Universidade de Aveiro Departamento de Biologia
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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Doutor em Biologia, realizada sob a orientação científica do Doutor Manuel António Silva Santos, Professor Associado do Departamento de Biologia da Universidade de Aveiro

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Dedico este trabalho à minha Mãe e ao meu Pai, pois finalmente reconheci a sua razão.

o júri

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palavras-chave Código genético, ambiguidade, *Candida albicans*, diversidade fenotípica.

resumo

A maioria dos organismos utiliza o mesmo código genético, no entanto alterações a este código padrão foram descobertas em procariotas e eucariotas. A maior parte das alterações ao código genético ocorre em mitocôndrias. No citoplasma eucariótico, o único exemplo conhecido de alteração ao código genético envolvendo a substituição de um aminoácido por outro aminoácido, ocorre em várias espécies do género *Candida*. Em *Candida albicans* o codão CUG é ambíguo, ou seja, pode ser traduzido como serina ou leucina, com predominância para o primeiro aminoácido. Na origem desta ambiguidade está um $tRNA_{CAG}^{Ser}$ de *C. albicans* que possui elementos de identidade para duas aminoacil-tRNA sintetases, nomeadamente a seril e leucil-tRNA sintetases, podendo, por isso, ser aminoacilado com serina e leucina. Este tRNA surgiu há cerca de 272 milhões de anos no antepassado das leveduras e introduziu dupla identidade (ambiguidade) no codão CUG que começou a ser descodificado como leucina e serina. As consequências biológicas desta ambiguidade e da alteração de identidade do codão CUG de leucina para serina são desconhecidas. O objectivo deste estudo foi elucidar a função biológica da ambiguidade do codão CUG que foi preservada em *C. albicans*. Pretendeu-se compreender porque é que a ambiguidade do codão CUG foi preservada e conhecer melhor os mecanismos de evolução ao código genético. Para tal, aumentou-se a ambiguidade do codão CUG, usando engenharia de tRNAs e estudaram-se as consequências de tal ambiguidade ao nível fenotípico. Os resultados demonstram de forma inequívoca que a ambiguidade do codão CUG é um gerador de diversidade fenotípica e sugerem que uma das funções da alteração ao código genético é potenciarem a evolução rápida de novos fenótipos. A ambiguidade do codão CUG induz a expressão de vários factores de virulência de *C. albicans*, nomeadamente variabilidade morfológica, alteração fenotípica, produção de hidrolases extracelulares e adesinas. Assim, a ambiguidade do código genético é fundamental para a biologia de *C. albicans.*

keywords Genetic code, ambiguity, *Candida albicans*, phenotypic diversity.

abstract Most organisms use the same genetic code, however several alterations to the standard code have been found in prokaryotes and eukaryotes. Most alterations occur in mitochondria and the only known case of a cytoplasmatic sense-to-sense codon identity change occurs in several species of the genus *Candida*. In *Candida albicans*, standard leucine-CUG codon is decoded mainly as serine but to a lesser extent as leucine. This is due the existence of a novel $tRNA_{CAG}^{Ser}$ that has identity elements for both the seryl- and the leucyl-tRNA aminoacyl synthetases and hence can be aminoacylated with serine and leucine. The $tRNA_{CAG}^{Ser}$ appeared 272 million years ago in the yeast ancestor, and created a CUG codon with double identity due to its decoding as both serine and leucine. The biological function of such ambiguity, which was preserved to the present day, is still unknown. The objective of this study was to elucidate the role of CUG ambiguity in *C. albicans* biology. An attempt was made to shed new light i) on the biological role of genetic code ambiguity, ii) on why CUG ambiguity was preserved and iii) on why genetic code alterations evolve. For this, highly ambiguous *C. albicans* strains were created through tRNA engineering techniques and the effects of such ambiguity were studied at phenotypic level. The data presented herein shows for the first time that genetic code ambiguity is a generator of phenotypic diversity and strongly suggests that genetic code alterations speed up evolution of new phenotypes. Ambiguous decoding of the CUG codon triggers expression of *C. albicans* virulence factors, namely morphogenesis, phenotypic switching, extracellular hydrolases production and adhesion, indicating that it plays a critical role on *C. albicans* biology.

Contents

ABBREVIATIONS

A (mA)	Ampere (milliAmpere)
Amp	ampicilin
APS	ammonium persulphate
Asn	asparagine
Asp	aspartate
ATP	adenosine 5'-triphosphate
bp (kbp)	base pair (kilobases)
Ci (µCi)	curie (microcurie)
cm (mm, nm)	centimeter (milimeter, nanometer)
CHAPS	3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate
CTP	cytidine 5'-triphosphate
Cys	cysteine
2D	Two dimensional
2-DE	Two dimensional electrophoresis
DEAE	diethylaminoethyl
DIC	differential interference contrast
DNA	deoxyribonucleic acid
DTT	dithiothreitol
EDTA	ethylenediamine tetracetic acid
g (mg, µg, ng)	gram (miligram, microgram, nanogram)
g	gravitational force
Glu	glutamate
Gly	glycine
h	hour
His	histidine
KDa	Kilodalton
l (ml, µl)	litre (mililitre, microlitre)
Lys	lysine
M	minute
M (mM, µM)	molar (milimolar, micromolar)

MCA	4-methylcoumaryl-7-amide
mRNPs	mRNA-protein complexes
NTP	nucleotide tri-phosphate
PEG	polyethylene glycol
Phe	phenylalanine
pm	picomoles
Pro	proline
RNA	ribonucleic acid
RNAse	ribonuclease
rpm	rotations per minute
s	second
SDS	sodium dodecyl sulphate
Tris	2-amino-2-hydroxymethyl-1,3-propanodiol
Triton X-100	octyl phenoxy polyethoxyethanol
Tyr	tyrosine
U	Units
UV	ultraviolet
w	weight
Val	valine
v	volume
V	volts

1. Introduction

PART A – Genetic code

PART B – *Candida albicans* biology

PART C - Objectives

PART A

1.1. Genetic code and its variants

The genetic code establishes a relationship between 22 amino acids, involved in protein synthesis, and the 64 possible combinations of the four ribonucleotides, adenosine (A), guanine (G), uracil (U) and cytosine (C), in form of triplets (codons) (Crick, 1966b). The biomolecule responsible for this link between nucleotides and amino acids is the transfer RNA (tRNA), as it carries an amino acid at its 3'-end and base-pairs with the messenger RNA (mRNA) codon through a triplet base sequence (anticodon) in the ribosome (Soll & RajBhandary, 1967; Crick, 1966b; HOAGLAND *et al.*, 1958). The universal genetic code established in the later 1960's showed degeneracy, i. e., more than one codon was assigned to each of the 22 amino acids, called "synonymous", where the third letter of the triplet is changeable. Thus, 61 codons are assigned to the 22 amino acids the remaining three are termination codons. In 1966, Crick proposed the "Frozen Accident Theory", which postulates that the genetic code was universal, that is evolved from a common ancestor with a complex proteome and that any alteration to it would cause major disruption and consequently would be lethal or strongly selected against (Crick, 1966a).

In the late 1970's, a genetic code variation was found by Barrel and colleagues (Barrell *et al.*, 1979) in human mitochondria, where the UGA-stop codon is decoded as tryptophan (Trp). Since then, a number of variations to the genetic code have been reported, not only in mitochondria but also in eubacterial and eukaryotic nuclear systems (Figure 1.1). Although all organisms share the same genetic code there are variations in a wide range of them (Santos & Tuite, 2004; Knight *et al.*, 2001b; Osawa *et al.*, 1992). Some genetic code changes are transversal to organisms with no phylogenetic relationship, namely diplomonads, ciliates and green alga *Acetabularia acetabulum*, where the termination codons UAA and UAG where reassigned to glutamine (Gln) (Osawa *et al.*, 1992). Animal and yeast mitochondria also reassigned the AUA codon from isoleucine (Ile) to methionine (Met)

(Andersson & Kurland, 1995). Despite this, similar changes happen in closely related lineages with consequent evolutionary implications. This is valid for yeasts (Sugita & Nakase, 1999), ciliates (Tourancheau *et al.*, 1995), the mitochondria of diatoms (Ehara *et al.*, 2000), algae (Hayashi-Ishimaru *et al.*, 1996) and metazoan (Castresana *et al.*, 1998). In yeasts, alternative CUG decoding originated a distinct clade in the hemiascomycetes group composed by several *Candida* species and *Debaryomyces hansenii* (Sugita & Nakase, 1999). Furthermore, *Candida glabata*, which posses a standard CUG decoding, is much more closely related to the *Saccharomyces sensu stricto* group than the other yeasts, including *C. albicans* (Dujon *et al.*, 2004). The study of deviant genetic codes and their phylogenetic implications have been carried out by mitochondrial cytochrome oxidase subunit I (*COXI*) gene sequence analyses in diatoms, algae and metazoan. Interestingly, the mitochondrial genetic code appears to be a useful genetic marker for classification (Ehara *et al.*, 2000).

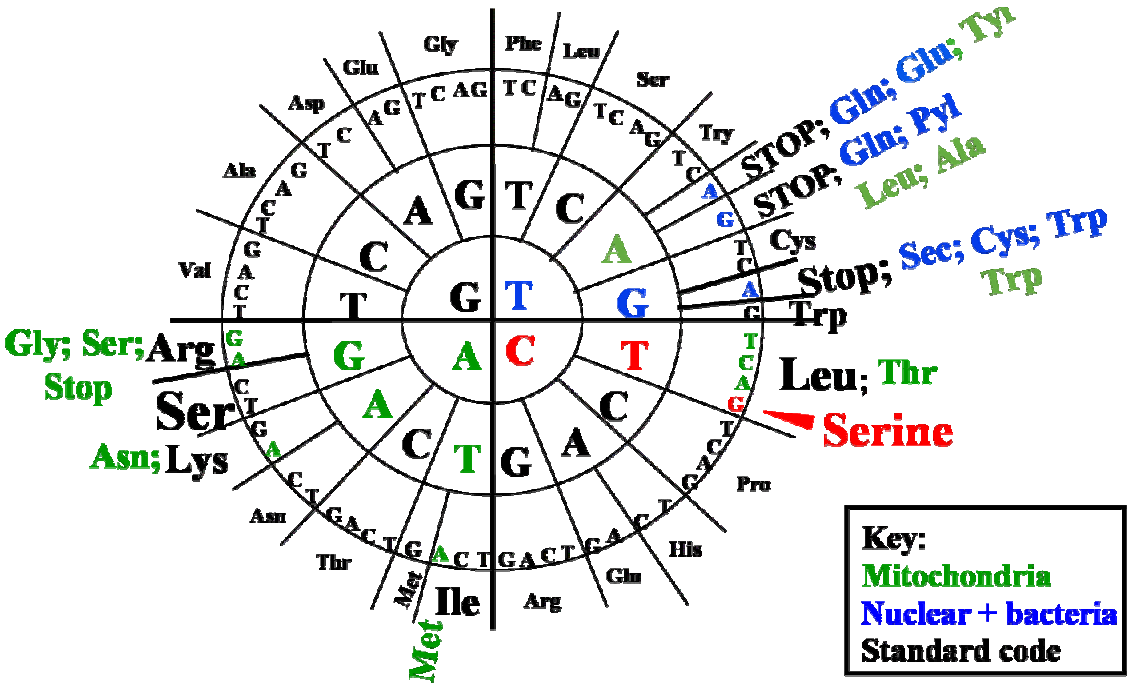


Figure 1.1. Genetic code in 2006. Standard genetic code and deviations found in archea, eubacteria, mitochondria and eukaryotic cytoplasmatic protein synthesis. In mitochondrial systems, there are sense and non-sense reassignments, while in prokaryotic and eukaryotic cytoplasmatic translation there is only non-sense to sense reassignments. The exception is the sense to sense reassignment of the CUG codon in *Candida* species (Santos *et al.*, 1997).

Mitochondrial genetic code changes are more frequent than cytoplasmatic ones. However, codons that have been lost or changed identity (reassigned) in cytoplasmatic translation systems have also been lost or reassigned in mitochondria (Knight *et al.*, 2001b).

Non-sense to sense codon reassignments, and vice-versa, are more frequent than sense-to-sense reassignments (Figure 1.1). For example the termination codon UAG has been reassigned to leucine (Leu), alanine (Ala), and Gln, while the AGG and AGA have been reassigned from arginine (Arg) to serine (Ser), glycine (Gly) and Stop. This is because non-sense suppressor tRNAs are frequently found in many organisms, release factors (RFs) mutate frequently and stop codons only appear once in each gene and consequently their reassignment causes minimal proteome damage (Osawa *et al.*, 1992).

Stop codons are also involved in expansion of the genetic code. In all three domains of life (with few exceptions), the UGA stop codon is reprogrammed in the selenoprotein mRNAs to selenocystein (21st amino acid) (Thanbichler & Bock, 2002; Bock, 2000; Ehrenreich *et al.*, 1992). In the archeon, *Methanosarcina barkeri*, the UAG stop codon is also reprogrammed to pyrrolysine (22nd amino acid) (Hao *et al.*, 2002; Srinivasan *et al.*, 2002). The mechanism of selenocysteine (Sec) insertion is known in detail. It requires a selenocysteine insertion sequence (SECIS element), which is an hairpin structure located in 3'-unstranslated regions (3'-UTRs) of selenoprotein mRNAs in eukaryota and archea, or immediately downstream of Sec-UGA codons in eubacteria (Hatfield & Gladyshev, 2002; Rother *et al.*, 2001; Low & Berry, 1996; Berry *et al.*, 1993; Bock *et al.*, 1991; Zinoni *et al.*, 1990; Leinfelder *et al.*, 1988; Zinoni *et al.*, 1987). SECIS is essential for Sec insertion since in its absence UGA is used as a normal stop codon (Fourmy *et al.*, 2002). SECIS elements function by recruiting *SBP2* (SECIS-binding protein 2), and both form a complex which binds to EFsec (Sec-specific elongation factor), recruits Sec tRNA$^{[Ser]Sec}$ and inserts Sec into nascent polypetides in response to UGA codons (Fagegaltier *et al.*, 2000; Tujebajeva *et al.*, 2000). Sec tRNA$^{[Ser]Sec}$ is the only tRNA that governs the expression of selenoproteins

and, therefore, plays a key role in selenoprotein byosynthesis (Ohama *et al.*, 1994; Bock *et al.*, 1991). It is the longest eukaryotic tRNA with an atypically long variable arm and the presence of 13 nucleotides in the acceptor and TψC stem helices instead of the 12 normally found in all other tRNAs (Amberg *et al.*, 1993; Diamond *et al.*, 1993). Biosynthesis of Sec occurs on its tRNA, Sec-tRNA$^{[Ser]Sec}$ is initially aminoacylated with serine in both prokaryotes (Bock, 2001b) and eukaryotes (Carlson *et al.*, 2001), which serves as the backbone for Sec synthesis. In *E. coli*, a pyrodoxal phosphate-dependent Sec synthethase catalyzes the removal of the hydroxyl group from serine to form an aminoacrylyl intermediate, which serves as the acceptor for activated selenium, resulting in the formation of selenocysteyl-tRNA$^{[Ser]Sec}$ (Bock, 2001a; Bock *et al.*, 1991).

Incorporation of pyrrolysine (Pyl) in monomethylamine methyltransferase (MtmB1) of *M. barkery* is mediated by a Pyl-tRNA with CUA anticodon *(*Hao *et al.*, 2002), which is specifically charged with Pyl by a PylRS (Polycarpo *et al.*, 2004; Blight *et al.*, 2004). Similarly to Sec it is thought that Pyl is inserted at UAG codons signalled by a hairpain structure, PYLIS (pyrrolysine insertion sequence), located immediately downstream the stop codon (Theobald-Dietrich *et al.*, 2005; Namy *et al.*, 2004; Schimmel & Beebe, 2004).

In some rare cases, entire codon families (4 codons), are reassigned through structural alteration of the translational machinery, which normally involves duplication and mutation of tRNAs. For example, in yeast mitochondria the leucine CUN codon family has been reassigned to threonine (Thr) *(*Bonitz *et al.*, 1980). Other cases involve split codon families, namely the arginine AGR codons (R is A or G), that were reassigned to serine, glycine and stop in many metazoans (Osawa *et al.*, 1989).

In the standard genetic code synonymous codons with the same initial doublet (e.g. CGN) can be either split or unsplit. The following codon families UCN (serine), CUN (leucine), CCN (proline), CGN (arginine), ACN (threonine), GUN (valine), GCN (alanine) and GGN (glycine) are unsplit since they code for the

same amino acid. Other codon families are 2/2 split between the pyrimidines (Y) and the purines (R). For example, UUY (phenylalanine)/UUR (leucine); UAY (tyrosine)/UAR (stop); CAY (histidine)/CAR (glutamine); AAY (asparagines)/AAR (lysne); AGY (serine)/AAGR (arginine) and GAY (aspartate)/GAR (glutamate). The AUN codon family is split 3/1 between Ile and Met and the UGN codon family is split in cysteine (Cys), Trp and stop. In variant codes, the main form of change involves 2/2 and 3/1 split codon families. This can be explained mechanistically by variation in chemical modification of the "wobble" base at the first position of the tRNA anticodon (Knight *et al.*, 2001b; Senger *et al.*, 1997; Muramatsu *et al.*, 1988), since G and U recognize both C/U and A/G, respectively at the 3rd position of the codon.

1.2. Mechanisms of codon reassignment

Genetic code changes are mediated through structural alterations of one or more components of the translational machinery, in particular tRNAs or termination RFs. However, these changes cannot comprise completely decoding fidelity. The later is achieved through different molecular mechanisms operating at three critical steps of protein synthesis (Figure 1.2):

i) Aminoacylation, where the aminoacyl-tRNA synthetase (aaRS) specifically recognizes and activates the amino acid and covalently links it to its cognate tRNA, which is also recognized with high specificity.

ii) tRNA selection at the ribosome A-site through correct codon-anticodon base pairing. In here, the extended "wobble rules" should be considered as standard decoding.

iii) Stop codon recognition by RFs. RFs contain a conserved amino acid sequence involved in stop codon recognition (peptide anticodon) (Lozupone *et al.*, 2001; Lehman, 2001; Bertram *et al.*, 2000). This is highly specific codon recognition. However, suppressor tRNAs and bad codon context result in readthrough of stop codons. Some nonsense to sense reassignment are caused by such lack of fidelity of the translational

machinery and mutations in the peptide anticodon of the termination factors (Lehman, 2001; Kuck *et al.*, 2000).

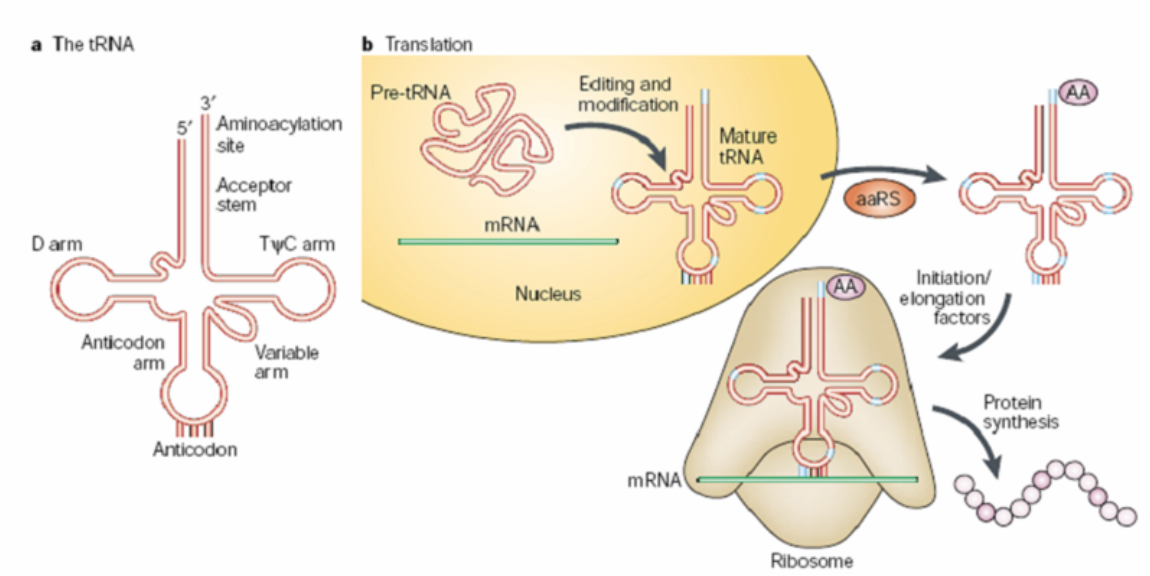


Figure 1.2. Representation of the mechanism and components involved in translation. a) Secondary structure of tRNA. The acceptor stem, where the amino acid is attached by the aminoacyl-tRNA synthetases (aaRS), the anticodon at the opposite end of the tRNA, which pairs with the mRNA codon in the ribosome. The D and $T_{\psi}C$ arms take their names from characteristic base modifications. b) tRNA and mRNA are transcribed from genes in the nucleous. Before translation, tRNA and mRNA can be edited by specific enzymes; in particular the tRNA can be modified in certain bases which could interfere with its decoding ability. In the cytoplasm, aaRS charge specifically the tRNAs with amino acids, and at the ribosome the three base anticodon pairs with the three–base codon in the mRNA. The amino acid attached to the tRNA is added to a growing peptide chain, which is extruded through a channel in the ribosome (Knight *et al.*, 2001b).

Large number of codon reassignments is due to structural alteration of tRNAs, ranging from single nucleotide substitutions to major alterations in the anticodon arm. These alterations result in the evolution of new tRNAs that decode codons in a non-standard manner. These structural changes may result from tRNA editing, alteration in tRNA base modification or by direct mutations of anticodon bases (Knight *et al.*, 2001b). Such alterations may alter aminoacylation or decoding fidelity. In order to understand how tRNA structural change may result in alterations of the genetic code one needs to understand the mechanisms of tRNA aminoacylation and decoding in detail.

Each tRNA possesses specific bases (identity bases) which are recognized by specific aaRS. For example, yeast LeuRS recognizes A_{35}, m^1G_{37} and A_{73} bases and makes direct contact with them through specific interactions with amino acids of its structure. Similarly, the yeast SerRS recognizes serine tRNAs only through direct contact with a run of 3 G-C bases located in the extra-loop and also with base G_{73} (Suzuki *et al.*, 1997; Soma *et al.*, 1996). Despite this, it is remarkable that changing a single base in the Leu-tRNAs, namely A_{73} to G_{73}, changes the identity of leucine-tRNAs to serine (Breitschopf *et al.*, 1995). In other words, a single base change may be sufficient to alter the genetic code of yeasts. A similar case occurs in yeast mitochondria where a novel tRNA and aaRS is responsible for the reassignment of leucine-CUN codon family to Thr. This novel ThrRS recognizes a novel mitochondrial $tRNA_{UAG}^{Thr}$ and is unable to recognize and aminoacylate the standard cytoplasmatic $tRNA_{UGU}^{Thr}$ (Pape & Tzagoloff, 1985).

Single mutations in the tRNA anticodon can effect aminoacylation and alter decoding efficiency (Saks *et al.*, 1998). The diversity of suppressor tRNAs indicates that these mutations may be more frequent than originally thought (Knight *et al.*, 2001a) and it has been assumed that mutations in anticodons lead to most codon reassignments (Jukes, 1990), since wobble pairing allows a single tRNA to decode multiple codons. Consequently, single mutations in tRNA genes may cause reassignments in two-codon sets. Other mutations far from the anticodon that cause structural alterations may also promote C/A or G/A mispairing at the third codon position or G/U misparing at the first codon position and explain some codon reassignment (Schultz & Yarus, 1996).

Those tRNA structural alterations, and their implications for codon decoding, led Schultz and Yarus (1996) to propose the "Ambiguous Intermediate Theory" to explain the evolution of genetic code alterations. This theory postulates that genetic code changes are driven by selection and that reassignment of codons is facilitated by translational ambiguity of the codon being reassigned. Such ambiguity arises by tRNA structural change, as described above (Schultz & Yarus, 1994). This theory is supported by the reassignment of non-sense (stop) codons in various species of ciliates and the leucine-CUG codon to

serine in many *Candida* species and finally by the reassignment of the leucine-CUN codon family to Thr in yeast mitochondria. In all these cases, tRNA, aaRS or RFs structural change is required for codon reassignment.

The other main theory for the evolution of genetic code alterations is the "Codon Capture Theory" (Osawa & Jukes, 1989). This neutral theory proposes that under directional mutational pressure (selective GC- or AT-biased pressure) certain codons can vanish from a genome (become unassigned) (Osawa & Jukes, 1989). These unassigned codons can, however, re-appear in the genome and be captured by mutant misreading tRNAs from non-cognate amino acid families. In other words, unassigned codons can be reassigned to other amino acid families upon re-entry into the genome. Unassigned codons have been discovered in genomes with strong G+C or A+T biases, namely the AGA and AUA codons in *Micrococcus luteus* (75% of genome is GC) and CGG codon in *Mycoplasma capricolum* (25% of genome is GC). In each case, biased genome G+C pressure is observed on the 3rd base of the codon (wobble base) and as this base changes in the direction of the bias it allows the first base of the anticodon free to mutate (Osawa & Jukes, 1989). This theory complements the "Ambiguous Intermediate Theory", but is distinct from the latter as it provides a neutral mechanism for the evolution of genetic code alterations. That is, it explains how genetic code alterations may evolve without affecting the proteome.

1.3. Genetic code in the genus *Candida* spp.

In the genus *Candida,* there are species that decode CUG codon as leucine, while other decode it as both leucine and serine or as serine only (Figure 1.3) (Sugita & Nakase, 1999; Suzuki *et al.*, 1997; Santos *et al.*, 1996; Sugiyama *et al.*, 1995; Santos & Tuite, 1995; Suzuki *et al.*, 1994b; Ohama *et al.*, 1993; Santos *et al.*, 1993; Yokogawa *et al.*, 1992). For example, in *C. glabata*, the CUG codon is decode in a standard manner (leucine), in *C. cylindracea* as serine only and in *C. albicans* and several other species as both serine and leucine (Suzuki *et al.*, 1994b; Yokogawa *et al.*, 1992).

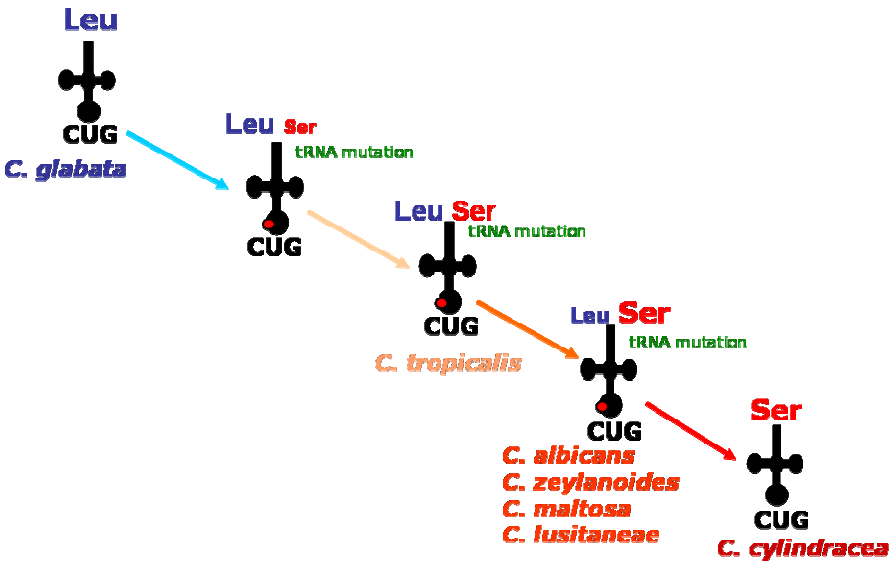


Figure 1.3. Evolutionary pathway and ambiguity of CUG decoding in *Candida* spp. The $tRNA_{CAG}^{Ser}$ evolved from a serine tRNA through an adenosine insertion in its intron sequence (Yokogawa *et al.*, 1992). This new $tRNA_{CAG}^{Ser}$ evolved in different ways in the various *Candida* species, which explains the differences in its aminoacylation. Adapted from Santos & Tuite (2004).

In *C. zeynaloides* and *C. albicans*, the $tRNA_{CAG}$ is charged, *in vivo*, with both serine (97%) and leucine (3%) (Suzuki *et al.*, 1997). Thus a single CUG decoding $tRNA_{CAG}^{Ser}$, can be recognized and aminoacylated by two different aminoacyl-tRNA synthetases, namely leucyl- and seryl-tRNA synthetases (LeuRS and SerRS) (Sugiyama *et al.*, 1995; Suzuki *et al.*, 1994b; Ohama *et al.*, 1993; Santos *et al.*, 1993; Yokogawa *et al.*, 1992), giving raise to two distinct tRNA species, i.e., ser-$tRNA_{CAG}^{Ser}$ and leu-$tRNA_{CAG}^{Ser}$. The CUG codon is also ambiguous in *C. maltosa*, and *in vitro* aminoacylation experiments using the *C. zeylanoides* LeuRS indicate that the CUG codon is also likely to be ambiguous in *C. lusitaneae* and *C. tropicalis* (Suzuki *et al.*, 1997). Thus, in a number of *Candida* species, the CUG codon appears to have two distinct assignments with serine predominating over leucine (Suzuki *et al.*, 1997). Probably, the level of CUG ambiguity is strictly related with the anticodon arm structure of the $tRNA_{CAG}^{Ser}$ which has subtle but important differences among the *Candida* species. The anticodon arm of the $tRNA_{CAG}^{Ser}$ besides being crucial for the recognition and affinity of the LeuRS (Figure 1.3) is also important for decoding efficiency (Perreau *et al.*, 1999; Suzuki *et al.*, 1997).

1.3.1. Evolutionary mechanism of CUG codon reassignment

The reassignment of the CUG codon in *Candida* spp. is viewed as a typical case of a genetic code change mediated by ambiguous decoding, as postulate by the "Ambiguous Intermediate Theory" (Knight *et al.*, 2001b; Santos *et al.*, 1996; Schultz & Yarus, 1996). As in many reassignments, the change of identity of the CUG codon was mediated through mutations in a $tRNA_{CGA}^{Ser}$ that occurred at 272±25 million years (My) ago (Massey *et al.*, 2003). These mutations led to the appearance of the novel $tRNA_{CAG}^{Ser}$. Molecular phylogeny studies have shown that this tRNA competed during approximately 100±10 My with the cognate $tRNA_{CAG}^{Leu}$ of the *Candida* ancestor for decoding of the CUG codon. This created ambiguity at CUG codons. Interestingly, such ambiguity resulted in disappearance of almost 30,000 CUG codons that existed in the *Candida* ancestor (Massey *et al.*, 2003). Of the original CUG codons only 0.2% remained in the *C. albicans* genome. Therefore, the 13,074 CUG codons present in the haploid genome of extant *C. albicans* emerged during the last 272 My from codons coding for serine or amino acids with similar chemical properties. In other words, these new codons are unrelated to the 30,000 CUG codons existent in the *Candida* ancestor (Figure 1.4) (Massey *et al.*, 2003).

The disappearance and the maintenance of this novel $tRNA_{CAG}^{Ser}$ marked the evolutionary divergence between *Saccharomyces* and *Candida* genus (Silva *et al.*, 2004; Massey *et al.*, 2003).

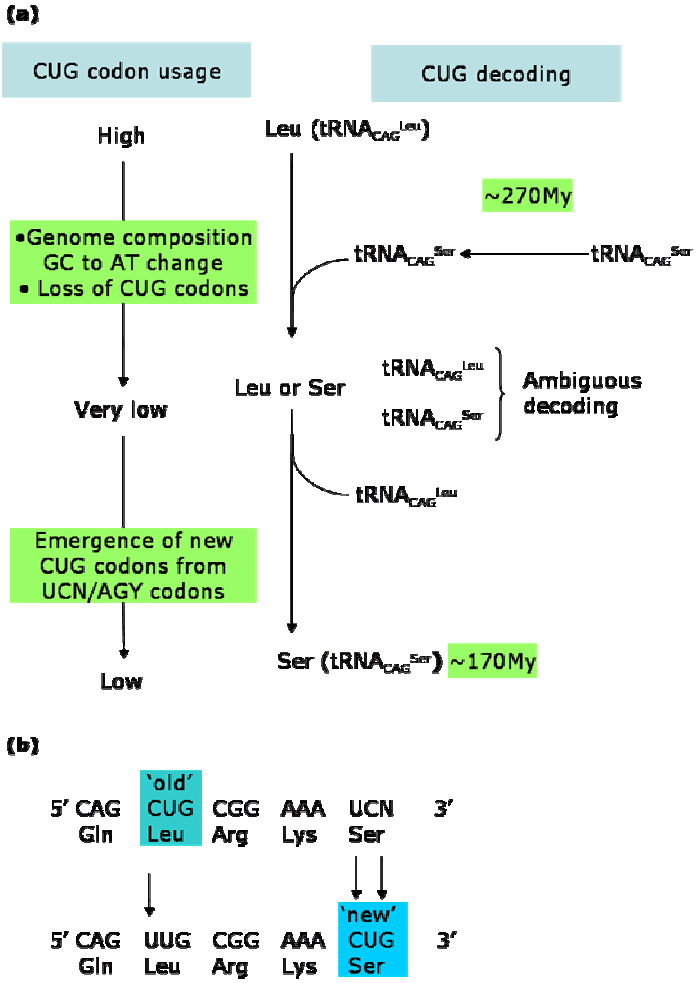


Figure 1.4. Evolutionary mechanism of CUG reassignment in *Candida* spp. (a) The novel tRNA$_{CAG}^{Ser}$ appeared approximately 270 My ago and during 100 My competed with the cognate tRNA$_{CAG}^{Leu}$ for CUG decoding. This ambiguous CUG decoding was the main force responsible for the decreasing CUG codon usage and consequent reorganization of the CUG in the genome. The 30,000 CUG codons present in the yeast ancestor disappeared, and "new" 13074 CUGs present in *C. albicans* genome evolved from UCN and AGY codons. For reasons not yet fully understood, the novel tRNA$_{CAG}^{Ser}$ was maintained while the cognate tRNA$_{CAG}^{Leu}$ was eliminated. **(b)** Ambiguous CUG decoding forced a mutational change of "old" CUG codons to the leucine codons UUG and UUA. Simultaneously, "new" CUG codons appeared via mutation in the UCN serine codons (Massey *et al.*, 2003). Adapted from Santos *et al.* (2004).

Molecular phylogeny studies have unequivocally shown that the *Candida* tRNA$_{CAG}^{Ser}$ evolved from a serine and not from a leucine tRNA. An analysis of the intron and anticodon sequences of serine tRNAs further showed that the ancestor tRNA was a tRNA$_{CGA}^{Ser}$ (Massey *et al.*, 2003). This newly created tRNA$_{CAG}^{Ser}$ then underwent several mutations of its anticodon arm which improved decoding efficiency and altered aminoacylation specificity. Sequence

alignments of the anticodon arm of the $tRNA_{CAG}^{Ser}$ from various *Candida* species and structural probing of the tRNA in solution showed that this class of $tRNA_{CAG}^{Ser}$ have atypical and divergent anticodon-arms, which are under strong mutational bias (Perreau *et al.*, 1999). The parallel evolution of anticodon arms of the various *Candida* $tRNA_{CAG}^{Ser}$ corresponds to different aminoacylation rates of the $tRNA_{CAG}^{Ser}$ with consequences for CUG ambiguity levels in each species (Figure 1.3). Several of these mutations play critical functional roles, namely the change of the conserved uridine at position 33 (U_{33}) for guanosine (G_{33}) in the anticodon arm of the tRNA. This mutation lowers decoding efficiency of the tRNA and simultaneously decreases its leucilation efficiency by the LeuRS (Suzuki *et al.*, 1997) (Figure 1.5 A and B), minimizing deleterious proteome effects during the early stages of CUG reassignment (Silva *et al.*, 2004). In *C. tropicalis*, the U_{33} was replaced by a cytosine (C_{33}), which suggests that in this species the level of CUG ambiguity is higher than in *C. albicans* or *C. zeylanoides*, since C_{33} does not distort the anticodon arm and consequently the LeuRS should recognize this tRNA more efficiently (Suzuki *et al.*, 1997).

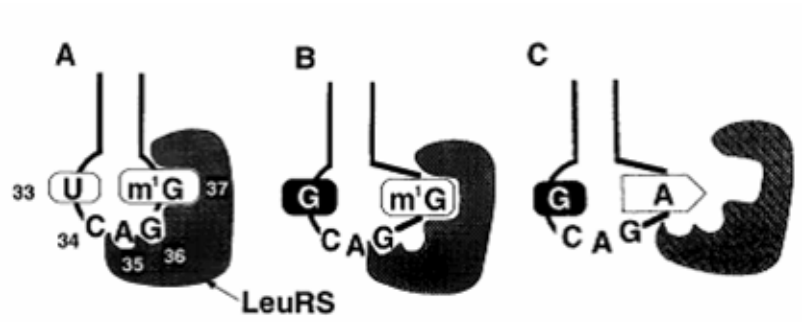


Figure 1.5. Schematic representation of the interaction of the LeuRS with the anticodon-loop of various *Candida* $tRNA_{CAG}^{Ser}$. The diagram highlights the importance of position 33 and 37 of the anticodon arm of the $tRNA_{CAG}^{Ser}$ for aminoacylation by LeuRS. (A) Anticodon arm with the correct conformational loop provided by the presence of a uridine. (B) Distortion of the anticodon arm of the tRNA caused by the presence of G_{33}, which lowered the affinity for the LeuRS. (A) and (B) the presence of m^1G_{37} is a key element for LeuRS recognition. (C) The presence of A_{37} blocks the binding of the LeuRS to the tRNA, preventing its charge with leucine (Suzuki *et al.*, 1997).

Finally, the presence of the m^1G_{37} together with A_{35} is responsible for mischarging of the tRNA with leucine in *C. zeynaloides* (Suzuki *et al.*, 1997). This mutation can be considered in two different perspectives: as an attenuator of the detrimental effect caused by the reassignment of the CUG codon from leucine to serine (Suzuki *et al.*, 1997), or as pre-requisite for improving decoding accuracy by the 5'-CAG-3' anticodon (Figure 1.5 A and B). This mutation is a decoding accuracy imperative since 5'-CAG-3' anticodons frameshift without m^1G_{37}. Interestingly, this modified base is an identity element for the LeuRS and explains why the $tRNA_{CAG}^{Ser}$ is charged with leucine, i.e., has double identity (Suzuki *et al.*, 1997).

1.3.2. The structure of the *C. albicans* $tRNA_{CAG}^{Ser}$

As mentioned above, the $tRNA_{CAG}^{Ser}$ from various *Candida* species is a unique molecule that can be aminoacylated with both serine and leucine. In other words, this is a hybrid tRNA. Cognate aaRS and non-cognate aaRS recognize tRNAs through a combination of positive and negative identity elements, which ensure that tRNAs are accurately charged.

One of the most interesting aspects of the *Candida* spp. genetic code change is that it involves two class-II tRNAs, namely serine and leucine. Eukaryotic and prokaryotic tRNAs are divided into two distinct structural classes (I and II), which are distinguished by the length of the extra-arm. In prokaryotes, class-II tRNAs belong to leucine, serine and tyrosine amino acid families, while in eukaryotes they belong to serine and leucine amino acid families only. Class-II tRNAs have more than 4 or 5 nucleotides in the extra-loop, which has a variable length, and normaly they have between 8 and 20 nucleotides (Dirheimer *et al.*, 1995). Interestingly, these tRNAs have very similar structures and conversion of one type of tRNA into another is relatively easy to accomplish. For example, yeast and human leucine tRNAs can be converted into serine tRNAs by mutating the discriminator base A_{73} (Figure 1.6), which is conserved in leucine tRNAs, into a G_{73}, conserved in serine tRNAs (Himeno *et al.*, 1997; Breitschopf *et al.*, 1995). In the case of the *Candida* genetic code

change the alteration of identity of the CUG codon from leucine to serine, involved an adenosine insertion in the middle position of the 5'-CGA-3' anticodon of a $tRNA_{CGA}^{Ser}$ that created the 5'-CAG-3' anticodon ($tRNA_{CAG}^{Ser}$). This was possible because the SerRS does not contact the anticodon-arm of serine tRNAs, rather it recognizes the discriminator base G_{73} and the run of G-Cs in the extra-arm (Figure 1.6). In other words, the anticodon alteration from 5'-CGA-3' to 5'-CAG-3' did not interfere with the recognition of the tRNA by the SerRS but altered its decoding properties, as the tRNA became a CUG decoder.

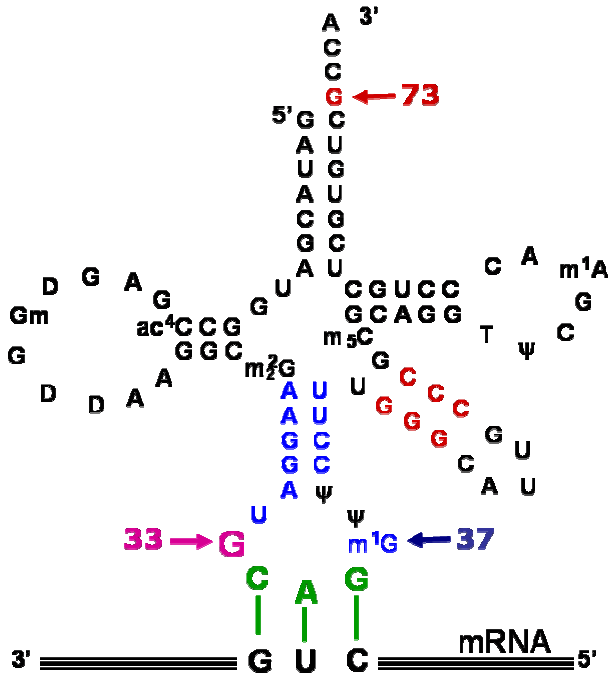


Figure 1.6. The *C. albicans* Ser-$tRNA_{CAG}$ which decodes the CUG codon. In red are the positive identify elements for the SerRS, while in blue are the identify elements for the LeuRS. The conserved position 33 is mutated to G_{33} is also highlighted.

1.3.3. Genomic and physiological significance of CUG reassignment

As referred above the 30,000 CUG codons present in *S. cerevisiae* are not related with the 13,074 CUG codons present in the *C. albicans* genome (haploid genome) (Moura, unpublished) suggesting that CUG identity reassignment evolved through a dynamic molecular mechanism that

reprogrammed the position of CUG codons on a wide scale (Massey *et al.*, 2003). This novel pathway of codon identity redefinition reveals that organisms can be highly tolerant to negative impact of proteome disruption caused by codon identity redefinition (Silva *et al.*, 2004). In particular, *C. albicans* has been able to tolerate an unstable proteome since the appearance of its novel $tRNA_{CAG}^{Ser}$, i. e., during the last 272±25 My. Therefore, this raises the puzzling question of "what selective advantages could have this genetic code change brought about to allow for its selection?"

In order to shed new light into the above question, Santos and colleagues (1996) reconstructed the *Candida* genetic code change in *S. cerevisiae* by engineered CUG ambiguity in this yeast. For this, the *C. albicans* $tRNA_{CAG}^{Ser}$ gene was transformed into a *S. cerevisiae* strain that maintained its $tRNA_{UAG}^{Leu}$ gene, which decodes the CUG codon as leucine (Santos *et al.*, 1996). Phenotypic characterization of this recombinant yeast strain showed that CUG ambiguity induced tolerance to stress agents such as oxidants, heavy metals, and drugs. It was rather remarkable that this recombinant ambiguous yeast could grow in extreme environmental stress conditions (Santos *et al.*, 1999). This result reveals that the growth rate disadvantage that resulted from CUG ambiguity could be easily overcome by tolerance to stress and increased potential to adapt to new ecological niches (Silva *et al.*, 2004; Santos *et al.*, 1999).

Thus, *S. cerevisiae* proved to be an excellent model organism to elucidate the physiological and evolutionary importance of genetic code alterations. The latter, result in large scale synthesis of mutant proteins that accumulate in the cytoplasm in an aggregated form or are targeted for degradation by the proteasome-ubiquitin degradation system (Silva *et al.*, 2004; Pickart, 2000). Interestingly, synthesis of such proteins triggered the stress response, namely up-regulation of a number of molecular chaperones (Hsp104p and Hsp70p), thus explaining the acquisition of tolerance to stress (Figure 1.7).

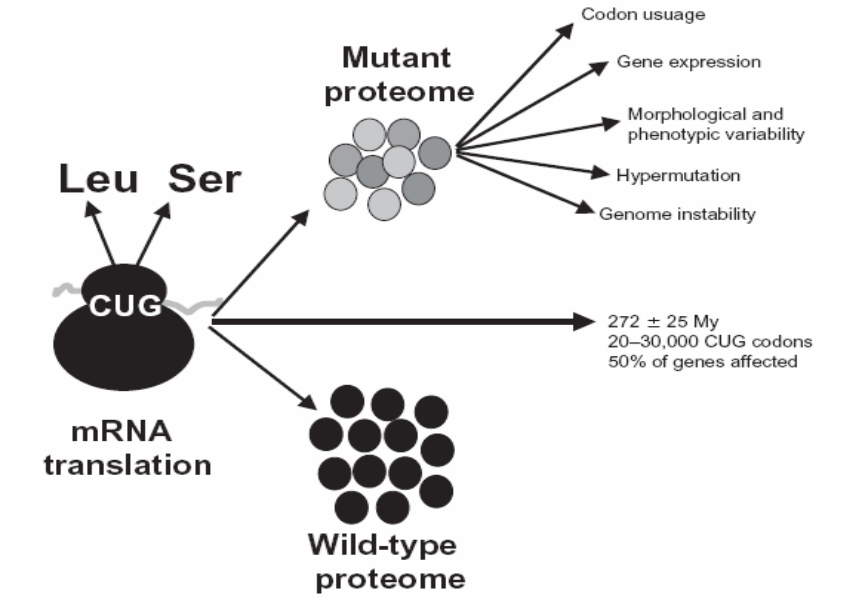


Figure 1.7. Global impact of CUG ambiguity in *S. cerevisiae*. Ambiguous CUG decoding in *S. cerevisiae* was achieved by introduction of the *C. albicans* $tRNA_{CAG}^{Ser}$, gene in the former (Santos *et al.*, 1997). This heterologous tRNA was expressed, correctly processed and charged and was able to compete for CUG decoding with the endogenous *S. cerevisiae* cognate $tRNA_{UAG}^{Leu}$. Since *S. cerevisiae* has about 30,000 CUG codons spread in 50% of its genes, a large amount of mutated proteins were produced by ambiguous CUG decoding. The mutant proteome had a global impact special on gene expression, genome stability, and morphological diversity (Silva *et al.*, 2004).

PART B

1.4. *Candida albicans* biology

The genus *Candida* belongs to the Deuteromycetes class, which includes fungi that do not reproduce sexually (anaphorm state). The anaphorm sate (asexual stage) has been commonly used to distinguish *Candida* species, even after the sexual states (teleomorph) have been identified in some of *Candida* species (Calderone, 2002). However, for those species of *Candida* for which a teleomorph stage has been described, they are ascomycetous (Kurtzman & Fell, 1998). Comparative sequence data from a number of studies support the idea that both the sexual and nonsexual *Candida* species are related phylogenetically to the Ascomycete rather than to the Basidiomycete class of fungi.

The genus *Candida* contains approximately 150 species some of which are pathogens to humans (Schauer & Hanschke, 1999), with considerable medical importance. Over the last decades, there has been a significant increase in the number of reported infections caused by yeasts of the genus *Candida*. These species can cause various human diseases ranging from superficial mucosal infections, such as vulvovaginal (VVC) and oropharyngeal (OPC) candidosis, to life-threatening invasive infections (Sullivan *et al.*, 2004). *C. albicans* is the most frequently isolated *Candida* spp., being the fourth most common hospital-acquired infection in the USA, whose treatment costs more than US $1 billion annually (Miller *et al.*, 2001; Beck-Sague & Jarvis, 1993). Approximately 75% of adult women experience at least one episode of VVC during their lifetime, among which approximately 40 to 50% will experience further episodes and 5% will develop the recurrent type of candidiasis (RVVC). Additionally, studies show that 20 to 25% of healthy and totally asymptomatic women exhibit positive vaginal secretion cultures for *C. albicans* (Fidel, Jr. *et al.*, 1997). About 80 to 90% of VVC cases are caused by *C. albicans*, while only 10 to 20% are due to other non-*C. albicans* yeasts, namely *C. tropicalis*, *C. glabrata*, *C. krusei*, *C. parapsilosis*.

1.4.1. The *C. albicans* genome

C. albicans is diploid and its genome has about 32 Mbp of nuclear DNA per cell (Magee & Chibana, 2002; Riggsby *et al.*, 1982), containing 6,354 ORFs (Open reading frames) (Braun *et al.*, 2005; Jones *et al.*, 2004) which are extremely rich in short sequence repeats compared with *S. pombe* and *S. cerevisiae* genomes (Braun *et al.*, 2005). There are strong similarities to the model organism *S. cerevisiae* in the number of genes with introns and the number of gene families. However in contrast to *S. cerevisiae*, *C. albicans* has a large number of genes for expanded metabolic capacity, including oligopeptide transporters, amino acid catabolizing enzymes, and lipid degradation enzymes. This difference could be related to the large number of niches in which *C. albicans* exists (Jones *et al.*, 2004).

In standard karyotype analysis, the number of *C. albicans* chromosome bands is 8, hence the diploid chromosome number is 16, but chromosomes contain largely homologous genetic information that migrate together on pulse-field gels (Magee *et al.*, 1992).

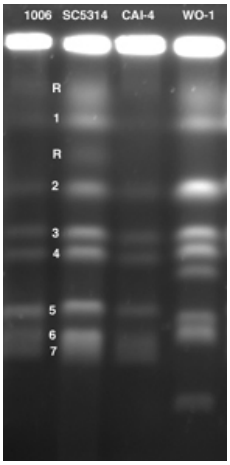


Figure 1.8. Contour-clamped homogeneous electric field (CHEF) pulse-field separations of *C. albicans* chromosomes. Chromosomes of four *C. albicans* strains (1006, SC5314, CAI-4 and WO-1) where separated by pulse field gel electrophoresis (PFGE). The chromosomes of 1006 and SC5314 are identified by the labels. Adapted from www.candidagenome.org.

The *C. albicans* karyotype is highly variable (Lasker *et al.*, 1989; Magee *et al.*, 1988; Snell *et al.*, 1987). This genome variability reflects a variety of genomic alterations, such as: translocation, deletion, amplification, and loss of particular chromosomes (Iwaguchi *et al.*, 2000; Navarro-Garcia *et al.*, 1995; Rustchenko *et al.*, 1994; Chu *et al.*, 1993; Thrash-Bingham & Gorman, 1992), aneuploidy and ploidy changes (Perepnikhatka *et al.*, 1999; Janbon *et al.*, 1998; Magee & Magee, 1997; Suzuki *et al.*, 1994a) and chromosome length polymorphisms (CLP) (Chibana *et al.*, 2000).

Many of the karyotype alterations found in *C. albicans* isolates are associated with translocations between heterologous chromosomes (Chibana *et al.*, 2000; Chu *et al.*, 1993), involving an intermediate repeat element called major repeat sequence (MRS) (Thrash-Bingham & Gorman, 1992). This is an intriguing feature of the *C. albicans* genome with no known homologues in the eukaryotic world (Lephart *et al.*, 2005). There is a sequence variation in the separate copies of the MRS but it maintains a structure composed by three

elements (Chindamporn *et al.*, 1998; Chindamporn *et al.*, 1995; Iwaguchi *et al.*, 1992), namely: i) HOK, which is about 8 kbp sequence, has 35% GC content and three small ORFs, that seem to be pseudogenes since they contain many stop codons (Chindamporn *et al.*, 1998); ii) a repetitive sequence (RPS), which varies in size from 2 to 2.9 Kbp and is a tandemly repeated sequence present in the MRS, whose number varies from few up to 50 repeats (Magee & Chibana, 2002; Iwaguchi *et al.*, 1992). Within the RPS, there are smaller tandem repeat called *alts*, which are composed by a variable sequence followed by a highly conserved sequence which contains a site for the restriction enzyme, *SfiI*. RPS size depends on the number of *alt* repeat units within it. The repeated *alt* region is flanked by DNA with high homology between the different RPS elements. The RPS element serves as both a breakpoint for chromosomal translocations and as a size expansion mechanism increasing the number of internal repeats. This is a major source of size variation between homologues (Chibana *et al.*, 2000) and gives rise to length polymorphisms in *C. albicans* chromosomes; iii) finally, RB2 is approximately 6 kbp of conserved sequence found on every chromosome, including chromosome 3, where apparently exists without an RPS or HOK unit (Chindamporn *et al.*, 1998).

