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THE ROLE OF THE GCN PATHWAY IN ACID STRESS TOLERANCE

Diplomarbeit
zur Erlangung des akademischen Grades
Diplomingenieur

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Matrikelnummer 0140289

22.10.2007

ACKNOWLEDGEMENTS

After more than six years of good life and hard work I have finally archived the aim and this is reason enough to say thank you to a lot of people.

First, I would like to thank my family. Thank you for making it possible, for always being there for me, for going to Vienna a hundred times, for supporting me financially and for everything else.

Second, I thank all my friends in Vienna and Valencia for having spent a good time together and for all the hours of conversation and party, remember the "LBT-Stammtisch"! Thanks to you I know what "real" student life is.

Third, I would like to thank Ramón Serrano and Mariche Montesinos who gave me the great chance to realize my Diploma Thesis in their laboratory. It was an honour to be surrounded by such brilliant people, thank you for everything!

Fourth, I would like to thank my tutor Guillem Hueso. You are a great instructor and a great person. I am sure that you will be an important scientist soon, and even sooner a famous DJ! I learned a lot and enjoyed the time we spent together.

Fifth, I would like to thank Jose Ramón Murguía for fruitful discussions, particularly about western blot analysis.

Sixth, as for me feeling comfortable at work is very important, I was very happy to be surrounded by you, my laboratory colleagues! Bea, Edu, Gaetano, JM, Lola, Regina, Rosa, Samy, Santi and all the others – thanks for making me feel like "one more".

Seventh, I would like to thank my "Erasmus coordinator" Dietmar Haltrich. Thank you very much for supporting my decision of staying abroad longer than

usual and for helping me a lot in order to being able to finish my studies without problems.

Last but not least, Jorge. You are my engine and my motivation; without you I would not be at this point yet and I would not be that happy. Thank you for being by my side; this work is yours.

ABSTRACT

Microbial spoilage of food causes losses of up to 40 % of all food grown for human consumption worldwide. Although most types of yeast are beneficial for many aliments, some harmful species exist and cause important losses of food production. Weak acids such as benzoic, acetic, propionic and sorbic acid are used in food industry as organic acid food preservatives, but many microorganisms have developed resistance.

Weak acid toxicity is based on intracellular acidification, which inhibits amino acid uptake in yeast. Amino acid starvation triggers the GCN pathway to induce amino acid biosynthesis/uptake from the medium. The pathway starts with activation of the Gcn2 protein kinase, phosphorylation of the eukaryotic translation initiation factor 2 (subunit α;eIF-2α), and the selective translation of the Gcn4 transcription factor. Gcn4 then induces expression of amino acid biosynthetic/transport genes.

The origin of the present project can be found in previous investigations conducted by eng. Guillem Hueso. He performed an overexpression screening to identify genes involved in yeast tolerance to acid stress. The most frequently isolated gene was *LEU2*, complementing leucine (*leu2*) auxotrophy of the strain used.

According to the results of our investigations, we hypothesize that intracellular acidification inhibits not only leucine transport but also leucine-tRNA^{leu} synthetase, explaining the presence of an intracellular pool of leucine and activation of Gcn2 by uncharged tRNA^{leu}. Gcn2 would activate leucine transport by a mechanism independent of Gcn4.

TABLE OF CONTENTS

TABLE OF FIGURES

1. INTRODUCTION

1.1. GENERAL INTRODUCTION

Weak acids such as benzoic, acetic, propionic and sorbic acid are the major organic acid food preservatives used in food industry (Primo Yúfera, 1997). Their effect over bacteria and fungi is directly related to the concentration of the undissociated form of the acid. Hans Krebs and Alberto Sols demonstrated in 1983 in yeast that the mechanism of action of acid preservatives is based on the diffusion of the protonated weak acid into the microbial cells and its posterior dissociation inside them, causing intracellular acidification (Krebs et al., 1983). These findings were later confirmed in bacteria (Salmond et al., 1984).

The tolerance of microorganisms to these weak acids can have various components such as permeability of the membrane for the protonated acid, susceptibility of different cellular systems to intracellular acidification and even active expulsion of protons and anions by cellular transport systems. Although metabolic enzymes like the phosphofructokinase have been most studied as targets of toxicity of acidification (Krebs et al., 1983), cell division cycle is inhibited by acidification (Gillies, R.J. et al., 1981; Epel and Dubé, 1987; Portillo and Serrano, 1989) while programmed cell death is activated by acidification (Gottlieb et al., 1996; Ludovico et al., 2001).

The present work is part of the doctoral thesis of engineer Guillem Hueso Lorente, which aims at elucidating the principal defences and targets of intracellular acidification caused by weak organic acid preservatives by using molecular biological tools and *Saccharomyces cerevisiae* as model organism.

