Universidade de Aveiro Departamento de Biologia
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mRNA mistranslation in *Saccharomyces cerevisiae*

Erros na tradução do mRNA em *Saccharomyces cerevisiae*

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Aplicada ramo Biologia Molecular e Celular, realizada sob a orientação científica do Dr. Manuel António da Silva Santos, Professor Associado do Departamento de Biologia da Universidade de Aveiro

“Every day you may make progress. Every step may be fruitful. Yet there will stretch out before you an ever-lengthening, ever-ascending, ever-improving path. You know you will never get to the end of the journey. But this, so far from discouraging, only adds to the joy and glory of the climb.” (W. Churchill)

o júri

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palavras-chave

Ambiguidade de codão, erros na tradução, mRNA, tRNA, expressão genética, agregados proteicos, resposta ao stress, estudos fenotípicos

resumo

O código genético pode ser definido como uma série de reacções bioquímicas que estabelecem as regras pelas quais as sequências nucleotídicas do material genético são traduzidas em proteínas. Apresenta um elevado grau de conservação e estima-se que tenha tido a sua origem há mais de 3.5 mil milhões de anos. Ao longo dos últimos anos foram identificadas várias alterações ao código genético em procariotas e eucariotas e foram identificados codões ambíguos, sugerindo que o código genético é flexível. Contudo, os mecanismos de evolução das alterações ao código genético são mal conhecidos e a função da ambiguidade de codões é totalmente desconhecida.

Nesta tese criámos codões ambíguos no organismo modelo *Saccharomyces cerevisiae* e estudámos os fenótipos resultantes de tal ambiguidade. Os resultados mostram que, tal como seria expectável, a ambiguidade do código genético afecta negativamente o crescimento, viabilidade celular e induz a produção de agregados proteicos em *S. cerevisiae*. Contudo, tal ambiguidade também resultou em variabilidade fenótipica, sendo alguns dos fenótipos vantajosos em determinados condições ambientais. Ou seja, os nossos dados mostram que a ambiguidade do código genético afecta negativamente a capacidade competitiva de *S. cerevisiae* em meio rico em nutrientes, mas aumenta a sua capacidade adaptativa em condições ambientais variáveis.

Os efeitos negativos da ambiguidade do código genético, nomeadamente a agregação de proteínas, sugerem que tal ambiguidade poderá ser catastrófica em organismos multicelulares em que a taxa de renovação celular é baixa. Esta hipótese é suportada pela recente descoberta de uma mutação na alanil-tRNA sintetase do ratinho que induz ambiguidade em codões de alanina e resulta numa forte perda de neurónios de Purkinge, neurodegeneração e morte prematura. Ou seja, a ambiguidade do código genético pode ter consequências negativas ou positivas dependendo do tipo de células e das condições ambientais.

keywords

Codon ambiguity, mistranslation, mRNA, tRNA, gene expression, protein aggregation, stress response, phenomics

abstract

The genetic code is defined as a series of biochemical reactions that establish the cellular rules that translate DNA into protein information. It was established more than 3.5 billion years ago and it is one of the most conserved features of life. Over the years, several alterations to the standard genetic code and codon ambiguities have been discovered in both prokaryotes and eukaryotes, suggesting that the genetic code is flexible. However, the molecular mechanisms of evolution of the standard genetic code and the cellular role(s) of codon ambiguity are not understood.

In this thesis we have engineered codon ambiguity in the eukaryotic model *Sacharomyces cerevisiae* to clarify its cellular consequences. As expected, such ambiguity had a strong negative impact on growth rate, viability and protein aggregation, indicating that it affects fitness negatively. However, it also created important selective advantages in certain environmental conditions, suggesting that it has the capacity to increase adaptation potential under environmental variable conditions.

The overall negative impact of genetic code ambiguity on protein aggregation and cell viability, suggest that codon ambiguity may have catastrophic consequences in multicellular organisms. In particular in tissues with low cell turnover rate, namely in the brain. This hypothesis is supported by the recent discovery of a mutation in the mouse alanyl-tRNA synthetase which creates ambiguity at alanine codons and results in rapid loss of Purking neurons, neurodegeneration and premature death. Therefore, genetic code ambiguity can have both, negative or positive outcomes, depending on cell type and environmental conditions.

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Abbreviations

aaRS - aminoacyl-tRNA synthetases;

aa-tRNA - aminoacyl-tRNA;

AIMP – aminoacyl-tRNA synthetase-interacting multifunctional protein;

ApnA - Diadenosine polyphosphates;

ARE - AU-rich element;

A-site - Aminoacyl-tRNA site;

CFU – Colony forming units;

CMT - Charcot-Marie-Tooth disease;

EF - Elongation factor;

ER - Endoplasmic reticulum;

ERAD - ER-associated degradation;

E-site – Exit-tRNA site;

GAP - GTPase-activating protein;

GEF – Guanine exchange factor;

GFP – Green fluorescent protein;

Hsp104 - Heat-shock protein 104;

IC - Initiator complex;

IF – Initiation factors;

LB - Lysogeny broth;

MFC - Multifactor complex;

mQ - MiliQ;

mRNA - messenger RNA;

mRNP - messenger Ribonucleoproteins;

MTF - Methionyl-tRNA formyltransferase;

NMD - Nonsense-mediated mRNA decay;

PABP - Poly(A)-binding protein;

PCR - Polymerase chain reaction;

PQC - Protein quality control mechanisms;

P-site - Peptidyl-tRNA site;

PTC - Premature termination codon;

RF - Release factors;

ROS - Reactive oxygen species;

RPS1 - Ribosomal protein S1;

RRF - Ribosome recycling factor;

RRM - RNA recognition motif;

rRNA - ribosome RNA;

SD - Shine-Dalgarno sequence;

SEM – Standard error of the mean;

sRNA - small non-coding RNA;

TIR - Translation initiation region;

Tm - Melting temperature;

tRNA - transfer RNA;

tRNAi - initiator transfer RNA;

UPR - Unfolded protein response;

UPS - Ubiquitin-proteasome system;

UTR - Untranslated region;

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Chapter 1

1. Introduction

1.1. Genetic Code

1.1.1. Genetic Code – Principal Features

Established 3.5 billion years ago, the genetic code can be defined as a series of biochemical reactions that establishes the rules by which the nucleotides sequence of a genetic material (DNA or messenger RNA (mRNA)) is translated into proteins (Osawa, Jukes et al. 1992; Line 2002). Proteins are mainly composed by the same twenty amino acids specified by the genetic code in all known organisms. Amino acids are coded by a sequence of three adjacent bases, triplets, in the polynucleotide DNA or RNA sequence, namely, codons (Figure 1.1) (Woese 1965; Crick 1968; Knight, Freeland et al. 2001).

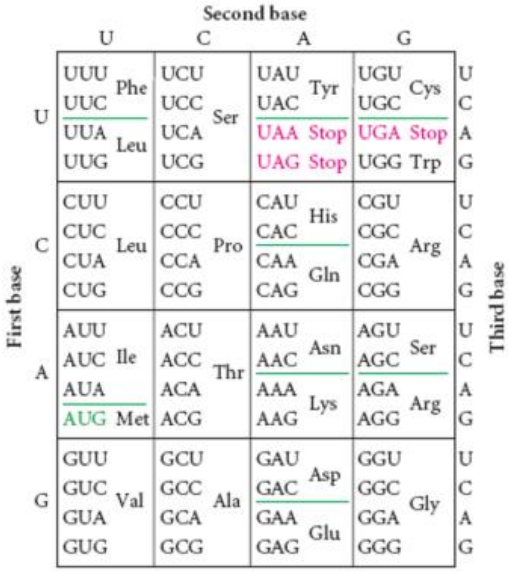


Figure 1.1: The genetic code. The genetic code is composed by 64 codons and the amino acids specified by them. Codons are written 5´to 3´, as they appear in the mRNA. Highlight for the initiation codon (AUG) and the three termination codons (UAA, UAG and UGA) (Smith 2008).

The first two letters of the code (A, G, C or U) create sixteen possible combinations each of which are represented in a separated “codon box”. Each “codon box” displays four possible combinations, which will give a total of sixty-four codons. Of these, sixty-one are recognized by transfer RNAs (tRNAs) for the incorporation of twenty-two amino acids existent in proteins, and three codons stop mRNA translation (Agris 2004; Agris 2008). The genetic code is therefore

degenerate because the great majority of amino acids have more than one codon. As a consequence, in all organisms codons out-number the tRNAs required to translate them.

The disparity among the utilization of synonymous codons is named codon bias. Codon bias is characteristic of each one of the codons and is linked to tRNA isoacceptor abundance in the cell (Ikemura 1985). In turn, tRNA abundance is correlated to environmental responses (Nazario 1972). The usage of rare codons in mRNA is associated with protein folding since there is a correlation between the codon usage in mRNA and topological features of the encoded proteins (Thanaraj and Argos 1996; Kimchi-Sarfaty, Oh et al. 2007).

Even before the triplet nature of the genetic code was established it was suggested that proteins must be produced by "starting at a fixed point and working along the sequence of bases three at a time" (Crick, Barnett et al. 1961). These unique points are the starting and end points and the ribosome is somehow able to faithfully maintain the reading frame during decoding (Crick, Barnett et al. 1961). A model that explains this is based on the fact that the anticodon of the tRNA consists of three nucleotides complementary to the codon and tRNA measure out the length of the codon using the anticodon as a yardstick (Holley 1965). More recent analysis reveal that when tRNA forms fewer than 3 base pairs with the mRNA, the interaction is insufficiently stable to preclude realignment of the tRNA with the peptidyl-tRNA site (P-site). A 4-nucleotide code was not adopted because of an insufficient need to encode more amino acids and/or a too stable, and irreversibility, tRNA-mRNA interaction (Farabaugh and Bjork 1999).

1.1.2. Amino Acids and Evolution

Amino acids are molecules that contain both amino ($-NH_2$) and carboxyl (-COOH) functional groups. Each amino acid has a different side chain (R group) that defines them. With few exceptions, all organisms are restricted to the twenty common amino acid building blocks for the biosynthesis of proteins. Based on chemical and/or structural properties of R group, the amino acids can be classified as: aliphatic, cyclic, hydroxyl or sulfur containing side chains, aromatic, basic and acid (Table 1.1) (Issaq, Chan et al. 2009). Besides the canonical amino acids, two more non-canonical amino acids have been discovered, namely selenocysteine (Sec) and pyrrolysine (Pyl) that are present in some organisms.

Early in the history of life only few of these twenty-two amino acids were encoded in the genetic code however, the composition of the initial group of amino acids remains a mystery.

Hypotheses to explain what are the ancient amino acids and the underlying genetic code are many and, one of the most famous is the theory of co-evolution. According to this, expansion of amino acids is achieved by biosynthetic transformation of precursor amino acids into product amino acids (Wong 1975). This hypothesis postulates that, the earliest proteins were constituted by prebiotic synthesized amino acids, namely, Gly, Ala, Ser, Asp, and Glu. Next three subsequent phases of amino acids incorporation into the code was followed (Wong 2005). Nevertheless, the theory of co-evolution is not unanimously accepted and the boundary between which were the ancient amino acids may be unrealistic because, with the exception of only three amino acids (Arg, Lys, and His), all others could be derived from prebiotic synthesis (Miller 1987). Another study showed that nine amino acids (Ala, Asn, Asp, Gly, His, Ile, Ser, Thr and Val) have a low frequency in proteins and could have been introduced early into the genetic code (once organismal diversification was in place) (Brooks, Fresco et al. 2002).

Table 1.1: The twenty natural amino acids directed specified by the genetic code. The table contains the list of the twenty amino acids specified by the genetic code, the abbreviation of names and the principal chemical features of the amino acids;

Amino Acid		Properties	Amino Acid		Properties
Alanine	Ala	aliphatic hydrophobic neutral	Leucine	Leu	aliphatic hydrophobic neutral
Arginine	Arg	polar hydrophilic charged (+)	Lysine	Lys	polar hydrophilic charged (+)
Asparagine	Asn	polar hydrophilic neutral	Methionine	Met	hydrophobic neutral
Aspartate	Asp	polar hydrophilic charged (-)	Phenylalanine	Phe	aromatic hydrophobic neutral
Cysteine	Cys	polar hydrophobic neutral	Proline	Pro	hydrophobic neutral
Glutamine	Gln	polar hydrophilic neutral	Serine	Ser	polar hydrophilic neutral
Glutamate	Glu	polar hydrophilic charged (-)	Threonine	Thr	polar hydrophilic neutral
Glycine	Gly	aliphatic neutral	Tryptophan	Trp	aromatic hydrophobic neutral
Histidine	His	aromatic polar hydrophilic	Tyrosine	Tyr	aromatic polar hydrophobic
Isoleucine	Ile	aliphatic hydrophobic neutral	Valine	Val	aliphatic hydrophobic neutral

1.2. Protein Biosynthesis

1.2.1. Protein Biosynthesis - General mechanisms

The cost of protein biosynthesis varies between the organisms, environmental conditions and resources availability, nevertheless this is arguably one of the most expensive universal cellular processes and a major component that sets metabolic rate (Waterlow 1984). Being divided in three major steps (amino acid synthesis, transcription and translation), protein synthesis expends at least 95% of metabolic energy in *Escherichia coli* (*E. coli*) and not much less in *Saccharomyces cerevisiae* (*S. cerevisiae*) (Jakubowski and Goldman 1992). Indeed, to synthesize the large ribosome RNA (rRNA) yeast cells spend approximately 60% of their energy (Li, Moir et al. 2000).

Translation is accomplished by ribosomes that act in concert with a large number of auxiliary factors in order to "translate" the information enclosed in mRNA. This is a multi-step process that can be, generally, divided into three phases: initiation, elongation and termination (Lackner and Bahler 2008). Some authors also consider a fourth phase named recycling (Kapp and Lorsch 2004). In each of these phases there are specific factors involved that are discussed bellow. The overall error rate in translation (10^{-3} and 10^{-4}) is a net accumulation of errors from several steps, including transcription ($\approx10^{-4}$), aminoacyl-tRNA (aa-tRNA) synthesis ($\approx10^{-4}$), and ribosomal decoding ($\approx10^{-4}$) (Ling, Reynolds et al. 2009). The phases of translation in eukaryotes are discussed bellow.

1.2.2. Initiation

Taking place in the cytoplasm or across the membrane of endoplasmic reticulum (ER), translation initiation is a major target of mRNA regulation during protein synthesis (Macdonald 2001; Dever 2002). Both in eukaryotes or prokaryotes initiation is the rate-limiting step and its efficiency depends on the sequence and structure of mRNA (Figure 1.2) (Skorski, Leroy et al. 2006; Nakagawa, Niimura et al. 2010).

1.2.2.1. Initiation in prokaryotes

In this section I we will provide the major characteristics of prokaryotic initiation. Taking *E. coli* as an example, the mRNA region important for translation initiation (translation initiation

region (TIR)) spans a few tens of nucleotides comprising the initiation codon, which is most frequently AUG (83%) and less common GUG and UUG, and the Shine-Dalgarno sequence (SD) (Shine and Dalgarno 1974; Shine and Dalgarno 1975; Ma, Campbell et al. 2002). This sequence (AGGAGG) is located approximately 4 to 15 nucleotides upstream of the initiation codon and pairs with a complementary sequence called anti-SD (CCUCC) located in the 3´ end of a 16 Svedberg (S) rRNA, the RNA from the 30S ribosomal subunit.

However, the strength of the pairing between the SD and the anti-SD correlates only weakly with the efficiency of translation. Other determinants within the TIR have more decisive contributions to the efficiency of translation (Lee, Holland-Staley et al. 1996). Among these determinants the most well characterized are the binding site for ribosomal protein S1 (RPS1), which is a A/U region that stretches upstream the initiation codon and enhances translation due to the binding to RPS1 (Boni, Isaeva et al. 1991; Zhang and Deutscher 1992). Secondary structure in the mRNA can also inhibit translation by hampering the access of the 30S ribosomal subunit (Nakamoto 2006). Besides this, *trans*-acting factors, such proteins, RNAs, or metabolites may also bind to the mRNA enhancing or repressing its translation (McCarthy and Gualerzi 1990; Milon, Konevega et al. 2008). A wide spread bacterial example of RNAs that act as regulator of gene expression are the small non-coding RNAs (sRNAs) (Urban and Vogel 2007). These molecules (50 and 200 nucleotides) act on *trans*-encoded target mRNAs influencing their translation and/or stability (Liu, Gui et al. 1997; Wassarman and Storz 2000). sRNAs normally have short and imperfect complementarity to their targets and this binding may result in either, the blockage of the ribosome entry or fusion of the inhibitory secondary structures. Regulation tends frequently to be linked to a nuclease-mediated cleavage of the mRNA (Masse, Escorcia et al. 2003).

The mechanism of translation initiation in prokaryotes, according to the minimal kinetic model, can be divided in two phases (Gualerzi, Risuleo et al. 1977). In the first phase mRNA and initiator $_{f}$Met-$_{t}$RNA^{fMet} bind to the 30S subunit with the help of initiation factors (IFs). SD and anti-SD pairing (SD interactions) and the subsequent formation of the 30S initiator complex (30S IC), composed by three IFs, mRNA and $_{f}$Met-$_{t}$RNA^{fMet}, ends this phase (Shine and Dalgarno 1974; Shine and Dalgarno 1975). In the second phase, the 50S subunit with the 30S IC associeted, GTP hydrolyses and the IFs dissociate. This phase is irreversible and results in the formation of 70S IC that is ready for the elongation phase (Gualerzi, Brandi et al. 2001).

1.2.2.2. Initiation in eukaryotes

Translation initiation in eukaryotes is accomplished in four steps: (i) formation of a 43S pre-initiation complex; (ii) recruitment of the 43S complex to the 5´ end of mRNA; (iii) scanning of the 5´ UTR (untranslated region) and start codon identification; (iv) formation of the complete 80S ribosome (Preiss and Hentze 2003).

i) Formation of a 43S pre-initiation complex

The first step of initiation is needed to promote the dissociation of the ribosomes into their subunits (Preiss and Hentze 2003). Conversely to what happens in bacteria, where the dissociation of free 70S ribosomes is known to be catalyzed in the presence GTP, by elongation factor G (EF-G), initiation factor 3 (IF3) and ribosome recycling factor (RRF) (Hirokawa, Demeshkina et al. 2006), in eukaryotes the process of dissociation is still unclear (Demeshkina, Hirokawa et al. 2007). Because physiological conditions favor the association of ribosomal subunits (40S and 60S), the intervention of initiation factors (IF) is required. Several have ribosome anti-association/dissociation activity, namely, eIF1, eIF1A and eIF3 (Preiss and Hentze 2003). At low free Mg^{2+} concentration (1 mM) eIF3, in cooperation with its poorly associated eIF3j subunit eIF1 and eIF1A, is responsible for the dissociation of the 80S ribosome into free 60S subunits, mRNA and tRNA-bound 40S subunits (Jackson, Hellen et al. 2010). More specifically, following this initially dissociation eIF1 promotes release of the tRNA and eIF3j mediates mRNA dissociation. The re-association of the 40S and 60S subunits is prevented by eIF3, and probably eIF1 and eIF1A, which remain associated with recycled 40S subunits (Jackson, Hellen et al. 2010).

The following step in initiation is the binding of eIF2 to the initiator transfer RNA (tRNAi), Met-tRNAi, in a GTP-dependent manner, forming a ternary complex composed by eIF2, GTP and methionine initiator tRNA (eIF2– GTP– Met-tRNAi) (Merrick 1992; Kapp and Lorsch 2004). The eIF2 factor is a heterotrimer of α, β and γ subunits with a molecular mass ranging between 35 to 52 kDa (Merrick 1992). The γ-subunit binds both GTP and Met-tRNAi. Subunit β is responsible for GTPase activity and modulates initiator tRNA binding of eIF2γ. For the formation of this complex the intervention of guanine nucleotide exchange factor (GEF) eIF2B is also needed. The eIF2B contains five polypeptides (α, β, γ, δ, and ε) with molecular mass not larger than 82 kDa. This factor favors the recycling of eIF2-GDP, a product of initiation, to eIF2-GTP in the end of each initiation cycle to allow a new round of initiation (Preiss and Hentze 2003). This happens because eIF2 was a 100-fold higher affinity for GDP than GTP (Kapp and Lorsch 2004) and the off-rate

constant for GDP release is slow. A similar situation happens in the bacterial elongation cycle with EF-Tu having also a 100-fold preference for GDP. Subunit α of eIF2 presents a central role as a regulator of translation initiation, being involved in the interactions between eIF2 and eIF2B. Indeed, phosphorylation of this subunit carried out by specific eIF2-kinases constitutes a major means of regulating translation in response to different forms of cellular stress (Preiss and Hentze 2003). After assembly the ternary complex, binds to the 40S with the help of at least, eIF1, eIF1A and the multisubunit factor eIF3. The resulting complex is called 43S (Kapp and Lorsch 2004). In yeast, an additional step occurs, independently of the ribosome. A multifactor complex (MFC) of eIF1, eIF2, eIF3, eIF5 and Met-tRNAi assembles. MFC may be an important functional unit during several stages of translation initiation (Asano, Clayton et al. 2000). eIF5 is a single polypeptide chain of 46 kDa in yeasts that presents a ribosome-dependent GTPase activity, appropriate for its proposed role in subunit joining (Merrick 1992). This translation factor is catalytically the most active.

ii) Recruitment of the 43S complex to the 5´ end of mRNA

The recruitment of the 43S pre-initiation complex to the 5´ end of mRNA begins with the assembly of the eIF4F complex on the 5´ cap of the mRNA and the, subsequent, unwinding of structures found in the 5´ UTR (Kapp and Lorsch 2004). eIF4F is an heterotrimeric complex composed by eIF4G, the cap-binding protein eIF4E and the ATP-dependent RNA helicase eIF4A (Preiss and Hentze 2003). eIF4G is a multivalent adaptor that functions like the core of eIF4F and appears to constitute the primary ribosome recruitment module. eIF4E is a factor that enhances the binding of the eIF4F complex to the 5´ end of the mRNA. The last factor of the eIF4F complex, eIF4A, is a DEAD box helicase that can unwind RNA secondary structures in an ATP-dependent manner (Pause, Methot et al. 1994). This helicase function of eIF4A is assisted by the RNA-binding protein eIF4B (homodimer that can bind RNA by virtue of an RNA recognition motif (RRM)) (Pause, Methot et al. 1994). Recently, another factor involved is this process was identified. That factor, eIF4H, has homology to the RRM domain of eIF4B and has been shown to stimulate ATPase and helicase activity of eIF4A (Preiss and Hentze 2003). eIF3 and poly(A)-binding protein (PABP) bound to the 3´ poly(A) tail are also required for binding of the 43S complex to the mRNA (Lamphear, Kirchweger et al. 1995).

iii) Scanning of the 5´ UTR and start codon identification

The scanning process requires ATP hydrolyses, although the ATPase involved has not yet been identified (Kapp and Lorsch 2004). After the assembly of the 43S complex near the 5´ end of the mRNA the complex needs to find the appropriate starting codon. Marilyn Kozak proposed a linear movement of the 43S along the mRNA 5´ UTR in search for the triplet, this mechanism is known as the scanning mechanism (Kozak 2002). This process is consistent with a large body of genetic and biochemical data, and explains the "first-AUG rule" - initiation should occur on the start codon closest to the mRNA 5´ end. Kozak proposed that factors eIF1 and eIF1A may play an important function during scanning, and recent biochemical data show that eIF1 plays an important role during selection in the selection of the start codon (Pestova and Kolupaeva 2002). According to Pestova and Kolupaeva (2002) 43S complex exists in two conformations, namely a closed conformation which is scanning-incompetent in absence of eIF1, and an open conformation which is scanning-competent when eIF1 is present. Only 43S open complex can establish stable interactions with the cognate AUG triplets located in a proper nucleotide context. The favorable nucleotide context is termed the "Kozak sequence" and is fundamental for initiation (Kozak 2002). When the 43S complex encounters correct codon-anticodon base pairing between the initiation codon and the initiator tRNA in the ternary complex the conformation of the 40S-subunit-bound eIF2 changes triggering GTP hydrolysis by eIF2, which is facilitated by the GTPase-activating protein (GAP) eIF5 (Sokabe, Yao et al. 2006). After GTP hydrolysis, the complex eIF2-GDP releases the Met-tRNAi into the P-site (peptidyl-tRNA site) of the 40S subunit and then dissociates. At this point eIF1, eIF1A, eIF3 and eIF5 also dissociate. Until recently, GTP hydrolysis by eIF2 was thought to be sufficient for 60S joining. However, *in vitro* experiments revealed that a second GTPase, eIF5B, is required (Lee, Pestova et al. 2002). For that, before or after the hydrolysis of GTP, eIF5B-GTP binds to the complex and stimulates 60S joining.

iv) Formation of the complete 80S ribosome

For the joining of the 60S subunit to the 40S scanning subunit, initiation factors have to dissociate from the latter (Kapp and Lorsch 2004). This last event, combination of 40S and 60S subunit, triggers GTP hydrolysis by eIF5B and then eIF5B-GDP dissociates from the complex, because of its low affinity for the ribosome (Pestova and Kolupaeva 2002). Although other steps may be required, this is thought to be the end of translation initiation. Briefly, the formation of an elongation competent 80S ribosome requires two distinct GTP hydrolysis steps that serve as checkpoints for proper identification of AUG codon and 80S (Preiss and Hentze 2003).

1.2.3. Elongation

The elongation machinery is highly conserved across the three kingdoms of life. For example, there is strong similarity between the different elongation factors (Merrick 1992), the human eEF1α and *E. coli* EF-Tu are 33% identical overall. Among EF2 homologs, EF2 from *Halobacterium halobium* (*H. halobium*) is 36% identical to eEF2 from *S. cerevisiae* and 30% to *E. coli* EF-G. Exceptions from the similarities are a variety of posttranslational modifications of both factors that only occur in eukaryotes (Kapp and Lorsch 2004). Consequently, mechanisms underlying elongation are similar in eukaryotes, bacteria and archaea. To explain the elongation mechanism many models have been proposed, but I will focus on the better-established bacterial model proposed by Moazed and Noller (Figure 1.3) (Moazed and Noller 1986).

According to that model, peptide chain elongation in eukaryotes begins with the initiator peptidyl tRNA firmly entrenched in the P-site of the 80S ribosome. The repetitive cycle for the codon-directed addition of aa-tRNAs begins with an aa-tRNA carried to the aminoacyl-tRNA site (A-site) of ribosome as part of a ternary complex composed by GTP and eEF1α (EF-Tu in bacteria). With the GTP and aa-tRNA, eEF1α catalyzes the binding of aa-tRNA to the ribosome, in a codon-dependent manner, through hydrolysis of GTP (Urban and Vogel 2007). To ensure that only the cognate tRNA is selected for the next cycle of elongation several processes that I will discuss later take place. In addition to the already mentioned codon-anticodon base pairing between the mRNA and the tRNA and GTP hydrolysis by eEF1α, conformational changes in the decoding center of the small ribosomal subunit must occur (Rodnina and Wintermeyer 2001). The codon-anticodon pairing induces three bases in the small ribosomal subunit rRNA to swing out interacting with the mRNA-tRNA duplex formed. Consequently, eEF1α (EF-Tu in bacteria) GTPase activity is activated and the resulting eEF1α – GDP releases the aminoacyl-tRNA into the A-site in a form that can continue with peptide bond formation (complex EF1α – GDP is released).

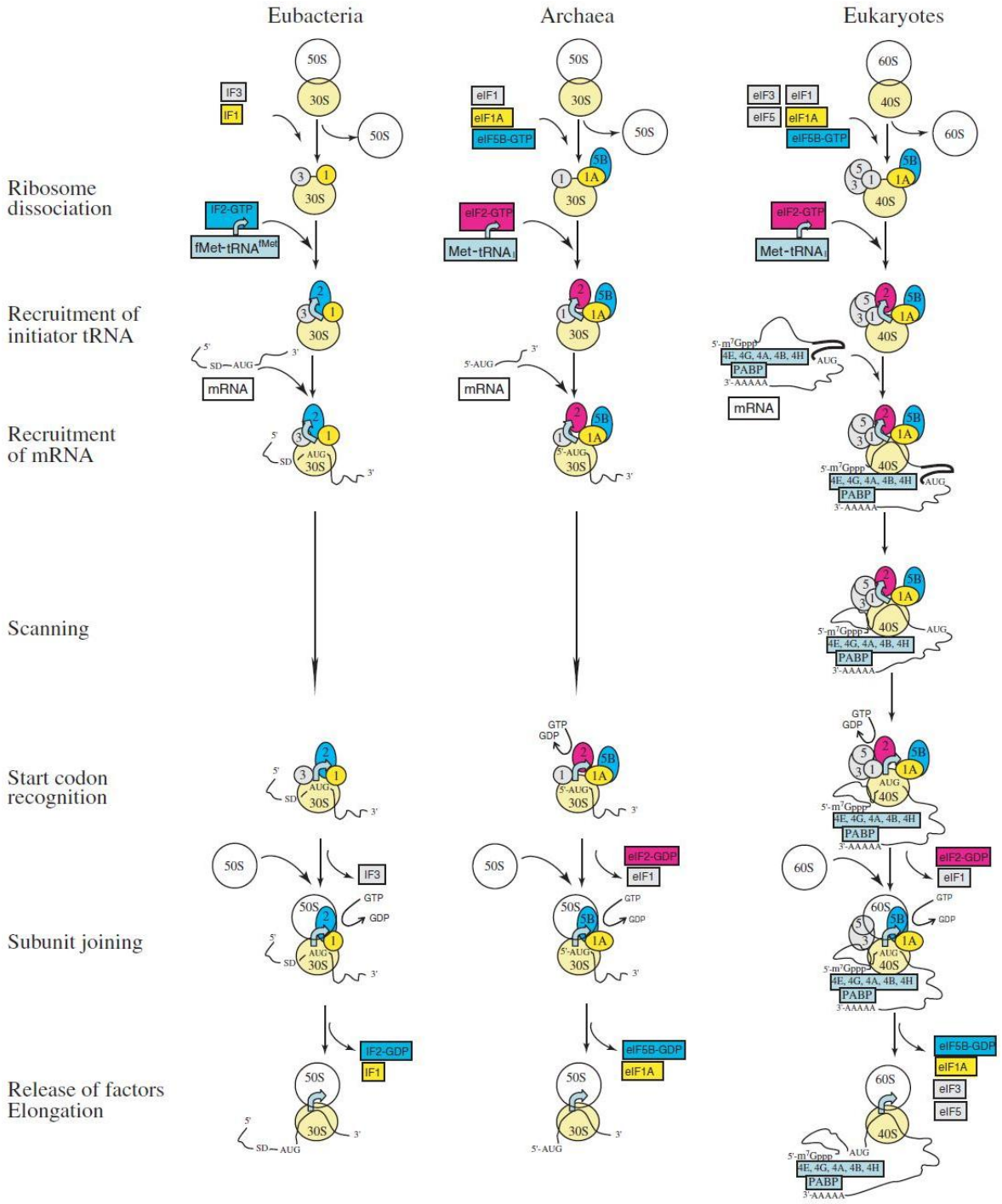


Figure 1.2: Translation initiation. Schematic overview of translation initiation in the three domains of life: bacteria, archea and eukaryote. Universally conserved factors IF1/eIF1A (yellow), IF2/eIF5B (blue) and archea and eukaryote eIF2 (red) are highlighted. The cap/poly-A-binding complex (only in eukaryotes) is in light blue. SD – Shine-Dalgarno sequence (Marintchev and Wagner 2004).

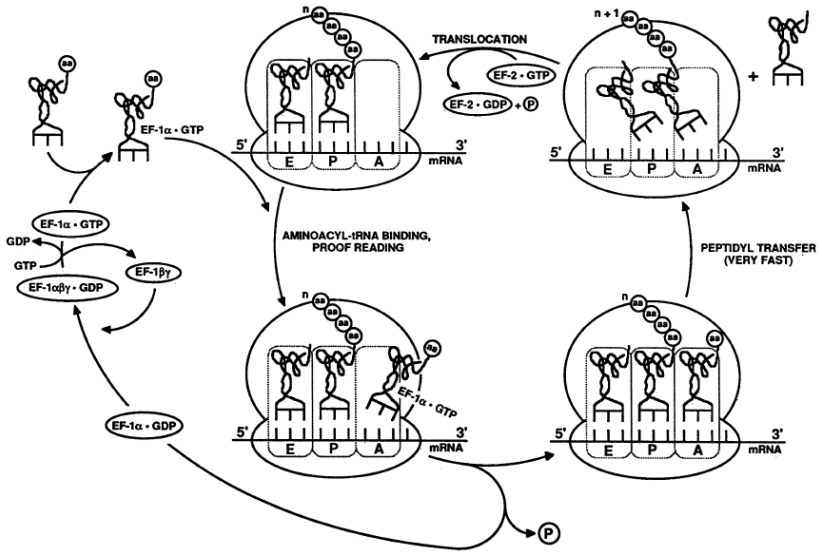


Figure 1.3: Elongation cycle in eukaryotes. This schematic representation follows the assumptions of the "half-site" model of Moazed and Noller and is based on the similarities between the structure and function of the eukaryotic and prokaryotic elongation factors. E – exit site; P – peptidyl-tRNA site; A – aminoacyl-tRNA site (Merrick 1992).

The presence of aa-tRNA in the A-site is very short lived because the ribosomal peptidyl transferase center quickly catalyzes the formation of a peptide bond between the incoming amino acid and the peptidyl-tRNA (Merrick 1992). Shortly, in this reaction, there is a nucleophilic attack by the α-amino group of the aminoacyl-tRNA in the A-site on the carbonyl of the ester linkage of the aa-tRNA (or peptidyl-tRNA) in the P-site. Resulting from that is a deacylated tRNA in a hybrid state with its acceptor end in the exit site (E-site) of the large ribosome subunit and its anticodon end in the P-site of the small subunit. In a similar hybrid situation is the peptidyl-tRNA with its acceptor end in the P-site of the large subunit and its anticodon end in the A-site of the small subunit. At this point a translocation must occur in such a way that the deacylated tRNA is completely in the E-site, the peptidyl tRNA fully in the P-site, and the mRNA moved by three nucleotides (one codon) so that a new codon is located in the A-site. This task is accomplished by eEF2 (EF-G in bacteria) in a GTP-dependent manner (Kapp and Lorsch 2004). As a consequence of this action, and in consonance with biophysical data, the growing polypeptide chain elongates but does not move (Odom, Picking et al. 1990). After the hydrolysis of GTP, and the release of eEF1α – GDP, this complex is recycled to its GTP-bound form so that it may participate in successive rounds of polypeptide elongation. This exchange is catalyzed by a multifactor complex called eEF1-βγ. For the eEF2 (and EF-G) there is no known guanine-nucleotide (Merrick 1992).

Energetically, the elongation is the step where most of the energy required for protein synthesis is consumed. Two high-energy phosphates (eEF1α and eEF2) are used per cycle and another two high-energy phosphates are used to generate each aa-tRNA (ATP + aa + tRNA -> AMP + PPi + aa-tRNA). Thus the formation of each peptide-bound costs four high-energy phosphates (Kapp and Lorsch 2004).

1.2.3.1. Elongation Factor 3 (eEF3) – An exception to elongation conservation

The major exception to the role of conservation in the translation elongation mechanism is the existence of elongation factor 3 (eEF3) exclusively in fungi (Figure 1.4) (Blakely, Hekman et al. 2001). This factor was discovered by Skogerson and co-workers (1976) and is required for *in vitro* protein synthesis by yeast ribosomes in addition to eEF1 and eEF2 (interchangeable with the eukaryotes homologous) (Skogerson and Wakatama 1976). Besides that function, some additional roles for eEF3 in fungi are currently known and, one of the most recent, is the association of the eEF3 with a calcium channel in the plasma membrane (Liu and Gelli 2008). Purified from several fungal species eEF3 consists of a single polypeptide chain with a molecular weight of about 125 kDa (Qin, Xie et al. 1990). The YEF-3 gene that codes for eEF3 is essential for viability in yeast and is located on chromosome XII.

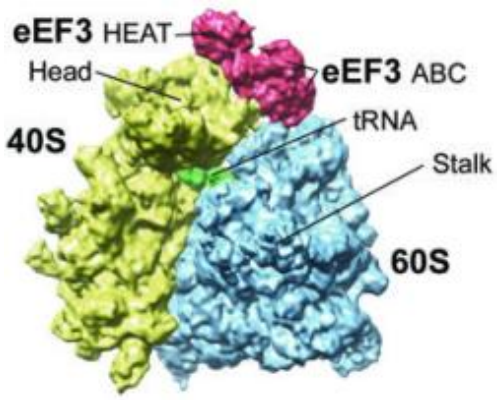


Figure 1.4: Elongation factor 3 (eEF3). Cryo-electron microscopy structure of *S. cerevisiae* ATP-bound form of eEF3 in complex with the post-translocational-state 80S ribosome. Highlighted one the constitutive amino-terminal HEAT repeat domain and two ABC-type ATPase domains (Andersen, Becker et al. 2006).

The eEF3 protein possesses ribosome-dependent ATPase and GTPase activities and contains a duplicated nucleotide binding motif homologous to those found in the typically membrane-associated ATP binding cassette (ABC) proteins (Belfield and Tuite 1993). It performs a vital role in protein translation, where it enables efficient binding of the eEF1A – GTP – aa-tRNA

ternary complex to the ribosomal A-site and facilitate the release of deacylated tRNA to the ribosomal E-site. In doing that eEF3 appears to maintain a balance between the protein translation rate and amino acid fidelity (Liu and Gelli 2008).

Mammalian ribosomes possess an intrinsic eEF3 like activity however no mammalian candidate has yet been identified as the homologue of fungal eEF3. In bacteria, *E. coli* has a soluble ribosome-dependent ATPase, RbbA, which require the hydrolysis of ATP for its functions and has some sequence similarity to eEF3 (Kapp and Lorsch 2004). Due to its unique occurrence in fungi and the lack of a homologue in the mammalian cells eEF3 is a promising drug target for the development of anti-fungals. Identification and characterization of this factor among diverse fungi of medical importance may be essential for the design of pharmacological inhibitors with broad specifity. Thus far, the major limitation relates to the fact that eEF3 has only been cloned and characterized from yeasts of the order Ascomycota as well as the fungus-like organism *Pneumocystis carinii* (Blakely, Hekman et al. 2001). Basidiomycete yeasts are distinct either in morphology and 18S ribosomal DNA sequence criteria (Qin, Xie et al. 1990).

