation sites at the junction between *secE* and *nusG* (**Figure 20**). Interestingly, no reads contained more

than one mutation. At the same time, in some evolving populations with multiple mutations to the *secE-nusG* operon, almost 100% of the reads contain at least one SNP in either *secE* or *nusG*.



Figure 20: Illumina reads mapping on the *secE-nusG* operon, from DNA extracted from Δ L9 evolving population line B. The SNPs causing the *nusG* Y6C and *secE* I120V are highlighted in dark grey and blue respectively.

These results together (SNPs in residues with no functional role, and with an effect on promoter activity) suggest that the main effect of the compensatory mutations is to regulate the gene expression of the operon, especially of *nusG*. From a previous study, it is known that while the two genes can be expressed from a single transcript, *nusG* can also be transcribed independently (Downing et al 1989). It also appears that the evolving cells still carefully regulate the balance of gene expression, with a single SNP necessary

and sufficient to provide a selective advantage, since no clone with more than one SNP in the operon was found.

We also found mutations to the ribosomal proteins *rpmB* (L28) and *rpsN* (S14). L28 is adjacent to L9 in the 70S ribosome and is known to be essential for ribosome assembly in *E. coli* (Nag et al 1991), but it is not known any specific role that perform in conjunction with L9. S14, in the small subunit, is located very far from L9, and direct interactions between the two are less probable.

The sequencing at 56 days showed a higher variability in mutations. New mutations arose that are known in *P. fluorescens* to appear as adaptation to an experimental evolution regime in microcosms, like to the *wss* and *wsp* operon. At the same time, new mutations to the *secE-nusG* operon arose, while others disappeared or decreased in frequency. This can suggest that while regulation of the operon is a quick and easily available route to recover fitness lost from a misfunction ribosome, with multiple suitable SNPs, there are multiple and possibly more optimal path.

3.2.6 *De novo* mutations in evolved Δ L24 mutants

De novo mutations in evolved Δ L24 mutants

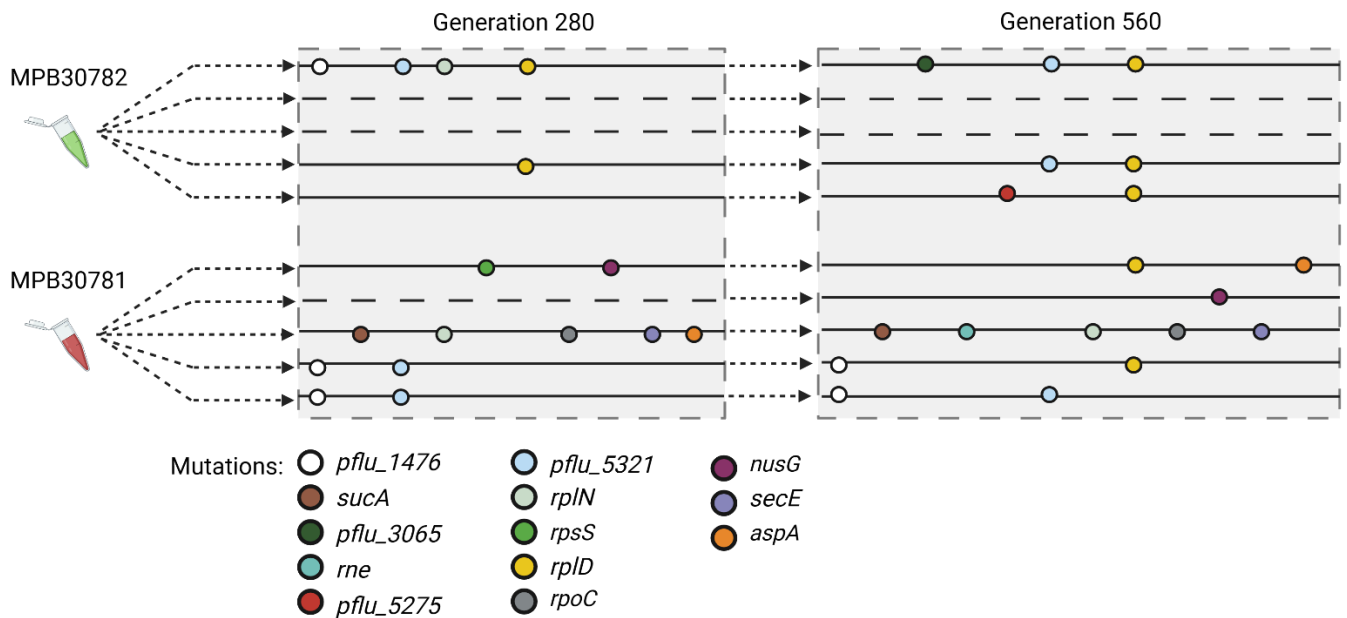


Figure 21: *De novo* mutations in evolved Δ L24 mutants. For each population, all genes with at least one mutation above 30% of frequency in at least one population are marked. In the left box are the mutations identified in populations evolved 280 generations; in the right box are the mutations at 560 generations. Continuous lines represent sequenced populations; dashed lines were not sequenced. Colored circles

represent SNPs to different genes. The order of the genes in the graph follow the order of the genes in *P. fluorescens* chromosome. The full list of SNPs and their chromosome positions are in supplementary materials (Supplementary Table 5).

In the evolved Δ L24 populations we also found mutations to the *secE-nusG* operon at high frequency (**Figure 21**). We found again the *secE* I120V mutation, but a different *nusG* mutation, Y6C. The same notes about these mutations in Δ L9 apply here.

We also found multiple mutations to the gene *rplD* and *rpsS*, coding for the r-proteins L4 and S19. L4 is an essential protein. It's not in direct contact with L24, but it is contact with the same rRNA section. L4, together with L24 and L3, is one of the main directors of ribosome assembly, guiding the folding of rRNA and addition of following r-proteins to the growing 50S subunit. For example, L24 and L4 work together to facilitate addition of L22 (Fox 2010).

S19 is in the small subunit and not in contact with L24. It is also interesting that the main known function of L24 is to drive the large ribosome subunit assembly, before the small and large subunit get joined in the 70S ribosome (Spillmann et al 1977). Recovery mutations to r-proteins in the small subunit suggest for some function of the 70S ribosome that gets affected after the loss of L24, in contrast with in *in vitro* experiments (Nishi et al 1985).

The mutations found at 56 days of evolution were overall similar, but we also found at high frequency a mutation to *rpoC* (D806G). *rpoC* codes for the RNA polymerase subunit β' . This mutation appears to affect a highly conserved domain known to be important in regulating the binding of the RNA polymerase complex on the DNA strand that is being transcribed. The mutation of a glutamate into glycine leads to a decrease in polarity that can possibly decrease the affinity of the protein for the (polar) DNA strand (Cohen & Hershberg 2022, Opalka et al 2010).

Lastly, each Δ L24 line showed a fixed mutation to *pnuC* (V55F). Since the mutation is always fixed and always caused by the same SNP, it is possible that the mutation was by chance in the ancestral SBW25 colony that acquired the mutant allele with the *rplX* deletion. *pnuC* codes for a nicotinamide ribonucleotide (an adenine precursor) transporter from the environment (Zhu et al 1989).

3.2.7 *De novo* mutations in evolved Δ L27 mutants

De novo mutations in evolved Δ L27 mutants

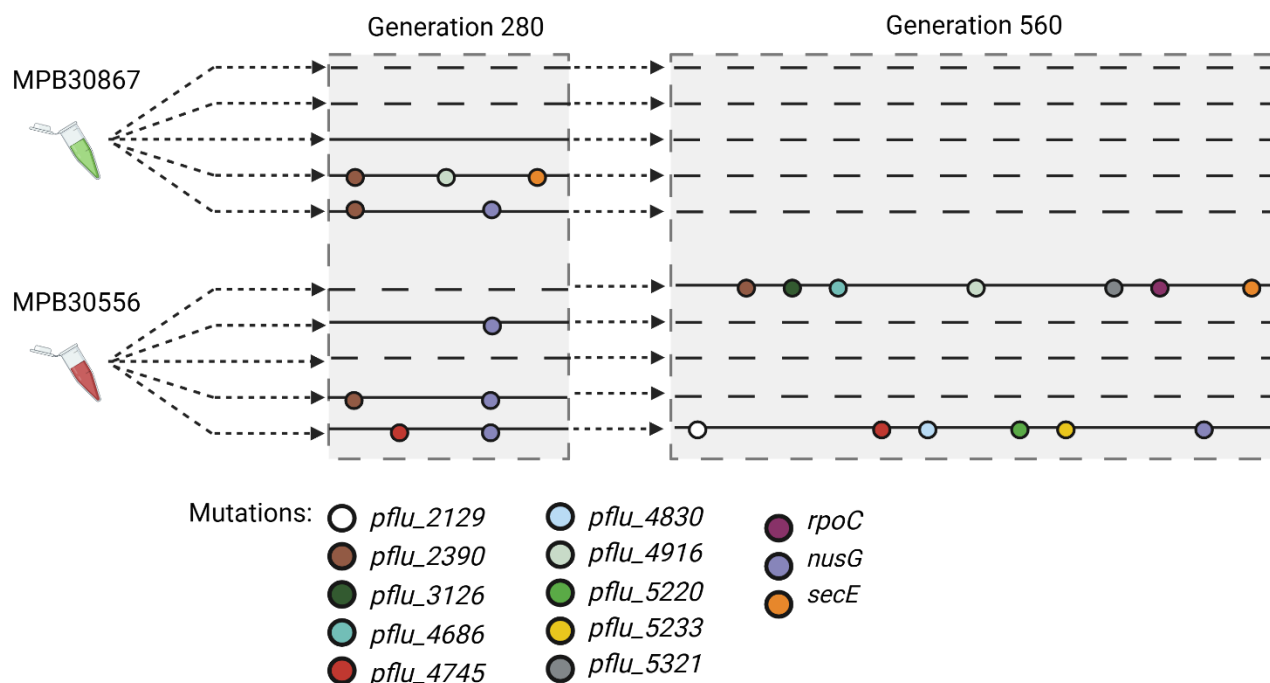


Figure 22: *De novo* mutations in evolved Δ L27 mutants. For each population, all genes with at least one mutation above 30% of frequency in at least one population are marked. In the left box are the mutations identified in populations evolved 280 generations; in the right box are the mutations at 560 generations. Continuous lines represent sequenced populations; dashed lines were not sequenced. Colored circles represent SNPs to different genes. The order of the genes in the graph follow the order of the genes in *P. fluorescens* chromosome. The full list of SNPs and their chromosome positions are in supplementary materials (Supplementary Table 6).

In the evolved Δ L27 populations the most prevalent *de novo* mutations were again in the *nusG* gene (*178W, Y6C, R4P) (Figure 22). The *178W mutation appear to produce an elongation of 20 amino acids with sequence LSKHPNPAALGCGVLSSLG. Looking for homologous peptides in PBLAST to the elongated NusG protein didn't find any match. At 56 days another *secE* SNP appeared (I120V).

Interestingly, we found a line with two mutations to *rpoC*, (T674A, V673A) with a combined frequency of 30%. The similar to the mutations to *rpoC* found in evolved Δ L24 strains, both mutant amino acids appear

to be involved in regulating the stability of the contact between RNA polymerase complex, DNA, and the growing RNA strand.

No mutation in any r-protein gene was detected.

3.2.8 *De novo* mutations in evolved Δ L33 mutants

De novo mutations in evolved Δ L33 mutants

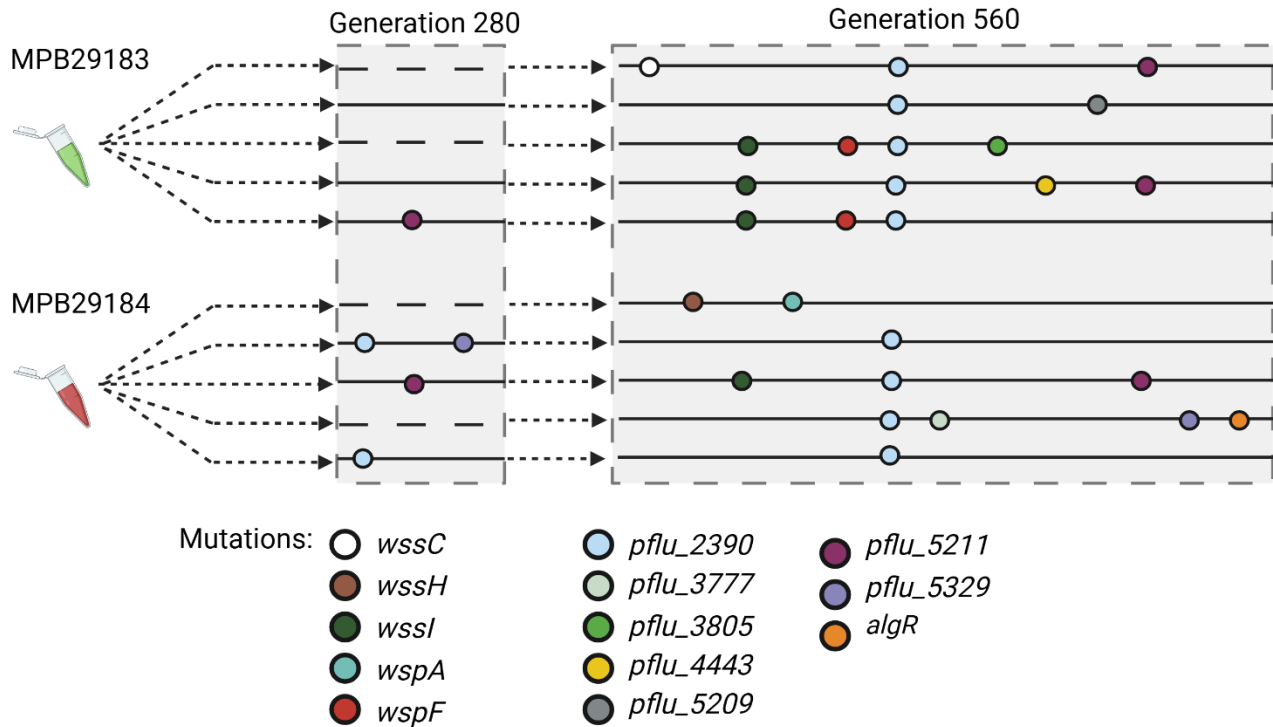


Figure 23: *De novo* mutations in evolved Δ L33 mutants. For each population, all genes with at least one mutation above 30% of frequency in at least one population are marked. In the left box are the mutations identified in populations evolved 280 generations; in the right box are the mutations at 560 generations. Continuous lines represent sequenced populations; dashed lines were not sequenced. Colored circles represent SNPs to different genes. The order of the genes in the graph follow the order of the genes in *P. fluorescens* chromosome. The full list of SNPs and their chromosome positions are in supplementary materials (Supplementary Table 7).

We didn't find any mutation with frequency above 63% in any line (**Figure 23**). This is consistent with the fact that the deletion of the gene *rpmG* (L33) produce a mutant with fitness still quite close to SBW25,

with the implication that not many mutations are easily available to increase it more. We found many low-frequency mutants of the wrinkly-spreader pathway, *wss* and *wsp*.

No mutants of r-proteins were detected.

3.2.9 *De novo* mutations in evolved Δ L36 mutants

De novo mutations in evolved Δ L36 mutants

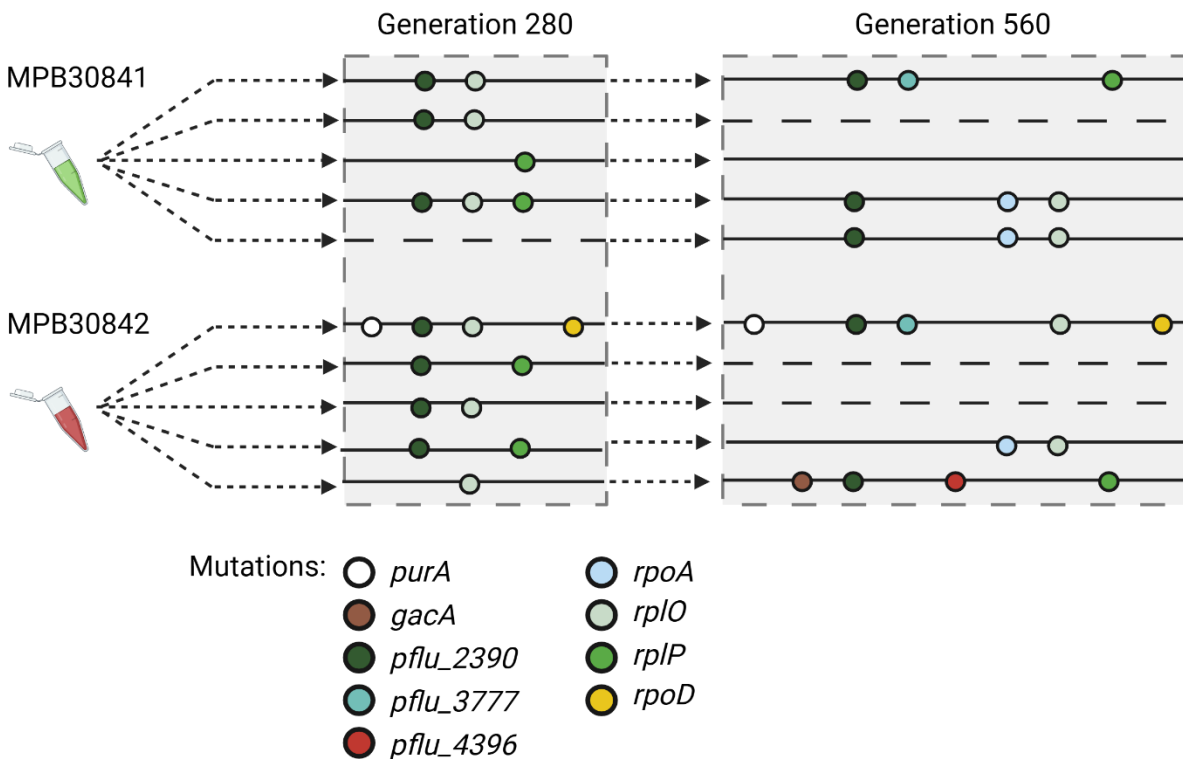


Figure 24: *De novo* mutations in evolved Δ L36 mutants. For each population, all genes with at least one mutation above 30% of frequency are marked. In the left box are the mutations identified in populations evolved 280 generations; in the right box are the mutations at 560 generations. Continuous lines represent sequenced populations; dashed lines were not sequenced. Colored circles represent SNPs to different genes. The order of the genes in the graph follow the order of the genes in *P. fluorescens* chromosome. The full list of SNPs and their chromosome positions are in supplementary materials (Supplementary Table 8).

We found various mutation to the genes *rplD* (Q29R), *rplO* (Q55H, T39N), *rplP* (H16R, T14K), coding the r-proteins L4, L15 and L16 (Figure 24). While L4 and L16 are essential in *E. coli* and *B. subtilis* (Akanuma 2012), L15 is not. L16 and L36 are in contact with opposite sides of the same rRNA loops. It's possible that

a mutation L16 helps stabilizing the loop and keeping it in position after the loss of L36. While neither L4 nor L15 are close to L36, they are actually close to each other. This could suggest that deletion of L36 has a deleterious effect on very far regions of the ribosome, that can be mitigated with other mutations of the local r-proteins. Moreover, both L15 and L16 are necessary to drive the assembly of the ribosome, but not essential in the activity of the complete particle (Franceschi & Nierhaus 1990). Similarly, L4 is essential for the initial steps of ribosome biogenesis (Fox 2010). It is possible that *de novo* mutations to L4, L15 and L16 indicates the necessity to recover ribosome assembly functionality after the loss of L36.

We also found mutations to *rpoA* (R283G, D249Y, C268S) and a mutation to *rpoD* (D216A). *rpoA* codes for the subunit α of RNA polymerase. The subunit is present as a dimer in the complete enzyme and is involved in RNA polymerase assembly, promoter recognition on the DNA, and transcription termination regulation (Liu et al 1996, Murakami et al 1997). *rpoD* codes for an RNA polymerase sigma factor and it is one of the main suppressors of transcription.

Two evolving Δ L36 populations were overcome by clones with hypermutator phenotype. This vastly increased the number of *de novo* mutations found in the populations. These mutations were excluded from our analysis. Other mutations were deletions in *pflu0636*.

3.2.10 R-protein double deletion

Since some compensatory mutations seems to help recover the fitness of multiple single deletions, I was interested to test how robust is their fitness recovery. So, I tried to produce additional r-protein deletions in an evolved mutant already carrying one. One such double-deletion mutant was constructed by performing a second two-step allelic exchange. The strain obtained consist in a deletion of L9 introduced into an evolved Δ L24 clone.

Measuring the fitness of the double mutant showed that it has higher fitness than both ancestral Δ L9 and Δ L24 strains (**Figure 25**). This corroborates the idea that identical compensatory mutations are responsible for the fitness recovery we saw in the populations evolved from different ancestral strains. It also suggests that in nature, after a first loss of a r-protein and subsequent fitness recovery by *de novo* mutations, the resulting mutant fitness landscape makes potentially easier to explore new mutations to multiple ribosome components.

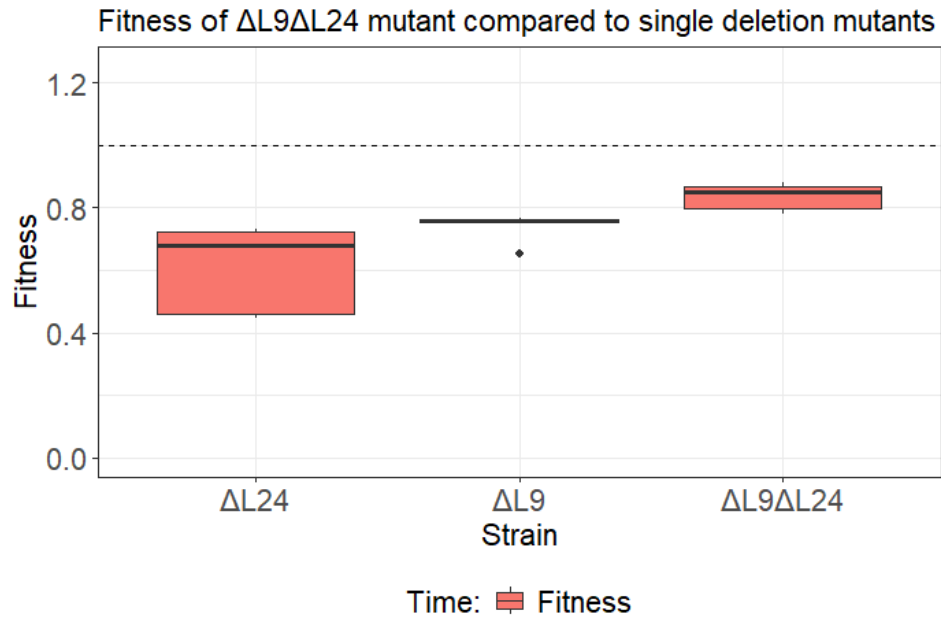


Figure 25: Fitness of r-protein double deletion mutant $\Delta L9\Delta L24$ and single deletion mutants $\Delta L9$ and $\Delta L24$, tagged with GFP. X-axis: mutant. Y-axis: competitive fitness against SBW25. Each measurement was repeated in 5 biological replicates. The colored boxes indicate the values between the first to third quartile. Error bars indicate the maximum and minimum values. Outliers are indicated as dots.

3.3 DISCUSSION

3.3.1 *P. fluorescens* r-protein deletion mutants are viable with lower fitness

The results of this chapter were aimed at elucidating the phenotypic and fitness effects caused by the deletion of different r-proteins on *P. fluorescens*, and to explore whether cells can acquire *de novo* mutations to compensate for deleterious effects. Comparative analysis of ribosomes from different species shows how the ribosome functionality can be mediated via considerably high structural plasticity, in which for example different r-proteins evolved in different lineages to fulfill the same roles (Klein et al 2004). The focus of this chapter is to better understand the evolution of such plasticity surrounding the genetic loss of r-proteins.