By now, growth of spoilage yeasts such as *Zygosaccharomyces bailii* and *Saccharomyces cerevisiae* can usually be retarded by weak organic acid preservatives; the inhibition often requires concentrations that are near the legal limits and is limited to a cytostatic effect (Lambert, R. J., and M. Stratford, 1999). Thus, adaptation responses eventually allow growth and spoilage by these organisms even at these high levels of preservatives (Deak, T. 1991 and Fleet, G. 1992).

The understanding of the mechanism of action of weak acids over microbial cells would allow a better design of food preservatives and a combat against adaptation responses of some contaminants. The results of this investigation could also be relevant for cancer investigation since on the one hand, the employed pharmaceuticals tend to cause cell death in tumours, availed by acidification and, on the other hand, tumour growth is associated with a high intracellular pH (Oberhaensli et al., 1986; Perona and Serrano, 1988). The development of new chemical compounds which regulate the pH in tumour cells could be inspired (Izumi et al., 2003).

After performing an overexpression screening in order to discover important genes involved in weak acid stress tolerance, Guillem Hueso identified *GLC7´* and *LEU2* (complementing leucine auxotrophy of the strain used) as significant for better growth. Mutants lacking *GCN2* exhibited extreme sensibility under acid conditions. The role of *GLC7´*, a truncated version of the protein phosphatase type 1, was investigated by Vanessa García in the same laboratory.

For this project, the following hypothesis was established: "Acid stress inhibits leucine uptake; the resulting intracellular leucine starvation drives to a higher concentration of uncharged $tRNA^{leu}$ which activates Gcn2p. The latter confers tolerance to acid stress by induction of Gcn4p, a master regulator of gene expression during amino acid starvation in yeast (Marton J. et al., 2001)."

The present work is aimed at confirming this hypothesis by studying the importance of leucine for the activation of the GCN pathway and the significance of that pathway itself for weak acid stress tolerance.

During more than 5 months of laboratory work, parameters like quantification of intracellular acidification, leucine transport, intracellular leucine levels, the localization of a specific leucine transporter, the sensibility of *Δgcn*-mutants and the control of activation of Gcn2p were investigated using a range of different molecularbiological methods.

1.2. ACIDS IN FOOD TECHNOLOGY

1.2.1. Historical introduction

Microbial spoilage of food causes losses of up to 40 % of all food grown for human consumption worldwide. Yeast growth is a major factor in the spoilage of foods and beverages that are characterized by a high sugar content, low pH, and low water activity, and it is a significant economic problem. Although most types of yeast are beneficial for many aliments, some harmful species exist. The latter are more resistant to stressful environmental conditions such as low water activity, low temperature, low pH values and antimicrobial agents (Orberá T., 2004). The resistance to acid pHs is important, due to the use of organic acids as food preservatives.

Actually, growth of spoilage yeasts such as *Zygosaccharomyces bailii* and *Saccharomyces cerevisiae* is usually retarded by weak organic acid preservatives, the inhibition often requires levels of preservative that are near or greater than the legal limits (Hazan R. et al., 2004).

In fact, the documented use of weak-acid preservatives to inhibit growth of micro-organisms in foods and beverages extends back many centuries. John Evelyn in 1670 described the use of sulphur dioxide from burning sulphur in the preservation of cider (Rose and Pilkington 1989). Sulphur dioxide and sulphites continue to be the method of choice for the preservation of wine. Other weak-acid preservatives include acetic acid in pickles, propionic acid in bread and sorbic and benzoic acids in soft drinks (Chichester and Tanner 1972).

1.2.2. Acid impact on *Saccharomyces cerevisiae*

Under aerobic conditions, the yeast *Saccharomyces cerevisiae* can use short-chain organic acids, such as acetic acid, as a carbon source (Thomas et al., 2002). As a consequence of the utilization of poor carbon sources, cells suffer metabolic reprogramming in order to adapt to the new situation (Paiva et al., 2004); translation machinery is activated, as well as mitochondrial biogenesis (Flores et al., 2000).

Microorganisms diminish their yield during acid stress because ATP is consumed for stabilizing their internal pH. In *Saccharomyces cerevisiae*, one mol of ATP is consumed for each mol of acetic acid which enters the cell. The growth inhibition caused by acetic acid depends on the pH, the buffering capacity of the media and the total amount of organic acid added (Thomas et al., 2002).