1.2.4. Termination

In the standard genetic code, sixty-one sense codons are decoded by cognate transfer RNAs aminoacylated by twenty natural amino acids (Seit-Nebi, Frolova et al. 2002). With these twenty amino acids a tremendous number of different polypeptide chains can be formed. The marker for translation termination comes with the appearance of one of the three stop codons (UAA, UGA and UAG). These three codons are also designated nonsense codons and were identified in the mid 60's - UAA and UAG (Weigert and Garen 1965) and UGA (Brenner, Barnett et al. 1967). These triplets in mRNA function *in vitro* as signals for release of polypeptide chains from ribosomes (Inge-Vechtomov, Zhouravleva et al. 2003). The existence of these three stop codons is true in organisms with a universal genetic code, whereas, in ciliates with variant code, only one or two codon(s) remain(s) (Seit-Nebi, Frolova et al. 2002).

The nonsense codons were recognized by specific termination recognition proteins called class 1 polypeptide release factors (RF) (Merrick 1992). The class-1 RF (which comprises prokaryotic RF1 and RF2 and eukaryotic eRF1) recognize stop codons and transfer the signal from the mRNA stop codon occupying the A-site of the ribosome to the peptidyl transferase center, inducing the hydrolysis of peptidyl-tRNA catalyzed by the peptidyl transferase center of the ribosome (Inge-Vechtomov, Zhouravleva et al. 2003). Despite having the same function,

eukaryotic and prokaryotic class 1 release factors do not present any obvious sequence homology. eRF1 is composed by three domains: N (or 1) that participate in stop codon recognition (Seit-Nebi, Frolova et al. 2002), M (or 2) responsible for the peptidyl transferase hydrolytic activity (Frolova, Tsivkovskii et al. 1999) and C (or 3) that are not involved in stop codon recognition but binds to the second termination factor eRF3 (Inge-Vechtomov, Zhouravleva et al. 2003). In 2002, Seit-Nebi and co-works postulated a novel decoding mechanism for the eukaryotic class 1 RFs and the principal findings are: (i) the recognition of the stop codon involves two distinct mini domains (Y-C-F and NIKS) of the N domain of eRF1; (ii) the recognition of some stop codons in organisms with a non-universal genetic code is limited by the existence of negative determinates (constraints) in eRF1 (Seit-Nebi, Frolova et al. 2002).

The class-2 RF (prokaryotic RF3 and eukaryotic eRF3) are GTPases which one not required *in vitro* for the peptide release reaction itself but stimulate the activity of class 1 regardless of which stop codon class 1 factor is engaged. RF3 and eRF3 functions in similar ways, however they present important structural differences between them (Kisselev, Ehrenberg et al. 2003).

So, in bacterial systems there are three RF, two of which have different codon specificities: RF1 (UAA, UAG) and RF2 (UAA, UGA). The third release factor, RF3, has no codon specificity and its function is to stimulate RF1 and RF2 activity (Inge-Vechtomov, Zhouravleva et al. 2003). Conversely to bacterial systems, eukaryotic, archea and mitochondria have only two RF (Kisselev, Ehrenberg et al. 2003): eRF1 (gene *SUP45* in *S. cerevisiae*) which governs the termination of translation recognizing the three nonsense codons and eRF3 (gene *SUP35*) which stimulates the activity of eRF1 (Inge-Vechtomov, Zhouravleva et al. 2003) (Figure 1.5). The action of eRF1 and eRF3 can extend besides translation termination. In *S. cerevisiae* a numerous number of pleiotropic effects of *sup45* and *sup35* mutations are identical. These findings are in consonance with the notion that these two genes operate together either in translational termination or in several other cellular processes. The transcripts of these genes interact with other proteins and some of the examples are components of the translation termination complex like PABP and a number of Upf proteins (Inge-Vechtomov, Zhouravleva et al. 2003).

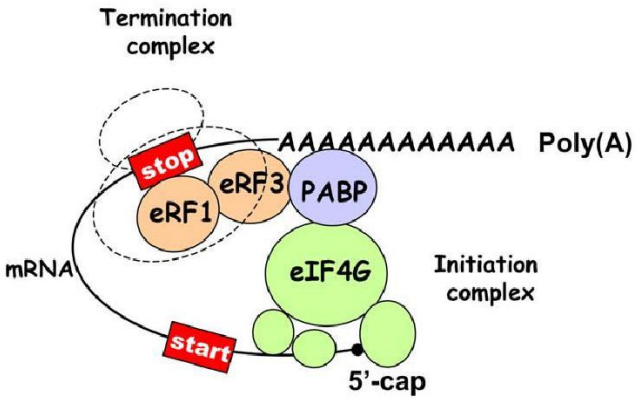


Figure 1.5: Translational termination complex. Scheme of the translational termination complex formed when the ribosome elongation machinery (dotes) finds a termination codon. Interaction between PABP and eIF (green) are fundamental to the ribosome recycling (Inge-Vechtomov, Zhouravleva et al.

1.2.4.1. [PSI$^+$] cells

Like many other proteins in yeast, eRF3 (*Sup35*) has prion-like properties. The aggregated form of *Sup35* results in a cytoplasmically inherited suppressor element called as [PSI$^+$]. One of the well-known phenotypic features of [PSI$^+$] is stop codon suppression. The Hsp104 protein plays an important role in prion formation as it was shown that it is required for formation and maintenance of [PSI$^+$] aggregates of eRF3. Its overproduction or inactivation cures cells of [PSI$^+$] (Cosson, Couturier et al. 2002).

1.2.4.2. Nonsense-mediated mRNA decay (NMD)

During the protein synthesis aberrant proteins can be produced. Some of these aberrant transcripts contain a premature termination codon (PTC) that codes for potentially deleterious protein fragments (Inge-Vechtomov, Zhouravleva et al. 2003). In these cases a surveillance complex forms and triggers rapid mRNA decay via the nonsense-mediated mRNA decay (NMD) pathway.

In yeasts, during premature translation termination the mRNA surveillance complex composing Upf proteins and release factors is assembled. This complex searches 3´ of the termination codon for specific signals that target the mRNA for rapid degradation. When such signal is found, the transcript is rapidly decapped by Dcp1p, followed by 5´ to 3´ decay by exonuclease Xrn1p (Wang, Czaplinski et al. 2001).

The identification of UPF homologs in different organisms suggests that NMD is an evolutionarily conserved pathway of RNA degradation in eukaryotes (Inge-Vechtomov, Zhouravleva et al. 2003). In yeasts, Upf1 protein (and SMG1 factor) interacts with both translation factors eRF1 and eRF3 (forming a complex named SURF), and likely influences translation termination efficiency (Funakoshi, Doi et al. 2007). On the other hand, Upf2p and Upf3p interact only with eRF3 and compete with eRF1 for binding eRF3 (Wang, Czaplinski et al. 2001). When eRF1 dissociates, it allows either Upf2 or Upf3 to bind to the eRF3-Upf1 complex. Since eRF3 blocks the ATPase-helicase activity of Upf1p, the activation of the surveillance complex requires dissociating of eRF3 from the Upf complex. Degradation by the NMD pathway is independent of prior deadenylation of the mRNA and Pab1p is unable to prevent the decay of mRNA subjected to NMD. Therefore, it is not likely that Pabp1 out-competes Upf proteins for binding to eRF3 (Cosson, Couturier et al. 2002).

1.2.5. Recycling

The fourth and final stage of translation is the recycling of the ribosomal subunits. In bacterial systems after termination of translation, one more protein factor, RRF, is involved. The function of RRF is to recognize the ribosome that was left on the mRNA with a deacylated tRNA. Probably this ribosome is in a P/E hybrid state in which the acceptor end of the tRNA is in the E-site of the 50S subunit and the anticodon end is in the P-site of the 30S subunit (Kapp and Lorsch 2004). With the help of the tRNA translocation factor EF-G and eIF3, RRF recycles the ribosome for a new round of initiation (Kisselev and Frolova 1999; Kisselev, Ehrenberg et al. 2003). The recycling of ribosomes is believed to occur by splitting the ribosome into its subunits promoting the dissociation and release of the tRNA and mRNA. In eukaryotic cells, no specific recycling factor has been identified so far and there is no evidence of a RRF ortholog (Kapp and Lorsch 2004). In the group of factors proposed to be involved in ribosome recycling, eIF3 is the most intriguing. A variety of experiments indicate that eIF3 binds to 40S subunits inducing a conformational change that could, potentially, increase the rate of subunit dissociation as well as lowering the rate of association. In order to explain recycling in eukaryotes the closed-loop model postulates that, contrary to the bacterial system, termination and recycling may not release the 40S subunit back into the cytoplasm (Kean 2003). Instead, 40S subunits may be shuttled across or over the poly(A) tail back to the 5´ end of the mRNA via the 5´ and 3´ end associated factors. The closed loop serves to facilitate re-initiation of translation rather than the first initiation event. The finding that eRF3 and PABP interact with each other, connecting the termination apparatus to the poly(A) tail

is in accordance with this proposal. Another argument in favor of this model is the finding that the disruption of the PABP-eRF3 interaction inhibits translation and that addition of eRF3 to *in vitro* translation assays stimulates the initiation process (Uchida, Hoshino et al. 2002). This hypothesis has not been verified *in vivo* but is in agreement with the size and properties of all proteins. In this study performed by Uchida and co-workers (2002) another argument in favor of this model was revealed. This study showed that GSPT (mammalian eRF3) associates with eIF4G through PABP and that the GSPT-PABP interaction is involved in many rounds of translation. Through the action of a complex composed of poly(A)-PABP-eIF4F-cap the mRNA is circularized and the effect is an enhancement in translation. In this hypothetical model a translation-terminating ribosome may be recruited to the next translation initiation. In situations where stop codons are distant from the poly(A) tail, a bridging protein is needed to physically couple them and allow the recycling of ribosomes. The fact that GSPT interacts with eRF1 and PABP at the same time suggests that GSPT may perform this function. In this model, the 3´ UTR that is located between a stop codon and the poly(A) tail could be looped out, and the terminating ribosome could be passed to the 5´ cap through a novel protein bridge consisting of eRF1, GSPT, PABP and eIF4F.

In Humans there are two distinct eRF3 genes, termed *GSPT1* and *GSPT2,* and structurally both subtypes consist of an N-terminal region and a C-terminal eEF1 α-like GTP-binding domain. C-domain interacts with eRF1 and is sufficient for the termination reaction. The N-domain associates with PABP and inhibits its multimerization (Uchida, Hoshino et al. 2002).

1.2.6. Poly(A)-binding protein

The poly(A)-binding protein (Pabp1 for yeasts) presents two major functions in the cell: mRNA stabilization regulates the poly(A) tail length during the polyadenylation reaction and prevents deadenylation and translation enhancement efficient translation requires the synergistic interplay between 5´ cap and 3´ end poly(A) tail of mRNA (Cosson, Couturier et al. 2002; Uchida, Hoshino et al. 2002). The latter appears to be independent of its binding to eIF4G and all these activities involve the N-terminal part of Pab1p. Another function assigned to Pab1p is an anti-suppressor effect *in vivo*. This effect requires the site of the binding of Pab1p to eRF3, suggesting that Pab1p has an anti-suppressor effect on translation termination through its interaction with eRF3. The interactions between eRF3 and PABP are evolutionarily conserved and occur in the N-terminal domain of eRF3 (Cosson, Couturier et al. 2002). In Human cells, when the eRF3-PABP interaction is inhibited the ribosome recycling is prevented. These data also supports the

existence of a protein complex including eRF1-eRF3-PABP-eIF4F, which mediate the coupling of termination and the initiation of protein synthesis. This complex may also be responsible for deciding what percentage of the 40S subunits may remain attached to the mRNA (Uchida, Hoshino et al. 2002).

1.2.7. Deadenylation

Control of mRNA decay is a fundamental step in the control of the amount of protein produced from the mRNA by translation. The decay is intimately linked and regulated by translation (Funakoshi, Doi et al. 2007). Defined as the exonucleolytic degradation of the 3´ poly(A) tail of eukaryotic mRNAs, deadenylation is an important step in both mRNA degradation and translational silencing (Dehlin, Wormington et al. 2000).

The major pathways of mRNA decay characterized so far in yeast and mammalian cells are: mRNA decay directed by AU-rich elements (AREs) in the 3´ UTR, decay mediated by destabilizing elements in protein-coding regions, NMD and decay directed by microRNAs (Zheng, Ezzeddine et al. 2008). The first major step in all these decay pathways is deadenylation. This process is the rate-limiting and the most efficient step in controlling mRNA decay (Funakoshi, Doi et al. 2007). Deadenylation can be divided in two phases. In the first phase poly(A) tails are shortened to approximately 110 nucleotides by the action of Pan2 in association with Pan3 (in yeast Pan3 does not exhibit poly(A) nuclease activity but is necessary for a proper function of Pan2) (Zheng, Ezzeddine et al. 2008). In the second phase of deadenylation, a complex composed of Ccr4 and Caf1 catalyze further shortening of poly(A) tail to oligo(A). Decapping by the Dcp1-Dcp2 complex may occur during and/or after the second phase of deadenylation (Zheng, Ezzeddine et al. 2008). In this processes two major mRNA deadenylase complexes (Caf1-Ccr4 and Pan2-Pan3) play a central role and both have been identified in yeasts and mammals (Funakoshi, Doi et al. 2007). The cytoplasmatic deadenylase complex Ccr4-Caf1 has significant homology with known 3´ to 5´ exonucleases and both proteins have catalytic activity. The other major complex, PAN, is a heterodimeric protein consisting of catalytic Pan2 and regulatory Pan3 subunits. Since the rate-limiting step for mRNA decay is the deadenylation, the enzymatic activity of these two deadenylases constitutes a major target for the control of mRNA decay. Deadenylation, and decay of a normal mRNA, is mediated by the action of eRF3 in a manner coupled to translation termination. Funakoshi and co-workers (2007) postulated that eRF3 mediates deadenylation through Ccr4-Caf1 and Pan2-Pan3 deadenylase (Funakoshi, Doi et al. 2007). The termination

complex eRF1-eRF3 and deadenylase complexes hPan2-hPan3 and hCaf1-hCcr4 interact with PABPC1. The binding of hPan2-hPan3 and hCaf1-hCcr4 with PABPC1 triggers the deadenylase activity (Uchida, Hoshino et al. 2004).

One consequence of deadenylation is the formation of nontranslatable mRNA protein complexes (messenger ribonucleoproteins - mRNPs). These nonstranslatable mRNPs may accumulate in P-bodies (composed by factors involved in translation repression, decapping and 5´ to 3´ degradation). In a 2008 study, Zheng and co-workers demonstrated that deadenylation is required for mammalian P-body formation and mRNA decay (Zheng, Ezzeddine et al. 2008). Pan2, Pan3 and Caf1 deadenylases were identified as new P-body components and show that Pan3 helps recruit Pan2, Ccr4, and Caf1 to P-bodies. When deadenylation is blocked P-bodies are not detected. The findings of this paper support the idea of a dynamic interplay among deadenylation, mRNP remodeling, and P-body formation in selective decay of mammalian mRNA. According to the model proposed Pan2-Pan3 and Ccr4-Caf1 complexes forms a super complex on mRNAs in the cytoplasm. In the first phase, 3´ poly(A) tail-PABP complex stimulates poly(A) shortening by the Pan2-Pan3 in the super complex. In this phase, the activity of Ccr4-Caf1 is inhibited and mRNP remodeling occur (dissociation of PABPs and some translation initiation factors and association of translation repressors). The remodeled mRNP (now in a nontranslatable state) may associate with existing P-bodies or nucleate formation of new P-bodies, in which the second phase of deadenylation by Ccr4-Caf1 and/or decapping would proceed. In alternative, a remodeled mRNP undergo the second phase of deadenylation outside P-bodies. This could induce another mRNP remodeling that determines whether the oligo(A)-mRNP would be degraded inside or outside of P-bodies. After those two general pathways occur, the mRNA is degraded in the 5´ to 3´ direction by Xrn1p in decapping complexes and in the 3´ to 5´ direction by exosomes. An important conclusion from these findings is that although P-bodies may be assembled via different mechanisms, deadenylation is always a necessary step that enables mRNPs to enter existing P-bodies or to initiate the formation of new ones (Figure 1.6).

1.3. tRNAs and aminoacyl-tRNAs synthesis

1.3.1. tRNA

Cellular life is based on the translation of the four-letter code of RNA into the twenty two-letter alphabet of proteins. A crucial feature of this process is the fidelity with which mRNA is

translated during protein synthesis. During this process the cell utilizes aa-tRNAs as building blocks, which are produced by aminoacyl-tRNA synthetases (aaRSs) and delivered to the ribosome by EF-Tu (in bacteria) or eEF1α (archaea and eukaryotes). To ensure the fidelity necessary in protein synthesis two main determinants exist: the availability of aa-tRNAs composed of cognate amino acid:tRNA pairs and the accurate selection of aa-tRNAs on the ribosome (Ling, Reynolds et al. 2009).

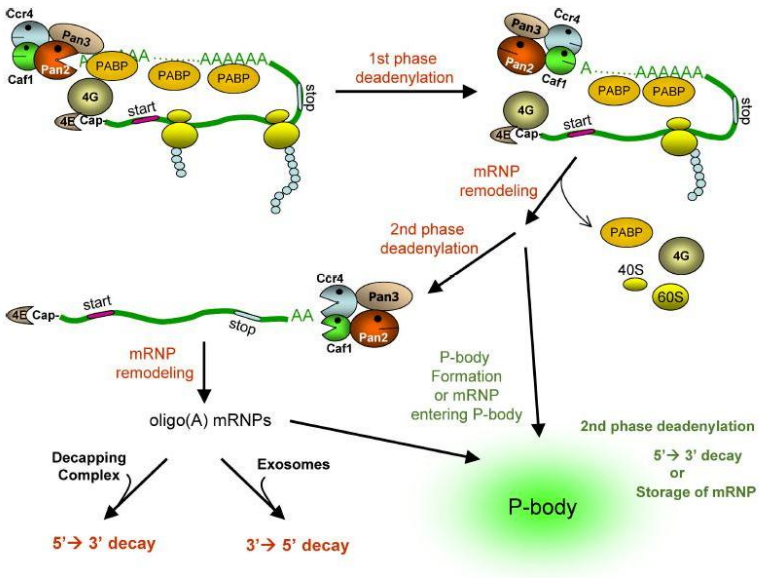


Figure 1.6: Model linking deadenylation, P-bodies, and mRNA decay. Schematic figure of the postulated relations between deadenylation of mRNA, the P-bodies formation and the mRNA 5′ to 3′ and 3′ to 5′ decay (Zheng, Ezzeddine et al. 2008).

Modern day proteins are synthesized in ribosomes, a complex molecular machine composed of proteins and RNA. The relatively small tRNA adaptors are central to this process (Sun, Li et al. 2008). tRNA is a molecule composed of RNA that functions as an adaptor in the cell to bridge the genetic code in mRNA with the twenty two-letter code of amino acids in proteins. Typically with a length of 73 to 93 nucleotides the role of tRNA as an adaptor is best understood by considering its three-dimensional structure. RNA forms double-helical structures with Watson-Crick base pairing but it contains many noncanonical base pairs, and even triplets, that allow it to fold into its unique three-dimensional structure. Conformational studies reveal a strong tendency for its strands to follow an A-helical pathway, even in non-base-paired regions (Noller 2005). A characteristic motif is the U-turn, of the anticodon loop of tRNA, which causes an abrupt reversal in direction of the RNA chain. tRNA structure also possess the coaxial stacking of RNA helices: the acceptor stem of 7 base-pairs (bp) stacks on the 5-bp T stem to form one continuous A-form

helical harm of 12-bp. The two other helices, the D stem and anticodon stem, also stack, although imperfectly, to form a second helical arm. The two coaxially stack arms are responsible for the distinctive L form of tRNA (Figure 1.7).

The cells devote a great deal of resources to the modification of RNA. Indeed, considerably more genetic information is allocated to tRNA modification than to tRNA genes (Gustilo, Vendeix et al. 2008). There is a great number, almost a hundred, of tRNA post-transcriptional modifications identified so far. Some of the modifications are as simple as methylations, whereas others involve multiple step additions of aromatic acid derivates and sugars (Agris 2004). The best understood modifications are those occurring in the anticodon stem and loop domain (ASL). For example, in the wobble position of tRNAs there are the following nucleosides - U, C, G or I (inosine), the later is derived from adenosine. Inosine appears to substitute A in the position 34 because I binds C and has a wobble capacity to A and U base pairing. Besides that, A in the wobble position of the P-site tRNA could destabilize the A-site anticodon-codon duplex (Agris 2004). The function of these modifications is not necessarily preventing the misreading of near-cognate codons but significantly increase the recognition of cognate codons. For example, in yeast genetic deletion of enzymes responsible for modifying tRNA wobble position of $tRNA^{Lys}$ is lethal (Gustilo, Vendeix et al. 2008). Modifications in the anticodon domain of tRNA, at purine-37 position, are also related with the maintenance of the reading frame. More precisely, natural modifications in the anticodon suppress frameshifting. For example, in the absence of the wobble-position modification queuosine, tRNA tyrosine or asparagine induce a +1 frameshifting and have a limited effect on -1 frameshifting. So, modifications in the anticodon loop at wobble position 34 and 37 appear to restructure the loop for correct decoding and are essential for the tRNA folding, secondary and tertiary structure, and thus tRNA function (Agris 2004; Gustilo, Vendeix et al. 2008).

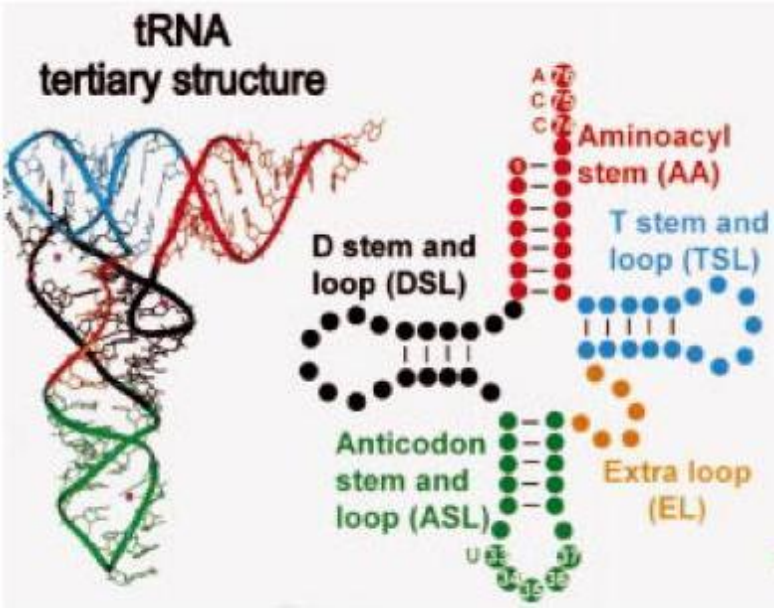


Figure 1.7: Structure of the tRNA. On the left a crystallographic structure representing a three-dimensional structure of the tRNA and, on the right, the cloverleaf secondary structure with the domains colored. Amino acid accepting stem (red), dihydrouridine stem and loop domain (DSL) (black), anticodon stem and loop domain (ASL) (green), extra loop (EL) (gold), ribothymidine stem and loop (light blue). Invariant U33 and amino acid accepting 3´ terminus (C74, C75 and A76) are show (Agris 2004).

1.3.2. Aminoacyl tRNAs synthetasis

The aa-tRNAs are formed by the action of the group of enzymes called aaRSs. The role of aaRSs in translation is defining the genetic code by accurately pairing cognate tRNAs with their corresponding amino acids (Ling, Reynolds et al. 2009). The aaRSs display an overall error rate of about 1 in 10,000. The recognition of correct tRNAs by aaRSs is made mostly by specific interactions with one or more of the discriminator base (N73), the acceptor stem, and the anticodon (Ibba and Soll 2000). However, tRNA identity is not only achieved with isolated interactions between single nucleotides and amino acids in aaRSs. The binding of aaRS can induce sequence-dependent alternative conformations in tRNA. The discrimination by aaRSs of amino acids is more problematic because some amino acids are chemically very similar. To prevent potential errors during translation, an intrinsic proofreading activity, called editing, serves to eliminate amino acids recognized by a noncognate aaRS (Ibba and Soll 2000). So, the specificity of synthetases necessary to keep errors in low level during translation is achieved by preferential binding of the cognate amino acid and selective editing of near-cognate amino acids.

Structurally, the aaRSs have separated active and editing sites, as proposed firstly in the double-sieve model (two-step substrates selection) (Fersht 1977). According to this, the active site

serves as the first “sieve”, activating cognate, isosteric and smaller amino acids but excluding the larger ones. The editing site is the second “sieve”, hydrolyzing misactivated or mischarged amino acids but excluding cognate amino acids on the basis of size or hydrophobicity. Regardless of their structural and conserved mechanisms of catalysis, aaRSs are divided into two unrelated classes (I and II), each with 10 synthetases (Woese, Olsen et al. 2000). This division is based on mutually exclusive sequence motifs that reflect distinct active site topologies. Structural studies show that in class I aaRSs the active site contains a Rossmann dinucleotide-binding domain and in class II aaRSs there is a novel antiparallel β-fold. Another major difference between the two classes is related to the binding of tRNA. Class I aaRSs approach the acceptor stem of tRNA from the minor groove side with the variable loop facing the solvent, on the other hand class II aaRSs approach the major groove side of the acceptor stem and the variable loop faces the synthetase. Structural and functional studies have shown that class I isoleucyl-RS, valyl-RS and leucyl-RS share a homologous editing domain, whereas class II aaRSs have divergent editing sites that share fewer common features (Ling, Reynolds et al. 2009). Another important domain in the aaRSs is the CP1. CP1 is a globular insertion domain (INS) positioned approximately 35 Å from the active site and is conserved among several class I aaRS. The function of CP1 is to recognize noncognate amino acids and edit substrate analogs. Mutations in the CP1 domain region reduce editing activities without affecting the aminoacylation efficiency (Ling, Reynolds et al. 2009).

Because the reactions require the capacity of aaRSs to recognize tRNAs as well as small chemicals such as amino acids and ATP, the structures of this enzymes are well equipped for interacting with diverse molecules (Park, Schimmel et al. 2008). In higher eukaroytes, several aaRSs form macromolecular complexes via multivalent protein-protein interactions with three aaRS associated proteins at the core. These nonenzymatic cofactors are known as aminoacyl-tRNA synthetase-interacting multifunctional protein (AIMP) 1/p43, 2/p38 and 3/p18 (Park, Choi et al. 2010). The main advantage of these complexes is improving aminoacylation efficiency by “channeling” substrates to the ribosome. Besides that, their propensity to form complexes appear to be related, in part, to the need of eukaryotic cells for a reservoir of regulatory factors that are involved in regulation of transcription, translation and a number of other signaling pathways. In the same way, the tRNA recognition capacity of bacterial threonyl-RS has been adapted for regulating translation by interacting with the 5´ and 3´ UTR regions of gene-specific transcripts (Park, Schimmel et al. 2008). Another example of the functional versatility of aaRSs is the fact that they are utilized in a large number of mechanisms. The roles of aaRSs in such processes can be divided in two groups, those that use the aaRSs ability to synthesize an aminoacylated RNA and

those that do not depend on aminoacylation. A well characterized example is the role of glutamyl-RS in porphyrin biosynthesis (where glutamyl-$tRNA^{Glu}$ functions as a precursor for 5-aminolevulinic acid) (Ibba and Soll 2000). Another example, is the catalytic activity for glycyl-RS, lysyl-RS, and tryptophanyl-RS that have been adapted to synthesize diadenosine polyphosphates (ApnA), which are involved in the regulation of glucose metabolism, cell proliferation, and death (Park, Schimmel et al. 2008). The expanded functions of aaRSs and their involvement in many mechanisms highlight the fact that they can be pathologically associated with various human diseases. A clear example is provided for Charcot-Marie-Tooth (CMT) disease. CMT is a neural disease caused by heritable dominant mutations in genes that encode for aaRSs causally associated with specific pathological condition (Park, Schimmel et al. 2008). Genes for the glycyl-RS and tyrosyl-RS are examples of the different genetic loci casually linked to the disease (Antonellis, Ellsworth et al. 2003; Jordanova, Irobi et al. 2006). Three aaRSs interacting components associated with the multisynthetase complex AIMPs have each been linked to signaling pathways relevant to cancers.

Overall, it is clear that aaRSs present an essential role for cell viability and the inhibition of a member of this family will produce deleterious effects on the cell. This assumption lead to the early realization that if inhibitors of synthetases could be found that differentiate between bacterial and/or fungal enzymes and their human homologs, such compounds might provide a means of developing antibacterial and antifungal agents. This fact together with the rapid rise of antibiotics-resistant pathogens has put considerable emphasis on the development of novel antibiotics, including the search for aaRSs inhibitors (Ibba and Soll 2000).

1.3.3. Aminoacylation

According to the RNA world hypothesis, in the RNA world the aminoacylation reaction was catalyzed by ribozymes (Lee, Bessho et al. 2000). Nevertheless, this happened during a relatively short time span as the aaRSs arose early in evolution (Park, Schimmel et al. 2008). The aminoacylation process by aaRSs occurs in two steps, namely the activation of amino acids with ATP (forming aminoacyl-adenylates – aa-AMPs) and the subsequent transfer of the activeted amino acid to tRNA (Figure 1.8) (Ibba and Soll 2000).

In the amino acid recognition and activation of class I aaRSs there is no involvement of any covalent or acid-base catalysis, but instead the binding energy provided by enzymes-reactant interactions to stabilize the transition state is used. In class II aaRSs the amino acids binding sites

are rigid templates that bind the amino acid and ATP substrates in the optimal positions for the formation of the transition state by in-line nucleophilic displacement. The selectivity of aaRSs for their cognate tRNAs is enhanced by a large contact surface area available during recognition and kinetic proofreading during aminoacylation. After its formation, aa-tRNAs are delivered by EF-Tu (or EF1α) to the ribosome for peptide synthesis. Elongation factors display selectivity for both the amino acids and the tRNA but bind all cognate aa-tRNAs equally well. This contrasts with near-cognate aa-tRNAs that are bound to elongation factors with different ranges of affinities (Ling, Reynolds et al. 2009).

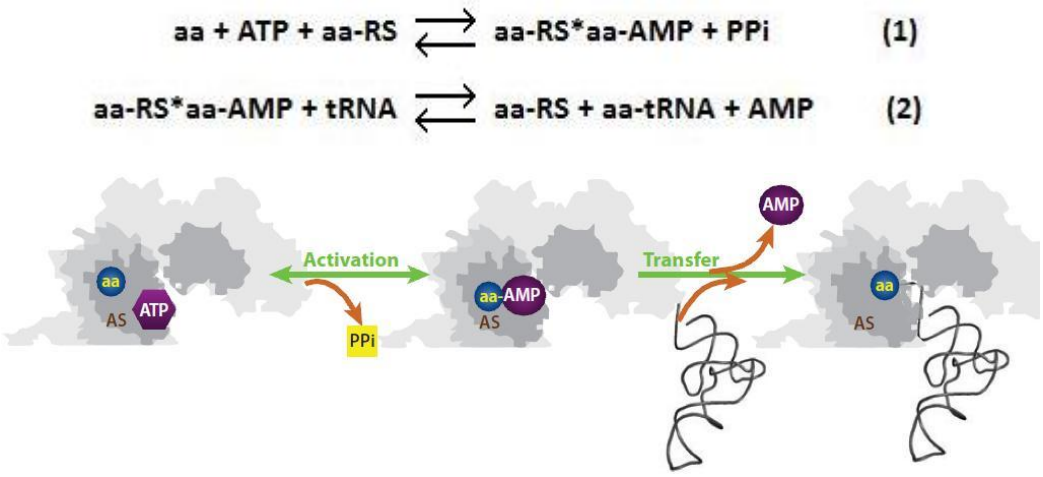


Figure 1.8: Aminoacylation reaction. Representation of the two steps of the aminoacylation reaction, namely, activation of amino acids (aa) by ATP at the aminoacyl-tRNA synthetase (aaRS) active site (AS) forming aminoacyl-adenylate (aa-AMP) (1) and subsequent transfer of the amino acid to the 3´ end of the tRNA (2). PPi – pyrophosphate. (Ling, Reynolds et al. 2009).

1.3.4. tRNA-dependent amino acid transformations

Besides aminoacylation, another pathway to form aa-tRNA occurs in all organisms. This is an indirect pathway which is called tRNA-dependent amino acid transformation (Woese, Olsen et al. 2000). The three main tRNA-dependent amino acid transformations are the formylmethionyl-tRNA synthesis, the selenocysteinyl-tRNA pathway and the pyrrolysine pathway (Sun and Caetano-Anolles 2008). The first pathway exists as a requirement of a special methionine tRNA to initiate protein synthesis. Bacteria and eukaryotic organelles (mitochondria and chloroplasts) use formylmethionine-$tRNA^{fMet}$ to initiate protein synthesis. The $tRNA_{fMet}$ is charged by methionyl-tRNA with methionine, which is then formylated by methionyl-tRNA formyltransferase (MTF) in the presence of the formyl donor N_{10}-formyltetrahydrofolate (FTHF). This reaction involves a highly specific protein-tRNA recognition process (Ibba and Soll 2000).

The other pathway is related to the formation of a nonstandard amino acid, called selenocysteine which is found in proteins of all three domains of life. Selenocysteine is co-translationally inserted into polypeptides under the direction of the codon UGA (recognized by the anticodon UCA) assisted by a distinct elongation factor and a structurally signal in the mRNA (Commans and Bock 1999). UGA is a typical stop codon which also codes for Cys and Trp (Sun and Caetano-Anolles 2008). For the modification and insertion a special tRNA species is required. This tRNA is named $tRNA^{Sec}$ and the gene encoding it has been characterized in organisms of all three kingdoms of life (Xu, Zhou et al. 1999). $tRNA^{Sec}$ is serylated by seryl-RS (usually with less efficiency than the $tRNA^{Ser}$) and then the resulting Ser-$tRNA^{Sec}$ is converted into selenocysteine-$tRNA^{Sec}$ in a two steps pathway (Ibba and Soll 2000). The evolutionary ancestry of Sec is explained for two strikingly opposing theories. On one hand, it was proposed that UGA originally codes for Sec, one of the earliest amino acids to be charged, and later evolved into the new coding functions. The other hypothesis argues that Sec evolved at later stages of the genetic code development (Sun and Caetano-Anolles 2008).

Finally, pyrrolysine is co-translationally inserted in response to the UAG codon (amber codon) and plays an essential role in the active site of a set of methylamine methyltransferases, which allows *Methanosarcineace* species, *Desulfitobacterium hafniense,* and glutless worm *Olavius algarvensis* to use methylamines as carbon sources (Heinemann, O'Donoghue et al. 2009; Silva, Duarte et al. 2009). The structure of this unusual amino acid is 4-methyl-pyrroline-5-carboxylate in amide linkage to the ε-amino group of l-lysine (Longstaff, Larue et al. 2007). The biosynthetic pathway of this amino acid was the last of the encoded amino acids to be discovered. A recent report, from Gaston *et al.* (2011), shows that pyrrolysine arises from two lysines in a *pylBCD*-dependent pathway. An S-adenosyl-l-methionine (SAM) protein, PylB, mediates a lysine mutase reaction whose the product is then linked to a second lysine by PylC previous to oxidation by PylD resulting in pyrrolysine (Gaston, Zhang et al. 2011).

1.3.5. Editing

Linus Pauling and Francis Crick were the first to foresee the need for a quality control pathway to distinguish similar amino acids providing a major safeguard against mistranslation (Ling, Reynolds et al. 2009). Some amino acids differ only in a methyl group making it difficult for aaRSs to distinguish them accurately. AaRSs rely on editing to efficiently discriminate cognate from structurally similar non-cognate amino acids. So, the editing activity plays a critical role *in*

vivo, decreasing the frequency of errors by the clearance of mischarged tRNA. Editing activity can potentially occur either before (pre-transfer editing) and/or after (post-transfer editing) the misactivated amino acid is attached to tRNA (Figure 1.9). Similar to aminoacylation, both tRNA-dependent pre-transfer and post-transfer editing require aaRSs to specifically recognize their cognate tRNAs. In addition to the editing activity that is part of the synthetase there are a few instances of freestanding editing domain homologs encoded by various genomes (Chong, Yang et al. 2008). Editing is present in approximately half of the aaRSs and the disruption of this activity in certain synthetases causes bacterial growth defects and apoptosis in mammalian cells (Ling and Soll 2010).

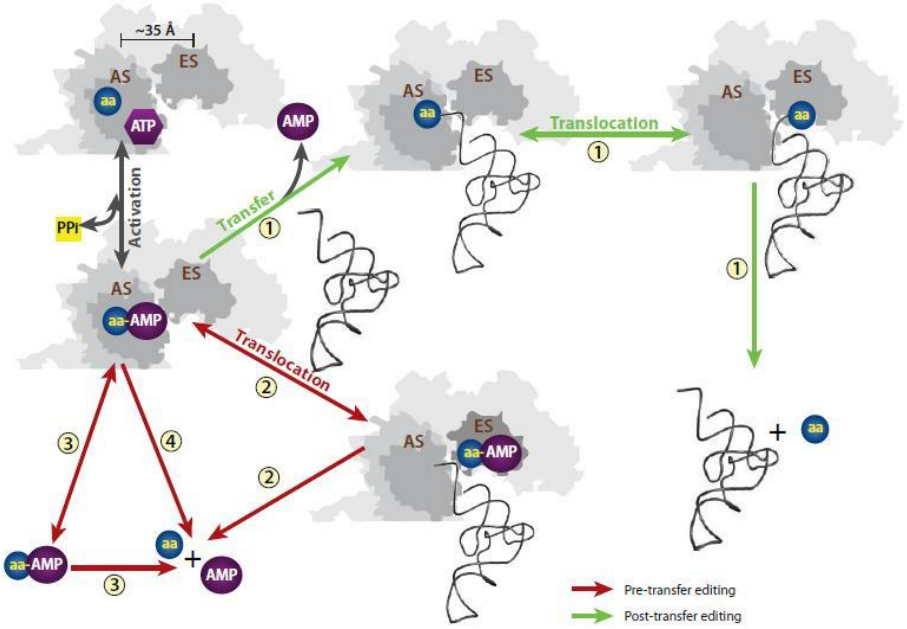


Figure 1.9: Pre- and post-tranfer editing pathways. Pathway 1 – post-transfer editing. The misactiveted amino acid (aa) is first linked to the tRNA and is subsequently translocated from the active site (AS) to the editing site (ES) where it is hydrolyzed. Pathways 2-4 – pre-transfer editing. 2 (translocation) – Misactivated aminoacyl-adenylate (aa-AMP) is directly translocated to the ES to be hydrolyzed. 3 (selective release) – Noncogante aa-AMP is release into solution and subjected to spontaneous hydrolysis. 4 (active site hydrolysis) – Noncognate aa-AMP is hydrolyzed, at the AS, before the release (Ling and Soll 2010).