To investigate this, I focused on a variety of r-proteins that show a broad range in a series of potentially interesting features like high conservation, interactions with functional centers in the ribosome, presence of homologs and paralogs in different lineages. Compared to other works of evolutionary cell biology that studied r-proteins with an experimental evolution approach, here working in parallel with different r-

protein deletion mutants grown in identical conditions allowed to obtain directly comparable conclusions about the fitness recovery and *de novo* mutations arising in different populations. As a consequence, this work both provides insights into the specific effect on fitness caused by each deletion, and to identify commonalities and differences in the evolutionary response to the loss of each r-protein. This will help to better understand the processes behind modifications over evolutionary time of the r-protein set of different organisms.

In this chapter I showed how the deletion of r-proteins L9, L24, L27, L33, L36 were performed in *P. fluorescens*. Although all proteins were previously proved to be non-essential in *E. coli* or *B. subtilis* (Akanuma 2012, Shoji et al 2011), functional studies of their role are still sparse. All the genes coding for these proteins are part of operons packed with other r-proteins and other essential genes, making experimental genetic alterations difficult to achieve. Furthermore, knock-outs from previous studies are often produced by inhibiting gene expression by inserting mutations to regulatory regions, instead of completely deleting the coding sequence.

The approach used in this work, based on clean deletion of the gene of interest, allows both to analyze the effects of each knock-out, and to perform an experimental evolution study without the risk of allowing the mutants to recover fitness just by recovering the gene functionality. The deletions were performed by carefully designing plasmids carrying the mutant alleles in such a way to reduce the risk of interfering with the expression of the neighboring genes of the target following the crossover insertion, to prevent transcription suppression of whole operons of r-proteins during the mutagenesis process.

After the experimental deletions, the fitness of the resulting mutants was measured. All the mutants showed a decrease in competitive fitness against the ancestral type. Each deletion caused a different amount of fitness defect. This variability aligns, to some extent, with both the results in other works and with the available information regarding how central is the activity performed by the different proteins, how widespread and how ancestral they are in the tree of life (Akanuma 2012, Lind et al 2010, Shoji et al 2011). As expected, deletions of r-proteins like L24 and L27 that are very ancestral, conserved, structurally close to functional centers of the ribosome and essential under certain conditions (like high temperature) produce a strong negative effect on fitness (Shoji et al 2011). Deletions of r-proteins less conserved and with less contacts with internal structures of the ribosome are less disadvantageous, like L9 and L36, to the extreme of the very peripheral and recently acquired L33, which deletion had almost no effect at all on fitness.

There are many ways in which the loss of an r-protein could cause a decrease in fitness mediated by a decrease of the protein translation rate; different r-proteins could be more or less involved in each of them. This is because there are multiple factors accounting for the ribosome activity. The loss of an r-protein could lead to a decrease in the total number of available ribosomes, by decreasing the ribosome assembly efficiency due to the r-protein role in driving the rRNA folding and subunit assembly (Spillman & Nierhaus 1978). For example, mutants lacking L24 show a decrease in ribosome assembly, causing lower fitness and temperature sensitivity. A similar phenotype is caused by the loss of L36, especially due to issues in the proper assembly of the L7/L12 stalk structure of the ribosome (Dabbs 1982, Zhong & Barrientos 2024)

Complete ribosomes could be less efficient at binding to available mRNA, possibly by missing an r-protein required for recognizing the ribosome binding site (RBS) (Subramanian 1983). The defective ribosomes could also be less efficient at initiating translation of the mRNA, due to issues with the interface between the LSU and SSU on the mRNA, or to incorrect placement of tRNA at translation initiation (Tobin et al 2010). The translation rate of active ribosomes could also be altered, again due to issues with the tRNA interaction, or with the codon-anticodon pairing on the mRNA, or with the catalytic activity of the PTC. This appear to be the case for the loss of L27, causing a number of issues: its absence leads to a disruption of the positioning of A-tRNA in the ribosome during translation, and to decreased ribosome assembly, due to insufficient assembly of L16, L20, L21. Both problems lead to a decrease in fitness and to temperature sensitivity (Wower et al 1998).

Finally, the translational accuracy could be affected, by altering the proof-reading mechanisms of the ribosome (Ehrenberg & Kurland 1984). This was proved to be the case with the deletion of L9, which also become unviable when coupled with secondary mutations of translation elongation factors such as EF-P. It was suggested that this is due to a regulation of translation speed (and thus accuracy) by L9 (Naganathan et al 2015). Many of the mechanisms listed above can affect each other by different feedback mechanisms. For example, a decrease in translational accuracy can result in further mutations of new r-proteins synthesized by defective ribosomes, which in turn can result in slower, defective ribosomes down the line (Ehrenberg & Kurland 1984). Much is still unknown; for example, it's still not clear what is the impact of L33 deletion on ribosome functionality (Maguire & Wild 1995)

3.3.2 Fitness can be recovered by *de novo* mutations

After measuring the fitness of each r-protein deletion mutant, I allowed them to recover fitness by allowing them of obtaining compensatory mutations in an experimental evolution study. Despite the relatively short duration of the experiment (56 cycles of transfers to fresh medium), most evolving populations recovered a significant fraction of the lost fitness. This result aligns with what expected from the literature (Knöppel et al 2020, Tobin et al 2010).

At the end of the experiment, different evolving lines from each ancestral deletion mutant showed different levels of total fitness recovery. It is interesting to see how some deletions were easier than other to recover from. Δ L33 populations quickly recovered the small amount of fitness they lost with the deletion, and then stabilized. Δ L24 and Δ L36 populations started from a very low fitness, between 40% and 60% compared to SBW25. Those populations showed a slow but steady recovery in fitness, though with substantial variation from population to population: some recovered the fitness level of SBW25, others remained within 10% of their initial state. Most Δ L9 populations, starting from a fitness around 80%, also recovered at or around the SBW25 level. No Δ L27 populations, starting at around 40% fitness, managed to recover close to the SBW25 level, with some populations still below 60% at the end of the experiment.

This result suggest that some r-proteins are more easily replaced than others, independently of the initial fitness cost of their deletion. A possibility is that the ribosome is more robust to the loss of r-proteins involved in a more dispersed structural or regulatory role, for which multiple of the other components involved can mutate to fix the genetic defect. This could especially be true for r-proteins far from functional centers. The opposite situation would happen after the deletion of r-proteins more directly responsible to provide functionality to the ribosome, thus more difficult to replace. An example is the role of L27 in interacting with the tRNA stalk in the PTC (Shoji et al 2011).

This first experiment is another confirmation that evolution can overcome the fitness cost of losing r-proteins. While it is known that multiple r-proteins have been repeatedly lost over many different lineages since even before LUCA (Desmond et al 2010, Fox 2010, Lecompte et al 2002), it is likely that in those instances the loss of the r-protein is itself positively selected for by providing an advantage. In this experimental setup, however, the loss of r-protein is deleterious and forced. Nonetheless, the confirmation that these mutations are viable and recoverable opens up the possibility for an organism to lose them spontaneously, if this is accompanied by other compensatory mutations. From this perspective, the loss of a r-protein can actually be considered as favorable: it is known from previous studies that the

de novo mutations arising in a population after r-protein deletion, while compensating the fitness of the strain with deletion, are actually disadvantageous on their own (Tobin et al 2010). But it is possible that also those secondary mutations, on their own, could be advantageous in other environmental conditions. A possibility is that the compensatory mutations identified via experimental evolution reflect the genetics of lineages predating the appearance of the most recent r-proteins. For example, L36 is not present in the ribosomes of Archaea, replaced by a hole (Maeder & Draper 2005). It is possible that the experimental deletion of L36 forces evolution to rediscover adaptive solutions present at the time before the separation of the two lineages and the appearance of L36.

A surprising result is the relatively high fitness of the evolved double mutant $\Delta L9\Delta L24$, in which the mutations that recovered fitness after the loss of L24 also appear to provide a tolerance to the loss of L9. One possible explanation is that both r-proteins could be involved in the same process, although there are no traces of it in the literature. Another possibility is that the compensatory mutations affect protein translation and ribosome activity as a whole. In this way, one single mutation could alter the fitness contribution of multiple r-proteins. This is more deeply explored in Chapter 4.

It is known that in nature many lineages lost multiple r-proteins (Yutin 2012). It is possible that such losses could be promoted following spontaneous mutations with broad effects like those identified here. Since the loss of ribosome complexity has been linked to loss of gene expression complexity, this result could possibly be obtained by moving regulatory steps of protein translation away from r-protein expression (Gray 2012). Further genome sequencing and experiments on the $\Delta L9\Delta L24$ mutant, together with deeper genetic analysis of such r-proteins-stripped lineages, could lead to more discoveries in this direction.

3.3.3 Three categories of genes are target of compensatory mutations

The next step was to characterize the evolved populations to detect *de novo* compensatory mutations. DNA was extracted from the populations at both 28 and 56 days of evolution, sequenced, and analyzed. Each population displayed a substantial variety of low-frequency mutations. In this work I focused only on high-frequency mutations that affected coding regions, or that were found recurrently in separate evolved populations. Through all the evolved populations from all the ancestral mutants, the most widespread *de novo* mutations are part of one out of three categories:

- 1- Mutations in other r-proteins. Mutations were identified in the genes *rpmB* (L28) and *rpsN* (S14) in $\Delta L9$ populations; *rplD* (L4) and *rpsS* (S19) in $\Delta L24$ populations; *rplD* (L4), *rplO* (L15) and *rplP* (L16) in $\Delta L36$. The best-known relationship among these pairs is between L4 and L24, known to be both part of

the first ribosome assembly group and as such being responsible for the proper assembly and folding of all the remaining components of the ribosome (Fox 2010). Previous work identified mutations in S19 as compensatory for the suppression of L24 (Dabbs 1982). The identification of S19 mutants in the current chapter proves that the function of such secondary mutations can't be explained as increasing the expression of the nearby *rplX* gene, as previously hypothesized, since in the current setup the gene is completely removed from the organism. Still, previous work showed how synonymous mutations in r-protein genes can affect fitness, proving that modifications in the mRNA sequence can alter expression; this is particularly relevant when considering that most r-protein genes are part of operons (Lind et al 2010). No other mechanistic interaction is known between the other r-proteins targeted by compensatory mutation and the deleted r-proteins that could suggest how the compensatory mutations contribute to fitness recovery. Many of the proteins involved are actually quite far from each other in the structure of the ribosome, sometimes even in the opposite subunit. This discovery could have different explanations. First, it could be a cue to a better understanding of the ribosome structural remodeling and movement that happen during the different phases of protein translation (Fox & Naik 2004). Second, it could suggest still unknown interactions between the proteins at the regulatory level, with the *de novo* mutations tuning the level at which r-proteins affect their own expression in cells experiencing stress (Ni & Buszczak 2024). Third, it could be caused by interaction with other factors external to the ribosome itself, such as RNA polymerases, translocons, chaperons, and other regulatory pathways. In a ribosome, many r-proteins have protein-protein interactions with each other, but such interactions usually involve only a much smaller area of their surface compared to the area involved in rRNA interactions (Klein et al 2004). The lack of compensatory mutations in r-proteins interacting with those deleted further confirm the secondary role of such interactions in the ribosome activity.

2- Mutations in transcription factors. Several mutations were identified in both RNA polymerase subunits α and β' , σ factors, and particularly in *nusG*. While the current results don't allow to determine whether these mutations increase or decrease the transcriptional activity, nor if the changes are global or gene-specific, two contrasting hypotheses can be made. In the first hypothesis, the disruption of the ribosome due to r-protein deletion decreases the translation rate, which becomes the bottleneck of the cell metabolism and synthesis of components necessary for the cell division. As a result, wild-type levels of transcription become wasteful. Accordingly, these mutations could decrease the transcription. In the second hypothesis, the disruption of the ribosome could be partially counteracted by further increasing transcription. Considering the mutant ribosome as a less efficient enzyme, a higher concentration of transcripts in the cell could drive the chemical reaction of protein synthesis towards the final product,

helping to restore the ancestral phenotype, albeit at the price of an increased cost in resources (Ehrenberg & Kurland 1984). The relative stoichiometry of the components required to allow efficient assembly of active ribosomes can be affected by the genes involved in the process, and as such is a phenotype upon which natural selection can act, as shown in previous works of experimental evolution of mutants with disrupted ribosomes (Knöppel et al 2020). Since mutations in RNA polymerase and NusG are the focus of the next chapter, more information about their genes, functions in the cells and their relationship with the ribosome are discussed in Chapter 4 introduction.

3- Mutations in genes of the cell metabolism. Many of the genes involved, like *sucA* or the *wss* operon, could be involved in the process of adaptation to the growth medium and experimental conditions. Mutations to the magnesium transporter *mgtE*, while potentially useful in a general way to better absorb ions from the environments, could also be explained as helping to regulate the amount of magnesium ions in the cell to a different level than in the; this may adjust the number of stabilizing interactions of rRNA in the ribosome after the loss of a structural r-protein (Hsiao et al 2009).

3.3.4 Conclusions

Between days 28 and 56 it appeared that the evolving populations acquired more and more varied compensatory mutations. In some cases, mutations that were present at high frequency at day 28 nearly disappeared from the same population at day 56, replaced by others. It is possible that some of the mutations seen at day 56 could not be selected before other mutations changed the genetic background of the cell in a permissive way. Another notable pattern is that different mutations don't appear together in the same clone, especially in the case of mutations to the *secE-nusG* operon, suggesting that the mutations being selected alters the balance of their product activity within a very narrow functional window. These two observations together show how the mutants are navigating and reshaping the fitness landscape as they evolve.

The sequencing analysis provided a backbone of information necessary to better understand the broad range of effects on the cell biology caused by the loss of a r-protein. The most striking finding of this work is how the deletion of different r-proteins, localized in very different parts of the ribosome and with very different known interactions and phylogeny, can still be recovered by mutations that in some cases are exactly the same across different lineages. This becomes even more apparent when considering how the deletion of a second r-protein, $\Delta L9$, become possible in an evolved $\Delta L24$ background, with a resulting fitness of the double mutant $\Delta L9\Delta L24$ still higher than that of either single deletion mutant. This

exemplifies how natural selection can shape and be shaped at the level of whole systems, in this case the system of genetic information processing.

CHAPTER 4 – Recovery of ribosome disruption by transcription regulation

4.1 INTRODUCTION

4.1.1 Study of compensatory mutations in *nusG*, *rpoC*, *rpoD*

In Chapter 3, I showed how the experimental deletion of different r-proteins in *P. fluorescens* causes a decrease in fitness, and that this decrease can be partially or fully recovered over time through compensatory mutations. To better understand the mechanistic effects of these compensatory mutations and the relationship between ancestral r-protein deletions and genes targeted by *de novo* mutations, it is necessary to better characterize the phenotypes of both ancestral and evolved mutants, and to draw on evidence about the function of the mutant genes.

As it was found in Chapter 3, the most common compensatory mutations affect genes in three categories: r-proteins, regulators of transcription and translation, and genes involved in energy metabolism. In this chapter I focus on the analysis of evolved mutants with mutations to genes in the second category: the *secE-nusG* operon, *rpoC*, *rpoD*.

This choice is based on several reasons. First of all, as discussed in the introduction to Chapter 3, our knowledge about many r-proteins functions is still limited. A lot of the breakthroughs in the field happened by obtaining more refined ribosome crystals at different steps of assembly or translation, allowing researchers to observe how the interactions between different ribosome components change over time; or by synthetic assembly and testing of ribosomes *in vitro* to directly quantify their functionality in different environments or with different available components (Kåhrström et al 2012, Wang et al 2018). Both types of studies are beyond the scope of this work. Second, many of the genes involved in energy metabolism and cellulose production appear to be adaptations to the specific experimental conditions to which the cells are exposed. Third, some of the *de novo* mutations in the *secE-nusG* operon appear at high frequency in more lines than any other single mutations, increasing the potential to obtain useful insight from the analysis of these mutants.

4.1.2 Impairing translation impacts cell metabolism

The detrimental effect on fitness caused by the deletion of a r-protein can be ascribed to the centrality of translation in the growth rate, and how the disruption of the ribosome can affect it. Translation, as a

whole, takes up 90% of the energy resources of a growing cell (Nord et al 2009). The energy is used both to synthesize the new proteins needed by the cell and to produce more ribosomes. The assembly of new ribosomes is a highly complex, regulated process (Lecompte et al 2002, Fox 2010). Disrupting this process by an r-protein deletion leads to a situation in which a decrease in the availability of ribosomes, caused by loss of a component needed for their assembly, directly produces an even lower number of ribosomes due to the now decreased global protein synthesis, in a self-reinforcing mechanism. It is important to note that the required activity of specific r-proteins differs for the translation of specific transcripts, such as in cases in which the r-protein is needed for directing the ribosome on specific mRNAs by sequence recognition or to allow synthesis of secreted peptides by directing the ribosome to the cell membrane. Other r-proteins have also specific functions in the cell even when free, not part of the ribosome, such as rRNA processing and damage response (Warner & McIntosh 2009). However, most experimental studies so far show that r-protein deletions tend to affect global translation (Shoji et al 2011, Tobin 2010).

As a consequence, what begins as a specific and localized issue in ribosome structure and functions directly becomes a problem that affects global translation levels of the cell. Given that transcription provides the main resource needed for translation, it is then no surprise how alterations in cell functionality and expenditure to one of the two processes drive the cell to acquire modifications to the other. Indeed, previous studies have shown that transcription and translation co-evolve to optimize their functional relationship and match their output (Irastortza-Olaziregi & Amster-Choder 2021).

The functions of the gene targeted by *de novo* mutations were already broadly outlined in the discussion of Chapter 3. To provide for a better background necessary for the study of *rpoC*, *rpoD* and the *secE-nusG* operon in the context of this work and to inform the rationale of the experiments of this chapter, a more in-depth literature analysis is presented in the following sections.

4.1.3 RNA polymerase

Table 4: *De novo* mutations in RNA polymerase genes

Gene	Mutation	Genome position	DNA change	Ancestral mutation
<i>rpoC</i>	D806G	6,055,399	T→C	ΔL24

<i>rpoC</i>	V673A	6,055,798	A→G	ΔL27
<i>rpoC</i>	T674A	6,055,796	T→C	ΔL27
<i>rpoD</i>	D216A	6,124,914	A→C	ΔL36
<i>rpoA</i>	R283G	6,035,804	G→C	ΔL36
<i>rpoA</i>	D249Y	6,035,906	C→A	ΔL36
<i>rpoA</i>	C268S	6,035,849	A→T	ΔL36

Column 1: mutant gene. Column 2: amino acid change. Column 3: SNP position on the chromosome. Column 4: nucleotide change. Column 5: ancestral mutant.

The RNA polymerase is the multimeric complex that performs transcription. The bacterial RNAP is divided into the subunits β and β' , coded by *rpoB* and *rpoC*; two copies of subunit α , coded by *rpoA*; and subunit ω , coded by *rpoZ*. Other transcription regulation factors join the holoenzyme as the σ family factors, such as those coded by *rpoD* and *rpoS* (Murakami et al 1997). The evolved r-protein deletion mutants analyzed in Chapter 3 showed *de novo* mutations in the genes *rpoA*, *rpoC*, *rpoD* (**Table 4**).

It is known from the literature that mutations to *rpoB* and *rpoC* are often observed in adaptation to minimal medium. Typically, cells growing slower produce lower levels of release factors and ribosomes, meaning more demands for release and recycling of the fewer molecules available. This makes the cells more sensitive to defects in the transcription termination regulation (Wannier et al 2018). Other studies of experimental evolution in *E. coli* show an increased activity of RNAP by mutations in *rpoC*. These mutations increase the elongation rate by decreasing the stop time at pause sites on the DNA, and by decreasing the open complex longevity (the state before transcription actually starts) at the rRNA promoter. Since rRNA operons are the most transcribed genes in the chromosome, the increase in their processivity frees up the RNAP to also transcribe other genes (Conrad et al 2010).

The *de novo* mutations found in *rpoA* affect amino acids in the DNA binding region of the protein like D249 and C268, in direct proximity of the specific amino acids providing protein-DNA interaction, while R283 is in the region in contact with transcription factor σ^{70} (Benoff et al 2002, Kim et al 2011). σ^{70} itself is an essential transcription factor that promotes global transcription and is coded by *rpoD*. The *de novo* mutation found in *rpoD* at the residue D216 is in a non-conserved region of domain 2; this domain performs both as promoter recognition on the DNA and is in contact with the RNAP (Gruber & Gross 2003). The β' RNAP subunit is coded by *rpoC* and, together with subunit β , is where the catalytic activity of RNA synthesis takes place. The amino acid D806 is in the helix bridge between the active site and the

trigger region of the protein; mutations in this region alter transcriptional activity due to its central location and function.

4.1.4 *secE-nusG* operon

Table 5: *De novo* mutations in the *secE-nusG* operon

Gene	Mutation	Genome position	DNA change	Ancestral mutation
<i>nusG</i>	R20P	6,065,036	C→G	ΔL9
<i>nusG</i>	I38N	6,064,982	A→T	ΔL9
<i>nusG</i>	W5C	6,065,080	C→G	ΔL9
<i>secE</i>	I120V	6,065,115	T→C	ΔL9, ΔL24
<i>secE</i>	I116V	6,065,127	G→C	ΔL9
<i>nusG</i>	Y6C	6,065,078	T→C	ΔL24, ΔL27
<i>nusG</i>	*178W	6,064,562	T→C	ΔL27
<i>nusG</i>	R4P	6,065,084	C→G	ΔL27

Column 1: mutant gene. Column 2: amino acid change. Column 3: SNP position on the chromosome. Column 4: nucleotide change. Column 5: ancestral mutant.

secE

The *secE-nusG* operon encodes the proteins SecE and NusG. In *P. fluorescens*, they are separated by 9 nucleotides. 111 nucleotides downstream of *nusG* is the *r*-protein operon encoding the genes *rplK*, *rplL*, *rplJ*, *rplL*. The two genes *secE* and *nusG* are known to be transcribed both together as a polycistronic mRNA and independently. When transcribed together, the two transcripts are cleaved by RNase III (Downing et al 1989).

SecE is a 123 amino acid-long protein part of the SecYEG translocon complex. The complex is a membrane channel for protein export. Targeting newly synthesized proteins to the membrane depends both on the protein sequence itself, and on the mRNA coding them: some mRNA coding secretory proteins, once transcribed, are directly moved to the membrane, where they are translated in direct proximity to the Sec complex. In the complex, SecY is the actual transmembrane pore and SecE is wrapping and regulating the opening of the pore, while SecG regulates the pore activity in concert with the ATPase SecA. Deletion of

the SecE domain that binds to SecY prevents SecE from performing its activity (Nijeholt et al 2013, Sarmah et al 2023). This does not completely inactivate the complex. Complete depletion of SecE, however, leads to stress response and cytosolic accumulation of proteins that should be secreted or localized in the outer membrane, though many secretory and membrane proteins can still reach their destination by using the remaining Sec translocon components, or by relying on Sec-independent secretory pathways (Baars et al 2008). The mutations identified the evolved mutants in Chapter 3 are all clustered in the last 8 amino acids of SecE and do not appear to affect amino acids of known function (**Table 5**).

nusG

NusG is a 178 amino acid-long universal transcription factor essential in most species. It covers a broad range of regulatory roles interacting with different cell components. Overall, NusG has four main functions: to silence the transcription of aberrant RNAs in cooperation with transcription terminator factor Rho; to help RNAP processivity; to join protein complexes that promote the synthesis and processing of rRNA; to protect mRNA from Rho silencing by bridging RNAP and ribosomes (Wang & Artsimovitch 2021). This complexity is compounded by the fact that in different organisms it appears to produce contrasting effects. In *E. coli* it regulates rho-dependent transcription termination, by putting in contact the Rho factor with the RNA polymerase (RNAP). It also increases the rate of transcription elongation by decreasing the pausing of the RNAP when not bound to Rho. On the opposite, in *M. tuberculosis* its function is to pause the RNAP (Eckartt et al 2024, Ingham & Furneaux 2000).