Many of the determinants of acetate resistance in *Saccharomyces cerevisiae* differ from the determinants of resistance to the more lipophilic sorbate and benzoate (Bauer et al., 2003). *Saccharomyces cerevisiae* is able to grow in the presence of sorbate due to the War1p transcription factor-dependent induction of a single ATP-binding cassette (ABC) transporter, Pdr12p (Piper P. et al., 1998 and Kren A. et al., 2003). Such is the strength of Pdr12p induction in sorbate-stressed cells, that levels of this transporter in the plasma membrane approach those of the most abundant plasma membrane protein, plasma membrane H^+-ATPase (Piper p. et al., 2001 and Kren A. et al., 2003). Pdr12p appears to be acting as an efflux pump for weak organic carboxylate anions. It has been directly shown that it lowers the intracellular levels of benzoate and fluorescein by catalysing an active efflux of these compounds from the cell (Kren A. et al., 2003 and Holyoak et al., 2000).

Pdr12p does not confer resistance to either acetate (Bauer et al., 2003) or highly lipophilic, long chain acids (Holyoak et al., 2000). Acetate is far less inhibitory to yeasts than the more lipophilic sorbate (trans,trans-hexanedienoate), even though these are two carboxylate compounds of identical pKa (4.76) (Piper et al., 1998; 65. Stratford, M. & Anslow, P.A., 1996; Stratford, M. & Anslow, P.A., 1998 and Bracey, D., Holyoak, C.D. & Coote, P.J., 1998). This is thought to be because the latter compound has a much higher capacity to dissolve in membranes and so disorder membrane structure (Piper et al., 2001). Nevertheless *S. cerevisiae* is often inhibited by the presence of high acetate levels in wine fermentations (Pretorius, I.S., 2000) partly through the glyoxylate cycle enzymes needed for the assimilation of acetate and propionate in aerobic cultures being glucose-repressed (Epstein, C.B., et al., 2001 and references therein).

In tryptophan biosynthetic pathway mutants, hypersensitivity to acetate and sorbic acid is suppressed by supplementing the medium with high levels of tryptophan or, in the case of sorbate sensitivity, by overexpressing the Tat2p high affinity tryptophan permease. Weak acid stress therefore inhibits uptake of aromatic amino acids from the medium (Bauer et al., 2003). In leucine auxotrophic mutants this hypersensitivity is also suppressed by overexpressing leucine (Hueso G., data not published).

1.2.3. Weak acids: Mode of action

Weak-acid preservatives appear to share a common mode of action, despite their various chemical structures. All become increasingly potent as antimicrobial agents at more acidic pH values. In aqueous solution, weak-acids exist in pH-dependent equilibria between uncharged acid molecules and their respective charged anions, for example acetic acid/acetate. The proportion of undissociated acid increases as the pH declines; the pH value at which there exists equal proportions of molecular acid and charged anions, is termed the pKa. Proportions of dissociated and undissociated forms of weak acid preservatives at each pH can be calculated using the Henderson-Hasselbalch equation:

$$pH = pK_a + \log\frac{[A^-]}{[HA]}$$

It is generally agreed that only undissociated acids have antimicrobial activity, although some activity by anions has been suggested (Eklund 1989).

The mechanism of action of weak-acid preservatives is thought to involve diffusion of lipophilic acid molecules through the plasma membrane into the cytoplasm (Stratford and Rose 1986). There they encounter a pH value near to neutrality and are forced to dissociate into charged ions. Charged ions cannot return across the plasma membrane and anions are thus concentrated within the cell (**Figure 1**) (Lambert R.J. and Stratford M., 1998). Dissociation of each weak-acid molecule will release a proton and the cytoplasm will become increasingly acidic. Acidification of the cytoplasm may prevent growth by inhibition

of glycolysis (Krebs et al. 1983), by prevention of active transport (Freese et al. 1973) or by interference with signal transduction.