1.3.5.1. Pre-transfer editing

Pre-transfer editing was firstly demonstrated in studies with isoleucyl-RS and refers to the hydrolysis of misactivated aa-AMP prior to transfer of the aminoacyl moiety to the 3´ end of tRNA. Actually, three pathways explain the mechanisms of pre-transfer editing, namely, translocation,

selective release and active site hydrolysis (Ling, Reynolds et al. 2009). An individual aaRS may employ one or more pathways of pre-transfer editing.

The translocation model is one in which near-cognate aa-AMP is synthesized at the active site but is hydrolyzed at the editing site. This model has been extensively studied for isoleucyl-RS. Conformational changes in isoleucyl-RS may preferentially stimulate the translocation of valyl-AMP, but not the cognate isoleucyl-AMP, either because of tighter binding of isoleucyl-AMP to the active site or because of its rejection by the editing site. Selective release is characterized by cyclization followed by elimination of misactivated amino acids within the active site. More recent studies found that pre-transfer editing could also involve misactivated amino acids hydrolyzed in the active site in addition to those selectively released into solution. This notion comes from several studies as those which involve two class II aaRS, seryl-RS and prolyl-RS. Seryl-RS, from *E. coli* and *S. cerevisiae*, presents tRNA-independent pre-transfer editing against various near-cognate amino acids. In the presence of threonine and ATP, seryl-RS catalyzed the AMP formation at a rate 17-fold higher than the spontaneous hydrolysis. The seryl-RS not presents a distinct editing site, which implies that the aminoacylation active site both preferentially hydrolyzes and selectively releases near-cognate aa-AMP. In a similar study with prolyl-RS in *E. coli*, same results were achieved.

1.3.5.2. Post-transfer editing

The post-transfer editing was first demonstrated in isoleucyl-RS and phenylalanyl-RS by Eldred and Schimmel, and by Yarus (Eldred and Schimmel 1972; Yarus 1972). They found that isoleucyl-RS and phenylalanyl-RS catalyzed the hydrolysis of misacylated Val-tRNA^{Ile} and Ile-tRNA^{Phe}, respectively. The mechanism of post-transfer editing requires that the 3´ end of mischarged aa-tRNAs travels from the active site to the editing site, which is 30-40 Å away (Ling, Reynolds et al. 2009). Structural and modeling studies suggest that acylated 3´-CCA end could be directly translocated between the two sites, while the rest of the tRNA molecule remained attached to the aaRS. Class I aaRSs may retain misacylated tRNA long enough for translocation to occur, whereas class II are more likely to release it into solution and edit in *trans*. According to the double-sieve model, 3´ end of both cognate and near-cognate aa-tRNAs traffic to the editing site however, only near-cognate species can form productive interactions that result in hydrolysis. The principal reason for maintaining this editing site specificity is that it is critical for aaRSs to correct mistakes without compromising the synthesis of cognate aa-tRNAs. The fidelity of this process is

obtained by the discrimination of substrates on the basis of size or hydrophobicity made by editing site of aaRS. To avoid accumulation of the misacylated tRNA, the editing site needs to catalyze hydrolysis at a rate slightly higher than that of aminoacylation. For that the labile ester bond of aa-tRNAs requires low activation energy for hydrolysis.

1.3.5.3. *Trans*-editing

Besides pre-transfer and post-transfer editing the accuracy of aminoacylation is accomplished from another mechanism. This pathway is called *trans*-editing and is based on the action of distinct domains (editing domains) corresponding to particular structural modules within the characteristic synthetase multidomain structure (Ahel, Korencic et al. 2003). Recent studies postulate that editing domains of aaRSs may have existed previously as autonomous proteins that were incorporated into the respective aaRSs genes later in evolution. The reason for the incorporation is likely due to lower specificity of freestanding modules compared with complete aaRSs, which can exploit more intricate tRNA contacts. A widespread (from bacteria to humans) example of *trans*-editing system is the homolog of the editing domain of alanyl-RS, AlaXp. The editing activity of this small protein clears $tRNA^{Ala}$ mischarged with serine and glycine either before translocation to or re-sample from the ribosome (Chong, Yang et al. 2008; Guo, Chong et al. 2009). The fact that this genome-encoded protein is one of the most distributed through the all three kingdoms of life suggests that overcoming the confusion of Gly or, principal, Ser for Ala is a major challenge to the cell.

1.4. mRNA Mistranslation

1.4.1. Basal error rate in translation

In the course of protein translation two possible classes of errors can happen. Firstly, there are missense errors which are due to the replacement of one amino acid by another (Gustilo, Vendeix et al. 2008). Most missense errors have little effect on protein function (termination codon read-through is a special case of this type) (Farabaugh and Bjork 1999). The second type of error is in processivity. The two consequences of this kind of error is premature termination of translation and a translational frameshifting (Gustilo, Vendeix et al. 2008).

Missense errors in translation occur at rates of one per 10^3-10^4 codons (Drummond and Wilke 2008). If we take as an example a protein with an average length (approximately 400

codons), at an error rate of 5 x 10^4, 18% of the molecules of this protein contain, at least, one missense substitution. Missense errors occur during aa-tRNA selection either because of reading by mischarged cognate-tRNAs or by a non-cognate tRNA. These types of errors are enhanced by amino acid starvation proving that competition between cognate and non-cognate aa-tRNAs determines in part the frequency of errors (Holland, Ghosh et al. 2010). Examples of this phenomenon comes from studies carried out by Holland and co-workers (2010) who showed that during chromate (VI) exposure, the depletion of methionine and cysteine are responsible for elevated mRNA mistranslation. However, most amino acids substitutions are not harmful to the cell. The reason is that they do not eliminate protein function. In contrast, processivity errors result in truncated and usually non-functional proteins (Farabaugh and Bjork 1999). From the two types of processivity errors premature termination is by far the most common. The mechanism by which it occurs is called ribosome editing and is characterized by a spontaneous dissociation of peptydil-tRNA from the ribosome ("drop-off") (Menninger 1977). The reason for drop-off of the ribosome from mRNA is the weak interaction between the peptidyl-tRNA with a non-cognate amino acid and the mRNA. In the case of translational frameshifting, it precludes completion of a nascent peptide chain causing an alteration in the normal reading frame and enhancing the probability of the ribosome to encounter a premature termination codon. Besides the spontaneous frameshifting and the frameshifting caused by mutant tRNA, there is another type called programmed frameshifting (Farabaugh and Bjork 1999). There are genes that have evolved sequences that allow the mRNA to re-program the ribosome to change the frame in which it initially was set to read. In situations like that the frequency of frameshifting that occurs at a programmed site is, approximately, 100% (Gustilo, Vendeix et al. 2008). The first bacterial gene that was found to require +1 frameshifting was the *E. coli* gene *prfB*, which encodes release factor 2 (RF2) (Hansen, Baranov et al. 2003). In this case the frameshifting occurs by slippage of peptidyl tRNA during a translational pause caused by poor recognition of the codon in the ribosomal A-site.

It has long been noted that residues directly involved in protein function tend to not tolerate substitutions (Drummond and Wilke 2008). Because of that, it has been postulated that slow-evolving proteins have more sites committed to function or are more functionally important. Alternatively, the reasons that determine the rate of gene evolution are related to their regional variation or mutation rate. In fact, some studies reveal that the functional importance of proteins, such as essentiality or the number of protein-protein interactions correlates surprisingly weakly with evolutionary rate (Drummond, Bloom et al. 2005).

1.4.2. mRNA Mistranslation – Negative aspects and positive features

Intuitively mRNA mistranslation is associated only with a bunch of negative consequences to the cells and organisms however, in reality, this is not the case. If codon-decoding ambiguity only causes proteome disruption and reduces fitness, natural selection should eliminate it (Silva, Paredes et al. 2007). However, mistranslation can bring deleterious consequences but certain advantageous too.

Using *S. cerevisiae* was a model, Santos and colleagues (1999) have demonstrated that cells with ambiguous genetic codes were able to outcompete wild-type cells under stress (Santos, Cheesman et al. 1999). Furthermore, Silva and co-workers (2007) showed that gene expression and physiological remodeling is responsible for stress cross-protection and pre-adaptation of cells to severe environmental alterations, allowing them to survive and explore new habitats (Silva, Paredes et al. 2007). In this same paper, the response to protein misfolding and the subsequent elimination or recovery of aberrant proteins was also characterized and up-regulation of proteasome activity, induction of stress proteins, cell-wall remodeling and accumulation of trehalose and glycogen are observed.

An additional study in *C. albicans* (2007) showed that genetic code ambiguity generates an unanticipated proteome expansion which can bring some advantageous phenotypes to the cell (Gomes, Miranda et al. 2007). This study concluded that, at least in unicellular organisms, negative effects of the codon ambiguity can, under some circumstances, be supplanted by its capacity to generate new adaptive traits. These conclusions emphasize the hypothesis advocated by recent reports that organisms are highly tolerant and readily adapt to genetic code ambiguity. Another theory compatible with these results is the co-evolutionary theory of the genetic code, a theory as described in this introduction, which postulates that amino acids were gradually incorporated into the genetic code.

Besides these potential advantages related to mRNA mistranslation the negative effects are always presents. Indeed, flow cytometry analysis and DNA-microarrays, showed that mRNA mistranslation destabilizes the genome and increases ploidy (Gomes, Miranda et al. 2007). In a study with *Schizosaccharomyces pombe* (*S. pombe*), mRNA mistranslation of threonine ACA codons as alanine resulted in a series of deleterious effects, namely, mitotic defects, abnormal chromosome segregation, aneuploidy and decreased cell viability (Kimata and Yanagida 2004; Silva, Duarte et al. 2009). Various studies with *S. cerevisiae* were also performed and the major

deleterious effects, besides the fitness costs, are increased ploidy (up to 4N), blocked mating and sexual reproduction, and altered expression of chaperones, proteasome activity and carbohydrate metabolism (Moura, Paredes et al. 2010). In a more recent paper (Silva, Duarte et al. 2009) a correlation between mRNA mistranslation/protein misfolding and the up-regulation of the longevity gene PCN1 was found. The principal insight that comes from this is that Pnc1p can be used as a molecular marker of mistranslation that can be used to monitor mistranslation, for example, in tumors, aging cells or neurodegenerative diseases.

Therefore, mistranslation above certain levels disrupts the proteome, decreases cell fitness and increases cell death (Moura, Carreto et al. 2009). Nevertheless, codon ambiguity can also increase proteome diversity which generates genetic and phenotypic diversity. This diversity can be explored by natural selection for development, metabolic and regulatory innovation. All of this is only possible because both bacteria and eukaryotes are more resistant to mistranslation than previously thought (Moura, Carreto et al. 2009; Moura, Paredes et al. 2010). Associated with this high level of mistranslation tolerance are the evolution of proteomic and phenotypic novelty.

1.5. Protein folding and quality control systems

1.5.1. General features

The folding of the majority of proteins in eukaryotic cells occurs either in the cytosol or in the ER. In both compartments the general principles are the same but idiosyncratic pathways exist (Sitia and Braakman 2003). Folding in the ER lumen occurs in conditions which are similar to the extracellular space in terms of high calcium concentrations but is more oxidizing than the cytosol. A set of specialized chaperones and enzymes are also displayed. Many of the proteins that suffer maturation in the ER require several post-translational modifications which turn the process slower and inefficient.

In association with the folding and with the task of minimizing harmful effects from aberrant proteins, cells possess a set of protein quality control mechanisms (PQC) (Gardner, Nelson et al. 2005). The reasons for the existence of these mechanisms are easy to understand especially in multicellular organisms where development relies on the fidelity of protein secretion (Sitia and Braakman 2003). To maintain the efficiency of quality control mechanisms either in the cytosol or ER under diverse physiological conditions, living cells develop regulatory circuits that monitor levels of available chaperones. Protein chaperones exist in all major cellular

compartments and function either by refolding misfolded proteins (for example, Hsp70 and Hsp90), binding to misfolded proteins and preventing aggregation (for example, Hsp40), or disrupting protein aggregation (for example, Hsp104) (Gardner, Nelson et al. 2005).

Chaperones are the first line of defense against aberrant proteins. Once a potentially damaging protein has been identified, the cell responds in three ways. First, cellular factors try to rescue misfolded conformations by refolding them. Secondly, the cell sequesters misfolded proteins in an attempt to prevent toxicity interactions (aggregates formation) (McClellan, Tam et al. 2005). When the proteins cannot refold they must be eliminated by the ubiquitin-proteasome system (UPS) in the cytosol. PQC degradation is primarily brought about by protein-ubiquitination complexes that mark proteins for proteasomal degradation. Repair and degradative PQC systems may not be mutually exclusive (Gardner, Nelson et al. 2005). Quality control systems maintain balance between retaining and degrading potentially harmful products and not preventing export of biologically active proteins. The role of this mechanism in living cells is of great importance because disease can originate from defective degradation. If the rate of synthesis of a protein exceeds the combined rates of folding and degradation, a fraction of it will accumulate intracellularly. The true action of these aberrant structures is not clear and is unknown if these structures are toxic *per se* or whether they represent a defense mechanism that segregates dangerous proteins into specialized ER subcompartments (Sitia and Braakman 2003). In this section I will review the principal insights of quality control in the cytosol, ER and a novel pathway in the nucleus.

1.5.1.1. Protein quality control systems in the cytosol

Misfolded proteins can be degraded by various proteases and in different cell compartments. However, the main intracellular pathway for the elimination of misfolded proteins in eukaryotic cells is the UPS (McClellan, Tam et al. 2005; Yerbury, Stewart et al. 2005). UPS is a highly complex and tightly regulated process that plays major roles in a variety of basic cellular processes. For its action proteins are targeted through the conjugation of multiple ubiquitin molecules with substrates to generate a polyubiquitin degradation signal. Secondly, the destruction of tagged proteins by 26S proteasome complex. The latter is composed by two sub-complexes: a 20S core particle (CP) that carries out the catalytic activity and a regulatory 19S particle (RP) (Ciechanover and Brundin 2003). The reaction of the 26S complex is catalyzed by deubiquitinating enzymes (DUBs) and the product is a free and reusable ubiquitin. Polyubiquitin

chains can be attached to misfolded proteins in the ER lumen but this predominantly occurs through the action of ubiquitin ligases and cofactors in the cytosol (Yerbury, Stewart et al. 2005). With few exceptions only polyubiquitined substrates are able to reach its proteolytic core. Ubiquitination is carried out by three enzymes: an E1 ubiquitin-activating enzyme, E2 ubiquitin-conjugating enzymes and E3 ubiquitin ligases. Off these three enzymes E3 seems to play a key role in the proteolytic cascade since it serve as the specific substrate recognition factors of the system. In some proteins an E4 enzyme is required. This enzyme cooperates with E3 ligases to extend the polyubiquitin chain (McClellan, Tam et al. 2005).

1.5.1.2. Protein quality control systems in the ER

Additionally to its function in the folding of proteins, ER plays vital quality control roles (Sitia and Braakman 2003). Rapid disposal of folding-incompetent polypeptides produced in the ER lumen is instrumental to maintain ER homeostasis (Hebert and Molinari 2007). Before the action of quality control mechanisms *per se* it is necessary to distinguish between native and non-native proteins. For such, folding or assembly intermediates expose hydrophobic surfaces which will interact with ER resident chaperones, as a consequence they are retained in the ER or retrieved from the Golgi complex (Sitia and Braakman 2003). To protect unstructured nascent chains synthesized in the ER lumen the multisubunit complex responsible for protein degradation, the 26S proteasome, has been positioned in the cytoplasm (Hebert and Molinari 2007).

In the ER, proteins are subjected to two quality control mechanisms that respond to the presence of misfolded proteins. The primary mechanism is an ER-dedicated stress response pathway termed the unfolded protein response (UPR) pathway, which acts to remodel the ER so as to increase its folding capacity (Bukau, Weissman et al. 2006). A secondary mechanism, termed ER-associated degradation (ERAD), relies on cell-specific factors and facilitates export of individual proteins or classes of proteins (Sitia and Braakman 2003). Specifically, it recognizes terminally misfolded proteins and retranslocates them across the ER membrane into the cytosol for degradation by the ubiquitin-proteasome degradation machinery. Recent studies argue that ERAD encompasses a number of different systems, each responsible for the degradation of a certain sub-type of proteins. In yeast, for example, there are at least two different surveillance mechanisms. The ERAD-L detects membrane and soluble proteins with luminal lesions and ERAD-C inspects membrane proteins with misfolded cytoplasmic domains (Vashist and Ng 2004). UPR and ERAD are intimately related: UPR induction increases ERAD capacity and loss of ERAD leads to

constitutive UPR induction (Bukau, Weissman et al. 2006). Even though some proteins can be rerouted to and degraded in the endolysosomal vesicle, the main ER function is to act as a test bench where molecules destined for the extracellular space are tested for their potential toxicity (Sitia and Braakman 2003). ER quality control seems to monitor primarily local structures within protein domains. Although with potential deleterious effects to the cells that ultimately can lead to proteins aggregation, a certain degree of freedom from quality control is essential for the evolution of proteins.

1.5.1.3. Protein quality control systems in the nucleus

Contrasting with ER, cytoplasm and mitochondria very little is known about PQC in the nucleus. Besides the identification of chaperones implicated in protein refolding and disaggregation in the nucleus, no clear PQC systems have been established (Gardner, Nelson et al. 2005). However, nuclear proteins can be damaged by the same stresses that occur in the other cell compartments so the cell must have some mechanism to deal with aberrant proteins that arise within the nucleus. In fact, aberrant protein accumulation in the nucleus likely underlies the pathology of Huntington´s disease (HD) and oculopharyngeal muscular dystrophy (OPMD) (Brais 2003; Jana and Nukina 2003). A recent study by Gardner *et al.* (2005) provides evidences for the existence of a PQC system that resides within the nucleus and degrades aberrant proteins. The defining member of this degradation system is Sanp1, an ubiquitin-protein ligase that mediates the ubiquitination of aberrant nuclear proteins. According to this model the action of San1p is helped by ubiquitin-conjugating enzymes (probably, Ubc1p and Cdc34p) which ultimately lead to the degradation of aberrant proteins by the proteasome (Gardner, Nelson et al. 2005).

1.6. Protein misfolding and disease

1.6.1. Diseases

Aberrant proteins and their persistence within the cell can often have deleterious effects like, loss of regulation, formation of inactive complexes that compete with functional ones, may introduce activities if mislocalized or may assemble into aggregates that eliminate protein function and/or cause toxicity (Gardner, Nelson et al. 2005). Such proteins can originate from a large number of processes like mutations, transcriptional or translational errors, incorrect folding,

imbalance subunit synthesis, improper trafficking or damaging caused by environmental conditions or metabolic byproducts (Sitia and Braakman 2003; Gardner, Nelson et al. 2005).

One type of inherited human diseases is caused by mutations in the sequence of a specific proteins leading to folding defects. Several of these mutations do not affect the activity of protein but significantly slows the polypeptide folding. As a consequence a loss of function conditions occurs, in which a membrane or secreted protein is retained and subsequently degraded. Examples of this type of diseases are cystic fibrosis, Fabry disease, and nephronegic diabetes insipidus (Hebert and Molinari 2007). Therapeutic treatment of these diseases is based on the use of chemical pharmacological chaperones that promote and/or accelerate productive folding and inhibit ER retention. Misfolding in the ER also causes a variety of congenital defects associated with ER storage. These mutations can be found in the secretory cargo leading to its degradation or creation of a toxic aberrant by-product which will interfere with the homeostasis of ER environment (Hebert and Molinari 2007).

Accumulation of intracellular aberrant proteins is associated with a number of neurodegenerative diseases such as Alzheimer, Huntington or Parkinson´s (Gardner, Nelson et al. 2005; Bukau, Weissman et al. 2006). This prevalence is explained because post-mitotic cells such neurons tend to accumulate toxic stress species and cannot dilute them during cell division (McClellan, Tam et al. 2005). Paget´s disease is also a condition caused by intracellular accumulation of aggregates. In this particular case aggregation is caused by missense mutations of the p97/Cdc48/VPC gene that affect the capacity of cells to degrade misfolded proteins (Hebert and Molinari 2007). Inclusion body myopathies are associated with severe weakness caused by muscle cell accumulating cytoplasmatic aggregates. The clearance of aggregates appears to occur through the action of ubiquitin modifications that recruit aggregates species via an independent mechanism called autophagy. Autophagy is a process characterized by recognition and engulfment of targeted proteins into autophagosome vesicles that will fuse with lysosomes, where either the vesicles or their content are broken down (Bukau, Weissman et al. 2006). An additional factor, that complicates the foundation of a link between these diseases caused by aggregates is the factor that they cannot be considered etiopathological entities, but rather syndromes with diverse etiologies (Ciechanover and Brundin 2003).

1.7. *S. cerevisiae* as a biological model

1.7.1. *S. cerevisiae* as a biological model – General features

Saccharomyces cerevisiae is a yeast species with large industrial applications, like baking or brewing, which was first isolated from the skin of grapes (Pretorius 2000). Morphologically, *S. cerevisiae* cells are round, with 5-10 micrometers in diameter, and her reproduce by a process known as budding.

With 6,275 genes organized in 16 chromosomes, *S. cerevisiae* was the first eukaryotic organism with the genome completely sequenced (sequence was released in 1996). It is also one of the most intensively studied eukaryotic model organisms in molecular and cell biology (Karathia, Vilaprinyo et al. 2011). Like mammalian cells, yeast cells are distinguished from bacteria and archaea by the presence of membrane bound organelles, including a nucleus. Because of these morphological similarities yeast cells recapitulate fundamental aspects of eukaryotic biology, including "a distinctive process of cell division and genetic transmission, transcriptional regulation, biogenesis and function of cellular organelles, protein targeting and secretion, cytoskeletal dynamics and regulation, and cellular metabolism"(Khurana and Lindquist 2010).

The conservation of homologous genes in yeast fulfilling the same function is a key factor that contributes by its use as a model in several human studies like, aging, regulation of gene expression, apoptosis or neurodegenerative disorders (Karathia, Vilaprinyo et al. 2011). Because of all these studies, among all the eukaryotes, the interaction network of *S. cerevisiae* is the best understood and the most complete (Skrzypek, Myers-Morales et al. 2003). Another fact which is of great importance is that, up to 30% of genes implicated in human diseases may have orthologs in the yeast proteome (Karathia, Vilaprinyo et al. 2011). Some of these proteins are already categorically associated with a vast group of disorders such as cancer, metabolic disorders or neurological and malformation syndromes (Skrzypek, Myers-Morales et al. 2003). A characteristic study with *S. cerevisiae* is the expression of human proteins in yeast. An important example, in the context of this thesis, is the expression of human α-synuclein protein (implicated in neurodegenerative disorders) in *S. cerevisiae*. As a result of this study, molecular insights into the pathways underlying normal biology of this protein, and the pathogenic consequences of its misfolding, were revealed (Zabrocki, Pellens et al. 2005).

Besides being biochemically and genetically well understood, the relevance of *S. cerevisiae* to human disease is well established by its conserved genome and cellular biology. *S. cerevisiae* is easily manipulated, grows quickly and is one of the few organisms for which many of the –omics techniques have been develop and applied on a global scale (Skrzypek, Myers-Morales et al. 2003; Khurana and Lindquist 2010). Because of this, *S. cerevisiae* is a natural choice for genetic studies, like this, associated with mistranslation and production of protein aggregates.

1.7.2. *S. cerevisiae* as a neurodegenerative model

Neurodegenerative diseases are a serious public health concern due to ageing populations in developed nations. During most of the last century the study of the neurodegenerative diseases was restricted to relating the clinical phenotype of the diseases to their postmortem neurophatology. Although these observations leads to the identification of phatological proteinacious aggregates and patterns of different neuronal vulnerability this methodology presented many limitations that only more complex studies recurring to the use of eukaryotic models can overcome. These new studies permitted, over the past 15 years, the identification of disease causing mutations and misfolded proteins that enabled the creation of cellular and animal models of neurodegenerative diseases. Among the cellular models yeasts captured key aspects of cellular pathology and provided novel gene-environment connections and therapeutic targets in an unparalleled scale (Khurana and Lindquist 2010).

As already mentioned neurodegenerative diseases, namely, Alzheimer (AD) and Parkinson´s diseases (PD) are associated with intracellular porteinacious aggregates. A wide range of evidences intimately associated protein misfolding, oligomerization and aggregation with neurodegeneration. The reason by which theses processes can be studied in yeast is because of the high conservation of the cellular PQC (Scheper and Hoozemans 2009). Another important dysfunction related with neurodegenerative diseases is the mitochondrial dysfunction and oxidative stress (Knott, Perkins et al. 2008). As in mammalian cells, the yeast mitochondrion is the organelle responsible for the production of reactive oxygen species (ROS). The capacity of yeasts to grow, or not, in fermentative states allows for the analysis of mitochondrial defects that can have deleterious impact in mammalian cells. Besides this, yeast also presents conserved mechanisms of cell death and survival that are related with neuronal loss.

However, as a unicellular organism with cell wall, yeast cells present some limitations as a model and, specifically, as a model system for neurodegenerative diseases. The principal one is

related with the analyses of aspects based on multicellularity and cell-cell interactions. As expected the basic elements of the UPR to ER stress are conversed, however, the response is far more complex in mammalian cells. Another example of the limitations is the neuronal specializations, like axonal transport or neurotransmitter release. These functions cannot be recapitulated in yeasts however the fundamental aspects of these proprieties may be conserved (Khurana and Lindquist 2010).

The objectives of this Master Thesis are:

1) Engineering of eleven mutagenic tRNAs which generated proteome errors and unfolded protein stress responses in *S. cerevisiae* cells.

2) Characterization of the transformants *S. cerevisiae* cells, evaluating the impact that mutagenic tRNA have in terms of growth rate in standard conditions and under conditions of stress, use of phenotypic screens to evaluate the impact that these mutations on respiratory capacity of the cells and function of mitochondria.

3) Study the impact of proteome mutagenesis on the viability of cells and evaluate whether mistranslation can lead to accumulation of protein aggregates (using GFP-HSP104).

Chapter 2

2. Material and Methods

2.1. Strains and growth conditions

2.1.1. Strains and genotypes

Escherichia coli

JM109, genotype: *recA1 SupE44 endA1 hsdR17* (rk–, mk+) *gyrA96 relA1 thi Δ(Lac-proAB) [F', traD36, proAB, lacI*q*lacZ ΔM15*]

DH5-α, genotype: *fhuA2 Δ(argF-lacZ)U169 phoA glnV44 Φ80 Δ(lacZ)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17*

Saccharomycies cerevisiae

YLL026W, genotype: *MATα, his3Δ, leu2Δ0; lys2Δ0, ura3Δ0; YLL026w::kanMX4.*

BY4734, genotype: *MAT*a/α, *his3Δ1/his3Δ1, leu2Δ0/leu2Δ0, met15Δ0/MET15, LYS2/lys2Δ0, ura3Δ0/ura3Δ0.*

2.1.2. Growth and maintenance of *E. coli* and *S. cerevisiae*

E. coli strains were grown at 37°C on nutritionally rich medium liquid broth (LB) [1% (w/v) peptone from casein, 0.5% (w/v) yeast extract, and 1% (w/v) sodium chloride (Merck)]. After transformation *E. coli* strains were grown in the LB-Ampicilin medium [LB and 100 µg/ml ampicillin sodium (Duchefa, Haarlem)]. Strains were stored at -80°C in 1.5 ml LB-Amp 20% (v/v) glycerol.

S. cerevisiae strains were grown at 30°C in either: rich medium in the case of BY4743 wild type strain (YPD – 2% glucose; 1% yeast extract and 1% bacto-peptone) or minimal medium in the case of YLL026W strain and transformed BY4743 (MM – 0.67% yeast nitrogen base without amino acids, 2% glucose, 0.2% drop-out mix with all the essential amino acids (annex A)). Variations in

drop-out mixes for the selection of specific were used. BY4743 transformed was grown in MM-LEU. Solid media were always performed by addition of 2% agar. Transformed strains were stored at -80°C in 1.5 ml of the corresponding minimal medium with 40% (v/v) glycerol. All media were sterilized by heat in an autoclave before use.

Variations in the MM-LEU medium and slight changes in the growth conditions were also utilized under different physiological conditions when the phenotypic screening with the BY4743 strain was carried out (Table 2.1).

Table 2.1: Description of the stresses that was tested in the phenotypic screening assay as well as the conditions for growth and changes to the medium associated. The standard growth conditions were temperature 30°C and MM-LEU.

Stress	Conditions for Growth and Changes in Medium
Heat	Incubation at 16°C, 30°C and 42°C
pH	MM-LEU with pH=6 ($NaHPO_4$ 0.2 M and Na_2HPO_4 0.8 M)
Carbon source alteration	MM-LEU without glucose Substitution of glucose (2%) by ethanol (2% and 5%) Substitution of glucose (2%) by galactose (2%) Substitution of glucose (2%) by glycerol (2%) Substitution of glucose (2%) by sorbitol (1.5 M) Substitution of glucose (2%) by acetate (2%) MM-LEU with pH 5.5 adjusted with lactose
ROS	Addition of ascorbic acid (80 mM) Addition of glutathione (40 mM)
Toxic compounds	Addition of cadmium chloride (50 µM and 100 µM) Addition of calcium chloride (0.5 M and 0.75 M) Addition of copper sulfate (2.5 mM) Addition of lithium chloride (0.15 M and 0.3 M) Addition of sodium chloride (0.5 M) Addition of caffeine (2 mM and 5 mM) Addition of EDTA (0.25 mM and 0.5 mM) Addition of geneticin (70 mg/l and 200 mg/l) Addition of menadione (90 µM and 150 µM) Addition of paromomycin (0.5 mM and 1 mM) Addition of SDS (0.015% and 0.01%)

2.2.1. Oligonucleotides

Oligonucleotides (Table 2.2) were purchased from IDT-Integrated DNA Technologies (Belgium) and were resuspended in ultra miliQ (mQ) water to a final concentration of 100 µM.

Table 2.2: The list of oligonucleotides bellow was used for site-directed mutagenesis, sequencing of transformants plasmids, PCR screening and Northern blot.

Oligo	Sequence (5´ → 3´´)	Tm (°C)
Site Directed Mutagenesis of $tRNA_{UGA}^{Ser}$		
UGA-CCU	GGTTAAGGAGAAAGACTCCTAATCTTTTGGGCTTTGC	62.6
UGA-AUG	GGTTAAGGAGAAAGACTATGAATCTTTTGGGCTTTGC	61.6
UGA-CUC	GGTTAAGGAGAAAGACTCTCAATCTTTTGGGCTTTGC	62.8
UGA-GCA	GGTTAAGGAGAAAGACTGCAAATCTTTTGGGCTTTGC	63.5
UGA-AAA	GGTTAAGGAGAAAGACTAAAAATCTTTTGGGCTTTGC	60.9
UGA-CCA	GGTTAAGGAGAAAGACTCCAAATCTTTTGGGCTTTGC	63.1
UGA-AUU	GGTTAAGGAGAAAGACTATTAATCTTTTGGGCTTTGC	60.4
UGA-CUG	GGTTAAGGAGAAAGACTCTGAATCTTTTGGGCTTTGC	62.8
UGA-CAU	GGTTAAGGAGAAAGACTCATAATCTTTTGGGCTTTGC	61.6
UGA-AGG	GGTTAAGGAGAAAGACTAGGAATCTTTTGGGCTTTGC	62.6
UGA-AUC	GGTTAAGGAGAAAGACTATCAATCTTTTGGGCTTTGC	61.6
Sequencing of transformed plasmids and PCR screening		
UGA F	CGCGTCGACGTCCAGGACTGATTTATGTGCATC	67
UGA R	CGCGGATCCCAGTATGGATTGCTAGTCCTAGAG	66
Northern blot with transformed plasmids		
tRNAUGASer	TTAACCGCTCGGACAAGTT	54.2
tRNACCCGly	GCGGAAGCCGGGAATCGAAC	61.4

2.2.2. Plasmids

2.2.2.1. Original Plasmids

In this work we used two plasmids to obtain the desired constructions: pRS315 and pUA261. Following are their description (plasmids maps and sequence of $tRNA^{Ser}$ are in annex B).

pRS315 Plasmid of 6018 bp containing the Amp^R and LEU2 gene, allowing for selection of transformants in LB media with ampicilin or in yeast MM-LEU media, respectively. Desired DNA fragments were inserted at unique restriction sites, namely *SalI* (3205) and *BamHI* (3160).

pUA261 Plasmid containing a copy of the *C. albicans* $tRNA_{UGA}^{Ser}$ inserted in plasmid pRS315. The tRNA gene fragment was inserted using the restriction sites of the enzymes SalI (3205) and BamHI (3160). *C. albicans* $tRNA_{UGA}^{Ser}$ (Assembly 21, Ca21chr1 335138-337219C (candidagenoma.org)) was amplified using the primers UGAF and UGAR.

2.2.2.2. Constructed plasmids

Using pUA261 as a template, we have created eleven new plasmids carrying mutant misreading tRNA genes (Table 2.3). Plasmids maps and sequence are in annex B.

Table 2.3: List of the eleven engineering plasmids constructed based on plasmid pUA261. Plasmid pUA261 contains a single copy of the *C. albicans* $tRNA_{UGA}^{Ser}$ gene.

Plasmids	Description
pUA801	Plasmid constructed by site directed mutagenesis of the anticodon of $tRNA_{UGA}^{Ser}$ for CCU. Primers UGA-CCU forward and reverse were used.
pUA802	Plasmid constructed by site directed mutagenesis of the anticodon of $tRNA_{UGA}^{Ser}$ for AUG. Primers UGA-AUG forward and reverse were used.
pUA803	Plasmid constructed by site directed mutagenesis of the anticodon of $tRNA_{UGA}^{Ser}$ for CUC. Primers UGA-CUC forward and reverse were used.
pUA804	Plasmid constructed by site directed mutagenesis of the anticodon of $tRNA_{UGA}^{Ser}$ for GCA. Primers UGA- GCA forward and reverse were used.
pUA805	Plasmid constructed by site directed mutagenesis of the anticodon of $tRNA_{UGA}^{Ser}$ for AAA. Primers UGA-AAA forward and reverse were used.
pUA806	Plasmid constructed by site directed mutagenesis of the anticodon of $tRNA_{UGA}^{Ser}$ for CCA. Primers UGA-CCA forward and reverse were used.
pUA807	Plasmid constructed by site directed mutagenesis of the anticodon of $tRNA_{UGA}^{Ser}$ for AUU. Primers UGA-AUU forward and reverse were used.
pUA808	Plasmid constructed by site directed mutagenesis of the anticodon of $tRNA_{UGA}^{Ser}$ for CUG. Primers UGA-CUG forward and reverse were used.
pUA809	Plasmid constructed by site directed mutagenesis of the anticodon of $tRNA_{UGA}^{Ser}$ for CAU. Primers UGA-CAU forward and reverse were used.
pUA810	Plasmid constructed by site directed mutagenesis of the anticodon of $tRNA_{UGA}^{Ser}$ for AGG. Primers UGA-AGG forward and reverse were used.
pUA811	Plasmid constructed by site directed mutagenesis of the anticodon of $tRNA_{UGA}^{Ser}$ for AUC. Primers UGA-AUC forward and reverse were used.

2.2.3. Site Directed Mutagenesis

With the goal of mutating the anticodon of the $tRNA_{UGA}^{Ser}$ gene inserted in plasmid pUA261, polymerase chain reactions (PCR) followed by incubation with a required restriction enzyme were prepared. The PCR reaction contained 10x Pfu buffer with $MgSO_4$, 5 µl, 1 µl dNTPs, 2.5 µl of each primer (diluted 1/10), 1 µl of *Pfu* and miliQ water to a volume of 50 µl. The thermocycler programme routinely used consisted of a starting incubation temperature of 95°C for 2:30 minutes followed by 18 cycles at 95°C during 30 seconds, 55°C during 1 minute and 68°C during 7 minutes. After this the PCR reactions were terminated at 37°C. Thermal cycling denatured the plasmid and annealed the mutagenic primers containing the desired mutation. Because the only plasmids of interest were those carrying mutations, the last step of site directed mutagenesis was a digestion of parental methylated and hemimethylated DNA with restriction enzyme *DpnI*, 2 µl.

After the digestion plasmids were quantified into a Nanodrop device, stored at -30°C and subsequently sent to sequencing (StabVida) with the respective primers containing the desired mutation.

2.2.4. Preparation of *E. coli* competent cells

For the preparation of *E. coli* competent cells, the TFB method was used. Buffers TFB I and TFB II were made prepared (annex C) and was used. *E. coli* JM109 and DH5-α cells were used. The protocol began by inoculating 200 µl of cells, from a 5 ml overnight culture, in 5 ml of LB media. This culture was incubated at 37°C with 180 rpm until an OD_{550} = 0.3 (approximately 2 h). When OD_{550} = 0.3 was reached, 4 ml of the anterior culture was inoculated in 100 ml LB and left to grow, again, at 37°C with 180 rpm until OD_{550} = 0.3 (almost 3h). After this, the cells were collected in two 50 ml falcons, placed in ice for 5 minutes and, centrifuged at 2500 rpm for 5 minutes at 4°C. Later, the supernatant was decanted and the two pellets were ressuspended, in 20 ml TFB I (cold) each. Under the same conditions, cells were centrifuged again, the supernatant was decanted and the pellets ressuspended on 5 ml TFB II (cold). Finally, the cells were incubated in ice for 5 minutes, distributed 200 µl aliquots of cells per cold Eppendorf tubes, putted the aliquots in liquid nitrogen and frozen at -80°C.

2.2.5. DNA Minipreps

Single colonies from a freshly streaked plate were picked and inoculated in 5 ml of LB medium with ampicilin. This culture was grown overnight at 37°C with agitation at 200 rpm. Bacterial cultures were harvested by centrifugation at 8000 rpm in a microcentrifuge for 2 minutes at room temperature. Supernatants were decanted and the entire remaining medium removed.