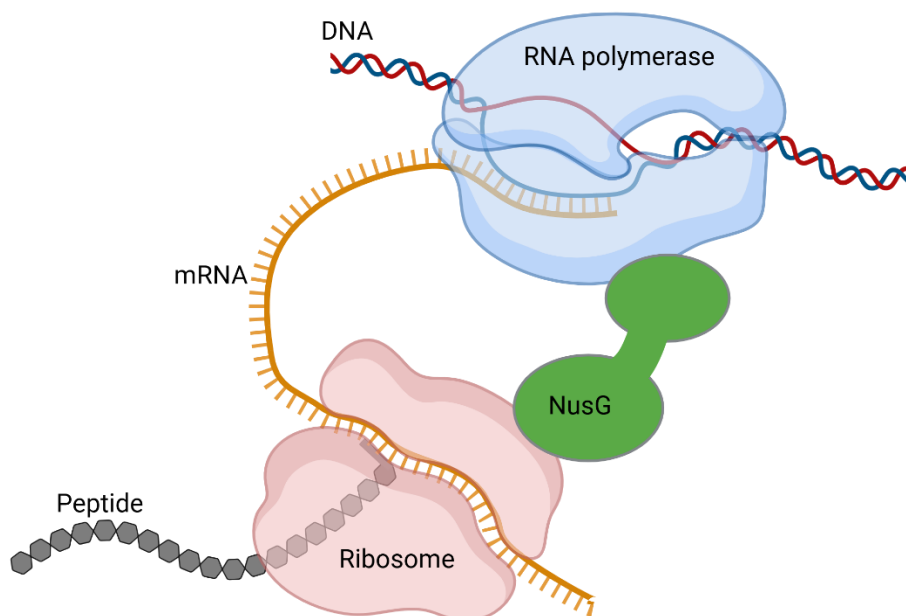


Figure 26: Model of interaction of NusG, RNA polymerase, and ribosome. NusG acts as a bridge on between RNAP and the ribosome, regulating both the processivity of transcription and coupling of transcription and translation. Image produced with Biorender. Not to scale.

NusG is one of the very few conserved universal transcription factors. This is evidence that its role in closing the RNAP clamp was already required in LUCA. However, since between bacteria and archaea and eukaryotes transcription factors mostly diverge and have no common origin (with the notable exception of the RNAP itself), and together with the fact that some phage RNAP does not require any transcription factor to function, this led to the hypothesis that most transcription factors were actually not present in LUCA (Iyer et al 2003, Werner 2012).

NusG is monomeric in bacteria, while in archaea and eukaryotes its homologue Spt5 forms dimers with Spt4 (Klein et al 2010, Werner 2012). It has two functional domains: the N-terminal domain is in contact with RNAP and the DNA, modulating transcription; the C-terminal domain contacts the ribosome through the r-protein S10 (also called NusE) and the transcription factor Rho (Burmam et al 2010, Eckart et al 2024, Mooney et al 2009). To allow the bridge between RNAP and ribosome to happen, the ribosome and the RNAP have to be within close distance (**Figure 26**). If this is not possible, there are also nus-independent interactions between the two (Irastortza-Olaziregi & Amster-Choder 2021).

nusG expression is self-regulated at the transcriptional level. Thus, it is possible that the *de novo* mutations in *nusG* identified in Chapter 3 can affect its expression both by decreasing the strength of its promoter or by altering how NusG can bind to its own transcript. Such self-regulation takes place after the transcription starts by contacting the region between -31 to +272 nucleotides of its start codon; this is the same region where we found most compensatory mutations in the *secE-nusG* operon. While promoter prediction in Chapter 3 also identified the region between *secE* and *nusG* as a promoter, other studies suggest that transcription regulation by *nusG* does not depend on this region (Downing et al 1989).

In this case, it could mean that the identified compensatory mutations are more specifically altering *nusG* self-regulation. Moreover, some mutations to RNAP can alter *nusG* self-regulation too (Downing et al 1989, Ingham & Furneaux 2000). Other mutations, such as SNPs on the residue F165 in *E. coli* (homologous to F162 in *P. fluorescens*) decrease ribosome binding activity of NusG. Mutation of this amino acid, decreases the coupling of transcription and translation. This mutant NusG can still suppress transcription pausing but it cannot anymore promote Rho termination (Saxena et al 2018).

All the *nusG* SNPs found in the evolved r-protein deletion mutants are in its N-terminal domain, which interacts with the ribosome (**Table 5**). Some of the SNPs found, such as Y6C and I38N, affect amino acids whose homologues in *E. coli* (R9 and V42 respectively) are directly involved in the NusG domain interacting with the RNAP (Klein et al 2010, Martinez-Rucobo et al 2011, Wang & Artsimovitch 2021, Werner 2012). I38 in particular is a conserved amino acid in various species. The non-polar amino acid V42 in *E. coli* NusG is part of a hydrophobic surface that is conserved and interacts with the RNAP clamp and exit gate; in *P. fluorescens* the change from the also non-polar amino acid isoleucine to the polar asparagine could alter the strength of the interaction. Mutations of *nusG* altering the interacting site with the RNAP are known to potentially lead to an increase in RNAP activity. These mutations are found when selected upon by evolution as a mechanism of antibiotic resistance against rifampicin, recovering the transcription rate (Eckart et al 2024).

Depending on during which transcription stage NusG binds the RNAP, it can produce different effects. It can lock the DNA in place closing the RNAP clamp around it, increasing the processivity of RNAP; or prevent new DNA from entering a free RNAP, thus decreasing transcription. Meanwhile, other transcription factors like TFE compete with NusG preventing it from blocking the free RNAP. This contributes to transcription regulation. Since RNAP can bind at the DNA randomly everywhere on the chromosome, not only at gene promoters, it appears that NusG prevents this binding of RNAP to the DNA at random sites, while TFE activity specifically promotes it around promoters (Wang & Artsimovitch 2021, Werner 2012).

The transcription regulation performed by NusG is modulated also by its own contact with the ribosome: the contact of NusG C-terminal domain with the r-protein S10 prevents NusG from interacting with Rho until the translation is over. This regulatory step is exemplified by how S10 itself can act independently from the ribosome as a transcription regulator when part of complexes with NusG and NusA, and by how missense mutations in S10 that prevent transcription antitermination can be recovered by secondary mutations to *nusG* (Burmah et al 2010, Werner 2012). In the same way, experimental depletion of NusG leads to defects in Rho-dependent termination. In contrast, the deletion of *nusG* is viable in cells lacking toxic RNAs or prophages, in which cases the cell does not require Rho termination to remove them (Wang & Artsimovitch 2021).

Beyond its function as a physical bridge between RNAP and ribosome, there are multiple ways in which NusG mediates the interaction of the processes of transcription and translation. The way in which S10 prevents NusG from interacting with the Rho factor causes the leading ribosome engaged in mRNA

translation to compete with Rho for NusG, preventing Rho-mediated termination. In other cases, if ribosomes do not bind NusG and the translation does not start right after the transcription does, NusG together with NusA and the Rho factor perform transcription termination (Burmann et al 2010, Irastortza-Olaziregi & Amster-Choder 2021).

Finally, NusG can modulate the biogenesis of the ribosome SSU. NusG in *E. coli*, together with RNases, interacts with sequences upstream and downstream of the transcribed pre-16S rRNA. In this role, as part of a complex with other transcription factors, NusG ensures rRNA transcription by its antitermination activity (Bubunencko et al 2014).

4.1.5 Transcription-translation coupling as a regulation mechanism

Both the function of NusG and the presence of many *de novo* mutations arising in the RNA polymerase suggested that the fitness defect caused by r-protein deletion can be rescued not only by directly recovering ribosome functionality, but also by altering transcription. Moreover, the presence of many mutations in the gene *nusG* suggests a role for the regulation of transcription-translation coupling (CTT) in the recovery of fitness (Stent 1964). Coupling of transcription and translation has multiple functions. It is necessary to ensure efficient use of the resources needed for transcription and translation. It helps to regulate gene expression.

CTT allows the cell to match the rate at which new nucleotides get polymerized into RNA, and amino acids in peptides. The genetic code itself requires three nucleotides in each mRNA codon to be translated to a specific amino acid; this three-to-one ratio is actually found as the relative rate of transcription and translation, and it is maintained through a broad range of cell growth rates and overall metabolic throughput (Werner 2012). Several different features of the genetic information processing apparatus evolved within this framework. First of all, the catalytic speed of RNAP and of the ribosome match: the ribosome can translate between 14 to 17 amino acids per second, while the RNAP transcribes mRNA at 42 to 49 nucleotides per second, about three times faster (Irastortza-Olaziregi & Amster-Choder 2021). An evidence of how the cell actively works to maintain this balance is provided by the fact the transcription of rRNA, which doesn't require further translation, is two times faster than mRNA transcription, at 90 nucleotides per second (Irastortza-Olaziregi & Amster-Choder 2021).

One of the mechanisms that perform this regulation is provided by the leading ribosome translating an mRNA strand. Since in bacteria the mRNA can be translated while it is still being transcribed, the first ribosome can have an active role in the transcription, both via NusG and other mechanisms. In this case,

ribosome and RNAP work together in what is called transcription elongation complex (TEC) (Irastortza-Olaziregi & Amster-Choder 2021). For example, it is known that ribosomes with mutations causing decreased elongation rates cause also a decrease in transcription by not preventing the RNAP from pausing and backtracking (Burmann et al 2010). In the opposite direction, the ribosome can push a slow RNAP. This forced transcription increases the risk of transcription termination, but it is hypothesized that ribosomes translating at normal speed stochastically push the RNAP from time to time, helping transcription to maintain the correct speed. This phenomenon can particularly promote fitness when in case of pause-prone RNAP (Eckart et al 2024, Proshkin et al 2010, Wang et al 2020, Wee et al 2023).

This relationship goes in both directions. If the translation slows down due to nitrogen limitation or oxidative stress, the regulation slows down the RNAP too without the need of contact with the ribosome; meanwhile in the case of essential genes, if the contact is present, then RNAP can in turn actually speed up the rate of translation to promote the correct balance of available amino acids and nucleotides in the cell and promote essential genes' expression (Irastortza-Olaziregi & Amster-Choder 2021, Werner 2012).

The assembly of the TEC is itself a regulatory step: if translation does not start immediately and the NusG-NusA complex is already on the RNAP, then the NusG C-terminal domain is not bound to the ribosome and is instead free to bind the rho factor, leading to premature transcription termination (Irastortza-Olaziregi & Amster-Choder 2021). The selective pressure favoring the evolution of such tight regulation becomes immediately clear when considering how phage RNAP like T7 can transcribe its target genes at the speed of 230 nucleotides per second. This increased speed, helpful for the phage to quickly replicate, is a danger for the host cell but also a target for self-defense mechanisms on which CTT regulation can act by promoting early termination before ribosomes can express the phage genes (Irastortza-Olaziregi & Amster-Choder 2021).

4.1.6 Evolutionary consequences of defective ribosomes

All the previous considerations directly tie to the study of ribosomal protein via experimental deletion. It is known that most mutations to ribosomes decrease the translation rate. In most cases, the mutations and deletion of r-proteins lead to a global decrease in translation rate, not by slowing the processivity of the ribosome but by decreasing the translation initiation rate (Kåhrström et al 2012).

This is a side effect caused by one safety mechanism that evolved to prevent failure of gene expression. The issue is that in the case of a stochastically defective r-protein or rRNA could decrease the overall accuracy of the ribosome, this would lead to propagation of more defective r-proteins and rRNAs.

Evolution found a solution to this potentially fatal feedback by the fact that non-functional ribosome components not only can decrease translation accuracy, but also translation speed; the decrease in translation speed in turn allows the rest of the ribosome components to perform increased proofreading that, in turn, brings back the accuracy. Accuracy of gene expression is then optimized, not maximized; the degree of optimization can then be selected by evolution (Ehrenberg & Kurland 1984).

The proofreading of the ribosome is optimized between the causes of loss of efficiency: producing mutant peptides, or having non-productive initiation complex cycles. This energetic cost is caused by the mechanism of translation initiation. Right before translation can begin, the initiation factor IF2 has to hydrolyze GTP allowing Met-tRNA to bind the mRNA in the SSU. Then the LSU can join the SSU-mRNA complex and translation can start. If it doesn't happen and translation doesn't start then, before a new cycle, IF2-GDP has to be phosphorylated back to IF2-GTP (Schneider et al 2020).

Most of gene expression errors happen at the translation level, with 75% of missense errors in *E. coli* proteins due to translation errors. This affects r-proteins too: as a consequence, ribosomes are heterogeneous, with between 1 and 10 missense substitutions in each bacterial ribosome. So, each cell carries a heterogeneous ribosome population with respect to the rate and accuracy of translation.

Since the efficiency of a pathway depends on the concentrations of metabolites and products, the role of some ribosome components can be limited to only influence the speed of ribosome assembly and direct the r-proteins and rRNA folding instead of catalysis of peptide synthesis. But this can already be enough to increase the efficiency of the ribosome mass, outweighing the cost of producing them (Ehrenberg & Kurland 1984).

The strength and direction of the selection for increased optimization of accuracy and speed depends on growth conditions. To complicate the picture, the rate of protein synthesis for ribosome mass is not constant, but decreases at lower growth rates. This means that cells growing slower not only have less ribosomes, but they are also less active (Churchward et al 1982, Maaløe 1970). This is also shown by a decrease in the concentration of tRNAs and elongation factors at low growth rates (Kjeldgaard & Kurland 1963).

In poor medium, optimum performance of the ribosome is when elongation factor molecules in the cell, like EF-Tu, are 25 times the number of ribosomes. The ratio decreases in rich medium. At high growth speed, the concentration of the tertiary complex (the ribosome ready for translation on the mRNA with all the initiation factors and tRNA) is minimized compared to the number of actively translating ribosomes.

One cause is that in poor medium the cost of synthesizing new amino acids for translation is comparatively higher compared to the cost of wasting inactive ribosome and GTP, so it pays off to decrease the error rate and more proof reading is advantageous. On the opposite, in rich medium, is better to increase the efficiency of the ribosomes even if this results in higher amounts of peptides with missense errors (Ehrenberg & Kurland 1984).

Finally, from the point of view of the cell, there is no difference between defective enzymes for nucleotide synthesis and an error-prone RNA polymerase as both decrease the efficiency of their pathway and growth rate. This becomes relevant when it comes to understand how compensatory mutations in the RNA polymerase and other transcription factors act.

4.1.7 Recovery mutation analysis

After the putative compensatory mutations that emerged during experimental evolution of *P. fluorescens* mutants with deletion of the r-protein L9, L24, L27, L33, L36 were identified in Chapter 3, my next step was to better characterize what is their effect. To do so, several tests were performed on both the ancestral deletion mutants and on a sample of single colony clones derived from the evolved populations. The goal was to measure how the deletion of r-proteins affects translation, to see if in the evolved mutants the recovery of fitness is driven by a recovery in translation, and to pinpoint the relationship of transcription, translation and fitness.

4.2 RESULTS

4.2.1 Isolation of mutant colonies

To better characterize the putative compensatory mutations found, it was tried to reconstruct them into the ancestral r-protein deletion mutant background from which they evolved. It was used the same method used to produce the deletion as described in Chapter 3, but without success. It was resorted to sample the final evolved populations looking for single colonies that carried our mutations of interest.

In order to isolate the required colonies, the evolved populations that showed higher frequency of the mutations of interest were grown from the frozen stock and plated for overnight growth. To decrease the chances of isolating clones with high number of mutations, we screened populations from day 28 of the

experimental evolution experiment. Single colonies were then screened by Sanger sequencing to confirm they carried the compensatory mutations of interest. The selected colonies were grown in liquid, sampled to produce frozen stock and the DNA was extracted for whole genome sequencing (**Table 6**). This sequencing allowed to choose the evolved colonies that both carried the compensatory mutation of interest and lowering the chances of further *de novo* mutations. Since the fitness of the Δ L33 mutant was already similar to the wild-type, and its evolved populations did not show any compensatory mutation at high frequency, it was not studied further in this work.

Table 6: Single colony isolates from evolved populations at day 28 of experimental evolution.

Name	Ancestor mutant	<i>De novo</i> mutations	Source population
MPB39184	Δ L9	<i>nusG</i> I38N, <i>tadG</i> Δ 1 bp (725263)	MPB32494 GFP E
MPB39189	Δ L9	<i>secE</i> I120V	MPB32495 RFP A
MPB39190	Δ L9	<i>secE</i> I120V	MPB32495 RFP A
MPB39300	Δ L24	<i>secE</i> I120V, <i>rpoC</i> D806G, <i>sucA</i> W10R, <i>pnuC</i> V55F	MPB32844 RFP C
MPB39387	Δ L24	<i>aspA</i> TRIA431T, <i>pnuC</i> V55F	MPB32842 RFP A
MPB39390	Δ L24	<i>aspA</i> TRIA431T, <i>pnuC</i> V55F	MPB32842 RFP A
MPB39391	Δ L24	<i>nusG</i> Y6C, <i>rpsS</i> P30R, <i>pnuC</i> V55F	MPB32843 RFP B
MPB39397	Δ L24	<i>nusG</i> Y6C, <i>pnuC</i> V55F	MPB32843 RFP B
MPB39201	Δ L27	<i>nusG</i> Y6C, <i>mgtE</i> R221P	MPB32519 RFP E
MPB39302	Δ L27	<i>secE</i> I120V, <i>rpoC</i> V673A, <i>N-acetyltransferase</i> D85E	MPB32515 RFP A
MPB39161	Δ L36	<i>rplO</i> Q55H, <i>rpoD</i> D216A, <i>purA</i> K293R	MPB32535 RFP A

Each clone genome was completely sequenced and the *de novo* mutations are listed. Column 1: ID of the isolated clone. Column 2: ancestral r-protein deletion. Column 3: *de novo* mutations identified in the clone after sequencing. Column 4: Evolved population from which the clone was isolated.

4.2.2 Translation rate measurement

While the fitness measurement provides a broad phenotypic characterization of our mutants, I was interested in studying more in detail how the loss of r-proteins can affect *P. fluorescens*. Previous studies

showed how deletions of r-proteins affect the ribosome activity at different levels, with various effects on translation (Shoji 2011, Kåhrström et al 2012, Shirakawa 2023). As a first test, the translation rate of the mutants was measured.

The usual method to obtain this measurement in bacteria usually relies on IPTG induction of the *lacZ* operon to assess the time before the colonies visibly change color, but *P. fluorescens* lacks the *lacZ* operon. As such, it was decided to measure the fluorescence recovery after photobleaching (FRAP).

Briefly, the fluorescent cells are inoculated on an LB-agar pad slide and imaged with a phase contrast microscope. Half of the cells under the field of view are then photobleached with the laser. This destroys the fluorophores, and the only way for the cells to recover fluorescence is to synthesize new fluorescent proteins. Repeating the observation of the cells at regular intervals allows to measure how long it takes the cells to recover at least 50% of the original fluorescence (**Figure 27**). This time is then compared between different strains and the wild-type to provide an estimate of protein synthesis rate and to calculate the corrected fraction of recovery (cFR) (Broder & Pogliano 2006, Wu et al 2006) (**Figure 28**).

FRAP experiments were first performed to the ancestral r-protein deletion mutants and SBW25, to know how the deletion of the different r-proteins affected the overall translation rate of the cell.

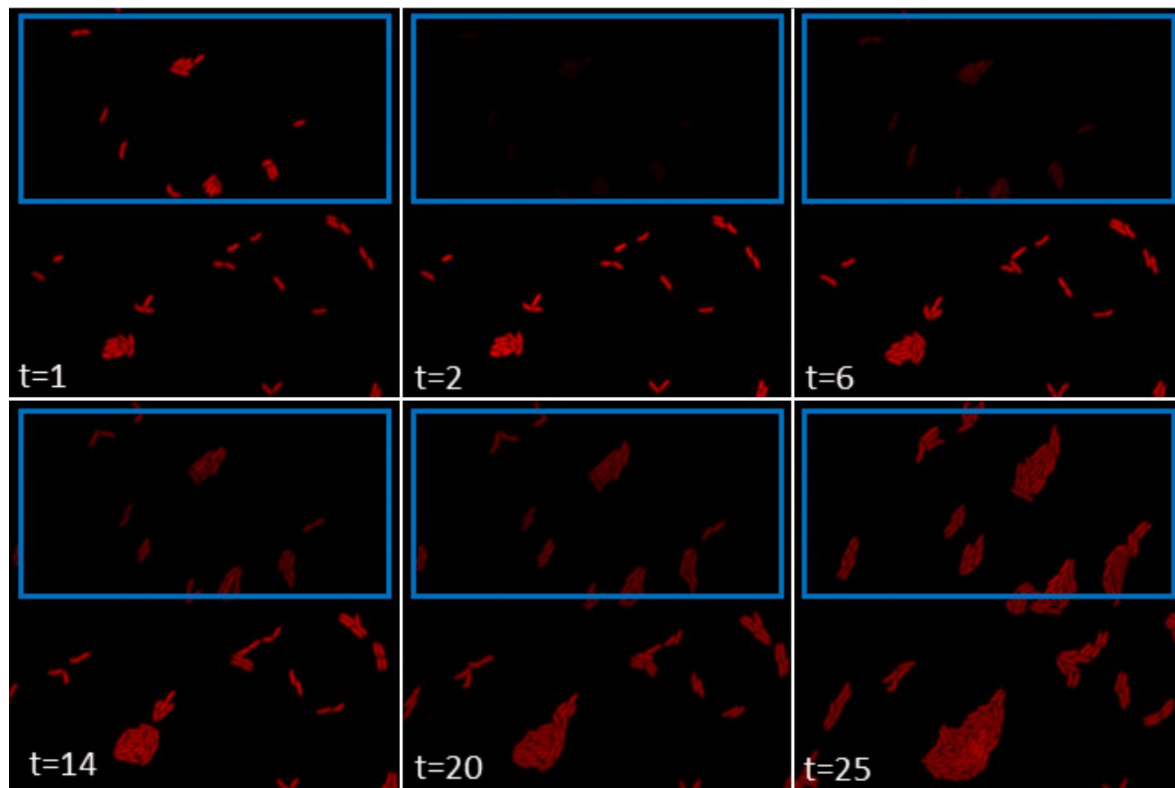


Figure 27: Example of FRAP imaging at 6 different timepoints. The red fluorescent cells in the blue square are photobleached between timepoints 1 and 2. The cells outside the square are not photobleached to use their fluorescence as a control. The recovery of fluorescence of the cells in the blue square is then measured.