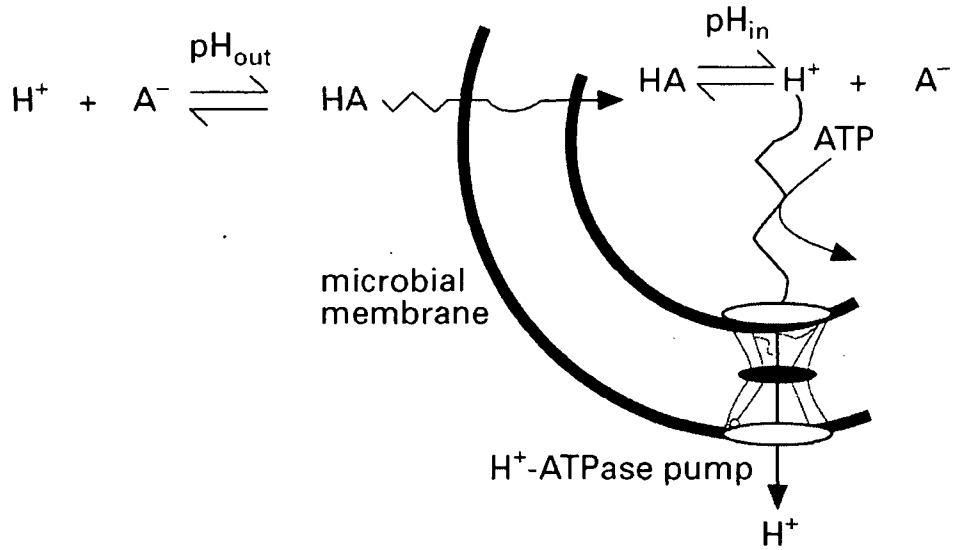


Figure 1: Predicted medium and cytoplasmic weak-acid/anion equilibria. Only uncharged weak-acid molecules (HA) can diffuse freely across the plasma membrane. Charged anions (A$^-$) and protons (H+) are retained within the cell; cytoplasmic protons are expelled by the membrane-bound H+-ATPase.

The internal pH is then, one of the most important factors in yeast physiology (Imai and Ohno, 1995).

1.3. SACCHAROMYCES CEREVISIAE

1.3.1. Yeast is a Model System

In "Getting started with yeast" (2002), F. Sherman justifies the use of the yeast *Saccharomyces cerevisiae* as a model system representing a simple eukaryote which genome can be easily manipulated. Some of the properties that make yeast particularly suitable for biological studies include rapid growth, dispersed cells, the ease of replica plating and mutant isolation, a well-defined genetic system, and most important, a highly versatile DNA transformation system (Gietz R.D., Woods R.A. 2002). Being nonpathogenic, yeast can be handled with little precautions. Large quantities of normal bakers' yeast are commercially available and can provide a cheap source for biochemical studies.

Strains of *S. cerevisiae*, unlike most other microorganisms, have both a stable haploid and diploid state, and are viable with a large number of markers. The development of DNA transformation has made yeast particularly accessible to gene cloning and genetic engineering techniques. Structural genes corresponding to virtually any genetic trait can be identified by complementation from plasmid libraries. Plasmids can be introduced into yeast cells either as replicating molecules or by integration into the genome. In contrast to most other organisms, integrative recombination of transforming DNA in yeast proceeds exclusively via homologous recombination. Cloned yeast sequences, accompanied with foreign sequences on plasmids, can therefore be directed at will to specific locations in the genome.

Genes can be directly replaced at high efficiencies in yeasts and other fungi, but only with difficulty in other eukaryotic organisms. Also unique to yeast, transformation can be carried out directly with short single-stranded synthetic oligonucleotides, permitting the convenient productions of numerous altered forms of proteins. These techniques have been extensively exploited in the analysis of gene regulation, structure–function relationships of proteins, chromosome structure, and other general questions in cell biology.

S. cerevisiae was the first eukaryote whose genome was completely sequenced (Goffeau A. et al., 1996). Subsequently, yeast became one of the key organisms for genomic research (Kumar A., Snyder M., 2001). The overriding virtues of yeast are illustrated by the fact that mammalian genes are routinely being introduced into yeast for systematic analyses of the functions of the corresponding gene products. Many human genes related to disease have orthologues in yeast (Ploger R. et al., 2000), and the high conservation of metabolic and regulatory mechanisms has contributed to the wide-spread use of yeast to as a model eukaryotic system for diverse biological studies. The ease to manipulate the genome of yeast is truly unprecedented for any other eukaryote.

1.3.2. Stress

As per Ian W. Dawes (2004), the unicellular organism yeast is exposed to many types of environmental insult, including sudden changes in temperature, desic-

cation, oxidative and osmotic stress, exposure to high salt or heavy metal ions or toxic chemicals. In order to combat these kinds of stress yeast cells have evolved a wide range of responses to many different types of stress, including osmotic, heat, oxidative, starvation, low pH and organic solvents (including the toxic effects of ethanol). Some of these defences are intrinsic (constitutive) and depend on the prior physiological or metabolic conditions under which the yeast has been grown, specially important are the growth phase and the stage of the organism in its life cycle. Other defence systems are inducible following stress.

The inducible responses differ from one stress to another, but it is now clear that these can be overlapping, so that acquisition of resistance to one form of stress leads to cross resistance to others. The existence of regulatory pathways leading to induction of resistance comes from a range of sensing systems, identified by signal transduction pathways and the transcription factors they influence; these form an important part of the stress responses in yeast and are presently very actively under study.

The present work focuses on stress induced by low pH, aiming at understanding the role of the GCN pathway which seems to be activated in response to this kind of stress.

1.4. GENERAL AMINO ACID CONTROL (GCN) PATHWAY

The capacity of adaptation to changes of intra- and extracellular conditions is a universal requirement for the survival and evolution of an organism (Rodrigues-Pousada et al., 2005). In response to environmental changes, all cells undergo a fast transcriptional reprogramming by mobilizing activators and transcriptional repressors (Hinnebusch and Natarajan, 2002). Simple cell organisms like *Saccharomyces cerevisiae* are able to adapt fast to extreme changes, like salinity or nutrients availability (Goossens et al., 2001).