The variability of composition of the MRS is the basis of a great deal of genomic variability of *C. albicans* (Chibana *et al.*, 2000), although it is not known how chromosomal translocation or RPS repeat expansion and contraction occur (Lephart *et al.*, 2005). Due to the observation made for chromosome 5, where a large MRS increases the frequency of loss of the chromosome homologue after growth in sorbose, it was suggested that large MRS alleles on homologous chromosomes are readily able to undergo beneficial mitotic non-disjunction events and adapt more rapidly to a new environment. On the other hand, strains that have smaller MRS alleles or non-functional MRS alleles may be at disadvantage, as they may not be able to adapt as quickly as the large-MRS strain (Lephart *et al.*, 2005).

Translocations also affect poliploidy and aneuploidy. Clones containing cells with high-ploidy, at certain frequency, have frequent chromosome translocations, namely, between chromosome 4 and 7 (Iwaguchi *et al.*, 2000). Aneuploid *C. albicans* cells have been reported since the early 1980 (Whelan & Magee, 1981), due to loss of one homologous chromosome (Janbon *et al.*, 1998; Magee & Magee, 1997; Barton & Gull, 1992). These aneuploid cells may represent a state of adaptation to certain growth conditions. For example, the loss of the homologue of chromosome 4 is associated with resistance to fluconazole (Perepnikhatka *et al.*, 1999). Also the loss of one homolog in the chromosome 5 occur in cells growing in the presence of L-sorbose, while growth in glucose selects strains that have two copies of this chromosome (Janbon *et al.*, 1998). Other cases related with translocation are the supernumerary chromosomes (snc) that appear in some strains (Iwaguchi *et al.*, 2000; Navarro-Garcia *et al.*, 1995; Chu *et al.*, 1993), and are associated with *C. albicans* phenotypic switching controlled by the *SIR2* (silent information regulator) gene (Perez-Martin *et al.*, 1999).

The frequency with which translocations are found in clinical isolates suggests that genomic rearrangements are neither a cause nor a result of the transition of *C. albicans* strains from commensal to pathogen. If translocations alter the pattern of gene expression, it is likely that such alterations are mediated through chromatin structures, since the unique DNA sequences are not altered (Magee & Chibana, 2002). Other CLPs may be due to variation in the size of telemores or to small translocations, which increase or decrease the chromosome size (Magee & Chibana, 2002).

The *C. albicans* genome also has a high level of heterozygosity (Forche *et al.*, 2004; Forche *et al.*, 2003), 89% of the heterozygous markers are located in intragenic regions with a potential to affect protein sequences or expression level (Forche *et al.*, 2004). This high level of heterozygosity could lead to differential gene expression as shown for the *SAP2* (secreted aspartic proteinase) gene (Staib *et al.*, 2002), which has a number of pentameric repeats in the promoter region that lead to differential regulation of the two alleles. Like aneuploidy, loss of heterozygosity (LOH), together with other

processes, could contribute to environmental adaptation. For example, LOH around the *ERG11* (ergosterol biosynthesis) gene, which is involved in the ergosterol biosynthesis pathway, suggested that homozigoty at this locus increases fitness in presence of fluconazole (Franz *et al.*, 1998; White, 1997).

1.4.1.1. The *C. albicans* chromosomes

The R chromosome is the largest with 3200 kbp in size and 961 predited ORFs (Moura, unpublished). It contains the ribosomal DNA (rDNA) cistrons, of which there are approximately 110 copies per diploid cell. The position of this chromosome on pulse-field gels is variable due to variation in the distribution of rDNA cistrons, which are found in tandem arrays whose copy differ in the two homologues of the R-chromosome (Rustchenko *et al.*, 1994; Iwaguchi *et al.*, 1992). This chromossome has four *SfiI* fragments, one of which contains the rDNA cistrons and thus varies in size both within and between strains. It contains at least one copy of the MRS.

Chromossome 1 is 3165 Kbp long, has four to five *SfiI* sites on each homolog and contains at least one MRS site located between two fragments involved in translocation with chromosome 5, in strain WO-1. There are 1339 predited ORFs assigned to chromosome 1 (Moura, unpublished).

Chromosome 2 is 2300 kbp in size and 961 predited ORFs (Moura, unpublished). It contains only two *SfiI* fragments separated by the MRS involved in translocation in several strains (Navarro-Garcia *et al.*, 1995).

Chromosome 3 is 1820 Kbp long, and it is the only *C. albicans* chromosome without a complete copy of the MRS, lacking the RB2 component, so no translocations occur with this chromosome (Chindamporn *et al.*, 1998). It has two *SfiI* fragments. In contrast to most of the other chromosomes, chromosome 3 is highly homozygous. There are 747 ORFs assigned to chromosome 3 (Moura, unpublished).

Chromosomes 4 (1700 Kbp) possesses two copies of the MRS sequence, in inverted orientations, and has four *SfiI* fragments. Translocations between chromosome 4 and 7 have been found in several strains (Chibana *et al.*, 2000; Chu *et al.*, 1993).

Chromosome 5 (1230 Kbp) has two *SfiI* fragments separated by the MRS (Chindamporn *et al.*, 1995), containing 50 repeats of the RPS (Magee & Chibana, 2002). This feature may be related with highly frequent translocations involving chromosome 5. Mating-type loci (MTL) are located in this chromosome (Magee & Magee, 2000; Hull & Johnson, 1999). MTL homozygozity corresponds to the mating-competent state (Miller & Johnson, 2002). This can result from loss of one homolog of chromosome 5 in cells growing in L-sorbose as sole carbon source (Janbon *et al.*, 1999; Janbon *et al.*, 1998). To metabolize L-sorbose, cells must express the gene *SOU1* (L-sorbose utilization) located in chromosome 4, but chromosome 5 contains a repressor gene which is dose dependent and insufficient to repress the *SOU1* gene in hemizygous strains. There are 508 ORFs assigned to chromosome 5 (Moura, unpublished).

Chromosome 6 (1100 Kbp) contains one copy of the MRS and two *SfiI* fragments, and it's involved in translocations with chromosome 5 in strain WO-1. 423 ORFs have been assigned to chromosome 6 (Moura, unpublished).

Finally, chromosome 7 the most completely characterized of the *C. albicans* chromosomes is 1 Mbp in size and contains 407 predicted genes (Chibana *et al.*, 2005). It is the only chromosome whose sequence has been completely finished and gene mapping had been carried out (Chibana *et al.*, 2005). Possesses four *SfiI* sites and two copies of the MRS. Translocations and variation in size of the MRS can lead to polymorphisms (Chibana *et al.*, 2000).

1.4.1.2. Relation between karyotype and phenotype

The phenotypic diversity displayed by *C. albicans* was initially related to karyotype diversity (Rustchenko-Bulgac *et al.*, 1990; Suzuki *et al.*, 1989) and

it was suggested that karyotype variation is a means to achieve genetic variability in *C. albicans*. In order to prove this idea, several studies were carried out to compare karyotypic and phenotypes variation, namely the ability to switch between white and opaque forms (Barton & Scherer, 1994; Ramsey *et al.*, 1994; Rustchenko *et al.*, 1993; Suzuki *et al.*, 1989), assimilation of carbon source (Rustchenko *et al.*, 1997; Rustchenko *et al.*, 1994; Wickes *et al.*, 1991), and resistance to fluconazole (Mori *et al.*, 1998).

In *C. albicans*, chromosome loss is rather frequent in certain environmental conditions, which also potentiate exposure of some phenotypes. Barton and Gull (1992) showed that when diploid *C. albicans* is grown on methyl benzimidazole carbamate, strains that have undergone non-disjunction and lack one chromosome arise 10 fold more frequently than in the absence of the drug. Also fluconazole-resistant mutants with 7 days exposure loose one copy of chromosome 4, where as long-term exposure to the drug could lead to trisomy of chromosome 3 (Perepnikhatka *et al.*, 1999). Finally, as mentioned above, *C. albicans* growing on sorbose loose frequently one homologue of chromosome 5 (Janbon *et al.*, 1998).

Despite the effort and the preponderance of variant karyotypes in clinical populations, no cause-and-effect relationship has yet been established between these variations and increased virulence or drug resistance. However, these findings have led to the notion that karyotype alteration leading to changes in phenotypes could function as an adaptation strategy for *C. albicans.* It also suggests that non-disjunction and probably translocation are frequent events among *C. albicans* isolates, and that the rate of karyotypic rearrangements is increased by stress conditions (Enloe *et al.*, 2000). An open and important question is whether rearrangements generate a subpopulation of cells that is fitter and selected *in vivo* (Magee & Chibana, 2002; Chibana *et al.*, 2000).

1.4.2. *C. albicans* cell wall

The cell wall provides a number of essential functions, namely protection against osmotic pressure, a permeability barrier, shape to the cell and in the case of pathogens is critical in the interaction with host cells. The *Candida* spp. cell wall is a dynamic structure whose composition changes during cell growth and transition between polymorphic states. For example, the transition from yeast to hyphal growth is accompanied by a four to five fold increase in chitin level as well as a number of differentially expressed proteins (Pitarch *et al.*, 2002; Chauhan *et al.*, 2002). However, the yeast cell wall remains relatively constant in composition during growth in different media and temperatures. The same is observed in mycelia, with the exception that glucose and mannose contents vary according to growth conditions (Chattaway *et al.*, 1968).

The cell wall of *C. albicans* is composed by approximately 80 to 90% of branched polymers of glucose (β-1,3 and β-1,6 glucose polymers [β-glucan]), unbranched polymers of β-1,4 N-acetyl-D (chitin) and polymers of mannose (mannan) with or without glucose covalently bound to protein (Chaffin *et al.*, 1998). Its basic organization is similar to that of *S. cerevisiae* and has a bilayered structure (Richard *et al.*, 2002a; Kapteyn *et al.*, 2000). The inner part consists mainly of β-1,3 glucan molecules, is a skeletal layer fortified by hydrogen bonds that extends through covalent binding to β-1,6 glucan and chitin chains. The outer part, which determines cell surface proprieties, consists mainly of mannoproteins which are covalently bound to β-1,6 or β-1,3 glucan (Figure 1.13) (Klis *et al.*, 2001).

Mannose polymers covalently associated with proteins are designated mannoproteins, and account for approximately 40% of the cell wall polysaccharide (Chaffin *et al.*, 1998). Mannoproteins are homopolymers of *N*- and *O*-glycosidic covalent linkages with polypeptide residues of asparagine (*N*-linked) and threonine/serine (*O*-linked) (Chauhan *et al.*, 2002).

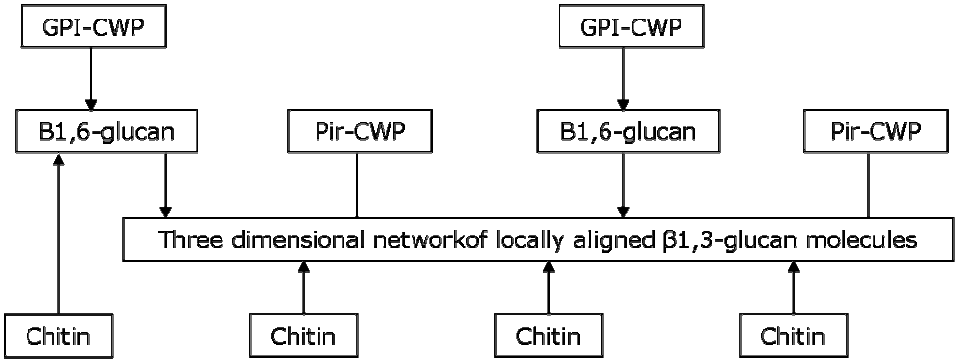


Figure 1.9. Squematic representation of *C. albicans* cell wall. Arrows indicate the orientation of each polysaccharide (chitin, β-1,6 glucan). β-1,3 glucan forms a three dimensional network that surrounds the cell to which most other components are attached (Kapteyn *et al.*, 2000).

In *C. albicans*, two types of covalently linked cell wall proteins (CWPs) have been discovered by immunoblot analyses and database searches (de Groot *et al.*, 2004; de Groot *et al.*, 2003; Sundstrom, 2002; Kapteyn *et al.*, 2000; Kandasamy *et al.*, 2000). The major class, glycosylphosphatidylinositol (GPI)-CWPs, is linked to the β-1,3 glucan network through a relatively short, water-soluble β-1,6-glucan moiety (Kollar *et al.*, 1997; Kapteyn *et al.*, 1996; Kapteyn *et al.*, 1995). GPI anchor occurs through a transamidation reaction in the endoplasmatic reticulum following proteolytic cleavage of a C-terminal propetide from the proprotein (Herscovics & Orlean, 1993). The second class, a small family of proteins with internal repeats named Pir proteins, is linked to β-1,3 glucan independently of the β-1,6-glucan through a linkage that is sensitive to 30 mM NaOH (Kapteyn *et al.*, 1999; Mrsa *et al.*, 1997). Chitin has been shown to be covalently linked to both types of β-glucan.

Transmission electron microscopy of *C. albicans* cell wall showed a layered structure similar to that of *S. cerevisiae*. The number of layers is dependent on the strain, morphology (yeast or filamentous forms), media, and the method of preservation used to fix cells (Chaffin *et al.*, 1998). The inner layer, adjacent to the plasma membrane is microfibrillar in appearance and is composed by polysaccharides, glucan and chitin. The outer layer is amorphous and is primarily composed of mannoprotein and proteins and in high-resolution appears densely fibrillar or floccular. The outer surface, also called fimbria, is

composed by a single, heavily mannosylated protein of about 66 KDa (Yu *et al.*, 1994).

1.4.3. Virulence factors

Over the past two decades, the increasing number of immunocompromised individuals has resulted in an increase of infections caused by opportunistic fungal pathogens. Of particular interest is candidiasis, a disease caused by members of the *Candida* genus which are the leading cause of life-threatening fungal disease and rank fourth among all bloodstream and urinary tract nosocomial infections in USA (Jarvis, 1995). *Candida* infections of the skin and of the mucous membranes may occur either in immunocompromised, such as HIV, cancer chemotherapy, and solid-organ and bone marrow transplantation patients, or in non-immunocompromised patients. This contrasts to systemic candidiasis (e.g. candidemia), which is only seen in severely immunocompromised patients.

Interestingly, *C. albicans* is an opportunistic commensal. It is a normal constituent of the gastrointestinal, genitourinary tract and to a lesser extent of the skin microflora. As an opportunist pathogen, *Candida* can establish an infection when immunologic and mechanical host defences are immunocompromised. This main feature implies a versatility and ability to survive in several anatomically distinct sites, each with its own specific set of environmental conditions. Besides of being able to compete among the mucosal flora the success of *C. albicans* as pathogen is related to its capacity to grow in a range of physiologically extreme conditions as well as to escape to immune response. The transition from commensal to pathogen is mediated by an array of weak virulence factors, namely morphogenesis, phenotypic switching, extracellular hydrolases, adhesion and growth at high temperature (Odds, 1994).

Although genetic manipulation of *C. albicans* has been difficult due to its diploid nature and the lack of a conventional sexual cycle, the contribution of specific genes to *C. albicans* pathogenesis has been dissected through

molecular genetics methodologies that permitted the development of techniques for targeted gene disruption (Calderone & Fonzi, 2001). These techniques include reverse-genetic approaches in which genes are first identified by their sequence and homology with *S. cerevisiae* homologues and then both genomic copies are sequentially deleted or mutated. Other strategy used to determine *C. albicans* gene function is to express genes in *S. cerevisiae* and identify the resulting phenotypes. However, this last approach is complicated by the non-standard genetic code of *C. albicans* (Santos & Tuite, 1995). Nonetheless, many wild type *C. albicans* genes are at least partially functional in *S. cerevisiae*, which facilitated their identification and function by complementation studies.

1.4.3.1. Morphogenesis

Morphogenesis refers to the transition between unicellular yeast cells to filamentous forms (Calderone & Fonzi, 2001). *C. albicans* is a polymorphic fungus (Calderone & Fonzi, 2001; Kobayashi & Cutler, 1998; Magee & Magee, 1997), since it is able to develop as single spherical cells, including typical yeast cells (or blastospores), and chlamydopores, or undergo morphogenesis and produce filaments in pseudohyphal and/or hyphal forms (Figure 1.10). Pseudohyphae are formed by chains of elongated buds (blastoconidia) and are characterized by constrictions at cell-cell junctions. Hyphae develop as a result of blastoconidia germination and are characterized by the presence of parallel walls at the point of germ tube emergence, the development of true cross walls or septa and the absence of constrictions. Thus, the term dimorphism, which traditionally is reserved for the yeast to hypha interconversion, designates the main theme of *C. albicans* and possibly fungal morphogenesis in general (Ernst, 2000). Budding-yeast cells can be induced to form true hyphae, which grow by continuous apical extension followed by septation. Pseudohyphae grow by unipolar budding: buds develop into elongated cells, which remain attached to mother cells, stop growth and resume budding. Chlamydospores are thick-walled spherical cells and develop on pseudohyphal support cells (Joshi *et al.*, 1993; Montazeri & Hedrick, 1984).

With the raising incidence of candidiasis in western countries, medical interest on *Candida* biology increased, and since morphogenesis enhances *C. albicans* virulence understanding how *C. albicans* regulates its cell shape has been intensively investigated (Sudbery *et al.*, 2004). Initially, *Candida* pathogenesis was thought to be dependent on hyphal-formation, since non-filamentous *C. albicans* mutants are avirulent in mouse models (Lo *et al.*, 1997). However, it is now clear that the different morphological forms of *C. albicans* are virulent, and may contribute in different stages to disease establishment and progression. Thus, it has been suggested that, yeast-like cells are necessary for dissemination and hyphae may be required to invade host tissue (Sudbery *et al.*, 2004; Brown, 2002a). Among *Candida* spp., only *C. albicans* and *C. dublinensis* form pseudohyphae and true-hyphae.

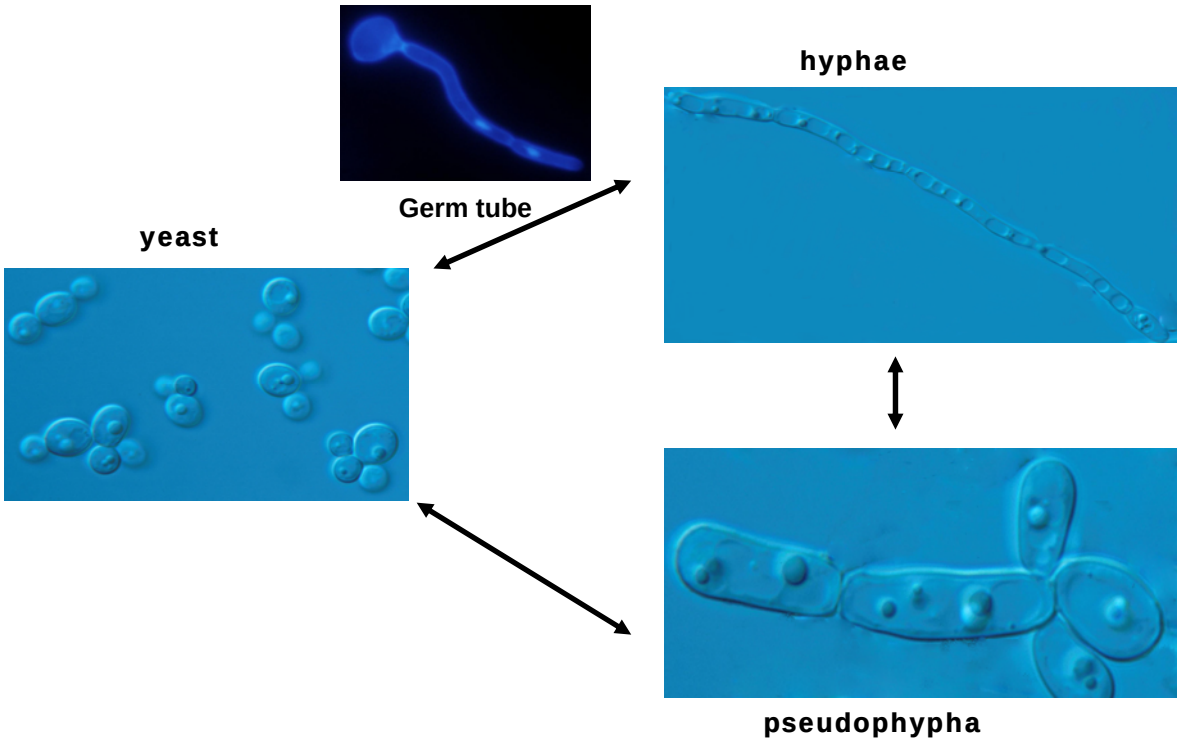


Figure 1.10. Transition between different morphological forms of *C. albicans*. Yeast-like forms can produce pseudophypha and true-hypha. According to some models, pseudohyphae can represent an intermediate stage between yeast and true-hypha, according to others could be formed via a separate pathway. Germ tube (outgrowths of yeast cells) grows by apical extension and septa are formed behind the growing tip of the hyphae.

C. albicans morphology is directly related to environmental conditions (Ernst, 2000). Hyphal development depends on two factors: i) the nature, number and intensity of environmental signals, and ii) activity of the signalling pathways including key transcription factors (cellular-response machinery). Environmental cues trigger separate signal-transduction pathways, which regulate common targets required to initiate hyphal growth. In *C. albicans*, a number of distinct signalling pathways appear to be responsible for transducing different types of signals. Some of these include cell density, temperature, medium pH, carbon starvation, nitrogen starvation, oxygen concentration, and some human hormones (Brown, 2002b). Most significantly to pathogenesis, morphogenesis can be induced by both human serum and human macrophages (Shepherd, 1982). Two known inducers of hypha formation, N-acetylglucosamine (GlcNAc) and proline may contribute to the serum effect since they are generated by degradation of serum (glycol-) proteins (Ernst, 2000). Amino acid starvation also leads to filamentous growth, especially of pseudohyphae formation rather than true hyphae formation (Tripathi *et al.*, 2002).

In vitro, *C. albicans* can thrive over a wide range of extracellular pH, from 2-10 (Davis, 2003). pH around neutrality (pH~6.5) favours filamentous growth of *C. albicans in vitro*, while low pH (pH<6.0) blocks hyphal formation and stimulates growth in the yeast form (Buffo *et al.*, 1984). In solid media (agar) glucose repression of morphogenesis is observed during the initial stage of growth, nevertheless glucose consumption allows filamentation after several days of growth. High osmolarity also inhibits hypha formation in agar plates (Alex *et al.*, 1998). The presence of easily utilizable nitrogen sources, such as ammonium salts, modulates hyphal development negatively to some degree (Ernst, 2000).

In the infected host, inhibition of filamentation by γ-interferon occurs when *C. albicans* contacts lymphocytes (Levitz & North, 1996; Kalo-Klein & Witkin, 1990). Some chemical inhibitors of filamentation are also known, namely diaminobutanone, amphotericin and azole anti-fungals (Hawser *et al.*, 1996; Martinez *et al.*, 1990).

Morphogenetic regulation in *C. albicans* is highly complex mainly because *C. albicans* has the capacity to inhabit different microenvironments, integrate numerous types of environmental cues and responds to external stimuli by altering its morphology. The polymorphic transition also involves program changes in cell metabolism and physiology, as well as in cell division and polarity. Hence, it distinguishes upstream regulatory pathways that receive and respond to morphogenetic signals, from downstream pathways that regulate the cellular processes that must respond to morphogenetic signalling (Brown, 2002b). Signal-transduction pathways that regulate morphogenesis are briefly summarized below. At least four positive and two negative pathways control morphological transition in *C. albicans*.

1.4.3.1.1. *EFG1* (enhanced filamentous growth) signalling pathway

Several morphogenetic processes are controlled by Efg1p, namely regulation of the yeast-to-hypha transition, determination of cell shape during white-opaque switching and generation of chlamydospores (Srikantha *et al.*, 2000; Sonneborn *et al.*, 1999a; Sonneborn *et al.*, 1999b; Lo *et al.*, 1997; Stoldt *et al.*, 1997). Efg1p performs a dual role in morphogenesis either as activator or as repressor of gene expression leading to reversible transition between non-filamentous and filamentous growth forms. Thus under standard hypha-induction conditions, serum, pH, N-acetylglucosamine, proline or spider medium, inactivation of *EFG1* blocks hyphal formation (Lo *et al.*, 1997; Stoldt *et al.*, 1997) indicating that Efg1p is a positive factor for hyphal development. However, *EFG1* could act as a repressor since its overexpression, in the absence of morphogenetic stimulus or under microaerophilic conditions (Giusani *et al.*, 2002; Brown, Jr. *et al.*, 1999; Sonneborn *et al.*, 1999a), also inhibits the formation of true hypha, inducing pseudohypha phenotype instead (Tebarth *et al.*, 2003; Stoldt *et al.*, 1997). In addition, *EFG1* expression is rapidly repressed by negative autoregulation after the onset of hyphal development (Tebarth *et al.*, 2003). Furthermore, generation of chlamydospores and yeast-cells (white cells) requires Efg1p as a positive factor (Sonneborn *et al.*, 1999a; Sonneborn *et al.*, 1999b). Therefore it seems

that the level of Efg1p activity has impact on *C. albicans* morphology, acting as activator and/or repressor of morphogenesis (Stoldt *et al.*, 1997).

Efg1p is a member of the APSES (Asm1p from *Neurospora crassa*, Pdh1p and Sok2p from *S. cerevisiae*, Efg1 from *C. albicans*, StuAp from *Aspergillus nidulans*) protein group. This is a conserved class of transcriptional regulators that are unique to fungi and regulate cellular differentiation in the ascomycetes (Stoldt *et al.*, 1997; Dutton *et al.*, 1997; Aramayo *et al.*, 1996; Ward *et al.*, 1995; Gimeno & Fink, 1994; Miller *et al.*, 1992). APSES proteins share 80-90% identical residues in a domain encompassing 100 amino acids that contains a basic helix-loop-helix (bHLH) motif, which might dimerize with another bHLH protein as a prerequisite for their binding to E-boxes (Stoldt *et al.*, 1997) in promoter regions of target genes (CANNTG) (Nasi *et al.*, 2001). E-boxes have been identified in the promoters of several hypha-specific genes, namely *HYR1*, *HWP1*, *ALS3*, *ALS8* and *ECE1* (Leng *et al.*, 2001), and the activation of these genes *in vitro* is dependent on *EFG1* under hypha-inducing conditions (Leng *et al.*, 2001; Braun & Johnson, 2000b; Sharkey *et al.*, 1999), although *in vivo* gene regulation by an E-box motif has not been demonstrated for any APSES protein.

Recently, an Efg1p-homologue was identified, named Efgh1p, which apparently modulates some of the functions of the Efg1p. This new protein functions as a transcriptional repressor (Doedt *et al.*, 2004). Like *EFG1*, *EFH1* overexpression leads to pseudohyphae formation in presence of Efg1p. In contrast, cells expressing *EFH1* at low levels do respond to serum, forming strongly aggregating germ tubes and true hyphae. However, hypha production is still blocked in *efg1* cells overexpressing *EFH1*, indicating that the requirement for Efg1p cannot be bypassed by *EFH1* overexpression. Efh1p enhances the activation and repression functions of Efg1p in morphogenesis, presumably by activation of *EFG1* expression (Doedt *et al.*, 2004).

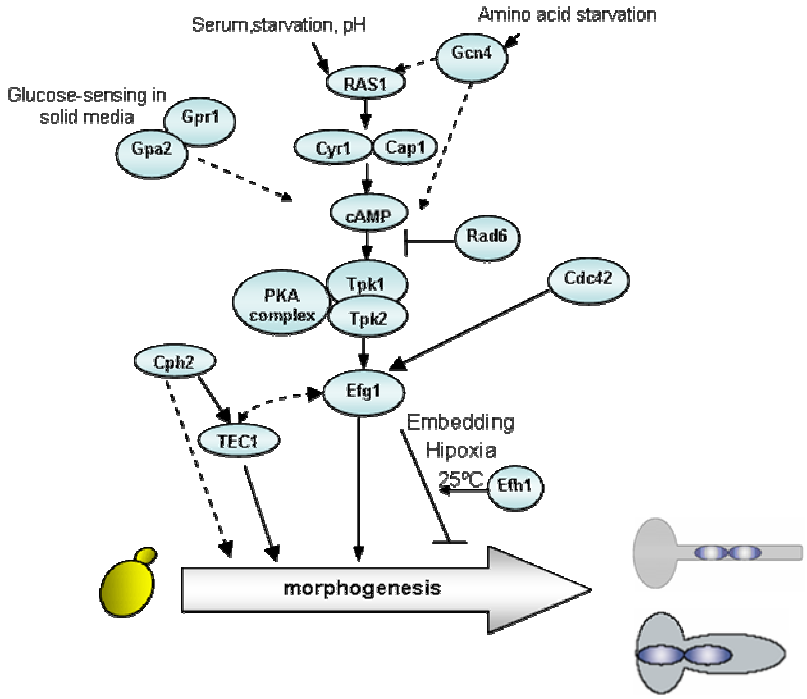


Figure 1.11. Efg1 morphogenetic signalling pathway. Interactions between the different known components are represented by straight arrows or bars, according to their activity, activation or repression respectively. Some of these interactions are not well defined (interrupted arrows). Adapted from Berman and Sudbery (2002).

Within the conserved APSES domain of Efg1p, but outside of the putative bHLH region, there is a stretch of threonine residues (T206-T208), which constitute possible phosphorylation sites for protein kinases. The PKA (protein kinases A) complex phosphorylates directly Efg1p (Figure 1.11) on T206 (Bockmuhl & Ernst, 2001). PKAs are structurally conserved, consisting of two catalytic subunits that are inactivated by binding of a homodimer of regulatory subunits. External cues elevate intracellular levels of cAMP (Adenosyne 3':5'-cyclic monophosphate), whose binding to the regulatory subunit liberates and thereby activates the catalytic subunits. *TPK1* (Takashi's Protein Kinase 1) and *TPK2* (Takashi's Protein Kinase 2) encode both isoforms of the PKA catalytic subunits. The levels of the two isoforms of PKA are important in morphogenesis under different environmental cues: Tpk1p is required mainly on solid induction media, whereas Tpk2p is of major importance in liquid media although, at lower temperatures, Tpk2p also contributes to hypha formation on solid media. At minimal PKA production levels, morphogenesis is

blocked in all induction conditions in an Efg1p dependent way (Bockmuhl & Ernst, 2001; Sonneborn *et al.*, 1999a).

Upstream of the PKA complex, intracellular cAMP levels control the frequency of bud-hypha transitions (Zelada *et al.*, 1998; Niimi, 1996; Chattaway *et al.*, 1981; Niimi *et al.*, 1980). Depletion of cAMP levels unable *C. albicans* cells to switch from the yeast to the hyphal form (Rocha *et al.*, 2001). Regulation of *C. albicans* cAMP levels is modulated by functional adenylyl cyclases: Cyrp (Cyclic AMP requirement) (Rocha *et al.*, 2001) and Cap1p (cyclase-associated protein) (Bahn & Sundstrom, 2001). *CAP1* was the first gene identified as adenylate cyclase activity regulator, directly related with cAMP levels, whose function has consequences in *C. albicans* morphogenesis and virulence (Bahn & Sundstrom, 2001), however phenotypes obtained were not as evident as those obtained for *CYR* (also named *CDC35*) gene. Deletion of both alleles of *CYR* abolished completely detectable levels of intracellular cAMP, suggesting that Cyrp is the only enzyme capable of producing cAMP in *C. albicans* cells since this mutant phenotype could be complemented by addition of exogenous cAMP. Cyrp is part of a sensory system involved in detecting environmental changes and sending signals to the morphogenetic machinery that controls the mode of growth in an Efg1p dependent manner (Rocha *et al.*, 2001). Cyrp contains a highly conserved Ras binding domain, leucine-rich repeat domain located in the central region of adenylyl cyclase (Suzuki *et al.*, 1990), suggesting that it has the potential to interact with Ras1p.

Ras1p is a member of a highly conserved family of GTP binding proteins that act as molecular switch in a variety of signal transduction pathways controlling cellular growth and differentiation (Bourne *et al.*, 1990), however and, in contrast to *S. cerevisiae*, the *C. albicans* RAS homologue is dispensable for vegetative growth (Leberer *et al.*, 2001) In *C. albicans*, Ras1p functions as a sensor and a mediator of the environmental cues that trigger morphogenetic signalling pathways, namely Efg1 and Cph1. In the Efg1 signalling pathway, Ras1p stimulates adenylate cyclase for the production of cAMP, which in turn acts as an intracellular second messenger for bud-hypha transition. *C. albicans ras1/ras1* mutant exhibit strong hyphal defect which is reverted by *EFG1*

overexpression or cAMP addition (Leberer *et al.*, 2001). Moreover, constitutive induction of hyphal growth caused by overexpression of the dominant-active $Ras1p^{G13V}$ mutant version (Feng *et al.*, 1999) is attenuated by deletion of the *EFG1* alleles and completely blocked by homozygous deletion of *CYR* (Leberer *et al.*, 2001).

In *C. albicans*, amino acid starvation promotes pseudohyphal growth in an Efg1p dependent manner (Tripathi *et al.*, 2002). This morphogenetic response is mediated by Gcn4p (general control nonderepressible), belonging to the family of bZIP transcription factors, whose sequence conservation is restricted to their DNA-binding domains (Murad *et al.*, 2001; Braun *et al.*, 2001; Kadosh & Johnson, 2001). Following amino starvation, Gcn4p interacts with GCRE-like (general control response elements) sequences (TGACTC) in the promoters of their target genes to activate transcription of genes on all amino acid biosynthetic pathways (GCN response-general amino acid control). Gcn4p may activate morphogenesis, only in response to amino acid starvation, acting downstream Ras1p and being strongly dependent upon Ras1p (Tripathi *et al.*, 2002).

The transmission of external signals in eukaryotic cells involves the small GTP-binding protein Ras and G proteins-coupled receptors (GPCRs). Typically, a ligand-bound GPCR activates or inhibits heterotrimeric G-protein complexes and transmits signals to downstream effectors, including adenylate cyclase, phospholipases, or protein kinases (Neer, 1995). In *C. albicans*, the Gpr1p and the G protein alpha subunit α-subunit Gpa2p regulates morphogenesis through a cAMP-dependent mechanism (Maidan *et al.*, 2005; Miwa *et al.*, 2004). *GPA2* and *GPR1* are required for a glucose-dependent increase in cAMP (Miwa *et al.*, 2004) and the former acts as mediator of the stimulatory role of the Gpr1p on the cAMP pathway (Maidan *et al.*, 2005).

Cdc42p (cell division cycle) is a Rho-type GTPase necessary for the establishment and maintenance of polarized growth in *C. albicans* (Ushinsky *et al.*, 2002) and in many, if not all, eukaryotic cell types (Johnson, 1999). In

strains with Cdc42p specific mutations, it was possible to examine the function of Cdc42p in the bud-to-hypha transition without altering the mitotic functions of the protein, which are necessary for cell viability and growth (VandenBerg *et al.*, 2004). These *cdc42* mutants display serious defects in hyphal formation, on a variety of hypha-inducing media, due to the reduced level of expression of *EFG1*, as well as, other Efg1p-dependent transcripts, namely *ECE1* (Extent of cell elongation), *HWP1* (hypha wall protein) and *SAP6* (secreted aspartic proteinase). Thus, Cdc42p interacts with Efg1p regulating the expression of hypha-specific genes (VandenBerg *et al.*, 2004).

The Efg1 pathway appears to be down-regulated by Rad6p (Radiation sensitive) (Leng *et al.*, 2000), which represses yeast-hypha morphogenesis in Efg1 dependent manner. Rad6p depletion promotes hyphal development under budding conditions and *RAD6* overexpression inhibits hyphal growth. In *S. cerevisiae*, Rad6p is an ubiquitin-conjugating enzyme that targets specific proteins for rapid degradation via proteasome (Varshavsky, 1997; Jentsch *et al.*, 1987). Therefore, in *C. albicans* Rad6p probably targets a component of the Efg1 pathway for ubiquitin-mediated protein degradation (Leng *et al.*, 2000; Madura & Varshavsky, 1994).

Other transcription factor related with Efg1p is Tec1p (Ty1 enhancement control). *C. albicans* Tec1p is a member of the TEA/ATTS family of transcription factors whose function is related with morphogenesis and virulence (Schweizer *et al.*, 2000), but it is not yet clear in which pathway it is involved. *tec1/tec1* and *efg1/efg1* mutants display the same phenotype, however, *EFG1* transcription is not regulated by Tec1p indicating that Tec1p acts independently or downstream of Efg1p (Schweizer *et al.*, 2000). Nevertheless, Tec1p is required for activation of genes involved in hypha formation, namely *SAP4-6* genes that possess promoter regions containing a head-to-tail spaced arrays of TCS (TEA consensus sequence; 5'-CATTCY-3') motifs, which are the binding sites for fungal TEA/ATTS transcription factors (Andrianopoulos & Timberlake, 1994). *TEC1* transcription is regulated by the bHLH protein Cph2p (*Candida* pseudohyphal regulator) which specifically binds to two SRE-1 (sterol regulatory element 1) elements upstream *TEC1*.

Furthermore, Cph2p also binds to E-box motifs being potentially able to regulate the expression of some hypha-specific genes. Nevertheless, since all known hypha-specific genes have *TEC1* potential binding sites, Cph2 plays a role of activator of hypha-specific genes by acting directly or through *TEC1* (Lane *et al.*, 2001).

1.4.3.1.2. Cph1 (Candida pseudohyphal regulator) signalling pathway

Extracellular signal-regulated protein kinase (ERK, or mitogen-activated protein kinase [MAPK]) regulatory cascades in fungi turn on transcription factors that control developmental processes, stress responses, and cell wall integrity (Csank *et al.*, 1998). Cph1p is the terminal effector of the MAP (mitogen-activated protein) kinase pathway (Figure 1.12), which was the first morphogenesis signalling pathway identified in *C. albicans* and whose components display high homology with the MAPK pathways in *S. cerevisiae* and other fungi (Banuett, 1998; Gustin *et al.*, 1998). Cph1p regulates filamentous growth in response to nitrogen starvation and is a functional homolog of the transcription factor Ste12p (Sterile), which lies at the bottom of the pseudohyphal and pheromone signalling pathway in *S. cerevisiae* (Liu *et al.*, 1994). While in *S. cerevisiae*, Ste12p interacts with Tec1p to activate the transcription of the genes carrying the filamentous response element (FRE) (Madhani & Fink, 1998), in *C. albicans*, no relation was demonstrated between Tec1p and Cph1p. Epistasis experiments place the transcription factor Cph1 downstream the MAPK module (Csank *et al.*, 1998; Leberer *et al.*, 1996).

The MAPK cascade is composed by MAPK Cek1p (*C. albicans* ERK-like kinase), MAPKK Hst7p (homolog-STE7), MAPKKK CaSte11p (*C. albicans* STE11) and MAPKKKK Cst20p (*C. albicans* STE20). These sequentially acting kinases activated by phosphorylation of tyrosine and threonine residues, transduce signals from the cell surface to the nucleus for a wide variety of responses in eukaryotic cells (Leberer *et al.*, 1997; Robinson & Cobb, 1997). Cek1 has high level of sequence identity with the *S. cerevisiae* kinases *FUS3* (cell fusion) and *KSS1* (Kinase Suppressor of Sst2 mutations) and to a variety of mammalian kinases of the ERK (extracellular signal-regulated kinases) class (Whiteway *et*

al., 1992) and is probably activated by MAPKK Hst7p, which is a functional homolog of Ste7 (Csank *et al.*, 1998; Kohler & Fink, 1996; Leberer *et al.*, 1996; Clark *et al.*, 1995). The MAPKKK CaSte11p has not been formally identified although a sequence homolog of *S. cerevisiae*, Ste11p, is present in *C. albicans* genome (Csank *et al.*, 1998).

Cst20p is an homolog of the *S. cerevisiae*, Ste20p (Sterile), a member of the serine/threonine kinases family, which are involved in triggering morphogenetic processes in response to external signals in a wide range of organisms from yeast to mammals (Leberer *et al.*, 1997; Sells *et al.*, 1997; Lim *et al.*, 1996). Upstream of the MAPK module are Cdc42p and Ras1p, external signals transducers, signalling two morphogenesis pathways, Efg1 and Cph1. *C. albicans* Cdc42p signals through Cst20p to the Cst11-Hst7p-Cek1 mitogen-activated protein kinase cascade, leading to the activation of the Cph1p transcription factor (Ushinsky *et al.*, 2002; Csank *et al.*, 1998). Cdc42p as a Rho-type GTPase acts as molecular switch that is inactive when bound to GDP and active when bound to GTP, thereby enabling it to interact with downstream effectors proteins. The defect in the budded-to–hyphal-form transition was apparent in numerous different hypha-inducing media, suggesting that Cdc42p is a convergence point for the regulation of a variety of upstream signals that induce hyphal growth. Since Cdc42p is also responsible for modulating rearrangements of the actin cytoskeleton in numerous cell types, it is surprising that Cdc42p was required to specifically regulate the proper formation of hyphal cells (VandenBerg *et al.*, 2004).

The small Rho-like G-protein Cdc42p carry the signal from Ras1p to Cst20p, which has a Cdc42p binding site (Leberer *et al.*, 1996). Ras1p is a component of the sensor machinery that links environmental signals to morphogenetic processes (Leberer *et al.*, 2001).

A balance between MAP kinase activation by kinases and their inactivation by phosphatases is likely to be important for decisions which govern developmental processes and virulence in *C. albicans*. Because dephosphorylation of threonine/tyrosine residues of a MAP kinase can result in

its inactivation, the tyrosine specificity of Cpp1p (Candida protein phosphatase) was found to perform a role in MAP kinase inactivation in *C. albicans* (Csank *et al.*, 1997). Cpp1p is a member of the VH1 family of dual-specificity phosphatases and is most similar to the *S. cerevisiae* MAP kinase phosphatase Msg5p (Doi *et al.*, 1994). Cpp1p functions as a MAP kinase phosphatase in *C. albicans* and acts to block the yeast-to-hyphal transition under noninducing conditions through inactivation of the MAPK Cek1p, since the disruption of *CEK1* completely suppresses the *cpp1* mutant phenotype (Csank *et al.*, 1997). Under hyphal-inducing conditions, namely at 37°C, Cpp1p is a potent suppressor of serum-induced hyphal gene expression (Schroppel *et al.*, 2000).

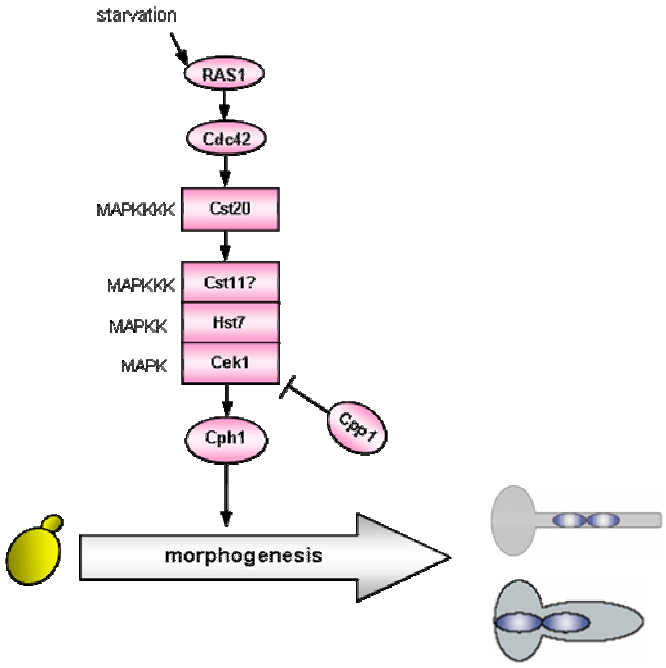


Figure 1.12. Cph1 morphogenetic signalling pathway. Interactions between the different known components are represented by straight arrows or bars, according to their function, activation or repression respectively. Some of these interactions are not well defined (interrupted arrows). Adapted from Berman & Sudbery (2002).

Finally, strains containing deletions in genes encoding the MAP kinase kinase kinase kinase, *CST20*, the MAP kinase kinase, *HST7*, *CEK1* MAP kinase gene, and the transcription factor, *CPH1*, are defective in hyphal growth on solid

substrate in response to nitrogen starvation (Leberer *et al.*, 1997; Kohler & Fink, 1996; Leberer *et al.*, 1996; Liu *et al.*, 1994).

1.4.3.1.3. Czf1 (Candida zinc finger) signalling pathway

Although homozygous double *efg1/cph1* mutants have a drastic block in hypha formation under most standard inducing conditions, considerable filamentation occurs under embedded/microaerophilic conditions (Brown, Jr. *et al.*, 1999; Sonneborn *et al.*, 1999a). These results indicate that under limited supply of oxygen an alternative filamentation pathway is activated independent from cAMP-PKA and MAPK pathways. The putative transcription factor Czf1p (Figure 1.13) is the key element of an alternative morphogenesis pathway in *C. albicans* (Brown, Jr. *et al.*, 1999). The central portion of Czf1p contains four clusters of glutamine residues and the C terminus contains a cysteine-rich region similar to zinc-finger elements.

Overexpression of *CZF1* stimulates filamentous growth, but only under embedded conditions and in certain media lacking glucose. Homozygous *czf1* mutant filament normally under standard induction conditions and is defective in hyphal growth when embedded in certain agar media. The defective phenotype of *czf1* mutant is exacerbated by the presence of *cph1* mutation which suggests that other factors, namely Cph1p, contribute to filamentation under embedded conditions. Hyperfilamentation of the *efg1* single and *efg1 cph1* double mutants suggests that Efg1p is a negative modulator of the Czf1 pathway under microaerophilic/embedded conditions (Figure 1.13). These data confirms the dual role of Efg1p in morphogenesis, as activator or as repressor, according to the environmental conditions. The ability of *C. albicans* to sense the presence of surrounding matrix may play a role during infection, probably Czf1p and Efg1p collaborate to allow filamentation in different host environments, as in blood (serum), and during the passage of tissues within the host cells, at low oxygen partial pressure (Ernst, 2000).

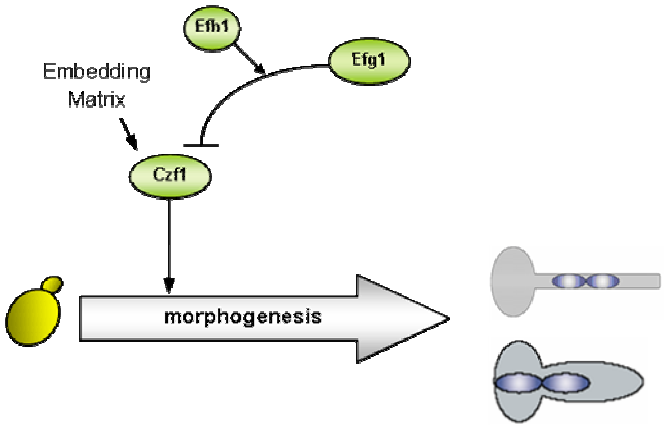


Figure 1.13. Czf1 morphogenetic signalling pathway. Interactions between the different known components are represented by straight arrows or bars, according to their function, activation or repression respectively. Some of these interactions are not well defined (interrupted arrows). Adapted from Berman & Sudbery (2002).

1.4.3.1.4. pH signalling pathway

In vitro, *C. albicans* can grow in a wide range of extracellular pH (pH 2-10), with implications at morphological level: neutral pH favours hyphal growth while acidic pH stimulates a yeast-like form growth. The ability of fungi to respond to alkaline environments is mediated by a conserved signal transduction pathway. This pathway governs pH responses trough the activity of the zinc finger transcription factor, Rim101p (Regulator of IME2) (Figure 1.14) (Tilburn *et al.*, 1995; Su & Mitchell, 1993). Rim101p promotes changes in gene expression which is controlled by proteolytic processing, involving the removal of the C-terminal domain and the activity of several upstream gene products, including Rim13p, Rim20p, Rim8p, Rim21p and Rim9p. At acidic pH, Rim101p is found predominantly in the full-length (non-functional) form. At alkaline pH, it is found predominantly in the processed (active) form (Li & Mitchell, 1997; Orejas *et al.*, 1995). Rim13p has homology to calpain proteases and is thought to be the proteolytic enzyme that processes Rim101p. Rim20p interacts with the C-terminal domain of Rim101p and may act as a scaffolding protein to bring Rim13p into position to cleave full-length Rim101p (Xu & Mitchell, 2001). The function of Rim8p is not known and it has no homology to proteins with known function. Rim9p and Rim21p appear to encode proteins with four and seven transmembrane-spanning domains

respectively and thus may be localized at the plasma membrane and serve as the sensors for extracellular pH (Davis, 2003).

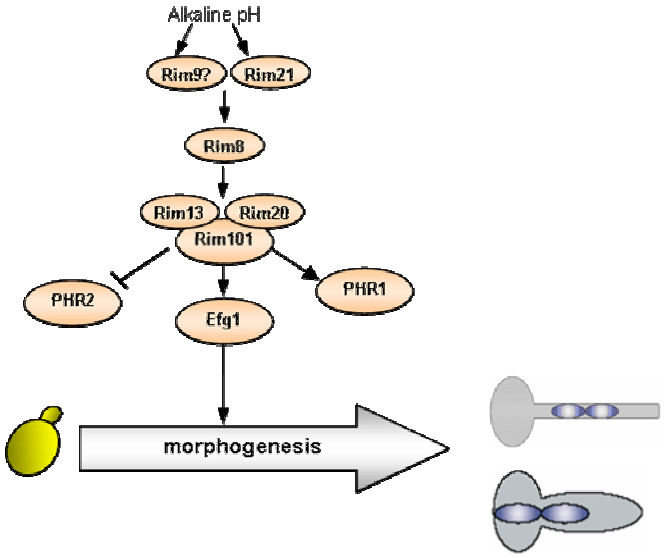


Figure 1.14. pH morphogenetic signalling pathway. Interactions between the different known components are represented by straight arrows or bars, according to their function, activation or repression respectively. Some of these interactions are not well defined (interrupted arrows). Adapted from Davis (2003).

The RIM101p has a central role in pH-dependent regulation by inducing expression of alkaline-expressed genes (*PHR1* and *PRA1*) and repressing acid-expressed genes at alkaline pH (*PHR2*) (Ramon *et al.*, 1999). *PHR1* (pH responsive) gene encodes a cell wall protein with β-1,3-and β-1,6-glucan crosslinking activity (Fonzi, 1999; Saporito-Irwin *et al.*, 1995), is expressed at pH≥5.5 and is essential for wild-type growth under these conditions (Saporito-Irwin *et al.*, 1995). *PRA1* (pH regulated antigen) gene encodes a cell wall protein originally identified as a hypha-specific antigen, is also expressed in more alkaline environments at pH≥6 (Sentandreu *et al.*, 1998). *PHR2* gene encodes a protein that is 71% identical to Phr1p and is expressed at pH≤5.

The alkaline response gene *PHR1* is essential for virulence in an hematogenously disseminated model of infection (Ghannoum *et al.*, 1995). The neutral/slightly alkaline pH of blood (pH 7.4) and many organs favour *PHR1* but not *PHR2* expression. Conversely, *PHR2* is required for virulence in

vaginal and stomach model of infection (de Bernardis *et al.*, 1998). The activation of hyphal growth is dependent on Efg1p (El Barkani *et al.*, 2000).