For the purification of plasmids, a kit (Fermentas – GeneJET^{TM} Plasmid Miniprep Kit) was utilized and the manufacture instructions were followed. After purification the plasmids were quantified using a NanoDrop.

2.2.6. Transformation of *E. coli*

Transformation of *E. coli* was carried by the chemical SOC method (SOC solution description in annex C). First aliquots of competent cells were withdrawn from the -80°C freezer (200 µl competent cells/transformation reaction). 10 ng of plasmidic DNA were added to competent cells (DH5-α). The reagents were mixed and then incubated on ice during 30 minutes, followed by 90 seconds at 42°C and 2 minutes on ice. 800 µl of SOC were added to the reaction mix, incubated during 1 h at 37°C and centrifuged for 1 minute at 2500 rpm. Supernatants were removed (leaving approximately 50 µl) and the pellet was homogenized with a pipette. Finally, 50 µl of transformation reaction was spread on LB-Ampicilin plates. Plates were left on the bench for absorption of liquid excess and then were placed in an incubator overnight at 37°C (16-18 h).

2.2.7. Yeast-specific techniques

2.2.7.1. Transformation of yeast cells

Transformation of *S. cerevisiae* was carried out using the lithium acetate (LiAc) method (Gietz and Woods, 2002). For this, overnight cultures were diluted in 10 ml of medium to an OD_{600} of 0.05. The cultures were grown at 30°C, 180 rpm shaking, until an OD_{600} of 0.4 – 0.5. Cells were then harvested by centrifugation in 1.5 ml tubes for 1 minute at 4000 rpm. The supernatant of each tube was discarded and the transformation reagents were added to the pellet in the following order: 240 µl 50% (w/w) PEG 3500, 36 µl 1.0 M LiAc, 50 µl boiled single-stranded carrier

DNA (2 mg/ml) previously denatured and 50 µl of an aqueous solution of the plasmid of interest (containing 0.5 – 4 µg of plasmid). Tubes were vortexed until a homogeneous suspension was obtained and then incubated at 42°C, in a water-bath, during 40 minutes. The cells were then harvested by centrifugation at maximum speed for 1 minute, the transformation mixture was discarded and the pellet was carefully resuspended in 200 µl of sterile mQ water. Each suspension (10-100 µl) was plated in selective media plates and incubated at 30°C, until isolated transformants colonies were visible (3-4 days).

2.2.7.2. Colony PCR

To check for the correct transformation of yeast cells, a colony PCR was performed using intact cells from a colony as template. For this, individual colonies of transformed cells were picked from a selective plate, using sterile P10 micropipette tips, transferred into 0.2 ml PCR reaction tubes and heat-shocked at 95°C during 10 minutes with 5 µl of mQ sterile water. After that, the PCR reagents were added: per tube, 1x Taq buffer (0.2 M), dNTPs (0.04 M), R and F primers (0.04 M) and *Taq* polymerase (1 U/µl – 0.01 M) (Fermentas or Bioron) were added. The PCR program consisted of 30 cycles, of denaturing at 95°C during 30 seconds, annealing at 60°C for 30 seconds and extension at 72°C during 1:30 minutes. Reaction were performed in a MyCycler^{TM} thermal cycler (BIORAD) and ended with a single incubation at 72°C during 5 minutes.

2.2.7.3. Agarose gel electrophoresis

DNA molecules were fractionated on agarose gels. Multi-Purpose agarose (Boehringer Mannheim) was melted (4 minutes at 500 W) using a microwave oven in TAE 1x (annex C) at concentrations of 1% (w/v). When the agarose melted,ethidium bromide (EtBr) (Invitrogen) was added to a final concentration of 0.002 % (v/v). Gels were casted on BioRad casting systems. DNA samples were then mixed with 6x loading buffer (annex C), by adding 1 µl of buffer for 5 µl of PCR sample, loaded into the wells (samples and *ladder*) and eletrophoresed at 80 V (Power Pac 3000, Bio-Rad) for 10 minutes followed by 50 minutes at 100-120 V in submerged horizontal electrophoresis systems (Mini-Sub Cell GT, Bio-Rad). The eletrophoresed sample gels were exposed to ultraviolet (UV) light using a Gel Doc 2000 Gel Documentation System (BioRad) coupled to a PC. The images were acquired and analyzed with the Quantity One software (Bio-Rad).

2.3. Study of the impact of mutation

2.3.1. Determination of transformation efficiency

For the calculation of transformation efficiency in yeast, the number of cells in each suspension of the transformation procedure was quantified. For that, the cell culture that was grown until an OD_{600} of 0.4 – 0.5 was quantified using for a Neubaeur chamber/hemocytometer and a microscope. Identical number of cells were plated and the number of colonies per µg of DNA used per transformation (0.5 – 4 µg of plasmid) was determined after 3 or 4 days of incubation. Results were expressed in number of transformants/µg of DNA.

2.3.2. Growth curve

Cultures of transformed yeast cells were grown in an incubator at 30°C and 180 rpm until stationary phase. In Erlenmeyer´s of 100 ml, 20 ml of the respective media were added followed by the necessary volume of cells to achieve OD_{600} of, approximately, 0.02. With a microplate reader, OD_{600} of all the samples was measured (time 0).Cultures were grown at 30°C and 180 rpm for 6 or 7 hours and OD_{600} was determined at intervals of 1 hour until cultures reached stationary phase. This procedure was carried out with three different clones of each strain and performed in triplicate.

2.3.3. Light microscopy of yeast cells

Since the *S. cerevisiae* strain YLL026W is tagged at the carboxy-terminal end of the HSP104 gene with green fluorescent protein (GFP) it is possible to observe the presence of protein aggregates inducing by misreading tRNAs. Transformants were grown overnight at 30°C and 180 rpm. When they reached an OD_{600} of 0.5, preparations for microscopy observation were prepared using a layer of agarose. Using an epifluorescence up-right microscope Imager.Z1 (Zeiss), and 38 HE GFP filter, approximately 400 cells were observed for counting the presence of protein aggregates. This procedure was repeated three times and in each treatment each misreading tRNAs was monitored in three independent transformant replicates. Results were expressed in number of cells with aggregates/total number of cells.

2.3.4. Yeast cell survival

The plating efficiency of yeast cells allows for determination of survival rates since colonies arise from single cells (CFU – colony forming units). To determine survival rates cell density in liquid cultures in stationary phase was determined by counting the number of cells using a Neubauer chamber/hemocytometer. Cultures were allowed to grow at 30°C and 180 rpm in an incubator and samples with 100 and 50 cells were taken every 4 days. Plates with the appropriated media were also incubated at 30°C until visible colonies (3 or 4 days) appeared. After this time CFUs were determined and compared with the number of cells plated (results was expressed in percentage).

2.3.5. Phenotypic screening

Phenotypic screening was performed with all the eleven BY4743 *S. cerevisiae* transformed strains and the control strain which was transformed with pUA261 plasmid. Screening plates (50 ml of medium) containing the compounds that are described in the section 2.1.2. were prepared. For each condition tested prepared 9 plates were prepared.

Transformants were grown overnight and when the exponential phase was reached (OD_{600} between 0.5 and 0.8) cell density was determined by counting the number of cells using a Neubauer chamber/hemocytometer. In a 96 wells microtiter plate (called “mother plate”) 200 µl of each sample with $1x10^7$ cells and three subsequent dilutions of 1/10 were dispensed from the first wells row to the wells right bellow of the first row. Three “mother plates” each with two clones of the same transformation and four dilutions (each clone was tested twice) were prepared. A robot used those “mother plate” to print cells in three screening plates previously prepared. Screening plates were then incubated in different temperature conditions (16°C, 30°C, 37°C, 42°C) according to the chosen stress during 5 days. Finally, photos of all plates were taken and the growth and pattern of colonies was analyzed with the software package ImageJ and compared with the respective control (results were expressed in percentage of growth relative to the control).

2.3.6. Total RNA isolation from *S. cerevisiae*

Total RNA was extracted using a acidic hot-phenol based protocol. Initially, cells were grown overnight in controlled conditions until exponentially phase. 25 OD units (volume of the

culture x OD_{600}) were collected into 50 ml tubes and harvested briefly (4 minutes, 4000 rpm) in a single step centrifugation. Remaining supernatants were poured off and the tubes were immediately immersed in liquid Nitrogen (1 minute) and stored at -80°C.

Frozen cells were taken from the -80°C freezer and resuspended in 500 µl of Acidic Phenol Cloroform 5:1, pH 4.7 (Sigma), warmed up at 65°C before use. The same volume of TES-buffer (10 mM Tris pH 7.5, 10 mM EDTA, 0.5% SDS) was added and the tubes were vortexed hard for 20 seconds with the intent of resuspending the cell pellets. The tubes were incubated during 1 hour in a water bath at 65°C, with 20 seconds of vigorously vortexing every 10 minutes. Tubes content were transferred to 1.5 ml Eppendorf tube and centrifuged for 5 minutes at 14000 rpm at 4° C. 600 µl of the water-phase was transferred to a new Eppendorf tube, and 600 µl of Acidic Phenol Cloroform 5:1, pH 4.7 was added. Tubes were vortexed for 20 seconds and centrifuged for 10 minutes at 14000 rpm at 4°C. This last step was repeated with a single alteration – adding 500 µl of Acidic Phenol Cloroform 5:1, pH 4.7 instead of 600 µl. Another new Eppendorf tube was taken and filled with 400 µl of water-phase from the last step and 400 µl of Chloroform:Isoamyl-alcohol 25:1 (Sigma), vortexed for 20 seconds and centrifuged for 10 minutes at 14000 rpm at 4°C. Once more, the water-phase (350 µl) was transferred to a new Eppendorf tube filled with 35 µl of sodium acetate (3 M, pH 5.2) and 800 µl of Ethanol 100% (-20° C). The mix was incubated at -20° C for 1 hour. After RNA precipitation, tubes were centrifuged for 5 minutes at room temperature at 14000 rpm. Carefully, all the liquid was removed without touching the RNA-pellets. The pellet were washed with 500 µl of ethanol (80%, -20°C) and centrifuged shortly for 1 minute at room temperature, 14000 rpm. All traces of ethanol were removed by air drying of RNA pellet. In the last step, RNA-pellet was dissolved in sterile mQ water to a concentration of approximately 10 µg/µl. Samples were frozen and kept at -80°C freezer.

2.3.7. Polyacrylamide gel electrophoresis

RNA samples were quantified prior to polyacrylamide gel electrophoresis, using a Nanodrop. Polyacrylamide gels were prepared (gel composition in annex C), the electrophoresis device was assembled according to the manufactures instructions and approximately 250 µl of TEMED (tetramethylethylenediamine) was used to solidify the gel. Electrophoresis was carried out for 1 hour at 300 V and the samples were loaded in the wells (25 µg of RNA plus the same volume of urea loading buffer). Electrophoresis was carried out at 500 V for more 16 to 20 hours.

Gel exposure and RNA transfer for a nitrocellulose membrane was the next step. With this purpose the gel was taken from the electrophoresis tank and placed in a plastic sheet during 5 minutes with 50 ml of TBE1x and 10 µl of ethidium bromide. Following, the gel was exposed under UV light and cut leaving only the area containing RNA bands. Ten pieces of whatman paper with the same dimension was also cut and, five of them were embedded in TBE 0.25 M and placed in the transfer area of an electroblotter, the gel was placed on top of the paper and was covered with five other pieces of paper. The transfer was performed for 37 minutes with amperage equal to 0.8 times the area of the membrane (in mili ampers).

2.3.8. Northern blot analysis

Nitrocellulose membranes obtained after the polyacrylamide gel electrophoresis were subjected to a Northern blot. Firstly, probes labeling mix (annex C) were prepared and them the membranes were placed in a SCHOTT flask, 10 ml of hybridization buffer (annex C) was added, and incubated in an oven with agitation, at a temperature 5°C lower than probe melting temperature (Tm) for at least 30 minutes. Hybridization buffer was decanted; labelled probe prepared previously were added and incubated in an oven with agitation, at Tm -5°C for 12-20 h.

After hybridization membranes were washed. For this, labelled probes were decanted in 15 ml tubes, stored at 4°C and, 25 ml of pre-warmed washing buffer (annex C) at Tm -5°C was added and incubated in the oven at Tm -5°C with agitation for 3 minutes. The washing buffer was poured off and last step was repeated. Next, 25 ml of washing buffer at room temperature was added and incubated in the oven at Tm -5°C with agitation for 3 minutes. Once more, the washing buffer was wasted, the last step was repeated and finally the membranes were closed in a plastic bag.

Membranes were exposed to K-screens cleaned by exposing 15 minutes to Screen eraser-K (Biorad), at room temperature for 1 to 3 days and were scanned using a Biorad Scanner with the appropriate settings.

Chapter 3

3. Effects of mistranslation on yeast growth rate and morphology

3.1. Overview

The genetic code was initially postulated by Crick (Crick, 1968) as a “frozen accident” (Crick 1968), because all existing organisms use the same code that as originated from a single, closely interbreeding population. The universality of the genetic code is one of the essential characteristics of life (Suzuki, Ueda et al. 1997). Crick followed the assumptions of Hinegardner and Engelberg (Hinegardner and Engelberg 1963), and postulated that after the primordial genetic code expanded to incorporate all twenty amino acids, any alteration in the code would result in changes in protein sequences that would be lethal. To explain the origin and evolution of the code three main theories were proposed (Koonin and Novozhilov 2009). These three most important explanations are the stereochemical theory which postulates that codon assignments are dictated by physico-chemical affinity between amino acids and the cognate codons, the co-evolution theory postulates that the code structure co-evolved with amino acids biosynthesis pathway and, the error minimization theory postulates that the principal factor in the code evolution was selection to minimize the adverse effects of point mutations and translation errors. In a more comprehensive view, the evolution that leads to the standard code can be seen as a combination of a frozen accident with selection for error minimization. However, contributions from co-evolution (with metabolic pathways) and affinities between amino acids and nucleotides triplets cannot be neglected.

It is now known that the standard code is not literally universal as a large number of alterations modifications have been discovered. Although any change in the code alters the meaning of the code and would introduce translational errors, the idiosyncratic arrangement of codon assignments minimizes the impact of errors (Knight, Freeland et al. 2001). Nevertheless, the misfolding of proteins always imposes a fitness cost to the cell that may (or not) be related to protein function. Protein misfolding compel such costs as a direct toxicity, characterized by disruption of membrane integrity or inappropriate interactions, indirect toxicity, increased sensitivity to environmental stresses; or, indirect toxicity burdens, due to increased requirement of energy to synthesize and detect misfolded proteins (Drummond and Wilke 2008).

Any change in the genetic code needs to be explained in light of three assumptions: what are the sources of the variability, what limits the alterations (if any) and, what causes a variant code to become fixed in a lineage (Knight, Freeland et al. 2001). Genetic code modifications are traditionally divided in a number of non-universal codes that have been reported in various non-plant mitochondrial systems (more frequent), as well as in several nuclear systems (Suzuki, Ueda et al. 1997). Sometimes the same alteration occurs in different lineages as illustrated by the reassigned of UAA and UAG codons from Stop to Gln in some diplomonads, in several ciliates and in the green alga *Acetobularia acetabulum.* Termination codons are, in fact, particularly unstable (Knight, Freeland et al. 2001). For preserving the high degree of fidelity in protein synthesis the accuracy in tRNA aminoacylation is crucial (Ruan, Palioura et al. 2008). Therefore, the propriety of aaRS to discriminate precisely its cognate amino acid from other structurally related at the adenylation reaction step, and its cognate tRNAs from non-cognate ones is critical (Suzuki, Ueda et al. 1997). Another important feature is the base-pairs of the charged tRNA with the correct codon on the mRNA, at the ribosome. The original codon assignments in an RNA world may require direct stereochemical association between the tri-nucleotides and amino acids (Knight, Freeland et al. 2001).

The components of the translation apparatus have different capacities for adaptive changes. The majority of the non-standard codon engineering assignments have been traced to changes in tRNAs. The explanation is that, unlike other components, mutant tRNAs are easy to characterize because of their relatively stability and small size. Another important characteristic is that single nucleotide substitutions have direct and specific effects on decoding (Giege, Sissler et al. 1998). This precise assumption is on the base of this study as we will see in the following section. Other components of the translation machinery that create more problems are both tRNA-binding and amino-acid binding domains of the aaRS (mutations would probably alter the translations of multiple codons) or ribosome (mutations will likely have effect on all codon-anticodon interactions) (Arnez and Moras 1997). A well studied example of alteration to the genetic code is the ambiguous translation of the universal CUG leucine codon as serine in some *Candida* species (Ohama, Suzuki et al. 1993). The most remarkable feature observed in these $tRNA^{Ser}$ CAG is the alteration of the 5´ adjacent nucleotide to the anticodon (position 33) that is occupied by G residue (G33) instead of the conserved U residue (U33). This fact is more important if we take in consideration that, in all tRNAs reported so far, U33 is necessary for forming the U-turn structure of the anticodon loop (Suzuki, Ueda et al. 1997). Interestingly, when the *Candida*

tRNA is expressed in *S. cerevisiae* the result are the production of misfolded peptides that induce heat-shock proteins. These proteins allow the transformants to survive under various environmental stresses like heat, oxidation, cycloheximide and 1.5 M sodium chloride (Santos, Cheesman et al. 1999). This fact shows that, under certain circumstances, ambiguous translation is advantageous rather than deleterious. After all, some of the modifications reported so far change the basic notion of the genetic code postulated by Crick that the standard was fixed simply because all extant life forms share a common ancestor and, remained mainly untouched, because of the deleterious effect of codon reassignment (Koonin and Novozhilov 2009).

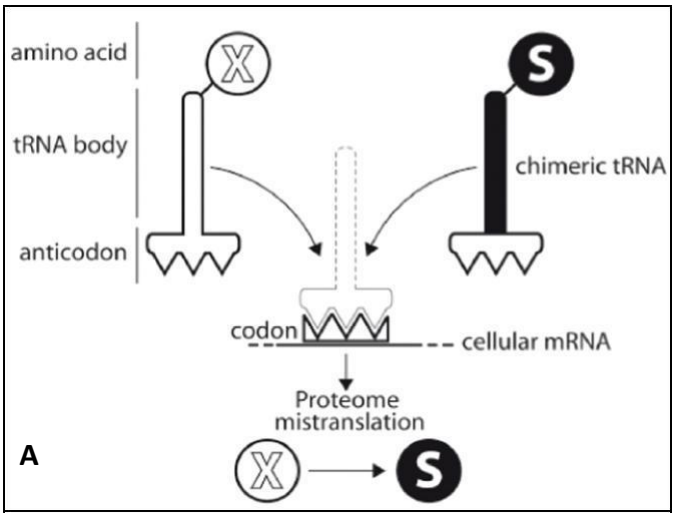
The experimental strategies chosen to introduce mistranslation and produce protein aggregates are based on the utilization of single mutant proteins known to aggregate, or the incubation of cells with analogous of proteinogenic amino acids (Suzuki, Ueda et al. 1997). In the present thesis, the strategy utilized to induce alterations and mistranslations on yeast cells is based on engineered tRNAs. When they actively integrate it in the translation machinery, general proteome substitutions occur. With this method, mutations in the proteome were introduced without exposing the cells to unnatural compounds that can trigger a number of collateral effects.

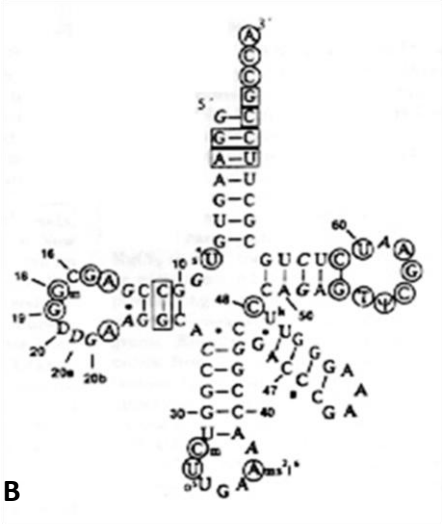
3.2. Results

3.2.1. Genetic system for the expression of $tRNA^{Ser}$ of *C. albicans* in *S. cerevisiae*

In this thesis mistranslation in *S. cerevisiae* yeast cells was induced by mutating the $tRNA_{UGA}^{Ser}$ from *C. albicans* gene, which was previously cloned in pRS315 vector (Mateus 2011), and was transformed into the yeast BY4743 and YLL026W strains. Eleven misreading tRNAs that introduce different mutations in the yeast proteome were engineered.

The seryl-tRNA synthetase (SerRS) does not interact with the anticodon of serine tRNAs and mutations in their anticodon create tRNAs that misincorporate serine at any chosen codons (Geslain, Cubells et al. 2010) (Figure 3.1).





Candida albicans tRNA ^{Ser}

Figure 3.1: Representation of the concept used to create quimeric tRNA^{Ser} (A) and figure of the *C. albicans* serine tRNA (B) (Geslain, Cubells et al. 2010). Chimeric tRNAs are engineered by mutation of the wild-type serine anticodon (UGA) in tRNA to specific triplets coding for other amino acids. Serylation occurs no matter what the anticodon sequence is. In consequence, expression of chimeric tRNA^{Ser} will produce mistranslated proteins.

The effectiveness of the site direct mutagenesis was validated by direct Sanger sequencing of the eleven engineered plasmids. Plasmids were them transformed into the *S. cerevisiae* strains YLL026W (haploid) and BY4743 (diploid). Colony PCR was carried out to guarantee that the plasmids contained the correct tRNA gene (annex D), and transformation efficiencies were determined (number of transformants per µg of DNA) (Figure 3.2).

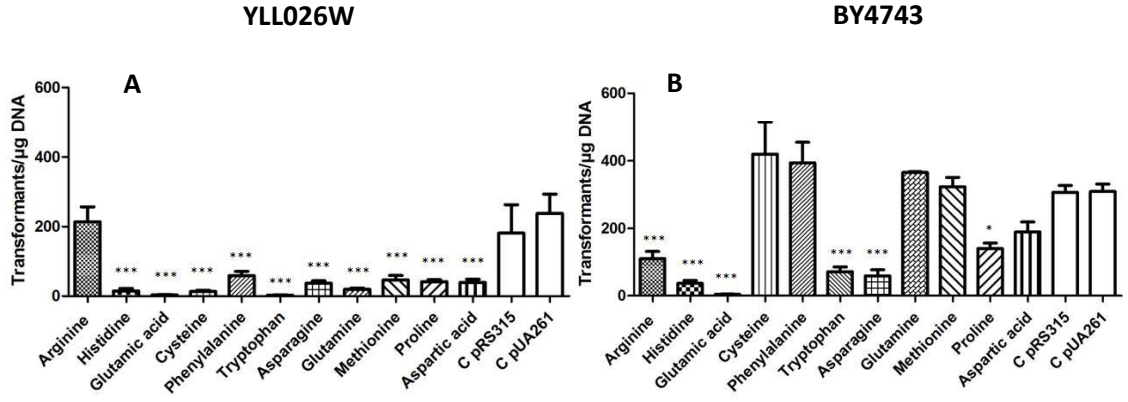


Figure 3.2: Transformation efficiency of S. cerevisiae strains YLL026W (A) and BY4743 (B) showing the mean number of transformants per µg of DNA utilized and SEM (8<n<2). For statistically analysis one-*way* ANOVAs were performed followed by a Dunnett's test to determine the significant differences between control pUA261 and tRNA expressing cells.

As can be seen in the figure 3.2A, for the haploid (YLL026W) strain only the plasmid Arginine, encoding serine $tRNA_{CCU}$ that misincorporates serine at arginine AGG codons showed similar transformation efficiency to the control cells. In the other cases the misreading tRNAs decreased transformation efficiency. The diploid BY4743 strain (Figure 3.2B) yielded higher number of transformants them the haploid strain and the plasmids carrying cysteine and phenylalanine misreading codons tRNAs produced higher number of transformants them the other plasmids. The plasmids carrying glutamine, methionine and aspartic acid codons misreading tRNAs yielded more transformants them whose carrying arginine, histidine, glutamic acid, tryptophan, asparagine and proline codons misreading tRNAs.

3.2.2. Impact of mistranslation on growth rate in yeast cells

The impact of the eleven engineered mutant $tRNA^{Ser}$, and the two control plasmids, on the growth rate of the YLL026W and BY4743 strains was also determined. Since the transformation efficiency results indicated that the strain BY4743 was less affected by the mutant tRNA the growth rate was determined in this diploid strain. The results of the growth rate were ploted in percentage of all transformants relative to the control pUA261 (Figure 3.3) and growth curves are displayed in annex (Annex E).

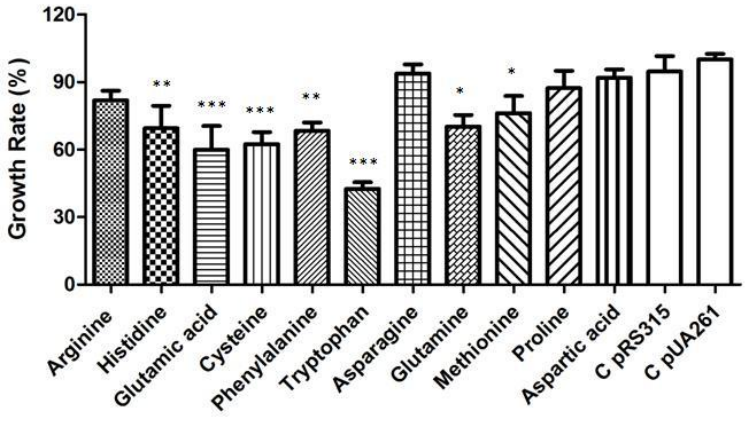


Figure 3.3: Mean values and SEM of growth rates of S. cerevisiae strain BY4743. The results were normalized considering the growth rate of the control pUA261 as 100%. For statistical analysis one-*way* ANOVA was performed followed by a Dunnett's test to determine the significant differences between control pUA261 mean and the remaining treatments.

As described in the literature changes in the meaning of the genetic code introduce translational errors that tend to imposes fitness cost to the cells (Keeling and Doolittle 1997). These deleterious effects may be related to the loss of protein function and/or direct and indirect toxicity (Drummond and Wilke 2008). From the wide range of deleterious effects, the energy expenditure in the production of misfolded proteins will affect negatively the growth rate. This assumption is observed in the growth rate results obtained (Figure 3.3), as the transformants carrying mutant tRNA showed lower growth rate them the control transformants. The most toxic tRNAs were those the misincorporated serine at glutamic acid, cysteine and tryptophan codons. Histidine and phenylalanine codons misreading tRNAs have affected growth rate, but to a lesser extent.

3.2.3. Mistranslations induce advantageous and deleterious phenotypic alterations

The results above demonstrate that mistranslation affect growth rate of yeast cells, what is consistent with the literature data which associates mistranslation with a wide range of deleterious effects, cell death and even diseases. However, as mentioned in the opening section of this chapter, under some conditions ambiguous translation can be advantageous. Examples of that come from previous studies that provide evidences that mistranslating cells are tolerant and grow under various environmental stresses like heat, H_2O_2, cycloheximide and salt (Santos, Cheesman et al. 1999).

In order to test if mistranslation can be advantageous under other stress conditions a phenotypic screening was carried out by growing the engineering *S. cerevisiae* strain BY4743 under a large range of environmental conditions. Different temperatures, ROS scavengers (ascorbic acid and glutathione), carbon sources (including non-fermentable carbon sources) and a wide range of environmental stressors were tested and a heat map was built (Figure 3.4). To facilitate the analyzes a figure compiling the significant differences between the cells transformed with mutant tRNAs and control cells with plasmid pUA261 was also made (annex F).

The results of the phenotypic screen were, like all the other results, analyzed by comparing cells transformed with mutant tRNA with control pUA261 transformants (figure 3.4). The data identified conditions where the transformants do not grow at all (namely, temperature 42°C, EDTA 0.25 mM and 0.5 mM, and menadione 150 µM), and conditions where the majority of the cells transformed with mutant tRNA grows faster them control cells (copper sulfate 2.5 mM,

SDS 0.01% and 0.015% and, lithium chloride 0.15 M and 0.3 M). This confirmed previous data from Santos and colleagues (1999) showing that under certain advantageous circumstances mistranslation can be advantageous. To better understand the results obtained an additional plot showing the data of all transformants that grow in copper sulfate 2.5 mM was built (figure 3.5).

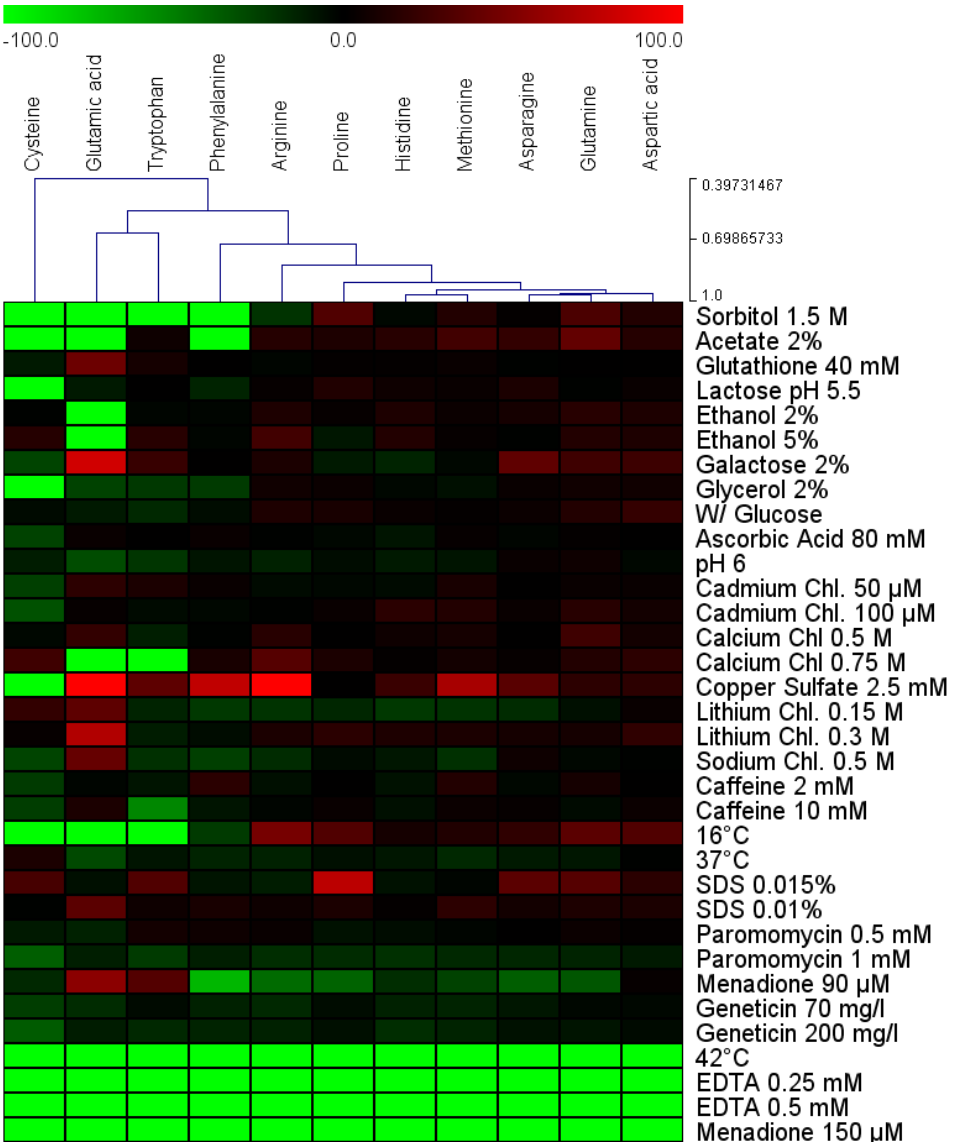


Figure 3.4: Heat-map of the phenotypic screen data. The size of the colonies obtained for all the transformed cells after 5 days of growth under the stipulated conditions was normalized to the control (size of the colony of the cells transformed with plasmid pUA261 was considered 0%). For statistical analysis one-*way* ANOVA was performed followed by a Dunnett´s test to determine the significant differences between control pUA261 mean and the remaining treatments (annex F). Differences to the control are represented in green when growth is below the control and red when growth is above the control. The final scoring was obtained considering the following values: *** = 3; ** = 2; * = 1 (green * are negative and red * are positive values). This graphic shows the transformed cells and treatments group by clustering analysis.

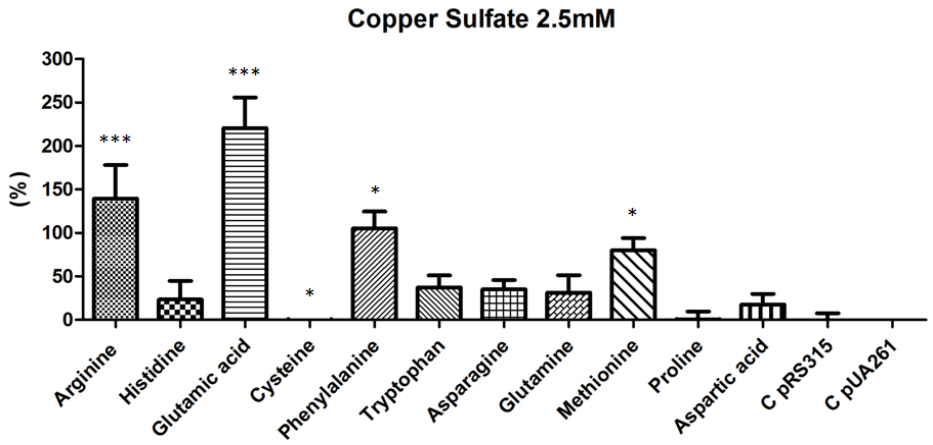


Figure 3.5: Graphic showing selective advantages induced by mistranslation. The results were normalized considering the size of the colonies of the control pUA261 as 0%. For statistical analysis one-*way* ANOVA was performed followed by a Dunnett´s test to determine the significant differences between Control pUA261 mean and the remaining treatments. Cells transformed with the tRNA misincorporating serine at cysteine codon (UGC) did not grow and her value was considered -100% relative to the control.

With exception of the cells transformed with the cysteine codon misreading tRNA the others cells grow faster them the control cells. Some of the mutant cells present, inclusively, large statistical differences relative to the control pUA261 namely, those that misread arginine and glutamic acid codons, while others showed more modest increases in growth rate, namely those that misread phenylalanine and methionine.

Grouping of treatments showed that temperature extremes (16°C and 42°C) and pH results in slower growth rates relative to control. Slower growth was also observed in cells exposed to cadmium chloride (50 and 100 µM), caffeine (2 and 10 mM), geneticin (70 and 200 mg/l) and, paromomycin (1 mM). Others environmental stressors, like menadione 90 µM, sodium chloride 0.5M or calcium chloride, showed also significant differences but, depending on the transformant, the growth was slower or faster them that of the control. Regarding the ROS scavengers, ascorbic acid (80 mM) and glutathione (40 mM), they showed a positive effect.

Growth on different carbon sources to analyze the respiratory capacity of cells and elucitade whether mistranslation interfered with the mitochondrial function were also performed. Yeast produces energy through glycolysis (absence of mitochondrial respiration) when the carbon sources are galactose or glucose (2%) (Fendt and Sauer 2010). With non-fermentable substrates such as ethanol (2% and 5%), sorbitol (1.5 M), acetate (2%), lactate and glycerol (2%)

yeast utilizes respiration to obtain energy (Karenlampi, Marin et al. 1981; D´amore 1992; Kiely, Cusick et al. 2011). The results obtained in the phenotypic screening for all these compounds showed that mistranslation had a big impact on growth in non-fermentable media. In galactose (2%) only the transformed with the glutamic acid codon misreading tRNA showed a statistically significant increased growth rate above that of the control strain. In non-fermentable substrates the impact of mistranslation was highly deleterious. The cells most affected were those transformed with tRNAs that misincorporated serine at glutamic acid, cysteine, phenylalanine and tryptophan codons which suggested that mitochondrial activity was somehow impaired in these cells. The treatments with paromomycin 0.5 mM and without source of carbon did not show significant differences between of the transformants.

In order to analyze the tRNA mutants individually a scoring system using the results of the statistical analysis (annex F) was performed. This scoring system only takes in to account significant differences between the distinctive mutagenic tRNA and discriminates the differences given points (in a scale of 1 to 3) if the differences is significant (1 point), highly significant (2 points) or extremely significant (3 points). When the difference corresponds to a faster grow relative to the control the values are positive, differences concerned to slow growth of transformants relative to the control pUA261 produced negative values. The values of the scoring system ranges from -38 to 4 indicating that the deleterious effects caused by mistranslation are larger than the advantageous effects. This analysis also identifies three groups of strains, namely those that misincorporated serine at asparagine and aspartic acid codons which did not show significant differences relative to the control. Transformants that misincorporated histidine, glutamine, methionine and proline codons whose overall score ranges from -1 and 3, which displayed significant differences in a maximum of three treatments, being, therefore, mildly affected by the mistranslation. And five transformants those misincorporation of serine at arginine, glutamic acid, cysteine, phenylalanine and tryptophan strongly affected growth rates in the phenotypic screen. These transformants had a minimum of five treatments with strong negative growth relative to the control and a score value ranging from 4 to -38. From this group, cysteine had a score of -38 and tryptophan -16, which were those with the highest sensitivity to the alteration of environmental conditions. Cells that misincorporated serine at glutamic acid codons also showed strong growth defects but had a mixed behavior with both negative and positive effects on growth. These groups were confirmed be the clustering analyses of the transformants displayed in figure 3.4.

3.3 Discussion

Mistranslation is supposed to have string impact on protein structure, inducing high level of proteome alterations, which should be reflected in the fitness of the cell because it will consume a lot of metabolic energy. Our results confirmed this hypothesis. Transformation efficiency of the *S. cerevisiae* strains YLL026W and BY4743 showed that the mutagenic tRNAs are toxic. The level of toxicity was dependent on the mutagenic tRNA introduced and the ploidy of the strain used. Indeed, the toxicity was more damaging in the haploid strain (YLL026W) than in diploid (BY4743) which suggests a possible effect of toxicity "dilution" with the increasing ploidy. In the BY4743 strain the copy number of tRNA genes is two-fold higher them in the haploid strain and this may explain those differences in the toxicity (Langkjaer, Cliften et al. 2003). In fact, the mean number of transformants obtained with the haploid strain was 36.71 colonies/µg of DNA which is much lower than the 139.8 colonies/µg of DNA verified in the BY4743 strain.