Protein phosphorylation is a reversible mechanism which plays an essential role in the release of responses to extracellular signals and in the subsequent regulation of its metabolic pathways. (Ballou and Fischer, 1986; Cohen, 1989). The phosphorylation state of a certain substrate is determined by the relative activities of the protein kinases and the protein phosphatases (Edelman et al., 1987; Hanks et al., 1988; Cohen, 1989).

The functioning of the GCN pathway depends on phosphorylation. Important proteins involved are Gcl7p, Gcn2 and Gcn4p.

1.4.1. GLC7

GLC7 gene is essential for *Saccharomyces cerevisiae* and encodes the catalytic subunit of the protein phosphatases serina/treonina type 1 (PP1). (Andrews and Stark, 2000). Glc7p interferes in numerous physiological processes, among others in glycogen metabolism, glucose repression, translation initiation, transcription, sporulation and mitosis control. (Wek et al., 1992; Cannon et al., 1994; Tu y Carlson, 1994; Black et al., 1995; Zhang et al., 1995; Ramaswamy et al., 1998). It needs subunits or no-catalytic domains in order to perform its wide range of physiological activities (Venturi et al., 2000).

The affinity model for each no-catalytic or regulating subunit is different, what suggests that the unions at the catalytic domain of PP1 could be competitive (Ramaswamy et al., 1998). A truncated version of the protein phosphatase (Glc7'p) inhibits the function of Glc7p and so the dephosphorylation of the translation initiation factor 2 (eIF-2α) (Wek R.C. et al., 1992).

1.4.2. Translation initiation factor 2 (eIF-2α)

The phosphorylation of the translation initiation factor 2 (eIF-2α) is an important mediator of the translational control in response to environmental stresses; it is conserved from yeast to mammals (Pain, 1994; Wek, 1994; Clemens, 1996; of Haro Et al., 1996).

Plants eIF2α is also specifically phosphorylated by Gcn2p (a protein kinase which has the opposite function of Glc7p) in vivo and regulates translation in a

similar way to yeasts eIF2α (Chang et al., 2000). This is another example for the conservation of the translation mechanism among eukaryotic organisms what stresses the utility of yeast as model organism for the analysis of the role of heterologous components in the translational control.

1.4.3. GCN2

Gcn2p ("General Control Nondepressible") is a protein kinase which works contrarily to Glc7p phosphatase, regulating the expression of genes related with amino acid biosynthesis in yeast (Wek et al., 1992), and modifying the phosphorylation level of eIF-2α. It is activated by uncharged tRNAs and the Gcn1p-Gcn20p complex (Kubota H, et al., 2001; Garcia-Barrio M, et al., 2000). This supposes a switch from an inhibited to a catalytic confirmation which causes direct contact between the protein kinase domain, the regulating region HisRS and the carboxi terminal end of Gcn2p (Qiu et al., 1998).

Structure and components of the protein Gcn2p are shown in **Figure 2**. As a result of its activation, translation of Gcn4p is stimulated.

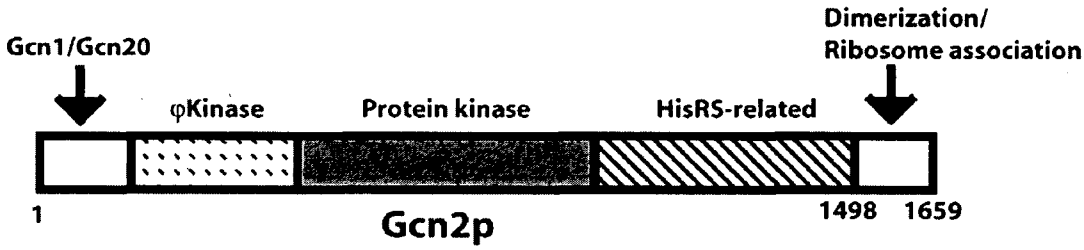


Figure 2: Structure of Gcn2p protein of Saccharomyces cerevisiae (Narasimhan Et al., 2004)

1.4.4. GCN4

GCN4 encodes a transcriptional activator (Hinnebusch A.G. and Fink G.R., 1983). Hinnebusch (2001) proposed that Gcn4p is a transcriptional activator of at least 539 genes, a quarter of them involved in amino acid biosynthesis. In addition to the derepression of genes involved in 19 out of 20 amino acid biosynthetic pathways, Gcn4p may directly or indirectly regulate the expression of genes involved in purine biosynthesis, organelle biosynthesis, autophagy, glycogen homeostasis, and multiple stress responses (Natarajan K, et al., 2001).