1.4.3.1.5. Tup1 (deoxythymidine monophosphate uptake) pathway

Besides low pH, low temperature and high cell density, high glucose concentrations down regulate hyphal development of *C. albicans* in liquid media. On solid media, osmolarity also inhibits hypha formation (Alex *et al.*, 1998). In *C. albicans*, the Tup1p transcription factor is an important repressor of morphogenesis. *TUP1* knockout strains become blocked in the pseudohyphal form (Braun & Johnson, 1997). A number of genes, termed repressed by *TUP1* (RBT), whose expression is regulated by Tup1p (Figure 1.15), encode secreted or cell surface proteins (Braun & Johnson, 2000a).

In *S. cerevisiae* Tup1p forms a complex with Ssn6p (Cyc8p-Cytochrome C) and represses the transcription of sets of functionally related genes, interacting with their promoters indirectly through specific DNA-binding proteins (Smith & Johnson, 2000; Park *et al.*, 1999; Treitel & Carlson, 1995). In *C. albicans*, Tup1p might operate in a similar way to repress the expression of hypha-specific genes and control morphogenesis, while Ssn6p has a dubious role either as activator or repressor of filamentous growth (Hwang *et al.*, 2003). The Tup1p transcriptional repressor may act in concert with other repressor proteins such as Nrg1p (negative regulator of glucose-repressor genes) and Rfg1p (repressor filamentous growth), to negatively regulate transcription of hypha-specific genes involved in the budded-to-hyphal form transition (Dhillon *et al.*, 2003; Murad *et al.*, 2001). Nrg1p is a DNA binding protein that represses transcription in NRE (NRG response element) present in the promoters of hypha-specific genes *ALS8*, *ECE1*, *HWP1* and *HYR1* (Murad *et al.*, 2001), however repression by Nrg1p is Tup1p dependent (Braun *et al.*, 2001).

A second putative Tup1-target protein regulates morphogenesis in *C. albicans*. Rfg1p is a putative DNA-binding protein that functions as a transcriptional repressor of hypha-specific genes via recruitment of the Ssn6-Tup1 complex

(Kadosh & Johnson, 2001) and regulates expression of at least eight genes, including cell wall or (in some cases) putative cell wall components that are specifically expressed when *Candida* grows in the filamentous form (*HWP1*, *RBT1*, *HYR1*, *ECE1*, *ALS1*, *RBT4* and *RBT5*).

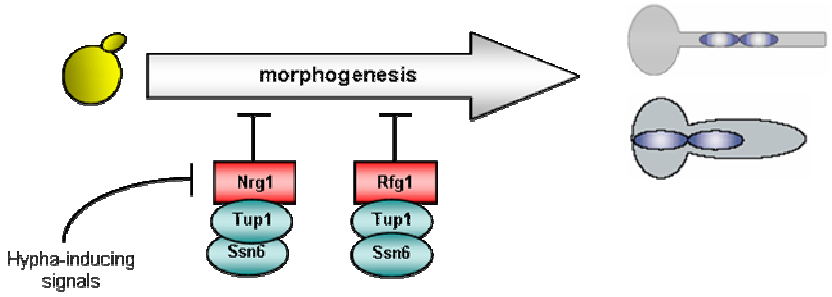


Figure 1.15. Repressor morphogenetic signalling pathway. Interactions between the different known components are represented by straight arrows or bars, according to their function, activation or repression respectively. Some of these interactions are not well defined (interrupted arrows). Adapted from Berman and Sudbery (2002).

1.4.3.1.6. Other factors

Apart from these genes mentioned above, several other genes and factors have been reported as required for *C. albicans* morphogenesis, some of which will be described briefly. *RBF1* (RPG binding factor 1) encodes a putative transcription factor of *C. albicans* that binds to the same consensus sequence as the *S. cerevisiae* transcription factor Rap1p. Mutation of *RBF1* stimulates filamentous growth, suggesting that *RBF1* plays a negative regulatory role in the yeast-to-hyphal-form transition (Ishii *et al.*, 1997).

SSK1 (suppressor of sensor kinase) encodes a response regulator in a two-component regulatory circuit involved in responses to osmotic stress. Double *ssk1* mutant's display reduced hyphal growth under serum stimulation (Calera *et al.*, 2000). By analogy with *S. cerevisiae* (Stanhill *et al.*, 1999) this might reflect an indirect effect of stress responses on morphogenetic regulation in *C. albicans*.

The yeast-to-hyphae transition involves changes in the cell wall structure and composition, including an increase in chitin levels in hyphae (Munro *et al.*, 1998). The *C. albicans* genome contains four chitin synthase (CHS) genes

(Munro *et al.*, 2003; Gow *et al.*, 1994; Sudoh *et al.*, 1993; Au-Young & Robbins, 1990) and three chitinase (CHT) genes (Nantel *et al.*, 2002; McCreath *et al.*, 1995). The regulation of chitin synthase and chitinase genes is poorly understood in *C. albicans* however two homologues of the *ACE2* and *SWI5* genes of *S. cerevisiae* were identified in *C. albicans* (Tzung *et al.*, 2001; O'Conallain *et al.*, 1999; King & Butler, 1998). *ACE2* (Activation of CUP1 expression) encodes a putative protein whose depletion blocks cell separation after division, thus increasing tendency to form pseudohyphae. *ace2* mutants also displayed altered adherence to plastic surfaces and form defective biofilms (Kelly *et al.*, 2004).

In fungi, proteins are *O*-mannosylated at serine or threonine residues during import into the endoplasmatic reticulum by protein mannosyltransferases (Pmt proteins). In *C. albicans*, Pmt proteins are encoded by a five *PMT* family genes whose deletion affects growth, morphogenesis and antifungal resistance. In standard inducing conditions, all *pmt* mutants suppress hyphal developmental with exception for *pmt5*. This morphogenetic defect could be due to lack of mannosylation of the Pmt-target proteins, which are required for the construction of hyphal tubes and can function as sensors that activate morphogenetic signalling pathways in response to environmental cues (Prill *et al.*, 2005; Timpel *et al.*, 2000; Timpel *et al.*, 1998).

Lipid rafts are membrane microdomains that contribute to morphogenesis due to their lipid composition, highly enriched in sterols and sphingolipids, which allows the partitioning of specific proteins into these domains, namely GPI-anchor proteins. A highly polarized rich-ergosterol domain is present specifically during hyphal growth, which are also enriched in proteins involved in signalling transduction pathways, cell adhesion (Als1p and Hwp1p) and other polarity processes (Martin & Konopka, 2004). The *CaGPI7* gene, involved in the biosynthesis of the GPI anchors, is also required for normal morphogenesis, virulence, survival in host and resistance to macrophages. *CaGPI7* lacking cells display defects in septation and consequently cells form aggregates. In these mutants, filamentous growth is suppressed in solid but not in liquid media. Therefore, the *CaGPI7* gene deletion results in the absence

of some GPI-anchored proteins involved in cell-signalling or cell-host interaction (Richard *et al.*, 2002b).

In many organisms, morphogenesis is tightly related with cell cycle progression and is regulated by the cyclin-dependent kinase (Cdk), thus many studies assessed the role in morphogenesis of genes that control cell cycle. Deletion of *FKH2* (Fork head homolog), a homolog of the transcription factors *FKH1,2* that regulate *CLB2* (Cyclin B) transcription in *S. cerevisiae*, caused constitutively pseudohyphal growth (Bensen *et al.*, 2002). A similar phenotype was reported for mutants deleted for *CDC5* (Cell division cycle) (Bachewich *et al.*, 2003) or *MCM1* (Minichromossome maintenance) (Rottmann *et al.*, 2003), genes having roles in cell cycle. The G1 phase of the cell cycle acts as an interface between environmental conditions and the decision to grow or to differentiate. In *S. cerevisiae,* start of a mitotic cell cycle is initiated by the G1 cyclin Cln3p in association with the Cdc28p kinase (Futcher, 1996), which then activate the transcription of two further G1 cyclins, *CLN1* and *CLN2*. Cln1p and Cln2p control the G1/S transition. *C. albicans* contains homologues of the G1 cyclins *CCN1* (*C. albicans* cyclin), *HGC1* (Hypha-specific expression and relatedness to G1 cyclins) and *CLN3* whose activity influences hyphal growth. *CCN1* seems to be strictly involved in the cell cycle since its deletion results in inability to maintain hyphal growth under certain conditions (Loeb *et al.*, 1999). The two other G1 cyclins play critical roles in morphogenesis but in opposite sides. *CLN3* is essential for budding and *cln3*-depleted cells spontaneously form hyphae, even under favouring yeast growth conditions (Lazo *et al.*, 2005; Bachewich & Whiteway, 2005). *HGC1* is required for hyphae formation and its deletion prevents hyphal growth under all hypha-inducing conditions (Zheng *et al.*, 2004). Neither factor is essential for cell cycle, suggesting that G1 cyclin in *C. albicans* evolved important roles in hyphal morphogenesis as opposed to cell cycle progression (Zheng *et al.*, 2004). Besides G1 cyclins, a protein kinase in the same Cdc2 subfamily as MAP kinases and Cdks was identified and designated *CRK1* (Cdc2-related kinase). *CRK1* deletion leads to defective hyphal formation since there was no induction of hypha-specific genes, while ectopic expression of *CRK1* catalytic

domain promotes the induction of hyphal colonies under yeast growth conditions (Chen *et al.*, 2000).

The *C. albicans INT1* (integrin-like protein) gene encodes a protein that is present at the septin ring of yeast and hyphal cells. When expressed in *S. cerevisiae*, *INT1* induces the formation of highly elongated cells that resemble hyphal germ tubes more elongated than *S. cerevisiae* pseudohyphae (Gale *et al.*, 1996). In these cells Int1p associates with septin proteins inducing formation of abnormal spiral structures (Gale *et al.*, 2001). *INT1* also affects morphogenesis and virulence in *C. albicans* (Gale *et al.*, 1998).

1.4.3.1.7. Hypha specific genes (HSGs)

Stimulation of one or more of the morphogenetic signalling pathways described above activates expression of filamentation specific genes. Molecular interactions between the various regulators of morphogenesis leads to coordinated control of hyphal development, but these interactions remain incompletely defined since no downstream target genes have been identified. It is assumed that the transition from one cell morphology to another is related to changes in the gene expression pattern. Microarray studies have uncovered genes that are activated during the yeast-to-hypha transition. The morphological change induced by serum and temperature (37°C), doubled the expression of at least 18 genes and increased more than 50% the expression of 56 additional genes (Nantel *et al.*, 2002), while a set of 46 genes were down-regulated. The HSG, *ECE1* (Birse *et al.*, 1993), *SAP4,5,6* (Monod *et al.*, 1994), *RBT1* (Braun & Johnson, 2000) and *HWP1* (Sharkey *et al.*, 1999) are all up-regulated and others have its expression highly up-regulated in the hyphae, namely *SOD5* (superoxide dismutase) (Martchenko *et al.*, 2004; Nantel *et al.*, 2002). The expression profile of the yeast-to-hypha transition is dependent on the inducing environmental signals applied (Nantel *et al.*, 2002). Morphogenesis induces expression of hyphal-specific genes, but some are expressed in all hypha-inducing conditions and others are expressed in response to specific inducing conditions. The latter are called conditional

hyphal-specific genes (Table 1.1), a good example is the *ALS1* (Agglutinin-like sequence) gene (Brown, 2002a).

Table 1.1. Hypha-specific and conditional genes in *C. albicans*.

Hypha-specific genes	Conditional hypha-specific genes
ALS3	ALS1
ALS8	SAP4
ECE1	SAP5
HWP1	SAP6
HYR1	
HGC1	

The *ALS1* gene is expressed when hyphal growth is induced in the RPMI tissue culture medium (Hoyer *et al.*, 1995) but not when hyphal growth is induced by pH (Hoyer *et al.*, 1998a). Asl1p localizes to the blastopore-filament junction suggesting that Asl1p could have a role at the beginning of the filamentation process. Also *ALS1* expression is regulated by Efg1p, which makes it a terminal effector of the PKA morphogenetic signalling pathway (Fu *et al.*, 2002).

Other members of the *ALS* family are hypha-specific genes (Table 1.1). *ALS3* shares 81% identity to *ALS1* and the gene product is expressed under serum-, N-acetylglucosamine-, and proline-inducing factors (Hoyer *et al.*, 1998a). *ALS8* gene encodes a hypha-specific cell surface agglutinin (Zhao *et al.*, 2004) and is activated under serum-, N-acetylglucosamine- and pH-inducing conditions. Its expression is controlled by the Efg1p signalling pathway and Tup1p repressor effect (Brown, 2002a). Despite *ALS3* and *ALS8* being considered hypha-specific genes required for viable hypha formation, *als3/als8* double mutants are still capable of forming hyphae although with reduced adhesion properties (Leng *et al.*, 2001).

Another family of genes whose expression depends on cell morphology is the *SAP* (secreted aspartyl proteinases) gene family (Hube *et al.*, 1994). Under

serum–inducing factor and neutral pH, the main proteinase genes associated with hypha formation are *SAP4*, *SAP5* and *SAP6* (White & Agabian, 1995; Hube *et al.*, 1994). *SAP4-6* have promoter elements with consensus sequences [CATTC(A/C)] for the TEA/ATTS transcription factor Tec1 (Schweizer *et al.*, 2000). Additionally, *SAP4-6* displayed increased expression in the hyper filamentous strain *cpp1* double mutant (Schroppel *et al.*, 2000), and decreased expression in *efg1/efg1* mutant, with strongly reduced ability to form hyphae (Felk *et al.*, 2002). Triple mutants of *SAP4-6* displayed reduced invasiveness but still produced hyphal cells (Sanglard *et al.*, 1997). *SAP5* expression is dependent on two morphogenetic signalling pathways (Efg1 and Cph1) but independent on the *C. albicans* growth form during infection (Staib *et al.*, 2002).

ECE1 was the first hypha-specific gene identified (Birse *et al.*, 1993). The transcript levels of *ECE1* are related to cell elongation, despite the induction conditions, suggesting that Ece1p perform a role in hyphal growth. However, Ece1p is not required for hyphal formation since *ece1* double mutants are able to form hyphae (Birse *et al.*, 1993). *ECE1* expression is regulated by Efg1 but is not affected by *TUP1* (Sharkey *et al.*, 1999).

HWP1 is a hypha-specific gene expressed in germ tubes and true hyphae (Staab *et al.*, 1996). It encodes an outer mannoprotein, with a cell surface exposed N-terminal domain and a C-terminal domain covalently integrated into cell wall through a GPI-anchor (Staab *et al.*, 1999; Staab *et al.*, 1996). The N-terminal domain has high similarity with mammalian TGase substrates (Staab *et al.*, 1996), suggesting the formation of stable attachments, by noncovalent adhesive forces, between germ tubes and BECs (buccal epithelial cells). Thus the primary role of Hwp1p is to serve as a substrate for the mammalian transglutaminases (Staab *et al.*, 1999). Expression of *HWP1* is activated by Efg1p and repressed by Tup1p. However, under serum-inducing conditions *hwp1* double mutants still form true hyphae (Sharkey *et al.*, 1999).

The *HYR1* gene has sequence similarity with genes encoding cell wall glycoproteins. *HYR1* is expressed during hyphal growth induced by serum, pH

and N-acetylglucosamine. However, *hyr1* double mutants are able to form normal hyphae and maintain the normal adhesion properties (Bailey *et al.*, 1996).

HGC1 gene was annotated by homology as encoding G1 cyclin, however its expression pattern and cellular functions suggests that *HGC1* diverged from other G1 cyclins having lost its function in cell cycle control but retaining the function of promoting apical bud extension (Zheng *et al.*, 2004). Hgc1p is produced during the cell cycle when cells are exposed to hypha-inducing signals and recruits a fraction of Cdc28p to promote hyphal morphogenesis, leaving the rest of the G1 and mitotic cyclins to control cell cycle progression. Despite having a critical role in morphogenesis its constitutive expression is not sufficient to cause hyphal growth, indicating that other proteins or cellular processes are also needed (Zheng *et al.*, 2004).

Most of the hypha-specific genes like *ALS3*, *ALS8*, *ECE1*, *HWP1*, and *HYR1* contain E-boxes in their promoter regions (CANNTG) (Leng *et al.*, 2001), which are binding sites for bHLH transcription factor APSES proteins (Massari & Murre, 2000), such as Efg1p. The hypha-specific promoters also contain STRE-like (C4T), YRE-like (RRTGACTC), and GCRE-like (TGACTC) sequences (Brown, 2002a), which mediate transcriptional responses to stresses and amino acid starvation in *S. cerevisiae* (Martinez-Pastor *et al.*, 1996; Wemmie *et al.*, 1994; Arndt & Fink, 1986).

1.4.3.2. Phenotypic switching

Phenotypic switching is a general mechanism which spontaneously originates a variety of phenotypes within a clonal population, giving rise to variability in clonal populations (Soll, 1992). Switching leads to a colony variant that in turn switches back to the original parental phenotype at high frequency. Spontaneous phenotypic switching can vary from 10^{-4} (low frequency mode) to 10^{-1} (in a high frequency mode) (Ramsey *et al.*, 1994; Slutsky *et al.*, 1985). This unique morphological phenomenon has eight general characteristics namely:

- occurs at higher frequencies than expected for point mutations (Bergen *et al.*, 1990; Rikkerink *et al.*, 1988);
- cells can be in a low frequency mode (basic smooth phenotype) or in high-frequency mode (other variants);
- is reversible at high frequency originating the original phenotype;
- can involve white-opaque transition (Slutsky *et al.*, 1987) or unmyceliated-myceliated transition (Vargas *et al.*, 1994; Soll *et al.*, 1987);
- different strains may display different switching systems related to colony phenotypes that are included in the switching repertoire (Soll, 1992);
- UV irradiation (Morrow *et al.*, 1989), temperature (Srikantha & Soll, 1993), age of colony (Soll *et al.*, 1989), white blood cells and oxidants (Kolotila & Diamond, 1990) stimulates switching;
- can occur without being noticed in the colony phenotype (Odds & Merson-Davies, 1989; Anderson & Odds, 1985);
- individual cells can have more than one switching system (Soll, 2002).

Two examples of phenotypic switching have been described in *C. albicans*: the white-opaque transition and the 3153A-type switching. The 3153A-type switching repertoire was described in cultures of the laboratory strain 3153A (Slutsky *et al.*, 1985) and it was also observed in other laboratory strains and clinical isolates (Soll *et al.*, 1987; Pomes *et al.*, 1985). This type of switching produces a repertoire of at least seven colony phenotypes that were classified as smooth, star, ring, irregular wrinkle, stippled, hat, fuzzy.

The white-opaque transition, involves the transition between white, hemispherical smooth colony and a grey, flat smooth colony morphology. This reflects a dramatic change in individual cells, occurs spontaneously at high frequency in both directions (white to opaque and vice-versa), and can be manipulated by temperature. High temperature favours the white cell while opaque cells are favoured by lower temperatures (Srikantha *et al.*, 2000; Srikantha & Soll, 1993; Rikkerink *et al.*, 1988; Slutsky *et al.*, 1987).

White budding phase cells are round to slightly ellipsoidal and have a smooth cell wall (Kvaal *et al.*, 1997). Opaque phase cells are elongated or bean shaped, contain a large vacuole, and have a discontinuous cell wall, with prominences called pimples (Kvaal *et al.*, 1997; Anderson *et al.*, 1989; Slutsky *et al.*, 1987). Besides morphological differences, white and opaque cells also differ in global gene expression profiles. These differences are mainly metabolic preferences, but also include adhesion and cell surface properties, secreted hydrolytic enzymes, stress and drug resistance and finally mating (Lan *et al.,* 2002).

Metabolic specialization found in white and opaque cells is tightly related to pathogenesis to exploit available nutrients on the site of infection. In opaque cells, glucose is converted to fatty acids providing substrates for β-oxidation, which is consistent with the idea that opaque cells are able to colonize skin, habitat rich in lipids and poor in free sugars. In contrast, white cells take advantage of glucose present in blood by expressing genes encoding low and high affinity glucose transporters (Lan *et al.*, 2002).

The transition between white and opaque phases involves the coordinated activation and deactivation of phase specific genes (Sonneborn *et al.*, 1999b; Balan *et al.*, 1997; Srikantha & Soll, 1993; Morrow *et al.*, 1992). As described in Table 1.2 there are white and opaque specific genes but there are also gene products that are more abundant in either one of the two phases.

Table 1.2. White- and opaque-specific genes expression in *C. albicans* strain WO-1.

White-phase	Opaque-phase
WH11	OP4
CDR4	SAP1
EFG1-3.2t	SAP3
HOS3-2.5t	CDR3
	EFG1-2.2t
	HOS3-2.3t

The opaque specific genes, *SAP1* (Morrow *et al.*, 1992; Hube *et al.*, 1991) and *SAP3* (Hube *et al.*, 1994) encode proteins belonging to the secreted aspartic proteinases. Other secreted enzyme, *LIP4*, a member of a secreted lipase family, is expressed at higher levels in opaque than white cells (Lan *et al.*, 2002). *OP4*, the second opaque gene cloned (Morrow *et al.*, 1993), encodes a protein with a hydrophobic N-terminus that may span the plasma membrane.

The initial screens for opaque-specific genes also uncovered an ABC transporter gene *CDR3*, which is a member of drug resistance family genes, (Balan *et al.*, 1997). Interestingly, the *CDR4* gene another member of the ABC drug resistance family is only expressed in white cells (Soll, 2002).

The first white specific gene cloned was *WH11* (Srikantha & Soll, 1993), which encodes cytosolic protein with homology to the heat shock protein Hsp12p of *S. cerevisiae* (Schroppel *et al.*, 1996). *WH11* expression is down-regulated not only upon switching to the opaque-phase but also during morphogenesis (Srikantha *et al.*, 1997; Srikantha & Soll, 1993), thus it was suggested that Wh11p might have a role in the genesis of spherical cell morphology (Kvaal *et al.*, 1997). Misexpression of *WH11* in the opaque-phase cells did not inhibit the formation of opaque cells and did not alter their phenotypic characteristics, however it did increase the switching rate from the opaque to white phase (Kvaal *et al.*, 1997), indicating that Wh11p is necessary for the maintenance of white phase (Schroppel *et al.*, 1996). Deletion of the *WH11* gene does not affect phase-specific cell and colony-morphology, expression of phase-specific genes (*SAP1* and *OP4*), and phenotypic switching (Park *et al.*, 2004). Since *WH11* is specifically activated in white cells, but is not required for phenotypic switching, it is likely that *WH11* and another genes differentially expressed in white and opaque phases, including metabolic genes (Lan *et al.*, 2002), are involved in the adaptation of white cells to specific environments (Park *et al.*, 2004).

Other genes have bigger transcript sizes in white than in opaque cells, namely *EFG1* and *HOS3*. *EFG1* is expressed in both types of cells, but its level of expression is 38-fold higher in white than in opaque phase cells (Srikantha *et*

al., 2000), being essential for maintenance of the round cell morphology characteristic of white cells. Functional analyses of the promoters of phase-regulated genes supported the hypothesis that switching involved complex regulation mechanisms. For instance, the promoters of the white-phase-specific genes *WH11* and *EFG1*-3.2 Kb contain unrelated white-opaque activation sequences, indicating that regulation of these co-ordinately expressed genes involved trans-acting factors containing different DNA-binding proteins (Lachke *et al.*, 2003; Srikantha *et al.*, 2000; Srikantha *et al.*, 1997). Some promoters of opaque-phase-specific genes contain a MADS-box binding site sequences at their activation sites (*OP4* and *CDR3*), while others do not (*SAP1*).

The white-opaque switching affects *C. albicans* physiology and virulence. Since white and opaque cells have distinct proprieties in terms of morphogenesis, adhesion, secretion of proteinases, antigenicity, sensitivity to neutrophils, and drug susceptivility (Lan *et al.*, 2002; Soll, 2002). Despite the discovery of white and opaque specific genes the molecular basis of phenotypic switching in *C. albicans* is not yet well understood and several switching mechanisms may exist. Several observations implicate the switching phenomenon with karyotype changes, particularly the ones that occur in the R-chromossome (Ramsey *et al.*, 1994). But no single karyotype correlates with a particular switch phenotype. No karyotype changes were observed in lineages of o-smooth cells and in white-opaque cell lineages of WO-1 strain (Zhao *et al.*, 2002; McEachern & Hicks, 1991). These results only demonstrate that an increase in the frequency of karyotype changes can accompany high-frequency phenotypic switching however a cause effect cannot be established (Perez-Martin *et al.*, 1999).

Disruption of the *SIR2* gene increases the frequency of phenotypic switching and promotes spontaneous morphogenesis and karyopyte changes. *SIR2* belongs to a family of silencers (repressors) and plays a prominent role in silencing at subtelomeric regions, mating type loci and ribosomal cistrons (Gartenberg, 2000; Rivier & Rine, 1992; Laurenson & Rine, 1992; Gottlieb & Esposito, 1989). Phenotypic switching is controlled by *SIR2* through a

mechanism similar to chromatin remodelling (Perez-Martin *et al.*, 1999). The silencing mechanism may involve positioning of genes that control colony morphology at chromosomal positions that exist in two alternative conformations: i) silenced state and ii) active state. Transition between the two states would result in changes in chromatin structure that once established, would be inherited. These *SIR2* studies were carried out in CAI4 strain however similar studies carried out in the WO-1 strain, showed that *SIR2* deletion has no effect in the white-opaque transition but wrinkled and myceliated phenotypes were observed (Soll, 2002). This suggests that the regulatory role of *SIR2* in phenotype switching is strain dependent or switching system dependent.

Histone deacetylases, also play an important role in switching since deacetylation suppresses it, as demonstrated by inhibition of deacetylation by trichostatin-A, which increases the frequency of white-opaque transition (Klar *et al.*, 2001). Deletion of two *C. albicans* deacytilase genes, *HDA1* (Histone deacetylase) and *RPD3* (Reduced potassium dependency), also showed that expression of these genes is affected by switching and that, while Hda1p plays a selective role in suppressing switching from white to opaque, Rpd3p suppresses the switch events in both directions (Srikantha *et al.*, 2001). It is not yet clear whether suppression of switching is directly mediated by deacylation at the site of the switching event or indirectly at the site of the gene that encodes an activator of the switch event.

Tup1p plays a role in both regulation of morphogenesis and transcription of a variety of genes, including genes involved in virulence (Braun *et al.*, 2000; Braun & Johnson, 2000; Braun & Johnson, 1997), but it also plays additional roles in phenotypic switching, is necessary for the phenotypic transition between the white and opaque phenotypes and is involved in the downstream regulation of phase specific gene expression and may participate directly in the switch event (Zhao *et al.*, 2002).

The MTLa and MTLα alleles located in chromosome 5 (Hull & Johnson, 1999), also affect white-opaque switching and mating in *C. albicans* (Miller & Johnson,

2002). Apparently, white-opaque switching is negatively regulated by the combined action of two homedomain proteins, Mtla1p and Mtlα2p. Loss of either gene products converts the CAI4 strain from a non-switching to a switching mode, which also triggers mating of opaque cells. Since mating between "a" and "α" cells in the opaque phase is 10^6 times more efficient than mating between these same cells in the white phase it was proposed that white-opaque switching and mating is controlled by the MTL locus in *C. albicans* (Miller & Johnson, 2002).

1.4.3.3. Extracellular hydrolases

Extracellular hydrolases are hydrolytic enzymes produced by *C. albicans* and include aspartic proteinases, phospholipases, and lipases. They are important virulence factors.

Secreted aspartic proteinases (*SAP*) are encoded by a large family of genes, *SAP1-10* that have an optimal activity at acidic pH and are inhibited by pepstatin A (Davies, 1990). All 10 *SAP* genes of *C. albicans* encode preproenzymes with about 60 amino acids longer than the mature enzyme. The N-terminal secretion peptide is cleaved by signal peptidase in the endoplasmatic reticulum, the pro-enzyme is transferred to the Golgi complex, where it is further processed at Lys-Arg sites by the Kex2 proteinase (Newport & Agabian, 1997; Togni *et al.*, 1996). After activation, the enzyme is packaged into secretory vesicles and transported to the plasma membrane or incorporated into cell wall (Naglik *et al.*, 2003a). The mature enzymes contain sequence motifs typical for all aspartyl proteinases, including the two conserved aspartate residues in the active site and conserved cysteine residues implicated in three-dimensional structure maintenance (Hube & Naglik, 2002). Despite having putative N-glycosylation sites, only Sap9p and Sap10p have a C-terminal consensus sequence for glycosylphosphotidylinositol (GPI) proteins and are glycosylated (White & Agabian, 1995; Hube *et al.*, 1994).

Despite being acidic proteinases, *SAP*s display different pH optima. For example, Sap1-3p has high activity at lower pH values, while Sap4-6p has maximum activity at higher pH values (Monod *et al.*, 1998; Monod *et al.*, 1994). These differences in pH optima are thought to confer versatility for *C. albicans* to survive and cause infections in different tissues or organs. Furthermore, Sapsp have different substrate and cleavage specificities (Koelsch *et al.*, 2000; Goldman *et al.*, 1995; Fusek *et al.*, 1994). Sap1-3p and Sap6p cleave peptide bonds between larger hydrophobic amino acids however Sap1-2p and Sap6p prefer phenylalaline while Sap3p prefers leucine.

C. albicans proteinases generate nutrients by cleaving extracellular proteins that are in contact with them. This allows for penetration and invasion and also evasion of the immune system (Naglik *et al.*, 2003a). There is a direct correlation between *SAP* genes, Sapp activity *in vitro* and *C. albicans* virulence (Monod *et al.*, 1994; Capobianco *et al.*, 1992). Furthermore, infected patients (oral or vaginal) harbour *C. albicans* strains displaying more proteolytic activity than those strains isolated from asymptomatic patients (Ollert *et al.*, 1995; Agatensi *et al.*, 1991; de Bernardis *et al.*, 1990; Cassone *et al.*, 1987). Sap proteins, localized on the cell wall, are produced *in vivo* during mucosal and systemic infection and are also immunogenic and elicit mucosal and systemic antibody responses (Naglik *et al.*, 2003a).

The relation between Sapsp and *C. albicans* virulence is complex mainly because *SAP* expression is also related to a number of other virulence factors including hypha formation, adhesion and phenotypic switching. As described previously, *SAP4-6* expression increases in hyperfilamentous strains and decreases in strains that are defective in hyphal-growth (Felk *et al.*, 2002; Schroppel *et al.*, 2000). This reduced expression of *SAP4* and *SAP6* is also related with an inability to invade or damage parenchymal organs including the liver and pancreas (Felk *et al.*, 2002). Sap proteins facilitate *C. albicans* adherence to many host tissues and cell types. Strains with strong proteolytic activity adhere more significantly to human buccal epithelium cells and colonize organs, namely liver, kidneys and spleen, more easily than strains that have reduced proteolytic activity (Abu-Elteen *et al.*, 2001; Ghannoum &

Abu, 1986). Furthermore, the opaque-specific *SAP1* confers increased adhesion and capacity to cavitate skin (Kvaal *et al.*, 1999).

A subset of *SAP* genes seems to be regulated by phenotypic switching, especially the *SAP1* gene. Transcripts of *SAP1* are abundant in specific-switching forms and are primarily responsible for the higher extracellular proteolytic activity observed in these switching states (Morrow *et al.*, 1994; Morrow *et al.*, 1992). *SAP3* and *SAP8* are also differentially expressed during switching (Hube & Naglik, 2002; Morrow *et al.*, 1993; White *et al.*, 1993).

Phospholipases refers to a heterogeneous group of enzymes that share the ability to hydrolyse one or more ester linkages in glycerophospholipids (Ghannoum, 2000). Although all phospholipases have phospholipids as substrates, each enzyme has the ability to cleave a specific type of ester bond, which permits differentiating them as type, A, B, C and D. *C. albicans* secrets three types of phospholipases: Lyso-PL (lysophospholipase), LPTA (lysophospholipase-transacylase) and PL (phospholipases A, B, C) (Ibrahim *et al.*, 1995; Banno *et al.*, 1985; Barrett-Bee *et al.*, 1985; Pugh & Cawson, 1977), although a phospholipase enzyme could have a dual hydrolase activity. Two extracellular *PLB* genes, namely *PLB1* and *PLB2* (Sugiyama *et al.*, 1999; Hoover *et al.*, 1998; Leidich *et al.*, 1998), three intracellular *PLC* genes coding for *PLC1* (Kunze *et al.*, 2005; Andaluz *et al.*, 2001; Bennett *et al.*, 1998) and one for *PLD* have been characterized in more detail (Hube *et al.*, 2001).

PLB1 contains a N-teminal signal sequence, a conserved motif (Gly-X-Ser-Gly) found in most lipolytic enzymes, but in contrast to other fungal phospholipases B, no GPI-anchored motifs were found (Hoover *et al.*, 1998; Leidich *et al.*, 1998). Like *PLB1*, *PLB2* does not contain any GPI-anchor motif at the C-terminal and has 65% of similarity with *PLB1* (Sugiyama *et al.*, 1999). Both yeast and hyphal forms express *PLB1*, but it increases in *tup1* double mutants that filaments, so *PLB1* transcription regulation is associated with morphogenesis (Hoover *et al.*, 1998). *C. albicans* morphological variation does not have any influence in *PLB1* expression, but environmental conditions, such as pH, induces *PLB1* expression (Mukherjee *et al.*, 2003). Studies in infected

tissues, using anti-Plb1 antibody showed that Plb1p is secreted during invasion of the gastrointestinal tract of mice and *plb1* mutants displayed attenuated virulence in disseminated candidiasis in a murine model. Insertion of the *PLB1* gene in the *plb1* double mutants restored the ability to invade tissues, such as liver (Mukherjee *et al.*, 2001). Recently, *PLB* expression was correlated with oral infection but not vaginal infection (Naglik *et al.*, 2003b), suggesting that candidal phospholipase activity may be required for dissemination of *C. albicans*, help in host cell penetration (Mukherjee *et al.*, 2001) and also interacting with host signal transduction pathways (Filler *et al.*, 1991).

1.4.3.4. Adhesion

Adhesins promote the adherence of *C. albicans* to host cells or host-cell ligands (Calderone & Fonzi, 2001). Adhesins are localized on the surface of cells where they are accessible to host ligands (Sundstrom, 2002). Adhesin genes share features of genes encoding highly glycosylated yeast cell wall proteins with N-terminal signal peptides and C-terminal features that mediate GPI anchor addition at an amino acid residue designated omega (ω) site (Hamada *et al.*, 1998).

The developmental regulated adhesin Hwp1p which is a hypha-specific gene found in germ tubes has all these structural features. As referred above, Hwp1 is a substrate for mammalian transglutaminases (TGases) and becomes cross-linked to TGase-expressed in the squamous surface of the oral mucosa (Staab *et al.*, 1999). Hwp1p is localized on the cell surface, is expressed during infection in host tissues and plays an important role in mucosal and systemic candidiasis (Sundstrom *et al.*, 2002; Staab *et al.*, 1999; Staab *et al.*, 1996).

Since *C. albicans* binds to several extracellular matrix (ECM) ligands, including fibronectin (FN), laminin and collagen I and IV (Chaffin *et al.*, 1998; Hostetter, 1994) and *C. albicans* proteins were recognized by integrin antibodies (Hostetter, 1994), it is likely that *C. albicans* expresses an integrin-like protein. Indeed, a putative integrin gene (*INT1*) with 18% identity with the ligand binding I-domain was identified in *C. albicans* (Gale *et al.*, 1996). Int1p

is a transmembrane protein that has an external EF-hand domain or divalent cation binding and a cytoplasmatic talin-interacting domain, which may interact with actin. Deletion of the *INT1* gene induces expression of multiple phenotypes, including reduction in adhesion to ephitelia, loss of virulence and inhibition of hypha formation (Gale *et al.*, 1998). Thus Int1p seems to have a dual role in adhesion and in morphogenesis (as described above).

The *ALS* gene family of *C. albicans* encodes large cell-surface glycoproteins some of which are implicated in adhesion to host surfaces. Each *ALS* gene shares a three-domain structure, including an N-terminal domain that is 55-90% identical across the family; a central domain containing a tandemly repeated motif; and a C-terminal domain, which is rich in serine and threonine. The latter is variable in length and sequence across the family but contains a GPI-anchor sequence (Zhao *et al.*, 2003; Hoyer, 2001). The size of the same *ALS* gene varies frequently between strains and within alleles of the same strain due to differences in the number of tandem repeats present in the central domain (Hoyer *et al.*, 1998a; Hoyer *et al.*, 1995). Also, gene sequence polymorphisms were found when comparing ALS sequences from different strains (Hoyer, 2001). The various *ALS* genes are differentially expressed in *C. albicans* under physiologically relevant conditions such as medium changes (*ALS1*) (Hoyer *et al.*, 1995), morphological forms (*ALS3* and *ALS8*) (Hoyer & Hecht, 2000; Hoyer *et al.*, 1998a) and stage of growth (*ALS4*) (Hoyer *et al.*, 1998b). *ALS1*, *ALS2*, *ALS3*, *ALS4*, *ALS5* and *ALS9* are expressed during infection in reconstituted human oral epithelium (RHE) model (Green *et al.*, 2004). In a murine disseminated candidiasis model, transcripts of *ALS1*, *ALS2*, *ALS3*, *ALS4*, and *ALS9* transcription were detected by RT-PCR (Green *et al.*, 2005). In human clinical samples, although expression of all *ALS* genes was detected across the set of clinical specimens, *ALS1*, *ALS2*, *ALS3*, and *ALS9* transcripts were detected most frequently (Cheng *et al.*, 2005).

Although being expressed during infection the role of ALS proteins in adhesion is still controversial, mainly due to the heterologous system used to study their function. The majority of the studies of the *ALS* gene family were carried

out in *S. cerevisiae*, which may not be suitable model (Sundstrom, 2002; Sundstrom, 1999). Nevertheless, studies with *C. albicans asl1* double mutants shown reduced (less 35%) adherence to human endothelial cells while *ALS* overexpression besides increasing adherence to endothelial cells, induced *C. albicans* flocculation (Fu *et al.*, 2002).

The *C. albicans* outer cell wall layer is enriched in mannoproteins, which are embedded in a matrix of structural polysaccharides, consisting of glucan and chitin (Kapteyn *et al.*, 2000). This layer is important in adhesion to host surfaces and their colonization (Dalle *et al.*, 2003; Kanbe & Cutler, 1994). Mannoproteins of *C. albicans* contain *N*- and *O*-linked oligosaccharides and glycosylation in *C. albicans* is relevant to adhesion since specific oligosaccharides, β-1,2-linked mannose have been directly implicated in adhesion (Hobson *et al.*, 2004). Pmt1p and Pmt6p are members of the *PMT* gene family, which is involved in transfer of mannose to serine or threonine residues in order to *O*-glycosylate cell wall proteins. Strains lacking Pmt1p and Pmt6p have reduced adherence to endothelial cells and to porcine aortic endothelial cells (PAEC), respectively (Timpel *et al.*, 2000). The addition of further mannose residues to the first *O*-linked mannose occurs in the Golgi and involves a range of mannosyltransferases. The *MNT* (Mannosyltransferase) gene family, *MNT1* and *MNT2*, encode α-1,2-mannosyltransferases which are responsible for the addition of the second and third mannose residues in *C. albicans O*-glycans (Munro *et al.*, 2005; Buurman *et al.*, 1998). Since both *mnt1*/*mnt1* and *mnt2*/*mnt2* strains showed defective adhesion to BECs and Matrigel, a role for *O*-mannosylation in adhesion was suggested (Munro *et al.*, 2005). A specific requirement for *O*-linked glycolysation in adhesion has been already reported in the binding of the cell surface mannoprotein mp58 to human fibrinogen (Casanova *et al.*, 1992).

Finally, the *ACE2* gene is involved in regulation of genes that control the chitin content in cell wall. Deletion of *ACE2* reduced adherence by two fold in comparison to the wild-type cells (Kelly *et al.*, 2004). *CSF4* (cell surface factor) gene which belongs to the yeast glycosidase family, is important for *C.*

albicans adhesion to mammalian cells *in vitro*, since *csf4* null mutants have reduced adhesive properties compared to wild type cells (Alberti-Segui *et al.*, 2004).

PART C

1.5. Objectives and main results of this thesis

During the last 30 years, a number of alterations to the genetic code have been discovered in various organisms, which invalidate the notion that the genetic code is universal. These natural variations invalidated the Frozen Accident Theory (Crick, 1968). The technical difficulties associated to the identification of genetic code alterations are a significant hurdle to gathering large scale information on the variability of the genetic code. It is likely that additional alterations will be discovered as the number of sequenced genomes increases and unconventional organisms are studied in more detail. Therefore, it is of fundamental biological importance to understand how and why genetic code alterations evolve. Elucidating these questions will provide new information on the flexibility of the genetic code, on how it evolved from a minimal number of amino acids to the 22 existent in extant organisms and on the global consequences of large scale proteome disruption.

We are using *C. albicans* as a model system to understand the evolution of genetic code alterations. In certain *Candida* species, the CUG codon is mainly decode as serine (97%) but can also be decode as leucine (3%) *in vivo* (Gomes *et al.*, unpublished). This unique codon decoding event is mediated by a novel $tRNA_{CAG}^{Ser}$ that acquired an anticodon arm with identity elements for the LeuRS and can be aminoacylated by both the LeuRS and its cognate SerRS, resulting in two distinct and competing tRNA species, namely leu-$tRNA_{CAG}^{Ser}$ and ser-$tRNA_{CAG}^{Ser}$. This $tRNA_{CAG}^{Ser}$ appeared 272±25 My ago in the ancestor of yeasts and is responsible for a unique evolutionary pattern of the CUG codons present in the *C. albicans* genome, which are new codons that evolved recently. The maintenance of the ambiguous $tRNA_{CAG}^{Ser}$ over that

evolutionary time scale is not yet understood. However, it is rather puzzling since serine is hydrophilic and leucine is hydrophobic, indicating that random insertion of serine and leucine at CUG sites gives rise to arrays of proteins creating a statistical proteome that is inherently unstable.

Since the change of identity of the CUG codon is a fundamental feature of *Candida* spp. biology we have developed an experimental strategy to elucidate the biological role of CUG ambiguity and uncover how the identity of the leucine CUG codon was redefined to serine without killing the *Candida* ancestor. Several studies have already contributed to the elucidation of the evolutionary mechanism of the *Candida* genetic code alteration (Santos *et al.*, 2004; Silva *et al.*, 2004; Massey *et al.*, 2003; O'Sullivan *et al.*, 2002). Also, reconstruction of the *Candida* genetic code change has already been attempted in the close relative *S. cerevisiae* (Silva *et al.*, 2004; Santos *et al.*, 1999). However, we still do not yet understand why the CUG codon remains ambiguous in several *Candida* species and why *Candida* evolved this genetic code alteration through ambiguous decoding.

The main questions that we have attempted to answer in this project were "how do *C. albicans* cells survived CUG ambiguity?" and "what are the biological consequences of CUG ambiguity?". The specific objectives of this project were the following:

1. Increase CUG ambiguity in *C. albicans* to expose any putative phenotypes associated to such ambiguity. For this, highly ambiguous *C. albicans* clones derived from the CAI-4 strain were created through tRNA engineering.
2. Carry out a detailed phenotypic characterization of the ambiguous cell lines. These studies focused on expression of virulence factors, namely morphogenesis, phenotypic switching, extracellular hydrolases production, and adhesion.
3. Evaluate the impact of increased CUG ambiguity in the *C. albicans* genome.

4. Evaluate the impact of increased CUG ambiguity on the *C. albicans* cell wall proteome.

The data obtained in this study shows that CUG ambiguity is a generator of phenotypic diversity, destabilizes the genome and has a major impact on the cell wall composition. Some of the phenotypes uncovered in this study have direct implication for *C. albicans* virulence, mating and adaptation, indicating that CUG ambiguity is a fundamental feature of the biology of this human pathogen. More importantly, the data has fundamental importance for our understanding of the evolution of genetic code alterations. The unanticipated discovery that CUG ambiguity generates phenotypic diversity indicates that genetic code expansion and alterations are in fact novel mechanisms for speeding up evolution of novel phenotypes. This fundamental brake with the traditional view of a rigid (frozen) genetic code highlights a novel concept of genetic code evolution through ambiguous codon identity and shows how its high selective potential minimizes deleterious effects of proteome disruption caused by codon decoding ambiguity.

2. Molecular manipulation of CUG ambiguity in *C. albicans*

2.1. Introduction

tRNA aminoacylation accuracy is critical to ensure high fidelity in protein synthesis. Aminoacyl-tRNA synthetases (aaRS) discriminate efficiently between cognate, near-cognate and non-cognate amino acids at the adenylation reaction step, and also cognate from non-cognate tRNAs (Kurland, 1992; Parker, 1989). The misacylation error in this process has been estimated to range from 10^{-4} to 10^{-5} (Okamoto & Savageau, 1984; Lin *et al.*, 1984). Discrimination of cognate tRNA from non-cognate tRNAs is achieved by positive and negative identity determinants located along the tRNA molecule (Normanly & Abelson, 1989; Yarus, 1988). These discriminatory elements allow aaRSs to assign accurate relationships between codons and amino acids, however some amino acids are chemically very similar (e.g., leucine and valine) and their discrimination is problematic (Meinnel *et al.*, 1995). To overcome this problem, some aaRS, namely the LeuRS, evolved editing mechanisms that eliminate closely related amino acids from their active sites (Hendrickson *et al.*, 2004).

The discovery of an ambiguous $tRNA_{CAG}^{Ser}$ in *Candida* spp. contradicts the established notion of aminoacylation with high fidelity (Suzuki *et al.*, 1997). This tRNA aminoacylation ambiguity is due to a hybrid nature of the $tRNA_{CAG}^{Ser}$ which has identity elements for both SerRS and LeuRS. The LeuRS recognizes A_{35}, m^1G_{37} and the anticodon-arm of the tRNA (which is typical of $tRNAs^{Leu}$), while the SerRS recognizes the extra-loop and the discriminator base G_{73}, which are typical of $tRNAs^{Ser}$ (Himeno *et al.*, 1997; Soma *et al.*, 1996; Saks & Sampson, 1996; Breitschopf *et al.*, 1995; Breitschopf & Gross, 1994; Achsel & Gross, 1993) (see introduction). For these reasons, the $tRNA_{CAG}^{Ser}$ is charged with both serine (97%) and leucine (3%) *in vivo*, thus introducing ambiguity at the CUG codon (Suzuki *et al.*, 1997). This tRNA decodes the leucine CUG codon as serine mainly but leucine is also inserted at CUG positions at low level.

The *C. albicans* $tRNA_{CAG}^{Ser}$ appeared approximately 272 My ago in the ancestor of yeasts. Interestingly, the genus *Candida* and *Sacharomyces* separated from

each other approximately 170 My ago, suggesting that the ancestor of these genera was ambiguous for about 100 My. Remarkably, ambiguous CUG decoding forced the disappearance of the leucine-CUG codons. These codons moved to the other major leucine codons, namely UUA and UUG, suggesting that codon ambiguity is a major evolutionary force shaping codon usage. On the other hand, the 13,074 CUG codons present in extant *C. albicans* evolved recently from codons coding for serine or amino acids with similar chemical properties to serine (Massey *et al.*, 2003), indicating that new tRNAs have the capacity to capture codons from cognate and near-cognate amino acids.

The dynamics of CUG evolution described above prompts the following questions: i) "Did the new CUG codons emerge randomly in the genome?, or ii) was this process directed to specific genes in order to bring some biological advantage?, and iii) what selective advantage may it be?". Apparently, the "new" CUG codons are distributed over the *C. albicans* genome in all functional classes of genes. However, there is some accumulation of CUG codons in regulatory genes that suggest that CUG ambiguity may have functional consequences. For example, a genome wide search for CUG codons in the *C. albicans* genome revealed that almost all genes related to phenotypic diversity have a significant number of CUG codons (Figure 2.1), suggesting that CUG ambiguity may increase the *C. albicans* potential to generate genetic diversity.

Considering that life evolved from a last universal common ancestor (LUCA), alternative genetic codes must have evolved from the genetic code used by LUCA. This raises a puzzling problem because code changes introduce amino acid substitutions in a large number, if not all, proteins and should be lethal or highly detrimental (Santos *et al.*, 1996). In *Candida,* the situation is even more difficult to explain because the ancestor of yeasts was a sophisticated organism encoding thousands of proteins and leucine and serine have different chemical properties. In other words, CUG reassignment must have caused major proteome disruption and should have been eliminated by natural selection.

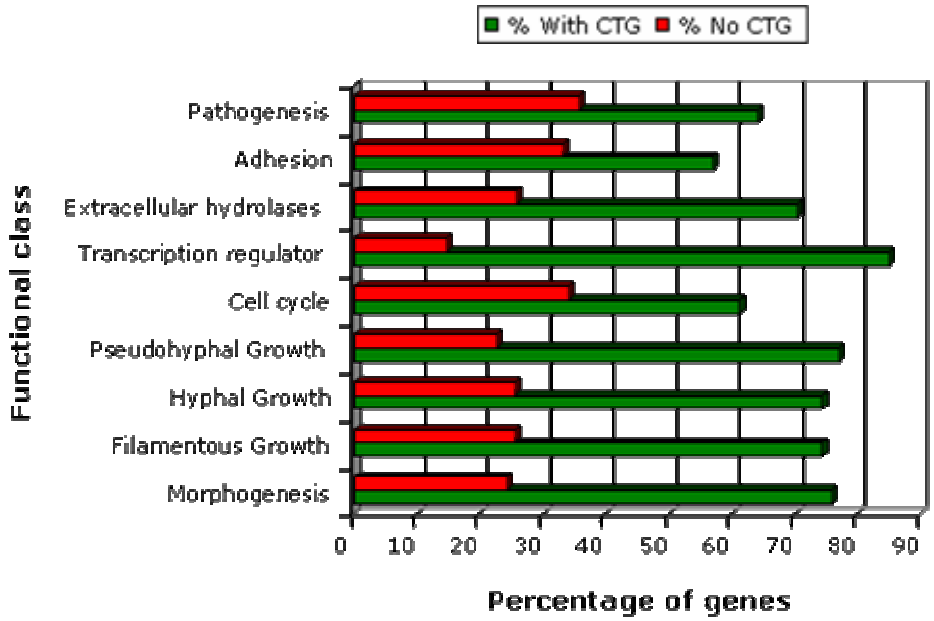


Figure 2.1. Distribution of CUG codons in the *C. albicans* genome by functional classes. The graph shows the percentage of genes containing one or more CUG codons for the gene categories indicated (green bars). The percentage of genes without CUG codons for the same functional categories is also shown (red bars). The data was collected from a survey of the entire *C. albicans* genome, assembly 19 (www.candidagenome.org).

In order to shed new light into this puzzling problem and better understand the impact of ambiguous CUG decoding in *C. albicans* biology, we have attempted to revert the identity of the *Candida* CUG codon from serine back to leucine, by engineering *S. cerevisiae* leucine tRNA genes and expressing them in *C. albicans.* These genes encode tRNAs that decode the CUG codon as leucine and not serine (Figure 2.2). tRNA engineering was based on the observation that: i) the internal transcriptional control promoter elements of heterologous *S. cerevisiae* tRNA genes are efficiently recognized by the *C. albicans* RNA polymerase III machinery and introns are correctly spliced (Leuker & Ernst, 1994); ii) different degrees of leucylation, as well as, efficiencies of CUG translation, can be achieved by manipulating the tRNAs anticodon-loop sequences (Figure 2.2); iii) decoding efficiency of the $tRNA_{CAG}^{Leu}$ can be lowered by mutating the uracyl at position 33 (U_{33}) in the anticodon-loop of tRNAs to guanosine (G_{33}). This is an important aspect of the

tRNA engineering process and results from the observation that position 33 is conserved in tRNAs and is critical for the correct U-turn of the anticodon loop (Sprinzl *et al.*, 1996). U_{33} is also important for the correct tRNA functioning in the ribosome, namely by ensuring accurate codon-anticodon interaction, proper GTP hydrolysis of the ternary complex and efficient codon decoding (Dix *et al.*, 1986). The presence of a G in position 33 functions as a negative modulator of the leucylation reaction since it alters the structure of the anticodon arm, which in turn prevents efficient binding of the LeuRS to the tRNA (Figure 1.5), but more importantly it decreases translation efficiency between 2- and 3-fold (Suzuki *et al.*, 1997).