Next, the degree of toxicity introduced by mistranslation was evaluated by its impact on growth rate. Mistranslation in *E. coli* causes a reduction in growth rate (Stoebel, Dean et al. 2008) and our data largely confirmed these studies. Indeed, the cells misincorporating serine at tryptophan codons had growth rate which was 42.53% of the growth rate of the pUA261 control cells. The explanation for this result is that mistranslation results in proteins that have less overall activity and the energetic costs of the proofreading requires a considerable investment of the cells resources, which will tend to reduce the growth rate. However, in this thesis, as in the study of Stoebel, cells could survive to the errors introduced into the proteome. This may be related to the plasticity of protein structure which permits multiple substitutions outside the catalytic active-site residues. This plasticity buffers against the deleterious effects of mutations, allowing for chaperones and proteases to take over in situations where mistakes cannot be corrected. Recent results indicate that up to 10% of protein mistranslation is not detrimental (Stoebel, Dean et al. 2008).

However, an ambiguous genetic code can produce selective advantages under certain environmental conditions (Gomes, Miranda et al. 2007; Silva, Paredes et al. 2007; Nevoigt 2008). Studies with *S. cerevisiae* reveal, inclusively, that approximately 2% of the total number of mutations can be beneficial (Joseph and Hall 2004). Our results demonstrate that under some stress conditions several of our ambiguous strains growth better than the control non-ambiguous strains. For example, cells misincorporating serine in glutamic acid codons grow better in lithium

chloride 0.15 M and 0.3 M (37.3% and 104.8%, respectively), them the control strains (annex G). Also, cells misincorporating serine at arginine and glutamic acid codons grow faster in copper sulfate 2.5 mM (120.3% and 220.6%, respectively) than the control cells. These results support the assumptions that some mutations that affect fitness under normal environmental conditions are beneficial under other environmental conditions. Nevertheless, beneficial mutations in laboratory conditions may be deleterious in nature. Indeed, in complete medium some metabolic pathways such as those involved in amino acid biosynthesis are repressed, but are used in natural conditions and therefore these metabolic differences may alter the outcome of genetic code ambiguity (Joseph and Hall 2004).

Another important finding was that mistranslation has a strong deleterious impact on respiratory capacity. Cells misincorporating serine at glutamic acid, cysteine, phenylalanine and tryptophan, were the most affect. These results are also consistent with others phenotypes since those cells displayed the highest loss of fitness under other environmental conditions. The impairment of mitochondrial activity likely has serious energetic consequences that affect growth rate. Interestingly, the mistranslating strains behaved similarly to strains harboring *petite* mutations (Wallis and Whittaker 1974; Joseph and Hall 2004). *Petite* mutants are characterized by substantial defects on growth rate that can be inclusively lethal on non-fermentable carbon sources. Indeed, the substrate can be responsible for these *petite* mutations. Of all the amino acids misincorporations tested that involved serine at cysteine codons showed the highest loss in respiratory capacity. These cells had a phenotypic screening overall scoring of -38.

Chapter 4

4. Mistranslation causes protein aggregation and cell degeneration

4.1. Overview

Protein synthesis evolved to a level of error rate of 1 in 10,000 (Nangle, Motta et al. 2006). To safeguard translational fidelity and limit errors several quality control mechanisms exist, an example being the proofreading performed by the aaRS; discrimination against misacylated aa-tRNA by EF-Tu or codon-anticodon alignment in the ribosome (Hendrickson, de Crecy-Lagard et al. 2004). Quality control mechanisms begin during protein synthesis and end with stress responses responsible to manage misfolded proteins throughout the cell (Geslain, Cubells et al. 2010). As reported in the *Introduction* the unfolded protein response (UPR) manages the accumulation of misfolded proteins in the lumen of the ER. The accumulation of these peptides will cause the release of ER-resident chaperone Grp78 (BiP), which induces three adaptive responses (Geslain, Cubells et al. 2010). If these responses do not reduce ER stress pro-apoptotic pathways are induced and cytochrome c is release from mitochondria by c-ABL kinase or calcium (Gow and Sharma 2003). In the cytosol unfolded proteins triggers the heat-shock response. So, the stress response is specific for the cellular compartment being affected.

The misincorporation of amino acids can ultimately lead to accumulation of misfolded proteins that may be related with protein aggregation and human disorders (Geslain, Cubells et al. 2010). The misfolding of proteins and its inherent cost to the cell can be reduced by some adaptations, namely: increasing properly translated proteins, decreasing proteins which misfold or unfold due to mistranslation, and decreasing properly translated proteins which rapidly lose their native structure (Drummond and Wilke 2008). The diseases associated with mutant proteins that cannot adopt stable three-dimensional conformations are collectively termed “conformational diseases” (Gow and Sharma 2003). Neurodegenerative diseases, such as Alzheimer or Parkinson´s diseases, represent a large class of conformational diseases. In fact, neuronal tissues are particularly sensitive to protein misfolding and accumulation of abnormal protein aggregates in and around affected neurons is a normal feature of neurodegenerative diseases. Contributing to this relationship are the elaborate ramified structures and ordinary cell length of many neurons, which confer a relatively high surface-area-to-volume ratio, increasing

the probability of disruptive protein-membrane interactions. This feature of the neurons is aggravated by the limited neuronal turnover, which makes cell loss more probable and more permanent under a chronic stress like the one induced by protein misfolding. These assumptions are in line with recent studies on mice which showed that protein misfolding has neurotoxic effects.

The objective of the study described in this chapter was to verify if mistranslation affects cell viability and formation of protein aggregates. The latter was possible due to the availability of the YLL026W strain which carried a GFP-Hsp104 reporter protein.

4.2. Results

4.2.1. Effect of regulated mistranslation on cell viability

In the previous chapter, we have shown that misincorporation of serine at various cognate codons affects yeasts growth rate and impair mitochondrial activity. The effects of mistranslation on cells viability were also studied by growing cells for a long period of time and plating them periodically in order to assess the percentage of CFU formed (Figure 4.1).

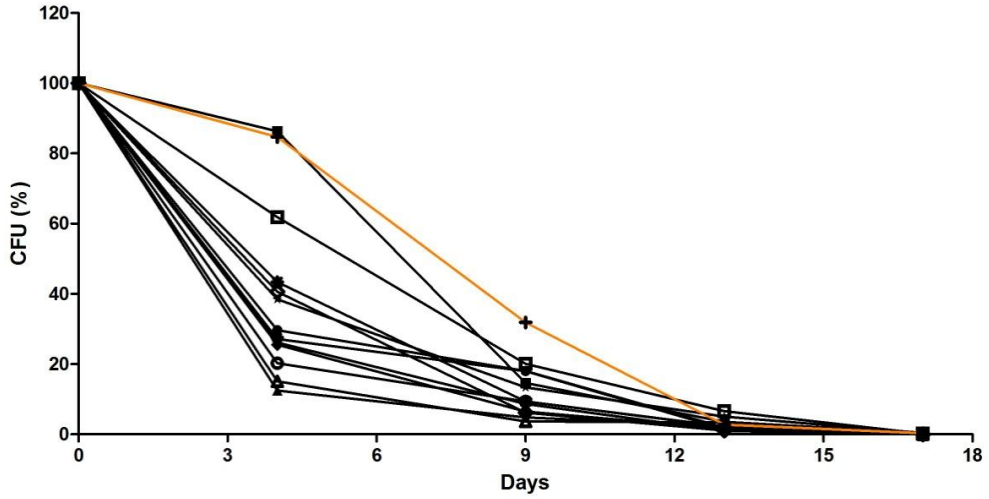


Figure 4.1: Mistranslation affects cell viability. In order to evaluate cell viability effects, CFUs were determined by counting the number of colonies that grow on agar plates (▬ control pUA261; ▬ control *wild type* and mutant transformants).

After 4 days of incubation, cell viability of mistranslation cells was always lower them that of control pUA261 cells, with the exception of serine misincorporation at histidine codons. Similar results were obtained after 9 days of incubation (Figure 4.2A, B).

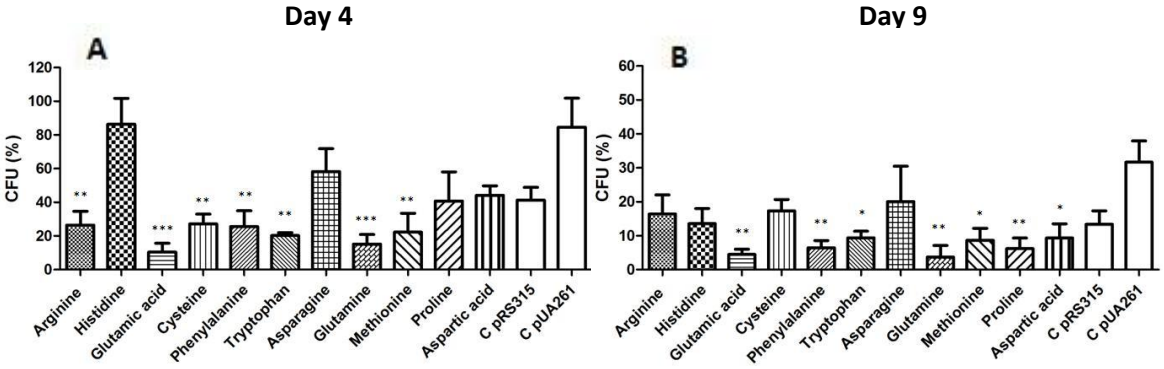


Figure 4.2: Effects of mistranslation on cell viability. The mean values and SEM for the percentage of CFU of *S. cerevisiae* strain BY4743 growing in minimal media at day 4 (A) and day 9 (B) are shown. For statistical analysis one-*way* ANOVA was performed followed by a Dunnett´s test to determine the significant differences between control pUA261 and the remaining treatments.

As it can be seen in the figure 4.2 (A and B) there are significant differences between the cell viability of control pUA261 and mistranslation cells. In all cases the mutant cells have lower viability. Interestingly, the differences between the control and the misreading cells tend to soften over time. At day 4 misreading cells that had significant differences relative to the control were glutamic acid and glutamine (***), arginine, cysteine, phenylalanine, tryptophan and methionine (**). At day 9 significant differences were found for misreading of glutamic acid, phenylalanine, glutamine and proline codons. Intriguingly, cells transformed with the pRS315 vector alone showed similar results to the misreading cells at days 4 and 9, suggesting that this plasmid was a negative impact on cell viability, which can be overcome by expression of the wild type serine tRNA. The reason for this unexpected phenotype is unknown.

4.2.2. Protein aggregates induced by mistranslation

In order to determine whether mistranslation induces formation of protein aggregates we have monitored such aggregates using *S. cerevisiae* strain YLL026W (haploid) which expresses the heat-shock protein 104 (Hsp104) tagged at the carboxyl terminus with GFP. This reporter was constructed by inserting the coding sequence of *Aequeorea Victoria* (S65T) gene in-frame with the yeast *HSP104* gene (Huh, Falvo et al. 2003). The choice of the molecular chaperone Hsp104 was

due to its involvement in the disaggregation of insoluble protein aggregates (Bosl, Grimminger et al. 2006; Doyle and Wickner 2009).

Yeast cells expressing the *HSP104-GFP* fusion reporter were transformed with the same mutant tRNA plasmids described previously. The recombinant cells were analyzed by epifluorescence microscopy in order to detect protein aggregates. This method does not affect tRNA expression and can be used to assess the physiological state of the cell. Another advantage of this method is that GFP fluorescence does not require external cofactors and this signal can be monitored using a fluorescence microscope without disrupting the cell. This permits the analysis of the timing and grade of stress responses induced by the different type of misreading tRNAs. Other common methods used to analyze protein localization in *S. cerevisiae* have depended on a transposon-mediated random epitope tagging and plasmid-based overexpression of epitope-tagged proteins (Huh, Falvo et al. 2003). However, both methods have the probability of leading to abnormal subcellular protein localization.

Transformed yeast cells were grown in liquid media to stationary phase and were observed using an epifluorescence microscope (Figure 4.3). Protein aggregation was detected in all transformants. Significant statistical differences between strains were observed in cells misincorporating serine at cysteine, phenylalanine (**), and proline (***) codons. The percentage of protein aggregates was also calculated by determines the number of aggregates per cell and the size of the aggregates (Figure 4.4).

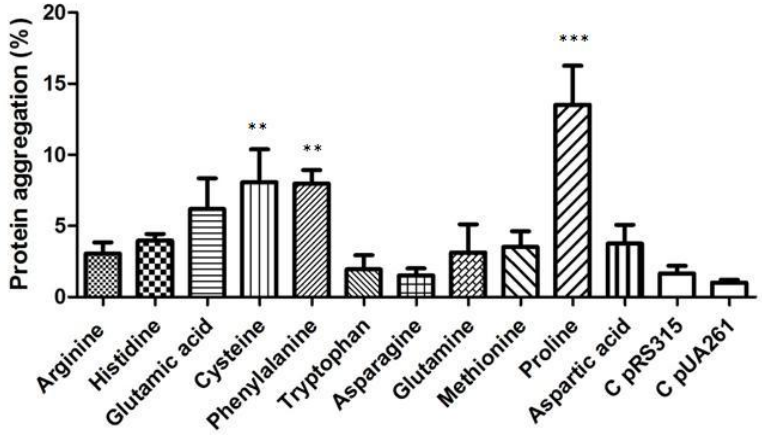


Figure 4.3: Mean values and SEM for the percentage of protein aggregates present in mistranslating *S. cerevisiae* YLL026W cells. Data were normalized considering the total number of cells counted (≈400) and the percentage of cells with aggregates. For statistical analysis one-*way* ANOVA was performed followed by a Dunnett´s test to determine the significant differences between control pUA261 mean and the remaining treatments.

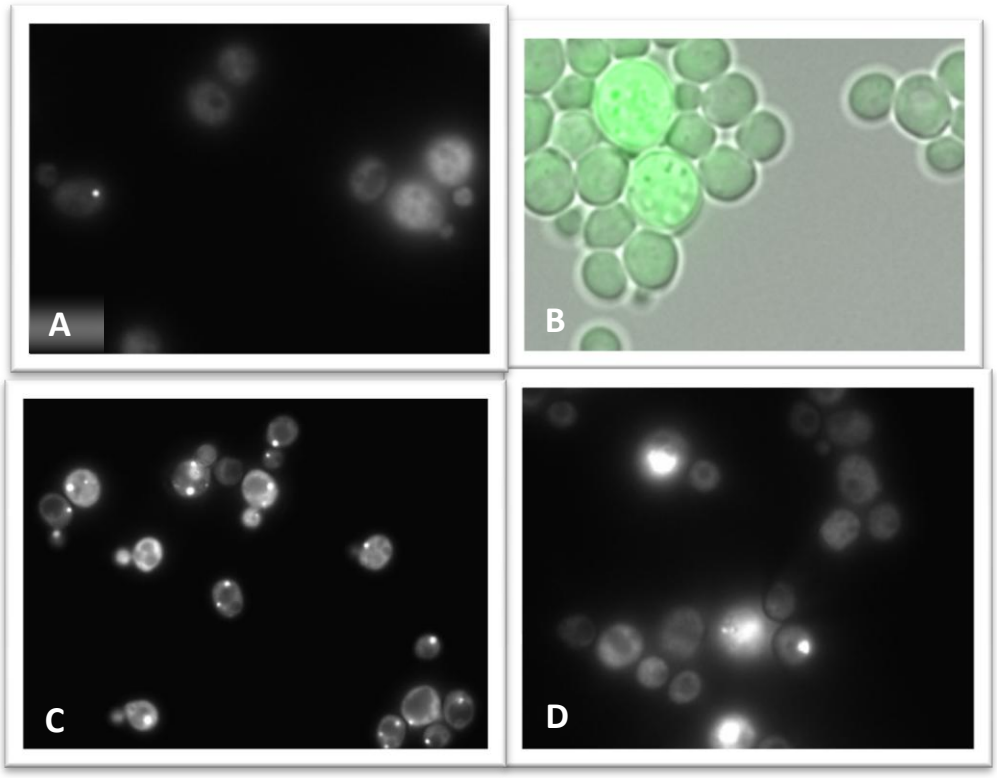


Figure 4.4: Representative images of the various types of protein aggregates detected by epifluorescence microscopy. A - Example of a standard protein aggregate observed (arginine). **B –** Image show increased size of cells misincorporating serine at arginine codons. **C** – Characteristic image of cells misincorporating serine at proline codons. **D –** Image of yeast misincorporating serine at cysteine codons. Yeast cells were observed using a fluorescence microscope (60-100x objectives).

The data (Figure 4.4) showed that different types of misreading produced distinct types of aggregates. For example, cells that misincorporated serine at proline codons produced more aggregates them the others and, in some cases, more them one aggregate was observed per cell (typically two or three). Various aggregates per cell were also observed in cells that misincorporated serine at glutamic acid codons. Interestingly, in dividing cells the proteins aggregates are normally present in the mother cells, and not in the daughter cells. This is a phenotype previously reported by Liu *et al.* (2010) and the explanation is that proteins that become damaged in the course of cell growth flow back into the mother cell and leave the young bud free of them (Liu, Larsson et al. 2010). The “retrograde transport” is carried out by actin

filaments, which grow from the growing bud to the mother cell. Such filaments are assembled at the tip of the bud in a structure that the authors called a “polarisome,” which is made up of core proteins plus some proteins involved in actin polymerization (formins). Finally, cells that misincorporated serine at cysteine, phenylalanine and proline codons produced large aggregates (Figure 4.4D).

Cells misincorporating serine at methionine codons showed a very strong flocculation phenotype (Figure 4.5). An important note is that self-flocculating yeast strains could be advantageous in industrial fermentation as part of the process in bioethanol production (Ma, Wakisaka et al. 2010). This finding will be most deeply analyzed in the discussion.

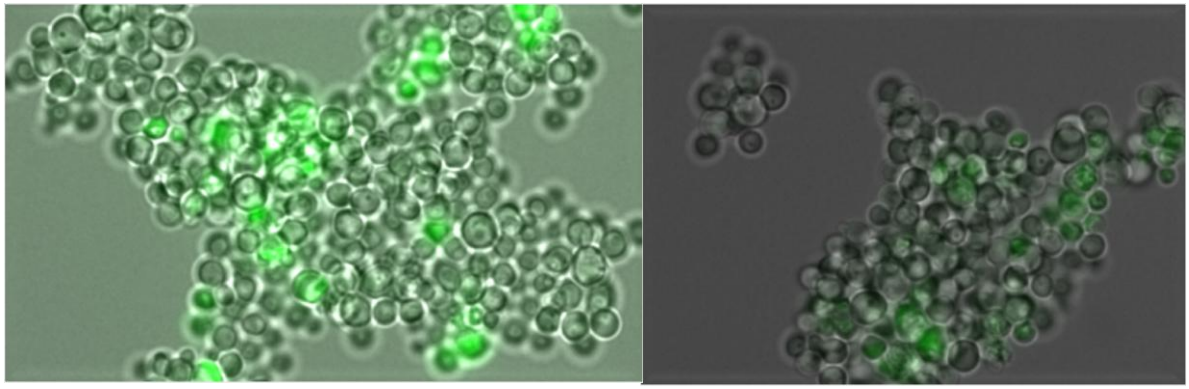


Figure 4.5: Representative pictures of yeast flocculation seen in cells that misincorporated serine at methionine codons.

4.2.3. Northern blot analyses of tRNA^{Ser} UGA expression

The expression of the mutagenic tRNA was validated using northern blot analysis. The northern blot was performed using total RNA extracted from the transformed cells of the strain BY4743 and YLL026W expressing the mutant tRNA that misread proline codons. Total RNA from *C. albicans* strain SN148 was used as a positive control. The original images are presented in the annex (annex H).

As can be seen in the figure 4.6, the positive control of the assay showed a strong signal indicating that the Northern blot technique was working. The internal controls and the negative control pUA315 also produced the expected results. Concerning to membrane hybridized with

serine probe, the control pUA261 showed higher RNA expression, which is due to the additional copy of $tRNA^{Ser}$. The mutagenic tRNAs were expressed at a lower level.

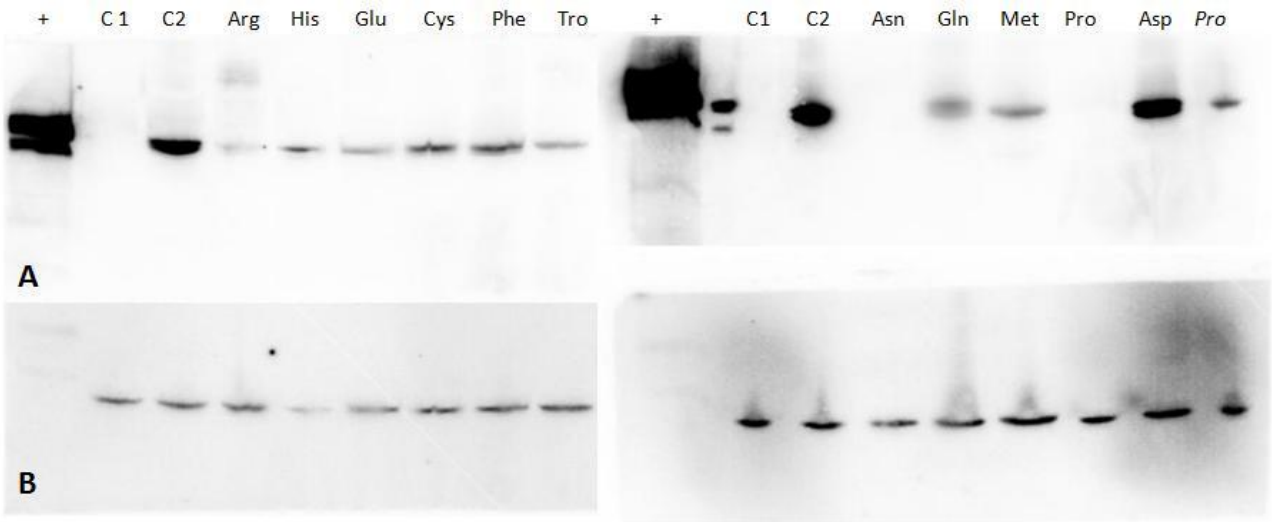


Figure 4.6: Northern blot analysis of the misreading tRNAs. Top left shows a membrane hybridized with the serine probe (**A**), below is the membrane hybridized with the control probe (glycine) (**B**). + positive control (RNA *C. albicans* SN148); C1 – Control pRS315; C2 – Control pUA261; *Pro*– RNA from transformant Proline of strain YLL026W.

4.3. Discussion

The results of cell viability showed that at day 9, the viability of the mistranslating strains was lower them that of control cells. After this time-point the viability showed no differences between mutagenic tRNA transformants and controls. Interestingly, the viability of the controls pUA261 and pUA315 was different. The additional gene copy of $tRNA^{Ser}$ is somehow important in the maintenance of cell viability even in none mutated cells.

Protein aggregation assays showed that mistranslation induces protein aggregation. In particular, tRNAs that misincorporated serine at cysteine, phenylalanine and proline were significantly different from the control. The protein aggregation suggests that the UPR was activated, confirming previous results from other laboratories (Geslain, Cubells et al. 2010). To confirm our results, the activation of proteins like BiP (gene *KAR2*) or other major components of the UPR need to be verified by RT-PCR or Western blot.

Besides the total number of protein aggregates in a cell population, other characteristics were studied, namely, the size of the aggregates and the number of aggregates per cell. The data shows that each type of mistranslation has slightly different phenotypes in terms of protein aggregation. Cells misincorporating serine at arginine and histidine codons were larger them other cells, indication of a possible increase in polyploidy, which may explain why these do not have significant differences in the percentage of protein aggregates. As already described, ploidy is a factor that influences toxicity and cells with increased ploidy are less susceptive to mistranslation and, specifically, will present a lower level of protein aggregation. Ploidy should therefore be checked by a flow cytometry.

As mentioned in the results, the most idiosyncratic phenotype observed was the flocculation of the strain that misincorporated serine at methionine codons. This self-flocculation may be advantageous in industrial fermentation, particularly in bioethanol production. The advantages are production of a higher cell concentration under proper conditions in the bioreactor and efficient separation of yeast cells in the fermenting mash (Ma, Wakisaka et al. 2010). Moreover the methionine transformants did produce significant protein aggregation formation. This is a very important feature once stress tolerance is crucial for yeast performance in any industrial processes (Nevoigt 2008). However, to verify the relevance of this transformant in industrial applications more and specific tests are necessary.

The levels of expression of the mutagenic tRNA were confirmed by northern blot. The data showed that our engineered tRNAs were expressed at lower level them the control $tRNA_{UGA}^{Ser}$, suggesting that mistranslation lowers the tRNA expression.

Chapter 5

5.1. General Discussion

Cells most affect by the deleterious effects of mistranslation at the level of growth rate, respiratory capacity, and cell viability were those that misincorporates serine at glutamic acid, cysteine, phenylalanine and tryptophan codons. The latter had the lowest growth rate (42.53%) and second lowest score in the phenotypic screening assay (-16). Serine misincorporation at glutamic acid and cysteine also showed significant differences in the phenotypic assay, although the former had advantageous growth under some environmental conditions. In the protein aggregation assay cysteine, phenylalanine and proline showed the highest percentage of aggregates. On the other hand, serine misincorporation at asparagines and aspartic acid codons did not result in significant difference relative to the controls in the conditions tested. The exception was their lower transformation efficiency.

To better comprehend the effects of the mutagenic tRNA on the transformant cells, the relation between the anticodon mutated and the amino acid that the mutant anticodon specified is showed in Table 5.1. The polar requirement, which is a property derived from paper chromatographic mobility of amino acid mixtures in pyrinidine-water, and an unidimensional characterization of the amino acids that seems to capture the essence of the way in which amino acids are related in the context of the genetic code (Woese, Olsen et al. 2000). This property is the simplest way to compare the amino acids and explain the different impact of the various mutagenic tRNAs. Indeed, the serine polar requirement is 7.5 and the polar requirements of the eleven amino acids that were substituted for serine indicate that cysteine, phenylalanine and tryptophan were those which serine replaced the amino acids with lowest polar requirements. In the cases of glutamic acid and proline the impact of the replacement was most difficult to explain based only on the polar requirement. More specifically, glutamic acid has a polar requirement of 12.5 and aspartic acid has 13. Serine misincorporation at aspartic acid and asparagine codons did not have negative effects in the conditions tested. Conversely, serine misincorporation at proline and methionine codons were those were the polar requirement was most similar (6.6 for proline and 5.6 for methionine). This indicates that the effects of mistranslation cannot be explained by the amino acids polar requirement only. If we considers the structure of the amino acids side chains, them phenylalanine, tryptophan and proline have distinct structures that may explain the

deleterious effects observed by serine misincorporations. Phenylalanine have a distinct phenyl group, tryptophan presents a unique indole ring and proline is the only amino acid that does not have a core structure because the alpha amino group is incorporated into the ring. In the case of the asparagine and aspartic acid codons, serine misincorporation did not cause significant cell alterations which may be explained be the relative similarity of the side chains of these amino acids with that of serine.

Table 5.1: Polar requirements of the amino acids codified by each mutagenic tRNA anticodon. The amino acids represented in this table were substituted for serine. The polar requirement was taken from Woese et al., 2000 (Woese, Olsen et al. 2000). Serine polar requirement is 7.5;

Transformant	Amino Acid	Polar requirement
pUA801	Arginine	9.1
pUA802	Histidine	8.4
pUA803	Glutamic acid	12.5
pUA804	Cysteine	4.8
pUA805	Phenylalanine	5.0
pUA806	Tryptophan	5.2
pUA807	Asparagine	10
pUA808	Glutamine	8.6
pUA809	Methionine	5.3
pUA810	Proline	6.6
pUA811	Aspartic acid	13

5.2. Conclusions and Future Work

The results achieved in this thesis show that the methodology used to introduce proteome-wide mutagenesis is effective. Mutant tRNAs induce formation of misfolded proteins that have serious impact on the fitness of yeast *S. cerevisiae*. Growth rate reduction, impairment of the respiratory activity of mitochondria, loss of cell viability and formation of protein aggregates which may activate the UPR response were observed. A relevant, and perhaps one of the most important findings established, was the relation between the effects that were discovered and the dysfunctions related with neurodegenerative diseases. As mentioned before (section 1.7.2.), neurodegenerative diseases are strongly associated with intracellular protein aggregation, mitochondrial dysfunction and cell death. This parallelism is an indication that, at least in *S. cerevisiae*, this methodology and the kind of mutations that were induced in this thesis, can be useful in the study of neurodegenerative diseases.

From all the mutagenic tRNA the most deleterious ones were the glutamic acid, cysteine, phenylalanine, tryptophan and proline. The deleterious effects can be explained, by the divergent characteristics of the amino acids relative to serine. Namely, the polar requirement property. Mistranslation can be advantageous in certain circumstances. For example, serine misincorporation at glutamic acid codons increased growth rate relative to *wild type* cells in presence of some environmental conditions. Interestingly, serine misincorporation at methionine codons produced a floculation phenotype and a series of others advantageous features that can be useful in some industrial applications.

The levels of mistranslation tolerated and UPR activation varies even within an organism. It will be interesting to test in future studies the response profile of the UPR and the identification of new UPR-related factors that may be induced in the response to the misfolded proteins. The ultimate goal will be the identification of all the components of cellular stress response and the elucidation of the functional and spatial connections between then.

The importance of this study is related to the involvement of misfolding proteins in neurodegenerative diseases in Humans. Studies in mice, which associetes editing-defective alanyl-RS to ataxia and neurodegeneration provide direct evidences for a role of mistranslation on human diseases. Inability to clear mischarged amino acids like serine and glycine in $tRNA^{Ala}$ causes misfolding of proteins in neurons and lead to disease. The misacylated aa-tRNA, with the exception of neurons, does not exhibit dramatic malfunctions in other tissues. This kind of studies allows the scientific community to study cystic fibrosis, Alzheimer diseases or cancer, which affect Humans. The connections between the aaRS and the mischarge of tRNA with human diseases make this a field of great importance. Future work should clarify the relations between the mischarging of tRNAs and identify the pathways involved in the responses. In a superior level, integration of this knowledge in eukaryotic multi-cellular organisms should be carried out to better understand the causes of neurodegenerative diseases. This will help to develop more efficient therapies.

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Annexes

Annex A

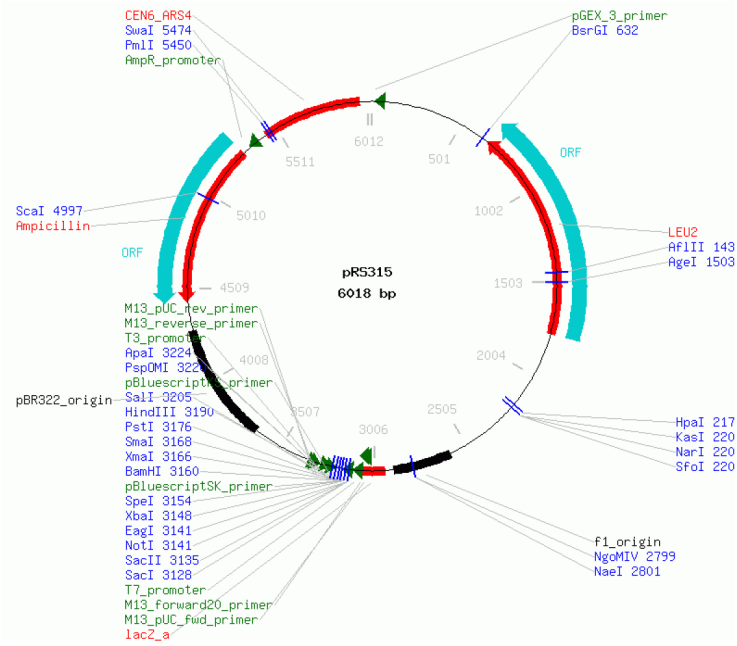
✓ *Drop-Out Mix:*

Adenine	0.25g
Alanine	1g
Arginine	1g
Asparagine	1g
Aspartate	1g
Cysteine	1g
Glycine	1g
Histidine	1g
Inositol	1g
Isoleucine	1g
Glutamate	1g
Glutamine	1g
Lysine	1g
Leucine	2g
Methionine	1g
Phenylalanine	1g
Proline	1g
Serine	1g
Treonine	1g
Tryptophan	1g
Tyrosine	1g
Uracil	1g
Valine	1g

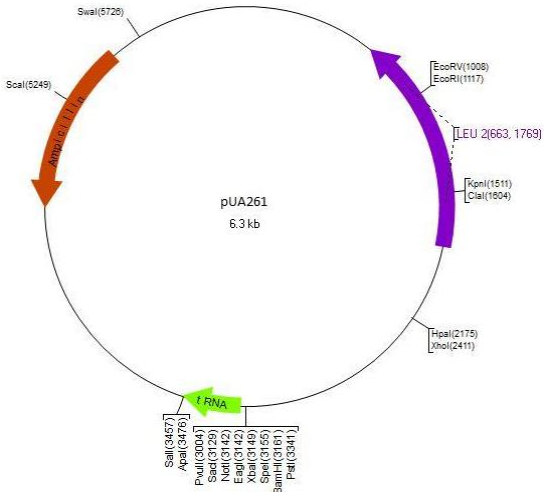
Annex B

✓ *Maps of the plasmids and sequence of $tRNA_{UGA}^{Ser}$ gene*

Maps of the plasmids pRS315 and pUA261



pRS315: Plasmid used by Mateus (2011) as vector for the mutation of $tRNA_{UGA}^{Ser}$.



pUA261: Plasmid constructed by Mateus (2011) based on pRS315. Plasmids pUA801 to pUA811, engineering during this thesis, have the same map. Unique difference is the anticodon that was differentially mutated.

Sequence of the $tRNA_{UGA}^{Ser}$ inserted gene into the pRS315 – Highlight for the anticodon

CAGTATGGATTGCTAGTCCTAGAGAAACCGTGGCCATTTTAGACGATTTAATTGATTTCATAGAAGGTTAG
ATAACAATATATATATATTTGCATGAGCTTTGTGAATCAGCTCCAAAATTGATGTAAAAAAAAAGAGGGCG
TTTGGCGGAGCAAAAAAAATAACGACAACTGCAGGACTCGAACCTGCGCGGGCAAAGCCCAAAAGATTTC
AAGTCTTTCTCCTTAACCGCTCGGACAAGTTGCCTAAATCTCGCTGAAATTAGGGATGCACATAAATCAGTC
CTGGAC

Anticodon of the $tRNA_{UGA}^{Ser}$ present into the eleven engineering plasmids (pUA801-pUA811).

Plasmids	Mutated Anticodon
pUA801	AGG
pUA802	CAT
pUA802	GAG
pUA804	TGC
pUA805	TTT
pUA806	TGG
pUA807	AAT
pUA808	CAG
pUA809	ATG
pUA810	CCT
pUA811	GAT

Annex C

✓ *Solutions and procedures*

Buffers TFB I and TFB II

TFBI (100ml)

- 0.3 g CH_3CO_2K;
- 1.2 g $RbCl_2$;
- 0.147 g $CaCl_2$ or 0.195 g $CaCl_2.2H_2O$;
- 1.0g $MnCl_2$;

The listed reagents were dissolved in $d2H_2O$ and 17.7 ml glycerol 87% was added. pH was adjusted to 5.8 with 0.2 M acetic acid. Solution was filtered and stored in 2.5 ml aliquots at -20°C.

TFBII (100ml)

- 0.24g MOPS Na;
- 1.1g $CaCl_2$ or 1.457g $CaCl_2.2H_2O$;
- 0.12 $RbCl_2$;

Reagents were dissolved in $d2H_2O$ and 17.7 ml glycerol 87% added. pH was adjusted to 6.5 with 0.5 M acetic acid NaOH. Solution was filtered and stored in 2.5 ml aliquots at -20°C.

SOC (100 ml)

- 2 g Tryptone;
- 0.5 g Yeast extract;
- 0.05 g NaCl;
- 1 ml KCl (250 mM);

Adjust the pH to 7.0 with NaOH (0.5 M) and distilled water added up to 80 ml. After autoclave, 20 ml of sterile filtered (0.22 µm) 1 M glucose were added.

TAE 1x

TAE50x

- Dissolve 242 g of Tris in 500 ml of dH_2O;
- Add 100 ml of Na_2EDTA (0.5 M and pH 8.0) and 57.1 of crystallized acetic acid. Adjust the volume to 1l adding dH_2O;

Add 20 ml of TAE50x to 980 ml of dH_2O. Store at room temperature;

Loading Buffer GB 6x

- 3.45 ml glycerol 87%;
- 6.5 ml dH_2O;
- 0.025 g Bromophenol Blue;

Probe Labelling mix

- 1 µl Oligo (1:10);
- 10 µl dH_2O;
- 3 µl [γ-32P] ATP;
- 2 µl Buffer kinase 10x;
- 2 µl Spermidine 0.1 mM;
- 1.5 µl T4 kinase;

Hybridization and washing buffer

20xSSPE

- 175.3 g NaCl;
- 27.3 g NaH_2PO_4;
- 9.4 g EDTA;

Adjust the volume to 1l adding dH_2O and set the pH to 7.4 (with NaOH);

50xDehneart

- 1 g Ficoll 400;
- 1 g Polyvinylpyrrolidone (PVP);
- 1 g BSA;

Adjust the volume to 100 ml adding dH_2O;

Hybridization buffer

- 3.3 µl 20xSSPE;
- 1 µl 50xDenheart;
- 4.7 µl dH_2O;
- 1 µl 10% SDS;

Washing buffer

- 50 µl 20xSSPE;
- 25 µl dH_2O;
- 425 µl 10% SDS;

Polyacrylamide gel

TBE10X

- 108 g Tris;
- 55 g Boric acid;
- 40 ml EDTA (0.5 M, pH 8.0);

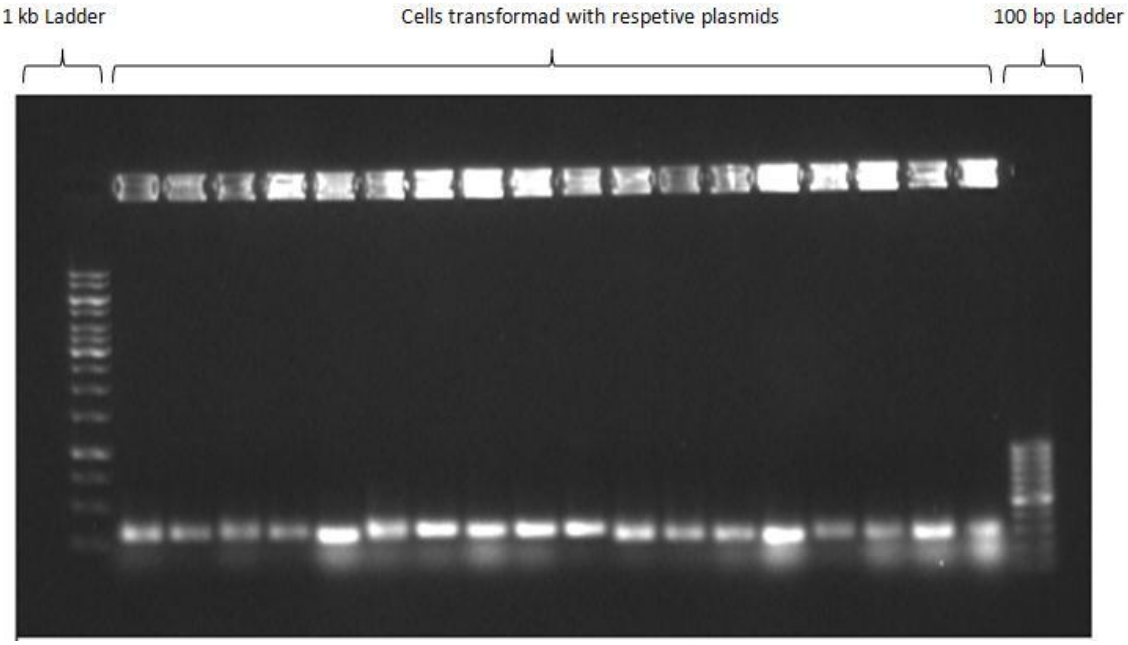
Gel

- 42 g urea;
- 37.5 ml acrylamide (19:1) Biorrad;
- 10 ml TBE10x;
- dH_2O to 100 ml;

Add 1ml of APS 10% and 100 µl of TEMED to catalyze the polymerization of acrylamide.