The level of Gcn4p is tightly regulated. Gcn4p is rapidly degraded by the ubiquitin pathway during conditions of non-starvation, but its half-life dramatically increases during amino acid starvation (Kornitzer D, et al., 1994; Meimoun A, et al., 2000). Translational regulation also contributes to the level of Gcn4p in the cell. Four small upstream ORFs (uORF1-4) in the 5' leader region of the GCN4 mRNA act as negative regulators of translation (Hinnebusch A.G., 1984). The ribosome initiates translation at uORF1 and becomes re-activated for translation at subsequent uORFs. Under environmental stresses, such as amino acid starvation, purine limitation, or nitrogen limitation, the translation of *GCN4* is induced in a *GCN2*-dependent pathway (Hinnebusch A.G., 1997); the scanning ribosome is not re-activated until it bypasses the uORFs and initiates translation at the *GCN4* ORF (Hinnebusch A.G., 1997).

Not all genes taking part in the amino acid biosynthetic pathways are regulated by Gcn4p. Nevertheless, not transcriptionally induced enzymes can be indirectly activated by Gcn4p through allosteric control (Natarajan et al., 2001; Hinnebusch y Natarajan, 2002). The deficiency of the nucleotide purine increases the *GCN4* expression as well, thanks to the action of Gcn2p what supports the idea that coordination between the amino acids and nucleotides synthetic pathways exists (Rolfes and Hinnebusch, 1993).

1.4.5. Acid stress response

Acid stress may prevent active transport (Freese et al. 1973), what leads to a higher concentration of uncharged tRNA. Gcn2p measures the amount of uncharged transfer RNA (tRNA) (Hoffmann et al., 1999), which causes the phosphorylation of eIF2α in serine 51. The function of eIF2 in translation is transferring its initiator Met-tRNA to the ribosome; with a binary complex eIF2-GTP the ribosome forms the ternary complex eIF2-GTP-Met.tRNA (Merrick, 1992).

Progressing with initiation, GTP hydrolyzes off the ternary complex and eIF2 is freed from its union to GDP and the ribosome (Dever, 1997). Thus, eIF2-GTP levels lower and the level of translational expression of Gcn4p is increased (Narasimhan et al., 2004). The mechanism of action of the GCN pathway is shown in **Figure 3**.