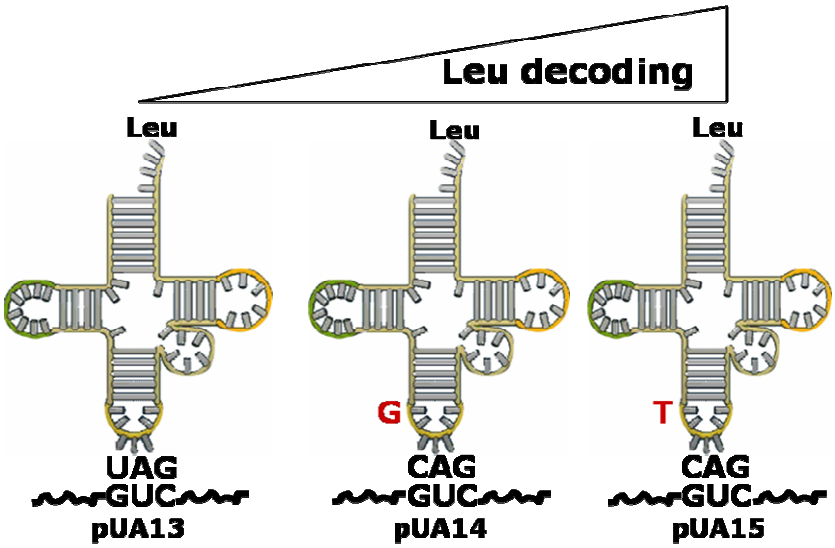


Figure 2.2. Wild type and mutant *S. cerevisiae* leucine tRNAs used to revert CUG identity in *C. albicans*. In *S. cerevisiae,* the CUG codon is decoded as leucine by the $tRNA_{UAG}^{Leu}$. The $tRNA_{CAG}^{Leu}$ are mutant tRNAs derived from the $tRNA_{UAG}^{Leu}$. These tRNAs were cloned and expressed in *C. albicans* using a low copy number plasmid, namely pUA13 (Leu-$tRNA_{UAG}$), pUA14 (Leu-$tRNA_{CAG}$, G_{33}) and pUA15 (Leu-$tRNA_{CAG}$, U_{33}). These tRNAs can decode CUG codons with different efficiency, resulting in different levels of CUG ambiguity.

The importance of U_{33} has also been confirmed *in vivo* in *S. cerevisiae* using the wild type and mutant forms of the $tRNA_{CAG}^{Ser}$. As expected, the tRNAs with pyrimidine at position 33, namely U_{33} and C_{33}, were efficient decoders while those with G_{33} or A_{33} were poor decoders (Santos *et al.*, 1996b). This was shown by monitoring growth rate and measuring the thermostability of β-

galactosidase, which gave an indirect measure of amino acid misincorporation levels. That is, β-galactosidase synthesized in the presence of C_{33} mutant tRNA displayed a faster rate of inactivation than β-galactosidase synthesized in the presence of wild-type G_{33}. The U_{33} mutant was lethal, thus confirming that purines at position 33 (G_{33} or A_{33}) interfere negatively with decoding efficiency.

Although it was already known that vectors encoding heterologous $tRNA^{Leu}$ could be transformed and expressed in *C. albicans*, as long as the standard genetic code rules were maintained (Leuker & Ernst, 1994), the *S. cerevisiae* $tRNAs^{Ser}$ (with the anticodons AGA and UGA) were used in this study as negative controls (Figure 2.3).

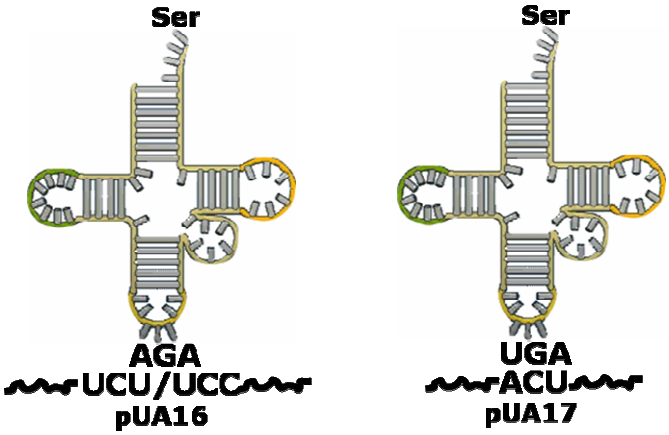


Figure 2.3. Heterologous *S. cerevisiae* serine-tRNA genes used as negative controls for the CUG identity reversion experiment carried out in *C. albicans* (see main text).

The above tRNAs were engineered by site directed mutagenesis. For this, their serine anticodons were modified to 5'-AAG-3' and 5'-TAA-3', to decode the CUC, CUU, CUA and UUA leucine codons. These nucleotide alterations on the anticodon of the tRNA were expected to induce partial sense-to-sense reassignments from leucine to serine, since the mutant tRNAs are charged with serine but contain leucine anticodons (Figure 2.4). This reassignment is not total because *C. albicans* cells maintain leucine tRNAs ($tRNA_{IAG}^{Leu}$,

$tRNA_{AAU}^{Leu}$) which compete for the same leucine codons. This strategy also ensured that ambiguous decoding was not lethal.

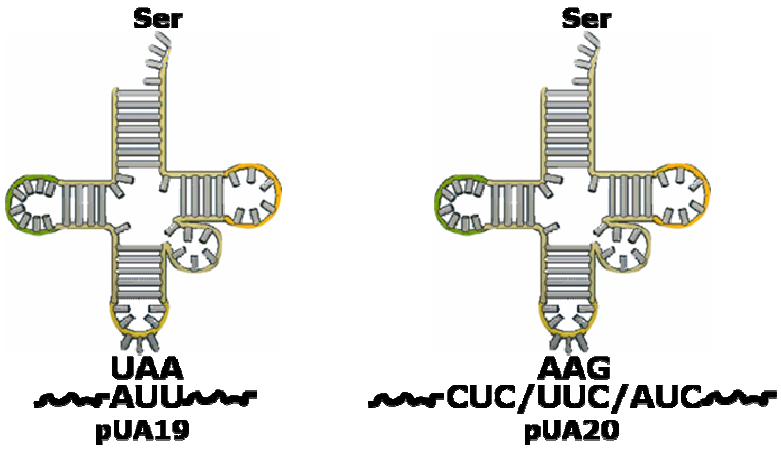


Figure 2.4. *S. cerevisiae* $tRNA_{AGA}^{Ser}$ and $tRNA_{UGA}^{Ser}$. The anticodons of these tRNAs were mutated to UAA and AAG, respectively, to decode leucine codons. tRNA genes were transformed in *C. albicans* CAI4 cells using plasmids pUA19 ($tRNA_{UAA}^{Ser}$) and pUA20 ($tRNA_{AAG}^{Ser}$).

Transformation of *S. cerevisiae* tRNAs into *C. albicans* introduced ambiguous decoding at two levels: i) at the CUG codon by increasing leucine misincorporation; ii) at leucine CUC, CUU, CUA and UUA codons by introducing serine misincorporation. Therefore, one was expecting that this would allow us to shed new light on how *C. albicans* copes with CUG ambiguity and also on why such ambiguity was preserved over 272 My in many *Candida* species. Since CUG ambiguity produces proteome diversity and exponentially increases the number of proteins that are synthesized by the 6,345 genes of *C. albicans*, one wondered whether CUG ambiguity would create phenotypic variability, in particular whether such ambiguity would affect expression of *C. albicans* virulence traits. In this chapter, I describe the impact of CUG ambiguity on *C. albicans* morphogenesis, phenotypic switching, adhesion and extracellular hydrolases production. The results show that CUG ambiguity does indeed create phenotypic variability, which is an important adaptive trait.

2.2. Materials and Methods

2.2.1. Strains, growth and maintenance

2.2.1.1. *Escherichia coli*

Escherichia coli (E. coli) strain JM109 (*recA1 SupE44 endA1 hsdR17 gyrA96 relA1 thi Δ(Lac-proAB) F'[traD36 proAB- lacI lacZ ΔM15*] was grown at 37°C on LB broth [1% (w/v) peptone from casein, 0.5% (w/v) yeast extract, 1% (w/v) sodium chloride; (Merck)] or on 2% (w/v) LB agar (Merck). For DNA manipulations, the transformed JM109 strain was grown in liquid LB or LB agar supplemented with 50 µg/ml ampicillin (Duchefa, Haarlem). *E. coli* strain JM109 as well as all the strains resulting from the plasmid transformation were preserved in 20% glycerol at -80°C.

2.2.1.2. *Candida albicans*

Candida albicans CAI4 (*ura3Δ::imm434/ura3::imm434*) was grown at 30°C in YPD (2% glucose; 1% yeast extract, 1% peptone) or minimal medium lacking uridine (MM-uri) (0.67% yeast nitrogen base without amino acids, 2% glucose, 2% agar and 100µg/ml of the required amino acids). Transformed and non-transformed cells were stored in 40% glycerol at -80°C.

2.2.2. Construction of plasmids for expression of heterologous tRNAs in *C. albicans*

2.2.2.1. Plasmid pRM1

The pRM1 vector (Figure 2.5), was constructed by Pla (Pla *et al.*, 1995), and is a *C. albicans* autoreplicative vector. It contains two ARSs (Autonomously Replicating Sequence), ARS2 (Cannon *et al.*, 1992) and ARS3 (Herreros *et al.*, 1992a), two auxotrophic yeast markers (*URA3* and *LEU2*) for replication and selection in yeasts, and ampicillin resistance marker for selection in *E. coli*.

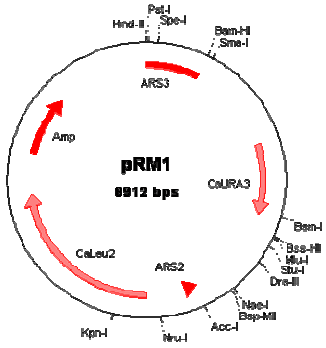


Figure 2.5. Diagram showing the structure of the autoreplicative pRM1 vector. It contains two ARSs (2 and 3), two yeast selectable markers (*URA3* and *LEU2*) and an ampicillin resistance marker of *E. coli*.

2.2.2.2. Plasmid pUA12

The pUA12 plasmid is a modification of the pRM1 vector and was used as a control plasmid and to express the tRNAs used in this study. The promoter region of the *CaLEU2* gene (*Candida albicans* isopropylmalate dehydrogenase) was digested with the restriction enzymes *NruI* and *EcoRV* to insert a Multi-cloning site (MCS) as shown in Figure 2.6.

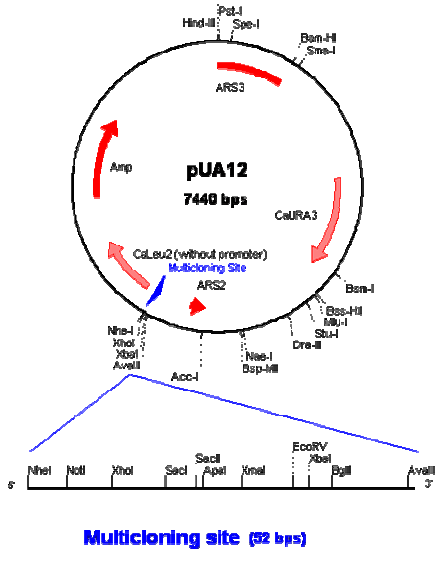


Figure 2.6. Diagram showing the structure of the control plasmid pUA12.

The MCS was inserted by digesting the pRM1 vector overnight (Figure 2.5). The digestion was then verified after loaded into a 0.8% agarose gel (MP, Merck) and electrophoresed at 100 V for 1 h. QIAEX II Agarose Gel Extraction Protocol (Quiagen) was used to separate the linearised plasmid, which was dephosphorylated for posterior ligation of the MCS oligonucleotide fragment. The latter fragment was obtained by hybridization of a pair of complementary primers, at 65°C for 1 h. The full sequence of the MCS oligonucleotide is the following: 5´ GCTAGCGGCCGCTCGAGCTCCGCGGGCCCGGCCGATATCTAGATCT ATGCAT 3`.

2.2.2.3. Plasmid pUA13

The pUA12 plasmid was used for the construction of plasmid pUA13. For this, the former was digested with the restriction enzymes *XhoI/XbaI* and, after dephosphorylation it was ligated with a purified *XhoI/XbaI* PCR fragment containing the *S. cerevisiae* $tRNA_{UAG}^{Leu}$ gene flanked by 300 bp upstream and downstream sequences. This fragment was obtained by PCR (polymerase chain reaction) amplification from *S. cerevisiae* genomic DNA using the following pair of primers: 5` CCGCTCGAGCGGCGACTGTCCAGACTTAGTAAAGCT 3` and 5' GCTCTAGAGCCCGCTG TCGCCAGCGTTAGC 3' (Figure 2.7).

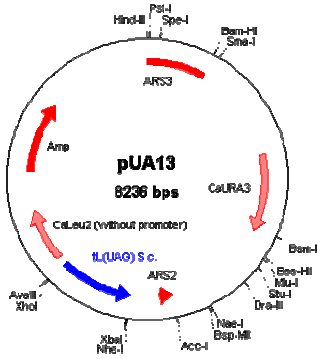


Figure 2.7. Diagram showing the primary structure of the pUA13 plasmid carrying the *S. cerevisiae* $tRNA_{UAG}^{Leu}$ with upstream and downstream 300 bp flanking sequences.

2.2.2.4. Plasmids pUA14 and pUA15

To construct pUA14 and pUA15 plasmids, *XhoI/ApaI* DNA fragment (250 bp), corresponding to the upstream flanking region of the *C. albicans* $tRNA_{CAG}^{Ser}$, was amplified by PCR from *C. albicans* genomic DNA using the primers: 5' CCGCTCGAGCGGGTATGCAATCGTTGTCTGTAATGTA 3' and 5' GCTATGGGCCC AAGCACAAATGGTTATGACAATTGATG 3'. The pUA12 vector was digested with the restriction endonucleases *XhoI/ApaI* and dephosphorylated. The linear plasmid was then ligated to the *C. albicans* DNA fragment. After this, the vector was further digested with the restriction enzymes *ApaI/AvaIII*, and dephosphorylated. The $tRNA_{GAG}^{Leu}$ gene was amplified by PCR using *S. cerevisiae* genomic DNA as template with the following set of primers: 5' GCTATGGGCCCTAGTTGCAACGGTACTCTGGCCGAGTGGTCTAAGGCGTCAGGTTC AGGTCC 3' and 5' GCTATGGGCCCTAGTTGCAACGGTACTCTGGCCGAGTGGTCTA AGGCGTCAGGTGCAGGTCC 3' as forward primers to obtain pUA15 and pUA14 respectively; and 5' ATGCATAAAAACAAAATTTGTTGAAA 3' as a reverse primer. This procedure introduced a G-to-C mutation at position 34 in the anticodon of the *S. cerevisiae* $tRNA_{GAG}^{Leu}$ in both plasmids (pUA14 and pUA15), and an additional U-to-G mutation was introduced in the pUA14 plasmid position 33. After purifying the DNA fragment, using the QIAquick PCR Purification Kit Protocol (Quiagen), it was digested with *ApaI/AvaIII* restriction enzymes and finally inserted in the intermediate plasmid, at the *ApaI/AvaIII* restriction site. Thus, two plasmids carry similar $tRNA_{CAG}^{Leu}$ genes with a single mutation at position 33 were obtained (Figure 2.8).

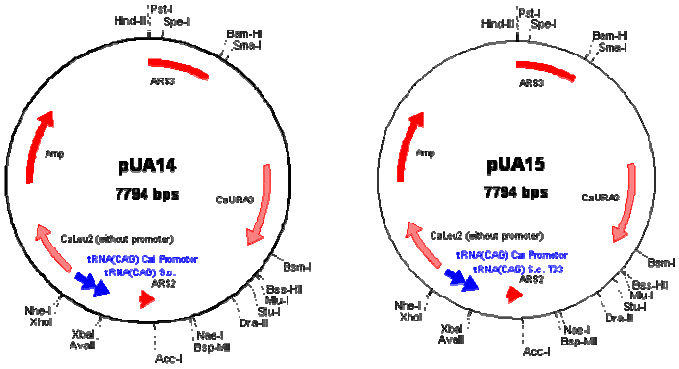


Figure 2.8. Diagram showing the primary structure of the pUA14 and pUA15 plasmids. Both plasmids carry the *S. cerevisiae* $tRNA_{GAG}^{Leu}$ gene mutated G-to-C at position 34. In pUA14 the $tRNA_{GAG}^{Leu}$ gene has G at position 33 while in pUA15 has T. This tRNA gene is flanked upstream by a sequence of 250 bp, identical to the upstream flanking region of the *C. albicans* $tRNA_{CAG}^{Ser}$ gene.

2.2.2.5. Plasmids pUA16 and pUA17

Using *S. cerevisiae* genomic DNA as template and the following two pairs of primers: 5' CCGCTCGAGCGGGTCATCTTCTTGCATCGTATATG 3' and 5' GGCTC GATGCATCATCACATGACCATGTTCGAGC 3'; 5' CGCTCGAGCGGGAGGATTCCTA TATCCTTGAGGAG 3' and 5' GGCTCGATGCATGCCAGGAAGAAATACACTGC 3'; a *XhoI/AvaIII* DNA fragment containing the $tRNA_{UGA}^{Ser}$ and the $tRNA_{AGA}^{Ser}$ genes flanked by a 250 bp upstream and downstream was amplified by PCR, digested with *XhoI/AvaIII* and inserted in the MCS of pUA12 as above (Figure 2.9).

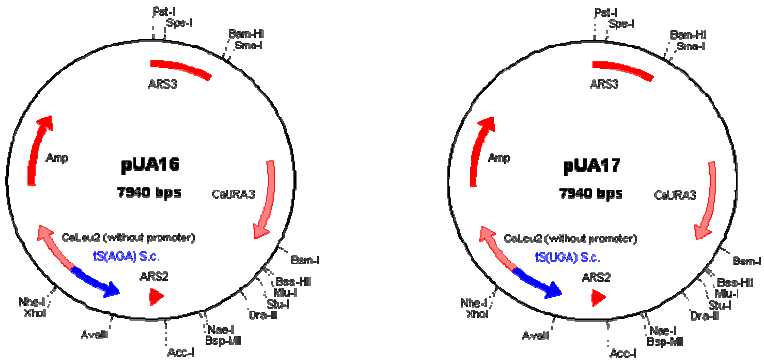


Figure 2.9. Diagram showing the primary structure of the pUA16 and pUA17 plasmids. These plasmids contain the *S. cerevisiae* serine tRNAs genes with the anticodon UGA and AGA, respectively.

2.2.2.6. Plasmids pUA19 and pUA20

The plasmids pUA19 and pUA20 were constructed by site directed mutagenesis using pUA17 and pUA16 plasmids, respectively. They contain the same *S. cerevisiae* serine tRNA genes but with mutated anticodons (Figure 2.10). The anticodon AGA of the serine tRNA gene of plasmid pUA16 was mutated to AAG and the UGA anticodon of plasmid pUA17 was mutated to UAA. For this, site directed mutagenesis was performed according to the manufacturer instructions of the QuickChange Site-Directed Mutagenesis Kit from Stratagene as described next.

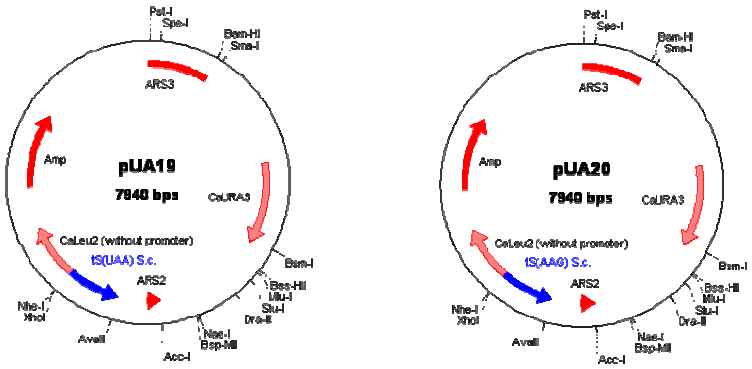


Figure 2.10. Diagram showing the primary structure of the pUA19 and pUA20 plasmids, containing the *S. cerevisiae* $tRNA_{UGA}^{Ser}$ and $tRNA_{AGA}^{Ser}$ genes with the anticodon modified, by site directed mutagenesis, to UAA and AAG, respectively.

Mutagenesis of the AGA (pUA16) to AAG (pUA20) anticodon of the *S. cerevisiae* serine $tRNA_{AGA}$ gene was carried out using the primers: 5' GGTTAAGGCGAAAGATTAAGAATCTTTTGGGC 3' and 5' GCCCAAAAGATTCTTAATCTTTCGCCTTAACC 3'. For mutagenesis of the UGA (pUA17) to UAA (pUA19), the primers were: 5' GGTTAAGGCGACAGACTTAAAATCTGTTGGGCTCTGCC 3' and 5' GGCAGAGCCCAACAG ATTTTAAGTCTGTCGCCTTAACC 3'. These two sets of primers were used in a PfuTurbo™ DNA polymerase PCR that replicates the two plasmid template strands (pUA16 and pUA17) with high fidelity. A 50 µl PCR was prepared as described in Table 2.1.

Table 2.1. – Reaction mixture for PCR amplification.

Component	Volume from stock solution	Final concentration
MilliQ H_2O	up to 50 µl	
Buffer 10x	5 µl	1x
dNTPs mix 10 mM	1 µl	1 mM
Primer F 100 pmol/µl	1 µl	0.1 µM
Primer R 100 pmol/µl	1 µl	0.1 µM
DNA template (100 ng/µl)	1 µl	100 ng/µl
PfuTurbo™	1 µl	2.5 Units

The PCR programme was as follow: cycle 1, 95°C for 0.5 m, cycles 2- 16, 90°C for 0.5 m, 55°C for 0.5 m, and 68°C for 8 m. For the mutagenesis, incorporation of the oligonucleotide primers generated a mutated tRNA gene containing staggered nicks. The PCR product was treated with a *DpnI* endonuclease at 37°C during 1 h. *DpnI* enzyme (target sequence: 5´-Gm6ATC-3´), which is specific for methylated and hemimethylated DNA, was used to digest the parental DNA template and to select for mutation-containing synthesized DNA. The nicked vector DNA incorporating the desired mutations was then transformed into Epicurian Coli® XL1-Blue supercompetent cells. The mixture of 5 µl of DNA with the supercompetent cells was incubated on ice for 30 m. A heat shock of 42°C for 45 s was carried out and then 0.5 ml of NZY+Broth [1%(w/v) NZ amine, 0.5% (w/v) yeast extract, 0.5% (w/v) NaCl, 0.4% (w/v) glucose, 12.5 mM $MgCl_2$, 12.5 mM $MgSO_4$, pH adjusted to 7.5 with NaOH] was added to the cell suspension. After incubation at 37°C with agitation (200 rpm) for 1 h cells were plated in LB/Amp agar. Transformation plates were incubated at 37°C overnight. To screen for positive clones, the plasmid was extracted and then sequenced as described in 2.2.3.8 and 2.2.3.9.

2.2.3. Procedures for cloning and analysing the *S. cerevisiae* tRNAs genes

2.2.3.1. Rapid isolation of genomic DNA from *C. albicans* and *S. cerevisiae*

Genomic DNA from *C. albicans* and *S. cerevisiae* strains was extracted as described in "Methods in Yeast Genetics" (2000). An overnight culture of 10 ml of YPD grown at 30°C, with shaking at 180 rpm, was centrifuged at 10,000 *g* (Centrifuge 5810 R, Eppendorf), for 5 m, at 4°C. Cells were washed in 0.5 ml of MilliQ H_2O and then resuspended in 0.2 ml of 2% Triton X-100, 1% SDS, 100 mM NaCl, 10 mM Tris-Cl (pH 8) and 1 mM Na_2EDTA. 0.2 ml of phenol:chloroform:isoamyl alcohol (25:24:1) (Sigma) and 0.3 g of acid-washed glass beads (Ø=0.5 mm, Sigma) were then added to the cell suspension. Cells were disrupted by vortexing for periods of 30 s intercalated with breaks on ice for 4 m. After centrifugation for 5 m at 16,000 *g* (Biofuge fresco, Heraeus Instruments), and 4°C, the aqueous layer was transferred to a new microfuge tube. 1 ml of 100% ethanol was added and the solution was mixed by inversion. A second centrifugation at 16,000 *g*, for 15 m, at 4°C, was performed, the supernatant was discarded and the pellet ressupended in 0.4 ml of TE (10 mM Tris-HCl, pH 8, 1 mM Na_2EDTA) plus 30 µl of a 1 mg/ml RNase A (Amersham Biosciences). After incubation for 3 h at 37°C, 10 µl of 1 mg/ml proteinase K (Boehringer Mannheim) was added to the DNA solution, which was incubated overnight at 65°C. The DNA was precipitated with the addition of 6 µl of 4 M ammonium acetate plus 1 ml of 100% ethanol and colleted by centrifugation at 16,000 *g* for 15 m at 4°C. It was then washed several times with 70% ethanol and resuspended in 200 µl of MilliQ H_2O.

These genomic DNAs were used as templates in PCR reactions for amplification of *S. cerevisiae* serine and leucine tRNA genes and the DNA fragment upstream of the *C. albicans* $tRNA_{CAG}^{Ser}$ gene.

2.2.3.2. Large scale preparation of plasmid DNA from *E. coli*

The several plasmids described above were extracted from *E. coli,* after transformation, using the QIAGEN Plasmid Midi Kit. The protocol for extraction of low copy plasmids was performed as described by the kit manufacturer.

2.2.3.3. Agarose gel electrophoresis of DNA

Agarose gels were prepared [0.8 to 1.5% (w/v)] using agarose "Multi-Purpose" or "Molecular Screening for small DNA fragments" from Boehringer Mannheim and 1xTAE (0.04 M Tris-acetic acid, 10 mM EDTA pH 8.0) containing 0.02 µg/ml ethidium bromide. DNA samples were dissolved in loading buffer [0.25% (w/v) of bromophenol blue, 0.25% (w/v) of xylene cyanol, 30% (v/v) glycerol] and run at 70 V (Power Pac 3000, Bio-Rad) in submerged horizontal electrophoresis systems (Mini-Sub Cell GT, Bio-Rad). When electrophoresis was completed, gels were exposed to UV light to visualize the DNA bands and an image was taken using the Quantity One software (Bio-Rad) coupled with Gel Doc 2000 Gel Documentation System with Motorized Zoom Lens (Bio-Rad).

2.2.3.4. Plasmid DNA digestion with restriction endonucleases

In order to prepare plasmid DNA for ligation, 5 µg of the required plasmid was digested with 5 U of the required restriction endonucleases for periods of time that ranged from 3 h to overnight at 37°C, in a 20 µl reaction. Restriction enzymes and buffers were purchased from Fermentas or Boehringer Mannheim and were used according to the manufacturer recommendations. Complete digestions were confirmed by running a small portion of the reaction in an agarose gel. The linear plasmid was purified using the QIAquick Spin Purification Procedure from the QIAquick PCR Purification Kit (Qiagen).

To screen for positive *E. coli* clones, restriction reactions were performed. After rapid plasmid DNA extraction, as described below (point 2.2.3.8), plasmid DNA was digested with specific restriction enzymes to confirm the insertion of the heterologous gene in the vector.

2.2.3.5. Amplification of *S. cerevisiae* and *C. albicans* genomic DNA by PCR

DNA amplification reactions (Table 2.2) were carried out in a Mastercycler gradient (Eppendorf) using a standard PCR programme. This included an initial incubation at 94°C for 2 m, and a program of 25 to 30 cycles: starting with 94°C for 30 s, followed by the melting temperature (Tm), which is dependent on the pair of primers used, and finally an extension temperature of 72°C for 30 s. A final extension of 2 m was also included in the PCR programme. After amplification, DNA fragments were purified from primers, nucleotides, polymerases and salts using QIAquick spin columns in a microcentrifuge. The QIAquick PCR Purification Kit protocol (Qiagen) was performed as described by the manufacture.

Table 2.2. – Reaction mixture for PCR amplification.

Component	Volume from stock solution	Final concentration
MilliQ H_2O	up to 100 µl	
Buffer 10x	10 µl	1x
dNTPs mix 10 mM	10 µl	1 mM
Primer F 100 pmol/µl	1 µl	10 pmol
Primer R 100 pmol/µl	1 µl	10 pmol
$MgCl_2$ 25 mM	6 µl	1.5 mM
DNA template	0 to 10 µg	500 ng
Taq Polymerase	1 µl	5 Units

2.2.3.6. Dephosphorylation and ligation of plasmid DNA

After restriction digestion, DNA vectors were treated with alkaline phosphatase to prevent recircularization. Reactions were prepared with 2 µg of restricted vector DNA containing 2 Units of shrimp alkaline phosphatase (SAP) (Roche) and 2 µl of 10x dephosphorylation buffer (0.5 M Tris-HCl, 50 mM $MgCl_2$, pH 8.5). The final volume of the reaction was 20 µl, which was adjusted with MilliQ H_2O. Dephosphorylation reactions were performed at 37°C for 1 h followed by inactivation of SAP at 65°C for 15 m.

Ligation of restricted DNA species was carried out using T4 DNA ligase (Gibco BRL or Fermentas). Routinely, 30-60 pmol of vector DNA was mixed with 90-

180 pmol of insert DNA fragments in reactions containing 4 µl of 5x Ligase Reaction Buffer [250 mM Tris-HCl (pH 7.6), 50 mM MgCl2, 5 mM ATP, 5 mM DTT, 25% (w/v) polyethylene glycol-8000], 5 Units T4 DNA Ligase. The volume of the reaction was adjusted to 20 µl with MilliQ H_2O. Ligations were performed overnight at 12°C or for 3 h at 22°C. The ligation mixture was then transformed into *E. coli* as described below.

2.2.3.7. *E. coli* transformation by plasmid DNA

Fresh single colonies were picked, inoculated into 5 ml LB and grown at 37°C, overnight, with vigorous shaking (250 rpm). 200 µl of this overnight culture was inoculated in fresh medium and allowed to grow, at 37°C, until the culture density reached an Optical Density ($OD_{550\ nm}$) of 0.3. To obtain highly competent cells, 100 ml of LB was inoculated with 4 ml of the previous culture, and incubated to an $OD_{550\ nm}$ of 0.3, at 37°C, with agitation. At this point, the culture was placed on ice for 5 m and centrifuged at 500 *g* for 5 m, at 4°C. The pellet was washed gently in 40 ml of cold TFBI [100 mM RbCl, 50 mM $MnCl_2.4H_2O$, 30 mM potassium acetate, 10 mM $CaCl_2.2H_2O$, 15% (w/v) glycerol, final pH 5.8]. Finally, the pellet was ressuspended in 5 ml TFBII [10 mM MOPS, 10 mM RbCl, 75 mM $CaCl_2.2H_2O$, 15% (w/v) glycerol, final pH 6.8] and distributed in 200 µl aliquots into ice cold microfuge tubes. Cells were directly used for transformation or frozen in dry ice and stored at -80°C.

For transformation, 200 µl aliquots of "competent" cells were incubated on ice, for 30 m, with 10-100 ng (10 µl of ligation reaction diluted 1:5). Cells were then submitted to a heat shock of 42°C for exactly 90 s and immediately transferred to ice for 2 m. Finally, 800 µl of SOC medium [20 mM glucose, 2% (w/v) tryptone, 0.5 % (w/v) yeast extract, 0.05% (w/v) NaCl, 2.5 mM KCl, pH adjusted to 7.0] were added and the mixture was incubated at 37°C, in a shaker, for 1 h. Cells were then centrifuged briefly (20 s), at low velocity (500 *g*), plated on LB/Amp and incubated overnight at 37°C.

2.2.3.8. Rapid isolation of plasmid DNA from *E. coli*

Screening of positive *E. coli* clones grown in LB/Amp was carried out by plasmid mini-preparations. For this, single colonies were picked from transformation plates and cells were grown overnight in 5 ml of LB/Amp at 37°C, 200 rpm. After this, 1.5 ml of culture were taken into a microfuge tube and centrifuged at high speed (15,000 *g*) at room temperature. Cells were washed with 1 ml of solution I (50 mM glucose, 25 mM Tris pH 8.0, 10 mM EDTA pH 8.0), the pellet was ressupended in 100 µl of solution I and 200 µl of solution II [0.2 M NaOH, 1% (w/v) SDS], were added followed by 150 µl of ice cold solution III [3 M potassium acetate (pH 5.0)]. The solution was mixed by inverting the tube, and then centrifuged at 15,000 *g,* at 4°C, for 5 m. This mixture was incubated on ice for 5 m and the supernatant was recovered into a clean 1.5 ml microfuge tube and 0.7 volume of isopropanol were added to the supernatant. The mixture was left at room temperature for 10 m and then centrifuged at 10°C, 15,000 *g* for 1 m. The pellet was washed with 1 ml of ice cold 70% (v/v) ethanol and the DNA pellet was dried at 37°C. For routine restriction digestion, the pellet was resuspended in a 20 µl of sterile MilliQ H_2O and 5 µl of DNA solution was digested to confirm the presence the insert of interest in the cloning DNA vector.

2.2.3.9. Procedures for DNA sequencing

For routine DNA sequencing, high quality plasmid DNA was prepared using QIAprep Miniprep Kit (Quiagen). Preparation of DNA samples was performed according to the DNA sequencing protocol described in the ABI PRISM® BigDye™ Terminator Cycle Sequencing Ready Reaction Kit, with AmpliTaq® DNA Polymerase, FS (*PE* Applied Biosystems). Briefly, each sequencing reaction was prepared in 200 µl PCR tubes containing: 200-500 ng of template DNA, 3.2 pmol of primer, 4 µl of dye Terminator Reaction Mix and sterile MilliQ H_2O to a final volume of 20 µl. Sequencing reactions were performed using the GeneAmp PCR System 9600 or 2400. PCR cycles were as follows: 96°C for 10 s, 50°C for 5 s and 60°C for 4 m. 2.0 µl of 3 M sodium acetate (pH 4.6) and 50 µl of 95% (v/v) ethanol were added to extension reactions mixed thoroughly. After precipitation (from 15 m to 3 h), tubes were spun for 20 m,

at 15,000 *g,* at room temperature. Supernatants were discarded, and pellets washed with 250 µl of 70% (v/v) ethanol, and dried at room temperature and finally resuspended in 20-25 µl of Template Suppression reagent. Before loading on the ABI Prism 377 DNA Sequencer (*PE* Applied Biosystems; University of Aveiro), samples were heated at 95°C for 2 m, to denature the DNA, and then chilled on ice.

2.2.4. Transformation of *C. albicans*

The transformation of *C. albicans* was carried out as described in the "Manual for the Preparation and Transformation of *Pichia pastoris* Spheroplasts" Version A, from Invitrogen. *C. albicans* CAI4 culture (200 ml) were grown at 30°C, 180 rpm, in YPD. Cells were harvested at an OD_{600} between 0.2 and 0.3, by centrifugation, at room temperature, for 5-10 m, at 15,000 *g,* and washed in 20 ml of sterile distilled water. At this point, the cell pellet was washed two times with 20 ml of fresh SED [19 ml of SE (1 M sorbitol, 25 mM EDTA, pH 8.0) + 1ml of 1 M DTT added at the moment], and then with 20 ml of 1 M sorbitol before resuspension in 20 ml of SCE buffer (1 M sorbitol, 1 mM EDTA, 1 mM sodium citrate buffer, pH 5.8). This suspension was divided in two tubes (10 ml each). One was used to monitor spheroplast formation and the other was kept at room temperature for later use.

Spheroplast formation was monitored as follows: 200 µl of cell suspension was added to 800 µl of 5% (v/v) SDS at times T_0, T_2, T_4, T_6, T_8, T_{10}, T_{15}, T_{20}, T_{25}, T_{30} m after the addition of 20 µl of 3 mg/ml Zymoliase 100 T (Seikagaku Corp.). The OD_{800} of the cell suspension mixed with SDS was determined using a solution of 800 µl of 5% (v/v) SDS and 200 µl of SCE buffer as blank. The percentage of spheroplasts for each time point was determined using the equation:

$$\% \text{ Spheroplasting} = 100-[(OD_{800} \text{ at time t}/OD_{800} \text{ at time 0}) \times 100]$$

For routine transformation, suspensions of 80% of spheroplasts were used. Spheroplasts were harvested by centrifugation at low velocity (750 *g*) for 10 m

at room temperature, and submitted to several isotonic washes. First, with 10 ml of 1 M sorbitol and then with 10 ml of CaS buffer (1 M sorbitol, 10 mM TrisCl, pH 7.5, 10 mM $CaCl_2$). Spheroplasts were resuspended gently in 0.6 ml of CaS and used immediately for transformation.

For each transformation, 100 µl of the spheroplast suspension were dispensed into 1.5 ml microfuge tubes. To each aliquot of spheroplasts, 3-12 µg of plasmid DNA plus 5 µl of 1mg/ml Yeast Maker Carrier DNA (Clontech) were added and incubated at room temperature for 10 m. During this incubation, a fresh PEG/CaT [1 volume of 40% (w/v) PEG 3350 solution for 1 vol of CaT (20 mM Tris, pH 7.5, 20 mM $CaCl_2$)], was prepared. To each transformation, 1 ml of fresh PEG/CaT solution was added, mixed gently and incubated for 10 m more at room temperature. After centrifugation, at 750 *g,* for 10 m, at room temperature, the PEG/CaT solution was completely removed by aspiration and the pellet was resuspended in 150 µl of SOS (1 M sorbitol, 0.3xYPD, 10 mM $CaCl_2$). Transformed cells were incubated at room temperature for more than 20 m and plated in selective media MM-uri. Agar plates were incubated at 30°C for a maximum period of 10 days.

2.2.5. Northern blot technique

2.2.5.1. Isolation of total RNA

For total RNA extractions, 250 ml cultures grown overnight in YPD or MM-uri medium were harvested at an OD_{600} of 0.7-0.9 and the pellets were frozen overnight at -70°C. Cells were ressuspended on ice in 5 ml lysis buffer (0.3M sodium acetate, pH 4.5, 10 mM EDTA) and 1 volume phenol equilibrated with sodium citrate pH 4.5. Approximately 3 ml of baked glass beads (Ø=0.4 mm) were then added to the cell suspension, which was vigorously shaken 8 times for 30 s with 1 m incubation on ice (Weygand-Durasevic *et al.*, 1994). This procedure was repeated 8 times. The aqueous phase containing RNAs was separated from the phenolic phase by centrifugation at 3,200 *g,* for 20 m, at 4°C, and then transferred to a new Falcon tube and re-extracted with fresh

phenol. The aqueous phase was separated by centrifugation at 3,200 *g,* for 20 m, at 4°C, and was frozen overnight at -20°C.

2.2.5.2. tRNA purification

Total RNA extracts were cleaned from contaminating rRNAs, mRNAs and protein on a 20 ml DEAE-cellulose column equilibrated with 0.1 M sodium acetate pH 4.5. tRNAs were eluted with 20 ml of 0.1 M sodium acetate/0.3 M sodium chloride and 20 ml of 0.1 M sodium acetate/1 M sodium chloride. These fractions were precipitated overnight at -20°C with 2.5 volumes ethanol, and then resuspended in 200 µl of 10 mM sodium acetate pH 4.5/ 1 mM EDTA and stored at -20°C for later use (Santos *et al.*, 1996a).

2.2.5.3. tRNA deacylation

For routine work, 50 µg of total tRNA was precipitated overnight at -20°C with 4 v of ethanol. The pellet was resuspended in 25 µl of Tris 1 M pH 8.0/1 mM EDTA and tRNAs were incubated at 37°C for 1 h. After a second ethanol precipitation deacylated tRNA were resuspended in 20 µl of 10 mM sodium acetate pH 4.5/1 mM EDTA and stored at -20°C, as described by Santos and colleagues (1993).

2.2.5.4. RNA analysis by gel electrophoresis

For routine work, glass plates (4 mm thick, 30 cm wide and 40 cm long) were cleaned with ethanol, dried and assembled between 0.8 mm thick spacers and held together with steel clips. Gel and stock solutions were prepared using MilliQ H_2O.

2.2.5.4.1. 10% Semi-denaturing polyacrylamide gel electrophoresis (PAGE)

Fractionation of total tRNAs was routinely carried out on 10% (w/v) polyacrylamide gels prepared from stock solutions of 40% (w/v) acrylamide

containing 1.6% (w/v) N´-N´-methylenebisacrylamide (bisacrylamide) (Bio-Rad). Solutions of 100 ml were prepared with 25 ml 40% (w/v) Acril:Bis (19:1) (Bio-Rad), 10 ml 10xTBE (0.9 M Tris, 0.9 M boric acid pH 8.3, 0.025 M EDTA), 7 M Urea and MilliQ H_2O up to 100 ml. Then, 1 ml 10% (w/v) ammonium persulfate (APS), and 35 µl N,N,N`,N`-tetramethylethylenediamine (TEMED) were added. Gel solutions were allowed to polymerize after filling the gel mould and introduce the slot former. The polymerization occurred at room temperature for 1 h and then gels were matured at 8°C, overnight.

Prior to sample loading, slot formers were removed and glass plates were inserted in an adjustable vertical 35 cm running system (ADJ3, Anagene). The buffer tanks were filled with 1xTBE and slots were washed with the same buffer. Samples of total tRNA were prepared by mixing 10 µg of tRNA with an equivalent volume of loading buffer [10 mM sodium acetate pH 5.0, 8 M urea, 0.05% (w/v) bromophenol blue, 0.05% (w/v) xylene cyanol]. In each gel slot, 20 µl of tRNA sample was loaded with a 50 µl Hamilton microsyringe. Electrophoresis was allowed to proceed at 700 V, at room temperature, until the bromophenol blue dye reached the bottom of the gel (Varshney *et al.*, 1991d). Fractioned tRNAs were stained with ethidium bromide solution and visualized by UV shadowing.

2.2.5.4.2. Acidic denaturing 7.5% PAGE

Acidic denaturing polyacrylamide gels (100 ml) were prepared by mixing 18.8 ml of 40% Acril:Bis (19:1), 8 M urea, 10 ml 1 M sodium acetate pH 5.0 and MilliQ H_2O. Finally, 1 ml 10% (w/v) APS and 100 µl of TEMED were added, just before pouring into the gel mold. A slot former was then introduced and the gel was allowed to polymerize, at room temperature, for 1 h, and then stored overnight, at 8°C. For electrophoresis, gels were placed in the running apparatus and the slot former was removed. Before sample loading, electrophoresis tanks were filled with 10 mM sodium acetate, pH 5.0, and sample loading slots were washed thoroughly with the above buffer. tRNA samples, containing 10 to 20 µg of diacylated and acylated tRNAs, were

dissolved in tRNA loading buffer and loaded into the gel with a 50 µl Hamilton micro syringe.

Acidic gels were run in a cold-room (4°C) at 100 V during 2 h, and then at 250-300 V until bromophenol blue reached the bottom of the gel (Varshney *et al.*, 1991c). Fractionated tRNAs were localized by UV shadowing, the portion of the gel containing tRNAs was cut and the tRNAs were transferred onto a membrane as described bellow.

2.2.5.5. Semi-dry blotting

The portion of gel containing tRNAs (see above) was measured and 10 sheets of 3 MM paper and a Hybond N membrane (Amersham Pharmacia) were cut to the size of the gel portion. The 3 MM paper sheets and the nylon membranes were soaked in 0.25xTBE placed into the anode of the Semy-Dry Trans Blot apparatus (Bio-Rad), as follows: 5 sheets of 3 MM paper, nylon membrane, gel and 5 sheets of 3 MM paper. tRNAs were transferred onto the membrane at 0.8 mA/cm^2 for 30-45 m (Varshney *et al.*, 1991b), and then fixed by both sides of the membrane UV with using the optimal crosslinking programme of the Stratalinker 1800 (Stragene).

2.2.5.6. Probe labelling and hydridization procedures

For hybridization, probes were labelled with [α-^{32}P]dCTP (Amersham) by PCR as described by Innis and colleagues (1990), with exception that the amount of dCTP that was reduced from 100 to 85 nM, and 5 nmol (30 μCi) 6000 Ci/mmol [α-^{32}P]dCTP were added to the reaction mixture. A 25 µl PCR reaction was prepared indicated in Table 2.3.

Table 2.3. Reaction mixture for PCR amplification.

Component	Volume from stock solution	Final concentration
MilliQ H_2O	up to 25 µl	
Buffer 10x	2.5 µl	1x
2 mM dNTPs (-C) mix	2.5 µl	0.2 mM
2 mM dCTPs	1 µl	0.08 mM
6000Ci/mmol [α-^{32}P]dCTP	3 µl	5 nM
Primer F 100 pmol/µ	1 µl	10 pmol
Primer R 100 pmol/µl	1 µl	10 pmol
25 mM $MgCl_2$	1.5 µl	1.5 mM
DNA template	0 to 10 µg	500 ng
Taq Polymerase	0.5 µl	2.5 Units

In order to decrease the background on the membranes caused by free radioactivity, 50 PCR cycles were performed to ensure maximum incorporation of [α-^{32}P]dCTP onto the PCR fragments.

The hybridization protocol was performed as described by Heitzler and colleagues (1992) using a hybridization-oven (HIR10M, Grant Boekel). For pre-hybridization, 5 ml of hybridization solution [50% (w/v) deionized formamide, 5xSSC (150 mM NaCl, 15 mM sodium citrate, pH 7.0), 1% (w/v) SDS, 0.04% (w/v) Ficoll, 0.04% (w/v) polyvinylpyrrolidone and 250 µg/ml of sheared salmon sperm DNA] were prepared for each 100 cm^2 of membrane. A volume of 10 mg/ml stock sheared salmon sperm DNA solution was boiled for 3 m and then added to the hybridization solution.

Pre-hybridization was performed for 5 to 8 h at 50°C, and after this the pre-hybridization solution was discarded and a fresh pre-hybridization solution was prepared, with exception that salmon sperm DNA was not included. The PCR-labelled DNA (see above) was boiled for 5 m before addition to the hybridization solution. A volume of 2.5 ml of hybridization solution was prepared for 100 cm^2 of membrane. Hybridization was carried out overnight at 50°C.

Membranes were then washed in the hybridization bottle at 50°C, with 1XSSC/0.1% (w/v) SDS for 30 m followed for another 30 m wash with

0.5XSSC/0.1% (w/v) SDS. Membranes were exposed to a K-screen from Bio-Rad at room temperature for periods of 3 h to 5 days. Screens were scanned using a Molecular Imager FX Pro Plus Multilmager System (Bio-Rad) and the image was analysed using the Quantity One software (Bio-Rad).

2.2.6. Optical microscopy

For analysis of cell morphology, a Zeiss Axioplan microscope equipped with phase-contrast optics was used. Images were captured through a Zeiss AxioCam HRc camera mounted on the microscope, using the Axio Vision software. Images of different colony phenotypes were acquired using the above camera coupled to Zeiss dissecting microscope. Image analysis was carried out using the software above.

2.2.7. Scanning electronic microscopy (SEM)

For scanning microscopy analysis, cells grown to mid-log phase in MM-uri liquid media were pelleted by centrifugation, washed once with double-distilled water, and fixed in 2.5% (w/v) glutaraldehyde in 0.1 M cacodylate buffer, for 30 m, and then washed in 0.1 M cacodylate buffer (pH 7.3). After this, cells were gradually dehydrated using a series of acetone solutions (15%, 30%, 50%, 70%, 90% and 100%), and finally any residual water content was removed using a Critical Point (Baltec CPD 030) apparatus. Samples were mounted on aluminium stubs, sputter-coated with gold palladium and scanned using L JSM 5400 scanning microscope.

2.2.8. Determination of the frequency of phenotypic switching

To compare the frequency of switching between clones, a MM-uri overnight culture grown at 30°C was serially diluted to 1000 cells per ml. From this dilution, approximately 50 cells were pipette and spread on each 10 MM-uri agar plates (8 cm diameter), and incubated at 30°C for 7 days. Sectored colonies were scored and an evaluation of the colony phenotype was carried out using a dissecting microscope. For each strain, the number of clones

studied varied from 6 to 20, and the data obtained was statistically tested using ANOVA statistical test. Colony images were acquired using a Zeiss AxioCam HRc system.

2.2.9. Monitoring production of extracellular hydrolases: secreted aspartic proteinases and phospholipases

The production of extracellular hydrolases was determined has described by Ibrahim and colleagues (1995), with minor adaptations. *C. albicans* strains were screened for production of extracellular phospholipase and secreted aspartic proteinase activity by growing cells on MM-uri agar supplemented with 10% egg yolk (Merck) or 10% bovine serum albumin (BSA) (Sigma), respectively, and measuring the size of the zone of precipitation using the method of Samaranayake (Samaranayake *et al.*, 1984). A 3x2 µl suspension of 10^7cells /ml in PBS (Phosphate Buffered Saline) (10 mM Na_2HPO_4 pH 7.0, 150 mM NaCl) was plated on the surface of the agar medium, MM/egg yolk and MM/BSA, in a 80-mm-diameter Petri dish and left to dry at room temperature. The culture was then incubated at 30°C for 3 days, after which the diameter of the colony and the precipitation zone around the colony was determined. Three different clones of pUA12, pUA13, pUA14 and pUA15 transformed cells were tested twice. The experiment was carried out on two different occasions. The extracellular hydrolase activity was calculated using the formula [(1/Pz)-1], where Pz value represents the hydrolyse zone, i. e., the cloudy-zone-around-plus-colony diameter divided by the colony diameter (Figure 2.11). Data obtained was submitted to an ANOVA statistical test.

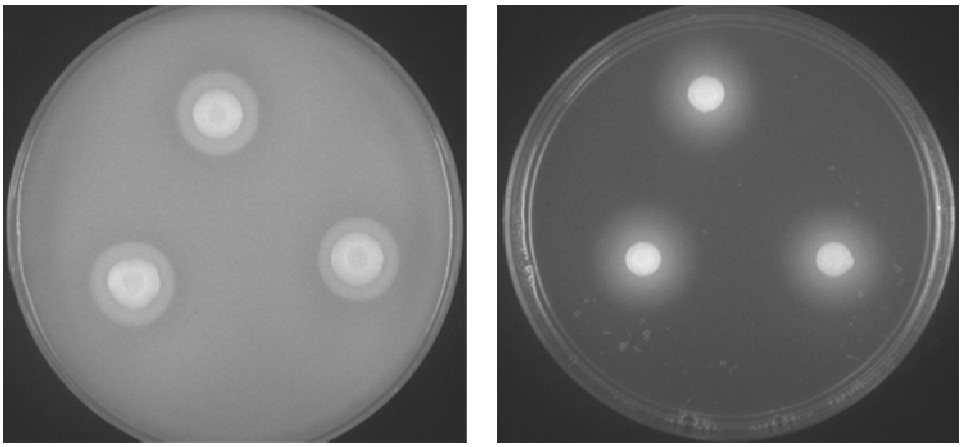


Figure 2.11. Production of extracellular phospholipases and secreted aspartyl proteinases. Hydrolytic activity was monitored by measuring the colony diameter and the precipitation zone around the colony. Measurements of the cloudy-zone-around-plus-colony diameter and the colony diameter were taken, in order to obtain the Pz value. Studies were carried out in MM supplemented with 10% of Egg-yolk and BSA, respectively.

2.2.10. Cell-cell and cell-surface adhesion

Cell-cell adhesion was evaluated in liquid cultures of MM-uri of cells grown at 30°C with agitation (180 rpm). Cell-surface adhesion was observed by streaking cells in MM-uri agar plates grown at 30°C for several days.