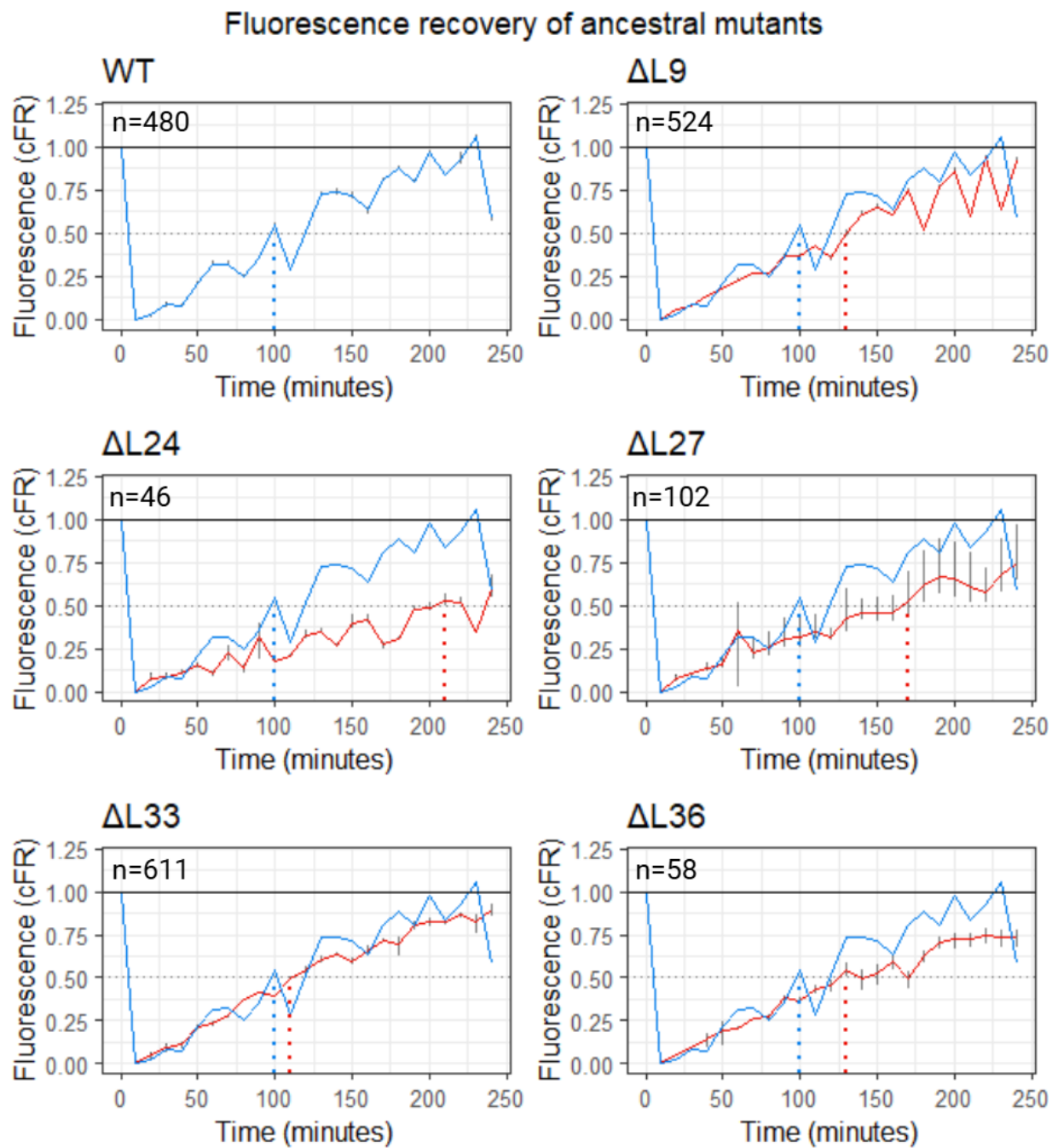


Figure 28: Analysis of FRAP of wild-type SBW25 and r-protein deletion strains. Clockwise from top-left: ancestral type SBW25; $\Delta L9$ and SBW25; $\Delta L27$ and SBW25; $\Delta L36$ and SBW25; $\Delta L33$ and SBW25; $\Delta L24$ and SBW25. X-axis: time in minutes. Y-axis: fluorescence of the bleached cells expressed as cFR. Blue line: ancestral type cFR. Red line: mutant cFR. The wild-type cFR has been over-imposed on every mutant plot as a reference. The colored dotted lines indicate the time at which SBW25 (in blue) and the deletion mutant (in red) recover 50% of fluorescence. The vertical bars represent standard deviation. The number of total cells analyzed is indicated for each sample.

This first experiment shows that the deletion of each r-proteins leads to a decrease in translation rate. In each mutant, the decrease in translation rate is proportional to the decrease in fitness previously measured (**Figure 29**). Cell size and count were also assessed (**Supplementary Figure 1, 2, 4**)

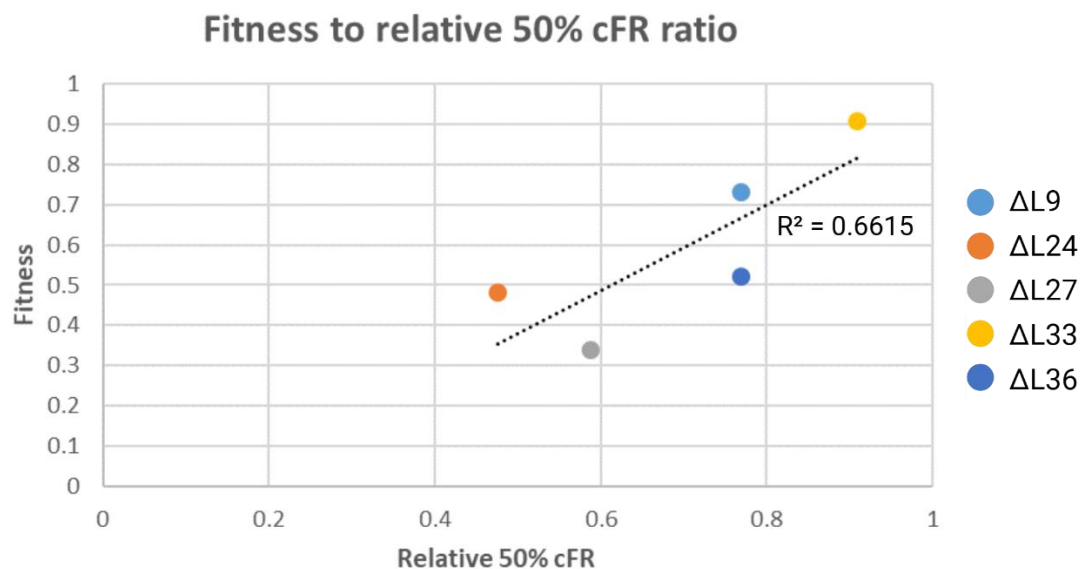


Figure 29: Comparison of fitness and cFR of ancestral r-protein deletion mutants. X-axis: relative cFR, expressed as ratio of time needed to recover 50% fluorescence between mutant and SBW25 (from data in Figure 28). Y-axis: mean competitive fitness (from data in Figure 17). Regression line in black.

Interestingly, while it was not the focus of the experiment, the microscope observation allowed to find out that $\Delta L27$ mutants show a filamentous shape. This is probably contributing to the very low fitness of the strain previously observed in Chapter 3 since it appears that during the time-lapse many filamentous cells lyse and die (**Supplementary Figure 3**).

4.2.3 Translation rate of evolved mutants

Having measured the translation rate of the ancestral r-protein deletion mutants, the same procedure was used to also measure the translation speed of the evolved mutants carrying compensatory mutations. The expectation was that, just as how the fitness of these mutants was recovered, also the translation rate would have been recovered. So, the FRAP experiment was repeated with the clones isolated from the evolved populations (**Figure 30**).

Surprisingly, most of the evolved strains did not show a change in translation rate compared to the ancestral mutants from which they originated. This seems to suggest both that the compensatory mutations we found do not directly fix the problem caused by the loss of the r-protein suggesting that their effect alters the balance between growth rate and translation rate. Finally, the double deletion mutant $\Delta L9\Delta L24$ show a profile of protein synthesis very close to the ancestral $\Delta L9$ mutant, suggesting the compensatory mutations acquired to recover from the loss of L24 prevent the secondary r-protein deletion of L9 from further disrupting protein synthesis.

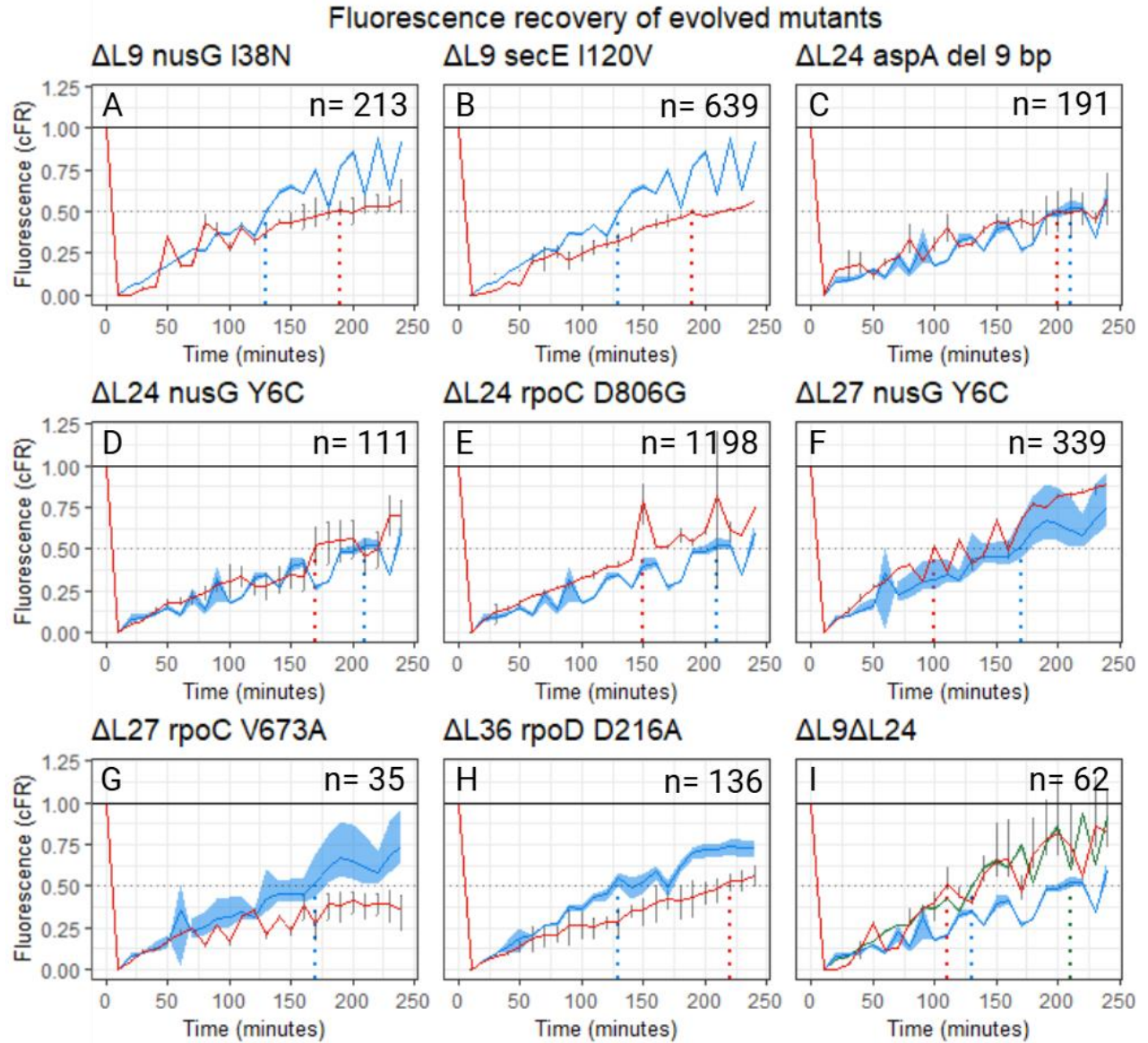


Figure 30: Analysis of FRAP of evolved strains. A, B: evolved Δ L9 mutants MPB39184 and MPB39190. C, D, E: evolved Δ L24 mutants MPB39387, MPB39391 and MPB39300. F, G: evolved Δ L27 mutants MPB39201 and MPB39302. H: evolved Δ L36 mutant MPB39161. I: double deletion mutant Δ L9 Δ L24 MPB36792. X-axis: time in minutes. Y-axis: fluorescence of the bleached cells expressed as cFR. Red line: evolved mutant cFR. The ancestor r-protein deletion mutant cFR has been over imposed on every plot as a blue ribbon as reference, as measured in Figure 28. The colored dotted lines indicate at which time the ancestral strain (in blue) and the evolved mutant (in red) recover 50% of fluorescence. For the double deletion strain, the cFR of the ancestral Δ L24 is in blue, and of the ancestral Δ L9 is in green. The bars represent standard deviation. The number of total cells analyzed is indicated for each sample.

4.2.4 Relative contributions of transcription and translation to fitness

Since the translation rate analysis disproved the hypothesis that the compensatory mutations helped to recover fitness by recovering the ribosome activity, I had to dig deeper for an explanation. The following focus was on the fact that most compensatory mutations modify genes involved in transcription and in the coupling of transcription and translation. Considering that the regulation of the two processes is linked (Stent 1964), it is plausible that a change to the transcription apparatus could help recover a fitness decrease caused by a defective ribosome.

As a preliminary exploration of this and following the work by Kafri et al 2016 I measured the fitness of the mutants when exposed to media composed of different amounts of nitrogen-based and phosphate-based metabolites. The rationale is that nitrogen is needed by the cell to synthesize the amino acids that are in turn used for translation whilst phosphate is required to synthesize nucleotides used in transcription (**Figure 31**). While there is an overlap between the use of the two elements for the two processes of transcription and translation, it was demonstrated that cultured cells suffer the lack of nitrogen and phosphate with gravity depending on the relative amount of transcriptional and translational activity the cell is performing.

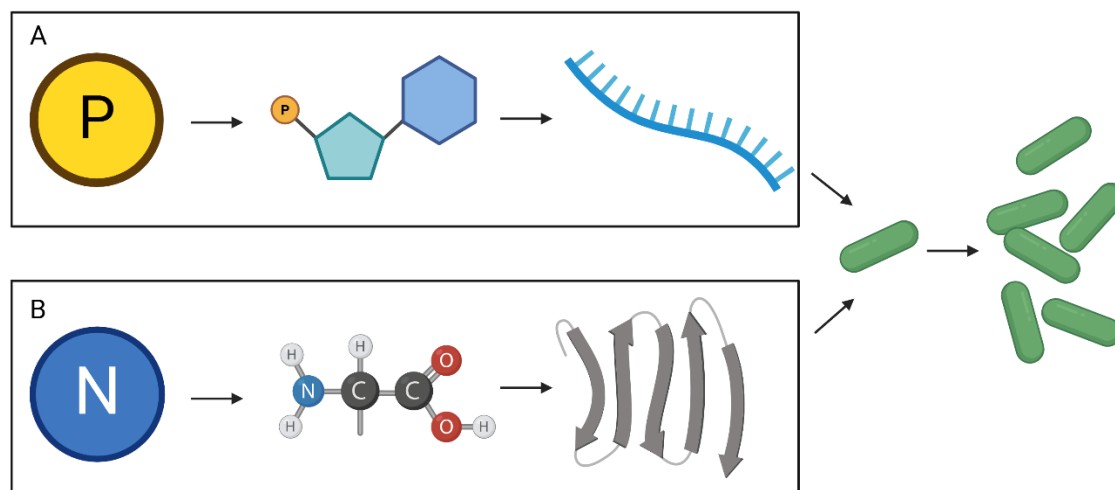


Figure 31: Usage of phosphate and nitrogen in transcription and translation. A: Sources of phosphate are used by the cell to synthesize nucleotides, which are then polymerized in RNA during transcription. B: Sources of nitrogen are used to synthesize amino acids, which are polymerized in proteins during translation. The two processes are required for cell growth.

As a first experiment, the fitness of the ancestral r-protein deletion mutants was measured when cultivated in different formulations of citric acid medium (CAM) (Al-Aoukaty et al 1991, Zhang & Rainey 2007). The media used were the standard formulation, then with a reduction of 70%[P], 40%[P], 40%[N], 20%[N]. All media pH was brought to 6.8 to compensate for the potential effect on fitness caused by the decrease in pH obtained from different concentrations of solutes.

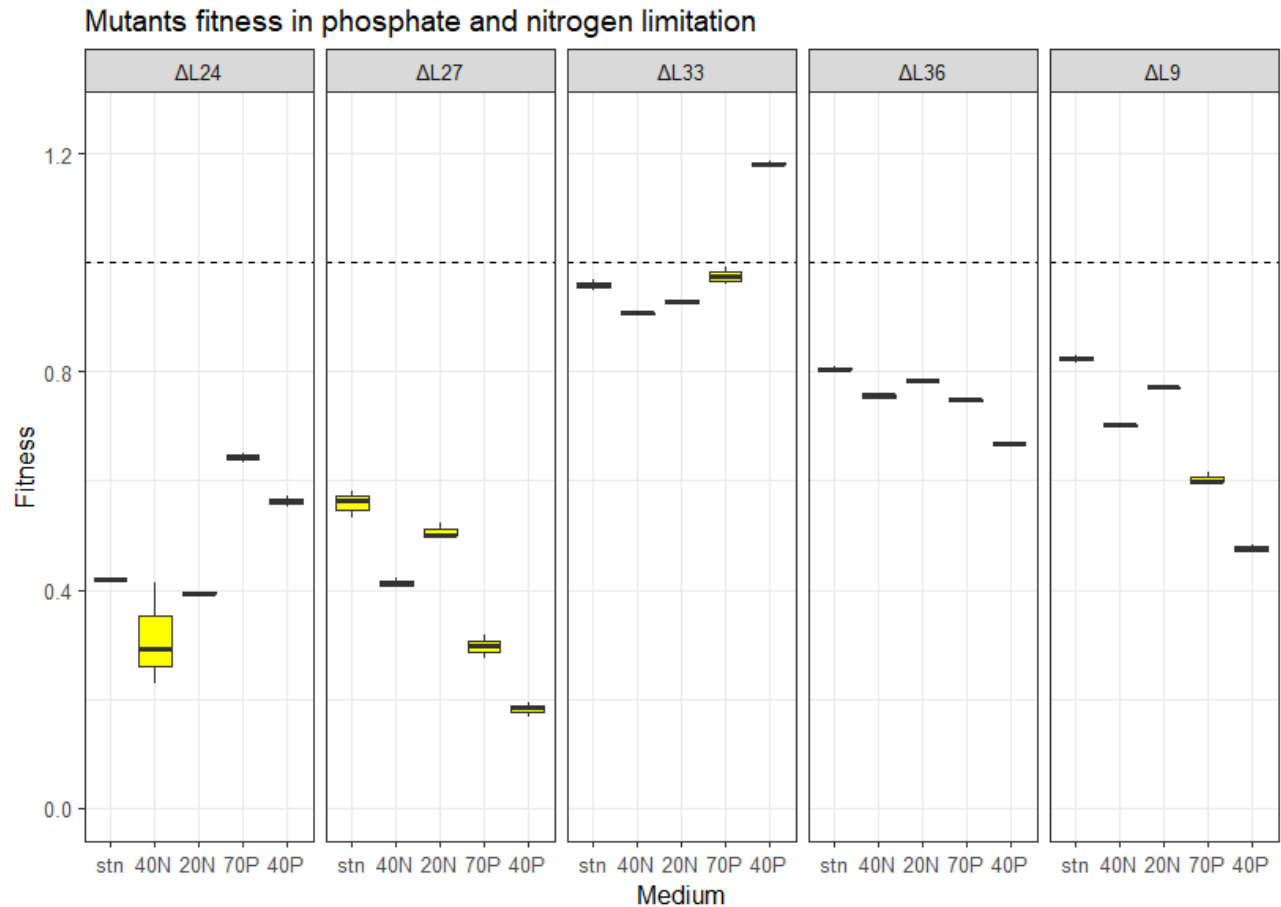


Figure 32: Fitness of ancestral r-protein deletion strain in CAM with different concentrations of phosphate- and nitrogen-based nutrients. From left to right: first panel: fitness in different media of $\Delta L24$, $\Delta L27$, $\Delta L33$, $\Delta L36$, $\Delta L9$. Y-axis: competitive fitness against SBW25. X-axis: medium where the fitness is measured (stn: standard CAM formulation. 40N: 40% of nitrogen. 20N: 20% of nitrogen. 70P: 70% of phosphate. 40P: 40% of phosphate). 3 biological replicates were measured for each sample. The bars represent minimum and maximum values; the boxes represent the mean and the data within 1st and 3rd quartile.

All r-protein deletion strains behaved in a similar fashion: the highest fitness was consistently obtained in the standard formulation of the medium (**Figure 32**). A small but significant decrease in fitness was observed by lowering the available nitrogen down to only 20% of the standard formulation. In contrast, a decline in the phosphate concentration to 70% of the standard already produced a stronger decrease in fitness, which becomes even greater when decreasing the phosphate concentration to 40% of the standard. Finally, in interpreting this result is important to note that, since what is measured is competitive fitness against the wild-type, the change of fitness produced by different growth media can be caused not only by changes in growth rate of the mutant but also in that of the wild-type.

The result suggests that the deletion of r-protein makes the cells more sensible to a secondary decrease in transcriptional activity. An explanation could be that a decrease in the total number of mRNA in the cell drives down even further the chances that an already defective ribosome successfully assembles on a given mRNA strand to perform translation. But we also note that this experiment does not discriminate between the different final products synthesized by the limited elements, such as mRNA from rRNA.

The next step was to measure the fitness in phosphate- and nitrogen-limited media of the evolved mutants. The fitness with phosphate or nitrogen deprivation was measured for each of the evolved clones previously isolated (**Figure 33**).

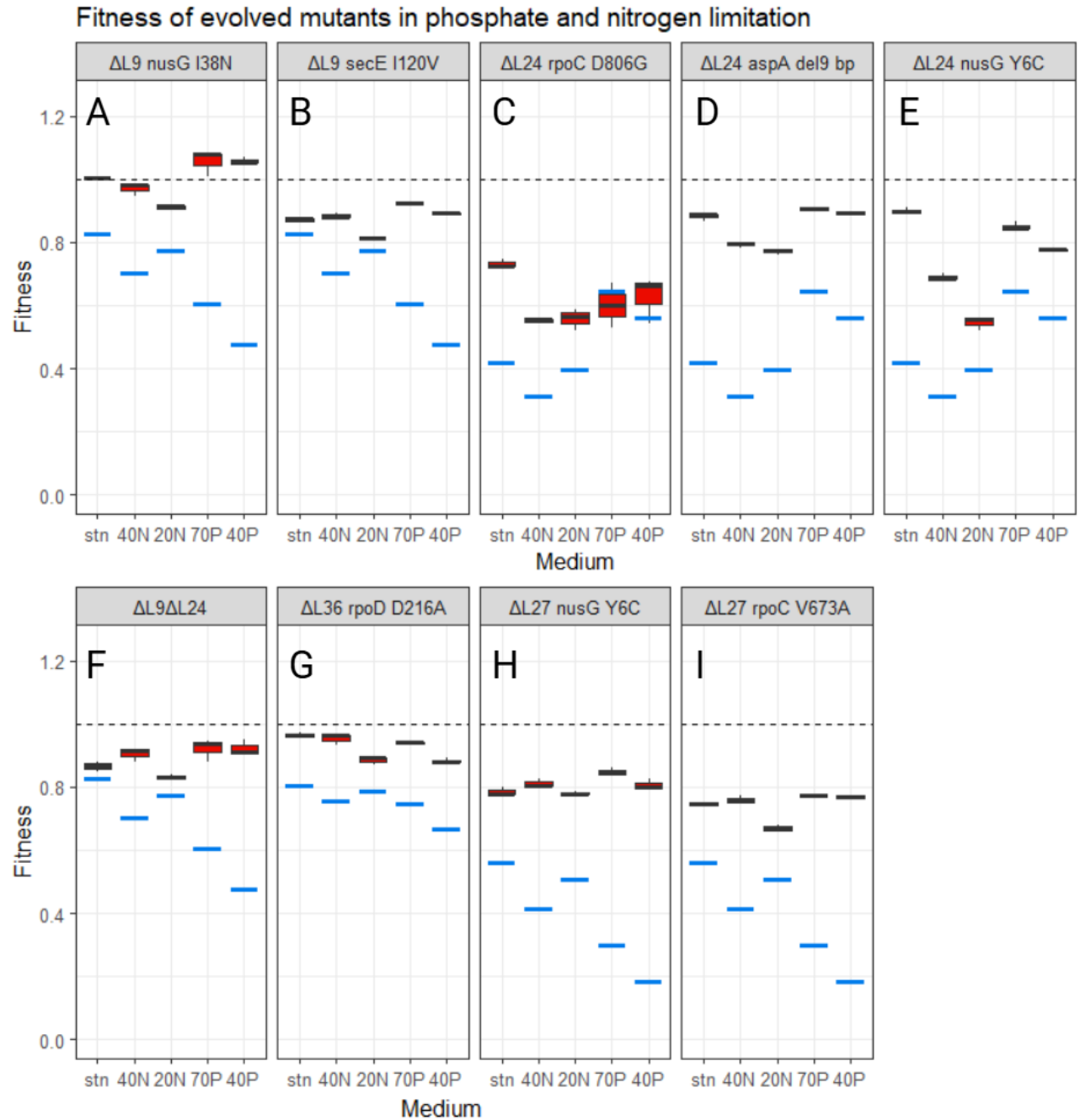


Figure 33: Fitness of single colony clones from evolved r-protein deletion populations in CAM with different concentrations of phosphate- and nitrogen-based nutrients. Top: A, B: fitness in different media of evolved $\Delta L9$ clones MPB39184, MPB39190. C, D, E: fitness of evolved $\Delta L24$ clones MPB39300, MPB39387, 39391. Bottom: F: fitness of double deletion mutant $\Delta L9\Delta L24$. G: fitness of evolved $\Delta L36$ clone MPB39161. H, I: fitness of evolved $\Delta L27$ clones MPB39201, MPB39302. Y-axis: competitive fitness against SBW25. X-axis: medium where the fitness is measured (stn: standard CAM formulation. 40N: 40% of Nitrogen. 20N: 20% of Nitrogen. 70P: 70% of Phosphate. 40P: 40% of phosphate). Red: fitness of the evolved clones. Blue: average fitness of

the corresponding ancestral r-protein deletion mutant in the corresponding medium. The compensatory mutation of interest of the evolved clone is listed above each panel. 3 biological replicates were measured for each evolved mutant sample. The bars represent minimum and maximum values; the boxes represent the mean and the data within 1st and 3rd quartile. For the ancestral mutants, only the mean (from the data of Figure 32) is indicated.