Annex D

✓ *Agarose gel confirming the efficiency of transformation*

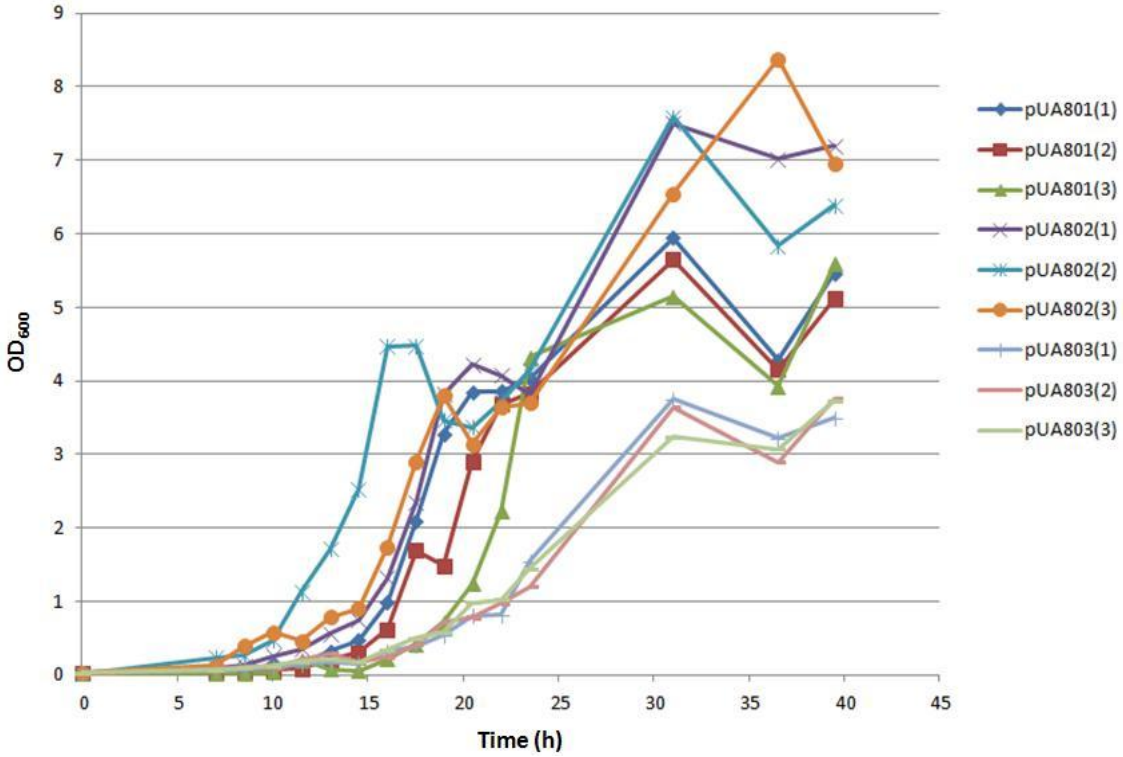


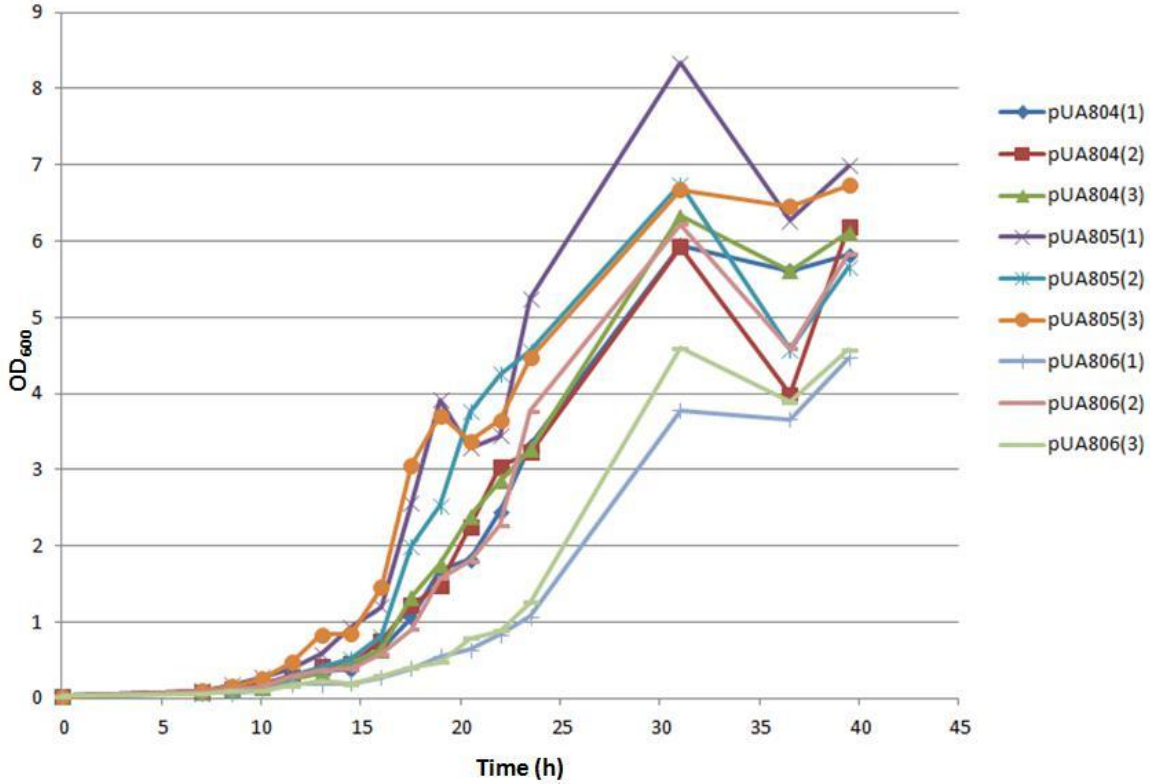
Agarose gel resulting from the electrophoresis performed with the transformed cells. The presence of the plasmid was confirmed;

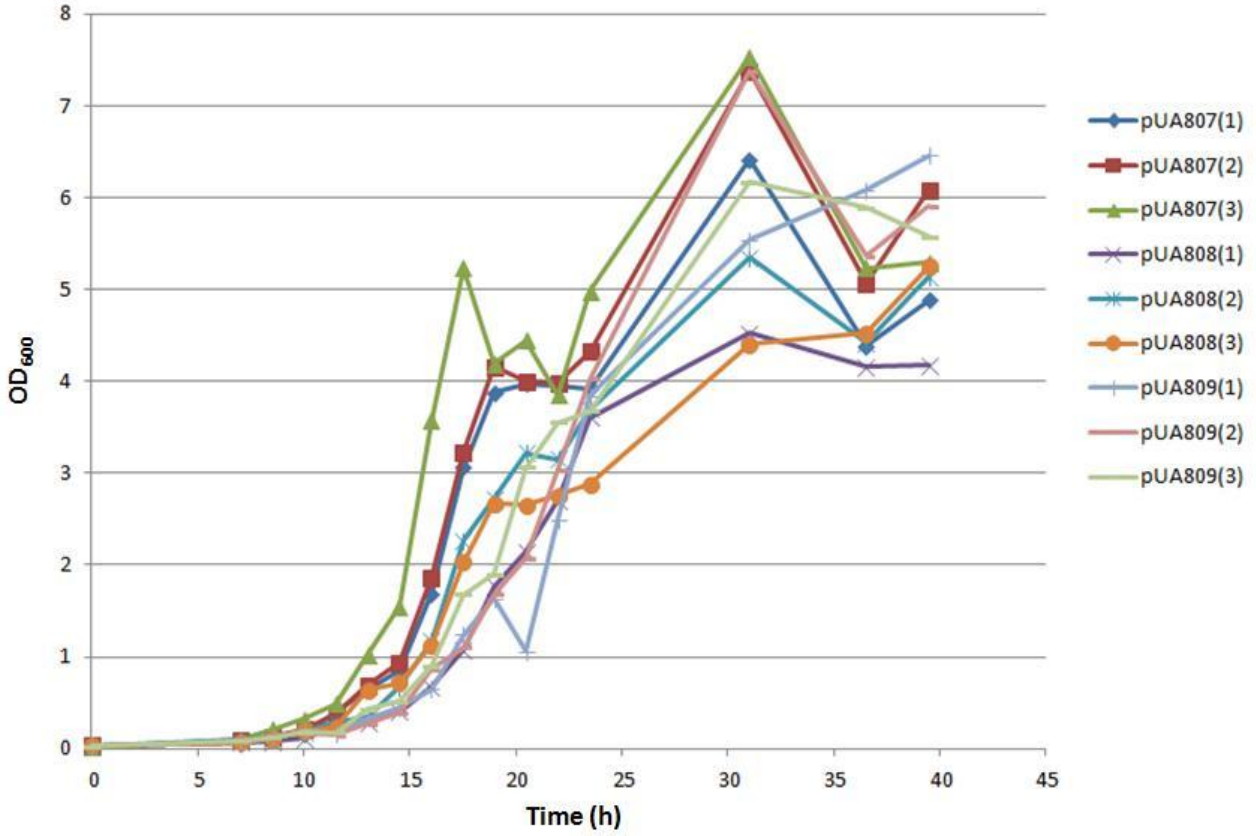
Annex E

✓ *Growth curves*

First replicated



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pUA807(2)
pUA807(3)
pUA808(1)
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pUA809(1)
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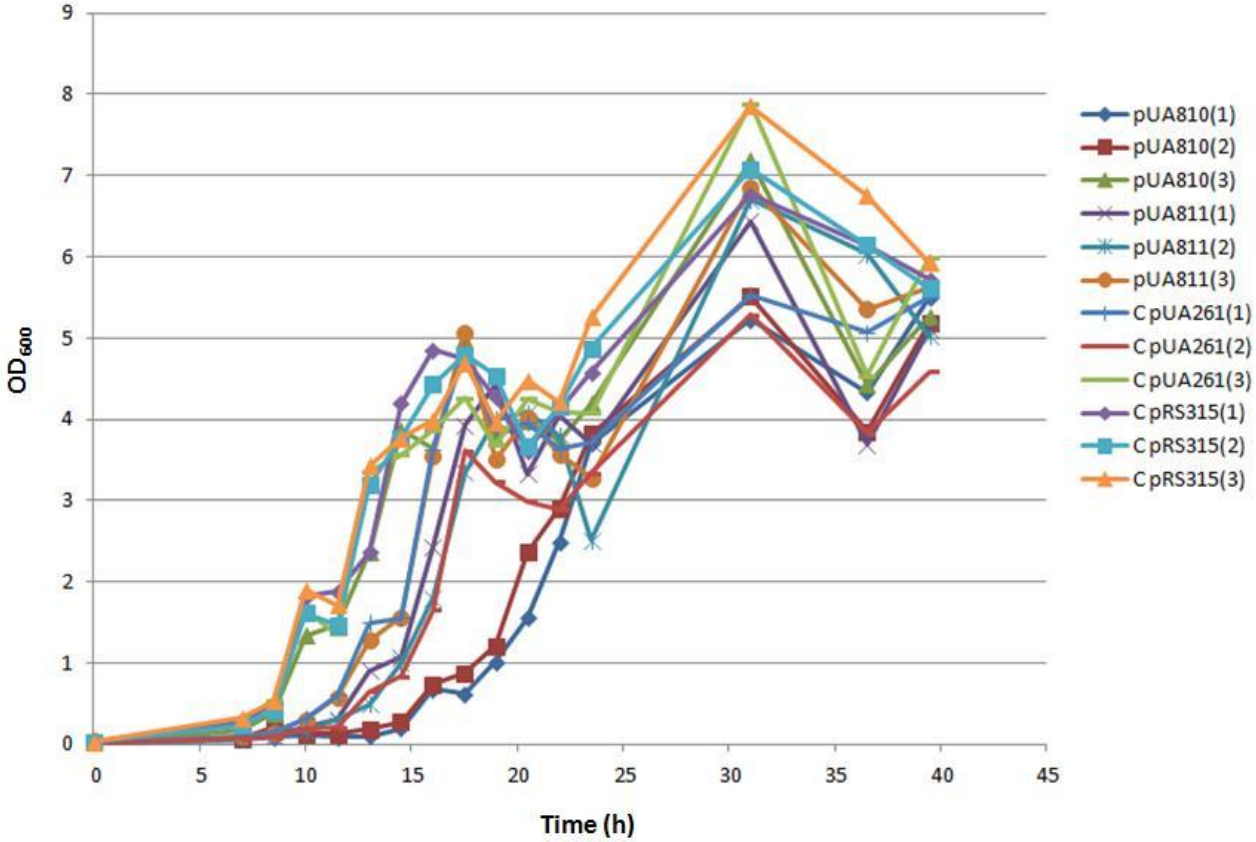
pUA809(3)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pUA811(2)
pUA811(3)
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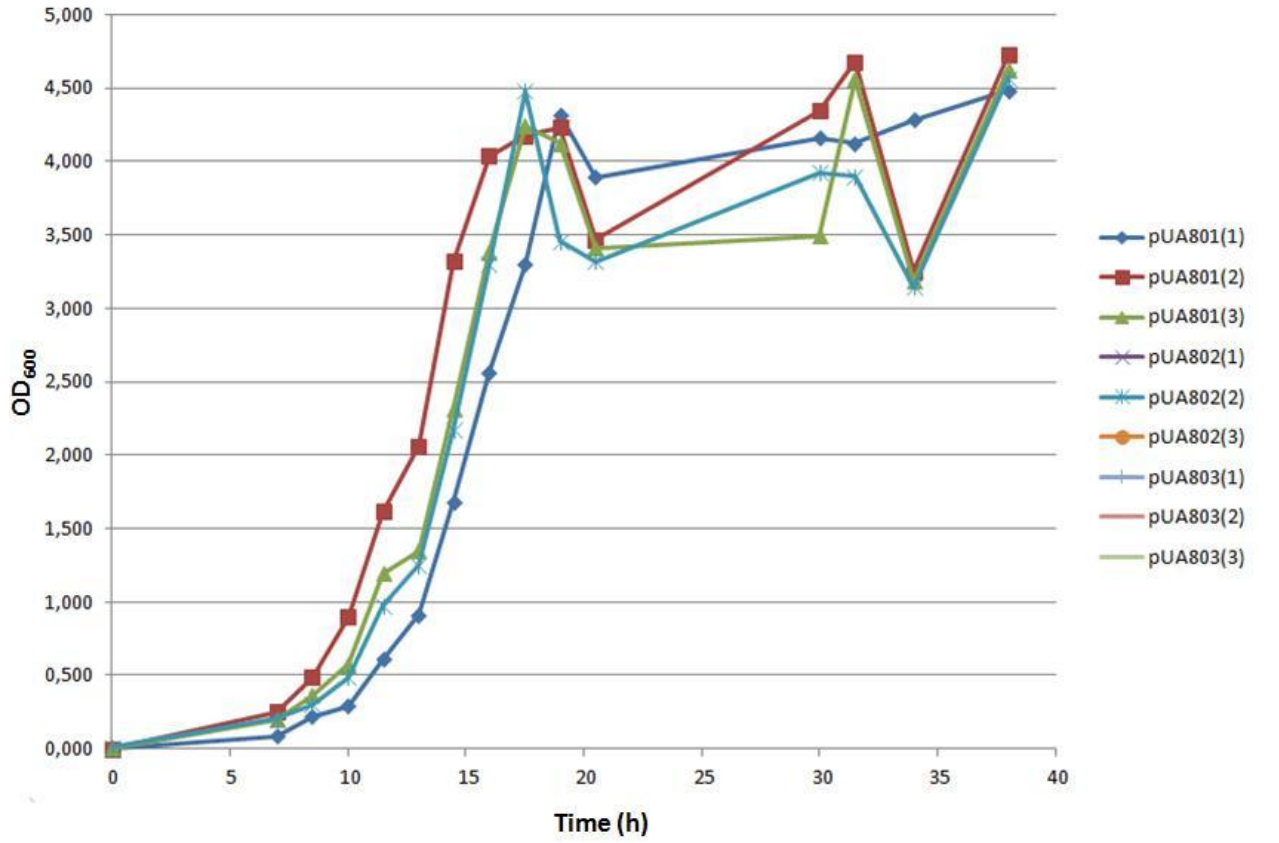
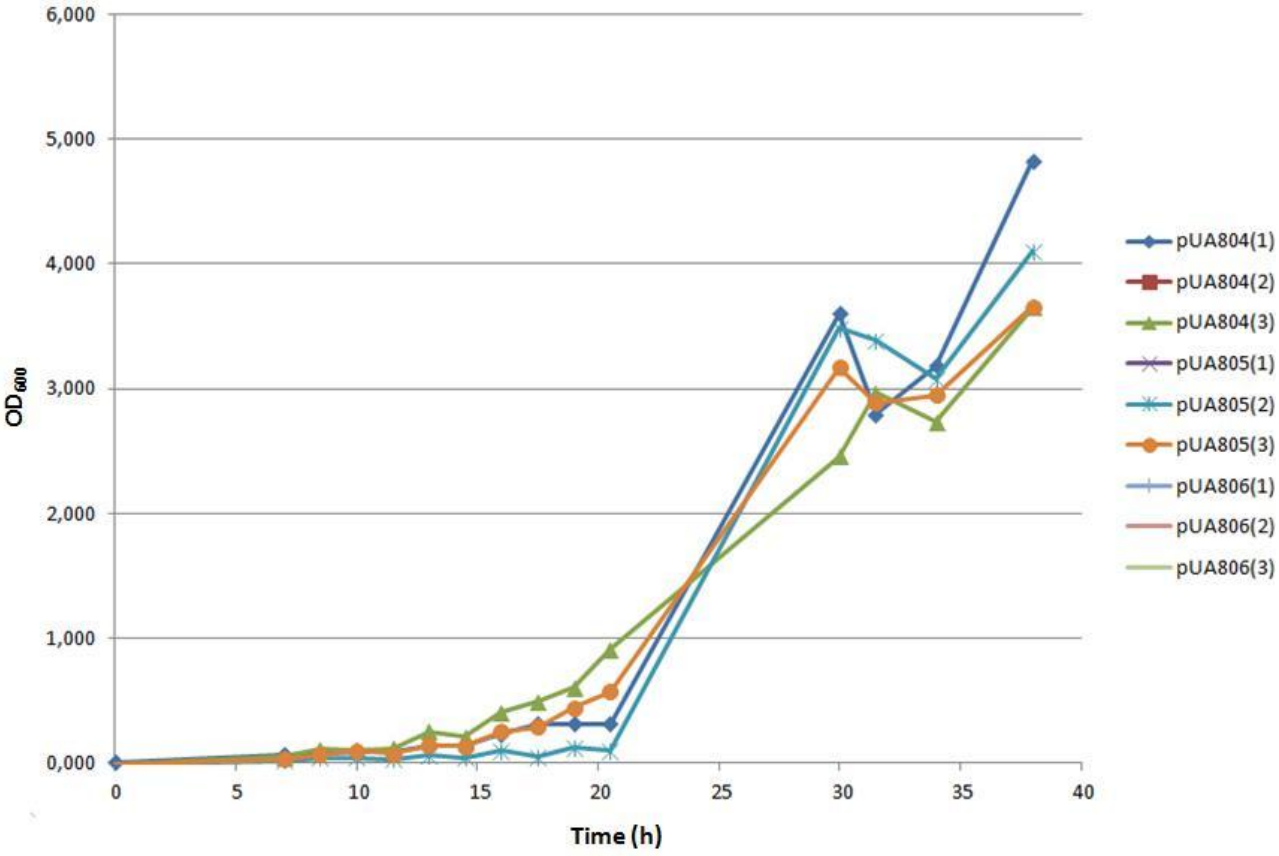
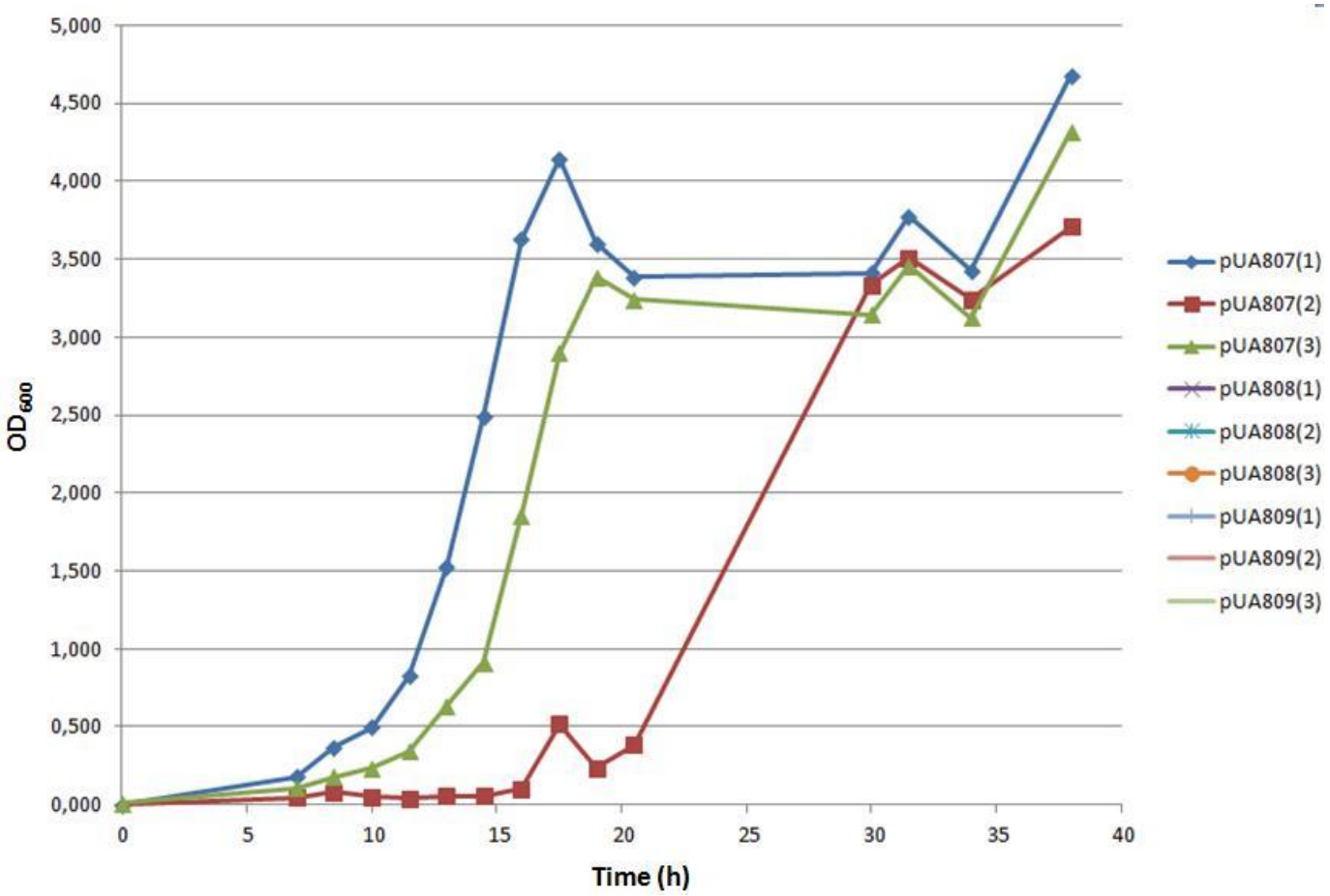
C pRS315(2)
C pRS315(3)

Second replicated



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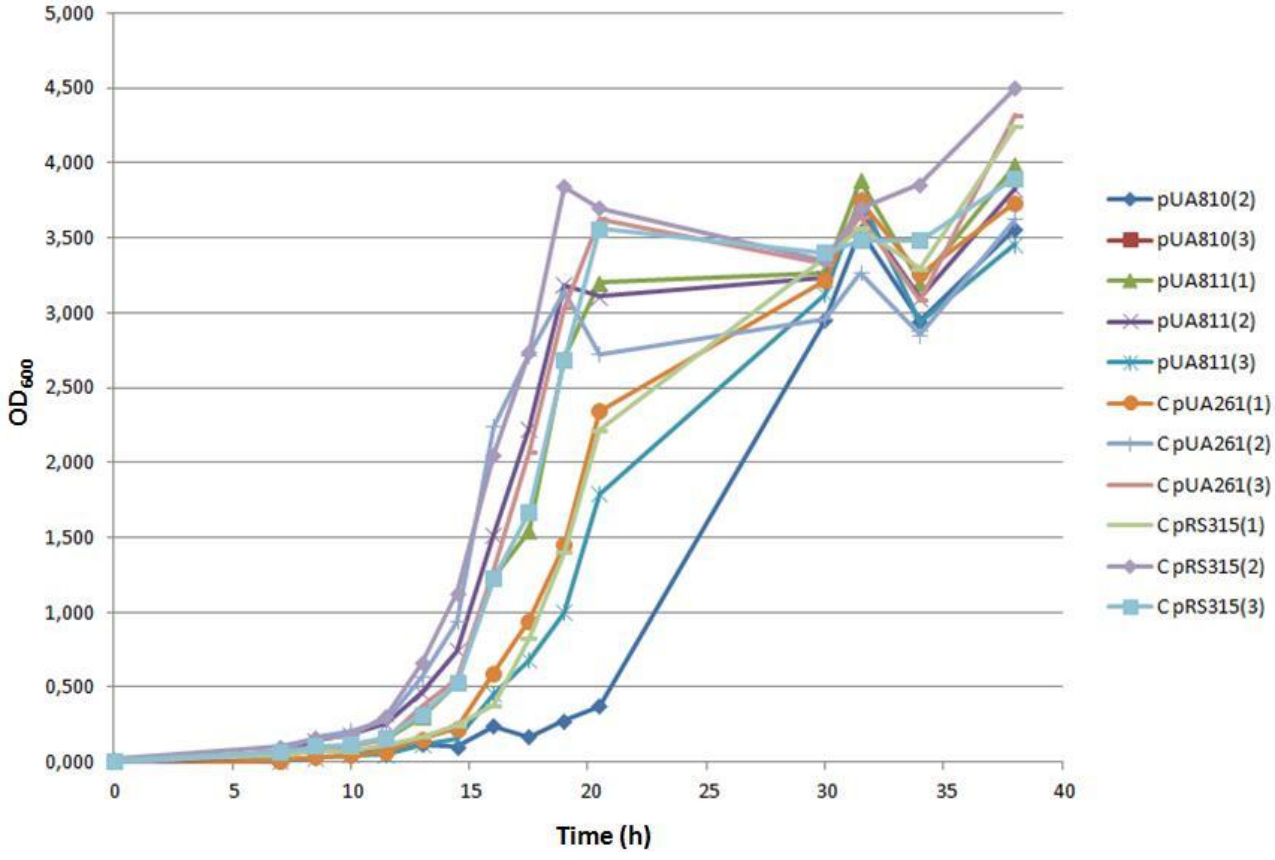
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Time (h)
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pUA810(3)
pUA811(1)
pUA811(2)
pUA811(3)
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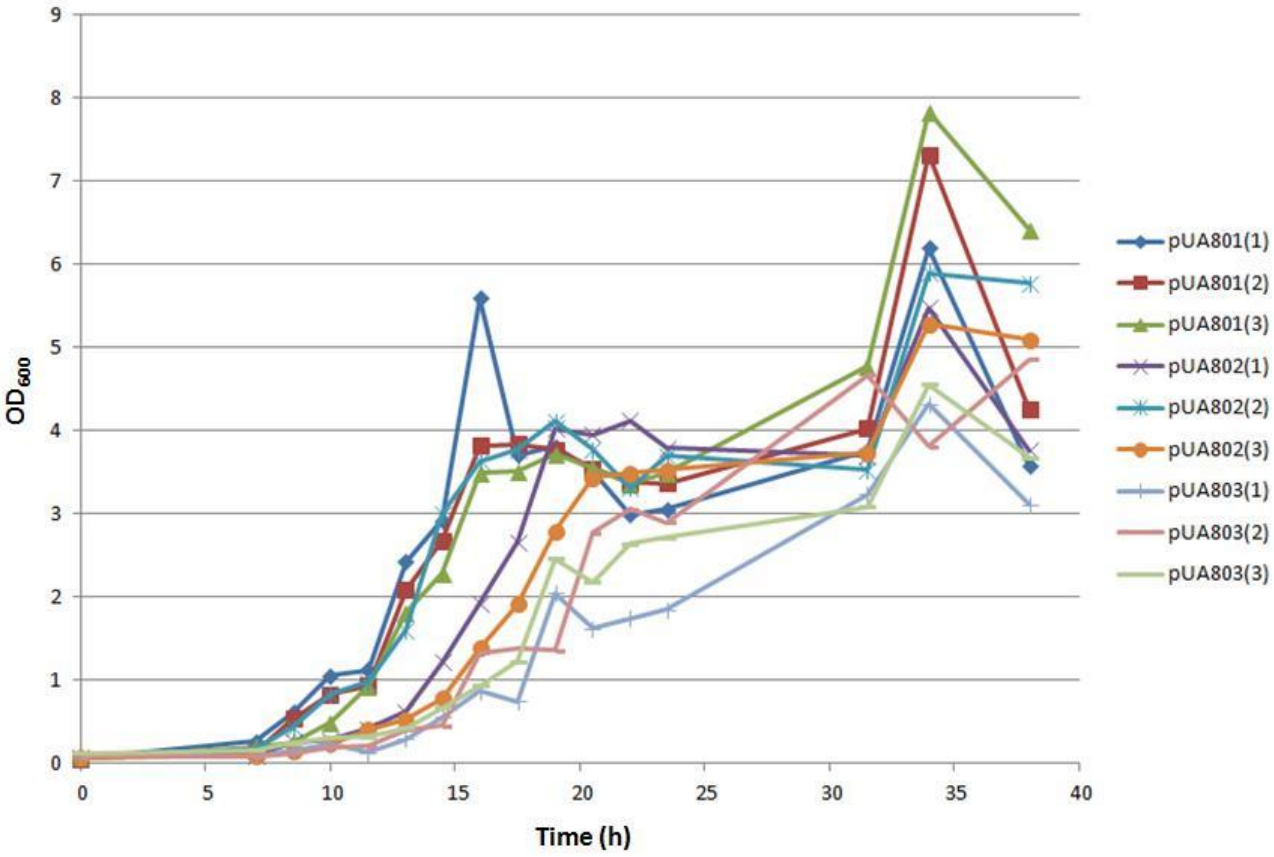
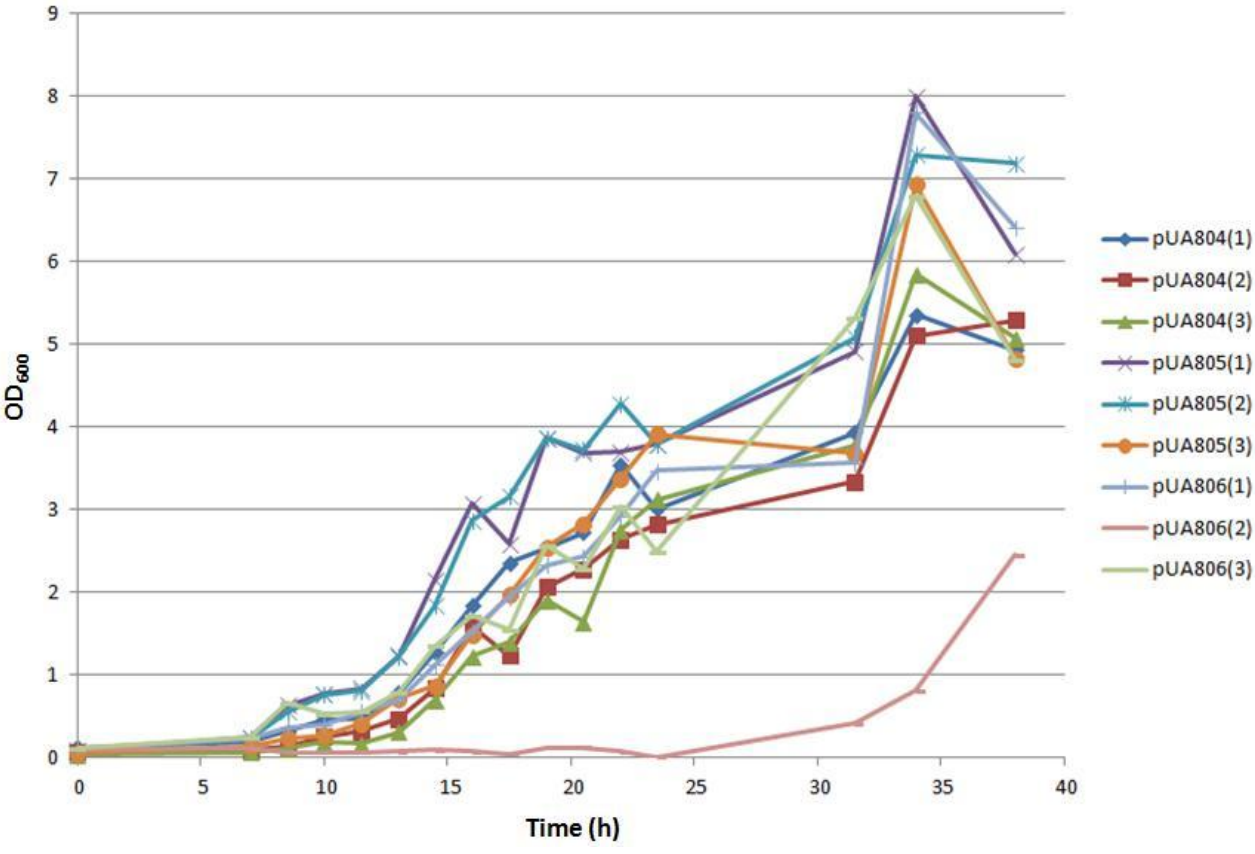
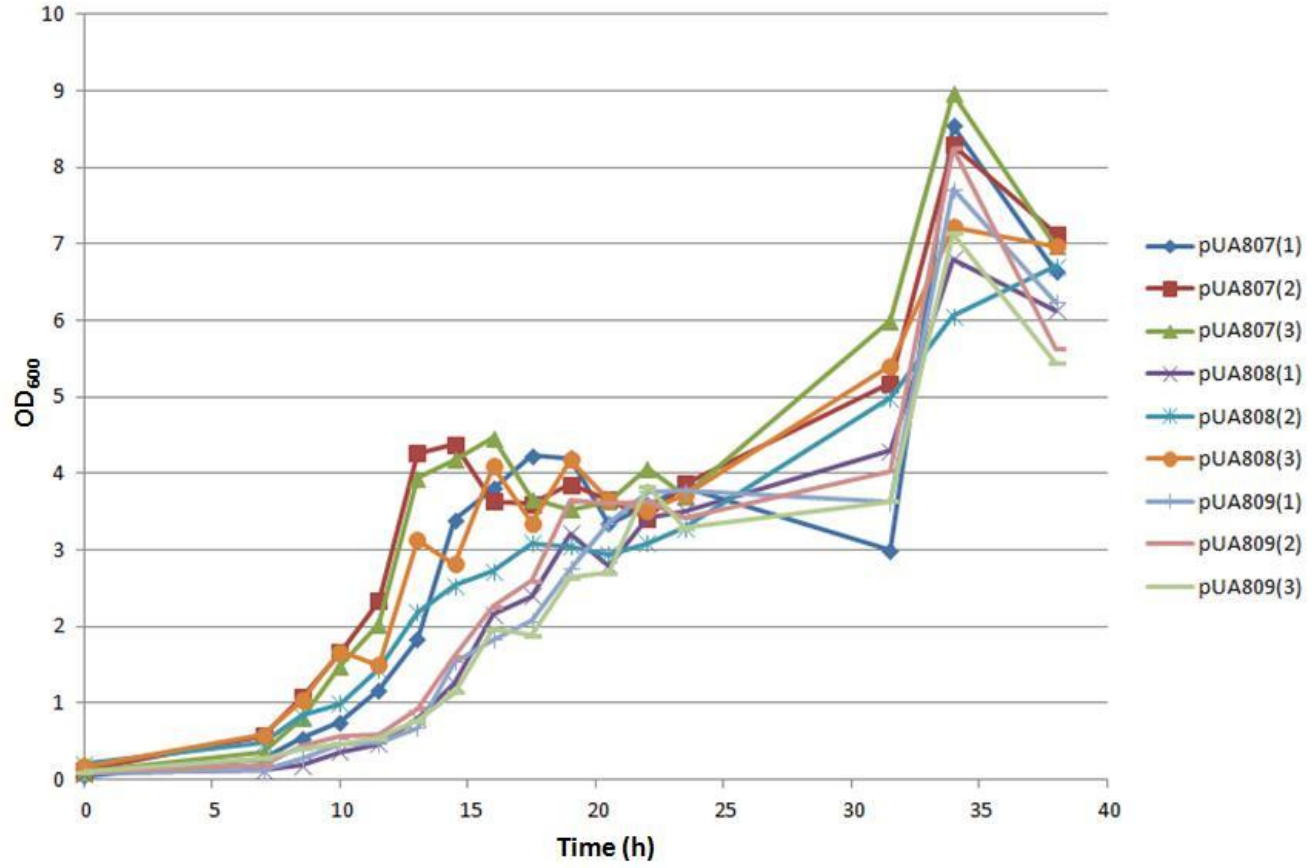
CpRS315(2)
CpRS315(3)