2.2.11. Virulence experiments

Virulence of pUA12 and pUA15 transformants of *C. albicans* CAI4 was tested by challenging 10 male Balb/c mice from the Harlan Interfauna Ibérica (Barcelona), with 7-9 weeks old, weighing between 20 g to 25 g, for each strain and inoculum density. For this, *C. albicans* cells were harvested from MM-uri agar plates, washed twice in PBS and diluted in the same buffer to obtain the following cellular densities: $2x10^7$ and $2x10^6$ cells/ml. Each mouse was injected with 0.5 ml of previous cellular solutions into the lateral vein of the tail. Mice survival was monitored daily for 30 days.

2.3. Results

2.3.1. CAI4 transformation efficiency

Leuker and colleagues (1994) found that transformation of *C. albicans* CAI4 cells with plasmids containing *S. cerevisiae* $tRNA_{UAG}^{Leu}$ and $tRNA_{CAG}^{Leu}$ genes, both tRNAs able to decode CUG codons, was lethal. Rare transformants were obtained but carried rearranged plasmids with deletions in the tRNA genes. Since then genetic manipulation of *C. albicans* have improved and new plasmids and transformation methods have emerged. Therefore, genetic code manipulation was carefully planed in order to decrease the toxicity caused by the incorporation of leucine at the CUG codon.

The plasmid used in all genetic transformations was a modified version of the pRM1 vector, herein named pUA12. pRM1 is a double-ARS shuttle vector (Pla *et al.*, 1995), that allows direct recovery in *E coli* of non-multimerized plasmids extracted from *C. albicans* transformants (Goshorn *et al.*, 1992). The ARS are supposed to represent chromosomal replication origins on the basis of their subcellular localization (nuclei), temporal replication (S phase) and spacing in chromosomes (Newlon, 1988). They are defined as DNA sequences which, when present in plasmids, allow their autonomous replication and increase the frequency of transformation from 2 to 4 orders of magnitude (Struhl *et al.*, 1979; Stinchcomb *et al.*, 1979; Hsiao & Carbon, 1979). The frequency of transformation obtained with plasmids carrying one ARS is about $1x10^3$ transformants/µg DNA. The incorporation of both ARS2 and ARS3 in a single plasmid does not improve the transformation efficiency ($5\text{-}10x10^3$), but decreases plasmid intracellular recombination, and also increases its copy number (around 4-8 copies per cell) and mitotic stability (Pla *et al.*, 1995).

pUA plasmids contain the *URA3* auxotrophic marker, that encodes orotidine 5'-monophosphate (OMP) decarboxylase. A variety of selectable markers have been used in fungi, however no dominant selectable marker has yet been found to improve *C. albicans* transformation. There are two major reasons for this: i) *Candida* species in general and *C. albicans* in particular, seem to be naturally resistant to most antibiotic selection markers available including

hygromycin, benomyl, cyclohexamide, mitomycim C, and tunicamycin (Beckerman *et al.*, 2001); ii) some *Candida* species decode the CUG codon as serine instead of leucine and many heterologous markers do not function in *C. albicans* unless CUG codons are first modified (Scherer & Magee, 1990).

Genetic manipulation requires a reliable and efficient method for DNA transformation. Electroporation is the fastest and simplest method of transformation. High-efficiency electroporation has been achieved in a variety of yeasts, such as *S. cerevisiae* (Simon, 1993; Manivasakam & Schiestl, 1993; Becker & Guarente, 1991; Delorme, 1989; Hill, 1989), *Hansenula polymorpha* (Faber *et al.*, 1994), *Candida maltosa* (Becher & Oliver, 1995; Kasuske *et al.*, 1992), and *Candida utilis* (Kondo *et al.*, 1995). However, *C. albicans* is difficult to transform by electroporation (Thompson *et al.*, 1998; Brown, Jr. *et al.*, 1996)) and transformation efficiencies are normally around 250 transformants/µg with linearized integrative DNA (De Backer *et al.*, 1999; Thompson *et al.*, 1998) and ~3 transformants/µg with circular integrative DNA.

Lithium acetate transformation (Ito *et al.*, 1983) and spheroplast transformation (Hinnen *et al.*, 1978) methods also suffer from significant limitations (De Backer *et al.*, 2000). Lithium acetate transformation of *C. albicans* is fast and simple but also gives transformation efficiencies in the order of 50-100 transformants/µg of replicative DNA (Sanglard *et al.*, 1996). Recently, an optimized heat-shock step slightly increased transformation efficiency (Walther & Wendland, 2003). However, a combination of the lithium acetate method followed by an electroporation pulse, increased transformation efficiencies to a level comparable with that of spheroplast transformation method: ≤300 transformants/µg with an integrative plasmids and ≤4500 transformants/µg with an *Candida* ARS-containing plasmid (De Backer *et al.*, 1999). Spheroplast transformation, although more efficient [~300 transformants/µg of integrative DNA and 10^3-10^4 transformants/µg of replicative DNA (Herreros *et al.*, 1992)], is more laborious and time consuming. A stable and efficient integrative transformation of *C. albicans* was achieved by a modified protocol used for transformation of the methylotrophic

yeast *Pichia pastoris.* This method gives transformation efficiencies of ~2000 transformants/µg of integrative DNA (De Backer *et al.*, 2001), and was chosen to transform *C. albicans* CAI4 cells.

Transformation efficiencies obtained for the pUA12 and other control vectors, namely pUA16 and pUA17, ranged from 25 to 29 transformants/µg of DNA. This represents a lower transformation efficiency from that described in the literature (Pla *et al.*, 1996). Apart from this, we also observed that the number of transformants obtained varied significantly between transformations, as indicated by the standard deviation (SP) shown in Figure 2.12.

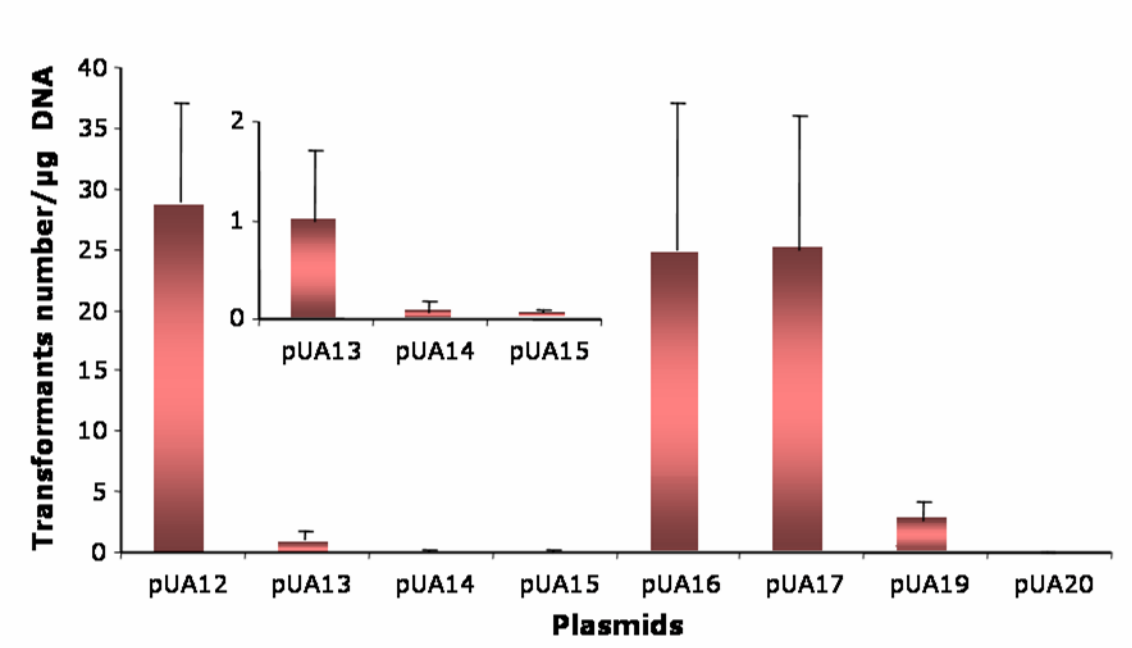


Figure 2.12. Transformation efficiencies of *C. albicans* CAI4. Transformations were carried out using by the spheroplast method. pUA12 - empty plasmid; pUA13 - plasmid containing *S. cerevisiae* $tRNA_{UAG}^{Leu}$; pUA14 - plasmid containing *S. cerevisiae* $tRNA_{CAG}^{Leu}$ (G_{33}); pUA15 - plasmid containing *S. cerevisiae* $tRNA_{CAG}^{Leu}$ (U_{33}); pUA16 – plasmid containing *S. cerevisiae* $tRNA_{AGA}^{Ser}$; pUA17 - plasmid containing *S. cerevisiae* $tRNA_{UGA}^{Ser}$; pUA19 - plasmid containing *S. cerevisiae* $tRNA_{UAA}^{Ser}$; and pUA20 - plasmid containing *S. cerevisiae* $tRNA_{AAG}^{Ser}$. The inset shows the transformation efficiencies of CAI4 obtained with pUA13, pUA14 and pUA15 plasmids.

Although *C. albicans* transformation was not a primary subject of this study its low efficiency was a significant problem for plasmids pUA13, pUA14, and pUA15, which encode tRNA genes that decode CUG as leucine and not serine.

Transformation of *C. albicans* with pUA20, which encodes the *S. cerevisiae* $tRNA_{AAG}^{Ser}$ yielded no transformants suggesting that this tRNA is apparently lethal. This serine tRNA, recognizes 3 leucine codons (CUC, CUU and CUA) and their decoding as serine is likely to be toxic for *C. albicans*. This is due to the fact that usage of those 3 leucine codons represents 16.1% of total number of codons in this species (CUC 2.5%; CUU 9.7%; CUA 3.9%) (Nakamura *et al.*, 2000). In addition, the heterologous *S. cerevisiae* $tRNA_{AAG}^{Ser}$, even when expressed from a low-copy plasmid, would be a strong competitor of the endogenous *C. albicans* $tRNA_{AAG}^{Leu}$ whose gene is present in single copy in the genome.

Although transformation efficiencies obtained with pUA13, pUA14, pUA15 and pUA19 plasmids were very low, transformants appeared on the transformations plates at frequencies of 1 (pUA13), 2.6 (pUA19) and 0.06 (pUA14 and pUA15) per µg DNA. The difficulties in transforming *C. albicans* with plasmids encoding leucine tRNAs with the anticodons 5'-UAG-3' (pUA13) and 5'-CAG-3' (pUA14 and pUA15), which decode leucine CUN codons and possibly UUR codons (Randerath *et al.*, 1979; Weissenbach *et al.*, 1977), were expected. Joachim Ernst 's group (Leuker & Ernst, 1994) tried a similar strategy and no transformants were obtained when *C. albicans* was transformed with plasmids encoding heterologous $tRNA_{CAG}^{Leu}$ and $tRNA_{UAG}^{Leu}$ suggesting that the insertion of leucine at CUG codons was lethal.

The toxicity of *S. cerevisiae* $tRNA_{UAG}^{Leu}$ (pUA13) and $tRNA_{UAA}^{Ser}$ (pUA19) may be due to their efficient decoding of CUG and UUA codons, respectively. The $tRNA_{UAG}^{Leu}$ inserts leucine in a major serine codon (CUG), while $tRNA_{UAA}^{Ser}$ decodes the UUA leucine codon as serine. The efficiency of transformation for both plasmids, pUA13 and pUA19, was almost identical despite significant differences in codon usage as well as in the number of endogenous tRNAs that are competing with the heterologous tRNAs. That is, the $tRNA_{UAG}^{Leu}$ decodes

the CUG codon, whose usage is 3.1% (Nakamura *et al.*, 2000) and competes with the *C. albicans* endogenous $tRNA_{CAG}^{Ser}$ whose gene copy number is one. The $tRNA_{UAA}^{Ser}$ decodes the Leu UUA codon, which is used ten times more frequently (33.5%) (Nakamura *et al.*, 2000), but its gene copy number is 3.

If transformation efficiency is an indicator of the toxicity caused by the introduction of *S. cerevisiae* tRNA genes in CAI4, then the $tRNA_{CAG}^{Leu}$ genes, both with G_{33} (pUA14) and U_{33} (pUA15), were the most toxic since both plasmids encoding $tRNA_{CAG}^{Leu}$ produced the lowest transformation efficiencies in this study.

2.3.2. Expression and aminoacylation of *S. cerevisiae* leucine tRNA genes in *C. albicans*

The expression of *S. cerevisiae* heterologous tRNAs in *C. albicans* was verified by Northern blot analysis (Figure 2.13), as described by Santos *et al.* (1996). The heterologous expression *S. cerevisiae* $tRNA_{UAG}^{Leu}$, $tRNA_{CAG}^{Leu}$, $tRNA_{AGA}^{Ser}$ $tRNA_{UGA}^{Ser}$, $tRNA_{UAA}^{Ser}$ encoded by plasmids pUA13 to pUA19 showed the expected expression patterns, both in terms of size and expression levels (Figure 2.13, lane 1 and lanes 3-6).

Plasmids in *C. albicans* are highly unstable, leading to wide variations in copy number and rapid loss of the plasmid in the absence of selection (Pla *et al.*, 1996). Indeed, the expression differences found may be related to a varying plasmid copy number, as was previously found by Leuker and colleagues (1992; 1994).

To test whether the *S. cerevisiae* heterologous tRNAs were aminoacylated *in vivo* in *C. albicans*, total tRNA samples were extracted under acidic conditions and fractionated in an acidic urea PAGE that separates charged from uncharged tRNAs (Varshney *et al.*, 1991). Charged and uncharged tRNAs were detected by Northern blot analysis, as before (Figure 2.14). As expected, the *S. cerevisiae* tRNAs expressed in *C. albicans* were fully charged, suggesting that mutations introduced in the heterologous tRNA genes did not affect

aminoacylation efficiency and that serine and leucine tRNA identity are identical in both yeasts.

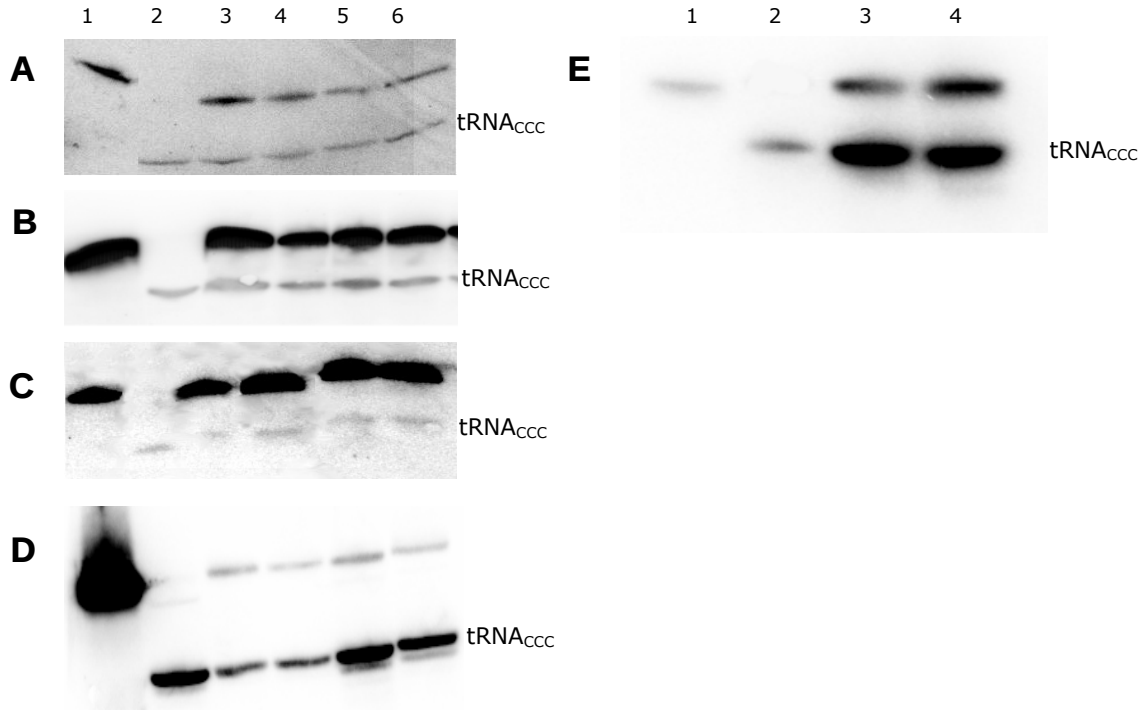


Figure 2.13. Northern blot analysis of *S. cerevisiae* $tRNA_{UAG}^{Leu}$ (A), $tRNA_{CAG}^{Leu}$ (B and C), $tRNA_{UGA}^{Ser}$ and $tRNA_{UAA}^{Ser}$ (D), and $tRNA_{AGA}^{Ser}$ (E) expressed in *C. albicans.* $tRNA_{CCC}^{Gly}$ was used as internal control for all assays. Total tRNAs extracted from *C. albicans* were fractionated on a 10% polyacrylamide-8 M urea gel and blotted onto Hybond N membranes. Detection of Leu-$tRNA_{UAG/CAG}$ and Gly-$tRNA_{CCC}$ was carried out using a [^{32}P]tDNALeu$_{UAG/CAG}$ and [^{32}P] tDNAGly$_{CCC}$ probe labelled by PCR. Lane 1 corresponds to tRNA extracted from *S. cerevisiae* (positive control). Lane 2 corresponds to tRNAs extracted from *C. albicans* transformed with empty pUA12 plasmid (negative control). Lane 3-6 correspond to tRNAs extracted from *C. albicans* transformed with pUA13 (A), pUA14 (B) and pUA15 (C), pUA16 (E), pUA17 and pUA19 (D).

Conversely to what has been previously reported (Leuker & Ernst, 1994), the data shown above indicates that *S. cerevisiae* tRNAs are functional in *C. albicans* and consequently the latter can tolerate increased levels of CUG mistranslation since these tRNAs decode the CUG codon as leucine and not serine. These results show for the first time that *C. albicans* tolerates CUG and UUA ambiguity *in vivo*.

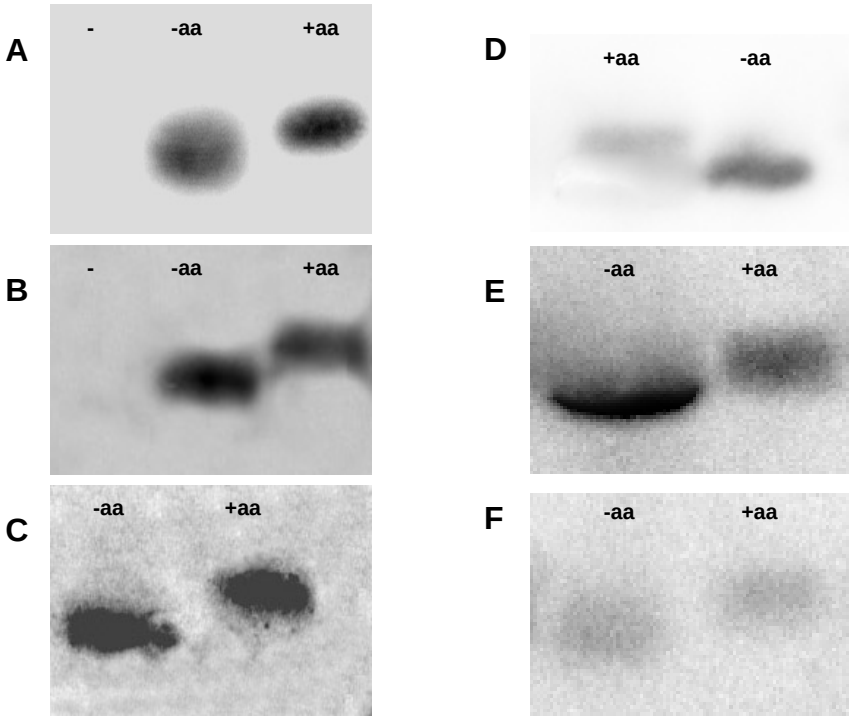


Figure 2.14. Aminoacylation of the *S. cerevisiae* leucine $tRNA_{UAG}$ and $tRNA_{CAG}$, serine $tRNA_{AGA/UGA/UAA}$ expressed in *C. albicans*. Total tRNAs were extracted under acidic conditions from *C. albicans* transformed with pUA12 (symbol – in A and B), pUA13 (A), pUA14 (B), pUA15 (C), pUA16 (D), pUA17 (E) and pUA19 (F). Separation of deacylated (-aa) and acylated (+aa) $tRNA_{UAG}^{Leu}$ (A), $tRNA_{CAG}^{Leu}$ (B and C), $tRNA_{AGA}^{Ser}$ (D), $tRNA_{UGA}^{Ser}$ (E) and $tRNA_{UAA}^{Ser}$ (F) which hybridize with the respective $[^{32}P]tDNA_{Leu/Ser}$ probe. tRNAs were fractionated by ACIDIC-PAGE, transferred to a nylon membrane using a semy-Dry Trans Blot apparatus.

The above mentioned ambiguity is in line with observations made by others that organisms are highly tolerant to genetic code ambiguity and manipulation either by incorporation of canonical or unnatural amino acids (Pezo *et al.*, 2004; Kiga *et al.*, 2002; Sakamoto *et al.*, 2002; Nangle *et al.*, 2002; Doring *et al.*, 2001; Wang *et al.*, 2001; Santos *et al.*, 1999). Genetic code ambiguity has previously been achieved by engineering various components of the translational apparatus, namely tRNAs and aaRS. This strategy takes advantage of the observation that several aaRSs do not have a mechanism for editing misactivated amino acids and consequently engineering the active site of these synthetases can be easily accomplished. On the other hand, deletion of the editing domain creates tRNA aminoacylation ambiguity that also results in genetic code ambiguity (Bacher *et al.*, 2005; Pezo *et al.*, 2004) (Hendrickson *et al.*, 2004).

2.3.3. Ambiguous CUG decoding triggers morphogenesis

C. albicans cells can grow in at least three different forms: yeast, pseudohypha and hypha. Morphological changes between yeast and filamentous forms occur in response to alterations in growth conditions. There is a wide range of physiological parameters that affect *C. albicans* morphogenesis (Brown, 2002b; Berman & Sudbery, 2002).

Interestingly, the heterologous expression of *S. cerevisiae* tRNA genes (see above) in *C. albicans* resulted in expression of distinct colony and cell phenotypes under normal growth conditions (in MM-uri at 30°C). As expected, cells from control strains (pUA12, pUA16 and pUA17) were ovoid, grew as yeast in liquid and solid media and formed white smooth colony phenotype (Figure 2.15).

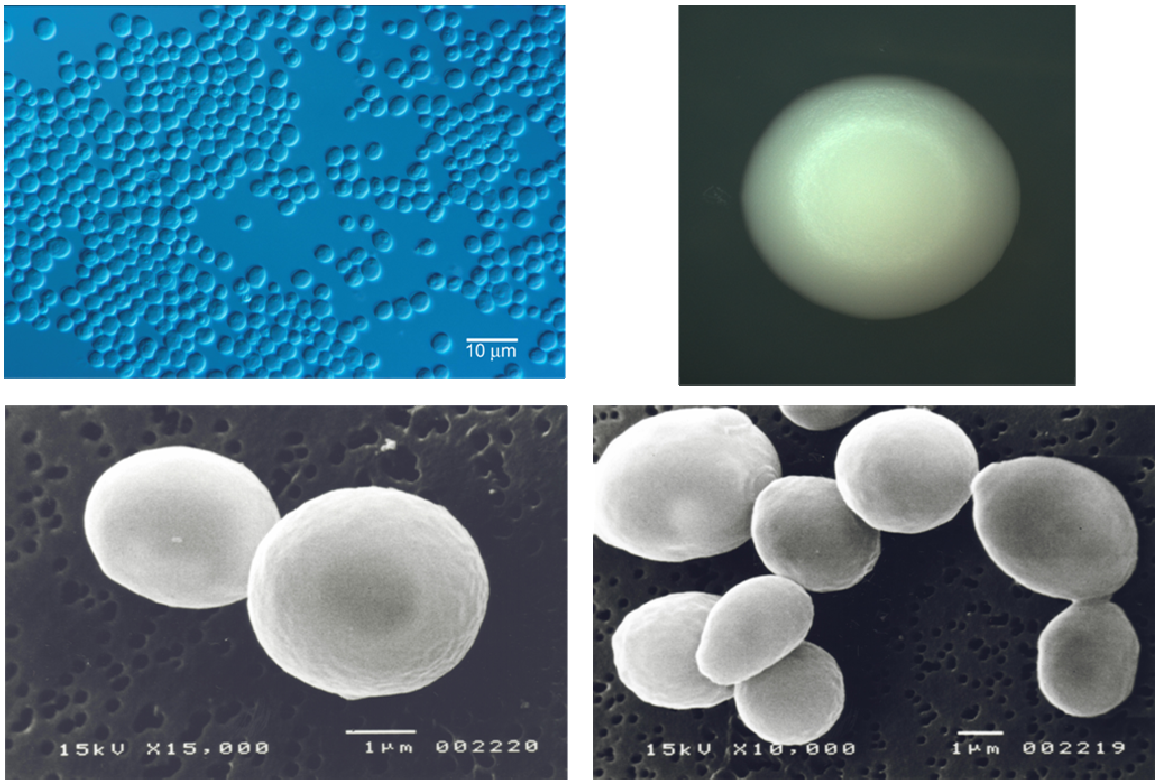


Figure 2.15. Wild type *C. albicans* cells. *C. albicans* grows in MM-uri at 30°C as yeast-like cells. The control pUA12 cell population was identical to wild type untransformed cells both in liquid (left up) and in solid media, displaying smooth colony phenotype (right up). The lower panels show typical *C. albicans* wild type cells under SEM. For SEM, cells were fixed with 2.5% (w/v) glutaraldehyde in 0.1 M cacodylate buffer. SEM image was taken in JEOL JSM 5400 scanning microscope. DIC and colony image were taken using a Zeiss AxioCam HRc camera mounted on Zeiss Axioplan microscope and a Zeiss dissecting microscope, respectively.

In contrast, *C. albicans* cells expressing *S. cerevisiae* $tRNA_{UAG/CAG}$ expressed from plasmids pUA13, pUA14 and pUA15 displayed extensive morphological variation. These cell populations were also highly heterogeneous and contained elongated ovoid cells, pseudohypha and true hypha (Figure 2.16). In order to distinguish the latter two forms, cells were stained with calcufluor white, which stains cell walls specifically. This permits observing chains of long cells divided by septa (hyphae) and chains of elongated cells with constrictions between them (pseudohypha).

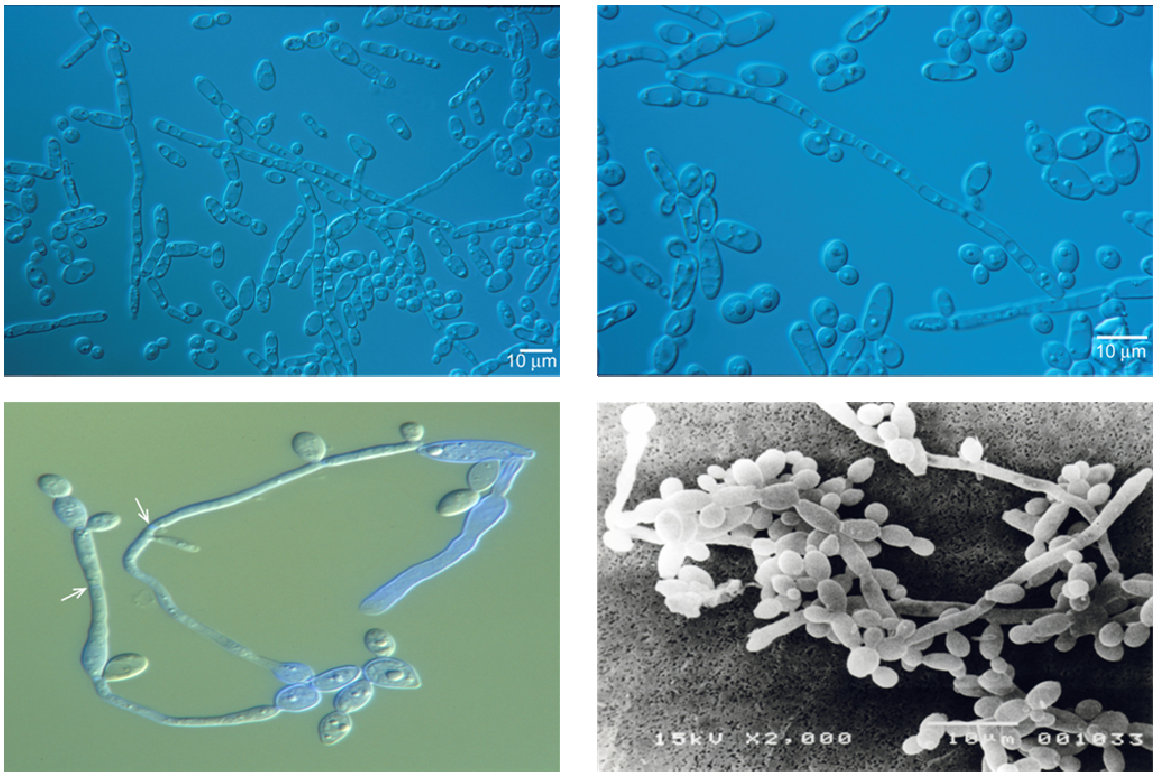


Figure 2.16. Morphological variability displayed by *C. albicans* transformed with plasmids pUA13, pUA14 and pUA15. Cultures of CUG ambiguous clones were grown in MM-uri liquid media overnight at 30°C and budding yeast-like, pseudohyphae and hyphae were observed. Filamentous forms, pseudohyphae and hyphae (septa pointed by arrows), were distinguished by calcufluor white staining (bottom left). DIC images were taken on a Zeiss Axioplan microscope equipped with a Zeiss AxioCam HRc. pUA15 cells were fixed with 2.5% (w/v) glutaraldehyde in 0.1 M cacodylate buffer and observed in JEOL JSM 5400 scanning microscope (bottom right).

The filamentous growth observed in liquid media was also found in solid media. After transformation with plasmids pUA13-15, *C. albicans* clones obtained were re-plated in selective medium. A variety of colony phenotypes constituted by aerial hypha occupying a sector or all colony were observed (Figure 2.17). Notably, morphological events that gave rise to these phenotypes happened spontaneously without the presence of any inducing factor. Therefore, the most likely explanation for these results is that CUG ambiguity induces morphogenesis in *C. albicans*.

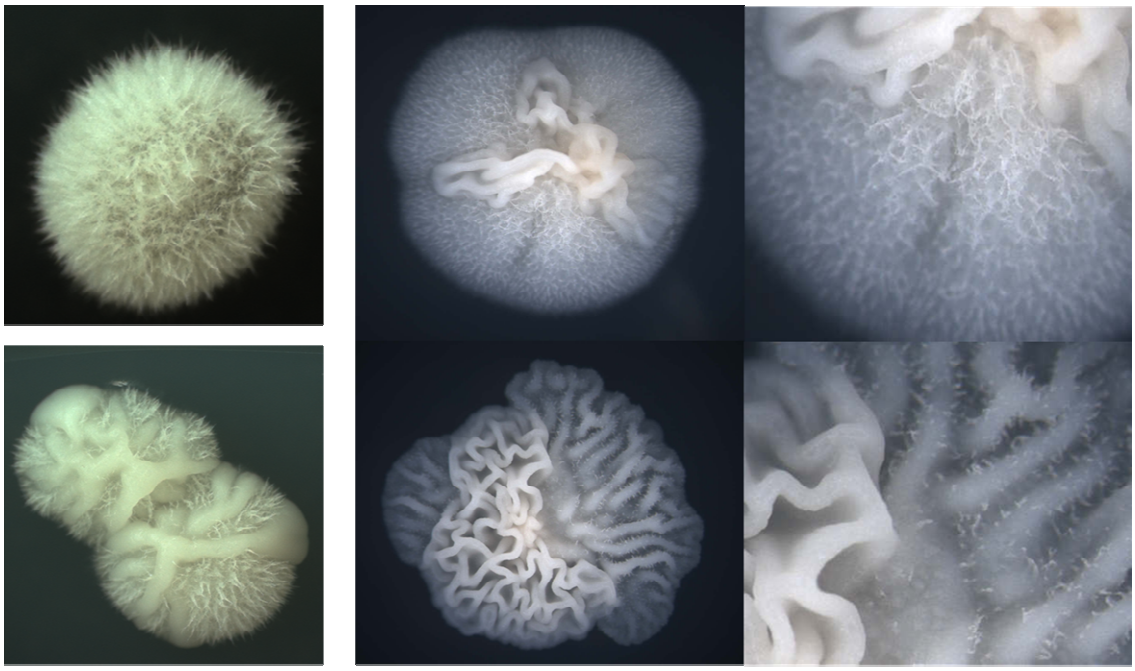


Figure 2.17. *C. albicans* morphological variation induced by ambiguous CUG decoding. *C. albicans* CAI4 transformed with pUA13, pUA14 and pUA15 plasmids were grown MM-uri agar plates at 30°C during 7 days. These cells formed colonies containing aerial hypha that were dominant or restricted to a sector colony. Images from the colonies were taken using a Zeiss AxioCam HRc camera mounted on Zeiss dissecting microscope.

Thus, all clones showed a high degree of morphological diversity however some clones produced hyphae mainly, demonstrating that CUG ambiguity triggered extensive morphogenesis (Figure 2.18). This observation was further reinforced when cells were grown at 25C° since, in contrast to previous results from other laboratories (Ernst, 2000), we have observed a sharp increase in hypha formation at this temperature. In order to confirm this unexpected

observation, an overnight culture grown at 30°C was used to inoculate fresh MM-uri plates that were further incubated at 25°C and 37°C. Remarkably, colonies grown at 25°C were entirely or partially composed of hyphal forms, while only a minor part of the colonies at 37°C presented aerial hyphae sectors. At this temperature, the predominant phenotype observed was the wrinkle phenotype (Figure 2.19).

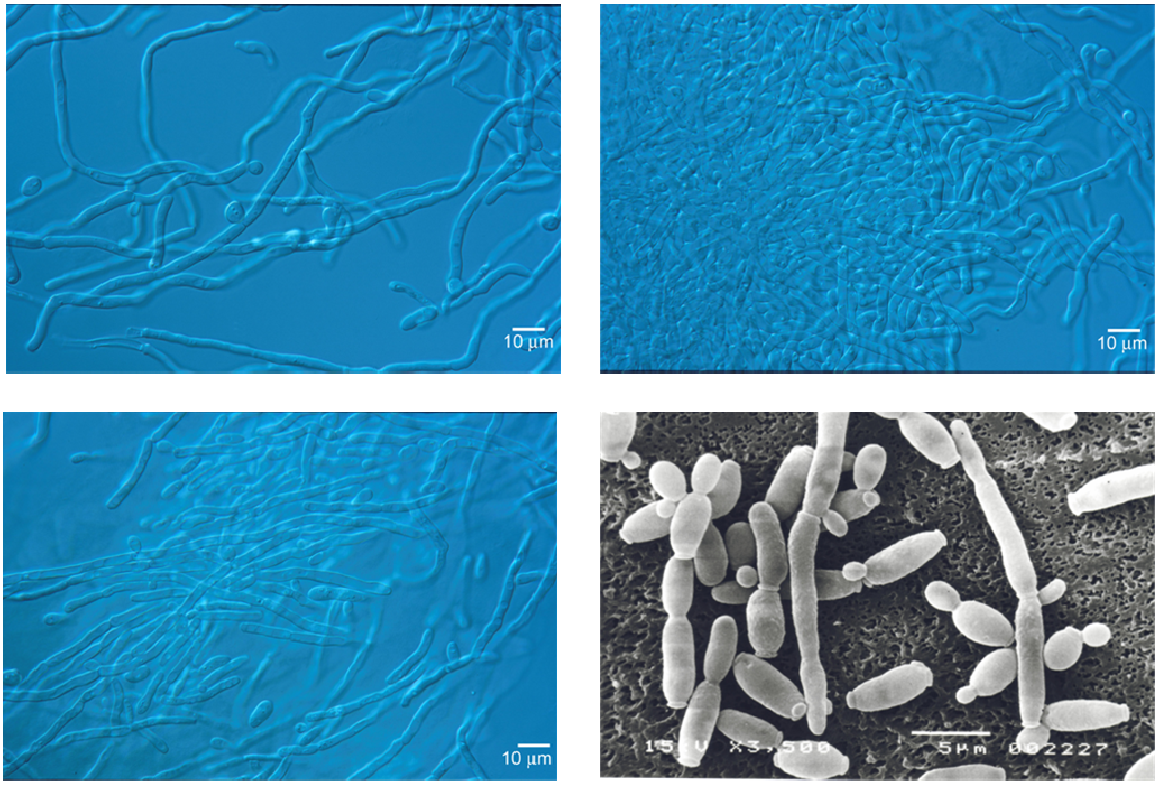


Figure 2.18. *C. albicans* morphogenesis triggered by CUG ambiguity. Transformation of *C. albicans* CAI4 with pUA15 plasmid induced morphogenesis at high rate. At 30°C, in liquid MM-uri, transformed cells grew exclusively as filaments and formed dense cell aggregates that flocculated in liquid media.

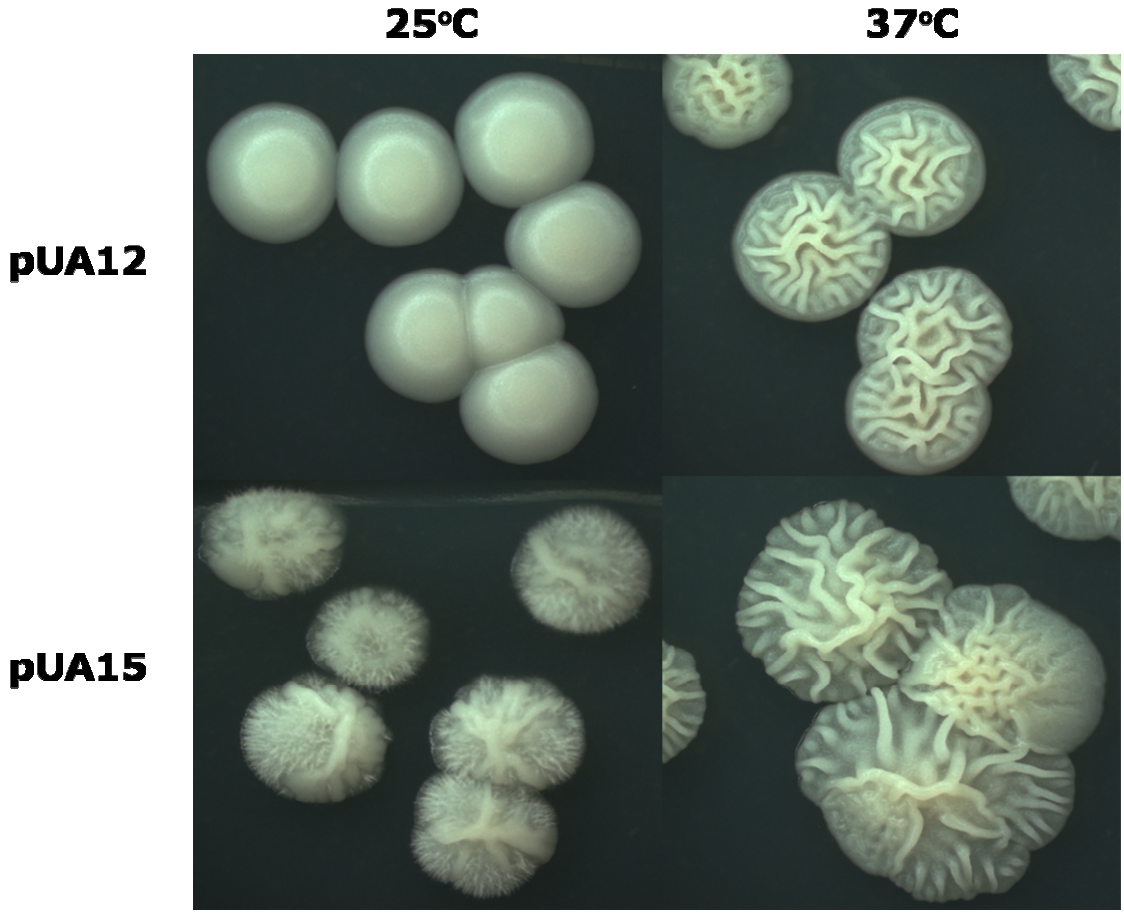


Figure 2.19. Growth temperature affects morphogenesis triggered by CUG ambiguity. The figure shows colonies of control (pUA12) and ambiguous cells (pUA15) grown at 25°C and 37°C. CUG ambiguity induces morphogenesis at both temperatures, however it was more extensive at 25°C. At 37°C, the colonies were wrinkled without aerial hypha. Control pUA12 cells did not display aerial hypha. Images from the colonies were taken after 5 days of growth using a Zeiss dissecting microscope equipped with Zeiss AxioCam HRc camera.

Interestingly, high temperatures (above 34°C) in conjugation with other factors such as neutral pH, serum or Lees medium (Berman & Sudbery, 2002), induce hyphal formation in most strains. How temperature affects morphogenesis in this particular case is not yet clear, however lower temperatures favours spontaneous protein folding and minimizes off-pathways reactions such as aggregation, allowing for expression of higher amounts of mutant proteins that may be unstable at higher temperatures and get degraded (Sangster *et al.*, 2004; Frydman, 2001). Therefore, it is likely that CUG ambiguity induced phenotypes may be favoured at lower and masked at higher temperatures. An alternative explanation may be that the metabolic rate is lower at 25°C than 37°C and consequently lower amounts of protein

being produced per unit of time may free molecular chaperones for recovering mistranslated proteins produced by CUG ambiguity. Also, at higher temperatures, cellular processes are accelerated and cellular environment is more crowded, which has a negative impact in folding process (Frydman, 2001). Therefore at higher temperature, large amount of mutant proteins are synthesized per unit of time, the majority of them are unable to fold correctly and become readily degraded since misfolded proteins are targeted to proteasome degradation by ubiquitin system (Kostova & Wolf, 2003). In fact, higher proteasome activity was found in *C. albicans* CAI4 cells transformed with pUA15 plasmid (see chapter 4).

2.3.4. Ambiguous CUG decoding increases phenotypic switching

Apart from morphogenesis, CUG ambiguity also induced phenotypic switching. The high diversity of colony phenotypes triggered by CUG ambiguity (Figure 2.20), and their instability on agar-plates forced us to establish unifying criteria to quantify phenotypic switching. For this, the two types of phenotypic switching, namely white-opaque and unmyceliated-myceliated, were quantified in colonies growing at 30°C, during 7 days, in selective medium MM-uri media. At least six clones of each plasmid were studied and more then 500 colonies of each clone were analysed. The percentage of sectored colonies was calculated for each clone and the data was analysed using the ANOVA statistical test in order to compare results obtained for the different transformants.

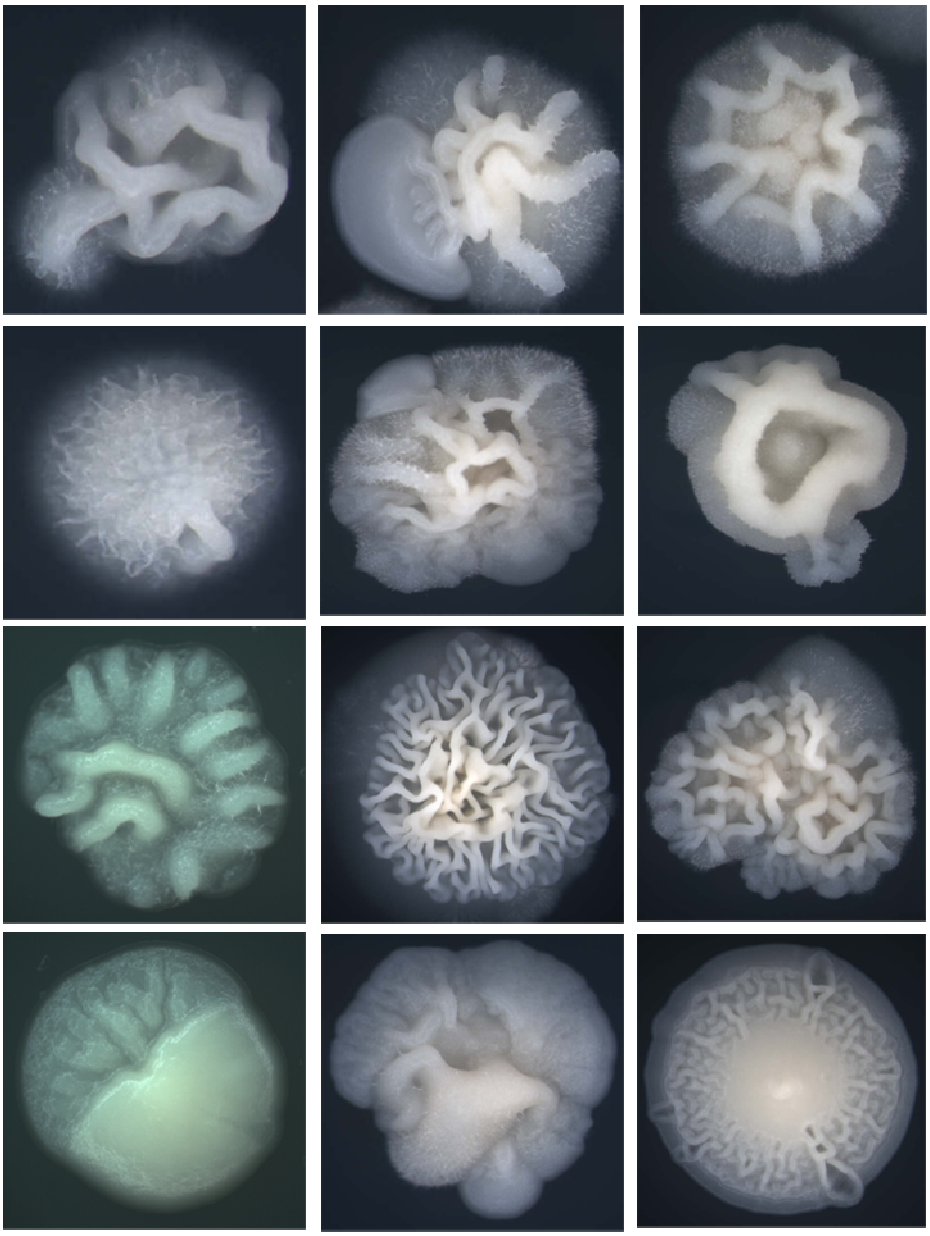


Figure 2.20. Phenotypic switching in *C. albicans* CAI4 transformed with plasmids pUA13, pUA14 and pUA15. Cells plated in MM-uri were allowed to grow at 30°C for 7 days. Colony diversity was a distinctive feature of CUG ambiguous clones. Two main features were evident, white-opaque switching, and presence of aerial hyphae. Only some of the colony phenotypes observed is shown. Images were acquired through Zeiss AxioCam HRc camera mounted in a Zeiss dissecting microscope.

According to Soll (2002), there are some confusion between morphogenesis and colony morphology switching. This confusion is related to the fact that switching occurs spontaneously while morphogenesis can be induced by a variety of environmental factors, as referred above. On the other hand, the

morphologies of *C. albicans* colonies often reflect the growth forms of the cells that compose the colonies. Indeed, different colony shapes are a direct consequence of different proportions of yeast and filamentous cells present inside the colony. For example, smooth colonies are composed entirely of yeast cells, wrinkled colonies are composed almost entirely of hyphal and pseudohyphal cells with very few blastopores (Braun *et al.*, 2000), semi-rough colonies consist of both yeast and true hypha, but in a minor proportion than that seen in wrinkled colonies (Radford *et al.*, 1994). Fuzzy colonies are composed by yeast, pseudohyphae and true hypha, with aerial hyphae in discrete areas. Finally, scallop colonies are composed entirely of pseudohyphal cells (Radford *et al.*, 1994). Thus, the criterion described above to quantify phenotypic switching is in agreement with phenotype switching definition (Soll, 2002), cells were not under morphology-inducing conditions and the various cellular morphologies present in a colony emerged spontaneously giving rise to distinct colony morphology. Finally, sectored colonies that underwent white-opaque (Slutsky *et al.*, 1987) or unmyceliated-myceliated transitions (Vargas *et al.*, 2000; Soll *et al.*, 1987) were scored. The 3153A switching system repertoire was not considered in this analysis, since many colony phenotypes obtained in *C. albicans* ambiguous clones are different from those described for the 3153A strain. Therefore, in order to analyse colony diversity induced by CUG ambiguity in a more extensive and simple manner only the white-opaque and unmycelated-mycelated switching systems were adopted for phenotypic switching quantification.

Considering the criteria described above, 88% of phenotypic switching was obtained for *C. albicans* CAI4 transformed with the pUA15, followed by pUA13 and pUA14, with percentages above 60% (Figure 2.21). In contrast, transformation with the control plasmids pUA12, pUA16 and pUA17 yielded no more than 10% of switching (Figure 2.21).

A partial reassignment of the UUA leucine codon to serine using the $tRNA_{UAA}^{Ser}$ encoded by plasmid pUA19, resulted in approximately 50% of sectored colonies (Figure 2.21). This high value indicates that codon ambiguity in

general (or mistranslation) may produce colony phenotypic diversity, including white-opaque and yeast-filamentous switching.

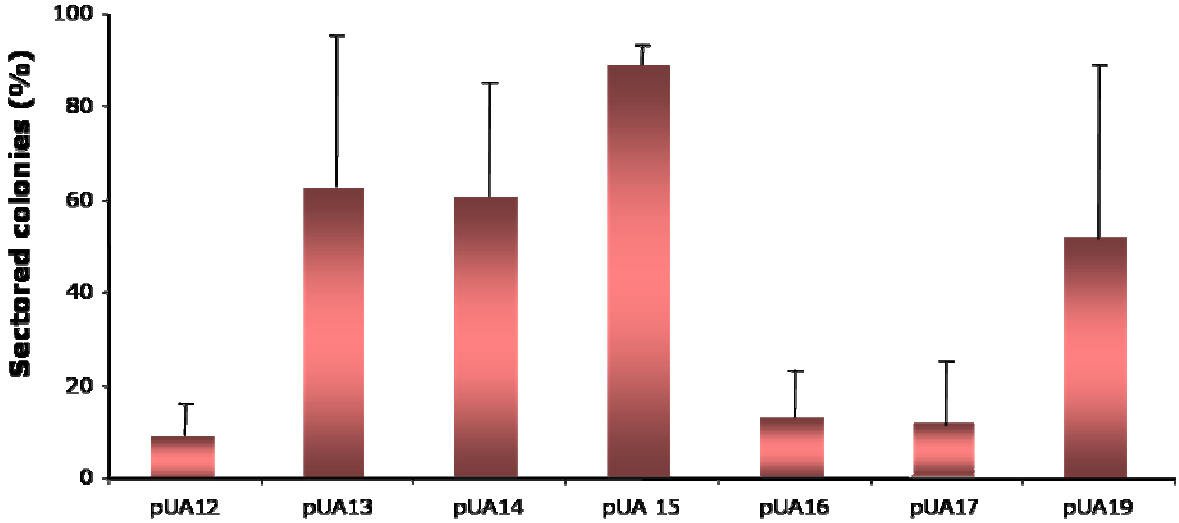


Figure 2.21. Phenotypic switching induced by genetic code ambiguity. Although high colony phenotype diversity was found in *C. albicans* CAI4 transformed with plasmids pUA13, pUA14, pUA15 and pUA19, a simple criterion was established to measure phenotypic switching. That is, sectored colonies were considered under switching process. For each plasmid, cells of 6-12 transformants were plated in MM-uri and allowed to growth for 7 days at 30°C, and then sectored colonies (white-opaque or white-opaque-hypha colonies) were scored using the Zeiss dissecting microscope.