Compared to the consistent effect on the fitness of different strains caused by deprivation of phosphate or nitrogen, this time there was much more variability in how different evolved mutant responded to the different environments. As expected, all evolved mutants showed higher fitness in the standard citric acid medium compared to their respective ancestral r-protein deletion mutant. While some evolved mutants (notably, all Δ L24 evolved mutants, **Figure 33 C, D, E**) show a slight decrease in fitness in low concentrations of nitrogen like the ancestor mutants, other evolved mutants (like both evolved Δ L27 mutants, **Figure 33 H, I**, and partially the evolved Δ L36 and Δ L9 mutants, **Figure 33 A, B, G**) show no decrease (or even an increase) in fitness in the same condition. More excitingly, most evolved mutants (all evolved mutants, except the evolved Δ L24 mutants) show to not particularly suffer any more in a low phosphate environment, a stark contrast with the ancestral mutants.

This result could indicate that the effects of the compensatory mutations alter the coupling of transcription and translation in a way that makes the process more robust to perturbations, both if this perturbation takes the form of a change in the available nutrient or of a defective ribosome.

4.2.5 Resistance to transcription inhibition

To further confirm a possible connection between transcription and translation at play, wild-type and ancestral r-protein deletion mutants were grown with different amounts of rifampicin, and their growth rate measured. Rifampicin is an antibiotic that specifically target transcription, by inhibiting the beta subunit of RNA polymerase. This is caused by binding of the antibiotics to the enzyme, preventing the elongation of RNA, followed by its premature release; it generally affects mRNA transcription more than tRNA (Mohanty & Kushner 2020, Mosaei & Harbottle 2019).

Minimum inhibitory concentration (MIC) of rifampicin in wild-type *P. fluorescens* SBW25 was determined by growth in KB medium with different concentrations of the antibiotic and measuring the OD600 after 24 hours (**Figure 34**). MIC was determined to be between 6 to 8 μ g/ml.

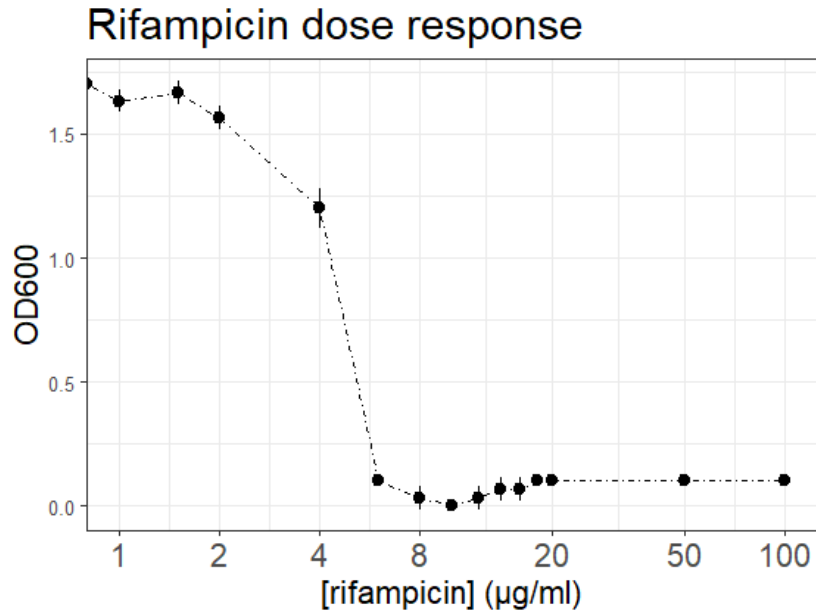


Figure 34: Growth curve of SBW25 in rifampicin. X-axis: concentration of antibiotic in 8 µg/ml. Y-axis: density of culture measured by OD600. Each data point is measured by three biological replicates. Black dots indicate the mean, the bars indicate standard deviation.

In the following experiment, the growth of SBW25 and mutants in different rifampicin concentrations was observed at 5 minutes intervals for 72 hours (**Figure 35**). Rifampicin slowed the growth of both the wild-type and the r-protein deletion mutants. At the same time, it appears to have a stronger inhibitory effect on wild-type cells. For both SBW25 and all the r-protein deletion mutants, the total yield of the cultures decreased at every time point proportionally to the concentration of rifampicin. While different mutants differed slightly in the effect of antibiotic on growth, none of them recovered growth rate when exposed at low doses of antibiotic. This disproved the idea that the effect of compensatory mutations in evolved clones provide benefit by directly slowing transcription on a global level.

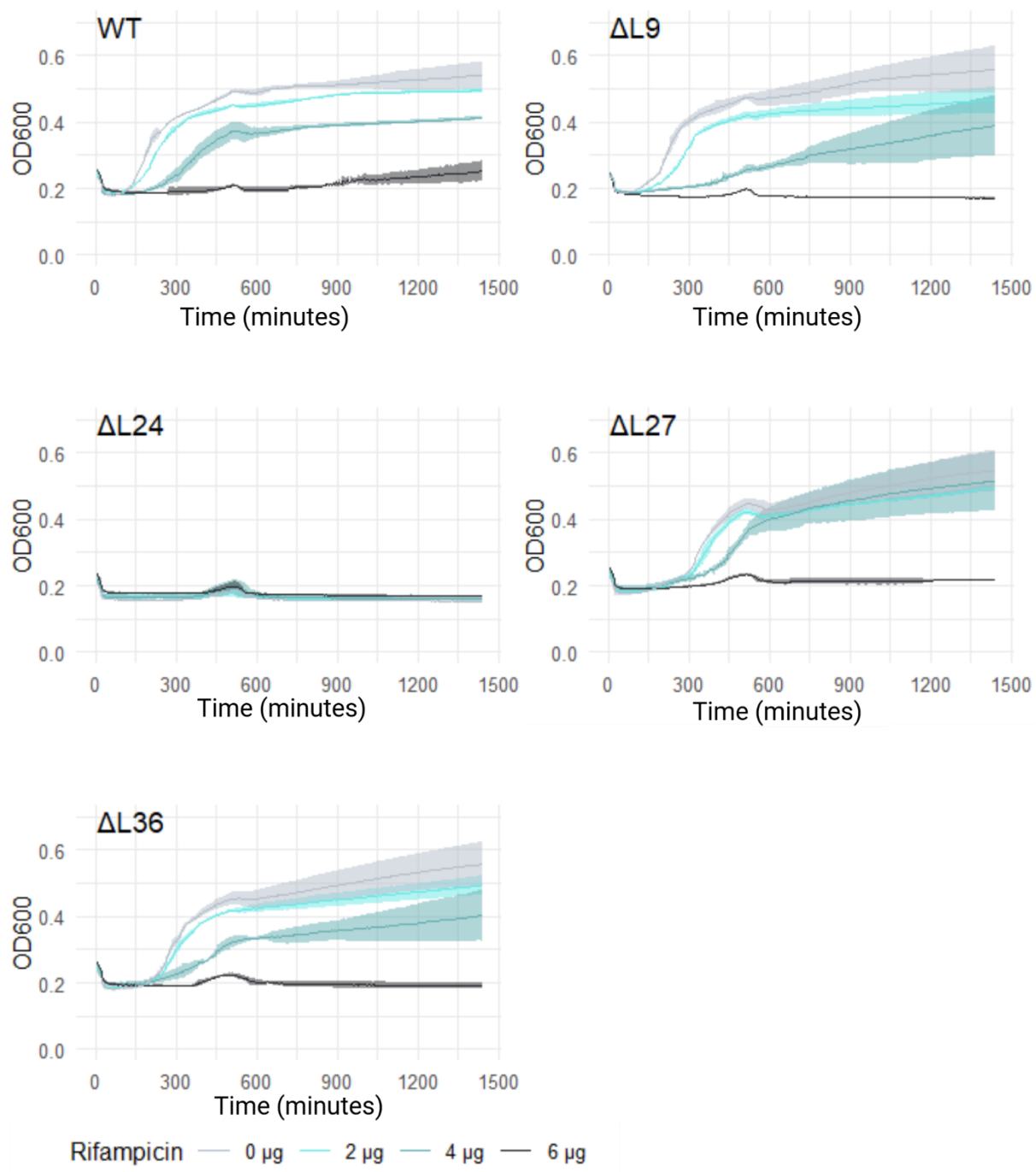


Figure 35: Growth of wild-type *P. fluorescens* and r-protein deletion mutants in different concentrations of rifampicin. X-axis: time in minutes. Y-axis: OD600. Each measurement was repeated in three biological replicates. The line represents the mean, the ribbon cover from the minimum to the maximum value.

4.3 DISCUSSION

4.3.1 Ribosomal protein deletion mutants show decrease in translation rate

It is known that bacteria have multiple mechanisms to regulate CTT; however, many of these mechanisms are still not well understood, nor is it well known exactly how they evolved (Blaha & Wade 2022). The results of this chapter suggest that mutations of the ribosome leading to changes in protein synthesis could drive part of this evolution.

The global translation rate of mutant *P. fluorescens* with single deletions of the r-proteins L9, L24, L27, L33, L36 was measured using FRAP. The results showed that r-protein deletions led to a decrease in translation, consistent with the decrease in fitness observed in Chapter 3. The decrease in translation following r-protein deletions was expected, from previous results of similar experiments in the literature (Kåhrström et al 2012, Shirakawa 2023, Tobin et al 2010).

In the same way, it was expected that the evolved mutants carrying compensatory mutations that recovered fitness would also have shown a recovery in translation rate. Surprisingly, the results here presented suggest that evolved mutants, despite growing at rates closer to the wild-type, still display translation levels similar to those of the ancestral deletion mutants.

It is well known that the major cost of cell division in bacteria is associated with protein synthesis. A large portion of this metabolic cost is dedicated to the expression of genes involved in gene expression itself (Bowman et al 2020, Briones & Amils 2004). Each cycle of cell division requires doubling not only the genome, cell membrane and wall, and metabolic enzymes, but also the number of ribosomes, in a mechanism of positive feedback. Altering ribosome functionality by r-protein deletion can alter this balance. Other studies involving r-protein deletion followed by experimental evolution have shown that one possible way cells can recover fitness is by restoring translational activity, thereby directly fixing this balance. In such studies, in many cases the compensatory mutations are SNPs to the remaining r-proteins; these SNPs usually provide a small recovery in fitness, around 10%, and are actually disadvantageous if introduced in the wild-type (Kåhrström et al 2012, Tobin et al 2010). As shown in Chapter 3, SNPs in r-protein genes were also present in the evolved mutant populations produced in this work, and it is plausible that such mutations provide an analogous phenotype by recovering translation rate. However,

since the single colony clones obtained and analyzed in this chapter had compensatory mutations in other genes, this cannot be confirmed yet.

In contrast, from the results in this chapter, one first conclusion is that another adaptation available to cells to overcome the loss of fitness caused by decreased ribosome efficiency is to maintain the lower level of protein synthesis and find other ways to recover growth speed, decoupling fitness from the translation level of wild-type cells.

This finding is partly consistent with the literature. In many cases, the high complexity of the ribosome is tied to the high complexity of gene expression regulation. This is evident in organisms specialized for growth in very stable environments, such as the endoparasites in the genera *Mycoplasma* and *Plasmodium*, or endosymbionts like mitochondria and chloroplasts. In these cases, a dramatic reduction in the number of r-proteins encoded by the genome is observed. This is explained as a product of selective pressure to reduce the significant amount of energy that would otherwise be required to synthesize a large number of r-proteins. In this situation, such proteins are no longer necessary for regulating gene expression of different genes under varying conditions, as the host cell already provides most of the resources needed for growth (Ehrenberg & Kurland 1984, Weber et al 2023). Still, the reduction in the expression of regulatory genes seen in these examples is not necessarily accompanied by an overall decrease in gene expression, as observed in this work.

4.3.2 Blocking transcription does not provide fitness recovery

To try and better explain the mechanisms by which compensatory mutations help recover fitness, I turned focus onto the information provided by the *de novo* mutations themselves. One of the interesting results from Chapter 3 consists in the fact that several lineages evolved from mutants with deletion of different r-proteins showed compensatory mutations in the same set of genes involved in transcription regulation and in genes associated with the coupling of transcription and translation, like *rpoA*, *rpoC*, *rpoD* and *nusG*.

This suggests that the deletion of r-protein genes has effects not only on translation but also on transcription, and on the balance between the two. The available literature did not provide sufficient information to pinpoint the specific effect on transcription caused by the evolved SNPs; a possibility was that the *de novo* mutations reduced the transcription level in the cell. A preliminary test to explore this connection consisted of growing the ancestral r-protein deletion mutants in various non-lethal concentrations of the antibiotic rifampicin. Rifampicin prevents RNA elongation by blocking the catalytic activity of RNAP, binding to a pocket in the DNA-RNA channel and inducing the release of an incomplete

RNA (Mosasei & Harbottle 2019). If the compensatory mutations acted by decreasing the level of transcription, then low concentrations of rifampicin could have mimicked it and helped the ancestral mutants to grow faster.

This hypothesis was disproved by the result; adding rifampicin to the growth medium reduced the yield of both wild-type and ancestral mutants. In all the mutants, the decrease was proportional to the concentration of the antibiotic. It appears that the reduction in yield caused by progressive increases of the antibiotic concentration is slightly stronger in the wild-type than in the deletion mutants, but still not enough to make the wild-type grow significantly slower than the mutants. This effect is stronger in the $\Delta L24$ mutant. While this result does not explain the functional mechanism of the compensatory mutations, it still suggests that such mutations do not act by directly decreasing global transcription in the cell.

4.3.3 Lack of nutrients differently affects mutants

Since the total level of transcription seemed not to be a target of compensatory mutations, the next step was to better understand how CTT regulation changed between ancestral and evolved mutants. To do so, the fitness of both ancestral and evolved mutants was measured in CAM with different available concentrations of phosphate and nitrogen. Both elements are used by the cell in several different pathways, but previous studies showed how changes in gene expression mechanisms are reflected in different costs to the fitness of the cell after deprivation of one of the two nutrients (Kafri et al 2016).

This is possible because different media allow to visualize the distinction in cost contribution to fitness between transcription and translation. In yeast, rapidly growing cells are not always limited by ribosome content, depending on the growth conditions. In phosphate limitation the fitness cost of gene expression scale with the copy number of a costly gene, so the transcription is limiting. In nitrogen-limited media, the fitness cost of a gene scales with protein expression level and product length, so translation is limiting (Kafri et al 2016).

The results of this chapter showed how the ancestral mutants $\Delta L9$, $\Delta L27$, $\Delta L36$ all showed a slight decrease in fitness following a decrease in nitrogen concentration. The fitness reduction was much stronger when the cells were exposed to low phosphate medium, with almost 40% of fitness lost. The $\Delta L24$ mutant showed the same slight sensitivity to low nitrogen, but actually showed an almost 20% fitness recovery in low phosphate medium.

This experiment was repeated with the evolved mutants. All the evolved mutants showed an increase in fitness in all the growth conditions, as expected, and also a much closer fitness between standard medium and low phosphate medium. This was observed in all the evolved mutants with compensatory mutations in the transcription apparatus, irrespective of the specific *de novo* or ancestral mutation.

The evolved $\Delta L9$, $\Delta L27$, $\Delta L36$ mutants all showed increased and more consistent fitness level in all the environments, relative to the fitness of their respective ancestral deletion mutants. Such situation appears also in the double mutant $\Delta L9\Delta L24$. The evolved $\Delta L24$ mutants all showed increased fitness in every environment, but interestingly in most cases the fitness in low nitrogen conditions lagged behind.

Three mutations of the *secE-nusG* operon appeared in the evolved clones analyzed: *nusG* I38N, *nusG* Y6C, *secE* I120V. Of these, *nusG* Y6C always appear in clones with more than 20% of fitness recovery in standard CAM. The mutation *secE* I120V provides a small recovery, and *nusG* I38N has an intermediate phenotype between the two. It is possible that the *secE* I120V mutation provides a little advantage by altering the expression of *nusG*, while the mutations in the coding sequence of *nusG* provide a stronger effect by changing NusG affinity to its ligands. All the *secE-nusG* operon mutants show a fitness in low phosphate comparable to the level in standard CAM.

A clue on the activity caused by these mutations is provided by the fact that the ancestral $\Delta L24$ and $\Delta L27$ mutants, while having similar fitness, show very different behavior in low phosphate: $\Delta L24$ appears to grow better than the wild-type in this environment, while $\Delta L27$ grows even worse. The evolved $\Delta L24$ clones MPB39391, MPB39397 and $\Delta L27$ clone MPB39201 all have the mutation *nusG* Y6C. In these mutants the pattern is switched: the evolved $\Delta L24$ have high fitness in standard CAM and in low phosphate medium, but low fitness in low nitrogen medium; the evolved $\Delta L27$ have high fitness in all media. This suggests how the contribution of L24 and L27 to protein synthesis relies on different mechanisms and possibly requires distinct components. It is known that L24 is crucial for ribosome assembly but is relatively less needed for the activity of assembled ribosomes (Spillmann & Nierhaus 1978). On the other hand, L27 directly interact with the peptidyl-transfer center of the ribosome during translation (Bischof et al 1995).

It appears the *nusG* Y6C mutation, while providing a fitness advantage to both mutants, specifically makes the $\Delta L27$ mutant less vulnerable to a decrease in transcription availability; at the same time, it does not provide a benefit as strong in $\Delta L24$ mutant when translation is also constrained by the low nitrogen levels. The difference makes sense when considering that compensatory mutations are selected during evolution

in rich medium. The variations in the effect of *de novo* mutations when presented to new growth conditions, like in low nitrogen, can be helpful to expose the interaction between *de novo* mutation and the ancestral r-protein deletion. It is possible that the difference in fitness between evolved L24 and L27 mutants with *nusG* Y6C mutations is caused by differences in the activity of ribosomes lacking L24 or L27. For example, it is possible that such mutation in *nusG* helps to recover processivity of a complete ribosome, but does not address the issues of reduced ribosome assembly due to loss of L24 (Klein et al 2004).

The evolved clones MPB39300 (evolved Δ L24) and MPB39302 (evolved Δ L27) carry *de novo* mutations in *rpoC*, respectively D806G and V673A; both clones also have the SNP *secE* I120V, which in the evolved Δ L9 clone MPB39190 appears to provide a small fitness recovery effect in standard CAM). Like in the case of the mutation *nusG* Y6C, mutations in *rpoC* appear to help recover a great amount of fitness to the Δ L24 mutant and a smaller amount of fitness to the Δ L27 mutant in standard CAM, while not contributing as much in low nitrogen in the case of MPB39300, while still showing a significant fitness recovery for MPB39302. This further supports that the deletion of L24 is more severe when the translational activity is lower and that the compensatory mutations in *nusG* and *rpoC* increase the overall fitness by providing more efficient transcription, which becomes evident when the phosphate is limiting and the transcriptional activity in the cell is compromised.

4.3.4 Transcription and translation contribute differently to fitness

Several explanations could account for these results. First of all, it is important to note that culturing cells in nutrient-limited media does not allow to directly distinguish between the different ways in which the nutrients are used. For instance, one primary function of phosphate in growing cells is to synthesize new ATP and GTP in sufficient amounts for both daughter cells, with up to 10^{10} new ATP molecules per cell (Lynch & Marinov 2019, Meyrat & von Ballmoos 2019). The phosphate limitation impact on transcription should also be divided between mRNA, rRNA and tRNA. In a cell there is usually a much higher amount of rRNA, with up to 20 times the mass of rRNA relative to mRNA (Milo & Phillips 2015). This is partially offset by the relative stability of rRNA in complete ribosomes and by the high turnover of mRNA, leading to a more balanced transcriptional load between the two, especially at slow growth rates (Bremer & Dennis 2008, Sulthana et al 2016). Despite this uncertainty, the results here presented and the insights from the known function of the mutant genes allow us to make some more hypotheses to explain how cells can adapt to the loss of a r-protein.

As discussed in this chapter introduction, deletions and deleterious mutations of r-proteins usually lead to a decrease in protein synthesis via a decrease in translation initiation rate. This occurs both by a delay in translation initiation and by mechanisms that slow down elongation rate to maintain a constant density of ribosomes on a mRNA through ribosome binding site occupation (Weber et al 2023). While slowing down translation has direct negative impact on cell growth, the close coupling of transcription and translation means that disrupting one process can readily affect the other.

One indication of this is how the ancestral $\Delta L9$, $\Delta L27$, and $\Delta L36$ mutants are more sensitive to a decrease in available RNA in a situation when the ribosome activity is already disrupted by r-protein deletion. It is possible that the compensatory mutations here identified allow for the ribosomes to quickly bind to the mRNA as soon as it is synthesized. This could be possible for example by an increased expression of *nusG*. In such a case, the cell would not waste the available ribosomes, which already require high energy investment to be produced. The mRNA too would avoid being wasted: in *E. coli*, on average, a mRNA is translated between 10 to 100 times before being degraded (Milo & Phillips 2015). Moreover, ensuring the contact of the ribosomes on the mRNA would decrease the risk of interrupted translation initiation, avoiding the costs needed to re-establish the phosphorylation status of translation initiation factors.

The regulation of the assembly of the translation initiation complex could even be at the level of ribosome assembly. It is possible that the compensatory mutations are actually altering the biosynthesis of rRNA. This is a possibility suggested both by the known function of NusG in regulating rRNA maturation and by the fact that in bacteria mRNA and rRNA are transcribed by the same RNAP. A stoichiometric increase in the abundance of one of the ribosome components, the rRNA, could potentially positively drive forward the ribosome assembly (Knöppel et al 2020).