Third replicated



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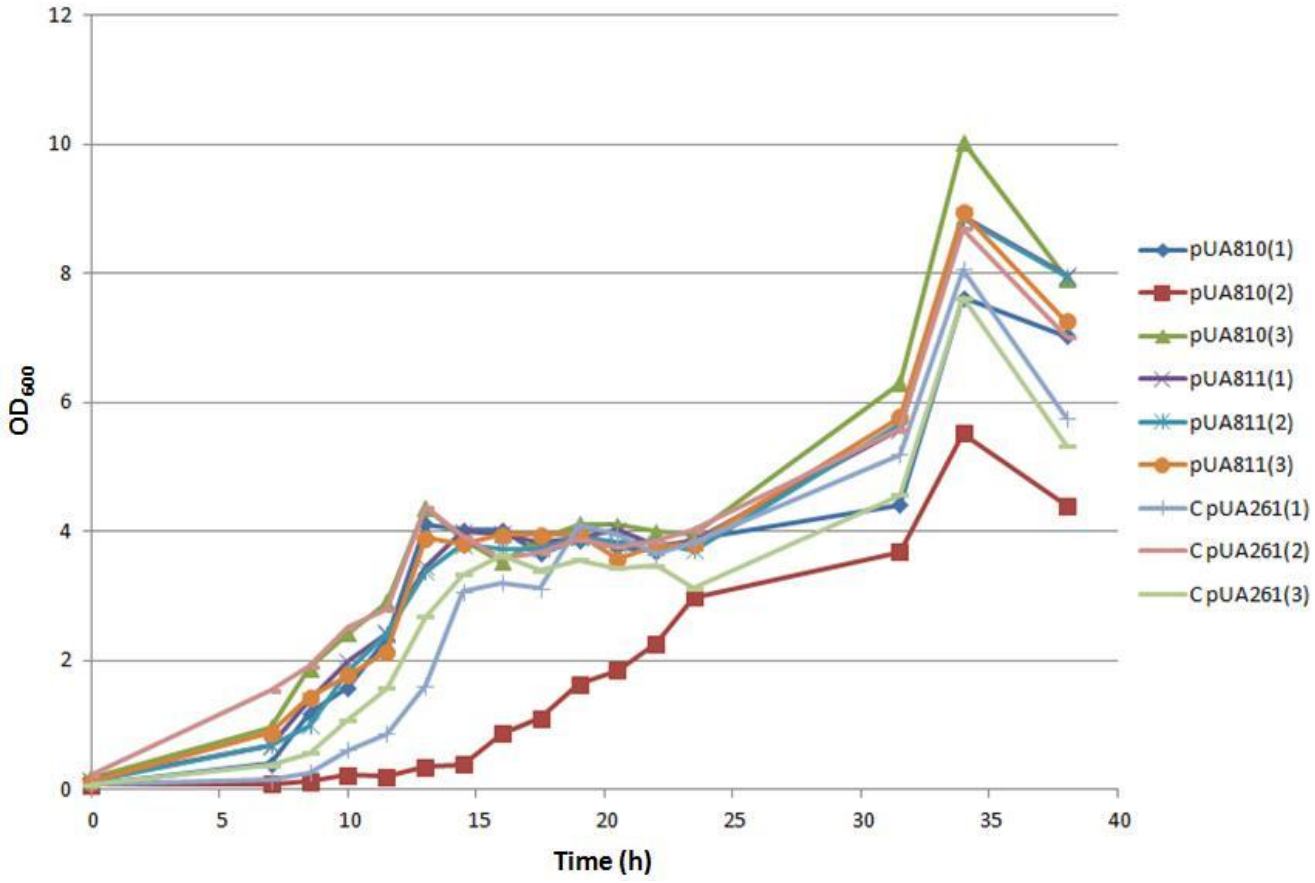
pUA809(3)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pUA810(3)
pUA811(1)
pUA811(2)
pUA811(3)
C pUA261(1)
C pUA261(2)
C pUA261(3)

Annex F

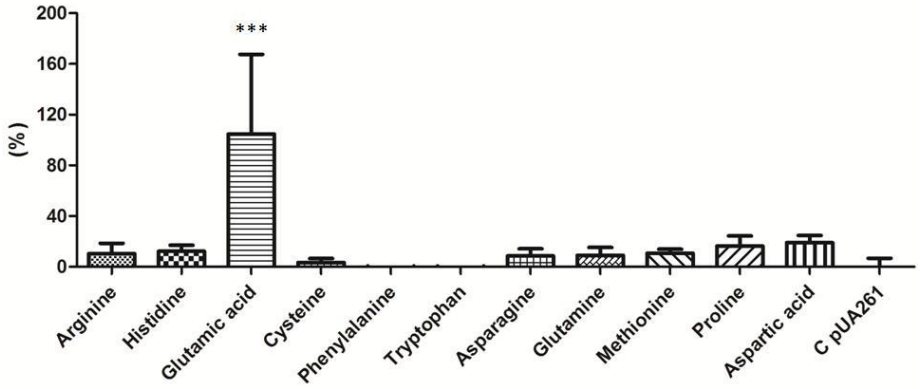
✓ *Statistically analyses of the data from the phenotypic screening*

Representation of the significant differences obtain after treating the data resulting from the phenotypic screening. The size of the colonies obtain for all the transformed cells was normalized with the control (size of the colony of the cells transformed with plasmid pUA261 was considered 0%). From statistical analysis one-*way* ANOVA was performed followed by a Dunnett´s test to determine the significant differences between control pUA261 mean and the remaining treatments. The significant differences are represented in green when represents a growth below the control and red representing growth above this. The final scoring was obtained considering the following values: *** = 3; ** = 2; * = 1 (green * are negative and red * are positive values).

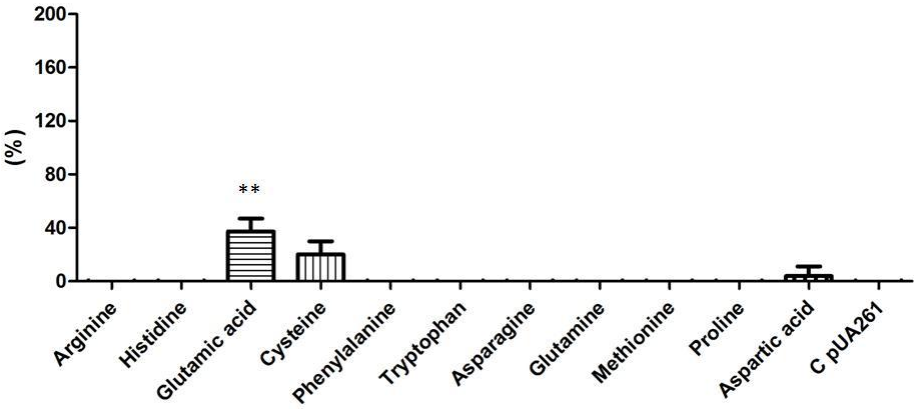
Annex G

✓ *Graphic illustration of the growth rates of transformed BY4743 cells in lithium chloride*

A)

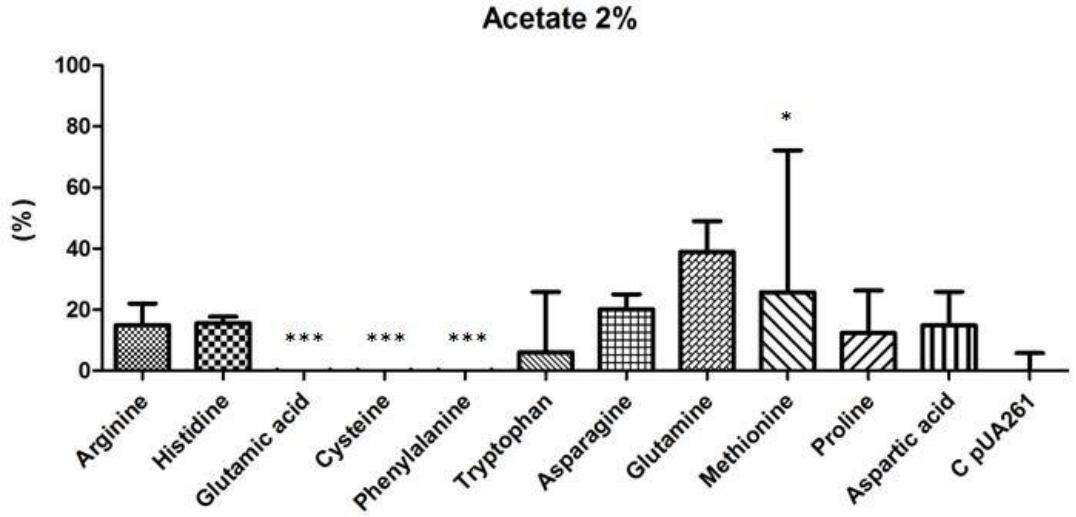


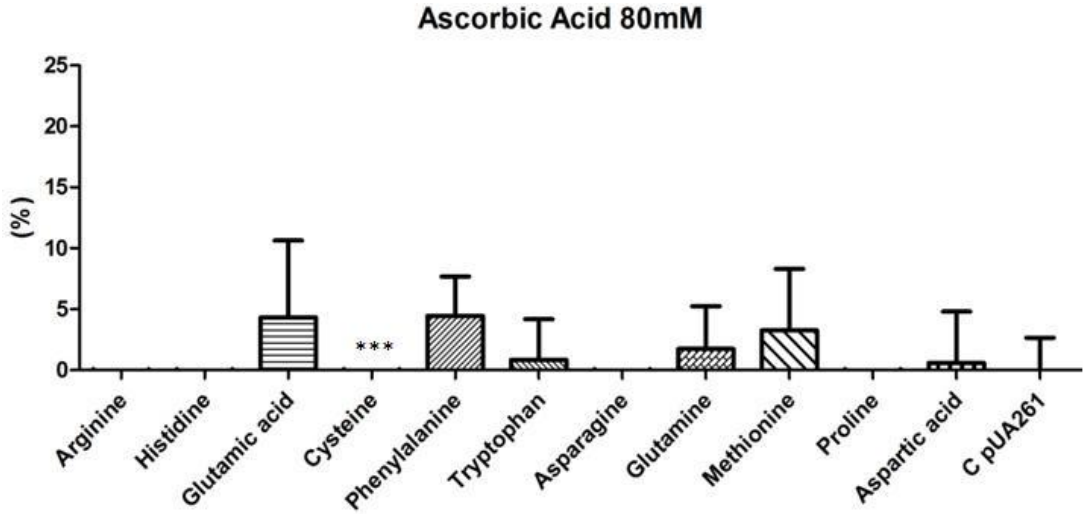
B)

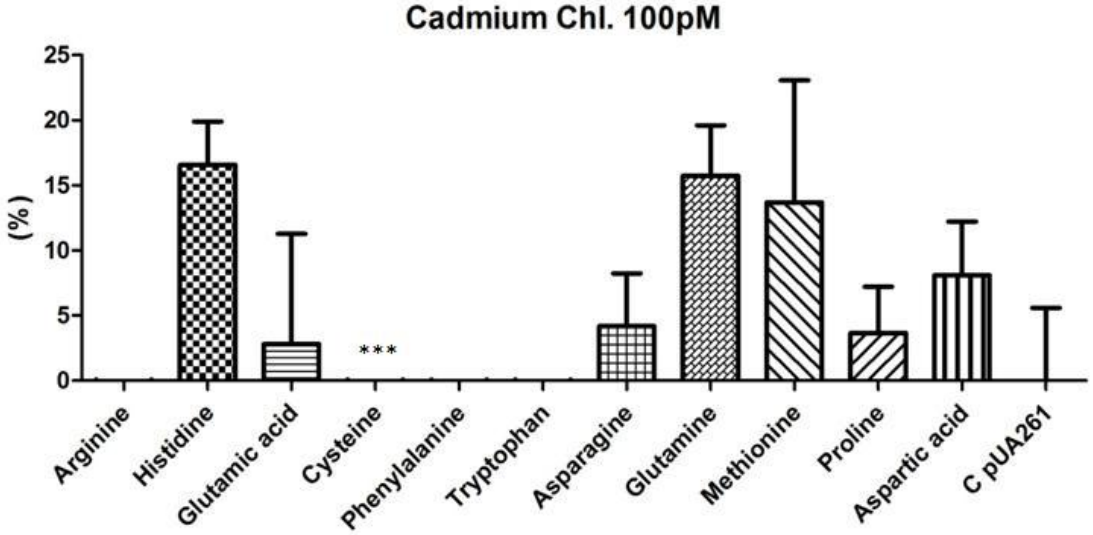


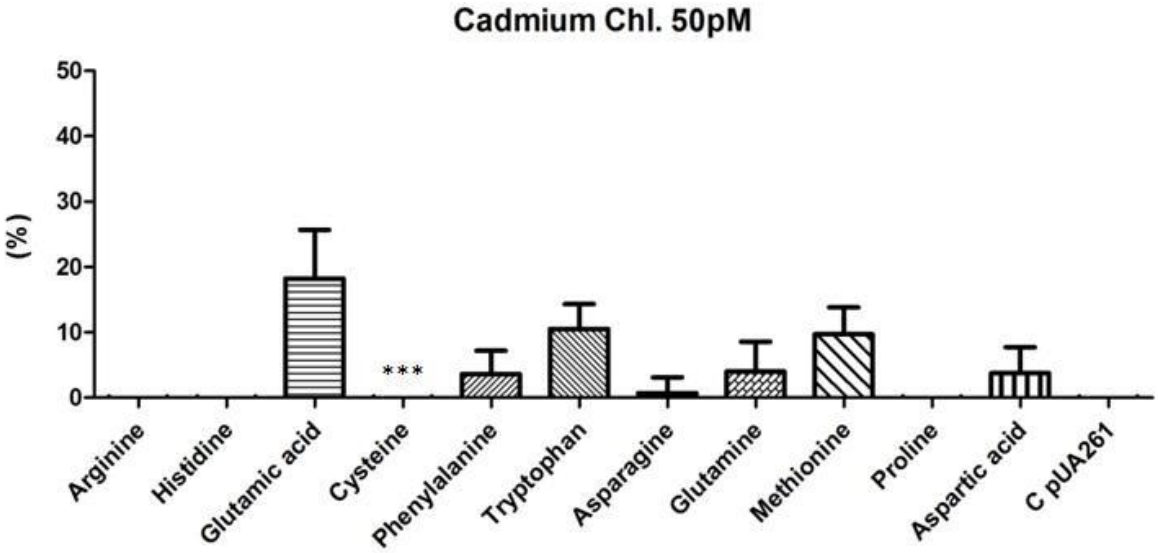
Mean values and SEM for the percentage of the size of the colonies, relatively to the mean values of the control pUA261, of *S. cerevisiae* strain BY4743 growing in regular media with the environmental stressor lithium chloride 0.15 M (A) and 0.3 M (B). The results were normalized considering the size of the colonies of the C pUA261 0%. From statistical analysis one-*way* ANOVA was performed followed by a Dunnett´s test to determine the significant differences between C pUA261 mean and the remaining treatments. Transformants that not grows her value is considering -100% than the growth of control cells.

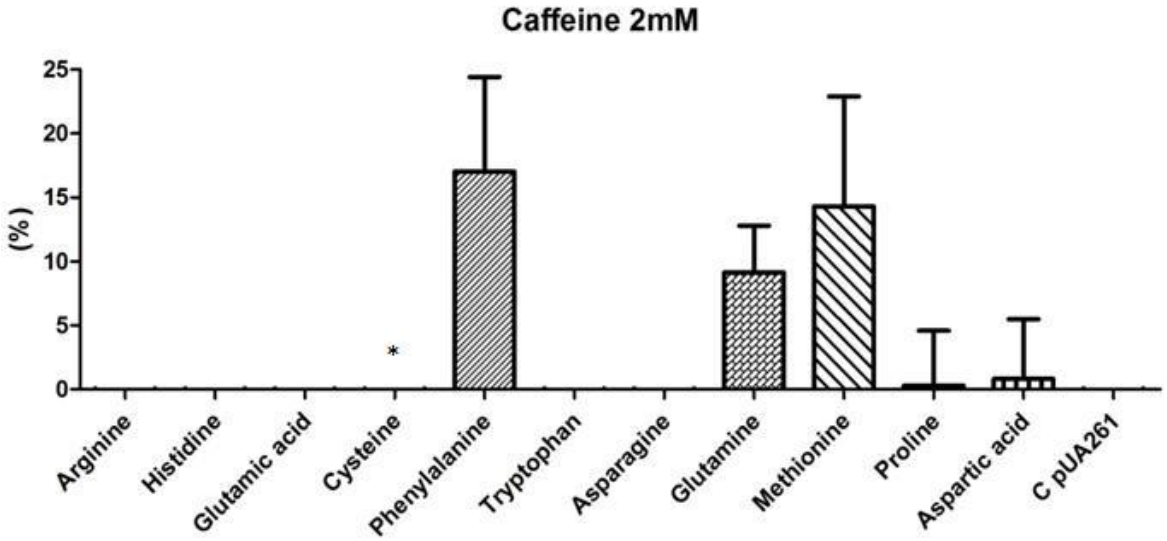
✓ *Complete list of the graphics that illustrate the growth of transformed BY4743 cells under various conditions tested in phenotypic screening*

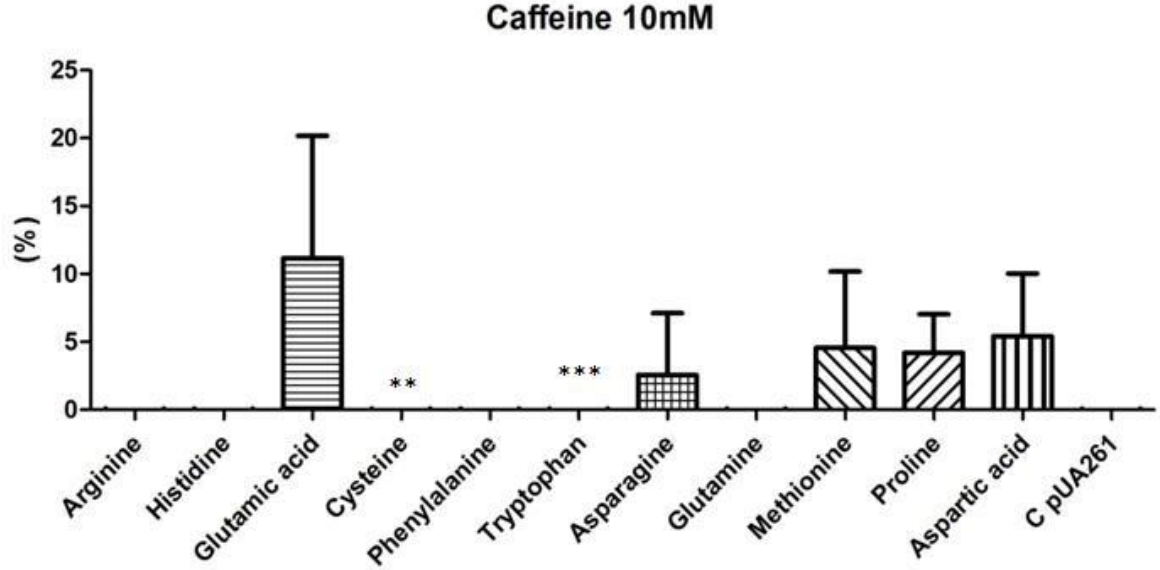


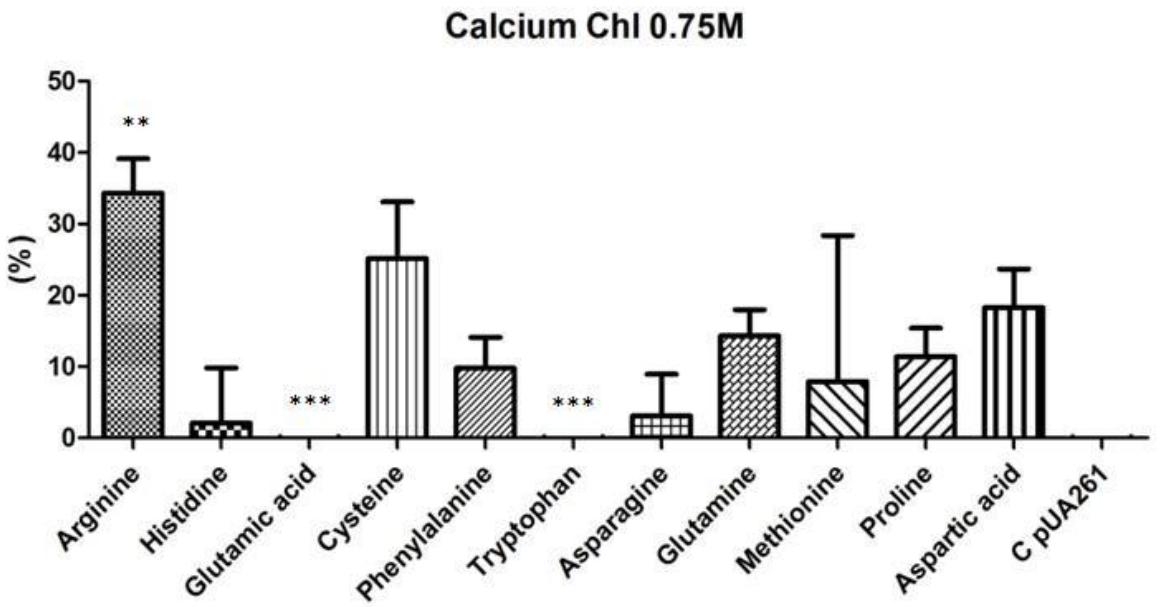


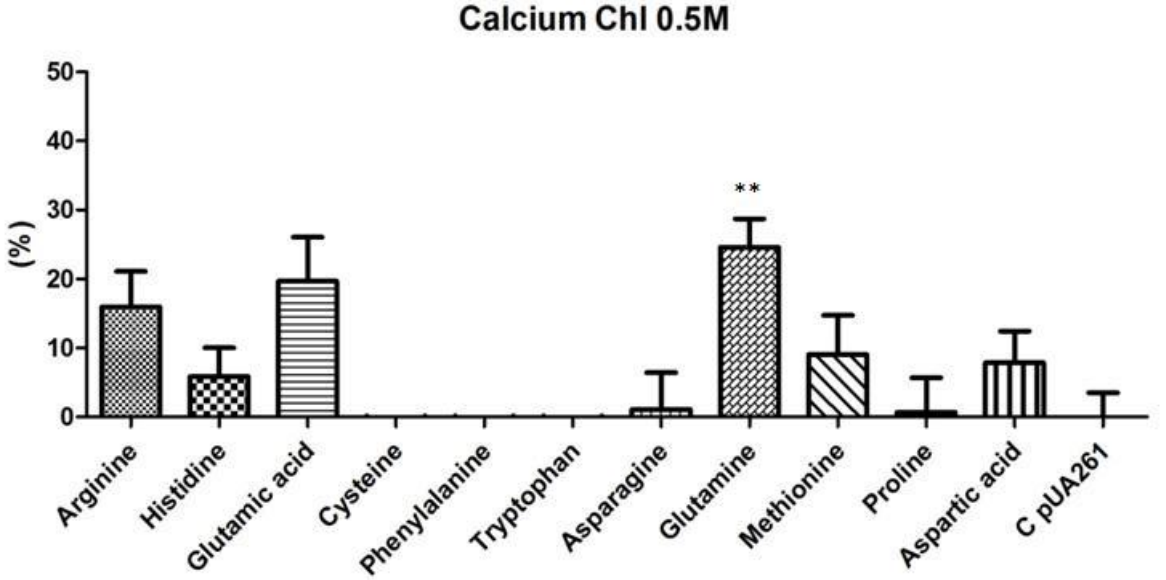


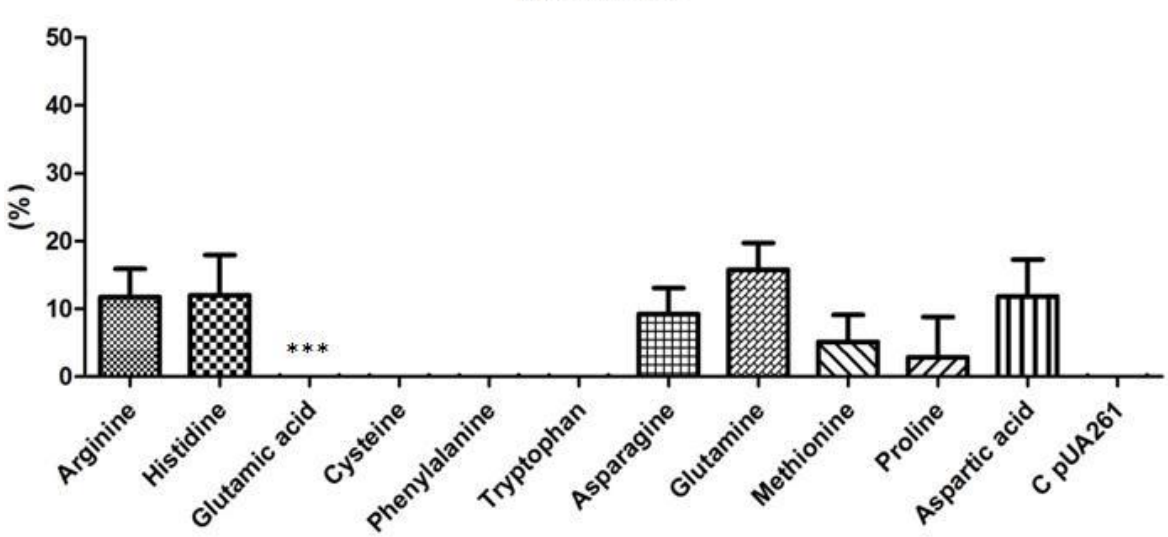

Cadmium Chl. 50pM
(%)
50
40
30
20
10
0
***
Arginine
Histidine
Glutamic acid
Cysteine
Phenylalanine
Tryptophan
Asparagine
Glutamine
Methionine
Proline
Aspartic acid
C pUA261

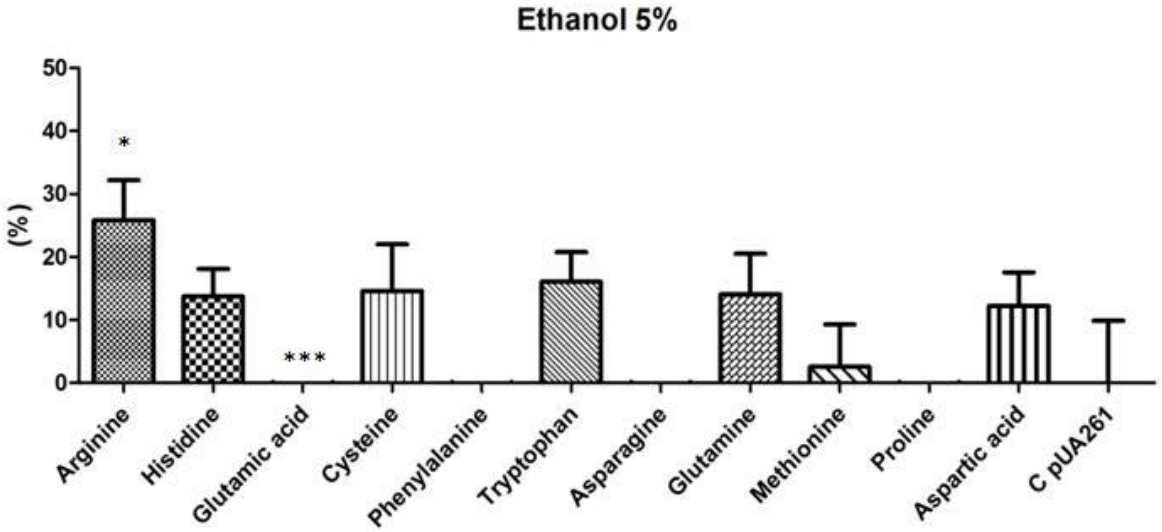

Caffeine 2mM
(%)
25
20
15
10
5
0
*
Arginine
Histidine
Glutamic acid
Cysteine
Phenylalanine
Tryptophan
Asparagine
Glutamine
Methionine
Proline
Aspartic acid
C pUA261

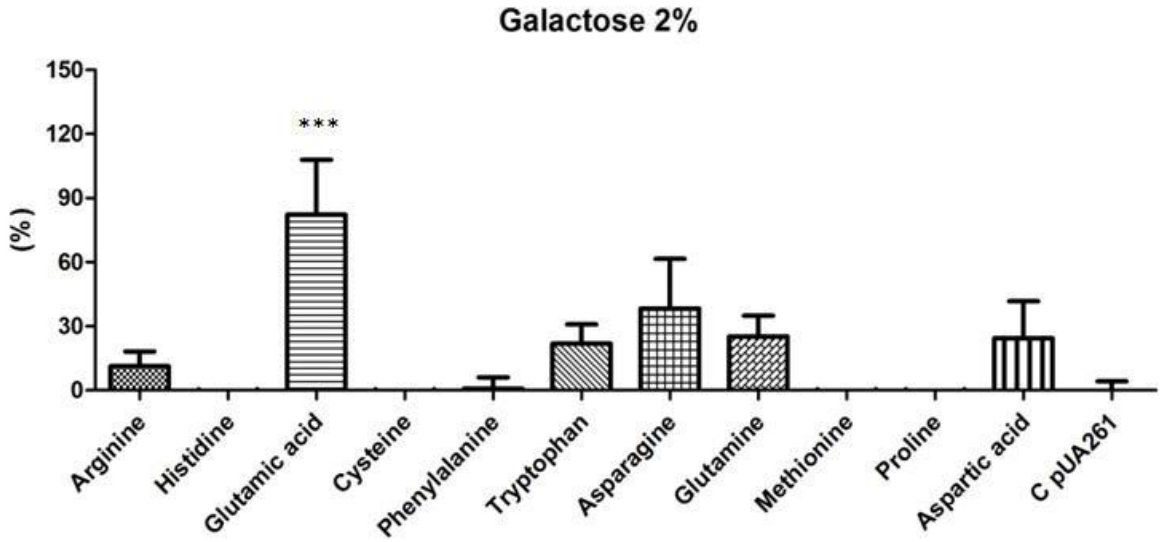

Caffeine 10mM
(%)
25
20
15
10
5
0
**
***
Arginine
Histidine
Glutamic acid
Cysteine
Phenylalanine
Tryptophan
Asparagine
Glutamine
Methionine
Proline
Aspartic acid
C pUA261

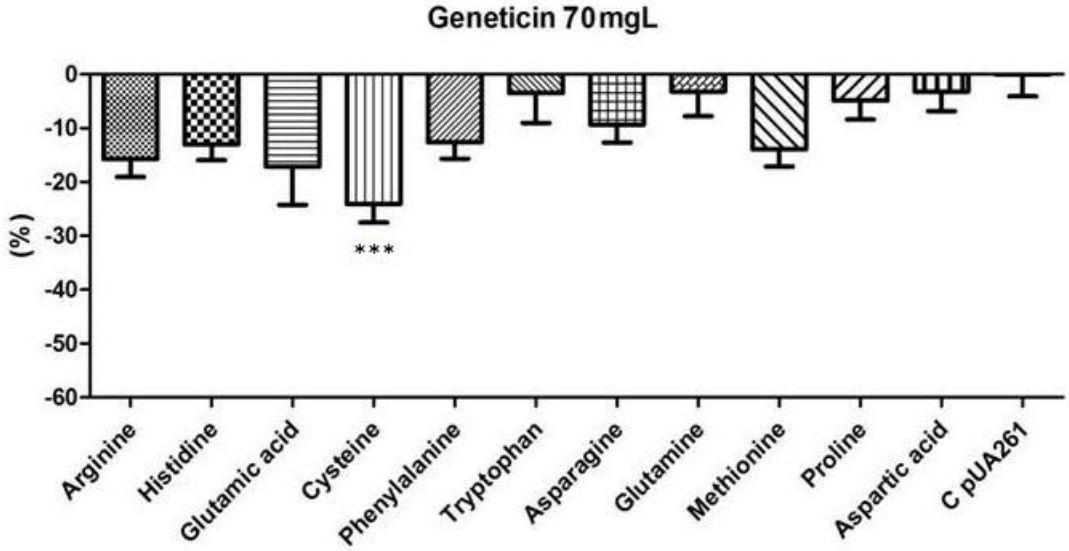

Calcium Chl 0.75M
(%)
50
40
30
20
10
0
**
***
***
Arginine
Histidine
Glutamic acid
Cysteine
Phenylalanine
Tryptophan
Asparagine
Glutamine
Methionine
Proline
Aspartic acid
C pUA261

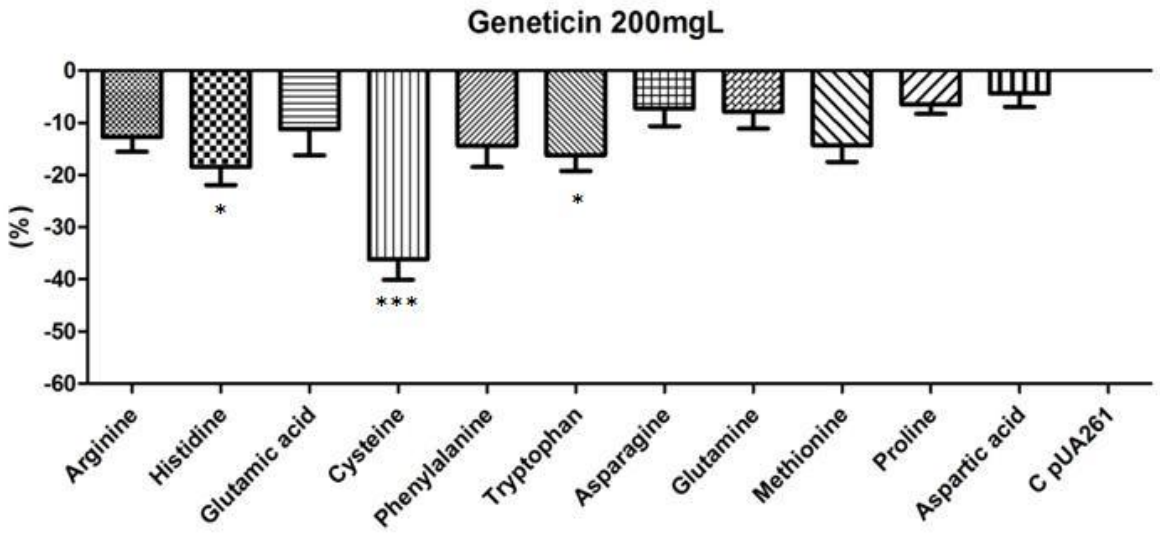

Calcium Chl 0.5M
(%)
50
40
30
20
10
0
**
Arginine
Histidine
Glutamic acid
Cysteine
Phenylalanine
Tryptophan
Asparagine
Glutamine
Methionine
Proline
Aspartic acid
C pUA261

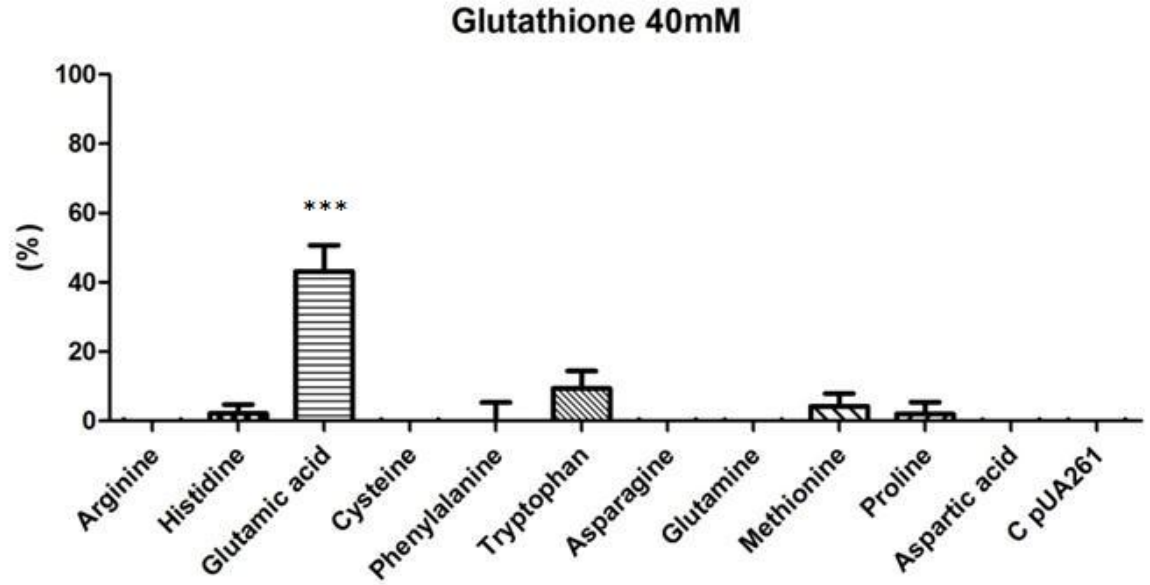

Ethanol 2%
(%)
50
40
30
20
10
0
***
Arginine
Histidine
Glutamic acid
Cysteine
Phenylalanine
Tryptophan
Asparagine
Glutamine
Methionine
Proline
Aspartic acid
C pUA261