Data from switching assays was analyzed statistically for differences between transformants using the ANOVA test with $\alpha \leq 0.05$. The results obtained were as follows: i) no statistical difference was obtained for switching between transformants of control plasmids pUA12, pUA16 and pUA17; ii) ambiguous clones transformed with plasmids pUA13, pUA14, pUA15 and pUA19 were statistically different from pUA12, pUA16 and pUA17 controls; iii) percentages of phenotypic switching from pUA13, pUA14 and pUA19 were not statistically different; iv) switching percentages of pUA13, pUA14 and pUA19 clones were statistically different from the pUA15 clone.

The differences in switching frequency permit dividing the transformants into 3 groups:

i) low phenotypic switching (control pUA12, pUA16 and pUA17);

ii) medium to high phenotypic switching (pUA13, pUA14 and pUA19);

iii) high phenotypic switching (pUA15).

These differences of phenotypic switching frequency seem to be directly proportional to destabilization of the proteome by genetic code ambiguity induced by the expression of heterologous tRNAs in *C. albicans* since these tRNAs were engineered to decode the CUG codon with different levels of efficiency creating different degrees of CUG ambiguity. As shown above, all the *S. cerevisiae* tRNAs were correctly expressed and aminoacylated and, therefore competed with the endogenous $tRNA_{CAG}^{Ser}$ for CUG decoding at the ribosome A-site.

The results described above provide indirect evidence for a direct link between CUG ambiguity and phenotypic switching, whose frequency is proportional to the expected misincorporation of leucine at the CUG positions. The latter is due to the fact that different *S. cerevisiae* tRNAs expressed in *C. albicans* are expected to decode the CUG codon with different efficiencies. For example, the $tRNA_{UAG}^{Leu}$ encoded by plasmid pUA13 decodes the CUG codon by wobble, while the $tRNA_{CAG}^{Leu}$ (G_{33}) encoded by plasmids pUA14 decodes it via 3 Watson-Crick base pairs but G_{33} reduces its decoding efficiency (Suzuki *et al.*, 1997). The $tRNA_{CAG}^{Leu}$ (U_{33}) encoded by plasmid pUA15 decodes the CUG codon more efficiently since it has the canonical U_{33} and decoding involves 3 cognate Watson-Crick codon-anticodon interactions. Finally, *S. cerevisiae* $tRNA_{AGA}^{Ser}$ and $tRNA_{UGA}^{Ser}$ encoded by plasmids pUA16 and pUA17 respectively decode cognate serine UCU and UCA codons in the standard fashion and, for this reason, do not induce phenotypic switching.

2.3.5. Ambiguous CUG decoding increases expression of extracellular hydrolases

The extracellular hydrolytic enzymes, including the secreted aspartic proteinase (*SAP*), phospholipase (*PLB*) and extracellular lipases (*LIP*) genes are among the few gene products that have been shown to directly contribute to *C. albicans* virulence (Stehr *et al.*, 2004; Mukherjee *et al.*, 2003; Naglik *et al.*, 2003a). Production of secreted aspartyl proteinases can easily be monitored on agar plates containing serum albumin since hydrolysis of BSA produces a halo around the colony whose size can be correlated to proteinase production. *C. albicans* cells transformed with pUA13, pUA14 and pUA15 plasmids produced higher levels of extracellular hydrolases than the control pUA12 cells (Figure 2.22).

Phospholipase production was also scored on agar plates containing egg yolk as substrate, whose hydrolysis also results in the formation of a precipitation halo around the colonies. As before, ambiguous clones produced higher levels of phospholipases than control clones (pUA12), but no difference was observed between clones transformed with plasmids pUA13, pUA14 and pUA15.

Since members of the *SAP* gene family (*SAP1*, *SAP7*, *SAP8*, *SAP9* and *SAP10*) and genes encoding phospholipases contain CUG codons, one would expect that CUG ambiguity would cause structural alterations with negative consequences to enzymatic activity. Interestingly, SAP and phospholipases activity was significantly increased ($\alpha \leq 0.05$) in the ambiguous *C. albicans* cells transformed with plasmids pUA13, pUA14 and pUA15 (Figure 2.22), suggesting that expression of the *SAP* genes is up-regulated.

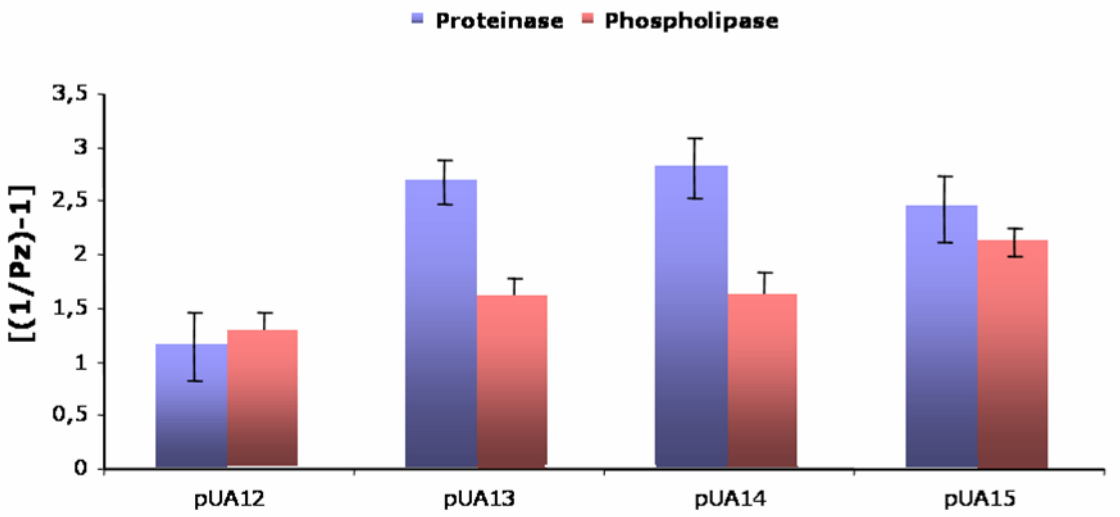


Figure 2.22. CUG ambiguity increases production of extracellular hydrolases. *C. albicans* CAI4 transformed with plasmids pUA13, pUA14 and pUA15 display higher hydrolytic activity than control pUA12 transformants. Secreted aspartic proteinase and phospholipase production was measured using BSA as substrate and egg yolk, respectively. Cells were prepared in PBS to a density of 10^7 cell/ml from a MM-uri overnight liquid culture. 2 µl of this cell suspension was plated on MM-uri on agar surface, supplemented either with 10% BSA or 10% egg yolk. Plates were incubated at 30°C for 3 days. Pz value was obtained by measuring the cloudy-zone-around-plus-colony diameter and dividing that value by the colony diameter.

2.3.6. Ambiguous CUG decoding increases adhesion

Apart from the phenotypes described above, CUG ambiguity also increased cell adhesion that could be easily observed in selective liquid cultures and agar plates. This was observed in clones transformed with plasmids pUA13, pUA14 and pUA15, however the adhesion phenotype was exacerbated in pUA15 clones (Figure 2.23). Interestingly, different clones of pUA13, pUA14 and pUA15 displayed significant differences of cell-agar and cell-cell adhesion proprieties. For example, three clones of pUA15 were extremely adherent to agar and displayed a strong flocculation phenotype (Figure 2.23).

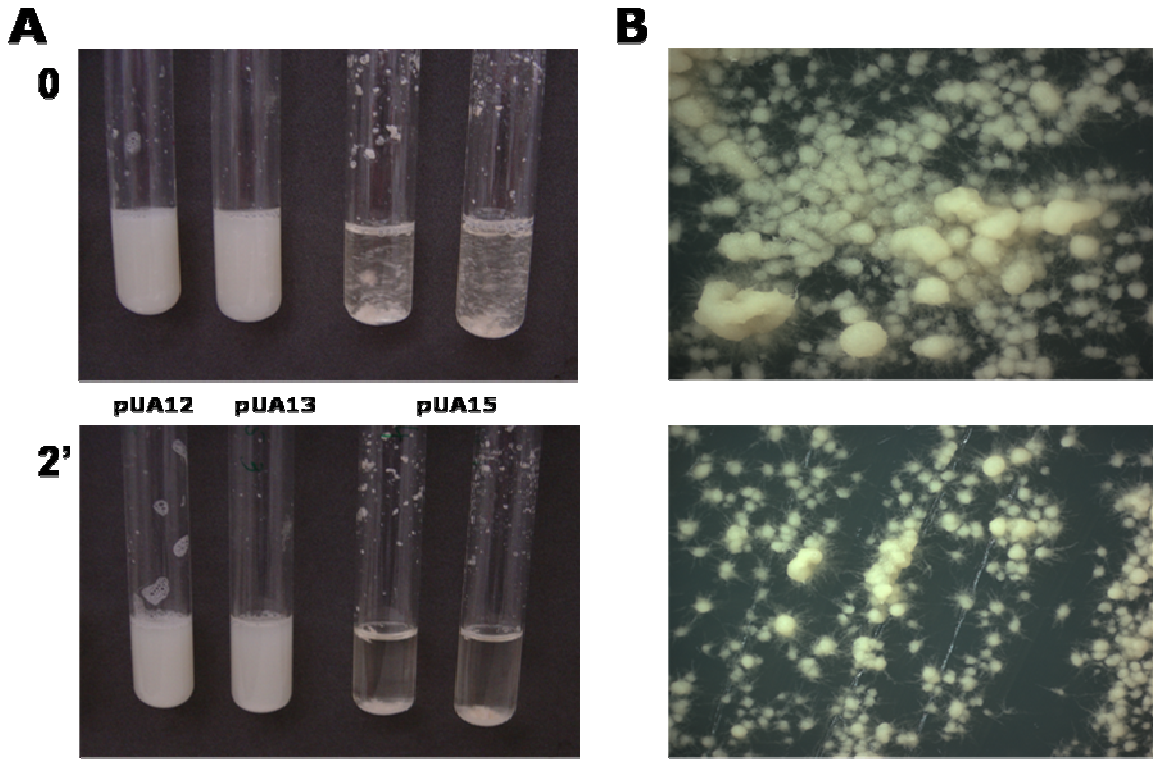


Figure 2.23. Cell-surface and cell-cell adhesion induced by CUG ambiguity. *C. albicans* CAI4 transformed with pUA15 plasmid exhibit strong adhesion phenotypes in liquid (A) and solid media (B). **(A)** Flocculation of pUA15 cells growing in liquid media for 2 days at 30°C, with agitation (180 rpm). Upper panel shows liquid culture immediately after resuspension by vortexing. Bottom panel shows the same cultures after 2 minutes without agitation. **(B)** *C. albicans* CAI4 transformed with pUA15 plasmid stroked into MM-uri agar displayed extreme adhesion proprieties, cell to cell and cell to agar surface.

Interestingly, more than 50% of the genes involved in adhesion contain CUG codons, in particular the members of the *ALS* gene family such as *ALS7* gene, which contains 18 CUG codons, *ALS5* and *ALS9* genes contain 12 CUG codons each. Whether these CUG codons are related to increased adhesion due to the hydrophobic characteristics of leucine, is not yet known. In any case, some of the pUA15 clones were extremely hydrophobic and flocculated in liquid media (Figure 2.23A), indicating that these cells are highly hydrophobic. This is of particular relevance to *C. albicans* biology because hydrophobicity increases adherence during recognition of both epithelial and endothelial cells, binding to ECM proteins and plastic devices (catheters). Furthermore, hydrophobicity is linked to increased virulence of *C. albicans* and speeds up germ tubes

formation (Masuoka & Hazen, 1999; Glee *et al.*, 1995; Hazen & Hazen, 1993; Hazen & Hazen, 1992).

2.3.7. Ambiguous CUG decoding affects *in vivo* virulence

The results described above showed clearly that CUG ambiguity triggered expression of a number of virulence factors, namely, morphogenesis, phenotypic switching, production of extracellular hydrolases and adhesion. This raised the hypothesis that the engineered ambiguous *C. albicans* clones were more virulent than wild-type ones. To test this hypothesis, male Balb/c mice were injected with either a lethal dose (10^6 cells) or a sub-lethal dose (10^5 cells) of pUA12 and pUA15 cells, in the tail vein. Interestingly, while a lethal dose of the control pUA12 clone was more virulent than pUA15 clone, injection of a sub-lethal dose of the pUA15 clone was more virulent than the control pUA12 clone (Figure 2.24).

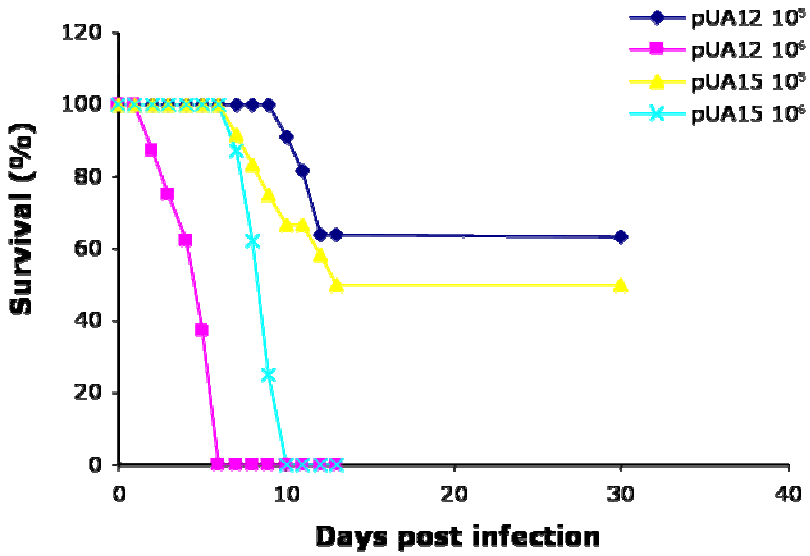


Figure 2.24. CUG ambiguity affects *C. albicans* virulence in murine model. Survival curves of mice (male Balb/c) infected with $1x10^5$ and $1x10^6$ cells of *C. albicans* CAI4 transformed with pUA12 and pUA15 plasmids. Ten mice were inoculated trough tail vein and mortality was determined over the course of 30-day experiment. Data were recorded as percent survival on each day.

Mice infections injected with 10^6 of pUA12 transformed cells resulted in 100% of mortality after 7th days post infection, while the same number of pUA15 transformed cells killed mice after 10 days post infection, revealing a slight difference in virulence between the clones. This shows that ambiguous pUA15 cells are less virulent than control pUA12 cells, thus contradicting the working hypothesis described above. Although this was a surprising result, it may be explained by two distinct factors, namely: i) white cells are more virulent than opaque cells in systemic infection (Kvaal *et al.*, 1999; Kvaal *et al.*, 1997; Tsuboi *et al.*, 1994) and pUA15 transformed clones have a very high percentage of opaque cells. The pUA15 clones have a heterogenous population of cells composed by large proportion of opaque cells, while pUA12 strain is mainly composed of white cells, and ii) the selective marker used (*URA3*) has one CUG codon and consequently its function may be affected by CUG ambiguity. This is relevant because *URA3* expression levels are directly correlated with OMP descarboxylase activity (Brand *et al.*, 2004), which in turn has consequences at phenotypic level, namely hyphal morphogenesis, buccal cell adherence and lethality of mice (Brand *et al.*, 2004; Cheng *et al.*, 2003; Sundstrom *et al.*, 2002; Bain *et al.*, 2001). Therefore, if increased CUG ambiguity destabilizes OMP descarboxylase (Ura3p) activity this may result in slow growth of *C. albicans* in infected mice, which could explain the weaker virulence of ambiguous clones.

Despite the negative impact on virulence, pUA15 transformed cells were slightly more virulent than control pUA12 cells when non-lethal inocula (10^5 cells) were used (Figure 2.24). After the 7th day post infection, pUA15 cells continued to kill mice, reaching 50% of mortality at the end of the experiment, while mice challenged with 10^5 of pUA12 transformed cells displayed 63% of survival. This also indicates that ambiguous decoding of the *URA* CUG codon may not affect *C. albicans* virulence. These results are clearly insufficient to clarify the role of CUG ambiguity in *C. albicans* virulence. Future virulence studies should also include pUA13 and pUA14 clones that express lower levels of CUG ambiguity, and other types of infection, such as cutaneous infections where opaque cells have stronger virulent phenotype. Also, such studies

should be carried out using selective markers without CUG codons and integrated in the genome to ovoid uncontrolled secondary effects of CUG ambiguity and plasmid instability.

2.4. Discussion

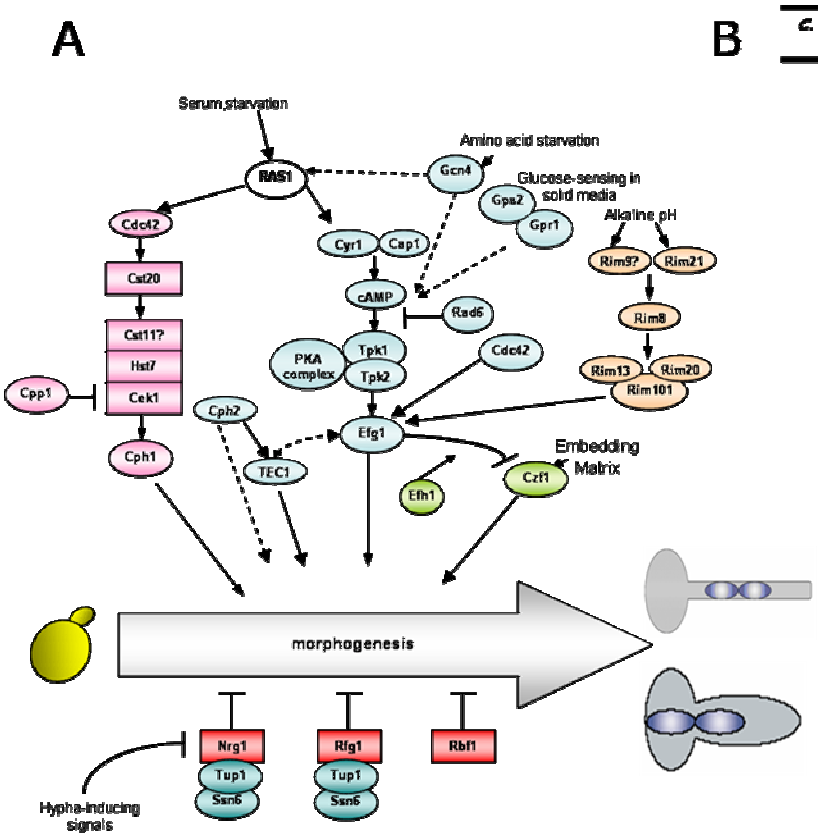
2.4.1. Effect of CUG ambiguity on morphogenesis

CUG ambiguity manipulation triggered the development of filamentous growth, in both liquid and solid media, during exponential and stationary growth phases, and more interestingly in the absence of any extra environmental signal. Therefore, this raises the question of how does CUG ambiguity trigger morphogenesis. As mentioned before, morphogenesis regulators have CUG codons and it is likely that CUG ambiguity may disrupt their function and, therefore, may affect the signalling pathways controlled by those regulators. However, morphogenetic activators contain CUG codons while the general morphogenesis transcription repressor *TUP1* does not, which makes spontaneous filamentous growth resulting from CUG ambiguity difficult to explain. The partial inactivation of components of the morphogenetic signalling pathways and absence of morphogenetic stimulus should result in a normal yeast-like colony and cell morphology phenotypes, with filamentation repressed (Figure 2.25). The disruption of protein structure caused by replacement of serine by leucine in the transformed clones of *C. albicans* CAI4, is likely to decrease cellular levels of the signalling proteins and hyphal transcription activators and consequently the extensive filamention observed is unexpected since it should have been inhibited. This is further supported by the non-existence of CUG codons, in the gene of the general repressor *TUP1* gene that, for this reason, escapes disruption caused by leucine CUG decoding.

Additionally, morphogenesis was spontaneous and exclusively related with CUG ambiguity, which contrasts with the activation of the signalling cascades that are triggered in response to environmental cues (Ernst, 2000). All the external signals described as morphogenesis inducers are directly linked to the

activation of a specific signalling pathway, for example: the cascade involving *RIM* genes is activated in response to alkaline pH (Davis, 2003; Buffo *et al.*, 1984) and the Czf1 pathway is triggered under microaerophilic conditions (Brown, Jr. *et al.*, 1999). Since CUG ambiguity has a pleiotropic effect certainly all morphogenetic signalling cascades, activators or repressors, are affected but the balance is clearly favouring filamention processes.

The molecular events that regulate morphogenesis are not yet fully understood, thus complicating the interpretation of the effect of CUG ambiguity on filamentation. The presence of CUG codons in the majority of genes that control morphogenesis (Figure 2.25) adds yet another level of complexity to the interpretation of the results. Despite this, our results support a model in which filamentous growth is regulated by a network of interacting signalling pathways and transcriptional regulators that extends down to the target genes without an intervention of a master regulator (Braun & Johnson, 2000; Brown & Gow, 1999; Mitchell, 1998). Despite the poorly understood network of morphogenetic pathways that led to filamentation, there are a couple of interesting genes that contain CUG codons, but further studies are necessary to understand the impact of CUG ambiguity on the structure and function of these genes. For example, *EFG1* contains one CUG codon and its ambiguous decoding may have important consequences since this gene is involved in multiple biological functions, namely regulation of transcription (Stoldt *et al.*, 1997), cell adhesion (Li & Palecek, 2003), cellular morphogenesis (Srikantha *et al.*, 2000), hyphal and pseudohyphal growth (Gow *et al.*, 2003; Braun & Johnson, 2000), host defence response to *C. albicans* (Dongari-Bagtzoglou & Kashleva, 2003), and pathogenesis (Gow *et al.*, 2003). Therefore, one can hypothesize that if CUG ambiguity destabilizes the Efg1p, a variety of phenotypes may arise since the protein is multifunctional.



B

C. albicans gene	CUG codons	Total codons
RAS1	1	291
CDC42	0	192
CST20	11	1231
HST7	4	590
CEK1	1	424
CPH1	3	657
CPP1	2	598
GCN4	0	324
GPA2	2	503
GPR1	3	824
CYR1	4	1691
CAP1	2	500
TPK1	2	411
TPK2	4	443
RAD6	0	105
EFG1	1	553
TEC1	4	744
CPH2	0	291
RIM9		
RIM21	5	786
RIM8	6	602
RIM20	5	786
RIM13	10	718
RIM101	2	605
CZF1	4	389
EFH1	6	721
TUP1	0	515
SSN6	6	1086
NRG1	1	311
RFG1	1	602
RBF1	1	528

Figure 2.25. Signal transduction pathways that regulate morphogenesis *in C. albicans*. A) At least four positive (arrowheads) and two negative (bars) pathways control morphological transitions: MAK-kinase pathway (pink), cAMP pathway (blue), Czf1 (green), pH response pathway (orange), Tup1 (red and dark blue) and Rbf1 (red) (Adapted from Berman & Sudbery, 2002). B) panel indicating the length and number of CUG codons in each morphogenesis regulatory gene.

Another interesting example of a gene involved in morphogenesis that contains CUG codons (5 CUGs) is the essential gene *MCM1.* The Mcm1p levels are crucial for determination of cell morphology and reduction of Mcm1p levels leads to constitutive induction of hyphae. Intriguingly, overexpression of *MCM1* also results in enhanced hypha formation (Rottmann *et al.*, 2003). As *MCM1*, the *SSN6* gene, which encodes a polypeptide of 1085 amino acids and

contains six CUG codons (Hwang *et al.*, 2003), functions as a repressor and activator of filamentation depending on the cellular concentrations and also on its interacting partners (Hwang *et al.*, 2003; Rottmann *et al.*, 2003). For example, *SSN6* mediates gene repression of some promoters through interaction with *TUP1* and *NRG1*, however it is not essential for repression of most Tup1p- or Nrg1p-regulated genes in *C. albicans* (Garcia-Sanchez *et al.*, 2005). This may be related to the capacity of acting independently of Tup1p and Nrg1p to regulate expression of some *C. albicans* genes, namely the white-phase specific gene *WH11* (Garcia-Sanchez *et al.*, 2005; Srikantha & Soll, 1993). Since *WH11* transcript levels were significantly elevated in *Δssn6* cells, *SSN6* may be involved in phenotypic instability, through regulation of expression of some phenotypic switching genes (Garcia-Sanchez *et al.*, 2005). This mechanism may also explain the high rate of phenotypic switching found in ambiguous *C. albicans* cells, since Ssn6p should be strongly affected by this manipulation. In particular, the cellular concentration of the Ssn6p should decrease creating a haplo-insuficiency phenotype that may affect expression of phenotypic switching genes.

Morphogenesis regulation as been recently associated with cell cycle control genes (Lazo *et al.*, 2005; Bachewich & Whiteway, 2005; Zheng *et al.*, 2004; Bensen *et al.*, 2002; Bai *et al.*, 2002). In *C. albicans*, the relationship between the G1 phase of the yeast cell cycle and differentiation of hyphae is complex (Bachewich & Whiteway, 2005). Nuclear division and septation occur with similar kinetics in hyphae and in yeast cells, and hyphal growth can be induced at any cell cycle stage (Hazan *et al.*, 2002), but germ tubes show hypha-specific localizations in septation and mitosis (Sudbery *et al.*, 2004; Hazan *et al.*, 2002). More than 60% of cell cycle related genes have CUG codons (Figure 2.1), and consequently, it is likely that CUG ambiguity influences this fundamental biological process. For example, the *CLN1/2* cyclins homologue *HGC1* (1 CUG codon) is required for hyphae formation promoting apical bud extension, however its constitutive expression is not sufficient to induce hyphal growth (Zheng *et al.*, 2004). On the other hand, *CLN3* contains 3 CUG codons and is essential for budding, but negatively regulates the yeast-hyphal

transition. Cln3-depleted cells spontaneously form hyphae, under conditions that favour growth in the yeast form (Lazo *et al.*, 2005). So, it is likely that CUG ambiguity has a major impact on cell cycle, complicating significantly the interpretation of the data on the morphogenesis signalling pathways described above. In other words, the effect of CUG ambiguity in morphogenesis may result from disruption of activation/repression events.

2.4.2. Effect of CUG ambiguity on phenotypic switching

The best known model system of phenotypic switching is the white-opaque transition (Soll, 1992). Interestingly, our results show that phenotypic switching is triggered by CUG ambiguity at high frequency (above 60%), in particular white-opaque and white-opaque-filamentous switching. Previous studies have shown that this transition occurs spontaneously at reasonable high frequency (about 1%) in both directions and that it affects both cellular and colony phenotype. Also, it can be induced in masse in one direction by increasing (favouring white type) or decreasing the temperature (favouring opaque type) (Srikantha *et al.*, 2000; Srikantha & Soll, 1993; Rikkerink *et al.*, 1988; Slutsky *et al.*, 1987). In our study, switching frequency was scored at 30°C without any temperature manipulation that would promote a specific type of morphology and yet we have observed high rates of switching.

Interestingly, we have also observed significantly higher frequency (about 10%) of phenotypic switching for the control cells (pUA12, pUA16 and pUA17) than those previously observed in other laboratories. Phenotypic switching frequencies can range from 10^{-4} to $5x10^{-1}$, depending on whether cells are in low-frequency or high-frequency switching modes (Soll *et al.*, 1993; Soll, 1992; Slutsky *et al.*, 1985). In several switching systems, the smooth colony phenotype represents a low-frequency mode, while the variants represent high-frequency modes (Soll, 2002). Therefore, the plasmid used in our genetic manipulations may some how increase switching frequency of our control clones, however the plasmid used contains two ARSs, which decrease plasmid recombination and increase mitotic stability (Pla *et al.*, 1995). *C. albicans* plasmids are inherently unstable and may integrate randomly in the genome

or replicate autonomously and one can not exclude that this plasmid instability may also contribute to a slight increase in switching frequency of the control clones.

The molecular basis of phenotypic switching in *C. albicans* is not well understood and several mechanisms may be relevant to it, namely genomic rearrangements (Ramsey *et al.*, 1994; Rustchenko-Bulgac & Howard, 1993; Rustchenko-Bulgac *et al.*, 1990), cell signalling, gene expression alterations and also epigenetic mechanisms (Soll *et al.*, 1989). In *C. albicans*, the histone deacetylase *SIR2* gene seems to control phenotypic diversity since *Δsir2* strains have a high frequency of switching (Perez-Martin *et al.*, 1999). However, the genes that are silenced by *SIR2* are not yet known and it is difficult to establish a clear link between CUG ambiguity and *SIR2*. Despite this, other *C. albicans* histone deacetylases are involved in chromatin structure regulation by selective histone deacetylation. For example the *C. albicans* Had1p and Rpd3p play roles in switching suppression, the former suppresses switching in the white-to-opaque direction and the later in both directions, white-to-opaque and opaque-to-white (Srikantha *et al.*, 2001).

Interestingly, morphogenesis and phenotypic switching induced by CUG ambiguity are rather similar to phenotypes displayed by *sir2/sir2* mutants (Perez-Martin *et al.*, 1999) and since *SIR2* gene has 2 CUG codons it is likely that the cellular concentration of Sir2p is significantly decreased in ambiguous cells due to replacement of serine by a leucine. This would not be too surprising since disruption of Sir2p structure would result in lower cellular concentration of this protein, which could create a haplo-insufficiency. In other words, ambiguous CUG decoding may trigger phenotypic diversity through *SIR2*. This hypothesis is further supported by our observation of frequent chromosome rearrangements in ambiguous cells (see chapter 3).

Recently, a molecular and functional link was discovered between switching and mating. The *C. albicans* genome encodes a mating-type locus (MTL) (Hull *et al.*, 2000; Magee & Magee, 2000), and the ability to undergo white-opaque switching is controlled by transcription factors Mtla1p and Mtlα2p (Miller &

Johnson, 2002). These regulators are two homedomain proteins that work together to repress white-opaque switching. Curiously, all the genes belonging to the MTL possess CUG codons with exception of *MTLa1* (discussed in Chapter 3).

2.4.3. Effect of CUG ambiguity on extracellular hydrolases production

CUG ambiguity is likely to play a role in host invasion, proliferation and antigenic variability. *In vivo* expression of *SAP* genes showed that individual members are differentially regulated and expressed at different stages, in different clinical forms of candidiasis *in vivo* (Naglik *et al.*, 2003b). Thus, *SAP1* (1 CUG codon), *SAP7* (3 CUG codon) and *SAP8* (2 CUG codon) are predominantly expressed in oral and vaginal infected patients while *SAP2* and *SAP4-6* (O CUG codons) are expressed in *C. albicans* carriers (Schaller *et al.*, 2005; Naglik *et al.*, 2003b; de Bernardis *et al.*, 1999; Naglik *et al.*, 1999). In cutaneous candidiasis similar expression profile was described, *SAP1* and *SAP8* are the most abundant transcripts (Schaller *et al.*, 1999). In this type of *C. albicans* infection Sap1p is in the limelight increasing the adherence and cavitation of skin (Kvaal *et al.*, 1999). Therefore, the increased activity of SAPs induced by CUG ambiguity may have important consequences for virulence.

Furthermore, proteinases production is also related with other virulence factors, namely morphogenesis and phenotypic switching. *SAP4* and *SAP6* expression is linked to hypha formation (Schroppel *et al.*, 2000), since *C. albicans tec1/tec1* clones that fail to produce hyphal cells *in vitro* do not express *SAP4* and *SAP6* (Schweizer *et al.*, 2000). Conversely, hyperfilamentous *C. albicans* strains harbouring deletion in the *CPP1* gene express high levels of *SAP4* and *SAP6* (Schroppel *et al.*, 2000). Therefore, the high amount of proteinase activity found in the ambiguous pUA13, pUA14 and pUA15 transformants, where morphogenesis and hypha formation were induced at high frequency may be due to an increase of *SAP4* and *SAP6* expression, but this remains to be determined experimentally. Phenotypic

switching also affects proteinase production (Soll, 1992). For example, *SAP1* expression increases during switching and is responsible for the higher extracellular proteolytic activity observed in these cells (Morrow *et al.*, 1992). Also, *SAP8* transcripts are present in opaque cells and absent in white cells (Hube *et al.*, 1997). Considering that CUG ambiguity induces switching at high level, it is likely that *SAP1* and *SAP8* expression is increased in ambiguous cells, especially in ambiguous opaque-phase cells.

2.4.4. Effect of CUG ambiguity on adhesion

Remarkably, the adhesion phenotypes obtained with CUG ambiguity are similar to those induced in *S. cerevisiae* expressing *ALS1* (5 CUG codons) (Fu *et al.*, 1998), *ALS5* (12 CUG codons) (Rauceo *et al.*, 2004; Gaur *et al.*, 2002) or *ALA1* (2 CUG codons) (Gaur *et al.*, 2002; Gaur *et al.*, 1999; Gaur & Klotz, 1997). It is not clear how expression of these *C. albicans* genes in *S. cerevisiae* increase adhesion so dramatically but it would be interesting to mutate the CUG codons present in these genes to other serine codons (to ensure correct synthesis of the recombinant proteins in *S. cerevisiae*) and determine whether these wild type proteins would also increase adhesion. Since adhesion is a very important virulence factor and leucine is a hydrophobic amino acid (while serine is polar), such experiment could either exclude or establish a direct connection between CUG ambiguity and adhesion mediated by *ALS* genes.

Switching affects adhesion since white cells are more adhesive to buccal epithelial cells than opaque cells (Vargas *et al.*, 1994). However, opaque cells are more hydrophobic than white cells (Kennedy *et al.*, 1988). Since CUG ambiguity induces morphogenesis and phenotypic switching, cell populations of *C. albicans* transformed with pUA13, pUA14 and pUA15 are composed by opaque-phase cells and filamentous forms, thus it is likely that adhesion and hydrophobicity are increased in these cells. Additionally, increased extracellular hydrolases activity also leads to increased adherence to buccal epithelial cells (Naglik *et al.*, 2003a; Abu-Elteen *et al.*, 2001; Ghannoum,

2000; Leidich *et al.*, 1998; Ghannoum & Abu, 1986), which establishes a link between increased adhesion and the higher hydrolytic activity found in ambiguous cells.

2.5. Conclusions

Previous studies revelled that genetic code alterations and mistranslation originate phenotypic variability (True *et al.*, 2004; Queitsch *et al.*, 2002; Rutherford & Lindquist, 1998). Despite decrease in fitness, organisms harbouring statistical or mutated proteomes have an enhanced adaptive capacity (Pezo *et al.*, 2004; Santos *et al.*, 1999). The heterologous expression of *S. cerevisiae* Leu-tRNA$_{UAG/CAG}$ and Ser-tRNA$_{UAA}$ in *C. albicans* CAI4 revelled that the later tolerates genetic code ambiguity, but this may be dependent on codon usage. Here we show that CUG codon, through its Leu/Ser ambiguous decoding, evolve to be a generator of phenotypic diversity modulating *C. albicans* evolution and biology.

3. Effect of CUG ambiguity on the stability of the *C. albicans* genome

3.1. Introduction

Translational errors occur at a frequency of about $3x10^{-4}$ during translation (LOFTFIELD, 1963), but this may be as high as $3x10^{-3}$ (Parker, 1989; Bouadloun *et al.*, 1983), which makes translation inherently error-prone compared with DNA replication (10^{-9} errors per bp per replication cycle). Mistranslation can be increased due to transcriptional errors, mRNA damage and modification, tRNAs and ribosome mutations, tRNA mischarging and exposure to certain antibiotics (Dorazi *et al.*, 2002). Under one of these conditions, an increase in translational error leads to translational stress-induced mutagenesis (TSM). Interestingly, the TSM phenotype is triggered by ribosomal (Balashov & Humayun, 2003) and genetic code ambiguity through expression of mutant tRNAs (Al Mamun *et al.*, 2002; Dorazi *et al.*, 2002). In *E. coli*, mutation in a $tRNA_{GCC/U}^{Gly}$ leads to a strong mutator phenotype (Murphy & Humayun, 1997; Slupska *et al.*, 1996), a single base substitution in the tRNA gene alters the anticodon in a way that the glycine mutant tRNA can decode aspartate codons (GAC/U) as glycine. Other studies indicate that general mistranslation is sufficient to induce a mutator phenotype, possible through enhanced protein misfolding and turnover, which triggers the expression of error-prone DNA polymerases (Dorazi *et al.*, 2002). How mistranslation induces mutagenesis is not yet clear, however two hypotheses have been discussed. In *mut*A cells, low level of Asp-to-Gly mistranslation creates a small fraction of inactive epsilon protein (proofreading subunit of DNA polymerase III holoenzyme), which when recruited into a functional holoenzyme leads to a transient mutator phenotype (Slupska *et al.*, 1998; Slupska *et al.*, 1996). Alternatively, mistranslation increases protein misfolding and turnover and may create proteins with novel functions (gain-of-function mutant proteins). Some of these may somehow decrease the replication fidelity of DNA polymerase III leading to a mutator phenotype (Al Mamun *et al.*, 2002; Balashov & Humayun, 2002; Dorazi *et al.*, 2002; Ren *et al.*, 2000; Al Mamun *et al.*, 1999; Ren *et al.*, 1999).

Besides the TSM phenotype there are further experiments demonstrating that codon ambiguity, and ultimately mistranslation, results in increased mutation

frequency. An *E.coli* auxotroph selected to grow continuously on high proportion of 4fW (4-flurotryptophan) accumulated 5 mutations in three genes responsible for tryptophan incorporation (Bacher & Ellington, 2001). Also in *E.coli,* mutants selected to incorporate cysteine at a valine codon, accumulated mutations in the editing domain of valyl-tRNA synthetase (Doring *et al.*, 2001). In bacteriophage Qβ a relatively small number of mutations (7) allowed unnatural amino acids (various tryptophan analogues) to be functionally incorporated into a highly interdependent set of proteins (Bacher *et al.*, 2003).

Ambiguous decoding of 13,074 CUG codons is, therefore, likely to induce hypermutagenesis in *C. albicans* because these CUGs are spread over 66% of the genome and each gene can give raise to an array of proteins (Gomes *et al.*, unpublished). In other words, most *C. albicans* proteins are not true chemical entities and the proteome has a statistical nature. In our case, heterologous expression of *S. cerevisiae* leucine tRNAs, encoded by plasmids pUA13-15, increased CUG ambiguity significantly and it is likely that such ambiguity may also trigger a mutator phenotype or destabilize the genome in some way. That CUG ambiguity can destabilize the genome is in line with our observations that CUG ambiguity triggers morphological variation, since karyotype rearrangements are also related with phenotypic diversity (Rustchenko-Bulgac, 1991; Rustchenko-Bulgac *et al.*, 1990; Suzuki *et al.*, 1989).

Interestingly, *C. albicans* genome instability is sometimes associated with increased ploidy (Storchova & Pellman, 2004). *C. albicans* is diploid yeast and it is frequently found as a polyploidy in nature. However, polyploidy yeast cells have significantly increased rates of chromosome loss and recombination (Mayer & Aguilera, 1990). For example, polyploidy fission yeast undergoes chromosome missegregation at high rate (Molnar & Sipiczki, 1993). More importantly, genetic code ambiguity in *S. cerevisiae,* induced through expression of *C. albicans* $tRNA_{CAG}^{Ser}$ (Santos *et al.*, 1999), promoted genome instability and genome duplication (Silva *et al.*, unpublished). Such tetraploidy in *S. cerevisiae* may be a mechanism of protection from genotoxic damage by

increasing gene copy number (Otto & Whitton, 2000). These considerations prompted us to investigate whether CUG ambiguity would destabilize the *C. albicans* genome in some way. In this chapter we describe a number of studies that confirm this hypothesis.

3.2. Material and Methods

3.2.1. Karyotype analysis

3.2.1.1. CHEF (contour-clamped homogenous electrical field)-plug preparation

DNA preparation for pulse field gel electrophoresis (PFGE) was essentially carried out as described by Chu *et al.* (1993). Briefly, a maximum of 2ml overnight culture grown in MM-uri was harvested at 15,000 *g* (Biofuge pico, Heraeus), at room temperature, and washed once in 1 ml of 50 mM EDTA (pH 7.5). Cells were resuspended in a solution of 160 µl of 50 mM Tris-HCl (pH 7.5) and 60 µl of 2.5 mg/ml Zymolyase 100T (Seikagaku Corp.) solution and 600 µl of 1.5% of low-melting agarose (LM-MP, Boehringer Mannheim) in 0.125 M EDTA (pH 7.5), kept at 50°C. The mixture was then transferred to a mold using 1ml syringes. Upon agarose solidification, the syringe body was broken and the agarose block was cut in slices and put in 15 ml Falcon tubes. The agarose blocks were incubated in 3 ml of LET buffer [0.5 M EDTA, 10 mM Tris-HCl pH 7.5, 1% (v/v) 2-mercaptoethanol] at 37°C for 1 day. The LET buffer was replaced with 3 ml of NDS [0.5 M EDTA, 10 mM Tris-HCl pH 7.5, 1% (w/v) N-laurosyl sarcosine, 0.2 mg/ml of proteinase K (Boehringer Mannheim)] and incubated at 50°C for 2 days. The plugs were washed twice with 0.5 M EDTA (pH 9.0) and stored at 4°C.

3.2.1.2. Pulse-field gel electrophoresis (PFGE) conditions

Chromosomes were separated in 0.6% agarose gels (Pulsed Field Certified Agarose Bio-Rad), at 14°C, in 0.5X TBE (0.045 M Tris, 0.045 M Boric acid, 1 mM EDTA) using the Bio-Rad CHEF DRII system. The separation conditions

were 120 to 300 s for 24 h, 420 to 900 s for 48 h at 80 V. In order to test for the presence of small chromosome fragments, another running condition was used: 60 to 120 s for 24 h, at 6 v/cm, in a 120° angle. For this, 0.9% agarose gels in 0.5xTBE were used. After electrophoresis, DNA was stained in 0.1mg/ml ethidium bromide solution for 1 h and then washed in MilliQ H_2O for 20 m to remove the excess of ethidium bromide. Gels images were acquired using a Molecular Imager FX Pro Plus Multilmager System (Bio-Rad), using the Quantity One software (Bio-Rad).

3.2.2. Fluorescent Activated Cell Sorter (FACS) analysis

3.2.2.1. DNA content analysis

FACS analysis of *C. albicans* DNA was performed as described by Fortuna *et al.* (2000). Briefly, cells from an overnight culture in MM-uri, at 30°C, were harvested by centrifugation at 10,000 *g*, washed twice in ice-cold MilliQ H_2O, counted using a hemacytometer and their concentrations adjusted to $1x10^7$ cells/ml in MilliQ H_2O. After centrifugation, cells were fixed overnight, at 4ºC, in 1 ml 70% (v/v) ethanol. The staining protocol involved washing the cell suspension with 50 mM sodium citrate buffer (pH 7.5) and resuspension in 750 μl of the same buffer. Then, cells were treated with 250 μg RNase A (Sigma) per $1x10^7$ yeast cells, for 1 h, at 37°C, followed by 1000 μg proteinase K (Roche) during 1 h at the same temperature. Cells were overnight stained with 20 μl SYBR Green I (Molecular Probes) working solution (1/10 diluted in Tris-EDTA buffer pH 8.0), at 6-8ºC and protected from light. Finally, Triton X-100 was added at final concentration of 0.25% (v/v). Samples were put on ice and sonicated before analyzes by flow cytometry. This was carried out using a Beckman Coulter-coulter Epics XL flow cytometer, using excitation at 488 nm and fluorescence emission collection at 525 nm.

3.2.2.2. Comparative analysis of cell size

Analyse of cell size was carried out by flow cytometry, as described by Pina-Vaz *et al.* (2001), with some adaptations. Cells were harvested in stationary phase (O.D.~4.0) after an overnight grown in MM-uri at 30ºC with 180 rpm, washed in PBS buffer (pH 7.0) and resuspended in 1 ml of the same buffer supplemented with 2% (w/v) glucose. Cells were then sonicated, counted in a Newbauer chamber, diluted to a density of $1x10^6$ cells/ml and analysed by flow cytometry (Beckman Coulter-coulter Epics XL).

3.2.3. Fluorescence staining

Cells were fixed with 70% (v/v) ethanol, washed once with distilled water, and stained with 4', 6'-diamidino-2-phenylindole (DAPI), at a final concentration of 1 µg/ml, for 15 m at 30°C. Staining solution was removed and cells were resuspended in PBS (Legrand *et al.*, 2004). Fluorescently labelled cells were visualized with using a Zeiss Axioplan 2 microscope.

3.3. Results

3.3.1. Ambiguous CUG decoding induces karyotype variation

C. albicans has an unstable karyotype characterized by frequent chromosome rearrangements, in particular of the chromosome R (Lasker *et al.*, 1989; Magee & Magee, 1987; Snell *et al.*, 1987). The karyotype variability observed by pulse field gel electrophoresis is a result of translocation events between various chromosomes. Apparently, chromosome 3 is resistant to such rearrangements (Chindamporn *et al.*, 1998). *C. albicans* transformed with plasmids pUA13, pUA14 and pUA15 showed high karyotype heterogeneity (Figure 3.1). Karyotype alterations observed in these clones were diverse but apparently affected the larger chromosomes, namely 1 and R. Control pUA12 cells showed normal karyotypes (Figure 3.1). Thus, ambiguous CUG decoding induced genome destabilization which may occured through several processes, namely translocation, deletion, amplification, chromosome loss or gain (Chu *et*

al., 1993). These karyotype rearrangements are similar to those observed in *C. albicans* clinical isolates.

Interestingly, most of the rearrangements observed involved the chromosome R, which contains the rDNA cistrons and is organized in tandem repeats. The latter can differ in the number of copies between the two homologs (Rustchenko *et al.*, 1994; Iwaguchi *et al.*, 1992) and in sister-chromatid exchange upon cultivation. Thus variation in the size between the two homologs of the chromosome R during serial cultivation (Rustchenko *et al.*, 1993; Iwaguchi *et al.*, 1992) is similar to that observed in the ambiguous cell lines (Figure 3.1).

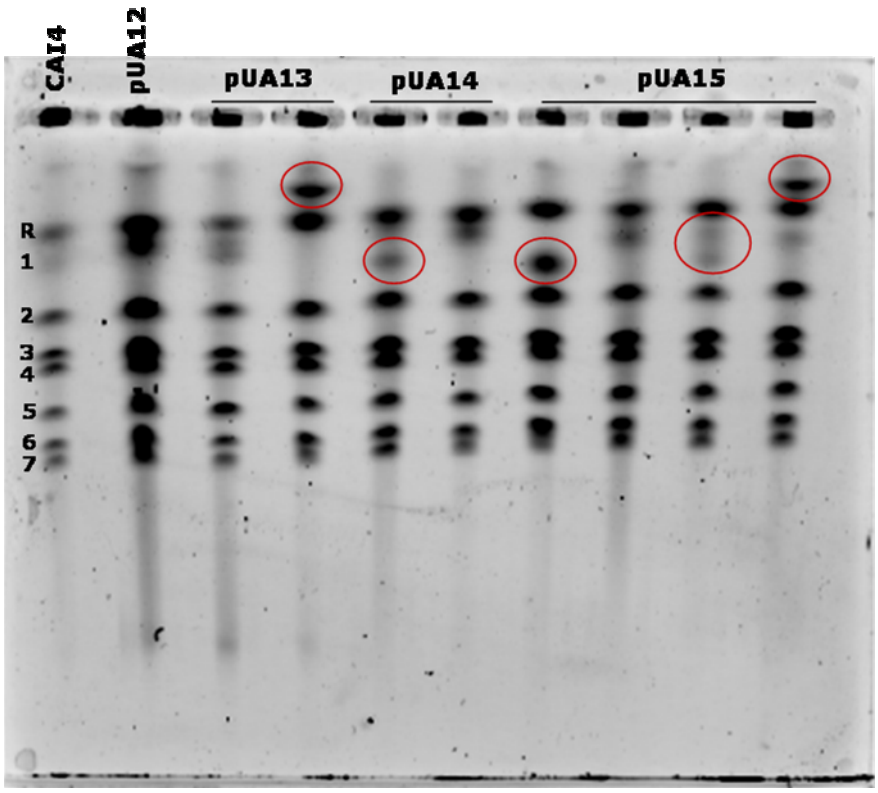


Figure 3.1. Karyotype rearrangements induced by ambiguous CUG decoding. Electrophoretic karyotypes of control clones, CAI4 untransformed and pUA12 transformed cells, and CUG ambiguous cell lines pUA13 (2 clones), pUA14 (2 clones) and pUA15 (4 clones). Chromosomes were separated by PFGE on 0.6% agarose gels under the following conditions: 120-300 s for 24 h at 80 V, then 420-900 s for 48 h at 80 V. The numbers 1-7 and R in lane 1 indicate *C. albicans* identity chromosomes. Circles highlight chromosomal rearrangements.

The instability of chromosome R has been associated with phenotypic switching, in particular with white-opaque transition (Ramsey *et al.*, 1994). In the ambiguous pUA13-15 cell lines a similar association can be established, since high phenotypic switching found in these mutants strains (pUA13, pUA14 and pUA15) correlates with the chromosomal rearrangements found in the R-chromosome. Thus, karyotype re-arrangements observed in CUG ambiguous clones fit with their high phenotypic diversity (Rustchenko-Bulgac & Howard, 1993; Rustchenko-Bulgac *et al.*, 1990; Suzuki *et al.*, 1989). In other words, some of the morphological variation observed in pUA13-15 clones may be a direct consequence of R-chromosome rearrangements.

3.3.2. CUG ambiguous decoding increases ploidy and cell size

C. albicans usually exists as diploid yeast (Scherer & Magee, 1990) however some clones produce a proportion of the cell population with a higher ploidy (Suzuki *et al.*, 1989; Suzuki *et al.*, 1986). Transformation of *C. albicans* CAI4 with pUA13, pUA14 and pUA15 plasmids also increased DNA content (Figures 3.2 and 3.3). In some cases, cells with 64N were observed. This phenotype of mixed ploidy states (Iwaguchi *et al.*, 2000) is characterized by a ploidy shift between two different ploidy states: diploid (or lower ploidy state) cells enter G2 arrest and then bypass the M phase, resulting in an up shift of ploidy; tetraploid (or higher ploidy state) cells engage in reductional nuclear division during the down shift of the ploidy state, producing diploid (or lower ploidy state) daughter cells (Iwaguchi *et al.*, 2000; Suzuki *et al.*, 1989; Suzuki *et al.*, 1986).

One of the main characteristics of the *C. albicans* transformed with pUA13, pUA14 and pUA15 plasmids was ploidy variability that ranged from 2N up to 64N (Figures 3.2 and 3.3). The ploidy variability between clones was very high and within the same clone ploidy-shift was higher than that previously described (Iwaguchi *et al.*, 2000; Suzuki *et al.*, 1994a). Interestingly, the highly ambiguous pUA15 clones showed high ploidy variation, which provides strong evidence that there is an indirect link between CUG ambiguity and genome destabilization.

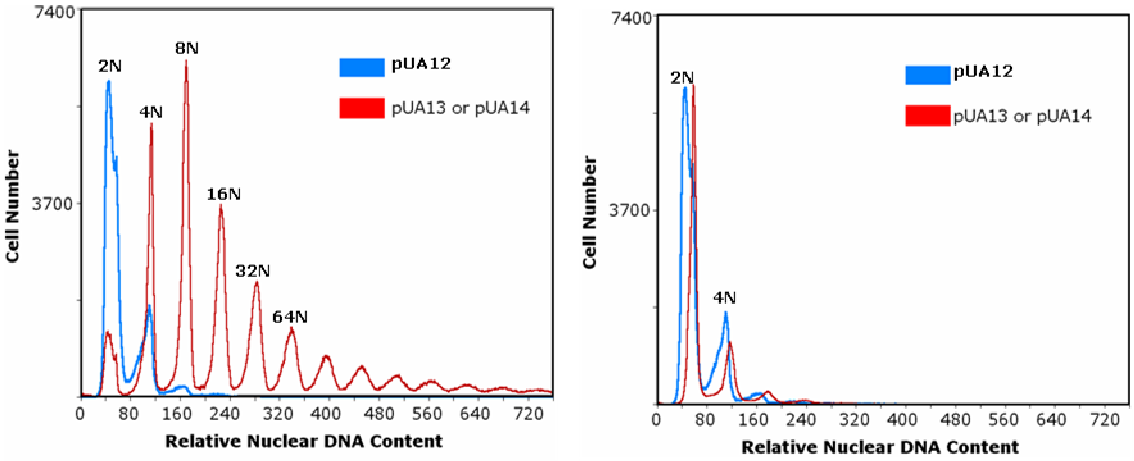


Figure 3.2. FACS analysis of the DNA content of *C. albicans* transformed with control pUA12 plasmid and with pUA13 and pUA14 plasmids carrying leucine tRNAs. CUG ambiguity increased DNA content per cell (left panel) or induced aneuploidy (right panel). Strains were analysed by SYRB Green I fluorescence-activated cell scan for DNA fluorescence.