4.3.5 Conclusions

The real challenge remains how to explain a recovery in fitness without a recovery in translation rate. It is possible that the evolved mutants are simply adapted to grow with lower amount of available proteins. Indeed, there is evidence that the cost to express a gene is inversely proportional to cell size and thus number of proteins in the cell (Lynch & Marinov 2015). However, cell size is largely consistent between ancestral and evolved mutants. The only visible cell morphology phenotype caused by r-protein deletion was the elongation observed in the $\Delta L27$ mutant. Such cells look extremely elongated and prone to lysis; this could partially explain their very low fitness, and it suggest for a previously unknown role of the gene *rpmA* in the regulation of cell division.

Alternatively, the inefficiency in translation caused by r-protein deletion might be strong enough, and the increased coupling of transcription and translation efficient enough, that the two effects on fitness balance each other. A good example for this explanation is the evolved Δ L24 clone MPB39391, with a recovery mutation in *nusG* that recover the fitness from around 40% to 90%. If such mutant is exposed together with the wild-type in a low nitrogen environment the result is that the compensatory mutations, not adapted to grow with an even lower protein synthesis rate, revert back to a low fitness level. On the opposite, when exposed to low phosphate, the mutant can keep producing all the required components as well as in the rich medium. This could mean that the mutant is always actively transcribing as much RNA as possible to maintain fitness, while the process of translation is still depending on the concentration of available amino acids.

Another example is given by the evolved Δ L27 clone MPB39201, with the same *nusG* Y6C mutation as MPB39391. In this case the evolved clone maintains the same fitness level independently of the limitations of nutrients for transcription and translation. The fact that the same mutation in the same gene appeared in two different r-protein deletion mutants and that these mutants behave differently in different media shows that evolution, by selecting *de novo* mutations aimed at increase growth rate, is actually blind to how to this growth rate is obtained, and to how successful the mutant organism will be in a different environment.

Overall, the results of this chapter show how the loss of different r-proteins, each with a different function, can be recovered, and how some of the easiest routes available to evolution to do so are through similar *de novo* mutations. This points to the genes targeted by such compensatory mutations, such as *nusG* and *rpoC*, as crossroads of regulatory and metabolic networks, and as potential hotspots of variability via adaptation despite their high degree of conservation across lineages.

CHAPTER 5 - CONCLUSION AND OUTLOOK

Chapter 2

In this dissertation I demonstrated how different cellular processes can be studied combining genetic and evolutionary approaches.

In Chapter 2, I demonstrated how the deletion of *rodA* leads to altered fitness and morphology of the cells, and how the fitness can be recovered through evolution of compensatory mutations in *ftsZ* and in the *min* operon. These results mirror the evidences found in previous studies of other components involved in the longitudinal synthesis of peptidoglycan in rod-shaped cells, like *mreB* (Yulo et al 2025). Performing studies of evolutionary cell biology on different, but related, genes, can inform us on the points of contact between the mechanistic pathways affected by such genes.

While the deletion of *mreB* and *rodA* in *P. fluorescens* affect similar synthetic pathways and produces similar phenotypes, the compensatory mutations that are selected during fitness recovery are different. The compensatory mutations in evolved $\Delta rodA$ mutants affect genes that guide the correct localization of the division septum, while the compensatory mutations in evolved $\Delta mreB$ mutants are in genes involved in undirected cell wall synthesis such as *pbp1a*. It is possible that in both cases the compensatory mutations provide regulation at the level of increasing the accuracy of the process of septation formation. Alternatively, the differences in compensatory mutations can be interpreted as evidences of the different interactions that MreB and RodA have with different systems; for example, it is known that MreB is involved not only in interacting with the elongasome, but also in some cases with the divisome, and in providing structural strength (Fenton & Gerdes 2013,).

More work is currently ongoing in Paul Rainey lab to extend on the results of Chapter 2. Work by David Rogers, Ellen McConnell and others has demonstrated how the *ftsZ* S281R and D176G SNPs, identified in this work, are directly responsible in providing a fitness recovery. Moreover, it's been possible to tag with a fluorophore FtsZ in both its wild-type and mutant alleles. This can allow to track the movement of FtsZ particles in the cells, together with the rate and localization of Z-ring assembly in both wild-type, $\Delta rodA$, and evolved $\Delta rodA$ cell types. This showed how $\Delta rodA$ round cells can properly assemble the Z-ring along the cell diameter if the cell carries a mutant copy of *ftsZ*. Similarly, it was proved that duplication of the operon *minCDE* recover fitness, although at a lower level than the *ftsZ* SNP mutants. Further studies could

be focused on better quantify the Z-ring assembly in different cell morphology, and to study how different species coevolved cell division and shape.

Interestingly, evolved $\Delta mreB$ population carried a SNP in *ftsZ*, D176A, in the same residue as one found in the Chapter 2, D176G (Yulo et al 2025). Introducing D176G in a $\Delta mreB$ mutant recovered fitness, similarly to what happens in a $\Delta rodA$ mutant. In parallel, the knock-out of *pbp1a*, that was found to recover fitness in $\Delta mreB$, does the same in $\Delta rodA$. These commonalities reveal two insights. First, the loss of multiple elongasome components lead to deleterious effects on fitness that can be recovered by identical *de novo* mutations. Second, depending on which elongasome gene is knocked out, the most common compensatory mutations differ, despite providing a similar benefit. It remains to be investigated what causes the commonalities and differences leading to these results. An interesting future step would be to perform evolutionary cell biology studies on the remaining elongasome components such as PBP2.

Chapter 3

In Chapter 3, I successfully deleted the r-proteins L9, L24, L27, L33, L36 in *P. fluorescens*. Following analyses showed how altering the ribosome decrease fitness, and how the fitness can be recovered by experimental evolution. As expected, the evolved mutants behaved differently depending on which r-protein gene was deleted. The differences were both in the magnitude of fitness recovered, and in the specific compensatory mutations identified. Despite this, an interesting find is how the same class of transcription and translation related genes, such as *nusG* and *rpoC*, was repeatedly targeted by *de novo* mutations in different evolved mutants. As in the case of parallel findings from the results of evolved $\Delta rodA$ mutants from Chapter 2 and those from evolved $\Delta mreB$ mutants, a deeper study on evaluating how much different compensatory mutations recover fitness in different genetic backgrounds could be done. This could explain why are different compensatory mutations selected if they provide similar amount of fitness, could better elucidate the commonalities and differences in the functions performed by different r-proteins

The results of Chapter 3 could also drive further research driven by the understanding of how the mutations needed to recover the fitness after the loss of ancestral, conserved and important genes such as the ribosomal proteins are targeting other ancestral, conserved and important genes such as *nusG* and RNAP genes. Such genes are usually central to multiple pathways. *De novo* mutations targeting them, while useful to recover the loss of other housekeeping genes, can possibly make multiple pathways more

fragile to further genetic changes or environmental changes that the organism may encounter. This could decrease the long-term fitness of the lineage and contribute to the low rate of mutation seen in such genes.

Another interesting result of Chapter 3 was the identification of compensatory mutations in r-proteins that are apparently not structurally or functionally related to the r-protein deleted in the experiment. More experiment could be done to explore the relationship between such r-proteins and their function in the ribosome activity, and to expand our current knowledge of interaction networks inside the ribosome at different stages of translation.

Chapter 4

In Chapter 4, I studied how the recovery mutations identified in Chapter 3 in the *secE-nusG* operon and in the genes *rpoC* and *rpoD*, appear to provide fitness recovery after ribosome disruption through translation and transcription regulation. It appears that the r-protein deletion mutants produced in Chapter 3 suffer a translation rate deficiency, but this deficiency is not recovered together with the fitness in the evolved mutants. Further experiments showed how the ancestral and evolved mutants with mutations in *rpoC*, *rpoD*, *secE*, *nusG* behave differently depending on the availability of nitrogen-based and phosphate-based nutrients. This further suggests that growth speed lost after translation impaired can be recovered through regulation of transcription.

Since the details of such mechanism of action are still not clear, more experiments can be done to better understand the picture. One possibility is to directly quantify transcription in ancestral and evolved r-protein deletion mutants. This would allow to understand if the fitness is being recovered by simply better matching transcription and translation rates. Such analysis could be also incorporated by measuring the activity of the wild-type and mutant genes targeted by compensatory mutations. Another possibility is to measure the translation initiation rate by ribosome profiling. Such analysis would explore the details of how the different r-protein deletion affect the working of the ribosome, which steps of translation are disrupted, and which are recovered after evolution of compensatory mutations. Finally, expanding on the result of how the limitations on transcription and translation differently affect ancestral and evolved mutants, the role of the ribosome in its own biosynthesis could be studied. Considering the involvement of transcription regulation and NusG in the fitness recovery of evolved r-protein deletion mutants, it is

possible that the cells are regulating the production of rRNA to the rate of translation, reaching the best possible balance of rRNA and r-proteins in the cytoplasm to allow optimal ribosome assembly.

Overall, the results in this dissertation express how evolutionary cell biology can be applied to diverse systems, and how it can be useful in studying complex molecular systems. Especially in cases of essential genes, which are difficult to manipulate and study for the experimenter, employing experimental evolution as a discovery tool can provide unexpected knowledge that can be used to understand previously unknown genetic network. The networks are both at the functional level, revealing interactions between proteins and how they act together; and at the evolutionary level, showing which genes tend to evolve following mutations to another.

CHAPTER 6 - MATERIAL AND METHODS

6.1 GENERAL METHODS

6.1.1 Growth conditions

All liquid cultures were grown in 6 ml microcosms shaking at 220 rpm. Unless otherwise specified in the text, *P. fluorescens* cultures were grown in KB at 28°, while *E. coli* cultures were grown in LB at 37°. Plates were produced by adding agar 15 g/l to the medium. To grow plasmid-carrying mutants, antibiotics were added to the growth medium to prevent plasmid loss by selecting for the antibiotic resistance gene present in the plasmid.

6.1.2 Media composition

KB: glycerol 10 ml/l, proteose peptone No.3 20 g/l, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 1,5 g/l, K_2HPO_4 1,5 g/l

LB: tryptone 10 g/l, yeast extract 5 g/l, NaCl 10 g/l

TYS10: tryptone 10 g/l, yeast extract 5 g/l, sucrose 100 g/l

TB: KH_2HPO_4 23,1 g/l, K_2HPO_4 125,4 g/l, tryptone 12 g/l, yeast extract 24 g/l, glycerol 4 ml/l

CAM was produced in five different formulations, with different levels of nutrient depletion:

Formulation	Citric acid	$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	Na_2HPO_4	KH_2PO_4	NH_4SO_4	NaOH 1M
Standard	4 g/l	0,2 g/l	6 g/l	3 g/l	1 g/l	40 ml/l
70% [P]	4 g/l	0,2 g/l	4,2 g/l	2,1 g/l	1 g/l	45 ml/l
40% [P]	4 g/l	0,2 g/l	2,4 g/l	1,2 g/l	1 g/l	50 ml/l
40% [N]	4 g/l	0,2 g/l	6 g/l	3 g/l	0,4 g/l	45 ml/l
20% [N]	4 g/l	0,2 g/l	6 g/l	3 g/l	0,2 g/l	47 ml/l

Antibiotics were added to the required media at the following concentration: tetracycline (Tet): 12,5 µg/ml; ampicillin (Amp): 100 µg/ml; kanamycin (Kan): 50 µg/ml; gentamycin (Gm): 20 µg/ml; nitrofurantoin (Nf): 100 µg/ml.

6.1.3 Molecular techniques

Plasmids were extracted by QIAgen Miniprep kit. Genomic DNA was extracted by QIAgen Blood and Tissue kit. PCR reactions were performed with NEB Q5 Master Mix or NEB Taq Master Mix. PCR reactions were confirmed by running gel electrophoresis of the sample on 1% agarose pads, with ThermoScientific

GeneRuler DNA ladder mix as a control to compare the amplified product size. Plasmid assembly was performed with NEB Gibson assembly Master Mix. The amount of DNA required was calculated with NEB biocalculator (<https://nebiocalculator.neb.com/#!/ligation>). Sanger sequencing was performed in-house with BigDye-Xterminator from Thermofisher.

6.1.4 Two-step allelic exchange

All the gene deletions were performed by two-step allelic exchange. The procedure used was adapted in-house by David Rogers and Ellen McConnell from Hmelo et al 2015. It requires a receiver strain, a helper strain, and a donor strain (**Supplementary Table 9**).

The donor strains were produced by transformation of competent *E. coli* with a plasmid including the mutant allele and a selection cassette. The plasmid is made of plasmid backbone, and upstream and downstream region of the gene required to delete.

To construct the plasmid used to perform a deletion, it is required to amplify by PCR the two genomic regions immediately upstream and downstream of the region to be deleted. The primers used for the two PCRs are designed to provide an overlap both between the inward extremities of the upstream and downstream regions facing each other, to provide a bridge over the deleted section, and outward, to allow the assembly of the two segments with the plasmid backbone (**Supplementary Table 10**).

The competent *E. coli* is then transformed by heat shock, and grown under selection in LB-Tet to screen for colonies that acquired a plasmid. Single colonies are screened by gel electrophoresis of PCR products obtained from primers within the upstream and downstream region to select those with a plasmid that contained both of them. Such colonies are then grown in LB 37°, then the plasmid is extracted and sequenced by Sanger sequencing around the integrated locus to confirm the plasmid assembly and transformation were successful.

To introduce the mutation in *P. fluorescens*, a single donor *E. coli* is grown in a conjugation spot with a receiver *P. fluorescens* and a helper *E. coli* strain. Each of the three strains is first grown alone overnight in liquid culture. The receiver strain is then heat-shocked at 45° 20', and then mixed with donor and helper. The mix is plated in a single spot in the center of a LB plate and grown overnight at 37° degrees. This allows the receiver strain to acquire the plasmid.

To select for receiver colonies that integrated the plasmid in the genome, the conjugation spot is scraped, mixed in LB, and plated on LB-Nf-Tet at 28° overnight. The nitrofurantoin kills all the *E. coli* cells (donor

and helper), while the tetracycline kills all *P. fluorescens* cells that didn't integrate the plasmid in the chromosome, since *P. fluorescens* is unable to replicate the free plasmid and transmit copies of it to daughter cells in a growing colony. The resulting colonies are harvested and streaked on TYS10 plates to select colonies that performed the second allelic exchange, excising the plasmid backbone together with one of the two alleles (randomly, the wild-type or the mutant). This is because TYS10 activate the suicide cassette inside the plasmid backbone.

The resulting colonies are screened by PCR with inward pointing primers outside the gene of interest to distinguish the colonies that kept the mutant allele from those that kept the wild-type allele: the expectation is that half the colonies that grow in TYS10 kept the mutant allele with the genetic deletion, and as such the PCR product is shorter. The successful colonies are then sequenced around the mutant locus by Sanger sequencing to confirm the successful mutagenesis. Mutants are stored at -80° in a tube comprising of 400 µl glycerol, 800 µl culture.

6.1.5 Insertion of fluorescent tag

Each mutant strain was separately tagged by both GFP and mScarlet. The fluorescent protein genes are introduced by Tn7 transposition insertion. The recipient mutants are heat-shocked at 45° 20', and mixed with donor strain (MPB158011 for GFP or MPB15801 for mScarlet) (**Supplementary Table 9**). The mix is centrifuged and resuspended in LB, then plated in a conjugation patch on LB. After overnight growth, the conjugation patch is scraped, resuspended in LB and then plated again on LB+Nf+0.1%arabinose+antibiotic (Km for GFP tagging, Gm for mScarlet tagging). Successful integration is confirmed by fluorescence and PCR of the transposon insertion.

6.1.6 Fitness assay

To measure the fitness of a mutant, its competitive fitness against the wild-type is measured. Each measurement is taken with three replicates. To make it possible, either the mutant or wild-type must be tagged with GFP, the other with mScarlet. Both strains are inoculated separately from frozen stock in KB and grown overnight. The two cultures are then mixed in a ratio of 10 volumes of mutant to 1 of wild-type. Since most of our mutants grow slower than the wild-type, this dilution ensures that there will be enough mutant cells in the following steps to ensure robust measurements. 60 µl of the mixture are inoculated in fresh 6 ml of KB and grown overnight. This first mixed culture is then sampled to count the cells at T0, and 60 µl are inoculated in fresh 6 ml of KB to grow overnight. Then the culture is sampled again count the cells at T1.

The cells in the cultures at T0 and T1 are counted with the flow cytometer. To do so, 200 µl of culture are 1:10000 diluted in PBS and inoculated in a 96-well plate for the read. The flow cytometer measure for each cell in the sample the level of fluorescence in the green and red spectrum. Each cell is classified as wild-type or mutant depending on their fluorescence.

To quantify the fitness of the mutant, the Malthusian parameter is calculated from the cell count at T0 and T1:

$$Fitness = \frac{\log\left(\frac{fMt1}{0.1 * fMt0}\right)}{\log\left(\frac{fWTt1}{0.1 * fMt0}\right)}$$

where fM is the frequency of mutant cells, fWT is the frequency of wild-type cells and t0 and t1 indicates the time of measurement. The result is the relative growth of mutant against wild-type.

6.1.7 Genome-wide sequencing and secondary mutations identification

DNA from a sample is extracted with QIAgen blood and tissue kit. The resulting DNA concentration is measured by Nanodrop and Qubit, and then sent for Illumina sequencing at Eurofins (www.eurofinsgenomics.eu), aiming for a sequencing depth of 100x.

The resulting reads were mapped on the SBW25 reference genome (from NCBI, reference AM181176.4) using the software BreSeq (Barrick et al 2014) and Geneious (Geneious Prime 2021.2.2) looking for *de novo* mutations at high frequency in a population (>60%), especially in coding regions, and for mutations that appeared in multiple populations.

The reference and mutant sequence of the *secE-nusG* operon was analyzed by the online software SAPPHIRE.CNN (Coppens et al 2022).

6.2 METHODS OF CHAPTER 2

6.2.1 Experimental evolution of *rodA* mutants

From each *rodA* mutant, ten populations were inoculated in 6 ml KB (**Supplementary Table 9**). Each population was grown overnight at 28° 220 rpm, followed by 1:1000 dilution in fresh medium. This regime averages to around 10 generations/cycle. Every 7 days the populations were sampled to produce a

glycerol stock to store at -80°, and to measure their fitness against the wild-type. The experimental evolution proceeded for a total of 69 days for all of the evolving populations. Each evolving population was sampled and sequenced at 34 and 69 days of evolution.

6.2.2 Microscopy

Each strain is grown overnight in LB from frozen stock. The cultures are then diluted 1:10 in fresh medium and grown another 2 hours to ensure the cultures is in growth phase. 2.5 µl of sample are then diluted 1:10 in LB. The microscope slides are prepared by covering them with a LB-2% agar pad of 350 µl, on which the sample is inoculated. After dehydration of the sample drop, it is covered with the cover slide. The slides were observed by phase contrast and fluorescence microscopy with a Carl Zeiss Axio Imager Microscope.

6.2.3 Colony size measurement

Ancestral and evolved populations at day 34 of the evolution experiments were co-cultivated. Every evolved population was mixed with the corresponding ancestral mutant tagged with a different color. After overnight growth, the culture was plated on LB. The plates were imaged after 3 days with Chemidoc. Each plate was imaged at wavelengths to separately excite both GFP and mScarlet to distinguish colonies of evolved and ancestral mutants. The fluorescent colonies were identified and measured by Fiji (Schindelin 2012).

6.3 METHODS OF CHAPTER 3

6.3.1 Experimental evolution of r-protein mutants

For each r-protein mutant, 10 populations were produced (5 tagged with GFP, 5 tagged with mScarlet) (**Supplementary Table 9**). Each population went through cycles of overnight growth in 6 ml TB at 28° 220 rpm followed by 1:1000 dilution in fresh medium. This regime averages to around 10 generations/cycle. Every 7 days the populations were sampled to produce a glycerol stock to store at -80°, and to measure their fitness against the wild-type. The experimental evolution proceeded for a total of 56 days for all of the evolving populations, with the exception of the Δ L27-RFP populations that went through 28 days of growth. Each evolving population was sampled and sequenced at 28 and 56 days of evolution.

After identification of *de novo* mutations of interest, it was tried to reconstruct them in the genetic background of the ancestral mutants by two-step allelic exchange, without success. It was instead decided to isolate single colonies from the evolved populations that carried the compensatory mutations. To do

so a sample of each evolved population at day 560 was plated, and the region of interest of single colonies was sequenced by Sanger sequencing. The genomic DNA was extracted from multiple colonies that carried the mutations of interest and sent it for full genome sequencing at Eurofins, to confirm the result and select for further experiments the colonies that carried the lower number of secondary mutations beside those of interest. In addition to the mutations described in the text, the chosen colonies carried between 0 and 5 SNPs in non-coding regions.

6.4 METHODS OF CHAPTER 4

6.4.1 Fitness assay in nitrogen- and phosphate-limited medium

The fitness of each mutant was compared against wild-type in citric acid media with different concentrations of nitrogen (N) and phosphate (P) components: standard formulation, 70% [P], 40% [P], 40% [N], 20% [N].

Each mutant and wild-type are first individually grown in KB overnight from frozen stock. 900 μ l of mutant culture are then mixed 100 μ l of wild-type culture. 60 μ l of the mixed culture are then inoculated in a microcosm of each different medium and grown overnight at 28° 220 rpm. After 24 hours, the first fitness measurement is taken, and 60 μ l of culture were transferred to fresh medium. After another 24 hours of growth, the second measurement is taken. The cell count and fitness measurement were performed as described above.

6.4.2 FRAP

Each strain is grown overnight in LB from frozen stock. 600 μ l of culture are transferred to 6 ml of fresh medium and grown another 2 hours to ensure the cells are in growth phase. 2.5 μ l of sample are then diluted 1:10 in LB and inoculated on the microscope slide. The microscope slides are previously prepared by covering them with a LB-2% agar pad of 350 μ l, with a channel of ~0.5 cm cut in the middle of the pad to allow for aeration. The sample is inoculated above the LB-agar pad besides the air channel, and covered with the cover slide after dehydration of the sample drop.

The slides were observed at the LSM Axion Imager 2 microscope using Zeiss software at 400x magnification. For each slide the target area is selected within 200 to 1000 μ m from the air channel to ensure the imaged cells can receive oxygen. Half of the field of view is then selected to be photobleached by the 561 nm laser set with a dwelling of 0.98 μ s/pixel, 6 iterations. One picture is taken in both

brightfield and 488 nm wavelength every 10 minutes for 250 minutes. The photobleaching is performed between the first and second picture of the timeseries are taken.

6.4.3 FRAP analysis

The FRAP time-lapse stacks of images were processed with Omnipose for segmentation and analysis. Each stack is split in bidimensional TIF images for each timepoint. Omnipose is used to perform segmentation and identify each cell in each slide. At each timepoint is separately measured in the bleached and non-bleached region of the picture the number of cells, the average size and the average fluorescence. Then the corrected fluorescence recovery (cFR) is measured as the ratio of fluorescence intensity of bleached and non-bleached cells at each timepoint compared to the normalized values before the bleaching (cFR=1) and right after the bleaching (cFR=0), using the formula from Wu et al 2006.

6.4.4 Rifampicin growth measurement

The viable range of rifampicin concentration was assessed first. A sample of SBW25 is grown overnight and then diluted 1:1000 in fresh medium with the addition of different concentrations of rifampicin. Three replicate cultures for each concentration are prepared. After overnight growth, the OD600 is measured for each sample with a manual spectrophotometer.