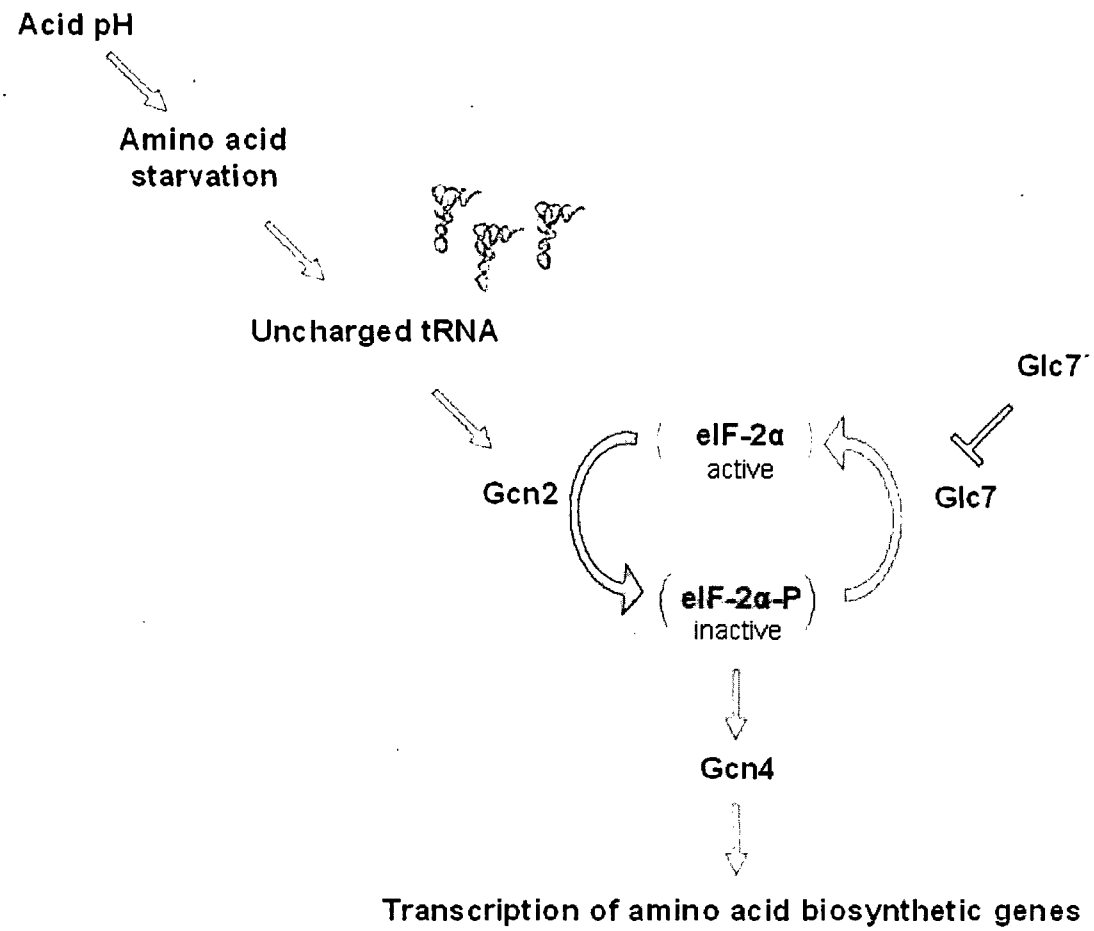


Figure 3: Mechanism of action of the genes GCN2, GCN4 y GLC7 in the general amino acid control.

During glucose starvation as well as during amino acid starvation, the accumulation of uncharged transfer RNA in the cell contributes to the activation of Gcn2p (Yang et al., 2000). Nevertheless, in response to both types of starvation there are remarkable differences in the mechanism of regulation:

Amino acid limitation:

- An alteration of its levels contributes to the induction of *GCN4*
- An association between the protein kinases Gcn1, Gcn2p and ribosomes is necessary for the phosphorylation of eIF-2 (Marton et al., 1997).

Glucose limitation:

- In order to phosphorylate the initiation factor, only the Gcn1p domain has to associate with the ribosomes (Vázques de Aldana et al., 1995)

- Amino acid levels decrease in the cytoplasm but augment in the vacuoles. They are nitrogen residues in form of amino acids, for future use.
- Glycogen levels decrease as an indirect consequence of the diminution of intermediate metabolites (Wek et al., 1992).

1.5. AMINO ACIDS

1.5.1. Classification and structure of amino acids

As per M.W. King (2006), all peptides and polypeptides are polymers of alpha-amino acids. There are 20 a-amino acids that are relevant to the make-up of mammalian proteins (see **Table 1**). Several other amino acids are found in the body free or in combined states (i.e. not associated with peptides or proteins). These non-protein associated amino acids perform specialized functions.

Figure 4 shows the general structure of amino acids. The a-amino acids in peptides and proteins (excluding proline) consist of a carboxylic acid (-COOH) and an amino (-NH2) functional group attached to the same tetrahedral carbon atom. This carbon is the α-carbon. Distinct R-groups, that distinguish one amino acid from another, also are attached to the alpha-carbon, except in the case of glycine where the R-group is hydrogen. The fourth substitution on the tetrahedral a-carbon of amino acids is hydrogen. There are two broad classes of amino acids based upon whether the R-group is hydrophobic or hydrophilic.