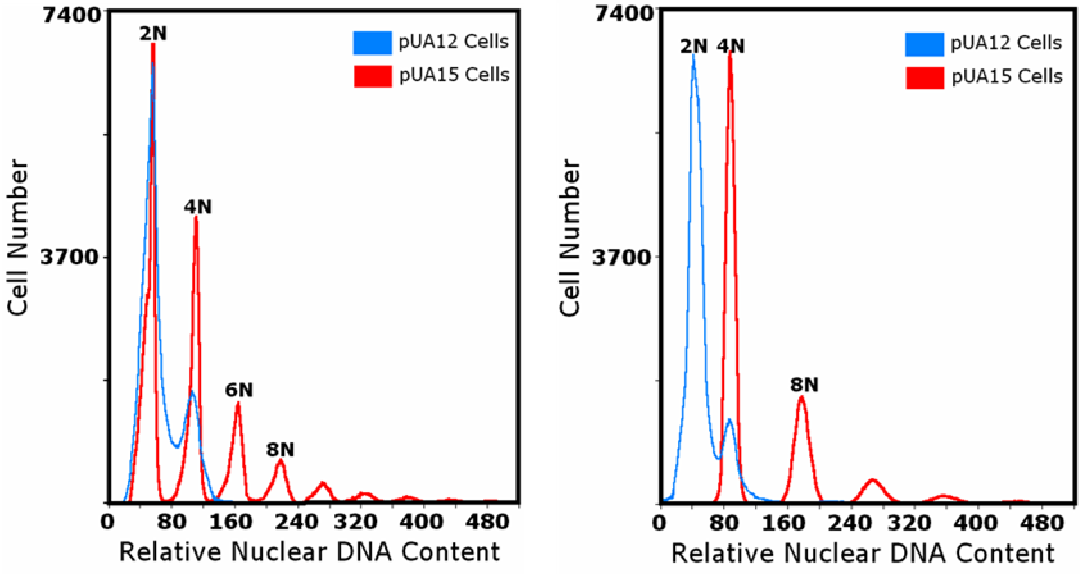


Figure 3.3. FACS analysis of the DNA content of the pUA12 control and pUA15 ambiguous clones. In pUA15 clones ploidy variability was predominant, some maintained stable tetraploidy (right panel), while other clones had a heterogeneous cell population (left panel). Cells were analysed by SYRB Green I fluorescence-activated cell scan for DNA fluorescence.

Since there is a link between white-opaque transition and mating (Miller & Johnson, 2002), and since CUG ambiguity induced production of opaque-phase cells at high frequency, we hypothesized that CUG ambiguous cells also mate at high frequency. This hypothesis was confirmed since ploidy histograms of

pUA15 clones showed a very high level of tetraploid cells (mating products) (Figure 3.3 left panel). Additionally, pUA15 cells stained with DAPI showed that a significant proportion of these cells are bigger than wild type cells and contain two or more nuclei (Figure 3.4). Furthermore, conjugation tubes were also observed in ambiguous cells, which strongly supported that mating occurs. Surprisingly, amplification of *MTLa1* and *OPBa* genes belonging to the MTLa proved very difficult even in the pUA12 control strain. Conversely, detection of MTLα genes (*MTLα1*, *MTLα2* and *OBPα*) was rather easy to achieve (data not shown). This may be due to aneuploidy of the chromosome 5 or lack of segment containing MTL locus as consequence of molecular manipulations (Selmecki *et al.*, 2005).

After mating, tetraploid *C. albicans* cells lose half of their chromosomes through an as yet unknown molecular mechanism (Legrand *et al.*, 2004; Bennett & Johnson, 2003; Hull *et al.*, 2000; Magee & Magee, 2000) and return to the diploid state. Interestingly, in some pUA15 clones and after several rounds of selection, the cell population maintained a stable tetraploid state (Figure 3.3). A similar phenomenon was also observed in *S. cerevisiae* in response to genetic code ambiguity (Rocha, personal communication). In other clones, ambiguity created tetraploid cells, which after several rounds of selection, became diploid or aneuploid (Rocha, personal communication). *C. albicans* has a remarkable tolerance to aneuploidy (gain or loss of whole chromosomes or chromosome fragments), which appears to provide a selective advantage under specific stress conditions (Selmecki *et al.*, 2005). For example, one copy of chromosome 5 is often lost in cells forced to growing in sorbose as sole carbon source (Janbon *et al.*, 1999; Janbon *et al.*, 1998). After growth in rich media, chromosome 5 reduplicates creating a homozygotic state that confers a growth advantage in that medium.

When observed by optical microscopy, ambiguous cell lines contained cells with variable sizes and when analysed by epifluorescence microscopy the DNA was concentrated in one or more nuclei or micro-nuclei distributed over most of the cytoplasm (Figure 3.4). Since poliploidy increase correlates with cell size

increase (Storchova & Pellman, 2004), in order to quantify the diversity of cells present in pUA15 cultures, cells were further analysed by FACS and their size difference was quantified by comparing relative size values between pUA12 and pUA15 clones. White and opaque cells from pUA12 and pUA15 clones were also compared. Ambiguous cell lines had an increase in cell size ranging from 17% to 24% compared with control pUA12 cells (Table 3.1). Therefore, CUG ambiguity increases ploidy and cell size.

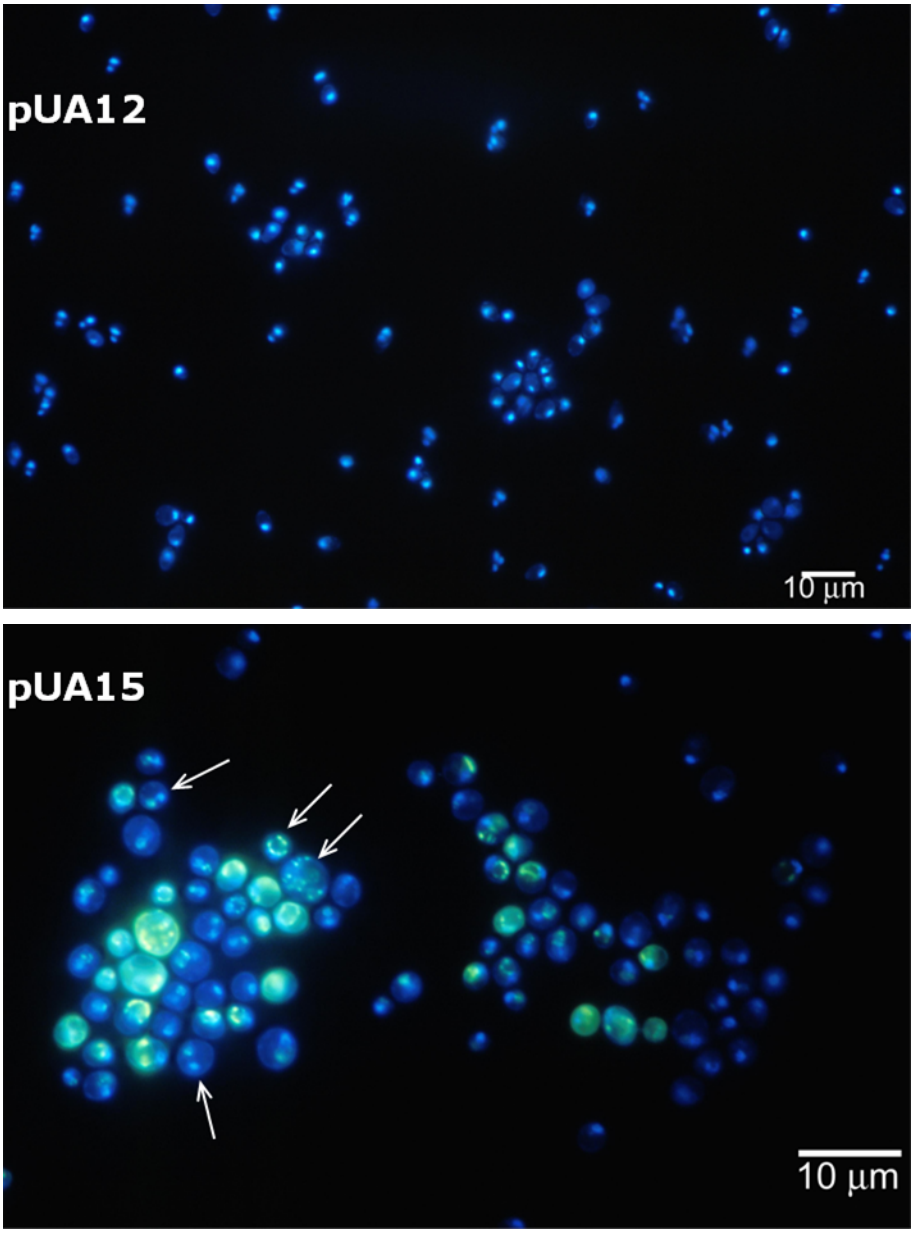


Figure 3.4. CUG ambiguity increases DNA content. DIC images of *C. albicans* cells transformed with plasmids pUA12 and pUA15 stained with DAPI. pUA12 control cells show a single nucleus while of pUA15 cells showed one or more nuclei or even micronuclei (arrow pointed).

Table 3.1. CUG ambiguous decoding increases cell size. Comparative analysis of cell size between pUA12 and pUA15 clones. This analysis done by FACS discriminates between white and opaque cells.

Strain	Relative size	pUA12/pUA15 (%)
pUA12 White	53.9	--------
pUA12 Opaque	59.6	--------
pUA15 White	62.9	17
pUA15 Opaque	74.2	24

3.4. Discussion

Approximately 40% of *C. albicans* isolates show significant variations in their electrophoretic karyotype (Asakura *et al.*, 1991). Karyotype variation among the clinical isolates seems to confer genetic variation since several groups have reported a correlation between karyotype diversity and colony morphology (Ramsey *et al.*, 1994; Rustchenko-Bulgac & Howard, 1993; Rustchenko-Bulgac *et al.*, 1990; Suzuki *et al.*, 1989). Our study further supports this relationship between karyotype rearrangements and phenotypic diversity, since both events were observed in ambiguous CUG decoding cells. In particular, the association between white-opaque transition and chromosome R variation (Ramsey *et al.*, 1994) was strong in pUA13-15 clones. Comparison of karyotypes of control (CAI4 and pUA12 strains) against pUA13, pUA14 and pUA15 transformed cells, showed clearly that only the larger chromosomes, in particular chromosome R, were responsible for karyotype variation induced by CUG ambiguity. Some of these rearrangements found in the R-chromosome corresponded to variation in size between the two chromosome homologs (Iwaguchi *et al.*, 1992) and chromosome translocations (Chu *et al.*, 1993).

In *sir2/sir2* mutants, who show similar phenotypes to those obtained by pUA13-15 transformants, high frequency karyotype changes resulted in appearance of "minichromosomes" (composed by rDNA) that arose by

recombination between rRNA genes (Perez-Martin *et al.*, 1999). It is difficult to establish a direct link between ambiguity and the chromosome R rearrangements however it is very interesting that mRNA decoding ambiguity induces rearrangements in the chromosome that contains the rRNA cistrons. This suggests that rRNA synthesis and ribosome assembly may be affected and it is likely that CUG ambiguity has a more profound effect on the translational machinery than originally thought.

The increase in ploidy induced by CUG ambiguity could be a stress-like response protecting cells against genotoxic and metabolic stress (Storchova & Pellman, 2004). *C. albicans* has a single copy of the $tRNA_{CAG}$ gene. When transformed with a low copy plasmid pUA13-15, encoding *S. cerevisiae* leucine tRNA, a pool of Leu- and Ser-tRNAs able to decode the CUG codon in *C. albicans* cytoplasm elevates the natural CUG ambiguity, which is toxic due to disruption of protein structure on a proteome wide scale. Poliploidy attenuates these toxic effects since it has the potential to increase the copy number of the $tRNA_{CAG}^{Ser}$ gene by increasing chromosome number and keeping plasmid copy number low. Since tRNAs are transcribed by RNA polymerase III and the promoters are intragenic, gene copy number has a direct impact on tRNA expression levels. Therefore, ploidy increase may provide a mechanism to increase the cellular level of $tRNA_{CAG}^{Ser}$, while maintaining the level of the heterologous leucine tRNAs expressed from plasmids pUA13, pUA14 and pUA15.

Increased ploidy induced by ambiguous CUG may have evolutionary implications, since increased ploidy is favoured when deleterious alleles are partially recessive; individuals with more than one allele per locus mask these deleterious effects (Otto & Goldstein, 1992; Perrot *et al.*, 1991). Conversely, lower ploidy is favoured when beneficial alleles are partially recessive as the beneficial effects of mutations are fully revealed in haploids (Orr & Otto, 1994). This is important because codon ambiguity in *E. coli* induces hypermutagenesis due to synthesis of mutant DNA polymerases (Al Mamun *et al.*, 2002; Balashov & Humayun, 2002; Dorazi *et al.*, 2002; Ren *et al.*, 2000; Al Mamun *et al.*, 1999; Ren *et al.*, 1999). Interestingly, the *C. albicans POL3*

(Polymerase III) gene which encodes for the large subunit of the DNA polymerase III contains 1 CUG codon. Thus, CUG ambiguity and mistranslation could also originate a mutant DNA polymerase with potential to generate mutagenesis. Furthermore, one may hypothesized that the unusual high level of heterozygosity, including more than 55,700 single-nucleotide polymorphisms found in *C. albicans* genome (Forche *et al.*, 2004; Jones *et al.*, 2004) could partially be due to mistranslation-induced mutagenesis.

In *S. cerevisiae,* ploidy regulates invasiveness (Galitski *et al.*, 1999), which is a developmental response important for fungal virulence that involves a change in budding pattern and formation of chains of yeast cells that invade agar substrate (Roberts & Fink, 1994), through *FLO11* (also called *MUC1* mucin-like protein) expression. The ploidy repression of *FLO11* gene was reflected in invasive growth. As ploidy increased, the expression of *FLO11* and invasiveness decreased. The connection between ploidy and morphology is due to ploidy-regulated genes, such as *FLO11* and G1 cyclins (Galitski *et al.*, 1999).

In *S. cerevisiae*, the increase in ploidy was associated with mutations in genes essential for the proper function of the spindle pole body (Vallen *et al.*, 1992; Winey *et al.*, 1991; Snyder & Davis, 1988; Rose & Fink, 1987; Thomas & Botstein, 1986; Schild *et al.*, 1981). Thus genes involved in cell cycle and DNA repair and replication are considered important for the maintenance of genomic stability (Chan & Botstein, 1993; Bender & Pringle, 1991; Palmer *et al.*, 1990). In *C. albicans*, more than 60% of the genes related to the cell cycle have one or more CUG codons, which could be an indicator of defective or even abortive cell cycle.

3.5. Conclusions

CUG ambiguity and the consequent production of a mutant proteome induced karyotype rearrangements accompanied by ploidy-shift, characterized by poliploidy and aneuploid states. Ploidy shift is associated with chromosomal rearrangements (Selmecki *et al.*, 2005; Iwaguchi *et al.*, 2000; Suzuki *et al.*,

1994) which, in turn, is related with colony morphology variation (Rustchenko-Bulgac & Howard, 1993; Rustchenko-Bulgac *et al.*, 1990; Suzuki *et al.*, 1989) and even with homozygoty at chromosome 5 (Legrand *et al.*, 2004). The data described in this chapter support that CUG ambiguity might trigger a cellular condition that results in an alteration in genome base composition and, via translational stress-induced mutagenesis, leads to an increased rate of genome evolution.

4. Effect of CUG ambiguity on cell biology

4.1. Introduction

The effect of genetic code ambiguity on *C. albicans* biology is difficult to predict since it affects the entire proteome. In other words, CUG ambiguity creates a statistical proteome, which is inherently unstable and should have pleiotropic biological effects. In this case, such effects should be exacerbated since serine and leucine have different biochemical proprieties: serine is a polar amino acid, which can be phosphorylated and glycosylated, while leucine is hydrophobic.

As shown in other organisms, genetic code ambiguity normally reduces growth rate (Bacher *et al.*, 2005; Pezo *et al.*, 2004; Bacher & Ellington, 2001; Santos *et al.*, 1996). This may be due to destabilization of the proteome and the accumulation/degradation of unfunctional misfolded proteins. Proteins destined for secretion, the plasma membrane or cell surface are translocated into the endoplasmatic reticulum (ER), in an unfolded state (Kostova & Wolf, 2003). During translocation, proteins are modified and folded to acquire their biological active conformation (Haigh & Johnson, 2002) and undergo a quality control procedure that discriminates between properly folded proteins and terminally misfolded species, as well as, unassembled protein subunits (Ellgaard *et al.*, 1999). Misfolded polypetides and orphan subunits are subsequently subjected to ER-associated degradation (ERAD). The ERAD process requires retrotranslation of the misfolded proteins across the ER membrane into the cytoplasm and subsequent degradation by the 26S proteasome (Brodsky & McCracken, 1999; Plemper & Wolf, 1999; Sommer & Wolf, 1997). Therefore, CUG ambiguity may interfere with various cellular functions and result in accumulation of aberrant or misfolded proteins. We have decided to investigate this hypothesis by studying the activity of the proteasome and analysing cell ultrastructure by transmission electronic microscopy (TEM). Considering that large scale synthesis of aberrant proteins is toxic and constitutes a form of stress, it is likely that cells respond to CUG ambiguity by triggering expression of stress proteins and accumulation of stress protective metabolites (Parrou *et al.*, 1997; Arguelles, 1997; Ruis & Schuller, 1995; Parsell *et al.*, 1994). This prompted us to investigate whether

C. albicans cells transformed with pUA13-15 plasmids accumulated glycogen and trehalose.

The other important cellular perturbation induced by CUG ambiguity is related to the cell wall. About 50% of the cell wall genes have CUG codons, therefore cell wall proteome should be strongly affected by CUG ambiguity. Since the *C. albicans* cell wall is the primary contact point with immune cells and cell wall proteins are highly modified at serine residues, CUG ambiguity may have significant impact on cell wall structure and antigenic variability. This is a rather interesting hypothesis because antigenic variability is an important mechanism that pathogenic and commensal organisms use to circumvent the immune system. For example, *Bacteroides fragilis*, which colonizes the intestinal tract, modulates its surface and alters surface antigens by producing arrays of capsular polysaccharides. Surface diversity, generated by arrays of distinct surface polysaccharide combinations allows maintaining long-term commensal relationship in the human colon (Krinos *et al.*, 2001). In order to evaluate the impact of CUG ambiguity on the cell wall we have used proteomics methodologies.

4.2. Material and Methods

4.2.1. Growth curves

C. albicans growth rates were studied by analysing the growth of two clones of pUA12, pUA13, pUA14 and pUA15 transformants. 50 ml of MM-uri were inoculated with 50 µl of stationary-phase culture in a 250 ml Erlenmeyer. Cultures were allowed to grow at 30°C, 180 rpm of agitation, and culture density was determined at $O.D._{600\ nm}$ (Beckman Du®530 Life Sciences UV/Vis spectrophotometer) and plotted versus period of incubation (h) in a logarithmic scale. Specific growth rate (µm) was determined following equation:

$$\ln O.D._t = \ln O.D._{t0} + \mu m t$$

$O.D._t$ and $O.D._{t0}$ corresponds to the O.D. measured in time t and t_0, respectively (Marison, 1988).

4.2.2. Transmission Electronic microscopy (TEM)

Samples were prepared as described by Robin Wright (2000). Briefly, cells grown overnight in MM-uri at 30°C were fixed in 2.5% (w/v) glutaraldehyde in 0.1 M cacodylate buffer for 2 h. Before and after a second fixation in 1% osmium tetroxide in 0.1 M cacodylate buffer for 1 h, cells were washed three times in 0.1 M cacodylate buffer. After completion of fixation, cells were dehydrated by incubation successively in 70%, 95% and 100% (v/v) of acetone solutions. Following dehydration, cells were rinsed twice with 100% propylene oxide and then incubated in an Epon resin:propylene oxide mixture for 1 h. Soaked samples were transferred to embedding capsules containing Epon resin and hardened at 70°C for 48 h. After thin sectoring of the block, serial thin sections were put in grids that were dipped in a solution of 2% uranil acetate for 20 m. After one wash with double-distilled water, samples were placed onto filter paper and allowed to dry. Finally, grids were dipped in plumb citrate for 2 m, washed with double-distilled water, and allowed to dry onto filter paper. Samples were observed and photographed in a JEOL 100SX electron microscope.

4.2.3. Flow cytometry analysis

4.2.3.1. Granularity analysis

Cells grown in MM-uri, at 30°C, overnight, with agitation (180 rpm) until stationary phase (OD_{600nm} ~ 4), were harvested at 10,000 *g*, for 5 m, at room temperature, and washed with PBS. Cells were resuspended in 1 ml PBS supplemented with 2% (w/v) glucose and sonicated to disperse cell aggregates. For each sample, the number of cells/ml was determined by counting cells using the Newbauer chamber and the appropriated dilution was made to obtain a concentration of $1x10^6$ cells/ml. Flow cytometry analysis were performed in Beckman Coulter-coulter Epics XL for the pUA12 and pUA15 clones.

4.2.4. Proteasome activity measurements

Proteasome activity was measured as described by Grune *et al.* (1998) and Demasi *et al.* (2003). For routine work, 100 µg of protein were diluted with MilliQ H_2O in order to obtain a final sample volume of 150 µl. 2 ml of assay buffer (10 mM Tris, pH 8; 20 mM KCl; 5 mM $MgCl_2$) and 10 µl of 50 µM proteasome substrate succinyl-leucine-leucine-valine-tyrosine-MCA (S-LLVY-MCA), were mixed. Proteasome activity was measured using a Perkin Elmer Luminescence Spectrometer (LS 50B) at 365 nm (excitation) and 435 nm (emission). The substrate degradation reaction was allowed to proceed for 60 m, at 37°C, with agitation and proteasome activity calculated by accumulation of free MCA. At least five independent measurements were performed for each clone.

4.2.4.1. Protein extraction

10 ml cultures from pUA12, pUA13, pUA14 and pUA15 clones were grown overnight in MM-uri, at 30°C, with agitation (180 rpm). Cells were harvested when the culture reached an OD_{600nm}=0.5. Pellets were washed with ice cold MilliQ H_2O and finally frozen at -80°C, overnight or for longer periods of time. Proteins were extracted on ice at 4°C to prevent degradation since protease inhibitors were not used. Cell pellets were defrosted on ice and resuspended in 150 µl of Lysis buffer (10 mM K-Hepes; 10 mM KCl; 1.5 mM $MgCl_2$). To the cell suspension an equal volume of glass beads (Ø=0.40-0.45 mm) was added and cells were disrupted using a Mini Bead-Beater (Biospec Products); for 10 cycles of 20 s intercalated with ice incubation for at least 20 s. The extract was collected with 300 µl of Lysis buffer, by washing the beads and centrifugation at 5,000 rpm, at 4°C, for 5 m. The lysate was re-centrifuged at 13,000 rpm, at 4°C, for 10 m. The protein extract was divided into aliquots and frozen at -80°C. For protein quantification, the BCA Protein Assay Reagent Kit (Pierce) was used. Protein quantification and integrity was further verified on 10 % Mini-gels using the Mini-PROTEAN 3 Electrophoresis Cell (Bio-Rad).

4.2.5. Determination of trehalose and glycogen concentration

Glycogen and trehalose were determined as described by Becker *et al.* (1978), Vandercammen *et al.* (1989) and Parrou *et al.* (1997). Cells from pUA12, pUA13, pUA14 and pUA15 clones were grown overnight in MM-uri. When cell cultures reached exponential growth phase (OD_{600nm}=0.6-0.8), 2 ml of culture were harvested at 5,000 rpm, at 4°C, for 5 m. Cell pellets were resuspended in 250 µl 0.25 M Na_2CO_3 and heated at 95°C for 4 h with agitation (600 rpm). Suspensions were adjusted to pH 5.2 with 150 µl 1 M acetic acid and 600 µl 0.2 M sodium acetate buffer pH 5.2 and divided into two equal volumes. 1.2 U of α-amyloglucosidase (Roche) was incubated overnight at 57°C with agitation (180 rpm), with ½ of the above mixture. The other half of the mixture was incubated overnight at 37°C, with agitation at 180 rpm, in the presence of 0.5 U of trehalase (Sigma). Samples were centrifuged at 5000 rpm, for 3 m, at 4°C and the glucose content of the supernatant was determined as described in the Glucose oxidase Assay Kit (Sigma, GAGO-20). Three independent measurements were performed for each clone.

4.2.6. 2D analysis of cell wall proteome

In order to evaluate the impact of CUG ambiguity on the *C. albicans* cell wall the cell wall proteome of white pUA12 and pUA15 clones were compared. Cell wall proteins extraction was performed as described by Pitarch *et al.* (2002). One white colony isolated from agar plates was resuspended in MilliQ H_2O and vortexed to obtain a suspension of cells that served as an inoculum for overnight cultures in MM-uri that grew at 30°C with 200 rpm of agitation. 6 litres cultures were prepared for each assay. When cultures reached an OD_{600nm} between 0.2 and 0.3, cells were collected by centrifugation at 5,000 rpm, at 4°C, for 10 m and washed with sterile MilliQ H_2O. Pellets were frozen at -20°C.

4.2.6.1. Cell disruption

Cells from above were washed five times with 10 mM Tris-HCl pH 7.4, ressupended in 12 ml of ice-cold lysis buffer (10 mM Tris-HCl pH 7.4, 1 mM

phenylmethylsulfonyl fluoride, 0.2 µg/ml leupeptine, 0.2 µg/ml pepstatine, 0.2 µg/ml antipaine) and an equal volume of glass beads (Ø=0.40-0.45) was added. Mechanical lyses were performed in a refrigerated cell homogenizer (Braun Biotech) using cycles of 30 s, with 1 m rest on ice. This procedure was carried out until complete cell breakage was achieved. This was verified by phase-contrast microscopy and failure of cells to grow on YPD plates supplemented with 10 mg/ml chloramphenicol. Glass beads were separated from suspensions of lysed cells by decantation by doing several washes with 10 mM Tris-HCl pH 7.4.

4.2.6.2. Purification of cell wall proteins – SDS/DTT fraction

Cell wall and soluble cytoplasmatic fractions were separated by centrifugation at 3,000 *g*, at 4°C, for 10 m. Supernatants were discarded and pellets, containing cell wall fractions, were submitted to several rounds of five washes, first with ice-cold MilliQ H_2O and then with the following ice-cold solutions: 5% (w/v) NaCl, 2% (w/v) NaCl, 1% (w/v) NaCl. Isolated cell wall extracts were washed five times with ice-cold MilliQ H_2O and resuspended in 10 ml SDS extraction buffer [50 mM Tris-HCl pH 8.0, 2% (w/v) SDS, 100 mM EDTA, 10 mM DTT]. Cell wall proteins were extracted by boiling for 10 m and separated by centrifugation at 3,000 *g*, at 4°C, for 10 m. The supernatant containing cell wall proteins (CWP) was transferred to corex tubes.

4.2.6.2.1. Protein concentration

Cell wall proteins extracted by SDS/DTT treatment were precipitated with trichloroacetic acid to a final concentration of 15% (w/v) and incubation on ice for a minimum period of 30 m. After centrifugation at 9,000 rpm, at 4°C, for 15 m, CPWs were washed several times with ice-cold acetone and dried at room temperature, then resuspended in 200 µl 0.1 M NaOH and immediately froze at -80°C. CPWs quantification was performed by the Bradford method using the Microassay Procedure from Bio-Rad as described by the manufacturer. Calibration curves were prepared ever time protein quantification was carried out. Samples were diluted 1:100, 1:500 and

1:1000, and 800 µl of each dilution were added to 200 µl of Bradford reagent (Bio-Rad). Mixtures were vortexed and incubated in the dark for approximately 30 m. Absorbance at 595 nm was measured for the three dilutions of each sample. The protein concentration (µg/µl) of each sample was estimated using the calibration curve (above). Before bi-dimensional gel analysis and in order to check the quantification and the quality of the CPW extracted, samples were electrophoresed in a 10 % Mini-gel using the Mini-PROTEAN 3 Electrophoresis Cell (Bio-Rad).

4.2.6.2.2. 10% Mini PAGE

For rapid analysis of protein extracted 10% (w/v) polyacrylamide gels, with a 4% (w/v) polyacrylamide stacking gel, were run at room temperature. 4 ml gel solutions were routinely prepared with 1.3 ml of the 30% (w/v) acrylamide solution, 503.5 µl of 2% (w/v) Bis, 1.25 ml 2.5 M Tris pH 8.9, 40 µl 10% (w/v) SDS, 1.0 ml MilliQ H_2O, 20 µl 20% (w/v) APS, and 10 µl TEMED. Gel solutions were poured to 8 mm from the top of the gel mould. After polymerization of the main gel, a 3 ml stacking gel solution was prepared with 400 µl 30% (w/v) acrylamide solution, 375 µl of 2% (w/v) Bis, 750 µl 0.5 M Tris pH 6.8, 30 µl 10% (w/v) SDS, 1.5 ml MilliQ H_2O, 15 µl 20% (w/v) APS, and 3 µl TEMED, was poured to fill the gel mold. A slot former was then introduced, the gel allowed to polymerize at room temperature. For routine analysis, 10 µg of protein was boiled for 5 minutes. The loading buffer [160 mM Tris pH 6.8, 2% (w/v) SDS, 10% (v/v) glycerol, 0.2 M DTT, 0.5 mg/ml bromophenol blue] was added in a 1:1 proportion, to the sample.

Gels were run at 150 V (Power Pac 300, Bio-Rad) submerged in electrophoresis buffer [25 mM Tris base, 192 mM glycine, 0.1% (w/v) SDS], for approximately 1 hour, until the bromophenol blue dye reached the bottom of the gel. Gels were stained using the Silver Staining Bio-Rad kit. Silver staining protocol was performed with the reagents provided by the kit and following the manufacture instructions.

4.2.6.3. Two-dimensional PAGE

4.2.6.3.1. First dimension

Samples containing 500 µg of protein were solubilised in a rehydration buffer [7 M urea, 2 M thiourea, 2% (w/v) CHAPS, 65 mM dithioerythritol, 2% (w/v) Pharmalyte pH 3-10 (Amersham Biosciences), bromophenol blue] and were applied onto the IPGphor strip holder (18 cm long, Amersham Biosciences). Immobilized pH gradient (IPG) strip (no-linear pH3-10, Amersham Biosciences) was pointed to anodic end of the strip holder and lowered carefully onto the solution, in a way that no bubbles were trapped under the IPG strip but making sure that the strip was completely wet. Care was taken to ensure that the gel contacted the strip holder electrodes at each end. To minimize evaporation and urea crystallization, each IPG strip was overlaid with IPG Cover Fluid (Amersham Biosciences). Isoelectric focusing was performed on an IPGphor system (Amersham Biosciences) at 15°C using the following program: passive rehydration for 16 h, 500 V for 1 h, 500 V to 2000 V for 1 h, and 8000 V for 5.5 h.

4.2.6.3.2. Second dimension

4.2.6.3.2.1. 10% polyacrylamide gel

Second dimension gels were prepared using the PROTEAN Plus precast system from Bio-Rad. A volume of gel solution (50 ml/gel) was prepared, and each gel, 1.5 mm thick, had the following composition: 0.8 M Tris-HCl pH 8.9, 10% (w/v) acrylamide, 0.17% (w/v) piperazine diacrylamide (PDA), 1% (w/v) SDS, 0.1% (w/v) ammonium persulphate (PSA), 0.077% (w/v) sodium tiosulphate and 0.13% (v/v) TEMED. Gels were overlaid with water saturated butanol for polymerization. After 2 h at room temperature gels polymerization was completed, butanol was replaced by MilliQ H_2O and gels were allowed to mature overnight at 4°C.

4.2.6.3.2.2. IPG strip equilibration and electrophoresis

After the first dimension electrophoresis, IPG strips were first equilibrated, for 12 m, in equilibration buffer [6 M urea, 50 mM Tris-HCl pH 6.8, 30% (w/v) glycerol, 2% (w/v) SDS] containing 2% (w/v) dithioerythritol, and then incubated for 5 m in 2.5% (w/v) iodoacetamide (Bjellqvist *et al.*, 1993). Equilibrated IPG strips were inserted between glass plates in contact with the top of the slab gel and embedded in 0.5% (w/v) agarose, in order to prevent the strip from moving or floating in electrophoresis buffer [25 mM Tris-Base, 192 mM Glycine, 0.1% (w/v) SDS]. Second dimension electrophoresis was carried out at 30 V/gel for 16 h using the PROTEAN Plus Dodeca Cell system (Bio-Rad).

4.2.6.3.3. Gel staining

Gels were silver-stained as described by Pitarch *et al.* (2002) with some modifications. After a brief wash with MilliQ H_2O during 5 m, gels were fixed in 40% (v/v) ethanol and 10% (v/v) acetic acid for 1 h and then in 5% (v/v) ethanol and 5% (v/v) acetic acid during 2 h or an overnight. Gels were washed three times with 7.5% acetic acid for periods of 30 m and soaked in 2.5% (v/v) glutaraldehyde for 30 m. After this, gels were extensively washed with water and stained with an ammonia silver nitrate solution [0.8% silver nitrate, 0.3% (v/v) ammonia, 20 mM sodium hydroxide] for 30 m. Gels were washed with water and developed in 0.01% (w/v) citric acid and 0.1% (v/v) formaldehyde. Staining was halted with 5% (w/v) Tris and 2% (v/v) acetic acid.

4.2.6.3.4. Image analysis

Two-dimensional images were captured by scanning the silver-stained gels using a GS-690 imaging densitometer (Bio-Rad) and digitized with Multi-Analyst software (Bio-Rad). Two-dimensional gel images were processed, including detection of volumetric quantification, matching, statistical analysis and editing of molecular masses and Isolectric point (pI) of the spots, using PDQuest software (version 7.2, Bio-Rad).

4.3. Results

4.3.1. Effects of CUG ambiguity on growth rate

Since CUG ambiguity is toxic and such toxicity decreases growth rate in *S. cerevisiae* (Santos *et al.*, 1996), we have determined the impact of the various heterologous tRNAs encoded by plasmids pUA13-15 on growth rate (μ). Surprisingly, growth rates of clones transformed with pUA13-15 plasmids were not significantly different from that of the control strain (pUA12). The calculated μm for the pUA12 was 0.35 h^{-1}± 0,02 h^{-1} slight above the ones obtained for pUA13 (0.21 h^{-1}± 0,1 h^{-1}), pUA14 (0.20 h^{-1}± 0,1 h^{-1}) and pUA15 (0.28 h^{-1}± 0,05 h^{-1}). These results contrast with those reported for genetic code ambiguity or mistranslation in *E. coli* (Bacher *et al.*, 2005; Pezo *et al.*, 2004; Baick *et al.*, 2004; Bacher & Ellington, 2001), *B. subtilis* (Wong, 1983), and *S. cerevisiae* (Santos *et al.*, 1999).

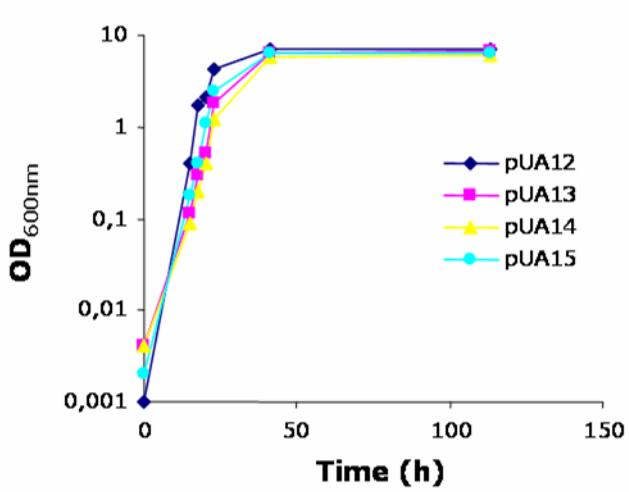


Figure 4.1. Growth curves of ambiguous clones of *C. albicans*. Growth curves of the pUA12, pUA13, pUA14 and pUA15 clones were determined in *C. albicans* transformants grown in MM-uri at 30°C with agitation (180 rpm). Optical Density (OD) at 600 nm was monitorized using a Beckman Du 530 spectrophotometer.

Considering that transformation efficiency of *C. albicans* with plasmids pUA13-15 was very low (see Chapter 2), suggesting that CUG ambiguity is indeed toxic, the lack of a growth phenotype was surprising. It suggests that *C.*

albicans does not tolerate a sudden burst of CUG ambiguity, but those cells that survive somehow adapt to CUG ambiguity and grow normally. How *C. albicans* copes with such ambiguity is not clear.

4.3.2. Structural cellular alterations induced by CUG ambiguity

Since CUG ambiguity should result in massive production of aberrant proteins that may misfold and aggregate we investigated whether accumulation of such aggregates could be observed by TEM. Large scale proteome disruption is also likely to affect the overall cell structure and we hypothesized that such alterations could also be detected by TEM.

Increased CUG ambiguity had a strong effect on cellular density (Figure 4.2-4.4), as determined by FACS analysis of relative intracellular granularity (Table 4.1). pUA15 cells, both opaque or white, had large and dense vacuoles (Figures 4.2-4.4), that contained very dense material, whose molecular nature is not yet understood. In white cells, the vacuoles were very large and the dense material was dispersed over the entire cytoplasm (Figure 4.3). Interestingly, this dispersion of dense material in the cytoplasm of white pUA15 cells did not correspond to higher relative granularity when compared to opaque pUA15 cells (Table 4.1).

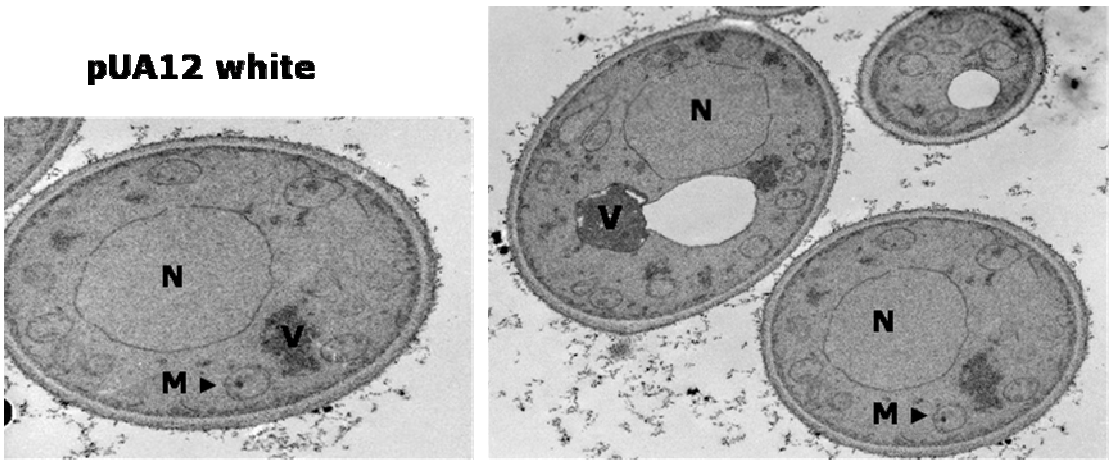


Figure 4.2. Ultrastructure of white phase pUA12 control cells. Cells were grown overnight in MM-uri, at 30°C, fixed with 2.5% (w/v) glutaraldehyde and embedded in Epon resin:propylene oxide. N=nucleous; V=vacuole; M=mitochondrion.

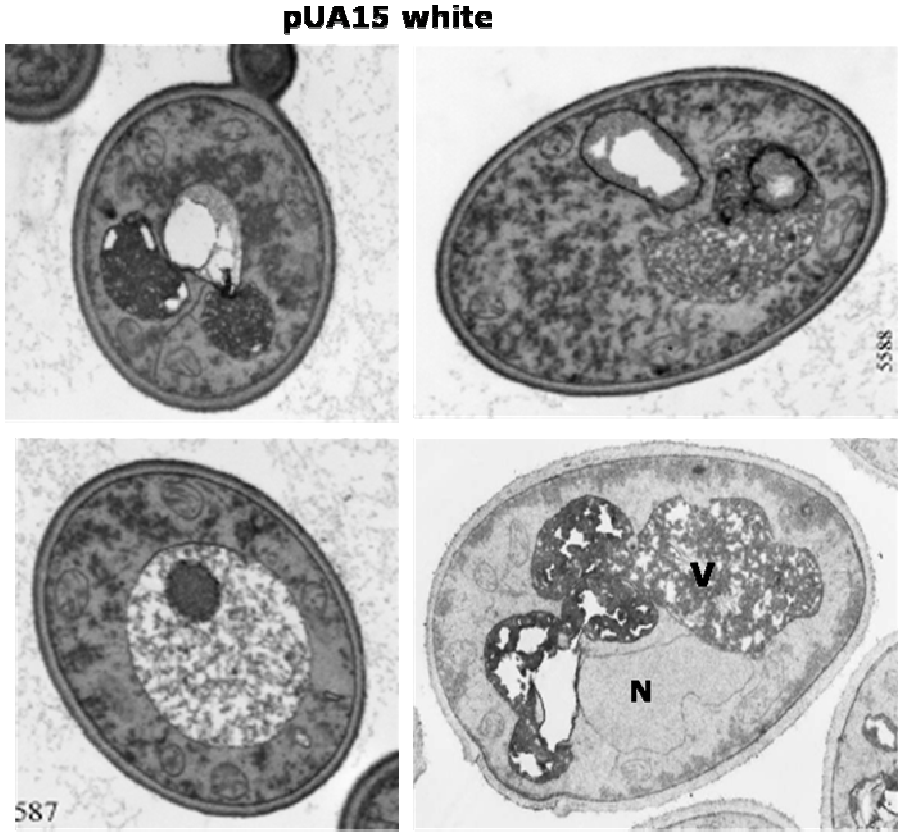


Figure 4.3. Ultrastructure of white phase pUA15 cells. pUA15 white cells have large vacuoles and large amounts of dense material dispersed in the cytoplasm, which may be aggregates of various components of the translational machinery, namely ribosomes. Similar structures are formed in other eukaryotic cells, namely plant and human cells exposed to stress (stress granules). Cells were grown overnight in MM-uri, at 30°C, fixed with 2.5% (w/v) glutaraldehyde and embedded in Epon resin:propylene oxide. N=nucleous; V=vacuole.

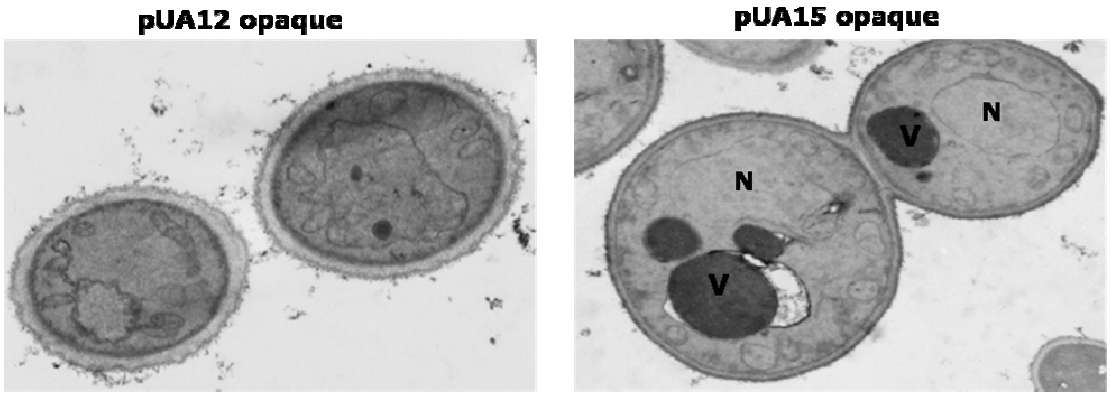


Figure 4.4. Ultrastructure of opaque phase pUA12and pUA15 cells. Opaque pUA15 cells have larger vacuoles than those observed in control pUA12 cells. These vacuoles contain very dense material whose molecular nature should be similar to that observed in white cells Cells were grown overnight in MM-uri, at 30°C, fixed with 2.5% (w/v) glutaraldehyde and embedded in Epon resin:propylene oxide. N=nucleous; V=vacuole.

The relative granularity of pUA12 and pUA15 transformed cells (both white and opaque) was also determined by FACS analysis using a protocol that measures light dispersion (Pina-Vaz *et al.*, 2001). These studies showed clearly that the relative granularity of pUA12 and pUA15, in both types of cells, was significantly different reaching 52% increase in opaque cells. This confirmed the TEM analysis.

Table 4.1. CUG ambiguity increases cellular granularity. Comparative cellular granularity was carried out by FACS for both white and opaque cells transformed with pUA12 and pUA15 plasmids. Differences between pUA12 white and opaque cells are mainly due to differences in cell shape and size, which interferes with the FACS analysis.

Strain	Relative Granularity	pUA12/pUA15 (%)
pUA12 White	18.7	--------
pUA12 Opaque	23.6	--------
pUA15 White	22.3	19
pUA15 Opaque	36.0	52

We have attempted to identify the molecular nature of the dense granular material observed in the cytoplasm of pUA15 cells. However, the thick cell wall of *C. albicans* prevented diffusion of fixative solutions (Bauer *et al.*, 2001) and precluded identification of that material. Despite this, it is likely that this material is composed by components of the translational machinery, such as various RNAs (mRNA, tRNA, rRNA), ribosomes and translational factors.

Eukaryotic cells exposed to environmental stress also form dense particles called stress granules (SGs), which assemble as a consequence of abortive translational initiation. When translation is initiated in the absence of eIF2-GTP-tRNAi^{Met}, the ternary complex that normally loads the tRNAi^{Met} onto the small ribosomal subunit, phosphorylation of eIF2α inhibits mRNA translation by reducing the availability of the eIF2-GTP-tRNAi^{Met} ternary complex (Anderson & Kedersha, 2002). Furthermore, eIF2 phosphorylation can be the final step of serine/threonine kinases signalling activated under stress

conditions, for example, when unfolded proteins accumulate in the ER (Patil & Walter, 2001; Harding *et al.*, 2000). Thus, pre-initiation complexes and their mRNA transcripts can be routed to the SGs in a process that requires proteins related to DNA-binding proteins (Kedersha *et al.*, 2002; Kedersha *et al.*, 2000; Kedersha *et al.*, 1999). Additionally, in yeast translationally repressed mRNAs are often localized in discrete cytoplasmatic granules, called processing bodies (P-bodies) (Teixeira *et al.*, 2005; Nakamura *et al.*, 2001). P-bodies are dynamic structures that vary in number and size during cellular responses to stress (Teixeira *et al.*, 2005).

4.3.3. Ambiguous CUG decoding does not induce the stress response

Mistranslation results in large scale synthesis of mutant proteins that misfold and aggregate or are degraded. In this sense, CUG ambiguity is similar to other forms of stress that create protein disruption, namely heat-shock and oxidative stress. These types of stress induce general stress and specific stress responses that up-regulate molecular chaperones, accumulation of metabolites and other physiological alterations. Since CUG ambiguity in *S. cerevisiae* triggered up-regulation of molecular chaperones, increased accumulation of trehalose and glycogen and resulted in tolerance to severe stress (Santos *et al.*, 1999), we have investigated whether increased CUG ambiguity in *C. albicans* also induced accumulation of storage metabolites.

Firstly, we have tested whether *C. albicans* transformed with pUA13-15 plasmids accumulated trehalose and glycogen. Interestingly, expression of *S. cerevisiae* $tRNA_{UAG/CAG}^{Leu}$ did not result in trehalose or glycogen accumulation in *C. albicans* (Figure 4.5), suggesting that CUG ambiguity does not induce a stress response (Parrou *et al.*, 1997). A possible explanation for this unexpected result could be that genes responsible for synthesis of these two stress metabolites contain CUG codons, and CUG ambiguity may disrupt the function of the proteins involved in trehalose and glycogen synthesis. Interestingly, similar results were found when *C. albicans* cells submitted to different types of stresses, namely thermal stress, corresponding to a temperature shift from 23 to 37°C, osmotic stress and oxidative stress, where

accumulation of reserve sugars was not found (Enjalbert *et al.*, 2003). Furthermore, no up-regulation of genes involved in the trehalose and glycogen metabolism was observed in those experiments and gene expression profiling using DNA microarrays of *C. albicans* cells exposed to hyperosmotic stress showed a slight up-regulation of genes involved in catabolism of storage metabolites (Enjalbert *et al.*, 2003). On the other hand, *S. cerevisiae* weakly induces production of trehalose in response to heat shock, hyperosmotic or oxidative stresses. This is due to increased turn-over of this metabolite (Parrou *et al.*, 1997). However, *S. cerevisiae* cells submitted to the same stress produced and accumulated glycogen (Parrou *et al.*, 1997).

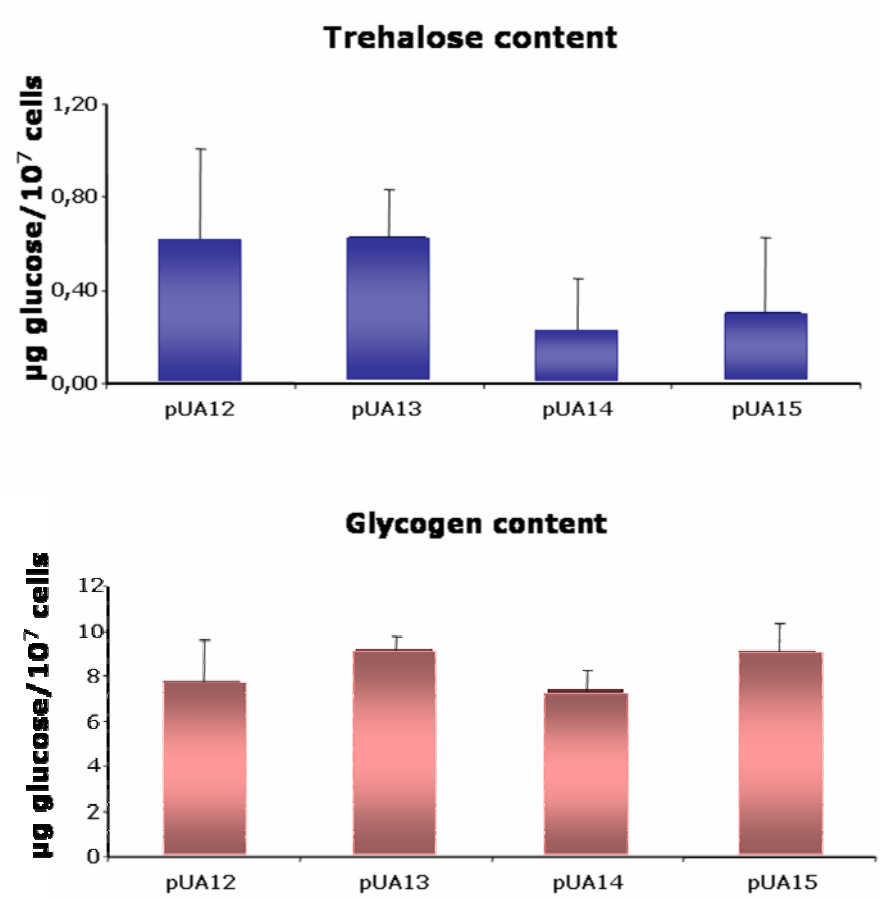


Figure 4.5. Effect of CUG ambiguity on glycogen and trehalose levels. CAI4 cells, transformed with plasmids pUA12-15, grown exponentially (10^7 cells/ml) in MM-uri, at 30°C, were harvested and assayed for glycogen and trehalose contents. For this, cells were mechanically disrupted and protein extracts were incubated at 95°C for 4 hours. Glucose content was determined after incubation of the extracts with amyloglucosidase at 57°C or with trehalase at 37°C.