To obtain the growth curves, the cells are cultured in KB overnight, followed by a 1:100 dilution in fresh medium with the addition of the required rifampicin concentration. For each rifampicin concentration treatment, three replicates were performed, plus three controls of LB without cells. 200 μ l of each sample were inoculated in a 96 wells plate, then sealed with transpiring membrane. The plate is then incubated in a BioTek Epoch 2 plater reader to obtain a kinetic read. The optical density at 600 nm of each well is measured every 5 minutes for 72 hours at 28°, with the plate shaking before every read. The results for each sample well are then normalized by subtracting the mean of the control wells.

ACKNOWLEDGMENTS

These last years were maybe not enough to produce a lot of great science, but they were plenty enough for much more. Getting to the point of writing these words was a struggle on so many levels, but lucky me, I am a strong proponent of the saying “better to have loved and lost than never to have loved at all”. Which metaphorically applies at basically every level of my experience in Plön. I got something out of it.

Thanks to the MPB people that helped me in this work. Especially to Dave for the always available support and for pointing me in fruitful directions. And to Charly for always being there and to give me back confidence in my work. And to Loukas for having so much patience. And to Paul for the support in producing and writing this thesis. And to Yansong and Jordan for the years spent on the other side of my messy desk, and the chill presence they brought to the office.

Still. All of the above, while nice and shiny and productive, hardly matters when compared to the rest. In the years I worked on this thesis I made so many personal connections and experiences that I treasure immensely. I came here feeling like an empty canvas; I will leave a watercolor. Thank you to Demetris, Vanesa, Camila, Corinna, Loukas, and Saumil. I grew up more in one year with you guys than in the rest of my life. And I am so happy that I met every other person that became a friend while adding color to my painting: Alec, Stella, Felipe, Charlotte, Louk, Vaibhvi, Quentin, Charly, Bea, Elio, Amor, Gisela. I always struggle to get close and to feel close to people, but I’m happy I managed (and managing) with all of you.

And of course, I’m happy I always had the support of my friends and family back in Italy, especially because of all the people listed here they are the only ones I don’t inevitably end up talking about work with. My cats are especially good at that. Grazie per esserci.

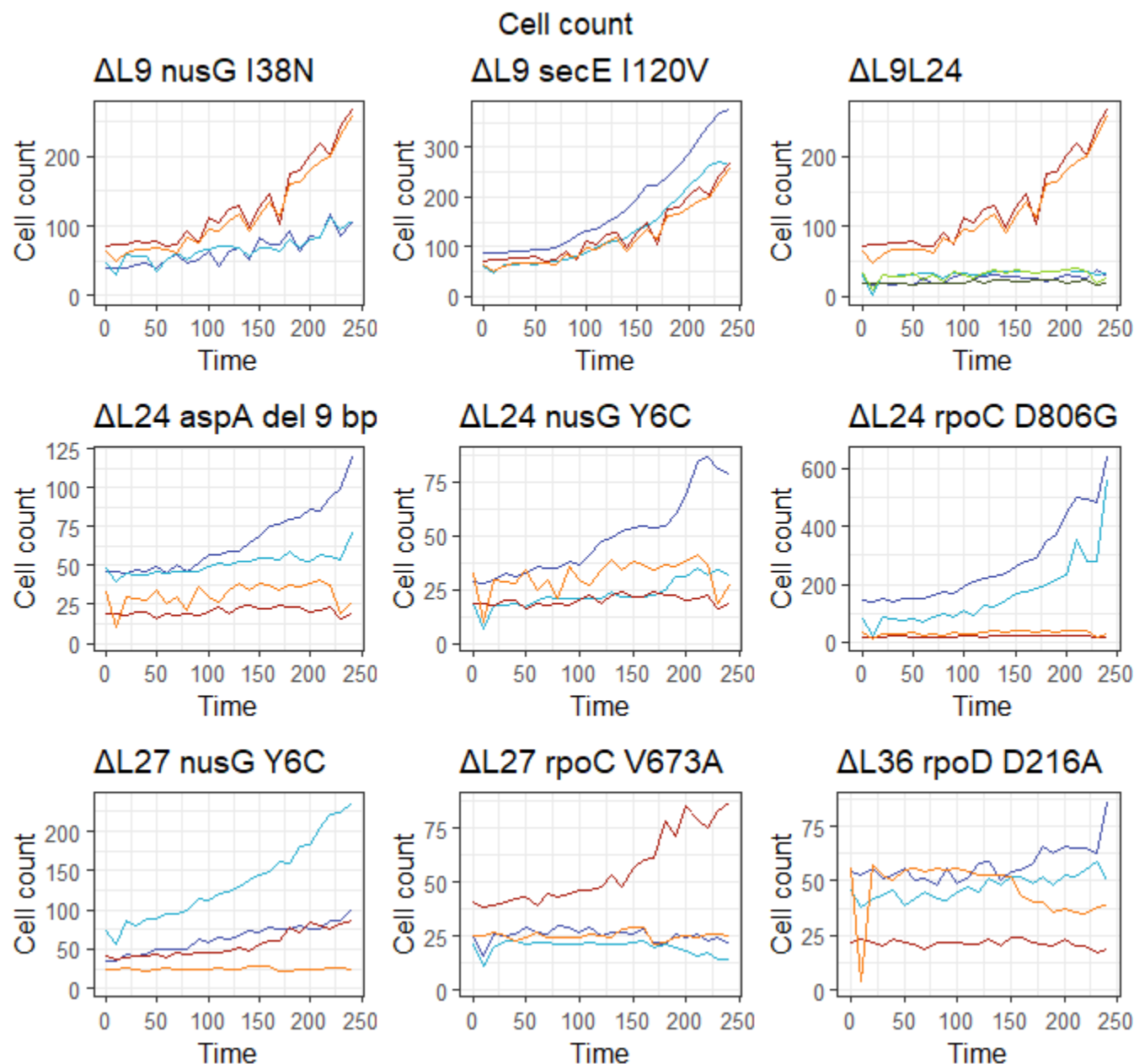
And thanks to you Karem! You are my team. You already know everything I want to say. I will stop writing now, I’m getting emotional.

From white, like ice

To colours, details, pictures.

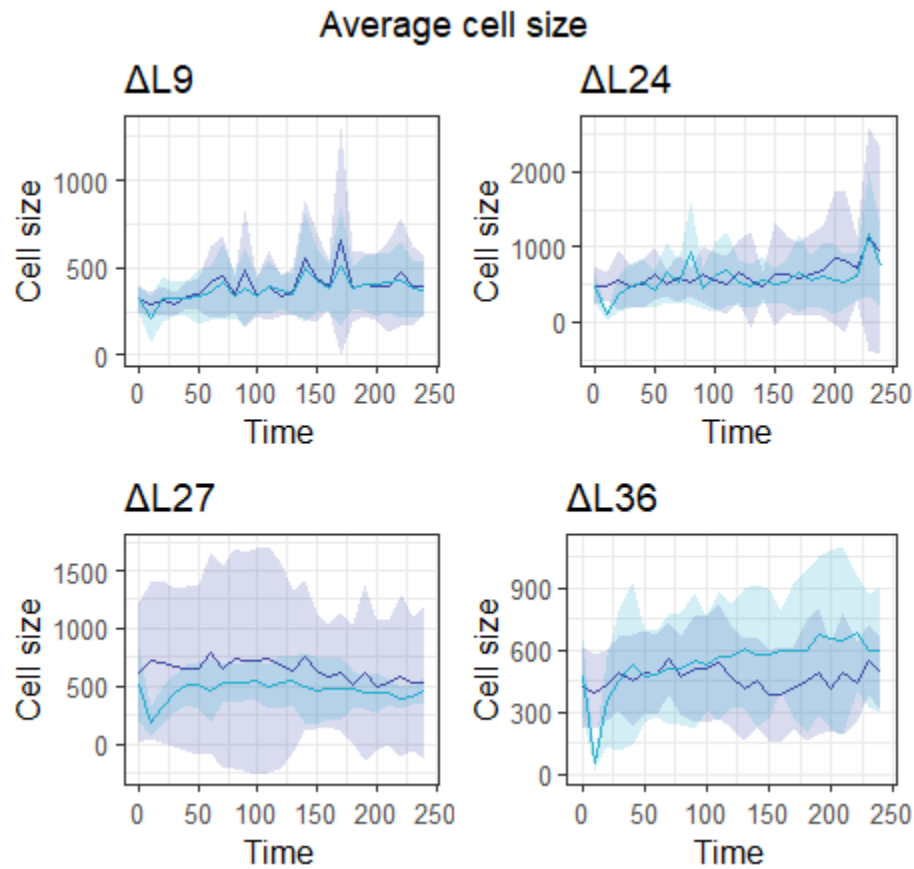
Many pages, through seasons.

SUPPLEMENTARY MATERIALS

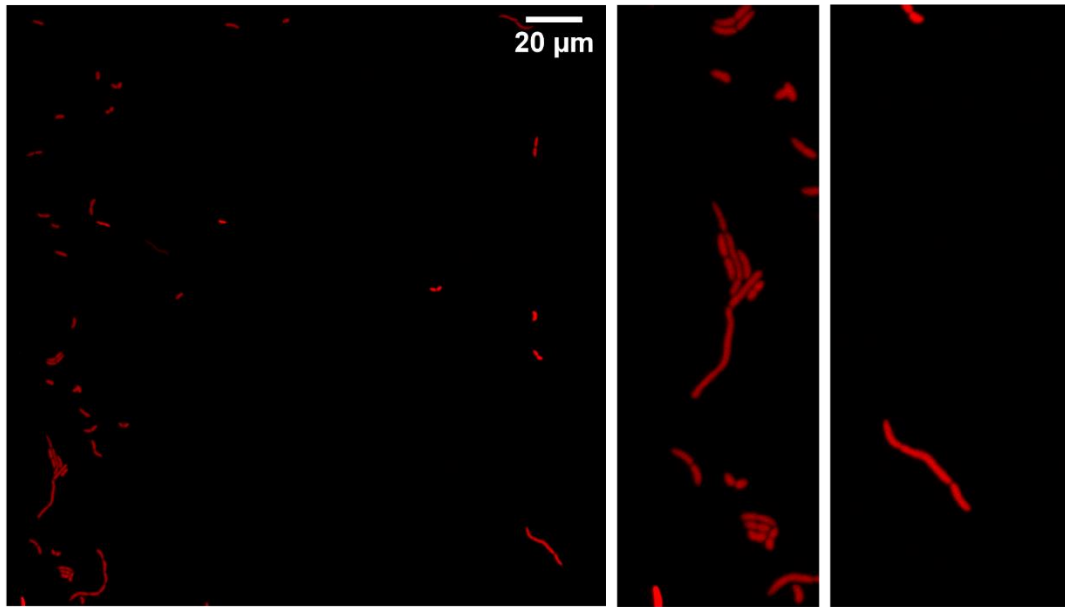


Supplementary Figure 1: cell count of evolved and ancestral r-protein mutants during the FRAP experiments. X-axis: time in minutes. Y-axis: total number of cells in the analyzed area. In each panel, the cell count of both the evolved and corresponding ancestral mutants are showed. Blue: evolved bleached mutant. Cyan: evolved control mutant. Red: ancestor control mutant. Orange: ancestor control mutant. In the $\Delta L9\Delta L24$ panel, the

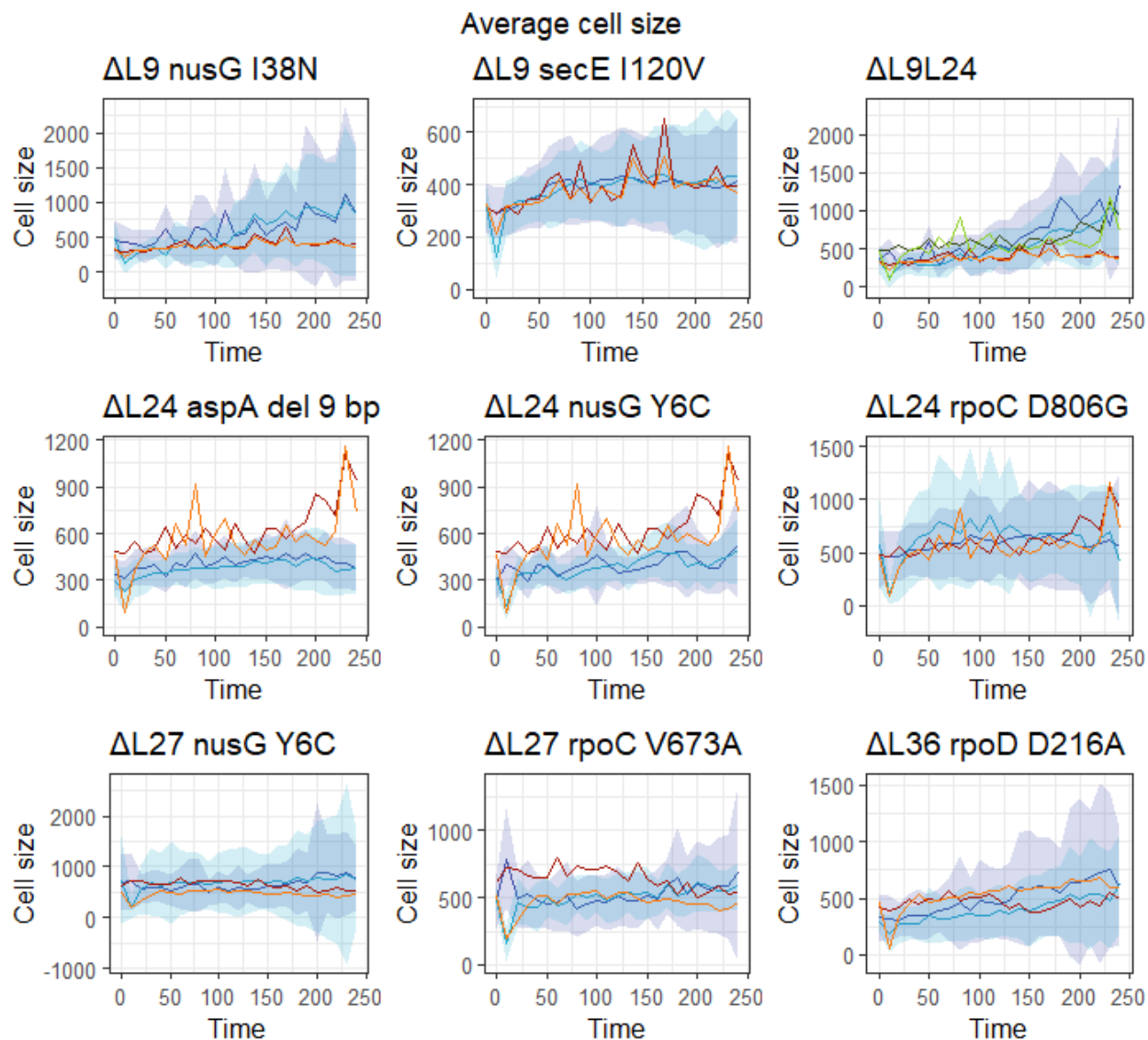
values of both ancestral mutant $\Delta L9$ (red: control, orange: bleached) and $\Delta L24$ (dark green: control, light green: bleached) are showed.



Supplementary Figure 2: cell size of ancestral r-protein mutant cells as measured during the FRAP experiments. X-axis: time in minutes. Y-axis: cell area measured in pixels. Cyan: bleached cells. Blue: control cells. The cyan and blue ribbon represent the average plus and minus standard deviation of respectively bleached and control cells.



Supplementary Figure 3: Δ L27 RFP mutant cells (left panel). Some cells grows without dividing, as evidenced in the zoomed in image (center and right panels).



Supplementary Figure 4: cell size of evolved r-protein mutant cells as measured during the FRAP experiments. X-axis: time in minutes. Y-axis: cell area measured in pixels. In each panel, the values of both the evolved mutant and of the corresponding ancestral mutant (from Supplementary Figure 2) are shown. Orange: ancestral bleached cells. Red: ancestral control cells. Blue: evolved bleached cells. Cyan: evolved control cells. The blue and cyan ribbon represent the average plus and minus standard deviation of respectively bleached and control evolved cells. In the $\Delta L9\Delta L24$ panel, the average values of both ancestral mutant $\Delta L9$ (red: control, orange: bleached) and $\Delta L24$ (dark green: control, light green: bleached) are showed.

Supplementary Table 1: *De novo* mutations in evolved partial *ΔrodA* mutants

Mutant	Gene	Mutation	Genome position	DNA change	Max. freq.	N. of lines	Population day
partial deletion	PFLU_4512	R488G	4979965	A -> G	71.10%	5	34
partial deletion	PFLU_4512	E489G	4979969	A -> G	70.30%	4	34
partial deletion	PFLU_4512	A487G	4979963	C -> G	69.20%	4	34
partial deletion	PFLU_4512	L490G	4979971	CTT -> GGG	68.00%	5	34
partial deletion	PFLU_4512	S515P	4980046	TCG -> CCC	82.40%	4	34
partial deletion	PFLU_4512	QA512PP	4980038	AAGCT -> CCCCC	82.40%	3	34
partial deletion	PFLU_2771	I73L	3064810	A -> C	67.90%	3	34
partial deletion	<i>ftsZ</i>	E242A	1057860	A -> C	96.50%	1	34
partial deletion	<i>ftsZ</i>	Q256P	1057902	A -> C	89.00%	1	34
partial deletion	<i>ftsZ</i>	D305A	1058049	A -> C	100.00%	1	34
partial deletion	<i>ftsZ</i>	N235K	1057840	C -> G	100.00%	1	34
partial deletion	<i>ftsZ</i>	S281R	1057976	A -> C	98.10%	1	34
partial deletion	<i>ftsZ</i>	D176G	1057662	A -> G	100.00%	1	34
partial deletion	PFLU_1247	H147P	1378712	A -> C	76.50%	3	34
partial deletion	PFLU_1247	L146P	1378709	T -> C	71.40%	3	34
partial deletion	PFLU_1247	R145P	1378706	G -> C	60.00%	1	34
partial deletion	PFLU_3017	del 144 bp (3480-3624)	3288727	deletion	100.00%	1	34
partial deletion	PFLU_3858	L466G	4253749	AAG -> CCC	65.90%	1	34
partial deletion	PFLU_5804	A410P	6358181	G -> C	66.70%	1	34
partial deletion	PFLU_3017	del 144 bp (3480-3624)	3288727	Deletion	100.00%	3	69
partial deletion	<i>ftsZ</i>	D176G	1057662	A -> G	96.00%	2	69
partial deletion	<i>ftsZ</i>	E242A	1057860	A -> C	100.00%	1	69
partial deletion	<i>ftsZ</i>	E87K	1057394	G -> A	98.60%	1	69
partial deletion	<i>ftsZ</i>	D252A	1057890	A -> C	98.40%	1	69
partial deletion	<i>ftsZ</i>	D305A	1058049	A -> C	100.00%	1	69
partial deletion	<i>ftsZ</i>	N235K	1057840	C -> G	100.00%	1	69
partial deletion	<i>ftsZ</i>	S281R	1057976	A -> C	100.00%	1	69
partial deletion	PFLU_4512	R494G	4979983	CGA -> GGG	68.40%	1	69
partial deletion	PFLU_4512	A487G	4979963	C -> G	65.80%	2	69
partial deletion	PFLU_4512	R488G	4979965	A -> G	68.40%	1	69
partial deletion	PFLU_4512	E489G	4979969	A -> G	66.70%	1	69
partial deletion	PFLU_4512	S515P	4980046	TCG -> CCC	66.70%	3	69
partial deletion	PFLU_3777	G447S	4173858	G -> A	91.10%	1	69
partial deletion	PFLU_0504	A281V	574010	C -> T	92.60%	1	69
partial deletion	PFLU_2771	I73L	3064810	A -> C	60.60%	1	69

Supplementary Table 2: *de novo* mutations in evolved full *ΔrodA* mutants

Mutant	Gene	Mutation	Genome position	DNA change	Max. freq.	N. of lines	Population day
full deletion	PFLU_4512	S515P	4980046	TCG -> CCC	75.80%	6	34
full deletion	PFLU_4512	E489G	4979969	A -> G	78.10%	6	34
full deletion	PFLU_4512	R488G	4979965	A -> G	76.50%	6	34
full deletion	PFLU_4512	Q512P	4980038	A -> C	65.80%	1	34
full deletion	PFLU_4512	A487G	4979963	C -> G	74.30%	6	34
full deletion	PFLU_4512	L490G	4979971	CTT -> GGG	75.80%	2	34
full deletion	PFLU_4512	QA512PP	4980038	AAGCT -> CCCCC	75.00%	4	34
full deletion	<i>ftsZ</i>	E306A	1058052	A -> C	99.30%	1	34
full deletion	<i>ftsZ</i>	P234L	1057836	C -> T	99.10%	2	34
full deletion	<i>ftsZ</i>	D176G	1057662	A -> G	98.90%	1	34
full deletion	<i>ftsZ</i>	S281R	1057976	A -> C	99.20%	2	34
full deletion	PFLU_3858	L467W	4253749	A -> C	66.70%	1	34
full deletion	PFLU_3858	L466G	4253749	AAG -> CCC	80.00%	3	34
full deletion	PFLU_1247	L146P	1378709	T -> C	66.70%	4	34
full deletion	PFLU_1247	H147P	1378712	A -> C	71.40%	4	34
full deletion	PFLU_2771	I73L	3064810	A -> C	81.00%	3	34
full deletion	PFLU_2771	R72P	3064808	G -> C	72.70%	1	34
full deletion	PFLU_3303	G367A	3657705	G -> C	62.20%	1	34
full deletion	PFLU_5804	A410P	6358181	G -> C	63.40%	1	34
full deletion	PFLU_0882	G171D	992325	C -> T	65.30%	1	34
full deletion	<i>ftsZ</i>	E306A	1058052	A -> C	100.00%	1	69
full deletion	<i>ftsZ</i>	P234L	1057836	C -> T	100.00%	1	69
full deletion	<i>ftsZ</i>	D176G	1057662	A -> G	100.00%	1	69
full deletion	<i>ftsZ</i>	S281R	1057976	A -> C	100.00%	3	69
full deletion	<i>ftsZ</i>	D270G	1057944	A -> G	76.00%	1	69
full deletion	PFLU_4512	R488G	4979965	A -> G	68.30%	4	69
full deletion	PFLU_4512	A487G	4979963	C -> G	67.90%	2	69
full deletion	PFLU_4512	S515P	4980046	TCG -> CCC	69.70%	4	69
full deletion	PFLU_4512	R494G	4979983	CGA -> GGG	64.10%	1	69
full deletion	PFLU_4512	E489G	4979969	A -> G	69.20%	2	69
full deletion	<i>gacA</i>	V4G	2372692	T -> G	63.40%	1	69
full deletion	<i>rpoN</i>	S4P	992827	A -> G	68.40%	1	69
full deletion	<i>ropE</i>	L654V	814265	C -> G	60.40%	1	69

Supplementary Tables 1, 2: only the mutations in coding regions that appear at >60% frequency in at least one evolving population are shown. Column 1: ancestral mutation. Column 2: mutant gene. Column 3:

amino acid change. Column 4: position in the chromosome. Column 5: nucleotide change. Column 6: highest frequency the mutation has been found. Column 7: number of populations that showed the mutation. Column 8: Age of the population where the mutation has been found.