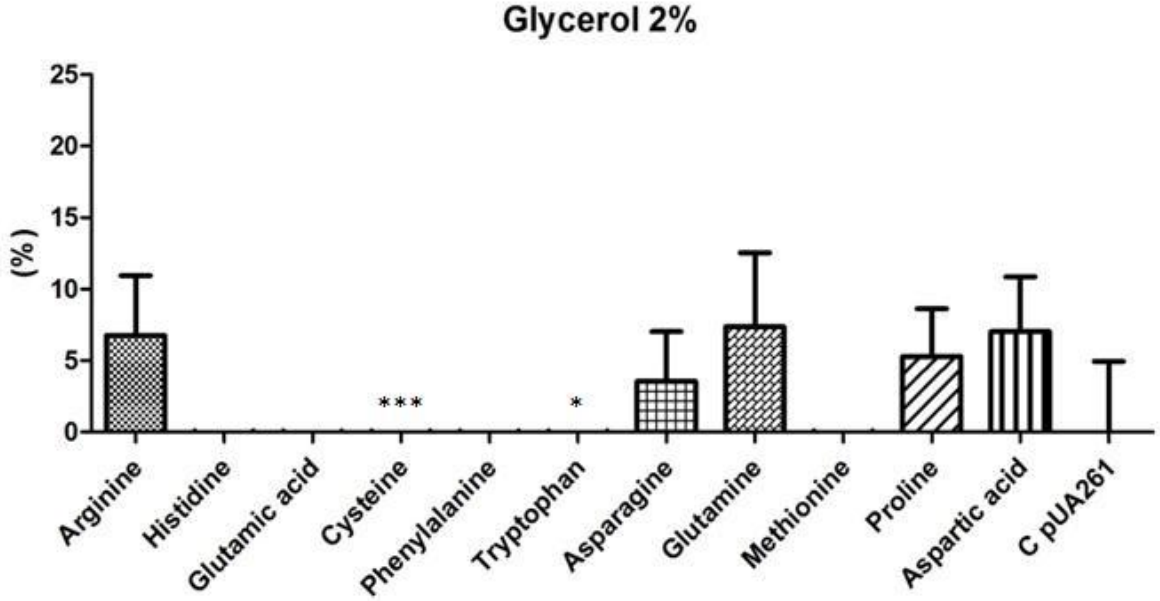

Ethanol 5%
50
40
30
20
10
0
(%)
*
***
Arginine
Histidine
Glutamic acid
Cysteine
Phenylalanine
Tryptophan
Asparagine
Glutamine
Methionine
Proline
Aspartic acid
C pUA261

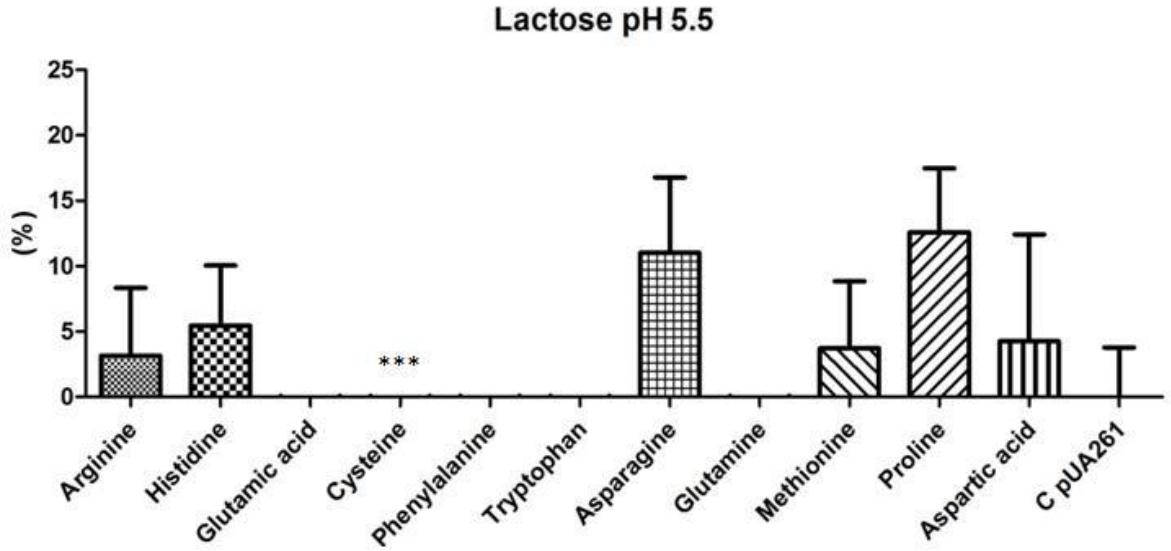

Galactose 2%
150
120
90
60
30
0
(%)
***
Arginine
Histidine
Glutamic acid
Cysteine
Phenylalanine
Tryptophan
Asparagine
Glutamine
Methionine
Proline
Aspartic acid
C pUA261

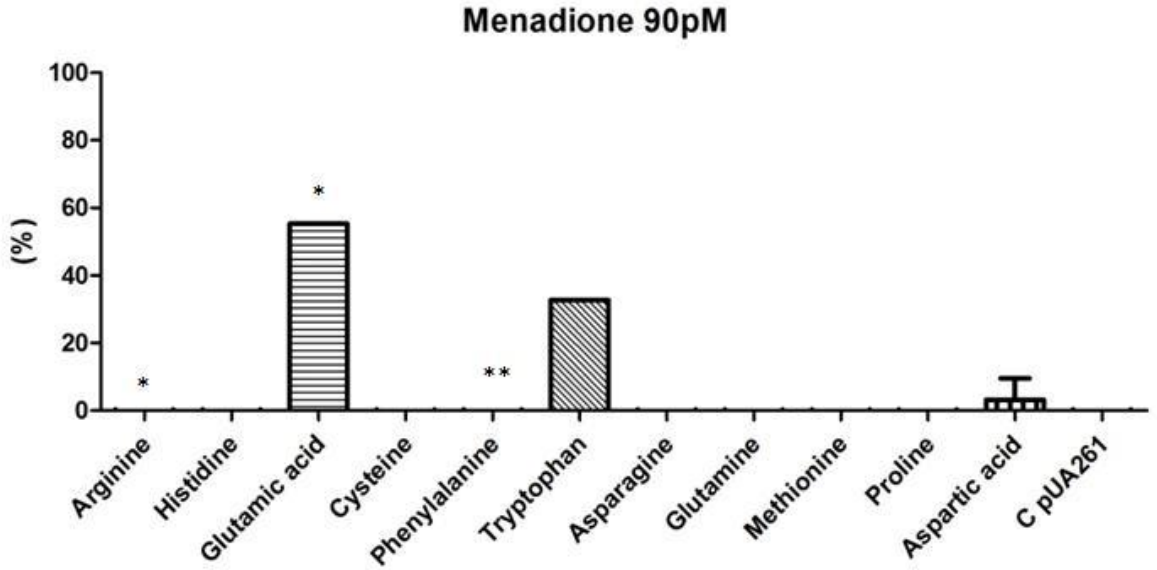

Geneticin 70mgL
0
-10
-20
-30
-40
-50
-60
(%)
***
Arginine
Histidine
Glutamic acid
Cysteine
Phenylalanine
Tryptophan
Asparagine
Glutamine
Methionine
Proline
Aspartic acid
C pUA261

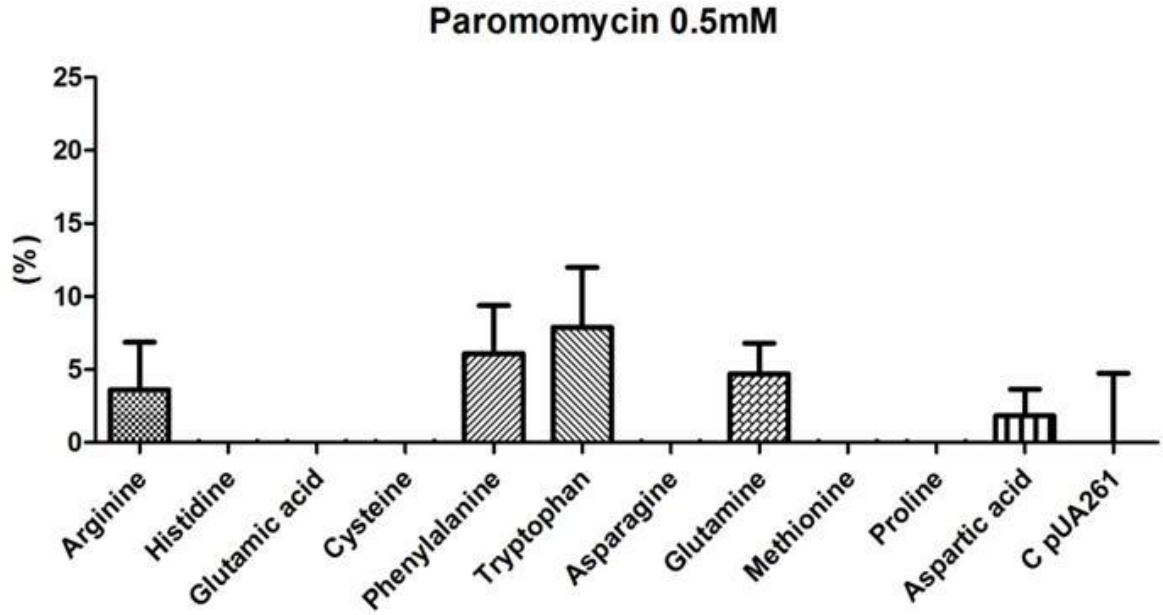

Geneticin 200mgL
0
-10
-20
-30
-40
-50
-60
(%)
*
*
***
Arginine
Histidine
Glutamic acid
Cysteine
Phenylalanine
Tryptophan
Asparagine
Glutamine
Methionine
Proline
Aspartic acid
C pUA261

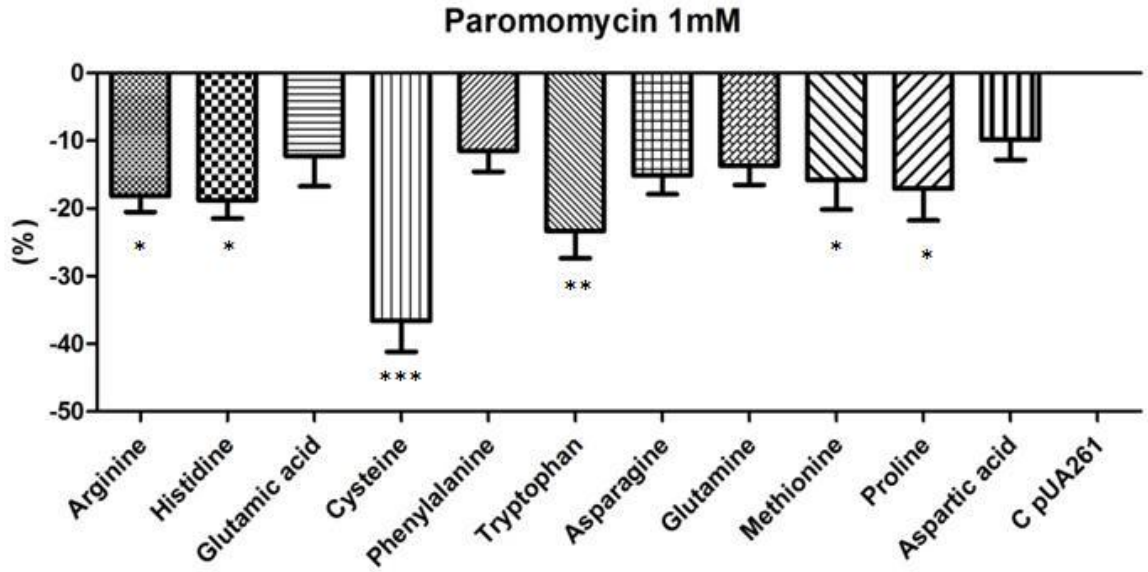

Glutathione 40mM
100
80
60
40
20
0
(%)
***
Arginine
Histidine
Glutamic acid
Cysteine
Phenylalanine
Tryptophan
Asparagine
Glutamine
Methionine
Proline
Aspartic acid
C pUA261

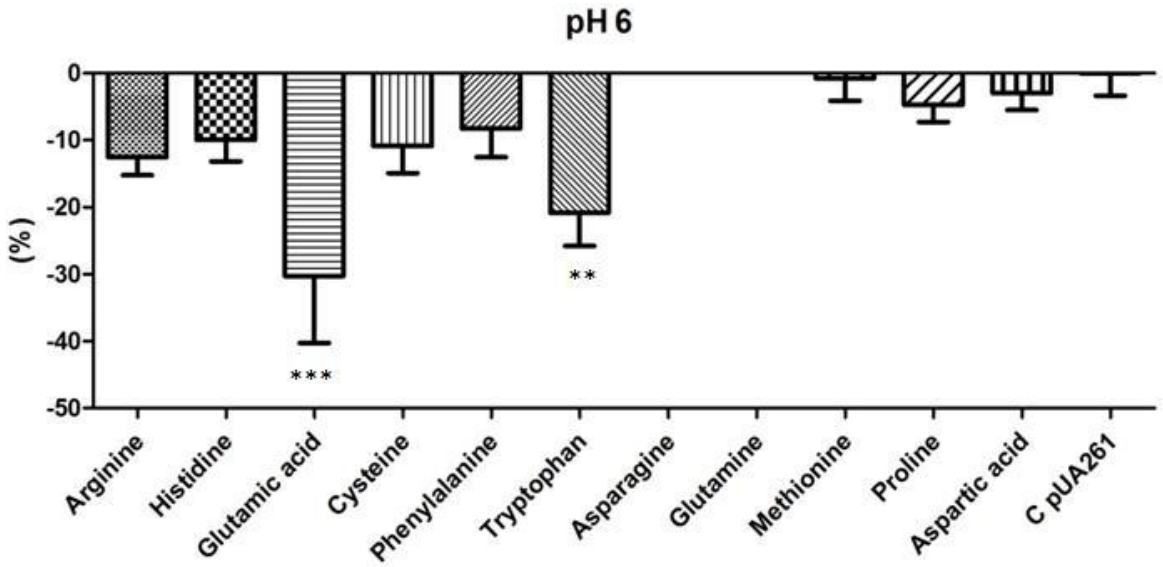

Glycerol 2%
25
20
15
10
5
0
(%)
***
*
Arginine
Histidine
Glutamic acid
Cysteine
Phenylalanine
Tryptophan
Asparagine
Glutamine
Methionine
Proline
Aspartic acid
C pUA261

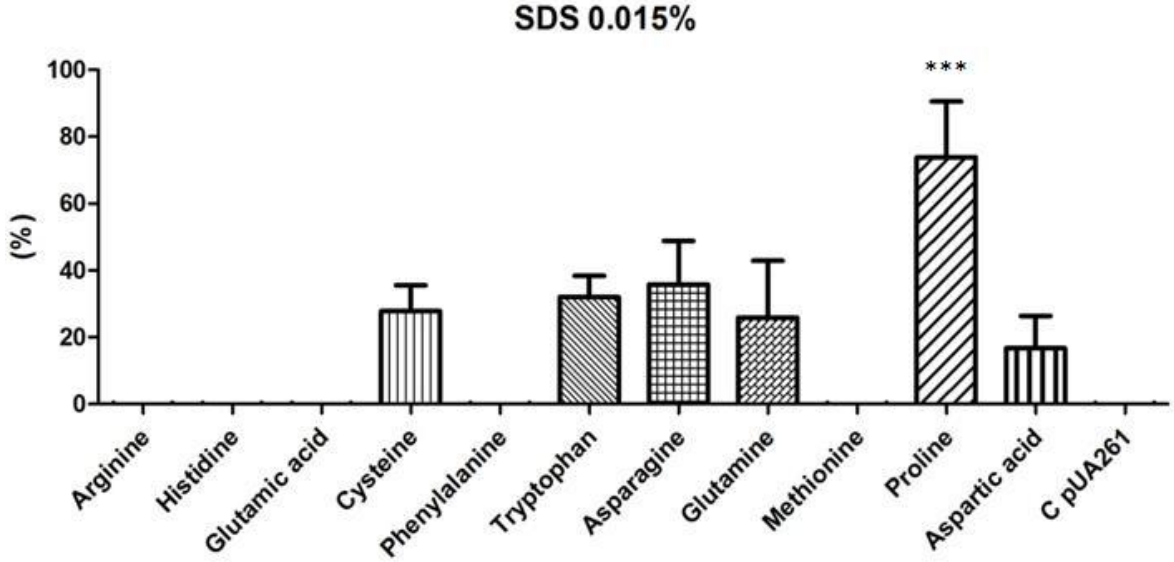

Lactose pH 5.5
(%)
25
20
15
10
5
0
***
Arginine
Histidine
Glutamic acid
Cysteine
Phenylalanine
Tryptophan
Asparagine
Glutamine
Methionine
Proline
Aspartic acid
C pUA261

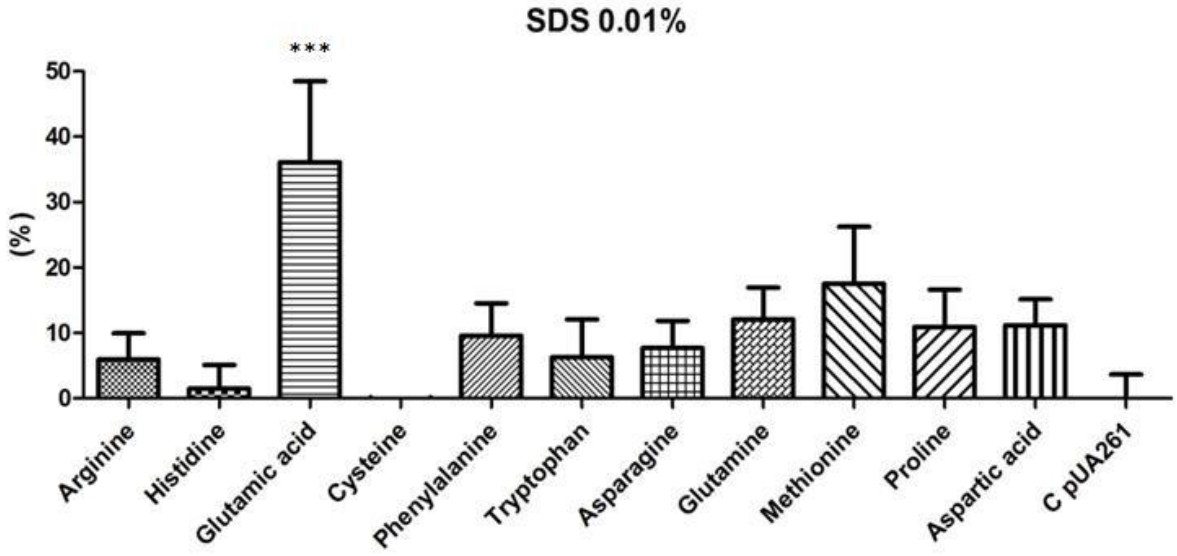

Menadione 90pM
(%)
100
80
60
40
20
0
*
*
**
Arginine
Histidine
Glutamic acid
Cysteine
Phenylalanine
Tryptophan
Asparagine
Glutamine
Methionine
Proline
Aspartic acid
C pUA261

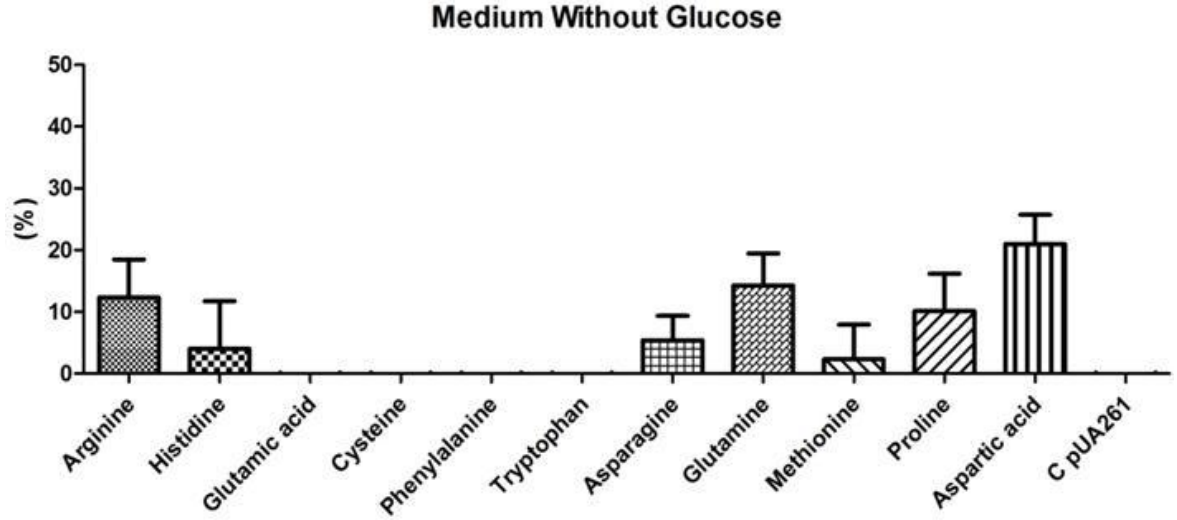

Paromomycin 0.5mM
(%)
25
20
15
10
5
0
Arginine
Histidine
Glutamic acid
Cysteine
Phenylalanine
Tryptophan
Asparagine
Glutamine
Methionine
Proline
Aspartic acid
C pUA261

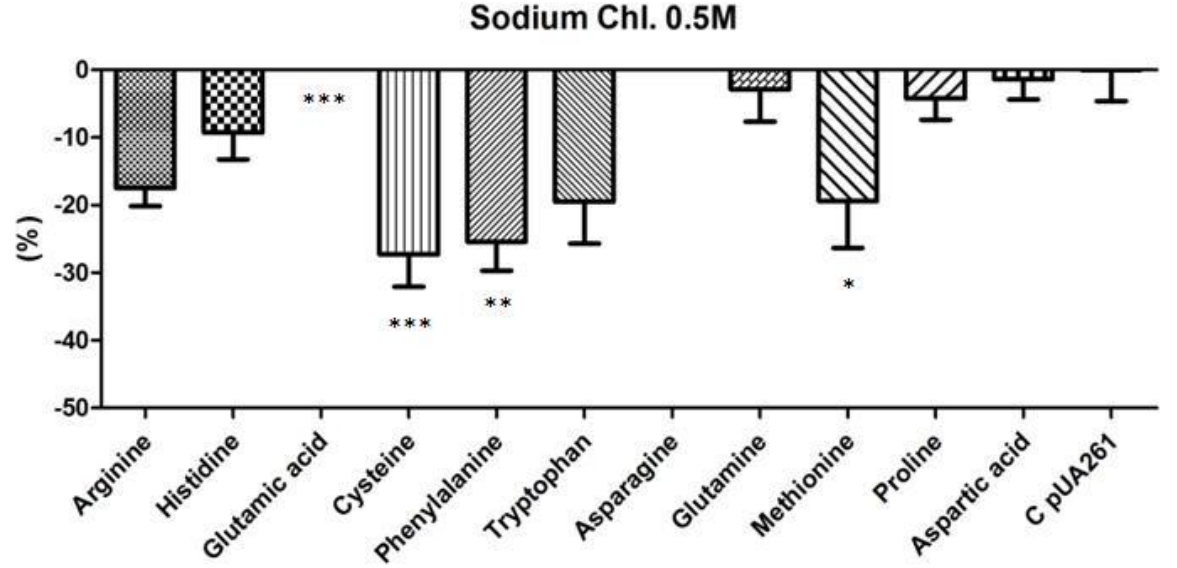

Paromomycin 1mM
0
-10
-20
-30
-40
-50
(%)
*
*
***
**
*
*
Arginine
Histidine
Glutamic acid
Cysteine
Phenylalanine
Tryptophan
Asparagine
Glutamine
Methionine
Proline
Aspartic acid
C pUA261

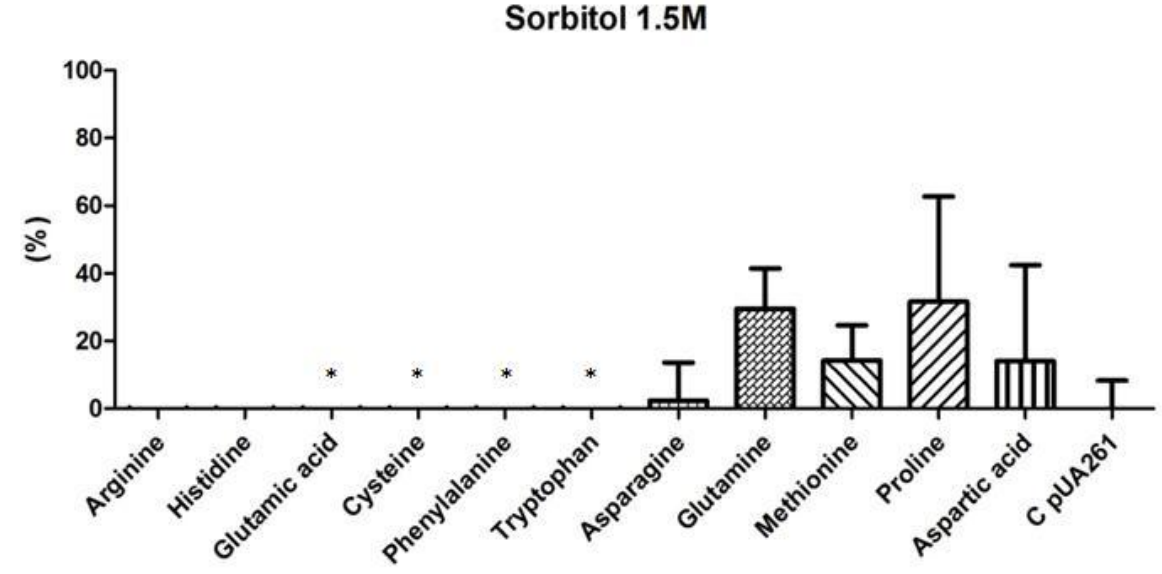

pH 6
0
-10
-20
-30
-40
-50
(%)
***
**
Arginine
Histidine
Glutamic acid
Cysteine
Phenylalanine
Tryptophan
Asparagine
Glutamine
Methionine
Proline
Aspartic acid
C pUA261

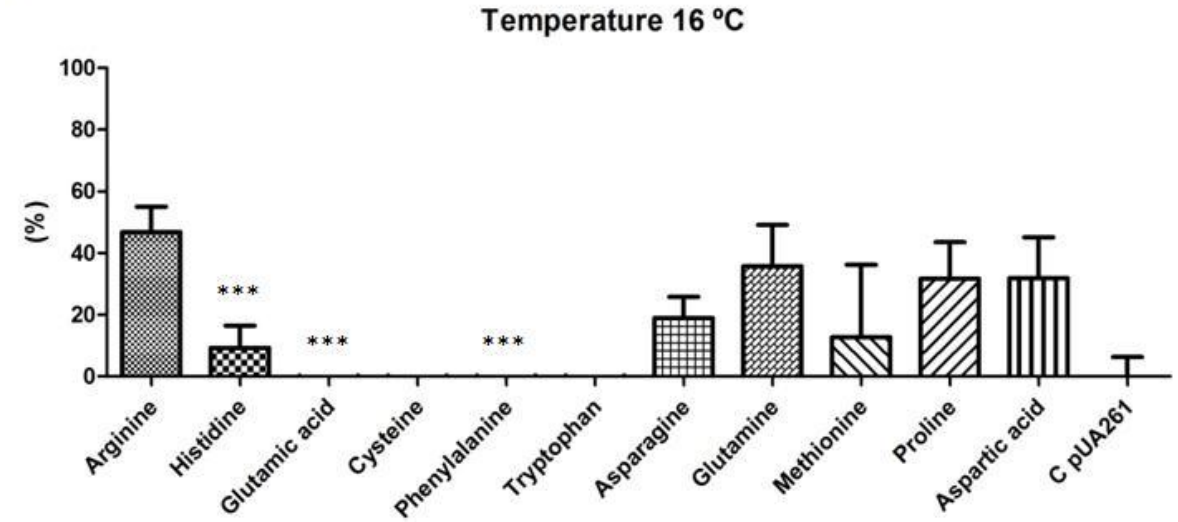

SDS 0.015%
100
80
60
40
20
0
(%)
***
Arginine
Histidine
Glutamic acid
Cysteine
Phenylalanine
Tryptophan
Asparagine
Glutamine
Methionine
Proline
Aspartic acid
C pUA261

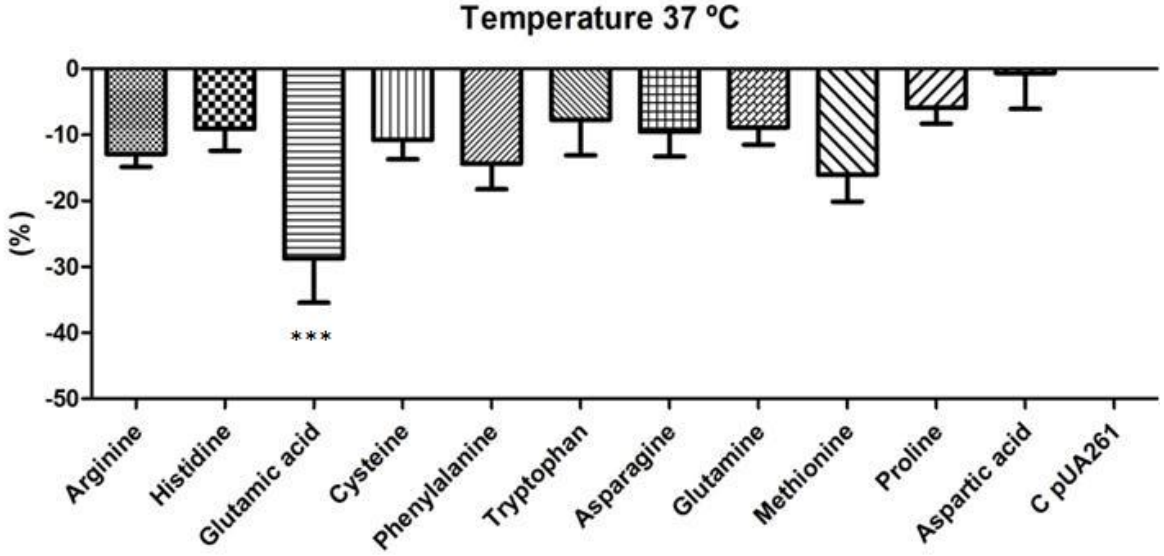

SDS 0.01%
***
50
40
30
20
10
0
(%)
Arginine
Histidine
Glutamic acid
Cysteine
Phenylalanine
Tryptophan
Asparagine
Glutamine
Methionine
Proline
Aspartic acid
C pUA261


Medium Without Glucose
50
40
30
20
10
0
(%)
Arginine
Histidine
Glutamic acid
Cysteine
Phenylalanine
Tryptophan
Asparagine
Glutamine
Methionine
Proline
Aspartic acid
C pUA261


Sodium Chl. 0.5M
0
-10
-20
-30
-40
-50
(%)
***
***
**
*
Arginine
Histidine
Glutamic acid
Cysteine
Phenylalanine
Tryptophan
Asparagine
Glutamine
Methionine
Proline
Aspartic acid
C pUA261


Sorbitol 1.5M
100
80
60
40
20
0
(%)
*
*
*
*
Arginine
Histidine
Glutamic acid
Cysteine
Phenylalanine
Tryptophan
Asparagine
Glutamine
Methionine
Proline
Aspartic acid
C pUA261

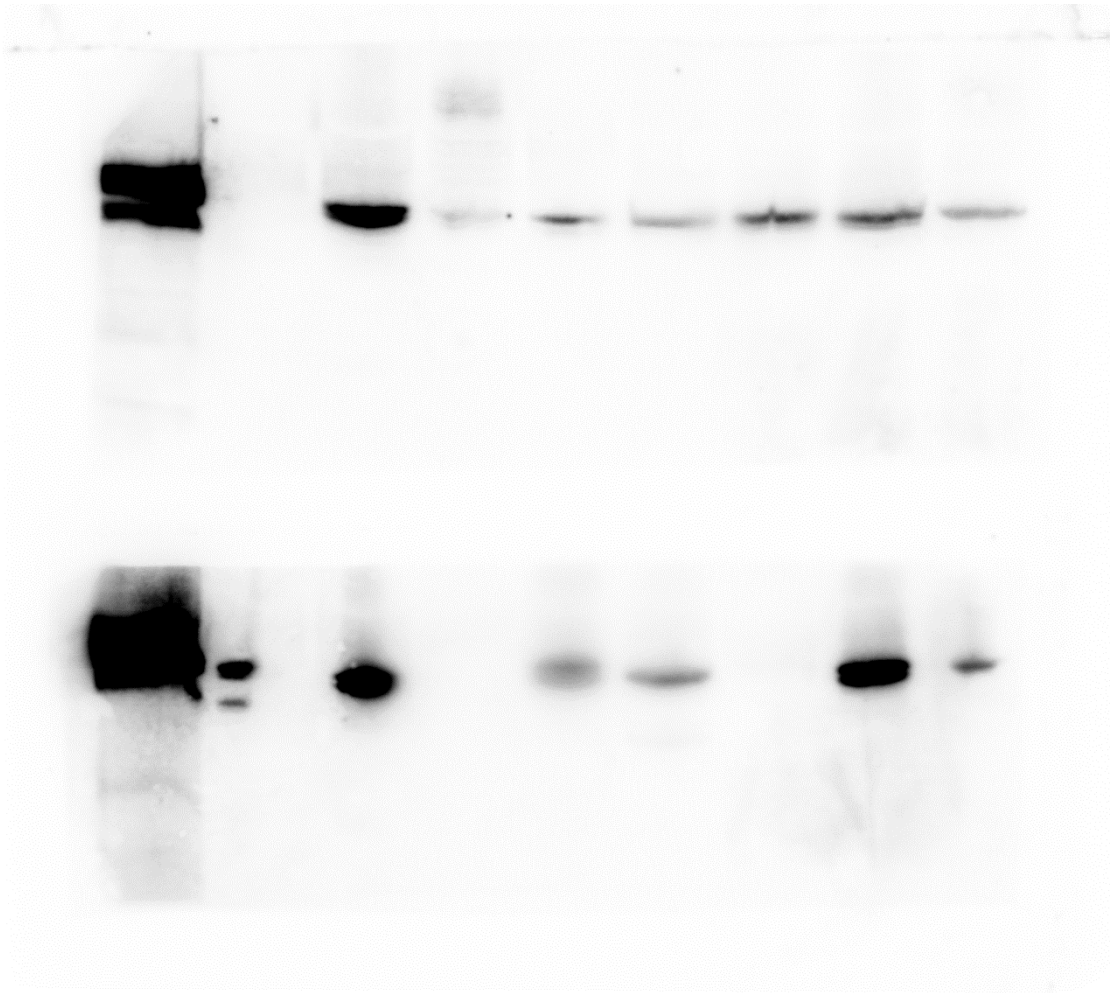

Temperature 16 ºC
100
80
60
40
20
0
(%)
***
***
***
Arginine
Histidine
Glutamic acid
Cysteine
Phenylalanine
Tryptophan
Asparagine
Glutamine
Methionine
Proline
Aspartic acid
C pUA261


Temperature 37 ºC
0
-10
-20
-30
-40
-50
(%)
***
Arginine
Histidine
Glutamic acid
Cysteine
Phenylalanine
Tryptophan
Asparagine
Glutamine
Methionine
Proline
Aspartic acid
C pUA261

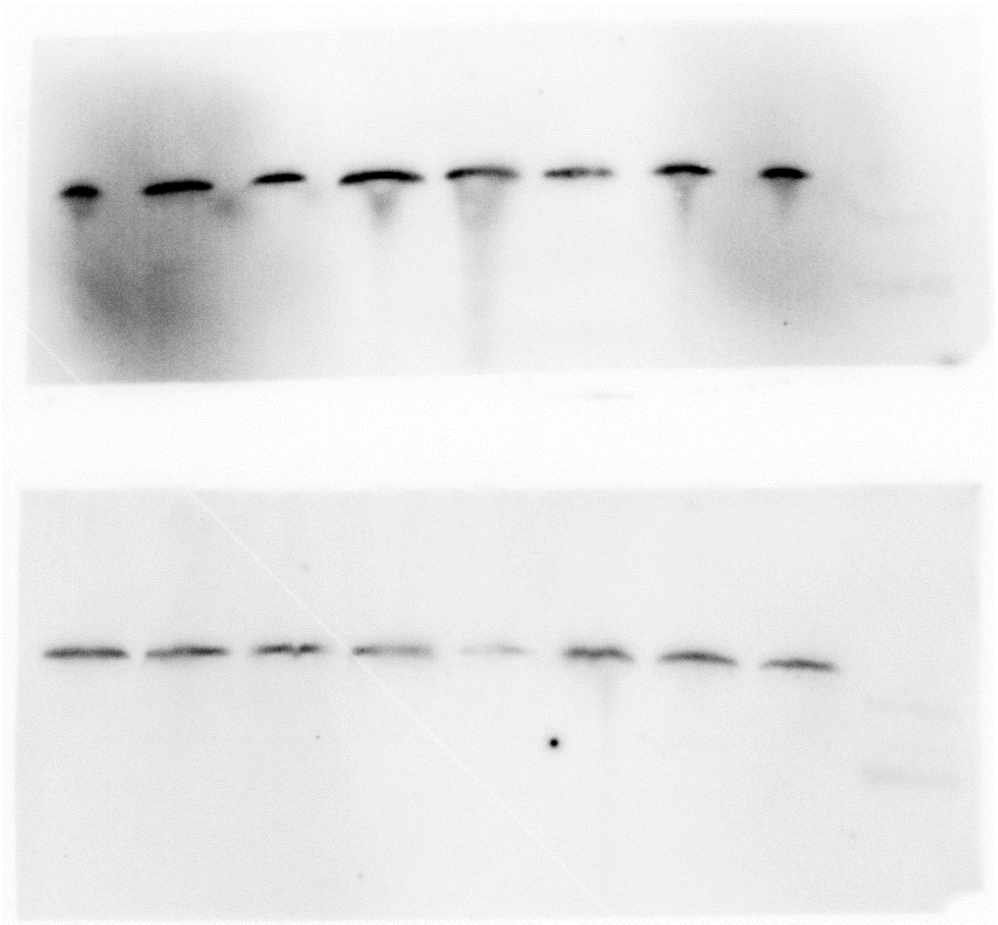
Annex H

✓ *Northen blot membranes – original images*

A)



B)



Images of the two membranes resulting from the northern blot performed with RNA from the transformants of *S. cerevisiae* strains BY4743 and YLL026W. Membrane hybridized with the serine probe (**A**) and the membrane hybridized with a control probe (glycine) (**B**).