In wild type *C. albicans* and *S. cerevisiae* cells growing under normal physiological conditions (Enjalbert *et al.*, 2003; Parrou *et al.*, 1997), the basal levels of glycogen is higher than trehalose. However, the difference that we observed between the two is higher (10 fold glycogen/trehalose) than that previously reported (2 fold than glycogen/trehalose) (Enjalbert *et al.*, 2003). The results obtained indicate that CUG ambiguity does not constitute a stress factor and confirms the absence of a general stress response in *C. albicans* (Enjalbert *et al.*, 2003). This is in sharp contrast to what was observed in *S. cerevisiae*.

4.3.4. Ambiguous CUG decoding increases proteasome activity

The results described above did not exclude the hypothesis that CUG ambiguity results in large scale production of aberrant proteins. In other words, *C. albicans* cells transformed with plasmids pUA13-15 should produce higher amounts of mutant proteins than control pUA12 cells. Since mutant misfolded proteins are targeted for degradation by proteasome via the ubiquitin pathway (Kostova & Wolf, 2003), we wondered whether proteasome activity was increased in pUA13-15 clones. To test this hypothesis, total protein extracts were prepared from control and ambiguous pUA13-15 cells and proteasome activity of each extract was quantified using a fluorogenic peptide as a specific proteasome substrate, as described in material and methods (4.2.4). Interestingly, proteasome activity in extracts from ambiguous clones pUA13 and pUA14 was very similar to that of the control pUA12 extracts. The pUA15 extracts had slightly higher proteasome activity, but these showed high standard deviation and the increase in activity is not significantly different from that of the other clones tested pUA12-14. It is not clear why extracts of the pUA15 clone had variable proteasome activity but since these clones display higher morphological variability it is likely that the cell population of this clone is more variable than that of other clones. In any case, proteasome activity is not significantly increased by CUG ambiguity in *C. albicans* (Figure 4.6)

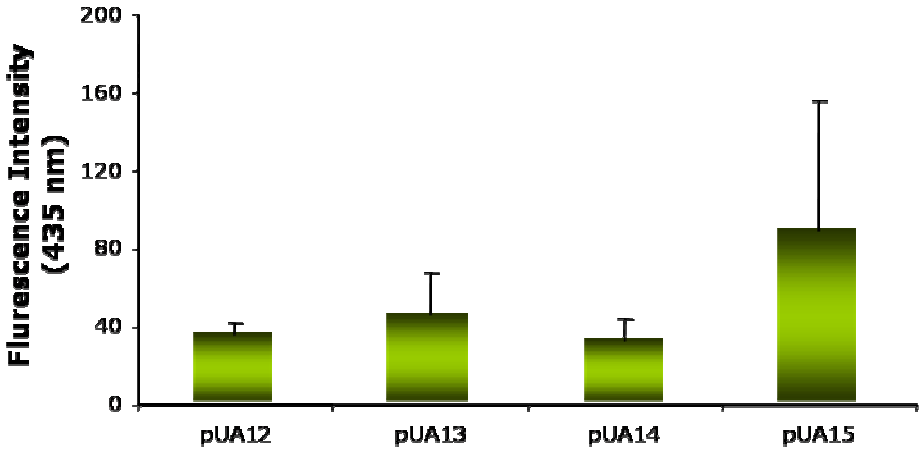


Figure 4.6. Proteasome activity is slightly increased in highly ambiguous pUA15 clones. Proteasome activity was quantified in total protein extracts prepared from *C. albicans* transformed with pUA12, pUA13, pUA14 and pUA15 plasmids by measuring degradation of the fluorogenic peptide SLLVY-MCA at 37°C, for 1 h, with agitation. Quantification of degraded MCA was calculated by measuring fluorescence intensity at 435 nm after 60 m. Values were corrected using fluorescence values at time zero.

The above results are rather surprising since *C. albicans* encodes 13,074 CUG codons (haploid genome) dispersed over 66% of its genes and the tRNAs expressed from pUA13-15 plasmids decode the serine CUG codon as leucine, strongly suggesting that these cells should indeed produce large amounts of aberrant proteins. However the slightly increased in proteasome activity and the dense intracellular structures that were observed in ambiguous cells by transmission electron microscopy (see above) indicate that CUG ambiguity is reprograms gene translation and promotes sequestration of mRNA or translation initiator complexes. This is similarly to what happen in stress conditions in mammalian, plants and yeast cells (Teixeira *et al.*, 2005; Anderson & Kedersha, 2002).

4.3.5. Ambiguous CUG decoding promotes cell wall proteome variability

Cell wall determines the shape of the cell and is essential for the osmotic balance, cell growth, cell division, morphogenesis, biolfilm formation and adhesiveness (de Groot *et al.*, 2004; Herrero *et al.*, 2004; Sundstrom, 2002; Klis *et al.*, 2001). It is also the first cellular organelle involved in host-fungus

interactions and protection against host defence (Calderone, 1993). Furthermore, the cell wall has been referred as a preferential antifungal target, since mammalian cells do not have a cell wall and consequently fungicides do not have secondary effects (Groll *et al.*, 1998).

About 50% of cell wall genes have one or more CUG codons (Miranda *et al.*, unpublished), thus cell wall proteome could be an interesting model to evaluate the impact of CUG ambiguity on cellular structure. To test this hypothesis, total cells wall proteins (CWP) were prepared using complete cell disruption by glass beads. Cells disruptions were confirmed either by microscopic examination or by YPD plating of the disrupted cell extract. Since glycolytic enzymes, namely Enop, Pgkp and Gapp were detected in CWP samples, high level of cell breakage efficiency and posterior extensive washes were carried out to remove any extracellular or cytosolic protein contaminants that could adhere to the cell wall through electrostatic forces (Pitarch *et al.*, 2002). Also, CWP samples of pUA12 and pUA15 clones were always treated in parallel. DTT and hot SDS-extracted proteins, corresponding to proteins loosely associated with the cell wall surface and a small fraction of membrane proteins (Pitarch *et al.*, 2002), were analysed by 2D-gel electrophoresis.

Normally, around 400 spots were detected (Figure 4.7), which correspond to a lower number of proteins than that previously reported by Pitarch *et al.* (2002) (700 spots). This disparity may be related to a combination of factors, namely detection techniques, gel staining, amount of protein loaded and differences in the cell wall of the strains used in both studies. While we used *C. albicans* CAI4 strain Pitarch and colleagues (2002) used SC5314 strain.

For comparative cell wall sub-proteome analysis, 6 replicas of each gel type were used for partially fractioned cell wall extracts of pUA12 and pUA15 clones. For data analysis, spots detected automatically using the PDQuest software package were confirmed manually to eliminate gel artefacts, namely background stains or streaks. Spot intensity was normalized using the total intensity of the spots present in the gel after subtracting background noise. The final spot volume corresponded to the average spot volume of the six gels

analysed. For final comparative analysis, pUA12 and pUA15 replicate gels were matched and qualitative and quantitative analyses were performed.

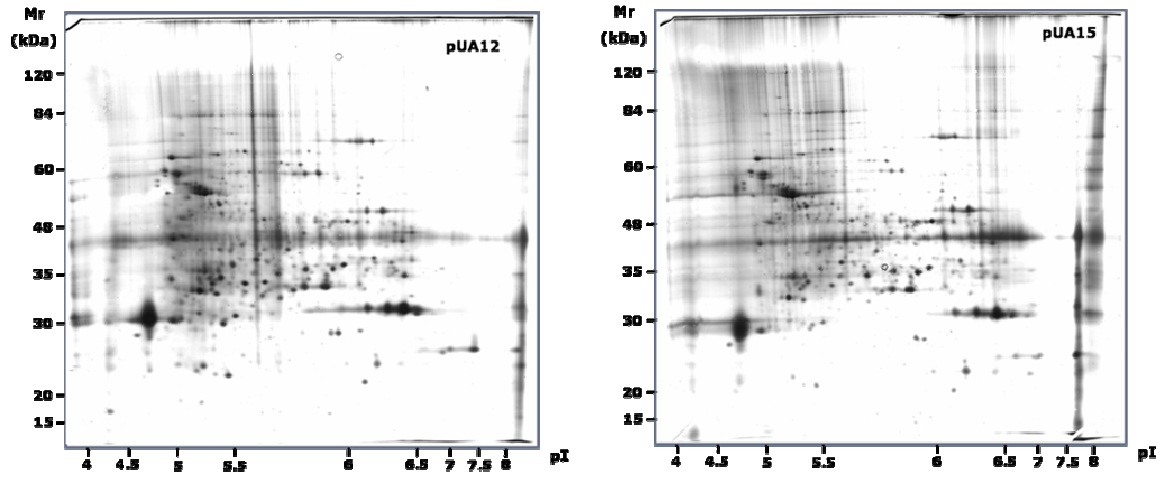


Figure 4.7. Silver-stained 2-DE gels of cell wall proteomes from pUA12 and pUA15 clones. CWPs were extracted with SDS- and DTT-extractable proteins from an early exponential phase culture. 500 µg of protein were applied onto Immobiline pH 3-10 non linear DryStrips. Isoelectric focusing was carried out using an IPGphor system with the following program: passive re-hydration for 16 h, 500 V, for 1 h, 500 V to 2000 V for 1 h, and 8000 V for 5.5 h. IPG strips were equilibrated and electrophoresed in 10% polyacrylamide gels at 30 V/gel for 16 h in a PROTEAN Plus Dodeca cell system.

Comparison of the 2DE-maps of the cell wall sub-proteome of white cells of pUA12 and pUA15 clones permitted identification of several proteins whose expression showed significant differences between the two types of clones. In particular, 74 spots present in the pUA12 map where absent in the pUA15 map while in pUA15 map detected 61 spots absent in pUA12 map (Figure 4.8). Apart from this, from a total of 217 spots, quantitative analysis of spot intensity revealed 101 spots whose normalized intensity did not change significantly ($0.5 \leq$spot intensity≤ 2) (Figure 4.8). Included in this group, were proteins encoded by genes containing CUG codons, namely Hsp104p and Adh1p (Figure 4.8). The former is a member of heat shock protein encoded by a gene containing one CUG codon and the second is alcohol dehydrogenase whose gene contains two CUG codons.

Quantitative analysis differentiated 116 spots, whose normalized intensity was two fold or more up-regulated or down-regulated, in relation to the control. Of these spots, 55% were up-regulated and 45% were down-regulated (Figure 4.8). The large majority of these spots were not yet identified by mass-spectrometry or comparative 2DE-map analysis.

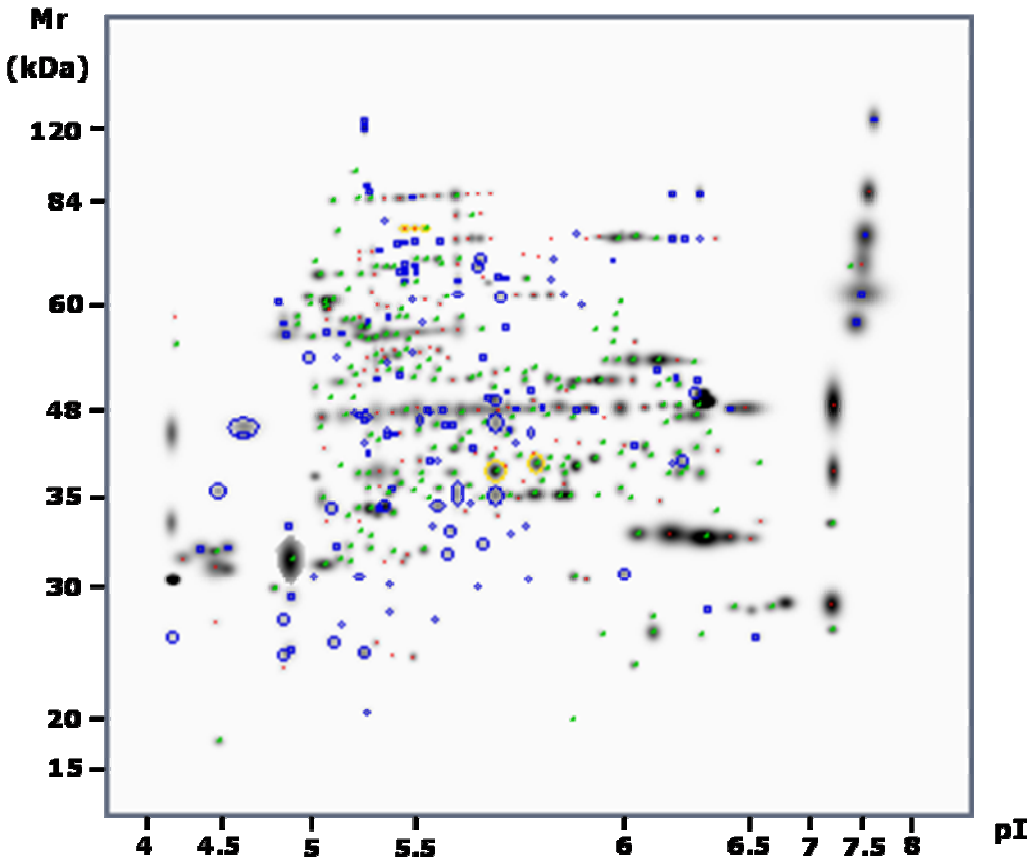


Figure 4.8. Comparative 2-DE map of cell wall proteins from pUA12 and pUA15 strains. 2DE-map of the cell wall sub-proteomes, obtained as described in the main text, were analyzed using the PDQuest software. Spots were submitted to qualitative and quantitative analysis (pUA12 *vs*. pUA15). Spots marked with blue square were exclusively found in pUA12 map. Spots marked with blue circle were exclusively found in pUA15 map. Marked in green are spots whose intensity was between 0.5≤spot≤2 fold. In red are spots whose intensity changed more than 2 fold (increased or decreased). Spots marked with a yellow circle correspond to proteins encoded by genes containing CUG codons, identified as Hsp104p and Adh1p by Pitarch *et al.* (2002).

Interestingly, the translation elongation factor, Tef1αp (Pitarch *et al.*, 2002), was up-regulated ten fold in ambiguous cells, suggesting that CUG ambiguity has a strong impact in the expression of other components of the translational

machinery. This is in line with our previous observation that CUG ambiguity induces rearrangements of the R-chromosome, which contains rRNA cistrons (see chapter 3). The appearance of the translation elongation factor Tef1αp in the cell wall is not too surprising since this is very abundant cytoplasmatic protein that has been found in the extracellular media or associated to the cell envelope in several organisms, namely in *C. albicans* (Pitarch *et al.*, 2002; Maneu *et al.*, 1996), *Trichoderma reesei* (Lim *et al.*, 2001), *Mycobacterium leprae* (Marques *et al.*, 1998), *B. subtilis* (Antelmann *et al.*, 2001), *Staphylococcus aureus* (Singh *et al.*, 2001) and *Arabidopsis thaliana* (Chivasa *et al.*, 2002). It is not clear why this translation factor is secreted but it may have an adhesion role or chaperone activity (Pitarch *et al.*, 2002).

4.4. Discussion

4.4.1. Overall effects of CUG ambiguity in *C. albicans* biology

The expression of *S. cerevisiae* tRNAs that decode the CUG codon as leucine, increasing CUG ambiguity, had rather surprising results. Firstly, the *C. albicans* $tRNA_{CAG}^{Ser}$, which decodes the CUG codon as serine, is a minor tRNA (Santos *et al.*, 1993) that decodes poorly due to the presence of G at position 33 (G_{33}) in the anticodon-loop (Perreau *et al.*, 1999). Consequently, those heterologous leucine tRNAs, which decode the CUG codon as leucine in *C. albicans*, compete efficiently with the $tRNA_{CAG}^{Ser}$ for CUG codons at the ribosome A-site. This was expected to have a profound impact on *C. albicans* biology since it encodes 13,074 CUG codons (haploid genome), dispersed over 66% of its genes at a frequency of 1 to 38 CUGs/gene (Gomes *et al.*, unpublished). Interestingly, CUG ambiguity affected transformation efficiency (see chapter 2) but did not have any significant impact on growth rate. When put together, these data suggests that a sudden burst of codon ambiguity, introduced immediately upon transformation (tRNA expression is constitutive in eukaryotic cells), is toxic and results in a strong decrease of transformation efficiency. However, cells that survive adapt quickly to such ambiguity and recover growth rate to normal levels. This is different to what has been observed in *E. coli* and *S. cerevisiae*, where high level codon ambiguity does

reduce growth rate significantly. For example, the expression of *C. albicans* $tRNA_{CAG}^{Ser}$ in *S. cerevisiae* led to 50% decrease in growth rate (Santos *et al.*, 1996). Overexpression of *B. subtilis* GluRS in *E. coli* mischarges the $tRNA_1^{Gln}$ (UUG) (Lapointe *et al.*, 1986) with glutamate and decreases growth rate and ultimately, in the absence of transamidation pathway for the formation of Gln-$tRNA^{Gln}$, if the amount of mischarged tRNA is excessive kills the cell (Baick *et al.*, 2004). Similarly, lack of editing by the IleRS and incorporation of an isoleucine analogue (norvaline) reduced growth rate more than 100-fold in *E. coli* (Bacher *et al.*, 2005). Finally, misincorporation of the tryptophan analogue (6-flurotryptophan) caused a >10-fold growth defect in the Qβ phage (Bacher *et al.*, 2003). Further studies are needed to understand why CUG ambiguity does not decrease significantly growth rate in *C. albicans* however it is likely that its "new" CUG codons evolved to tolerate such ambiguity.

The other surprising result was the lack of accumulation of trehalose and glycogen in ambiguous *C. albicans* (Figure 4.5). These two reserve metabolites are accumulated under stress conditions in *S. cerevisiae* (Parrou *et al.*, 1997) and in *C. albicans* (Van Dijck *et al.*, 2002; Arguelles, 1997), thus one was expecting to observe a strong accumulation in ambiguous cells. That result is consistent with the lack of an effect of CUG ambiguity on growth rate (Figure 4.1) and the weak increase of proteasome activity (Figure 4.6), but raises the intriguing question of how does *C. albicans* cope with the mistranslated proteins that should be produced due to CUG ambiguity. As mentioned above, CUG codons may be present in gene primary structure locations that evolved to minimize protein structure disruption due to the insertion of leucine instead of serine. However, it is very unlikely that all CUGs have been reprogrammed in the *C. albicans* genome since ambiguous cells accumulate dense aggregated material in the cytoplasm (see below), which may be formed by aberrant proteins.

The data mentioned above, shows clearly that CUG ambiguity does not constitute a stress similar to heat-shock, hyperosmotic or oxidative stresses and that *C. albicans* and *S. cerevisiae* respond in rather different manners to CUG ambiguity. Conversely to what happen in *S. cerevisiae* where these

metabolites accumulate in response to stress (Parrou *et al.*, 1997) and genetic code ambiguity (Silva, unpublished). However, in *C. albicans* this is a controversial subject since some authors demonstrated accumulation of trehalose in response to strong heat shock (Van Dijck *et al.*, 2002; Arguelles, 1997), while others have shown that accumulation of reserve sugars does not occurs in response to stress (Enjalbert *et al.*, 2003). Therefore, the stress response in *C. albicans* is not yet fully understood and may be significantly different to that of *S. cerevisiae*. Recent microarray data shows that *C. albicans* has a classical transient transcription profile in response to stress. However, each stress response is unique and apparently there is no cross-protection between different types of stress, as is the case of *S. cerevisiae* (Enjalbert *et al.*, 2003; Martinez-Pastor *et al.*, 1996; Schmitt & McEntee, 1996; Lewis *et al.*, 1995; Ruis & Schuller, 1995; Marchler *et al.*, 1993).

One observation that contradicts the lack of toxicity of CUG ambiguity in *C. albicans* was the accumulation of large protein aggregates in the cytoplasm of ambiguous *C. albicans* cells (Figures 4.3 and 4.4). These aggregates could correspond to RNA-protein complexes, translation machinery complexes, and aberrantly unfolded proteins. In animal and plant cells, environmental stress induces formation of stress granules (SGs), which also appear dispersed in the cytoplasm (Kedersha *et al.*, 1999; Scharf *et al.*, 1998; Nover *et al.*, 1989; Nover *et al.*, 1983). SGs are phase dense particles and harbour untranslated mRNAs that accumulate as a consequence of stress-induced translational arrest (Kedersha *et al.*, 1999). Phosphorylation of eIF-2α inhibits translational initiation (Srivastava *et al.*, 1998), allowing elongating ribosomes to run off their mRNA transcripts, which then accumulate in stress granules (Kedersha *et al.*, 1999). In cells allowed to recover from stress, stress granules-associated mRNAs rapidly move to polyribosomes (Nover *et al.*, 1989). In yeast cells, translational repressed mRNA are stored in discrete cytoplasmatic granules (Nakamura *et al.*, 2001) referred as P-bodies (Teixeira *et al.*, 2005; Sheth & Parker, 2003). Therefore, if translation reprogramming occurs in response to ambiguous CUG decoding, untranslated mRNA can be transferred to P-bodies or translation initiation complexes (mRNA, rRNA and RNA-binding proteins)

and become sequestrated in SGs. Unfortunately, technical problems did not allow us to identify the composition of the aggregates. This should be further studied since relative granularity of pUA15 cells, compared with the pUA12 cells, was increased 19% and 52% in white and opaque cells, respectively.

4.4.2. Effects of CUG ambiguity on the *C. albicans* cell wall proteome

Pathogenic and commensal microorganisms have evolved diverse mechanisms to generate phenotypic variation at the cell surface to cope with immune system challenges. For example, *B. fragilis* produces 8 distinct variable capsular polysaccharides that help it to establish and maintain long-term commensal relationship in the human colon (Krinos *et al.*, 2001). In other cases, surface phenotypic variation is accomplished through families of genes encoding cell-surface proteins, which are differentially expressed and help pathogens evade detection by the immune system or alter interactions with host tissues. For example, differential expression of *VSG* (variant surface glycoproteins) genes in *Trypanosome bruceii* (Pays *et al.*, 1994), *MSG* (major surface glycoproteins) genes in *Pneumocystis carinii* (Stringer & Keely, 2001), *var* genes in *Plasmodium falciparum* (Freitas-Junior *et al.*, 2005), all produce surface variability. In fungi, several gene families encoding cell-surface glycoproteins confer different adherence and immunogenic proprieties to the fungal cell wall. In *C. glabata*, *EPA* (Epithelial adhesion) genes encode proteins responsible for adherence to mammalian tissues (De Las *et al.*, 2003; Cormack *et al.*, 1999) and *FLO* (Flocculation) genes regulate the cell-surface properties in yeast (Halme *et al.*, 2004).

CUG ambiguity can alter directly or indirectly cell wall composition. Directly, since approximately 50% of the genes encoding cell wall proteins contain one or more CUG codons (Miranda *et al.*, unpublished), thus CUG ambiguity could be a mechanism generating surface variability. Outer CWP, i.e., proteins loosely associated with the cell wall, from the control (pUA12 clones) and ambiguous cells (pUA15 clones) were displayed in a 2-DE map and compared using the PDQuest software. Both strains displayed a similar general 2-DE protein pattern (Figure 4.7) nevertheless qualitative and quantitative

differences found may be sufficient to provide surface diversity. From the 400 spots detected, 61 proteins appear as a result of CUG ambiguity while 74 were absent. From the common 317 spots, 63% were quantitatively identical. Cell wall proteomics is a challenging task due to samples heterogeneity, low abundance, low solubility, hydrophobic nature, interactions with mannan, glucan and/or chitin. Furthermore, the small number of identified proteins in the 2-DE reference map makes a CWP too complex to analyse by 2-DE (Pitarch *et al.*, 2002; Niimi *et al.*, 1999).

CUG ambiguity also affects indirectly cell wall composition promoting haplo-insufficiency of proteins involved in cell wall biosynthesis encoded by genes containing CUG codons. Mannoproteins contain both *N*- and *O*-linked oligosaccharides. The *N*-linked glycans attach to the asparagines residues while the *O*-glycosylation occurs in serine or threonine residues of proteins. *PMT* gene family act to transfer mannose from dolichyl phosphate-activated mannose to serine or threonine (Gentzsch & Tanner, 1997; Gentzsch & Tanner, 1996). Deletion *PMT1* (1 CUG codon) or *PMT4* (2 CUG codons) genes alter markedly cell wall decreasing significantly mannoproteins content (Prill *et al.*, 2005). The second member of the *MNT* gene family, *MNT2* (3 CUG codons), which encodes a α-1,2-mannosyltransferase is responsible for the addition of the second and third mannose residues in *O*-glycans. Elimination of both Mnp1p and Mnp2p resulted in truncation of *O*-linked glycans (Munro *et al.*, 2005).

Another interesting example of how CUG ambiguity may affect indirectly cell wall composition, are the phenotypes obtained when both alleles of the *KRE5* (killer resistant) (11 CUG codons) gene are deleted. *C. albicans kre5/kre5* mutants have significantly reduced levels of β-1,6-glucan and more chitin and β-1,3-glucan and less mannoprotein than wild type (Herrero *et al.*, 2004). Furthermore, *SSK1* (4 CUG codons) and *CBK* (cell wall biosynthesis kinase) (3 CUG codons) are involved in regulation of expression of cell wall proteins (Chauhan *et al.*, 2003; McNemar & Fonzi, 2002), and the later also controls a two-component histidine kinase expression, *CHK1* (*C. albicans* histidine

kinase) (13 CUG codons), that regulates cell wall synthesis (Kruppa *et al.*, 2004).

GPI7 (2 CUG codons) gene is involved in the byosynthesis of GPI anchors and cell wall structure is modified in homozygous mutants, displaying hypersensitive to calcufluor white (Richard *et al.*, 2002b). Another component of cell wall is chitin which is synthesized by a multiple *CHS* genes (4). *CHS1* (4 CUG codons) encodes an essential chitin synthetase that is required for septum formation of both yeast and hyphae and also maintains the integrity of the lateral cell wall (Munro *et al.*, 2001). Despite of being viable, deletion of *CHS8* (2 CUG codons) causes a 25% reduction in chitin syntethase activity and mutant cells are hypersensitive to calcufluor white (Munro *et al.*, 2003).

Taken together the data reveals that many links between CUG ambiguity and cell wall composition and biosynthesis can be established. However, due to cell wall complexity and the large number of proteins resulting from CUG ambiguity this relation is extremely complex and far from being understood.

5. General Discussion

5.1. Genetic code evolution

During the last few years, deviations to the universal genetic code have been found in many prokaryotic and eukaryotic (Santos & Tuite, 2004). In order to explain how these alterations evolved, two theories have emerged, namely the "Codon Capture Theory" and the "Ambiguous Intermediate Theory".

The "Codon Capture Theory" (Osawa & Jukes, 1989) postulates that under strong GC- or AT-biased pressure, certain codons can disappear, allowing for subsequent loss of the corresponding tRNAs. At a later stage, these unassigned codons reappear by mutation and can be captured by mutant tRNAs from different isoacceptor families thereby introducing genetic code deviations. Important evidence supporting this theory is the presence of unassigned codons, in *Mycoplasma capricolum* (CGG) and *Micrococcus luteus* (AGA and AUA), which have 75% of AT and GC, respectively (Kano *et al.*, 1993; Osawa *et al.*, 1992). Since codons disappear from the genome, this theory is neutral as it does not introduce amino acid alterations into proteins upon codon reappearance in the genome (Osawa & Jukes, 1995).

The "Ambiguous Intermediate Theory" (Schultz & Yarus, 1994) postulates that tRNA structural change is the key element in any genetic code deviation. That is, mutations that allow tRNAs to recognize near-cognate codons will permit a single codon to be assigned to two different amino acids. This is an intermediate step but sets the stage for the mutant tRNA to capture codons being reassigned. Since codon ambiguity destabilizes the proteome, it must bring selective advantages to the organism, but the theory does not explain what kind of advantages may result from ambiguous codon decoding. A typical case supporting this theory is the reassignment of the CUG codon from leucine to serine in some species of the *Candida* genus (Sugita & Nakase, 1999; Sugiyama *et al.*, 1995; Santos & Tuite, 1995; Ohama *et al.*, 1993). In this case, the CUG codon is ambiguous due to a double identity of the $tRNA_{CAG}^{Ser}$, which is charged with both leucine and serine. For example, in *C. zeylanoides* the CUG codon is decoded 95-97% with serine and 3-5% with leucine (Suzuki *et al.*, 1997).

None of the above theories explains all known cases of codon reassignments, but the majority of them can be validated by the "Ambiguous Intermediate Theory" (Knight *et al.*, 2001; Schultz & Yarus, 1996). Since both theories are not mutually exclusive, recent *in silico* studies of the leucine CUG codon reassignment in *C. albicans* strongly suggested that evolution of this particular sense-to-sense reassignment has been driven by a combination of those two evolutionary mechanisms (Massey *et al.*, 2003). The $tRNA_{CAG}^{Ser}$ that decodes the CUG codon in *Candida* spp. evolved from a $tRNA_{IGA}^{Ser}$ through altered splicing of the intron of the later tRNA (Massey *et al.*, 2003; Yokogawa *et al.*, 1992). The $tRNA_{CAG}^{Ser}$ appeared approximately 272 My and for a period of 100 My competed with the wild type $tRNA_{CAG}^{Leu}$ for CUG decoding, creating CUG ambiguity (Massey *et al.*, 2003). When the *Saccharomyces* and *Candida* genus diverged the former lost the $tRNA_{CAG}^{Ser}$, re-acquiring standard CUG decoding, while *Candida* spp. maintained it and eliminated the cognate leucine tRNA. CUG codon ambiguity imposed a strong negative pressure on CUG usage, forcing it to mutate to UUG and UUA codons. Such negative pressure eliminated almost all CUG codons (only 0.2% remained) from the genome of the *Candida* ancestor. Simultaneously, the new $tRNA_{CAG}^{Ser}$ introduced a new selective pressure that led to capture of new CUG codons from serine UCN codons (Santos *et al.*, 2004a; Massey *et al.*, 2003).

Concerning CUG decoding, the *Candida* genus is heterogeneous. While *C. glabrata* maintained standard CUG decoding, *C. cylindracea* fully reassigned it to serine, while in other *Candida* species CUG is ambiguously decoded (O'Sullivan *et al.*, 2002; Sugita & Nakase, 1999; Suzuki *et al.*, 1997; Santos *et al.*, 1997). These differences among *Candida* species are due to differences in the structure of the $tRNA_{CAG}^{Ser}$ that decode the CUG codon in various *Candida* species. These tRNAs have different levels of misleucylation of the $tRNA_{CAG}^{Leu}$ (Santos *et al.*, 2004b). In *C. albicans*, the anticodon arm of the $tRNA_{CAG}^{Ser}$ has m^1G_{37} which together with the CAG anticodon, is responsible for its recognition by the leucyl-tRNA synthetase, with consequent aminoacylation with leucine. Interestingly, the presence of G_{33} reduces $tRNA_{CAG}$ leucylation efficiency (Suzuki *et al.*, 1997) but most of these tRNAs, including the *C.*

albicans $tRNA_{CAG}^{Ser}$ have identity elements for both seryl- or leucyl-tRNA synthetases (SerRS and LeuRS) and are charged with both serine and leucine. For this reason, in the cytoplasm of *C. albicans* and other *Candida* species there are two forms of the $tRNA_{CAG}$, namely Ser-$tRNA_{CAG}^{Ser}$ (charged by the SerRS) and Leu-$tRNA_{CAG}^{Ser}$ (charged by the LeuRS). As a consequence, the CUG codon is decoded as serine or leucine (O'Sullivan *et al.*, 2002; Suzuki *et al.*, 1997).

The replacement of polar serines by hydrophobic leucines in *C. albicans* proteins has consequences for their tridimensional structure and function. More importantly, *C. albicans* mRNAs containing CUG codons will encode arrays of proteins, certainly, with different structural and functional properties. In other words, the *C. albicans* proteome has a statistical nature since its proteins are not true chemical entities (Gomes *et al.*, unpublished). This raises the obvious questions i) "what could the selective advantage of having such a statistical proteome be? And ii) "Is CUG ambiguity an evolutionary intermediate step of CUG reassignment or does it have functional meaning?" The first experimental approach to tackle these questions was the partial reconstruction of the *Candida* genetic code change in *S. cerevisiae*, which was accomplished by expressing the *C. albicans* $tRNA_{CAG}$ in *S. cerevisiae* (Santos *et al.*, 1996). The latter, despite having a slower growth rate, was tolerant to heavy metals, drugs, ethanol, oxidants and sodium chloride (Santos *et al.*, 1999; Santos *et al.*, 1996), suggesting that these cells have a selective advantage under extreme environmental stress conditions. Since *S. cerevisiae* genome has 30,000 CUG codons (Massey *et al.*, 2003), their ambiguous decoding resulted in accumulation of aberrant unfolded proteins which triggered a general stress response that pre-adapted cells to exposure to severe stress (Santos *et al.*, 1999). This is not too surprising since there is cross-protection between different types of stress in *S. cerevisiae* (Enjalbert *et al.*, 2003; Schmitt & McEntee, 1996; Lewis *et al.*, 1995; Ruis & Schuller, 1995), but it is of relevance for genetic code ambiguity since it shows that the latter can be adaptive.

In order to understand genetic code evolution, a number of laboratories engineered codon ambiguity in *E. coli* (Bacher *et al.*, 2005; Pezo *et al.*, 2004; Nangle *et al.*, 2002; Bacher & Ellington, 2001), and *E.coli* phage Qβ (Bacher *et al.*, 2003). This affected growth rate but confirmed that codon decoding ambiguity creates important selective advantages that allow cells to explore new environmental niches or overcoming severe stress situations. These studies suggested that CUG ambiguity could also confer a selective advantage to *C. albicans*. However, the lack of a general stress response (Enjalbert *et al.*, 2003) in this yeast complicates the issue of the putative selective advantages induced by CUG ambiguity.

The genetic code alteration is a unique characteristic of the biology of *C. albicans* and of other *Candida* species, whose relevance we do not yet fully comprehend. In this study, we have tried to shed new light into how and why *Candida* spp. evolved a genetic code change by studying the phenotypic and physiological implications of CUG ambiguity. To accomplish this, *C. albicans* CAI4 cells were transformed with a low copy plasmid carrying *S. cerevisiae* leucine tRNAs genes. These tRNAs, with anticodons UAG (pUA13) and CAG (G_{33}/pUA14 and T_{33}/pUA15), are able to compete with the endogenous *C. albicans* $tRNA_{CAG}^{Ser}$ for CUG decoding with different levels of efficiency (Figure 2.2). The most evident and relevant outcome of such genetic code ambiguity was phenotype diversity. Whether this constituted a selective advantage for the preservation of CUG ambiguity during evolution is not yet clear. However, *C. albicans* is mainly asexual (mating is rare and involves diploids), commensal and pathogenic, with remarkable capacity for adaptation to different ecological niches, resistance to stress, and capacity to escape the immune system. This strongly suggests that phenotypic variability and cell surface alterations induced by CUG ambiguity are advantageous.

Interestingly, phenotypic diversity is also generated by reducing or abolishing the function of the molecular chaperone Hsp90 in *Drosophila melanogaster* (Rutherford & Lindquist, 1998) and *Arabidopsis thaliana* (Queitsch *et al.*, 2002). Therefore, it is likely that accumulation of misfolded/unfolded proteins

creates phenotypic plasticity that exposes morphogenetic variation in response to environmental change. Furthermore, *S. cerevisiae* has evolved a system to exploit hidden genetic variation via a conformational mutant of the translation termination factor Sup35p [PSI^+] (Uptain & Lindquist, 2002; Tuite & Lindquist, 1996). The [PSI^+] element confers short-term survival advantage over strains that lack it by increasing the synthesis of C-terminally extended proteins due to stop codon readthrough. Accumulation of these aberrant proteins also results in phenotypic variation and appearance of other beneficial phenotypes (True *et al.*, 2004). Taken together, these results show that proteome instability, independent of its origin, results in phenotype diversity.

5.2. Genomic implications of CUG ambiguity

Increased codon mistranslation leads to hypermutation in *E. coli*. A single anticodon mutation in the $tRNA_{GCC/U}^{Gly}$ gene creates a tRNA able to decode the aspartate codons (GAC/U) as glycine (Dorazi *et al.*, 2002; Slupska *et al.*, 1998) which originates genetic code ambiguity and hypermutagenesis (Zhao *et al.*, 2001; Murphy & Humayun, 1997; Slupska *et al.*, 1996). Similarly, the mutant $tRNA_{CAG}^{Ser}$ that appeared 272 My ago in the ancestor of yeasts mistranslated leucine codons as serine (Silva *et al.*, 2004; Massey *et al.*, 2003; Santos & Tuite, 1995). It is not yet clear whether CUG ambiguity also results in hypermutagenesis. However, this ambiguity had a massive negative impact on the usage of CUG codons present in the genome of the ancestor of yeasts. In other words, one of the important consequences of CUG ambiguity is codon usage reduction. In this sense, biased genome GC pressure and codon ambiguity have similar and synergistic effects on codon usage. Also interesting is the observation that the appearance of the novel $tRNA_{CAG}^{Ser}$ has been a major positive force converting serine UCN codons into CUG codons (Massey *et al.*, 2003).

Genome alterations occur also in response to chemical ambiguity since the incorporation of unnatural amino acids into proteins results in increased genome mutational change (Bacher *et al.*, 2003). Clearly, genetic code

ambiguity triggers alteration in genome base composition, via translational stress-induced mutagenesis, which conducts to an increased rate of genome evolution.

In this study, CUG ambiguous decoding promoted karyotype variability (Figure 3.1) and increased ploidy, up to 64 N (Figures 3.2 and 3.3). The increase in ploidy could be a cellular response to genotoxic and metabolic stresses (Storchova & Pellman, 2004) imposed by genetic code ambiguity, which would enable adaptation to higher CUG ambiguous decoding. In yeast, polyploid states are associated with mutations in cell cycle genes (Vallen *et al.*, 1992; Winey *et al.*, 1991; Snyder & Davis, 1988; Rose & Fink, 1987; Thomas & Botstein, 1986; Schild *et al.*, 1981). In *C. albicans*, 60% of the genes associated with cell cycle contain CUG codons and it is likely that the cell cycle is being affected by CUG ambiguity leading to an increase in ploidy. However, polyploidy was unstable since after several rounds of selection cells returned to the normal diploid state (Figure 3.2). This suggests that ambiguous *C. albicans* cells may mate at high frequency. In *C. albicans*, ploidy alterations promote chromosome rearrangements (Selmecki *et al.*, 2005; Iwaguchi *et al.*, 2000; Suzuki *et al.*, 1989), which in turn are related to phenotypic variability (Rustchenko-Bulgac, 1991; Rustchenko-Bulgac *et al.*, 1990; Suzuki *et al.*, 1989). Therefore, the data presented in this thesis strongly suggests an association between ploidy, chromosomal rearrangements, phenotype variability and CUG ambiguity.

CUG ambiguity triggers white to opaque transition, which was associated to the transition between mating-incompetent to mating-competent cells. According to Miller and Johnson (2002), mating occurs at higher rates (10^6), between opaque cells, which are homozygous for MTL. Mating products are tetraploid (Legrand *et al.*, 2004; Bennett & Johnson, 2003; Hull *et al.*, 2000; Magee & Magee, 2000) and probably return to the diploid state through a concerted random chromosome loss in which loss of one or more chromosomes predispose cells to lose others (Bennett & Johnson, 2003). Therefore, it is likely that CUG ambiguity induces mating at high frequency in

ambiguous cells lines. This is supported by the increase in ploidy and formation of conjugation tubes in ambiguous pUA15 clones. However, it is surprising that ploidy increased in some cells up to 64N. Such ploidy increase also increase cell size producing giant cells but our studies did not clarify whether these cells would be able to re-acquire a diploid status.

5.3. Cellular implications of CUG ambiguity

The low *C. albicans* transformation efficiencies obtained with pUA13-15 plasmids was the first indicator that *S. cerevisiae* Leu-tRNAs were being expressed and charged in *C. albicans* cells and also that CUG ambiguity manipulation was toxic (Figure 2.12). Northern blot analysis confirmed the expression and aminoacylation of the *S. cerevisiae* leucine tRNAs in *C. albicans* (Figures 2.13 and 2.14). Conversely to what as been reported by Leuker *et al.* (1994), CUG ambiguity is toxic but not lethal and its impact was not limited to cell biology but also extended to the genomic level. Interestingly, *C. albicans* transformed cells readily overcame toxicity induced by CUG ambiguity, since only a slight difference in growth rate between ambiguous and control clones was detected (Figure 4.1). Furthermore, the lack of accumulation of reserve sugars (trehalose and glycogen) (Figure 4.5), and the normal levels of proteasome activity found in pUA13 and pUA14 clones (Figure 4.6), suggest that *C. albicans* evolved to cope with high level of CUG ambiguity.

Apart from phenotypic variability, the strongest impact of ambiguous CUG decoding was on accumulation of dense material in the cytoplasm of *C. albicans*. In the pUA15 cells, dense aggregates dispersed in the cytoplasm (white cells, Figure 4.3) or concentrated in vacuoles (opaque cells, Figure 4.4) were easily observed. These aggregates resemble stress granules in plant and mammalian cells or P-bodies in yeasts observed under conditions of environmental stress (Teixeira *et al.*, 2005; Kedersha *et al.*, 2000; Kedersha *et al.*, 1999; Nover *et al.*, 1989). These granules are ribonuclear aggregates in which untranslated mRNAs accumulate during stress-induced translational arrest. These structures are sites of untranslated mRNA storage and sorting

either for reinitiation, degradation, or packing into stable non-polysomal mRNP complexes (Kedersha *et al.*, 2000; Kedersha *et al.*, 1999; Nover *et al.*, 1989). Thus, cytoplasmatic aggregates, formed when CUG ambiguity is raised to high levels (pUA15 clones), are probably composed by RNA and RNA binding proteins. This would be consistent with the hypothesis that CUG ambiguity induces translational arrest, which has not yet been studied. If so, it is likely that gene expression is reprogrammed at the translational level in highly ambiguous cells (pUA15), which may be an important molecular mechanism that *C. albicans* uses for protection against the deleterious effects of CUG ambiguity.

5.4. Consequences of CUG ambiguity for cell wall structure

The cell wall of *C. albicans* is a dynamic and complex multilayered structure, responsible for maintaining cell shape, acts as a permeability barrier with a nutritional role, and also mediates the interaction between the microorganism and the environment (Lopez-Ribot *et al.*, 2004). The major components (80-90%) of the *C. albicans* cell wall are carbohydrates: i) mannan or polymers of mannose covalently associated with proteins to form mannoproteins; ii) β-glucans, which are branched polymers of glucose containing β-1,3 and β-1,6 linkages; and iii) chitin. Proteins and lipids are present in minor proportion. β-glucans and chitin are the structural components that form a rigid microfibrillar network, and proteins and mannoproteins are bound to this skeleton as well as being present in the outer surface. The mannose polymers are linked to proteins by *N*-glycosidic bonds through asparagines residues and by *O*-glycosidic, alkali-labile linkages to threonine or serine residues (Kapteyn *et al.*, 2000; Chaffin *et al.*, 1998; Cassone, 1989). The major cell wall components that elicit a response from the host immune system are mannoproteins and proteins (Pitarch *et al.*, 1999; Martinez *et al.*, 1998).

Since 50% of cell wall related genes contain CUG codons, CUG ambiguity should have a strong impact on cell wall sub-proteome, namely creating surface variability, which is important for both commensal and pathogenic

microorganisms (Freitas-Junior *et al.*, 2005; Halme *et al.*, 2004; De Las *et al.*, 2003; Krinos *et al.*, 2001; Stringer & Keely, 2001; Cormack *et al.*, 1999; Pays *et al.*, 1994). In order to investigate the impact of CUG ambiguity on cell wall sub-proteome, we carried out a comparative 2-DE cell wall sub-proteome analysis between control pUA12 and highly ambiguous pUA15 clones. From the 400 spots detected by 2-DE PAGE, corresponding to proteins loosely associated to cell wall, 61 are unique to the pUA15 map. Also from the 217 common spots between pUA12 and pUA15 maps, 26% were up-regulated and 21% were down-regulated in ambiguous cells. If these qualitative and quantitative differences found between control and ambiguous cells generate surface antigenic variability is not yet clear and needs further study. In particular, the pattern of glycosylation of cell wall proteins should be investigated in detail since serine is glycosylated while leucine is not and this important difference may cause important antigenic alterations. Also, the proteins that appear *de novo* in the maps of pUA15 clones should be identified by mass-spectrometry. This would provide important new insights on the full impact of CUG ambiguity on the cell wall sub-proteome.

5.5. Implications of CUG ambiguity on virulence factors

Phenotypic diversity generated by CUG ambiguity is likely to have important implications for *C. albicans* pathogenesis. Morphogenesis, phenotypic switching, production of extracellular hydrolases and adhesion are the most important virulence attributes of *C. albicans* (Berman & Sudbery, 2002; Calderone & Fonzi, 2001). Interestingly, ambiguous CUG decoding triggered expression of the main *C. albicans* virulence factors. Ambiguous cell lines (pUA13-15) display high rate of morphogenesis and phenotypic switching (up to 88% in pUA15 clones), increase secreted aspartic protease and phospholipases production and exhibit cell-cell and cell agar adhesion.

In *C. albicans*, morphogenesis is triggered by a variety of environmental cues, including nutrient starvation, pH changes, serum, and growth in agar matrix, which activates specific signalling pathways that regulate the transition

between yeast to filamentous forms (pseudohypha and hypha). CUG ambiguity promoted spontaneous yeast-to-hypha transition. Our study did not show how CUG ambiguity induced morphogenesis, however 90% of the genes encoding transcription factors acting on morphogenetic pathways have CUG codons (Figure 2.25). This suggests the hypothesis that CUG ambiguity may disrupt their function leading to morphogenesis. However, the main morphogenetic repressor *TUP1* does not contain CUG codons, indicating that CUG ambiguity does not induce morphogenesis through derepression of hyphal gene promoters. Interestingly, the cell population of the pUA15 clones was heterogeneous, containing yeast, pseudophypha and hyphal cells, with predomination for the later. Therefore, spontaneous morphogenesis triggered by CUG ambiguity could be associated with insufficiency of functional cell cycle related proteins, like Cln3p (Lazo *et al.*, 2005; Bachewich & Whiteway, 2005).

As a result of morphogenesis and white-opaque transition triggered by CUG ambiguity, very high rates of phenotypic switching (reaching 88%), white-opaque and myceliated-unmyceliated switching, were obtained in CUG ambiguous clones (pUA13-15). Since similar phenotypes were obtained in mutants lacking *SIR2* gene (Perez-Martin *et al.*, 1999), phenotype switching in ambiguous cell lines could be due to the partial disruption of Sir2p since its gene contains 2 CUG codons.

CUG ambiguity also increased adhesion, namely cell-cell and cell-agar adhesion, which was extremely exacerbated in some pUA15 clones. Since CUG is a hydrophobic amino acid it is likely that increased adhesion may result from serine substitution by leucine at CUG positions. *C. albicans* encodes various adhesins, namely *HWP1* and the *ALS* gene family, whose up-regulation would increase adhesion. Interestingly these *ALS* genes are rich in CUG codons and misincorporation of leucine at these codons may strongly contribute to increased adhesion. This is corroborated by expression in *S. cerevisiae* of the *C. albicans* genes *ALS1* (5 CUG codons) (Fu *et al.*, 1998), *ALS5* (12 CUG codons) (Gaur *et al.*, 2002) or *ALA1* (2 CUG codons) (Gaur *et*

al., 2002; Gaur *et al.*, 1999; Gaur & Klotz, 1997) which result in a strong adhesion phenotype.

In saprophytic microorganisms, extracellular hydrolases are primarily secreted to breakdown or decompose complex materials into nutrients readily available to the cells or to compete with other environmental bacteria, parasites or fungi (Naglik *et al.*, 2004). However, pathogenic microorganisms (bacteria, parasites and fungi) adapted this biochemical property to carry out processes in the host. Direct virulence functions include hydrolysing substrates of the host membranes to facilitate adhesion and tissue invasion, or damaging cells and molecules of the host defence system to avoid or resist anti-microbial attack (Naglik *et al.*, 2003; Klemba & Goldberg, 2002; Monod & Borg-von Zepelin, 2002; Peschel, 2002; Rasmussen & Bjorck, 2002; McKerrow *et al.*, 1993; Ogrydziak, 1993). CUG ambiguity increases hydrolytic activity, as measured by activity of secreted aspartic proteinases and phospholipases. Since the expression of members of the *SAP* gene family are linked with morphogenesis and phenotypic switching and this phenomenon is triggered by CUG ambiguity, it is expected that *SAP4-6* (in hyphal forms) and *SAP1-3* (in opaque phase cells) expression would be up-regulated in ambiguous pUA15 clones.

5.6. Conclusion and future studies

The evolution of genetic code remains a mystery. The primitive genetic code is thought to have encoded statistical, ambiguous proteins in which more than one amino acid was inserted at a given codon. The relative vitality of organisms bearing ambiguous proteins and the selective pressures that forced development of the highly specific modern genetic code are unknown. The modern genetic code appeared more than 3 billion years ago (Ribas & Schimmel, 2000; Woese, 1973) and reached such complexity that is became inflexible for the large majority of organisms (Crick, 1968). However, genetic code alterations show that the genetic code is still evolving.

Our observation that statistical proteomes, produced by genetic code ambiguity, generate phenotypic variability and increase adaptive potential has direct implications for our understanding of the evolution of the genetic code since such ambiguity prevailed during evolution of early living systems (Pezo *et al.*, 2004; True *et al.*, 2004; Santos *et al.*, 1999). During 272 My of evolution, ambiguous CUG decoding imposed a strong negative pressure on CUG codons that led to their disappearance from the *Candida* ancestor genome (Massey *et al.*, 2003). Here, we show that *C. albicans* CUG ambiguity has important biological functions, since it creates phenotypic diversity and advantageous phenotypes. Our data strongly suggests that CUGs were reprogrammed in the *C. albicans* genome to minimize disruption of protein structure and function, thus preventing an "error catastrophe" (Hughes & Ellington, 2005). Increase in CUG ambiguity levels affect growth rate slightly and does not trigger a typical stress response (accumulation of trehalose and glycogen was not detected). However, synthesis of aberrant proteins resulted in accumulation of dense material in the cytoplasm of *C. albicans* suggesting that it may induce translational arrest and formation of stress-like granules (Teixeira *et al.*, 2005; Anderson & Kedersha, 2002; Kedersha *et al.*, 2002). Most importantly, CUG ambiguity generates phenotype and genome variability which is crucial for *C. albicans* pathogenesis and commensalism.

Our study validated our working hypothesis that increasing CUG ambiguity would expose phenotypes associated to such ambiguity and could provide new insight on why CUG ambiguity was preserved over 272 My of evolution. It also validated *C. albicans* as an excellent biological model to study the evolution of the genetic code. However, it raised more questions than answers. For example, does elimination of CUG ambiguity also abolishes phenotypic variability? Are non-ambiguous clones less virulent? Do they become stress sensitive? A biological comparison between ambiguous and non-ambiguous *C. albicans* clones would provide important insight on the answer to these questions. Other important questions that remain to be answered are: how does CUG ambiguity induce expression of phenotypic variability? Is CUG ambiguity random or is it sensitive to codon context? Do the LeuRS and SerRS

have a role in the regulation of CUG ambiguity? Is CUG ambiguity still evolving? Can the identity of the CUG codon be reverted from serine back to leucine? Answering these questions will most likely provide important new insight on the biological role of CUG ambiguity in *C. albicans* and will further help understanding how the genetic code evolves.

6. References

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