Supplementary Table 3: large scale duplications including the *minCDE* operon or *ftsZ* at 34 days of experimental evolution

Mutant	Start	End	N. of lines	Population day	Genes involved
partial deletion	4588623	4842133	1	34	<i>minCDE</i>
partial deletion	4041111	4876139	3	34	<i>minCDE</i>
partial deletion	4185345	4632667	1	34	-
partial deletion	4213289	4822189	1	34	<i>minCDE</i>
partial deletion	4313735	4823297	1	34	<i>minCDE</i>
partial deletion	1015730	1141917	1	34	<i>ftsZ</i>
partial deletion	4347812	4889344	1	34	<i>minCDE</i>
full deletion	4041111	4876139	1	34	<i>minCDE</i>
full deletion	4811312	4811449	1	34	-
full deletion	4320427	4853676	1	34	<i>minCDE</i>
full deletion	4392143	4836034	1	34	<i>minCDE</i>
full deletion	4591328	4820605	1	34	<i>minCDE</i>
full deletion	987772	1323773	1	34	<i>ftsZ</i>
full deletion	4296396	4864979	1	34	<i>minCDE</i>
full deletion	4233398	4834635	1	34	<i>minCDE</i>
full deletion	4372871	4822629	1	34	<i>minCDE</i>
full deletion	4286227	4833801	1	34	<i>minCDE</i>
partial deletion	4581470	4841908	1	69	<i>minCDE</i>
partial deletion	4354705	4856648	1	69	<i>minCDE</i>
partial deletion	4299476	4844506	1	69	<i>minCDE</i>
full deletion	4327527	4862574	1	69	<i>minCDE</i>
full deletion	4399202	4844545	1	69	<i>minCDE</i>
full deletion	4325768	4830829	1	69	<i>minCDE</i>
full deletion	4610863	4829932	1	69	<i>minCDE</i>
full deletion	4598073	4831242	1	69	<i>minCDE</i>
full deletion	4476368	4855509	1	69	<i>minCDE</i>

Supplementary Table 3: All the chromosomal duplications found in the evolved populations are listed. Column 1: Ancestral *rodA* deletion mutant. Column 2: Start of the duplication. Column 3: End of the duplication. Column 4: Number of evolving lines in which the duplication appears. Column 5: Age of the evolved population in which the duplication is found. Column 6: Genes of interest part of the duplication.

The operon *minCDE* is between chromosome positions 4818500 and 4820508; *ftsZ* is between 1057136 and 1058341.

Supplementary Table 4: Mutations in evolved Δ L9

Mutant	Gene	Mutation	Genome position	DNA change	Max. freq.	N. of lines	Population day
Δ L9	<i>nusG</i>	R20P	6,065,036	C→G	69%	1	28
Δ L9	<i>nusG</i>	I38N	6,064,982	A→T	29%	1	28
Δ L9	<i>secE</i>	I120V	6,065,115	T→C	100%	8	28
Δ L9	<i>secE</i>	I116V	6,065,127	G→C	80%	1	28
Δ L9	<i>rpmB</i>	D60A	6,537,281	T→G	46%	1	28
Δ L9	<i>wssE</i>	G1235S	337,646	G→A	37%	1	56
Δ L9	<i>wspF</i>	Q185*	1,359,168	C→T	48%	1	56
Δ L9	<i>gacA</i>	L63I	2,372,868	C→A	46%	1	56
Δ L9	<i>gacA</i>	R184L	2,373,232	G→T	49%	1	56
Δ L9	<i>PFLU_3777</i>	T684P	4,174,569	A→C	44%	1	56
Δ L9	<i>PFLU_3777</i>	K772N	4,174,835	A→C	36%	1	56
Δ L9	<i>PFLU_3777</i>	L334P	4,173,520	T→C	44%	1	56
Δ L9	<i>PFLU_3777</i>	V693M	4,174,596	G→A	37%	1	56
Δ L9	<i>PFLU_3777</i>	Δ 40 bp (1483-1522 nt)	4,174,002	Deletion	54%	1	56
Δ L9	<i>PFLU_3780</i>	E94K	4,177,142	G→A	44%	1	56
Δ L9	<i>PFLU_5211</i>	Δ 33 bp (223-255 nt)	5,707,411	Deletion	47%	1	56
Δ L9	<i>pflu_5329</i>	M926I	5,857,440	G→A	38%	1	56
Δ L9	<i>nusG</i>	R20P	6,065,036	C→G	68%	1	56
Δ L9	<i>nusG</i>	W5C	6,065,080	C→G	53%	1	56
Δ L9	<i>secE</i>	I120V	6,065,115	T→C	61%	3	56
Δ L9	<i>algR</i>	Δ 1 bp (372 nt)	6,493,727	Deletion	41%	1	56
Δ L9	<i>rpmB</i>	D60A	6,537,281	T→G	33%	2	56

Supplementary Table 5: Mutations in evolved Δ L24

Mutant	Gene	Mutation	Genome position	DNA change	Max. freq.	N. of lines	Population day
Δ L24	<i>sucA</i>	W10R	1,984,322	T→C	100%	1	28
Δ L24	<i>PFLU_1476</i>	T44P	1,620,963	T→G	39%	1	28
Δ L24	<i>PFLU_1476</i>	Δ 9 bp (203-211 nt)	1,620,882	Deletion	100%	2	28
Δ L24	<i>pnuC</i>	V55F	3,098,074	C→A	100%	10	28
Δ L24	<i>PFLU_5321</i>	Δ 12 bp (227-238 nt)	5,846,411	Deletion	42%	1	28
Δ L24	<i>PFLU_5321</i>	F740L	5,844,431	A→G	100%	1	28
Δ L24	<i>PFLU_5321</i>	Δ 1 bp (1462 nt)	5,845,187	Deletion	100%	1	28
Δ L24	<i>rplD</i>	D170G	6,047,372	T→C	29%	1	28
Δ L24	<i>rplD</i>	D170Y	6,047,373	C→A	94%	1	28
Δ L24	<i>rplD</i>	D170H	6,047,373	C→G	46%	1	28

ΔL24	<i>aspA</i>	Δ9 bp (1292-1300 nt)	6,626,699	Deletion	73%	1	28
ΔL24	<i>rpsS</i>	P30R	6,046,038	G→C	50%	1	28
ΔL24	<i>nusG</i>	Y6C	6,065,078	T→C	100%	1	28
ΔL24	<i>secE</i>	I120V	6,065,115	T→C	100%	1	28
ΔL24	<i>PFLU_1476</i>	Δ9 bp (203-211 nt)	1,620,882	Deletion	100%	2	56
ΔL24	<i>sucA</i>	W10R	1,984,322	T→C	100%	1	56
ΔL24	<i>rne</i>	A137V	5,189,068	C→T	88%	1	56
ΔL24	<i>PFLU_2807</i>	V55F	3,098,074	C→A	100%	10	56
ΔL24	<i>PFLU_5321</i>	D652Y	5,844,695	C→A	40%	1	56
ΔL24	<i>PFLU_5321</i>	F740L	5,844,431	A→G	100%	1	56
ΔL24	<i>PFLU_5321</i>	Δ23 bp (2118-2140 nt)	5,844,509	Deletion	61%	1	56
ΔL24	<i>PFLU_5321</i>	Δ1 bp (1462 nt)	5,845,187	Deletion	100%	1	56
ΔL24	<i>rplD</i>	D170H	6,047,373	C→G	84%	2	56
ΔL24	<i>rplD</i>	D170G	6,047,372	T→C	94%	3	56
ΔL24	<i>rpoC</i>	D806G	6,055,399	T→C	100%	1	56
ΔL24	<i>nusG</i>	Y6C	6,065,078	T→C	66%	1	56
ΔL24	<i>secE</i>	I120V	6,065,115	T→C	100%	1	56
ΔL24	<i>aspA</i>	Δ9 bp (1292-1300 nt)	6,626,699	Deletion	100%	1	56

Supplementary Table 6: Mutations in evolved ΔL27

Mutant	Gene	Mutation	Genome position	DNA change	Max. freq.	N. of lines	Population day
ΔL27	<i>PFLU_4745</i>	R221P	5,219,121	C→G	65%	1	28
ΔL27	<i>PFLU_4916</i>	D228G	5,395,002	T→C	39%	1	28
ΔL27	<i>nusG</i>	*178W	6,064,562	T→C	100%	1	28
ΔL27	<i>nusG</i>	Y6C	6,065,078	T→C	100%	3	28
ΔL27	<i>nusG</i>	R4P	6,065,084	C→G	38%	1	28
ΔL27	<i>PFLU_3126</i>	E85K	3,415,933	G→A	53%	1	56
ΔL27	<i>PFLU_4916</i>	Q231R	5,394,993	T→C	52%	1	56
ΔL27	<i>secE</i>	I120V	6,065,115	T→C	100%	1	56
ΔL27	<i>PFLU_4745</i>	R221P	5,219,121	C→G	65%	1	56
ΔL27	<i>nusG</i>	Y6C	6,065,078	T→C	100%	1	56

Supplementary Table 7: Mutations in evolved ΔL33

Mutant	Gene	Mutation	Genome position	DNA change	Max. freq.	N. of lines	Population day
ΔL33	<i>PFLU_5211</i>	Δ33 bp (223-255 nt)	5,707,411	Deletion	39%	2	28
ΔL33	<i>PFLU_5329</i>	Δ9 bp (3071-3079 nt)	5,857,733	Deletion	38%	1	28
ΔL33	<i>wssC</i>	Q567*	332,200	C→T	33%	1	56
ΔL33	<i>wssH</i>	Δ15 bp (473-487 nt)	339,620	Deletion	57%	1	56
ΔL33	<i>wssI</i>	Δ1 bp (375 nt)	340,950	Deletion	50%	2	56
ΔL33	<i>wssI</i>	Δ9 bp (372-383 nt)		Deletion	35%	1	56

ΔL33	<i>wssI</i>	Δ1 bp (363 nt)	340,938	Deletion	50%	1	56
ΔL33	<i>wspA</i>	A381V	1,353,411	C→T	60%	1	56
ΔL33	<i>wspF</i>	T274I	1,359,436	C→T	54%	1	56
ΔL33	<i>wspF</i>	Δ14 bp (322-335 nt)	1,358,937	Deletion	38%	1	56
ΔL33	<i>PFLU_3777</i>	L510Q	4,174,048	T→A	47%	1	56
ΔL33	<i>PFLU_4443</i>	Δ118 bp (585-692 nt)	4,907,863	Deletion	35%	1	56
ΔL33	<i>PFLU_5209</i>	Q40P	5,705,711	T→G	37%	1	56
ΔL33	<i>PFLU_5211</i>	Δ33 bp (223-255 nt)	5,707,411	Deletion	41%	2	56
ΔL33	<i>PFLU_5329</i>	S734L	5,856,863	C→T	63%	1	56
ΔL33	<i>algR</i>	R263C	6,493,393	G→A	56%	1	56

Supplementary Table 8: Mutations in evolved ΔL36

Mutant	Gene	Mutation	Genome position	DNA change	Max. freq.	N. of lines	Population day
ΔL36	<i>purA</i>	K293R	596,047	A→G	100%	1	28
ΔL36	<i>rplO</i>	Q55H	6,039,965	C→A	100%	4	28
ΔL36	<i>rplO</i>	Q55L	6,039,966	T→A	100%	1	28
ΔL36	<i>rplO</i>	T39N	6,040,014	G→T	100%	2	28
ΔL36	<i>rplP</i>	H16R	6,044,748	T→C	89%	2	28
ΔL36	<i>rplP</i>	T14K	6,044,754	G→T	100%	1	28
ΔL36	<i>rpoD</i>	D216A	6,124,914	A→C	87%	1	28
ΔL36	<i>purA</i>	K293R	596,047	A→G	100%	1	56
ΔL36	<i>gacA</i>	Δ76 bp (284-359 nt)	2,372,965	Deletion	100%	1	56
ΔL36	<i>PFLU_3777</i>	V581F	4,174,260	G→T	80%	1	56
ΔL36	<i>PFLU_3777</i>	E757K	4,174,788	G→A	30%	1	56
ΔL36	<i>rpoA</i>	R283G	6,035,804	G→C	100%	1	56
ΔL36	<i>rpoA</i>	D249Y	6,035,906	C→A	100%	1	56
ΔL36	<i>rpoA</i>	C268S	6,035,849	A→T	81%	1	56
ΔL36	<i>rplO</i>	T39N	6,040,014	G→T	100%	2	56
ΔL36	<i>rplO</i>	Q55H	6,039,965	C→A	100%	2	56
ΔL36	<i>rplP</i>	Q98K	6,044,503	G→T	100%	1	56
ΔL36	<i>rplP</i>	H16R	6,044,748	T→C	100%	1	56
ΔL36	<i>rpoD</i>	D216A	6,124,914	A→C	100%	1	56

Supplementary Tables 4,5,6,7,8: *De novo* mutations found in the evolving populations at 28 and 56 growth days. All the missense and nonsense mutations affecting coding regions that appear at least 30% frequency in at least one evolved population are listed. Column 1: ancestral mutant. Column 2: mutant gene. Column 3: amino acid change. Column 4: SNP position on the chromosome. Column 5: nucleotide change. Column 6: highest frequency the mutation has been found. Column 7: number of populations that showed the mutation. Column 8: Age of the population where the mutation has been found.

Supplementary Table 9: Strain list

ID	Name	Species	Notes	Source
MPB28225	SBW25 WT	<i>P. fluorescens</i>	SBW25	Rainey & Bailey 1996
MPB18630	pUlsacB_T4	<i>E. coli</i>	plasmid msfGFPtetA sacB oriT pBR322 origin Gibson landing, used as backbone for 2XO	Rainey lab
MPB28807	pRK2013	<i>E. coli</i>	Helper plasmid for tri-parental conjugation: tra, mob, Kan	Figurski & Helinski 1979
MPB15811	pMRE-Tn7-152	<i>E. coli</i>	plasmid pMRE-pSC101ori-oriT-Tn7-AraC-Pbad-tnsABCD-PnptII-sGFP2	Addgene
MPB15801	pMRE-Tn7-145	<i>E. coli</i>	plasmid pMRE-pSC101ori-oriT-Tn7-AraC-Pbad-tnsABCD-PnptII-mScarlet-I	Addgene
MPB15850	SBW25 GFP	<i>P. fluorescens</i>	SBW25 attTn7::Tn7-PnptII-sGFP2-Kan	Rainey lab
MPB15845	SBW25 RFP	<i>P. fluorescens</i>	SBW25 attTn7::Tn7-PnptII-mScarlet-Gm	Rainey lab
MPB21772	Δ rodA full 2XO construct in pUlsacB_T4	<i>E. coli</i>	Top10 + pUlsacB_T4 with insertion of upstream and downstream region of rodA	This work
MPB21769	Δ rodA partial 2XO construct in pUlsacB_T4	<i>E. coli</i>	Top10 + pUlsacB_T4 with insertion of upstream and downstream region of rodA signal peptide	This work
MPB21853	Δ rodA full	<i>P. fluorescens</i>	SBW25 Δ rodA	This work
MPB21843	Δ rodA partial	<i>P. fluorescens</i>	SBW25 Δ rodA(1-361)	This work
MPB23673	Δ rodA full GFP	<i>P. fluorescens</i>	SBW25 attTn7::Tn7-PnptII-sGFP2-Kan Δ rodA	This work
MPB23652	Δ rodA full RFP	<i>P. fluorescens</i>	SBW25 attTn7::Tn7-PnptII-mScarlet-Gm Δ rodA	This work
MPB23648	Δ rodA partial GFP	<i>P. fluorescens</i>	SBW25 attTn7::Tn7-PnptII-sGFP2-Kan Δ rodA(1-361)	This work
MPB23672	Δ rodA partial RFP	<i>P. fluorescens</i>	SBW25 attTn7::Tn7-PnptII-mScarlet-Gm Δ rodA(1-361)	This work
MPB18251	Δ minC	<i>P. fluorescens</i>	SBW25 Δ minC	Rainey lab
MPB16556	minC-GFP	<i>P. fluorescens</i>	SBW25 minC::GFP	Rainey lab
MPB28855	Δ L9 2XO construct in pUlsacB_T4	<i>E. coli</i>	Top10 + pUlsacB_T4 with insertion of upstream and downstream region of L9	This work
MPB29938	Δ L24 2XO construct in pUlsacB_T4	<i>E. coli</i>	Top10 + pUlsacB_T4 with insertion of upstream and downstream region of L24	This work
MPB29936	Δ L27 2XO construct in pUlsacB_T4	<i>E. coli</i>	Top10 + pUlsacB_T4 with insertion of upstream and downstream region of L27	This work
MPB28808	Δ L33 2XO construct in pUlsacB_T4	<i>E. coli</i>	Top10 + pUlsacB_T4 with insertion of upstream and downstream region of L33	This work
MPB29937	Δ L36 2XO construct in pUlsacB_T4	<i>E. coli</i>	Top10 + pUlsacB_T4 with insertion of upstream and downstream region of L36	This work
MPB28902	Δ L9	<i>P. fluorescens</i>	SBW25 Δ rplI	This work
MPB30471	Δ L24	<i>P. fluorescens</i>	SBW25 Δ rplX	This work
MPB30242	Δ L27	<i>P. fluorescens</i>	SBW25 Δ rpmA	This work
MPB28879	Δ L33	<i>P. fluorescens</i>	SBW25 Δ rpmG	This work
MPB30521	Δ L36	<i>P. fluorescens</i>	SBW25 Δ rpmJ	This work
MPB29185	Δ L9 GFP	<i>P. fluorescens</i>	SBW25 attTn7::Tn7-PnptII-sGFP2-Kan Δ rplI	This work
MPB29186	Δ L9 RFP	<i>P. fluorescens</i>	SBW25 attTn7::Tn7-PnptII-mScarlet-Gm Δ rplI	This work
MPB30782	Δ L24 GFP	<i>P. fluorescens</i>	SBW25 attTn7::Tn7-PnptII-sGFP2-Kan Δ rplX	This work
MPB30781	Δ L24 RFP	<i>P. fluorescens</i>	SBW25 attTn7::Tn7-PnptII-mScarlet-Gm Δ rplX	This work

MPB30867	ΔL27 GFP	<i>P. fluorescens</i>	SBW25 attTn7::Tn7-PnptII-sGFP2-Kan <i>ΔrpmA</i>	This work
MPB30556	ΔL27 RFP	<i>P. fluorescens</i>	SBW25 attTn7::Tn7-PnptII-mScarlet-Gm <i>ΔrpmA</i>	This work
MPB29183	ΔL33 GFP	<i>P. fluorescens</i>	SBW25 attTn7::Tn7-PnptII-sGFP2-Kan <i>ΔrpmG</i>	This work
MPB29184	ΔL33 RFP	<i>P. fluorescens</i>	SBW25 attTn7::Tn7-PnptII-mScarlet-Gm <i>ΔrpmG</i>	This work
MPB30841	ΔL36 GFP	<i>P. fluorescens</i>	SBW25 attTn7::Tn7-PnptII-sGFP2-Kan <i>ΔrpmJ</i>	This work
MPB30842	ΔL36 RFP	<i>P. fluorescens</i>	SBW25 attTn7::Tn7-PnptII-mScarlet-Gm <i>ΔrpmJ</i>	This work
MPB36792	ΔL24ΔL9 GFP	<i>P. fluorescens</i>	Evolved clone of MPB30782 <i>ΔrplI</i>	This work
MPB39184	ΔL9 GFP <i>nusG</i> I38N	<i>P. fluorescens</i>	Evolved clone of MPB29185	This work
MPB39190	ΔL9 RFP <i>secE</i> I120V	<i>P. fluorescens</i>	Evolved clone of MPB29186	This work
MPB39189	ΔL9 RFP <i>secE</i> I120V	<i>P. fluorescens</i>	Evolved clone of MPB29186	This work
MPB39397	ΔL24 RFP <i>nusG</i> Y6C	<i>P. fluorescens</i>	Evolved clone of MPB30781	This work
MPB39391	ΔL24 RFP <i>nusG</i> Y6C	<i>P. fluorescens</i>	Evolved clone of MPB30781	This work
MPB39390	ΔL24 RFP <i>nusG</i> Y6C	<i>P. fluorescens</i>	Evolved clone of MPB30781	This work
MPB39387	ΔL24 RFP <i>nusG</i> Y6C	<i>P. fluorescens</i>	Evolved clone of MPB30781	This work
MPB39300	ΔL24 RFP <i>rpoC</i> D806G	<i>P. fluorescens</i>	Evolved clone of MPB30781	This work
MPB39302	ΔL27 RFP <i>rpoC</i> V673A	<i>P. fluorescens</i>	Evolved clone of MPB30556	This work
MPB39201	ΔL27 RFP <i>nusG</i> Y6C	<i>P. fluorescens</i>	Evolved clone of MPB30556	This work
MPB39161	ΔL36 RFP <i>rpoD</i> D216A	<i>P. fluorescens</i>	Evolved clone of MPB30842	This work

Supplementary Table 10: Primers list

ID	Name	Sequence
DWR213	5' Tn7 insertion	CACCAAAGCTTTACCACCCAA
DWR214	3' Tn7 insertion	CAGCATAACTGGACTGATTTTCAG
DWR 234	5' pUlsacB_T4 backbone	ATTTGTCCTACTCAGGAGAGCGTTC
DWR076	3' pUlsacB_T4 backbone	AGCGTTCTGAACAAATCCAGATG
EF3	5' <i>ΔrodA</i> upstream region	gtacagctatcttggttcagACTACGATCACAATACCGTAACTG
EF4	3' <i>ΔrodA</i> upstream region	tgcatcatCACGTCCTCACTGGAGAGGATG
EF14	5' <i>ΔrodA</i> (1-368) downstream region	gtacaagtaaATAAGGTGAAGATGTCAATGCAAG
EF5	5' <i>ΔrodA</i> (1-361) downstream region	tgaggacgtgATGGATCGCACAGGTTTGAATAAG
EF7	3' <i>ΔrodA</i> downstream region	tagtcagttgatgtagtctGCTTGAAGTAGCTGGCCACG
EF44	5' <i>ΔL9</i> upstream region	gtacagctatcttggttcagTTGTCTGACCGTTTTTGAG
EF45	3' <i>ΔL9</i> upstream region	cctgacacgcTTAACCTTACCGTTTCGC
EF46	5' <i>ΔL9</i> downstream region	tgaaggttaaGCGTGTGAGGCAGTTAAC
EF47	3' <i>ΔL9</i> downstream region	tagtcagttgatgtagtctGTGACCGGTGTTGAGATG
EF105	5' <i>ΔL24</i> upstream region	gtacagctatcttggttcagCACCCGACGTTGAAAGTAC
EF106	3' <i>ΔL24</i> upstream region	gagatccgacACACTGCTAGGTAGAAGACC
EF107	5' <i>ΔL24</i> downstream region	ctagcagtgGTCGGATCTCCTTACAGC
EF108	3' <i>ΔL24</i> downstream region	tagtcagttgatgtagtctGTTAAACGACGGTGATG
EF121	5' <i>ΔL27</i> upstream region	gtacagctatcttggttcagAATGTCGGCGATCACGAAG
EF122	3' <i>ΔL27</i> upstream region	gaattgactcTTGCGAGATCGCTGGAAGG
EF123	5' <i>ΔL27</i> downstream region	gatctcgaaGAGTCAATTCTCTAGTGAGG
EF124	3' <i>ΔL27</i> downstream region	tagtcagttgatgtagtctCTTGCCACACAGCAACAC

EF52	5' ΔL33 upstream region	gtacagctatcttggttcagATCATGGTGGCTTTGCGTTTG
EF53	3' ΔL33 upstream region	aaggagctctCCTTGTGAAAAAAGCCCG
EF54	5' ΔL33 downstream region	tttcacaaggAGAGCTCCTTAGATCTTGC
EF55	3' ΔL33 downstream region	tagtcagttgatgtagtgctTCAGCGAGTTGTATTGCAC
EF153	5' ΔL36 upstream region	gtacagctatcttggttcagATTACACGCGGCGCTTCTTC
EF154	3' ΔL36 upstream region	ggagttggtgGTGTGATCCGCTTGAAGC
EF155	5' ΔL36 downstream region	cggatcacacCACCAACTCCTCGAACCTTATG
EF156	3' ΔL36 downstream region	tagtcagttgatgtagtgctTCACTGGTGACTTTGGCTTC

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