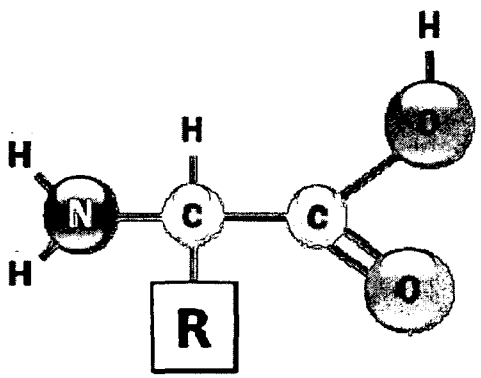
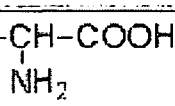
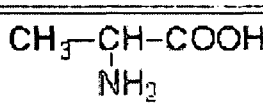
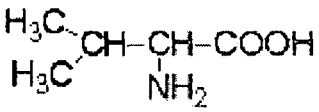
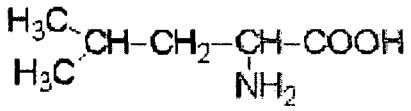
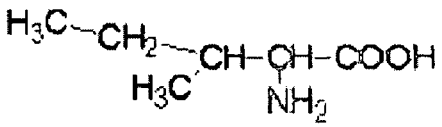
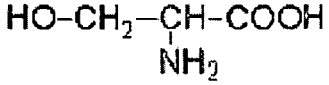
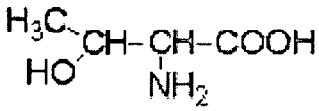
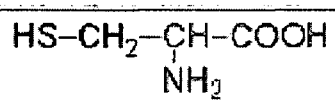
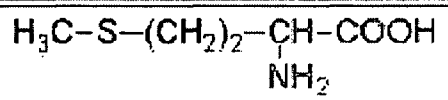
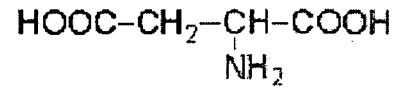
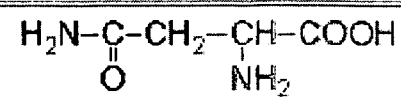
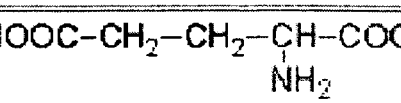
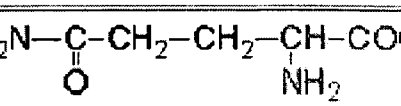
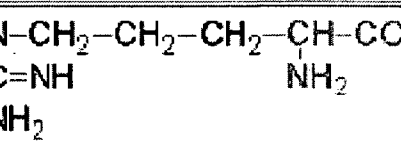
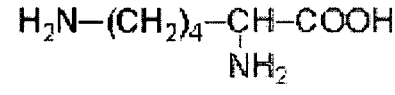
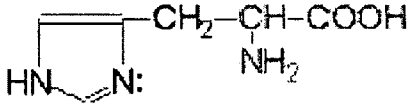
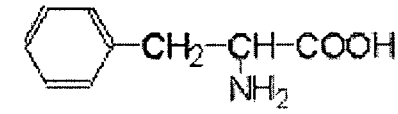
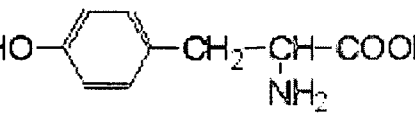
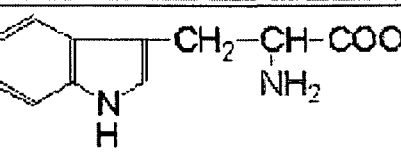
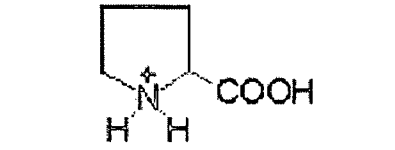


Figure 4: General amino acid structure.

Table 1: Classification and structure of amino acids.

Amino Acid	Symbol	Structure*	pK_1 (COOH)	pK_2 (NH_2)	pK R Group
Amino Acids with Aliphatic R-Groups					
Glycine	Gly - G		2.4	9.8	
Alanine	Ala - A		2.4	9.9	
Valine	Val - V		2.2	9.7	
Leucine	Leu - L		2.3	9.7	
Isoleucine	Ile - I		2.3	9.8	
Non-Aromatic Amino Acids with Hydroxyl R-Groups					
Serine	Ser - S		2.2	9.2	~13
Threonine	Thr - T		2.1	9.1	~13
Amino Acids with Sulfur-Containing R-Groups					
Cysteine	Cys - C		1.9	10.8	8.3
Methionine	Met-M		2.1	9.3	

Acidic Amino Acids and their Amides					
Aspartic Acid	Asp - D		2.0	9.9	3.9
Asparagine	Asn - N		2.1	8.8	
Glutamic Acid	Glu - E		2.1	9.5	4.1
Glutamine	Gln - Q		2.2	9.1	
Basic Amino Acids					
Arginine	Arg - R		1.8	9.0	12.5
Lysine	Lys - K		2.2	9.2	10.8
Histidine	His - H		1.8	9.2	6.0
Amino Acids with Aromatic Rings					
Phenylalanine	Phe - F		2.2	9.2	
Tyrosine	Tyr - Y		2.2	9.1	10.1
Tryptophan	Trp-W		2.4	9.4	
Imino Acids					
Proline	Pro - P		2.0	10.6	

Of the 20 standard proteinogenic amino acids, 10 are called essential amino acids because the human body cannot synthesize them from other compounds through chemical reactions, and they therefore must be obtained from food. Cysteine, tyrosine, histidine and arginine are considered as semiessential amino acids in children, because the metabolic pathways that synthesize these amino acids are not fully developed (Imura K., Okada A., 1998).

Table 2: Clasification of amino acids

Essential	Nonessential
Isoleucine	Alanine
Leucine	Asparagine
Lysine	Aspartate
Methionine	Cysteine
Phenylalanine	Glutamate
Threonine	Glutamine
Tryptophan	Glycine
Valine	Proline
Arginine*	Serine
Histidine*	Tyrosine

(*) Essential only in certain cases.

1.5.2. Leucine

1.5.2.1. Leucine biosynthesis

Leucine and valine are synthesized following the same superpathway. This superpathway starts from pyruvate which is transformed into 2-keto-isovalerate. At that point, leucine and valine biosynthesis pathway separates. Leucine synthesis is shown in **Figure 5**.

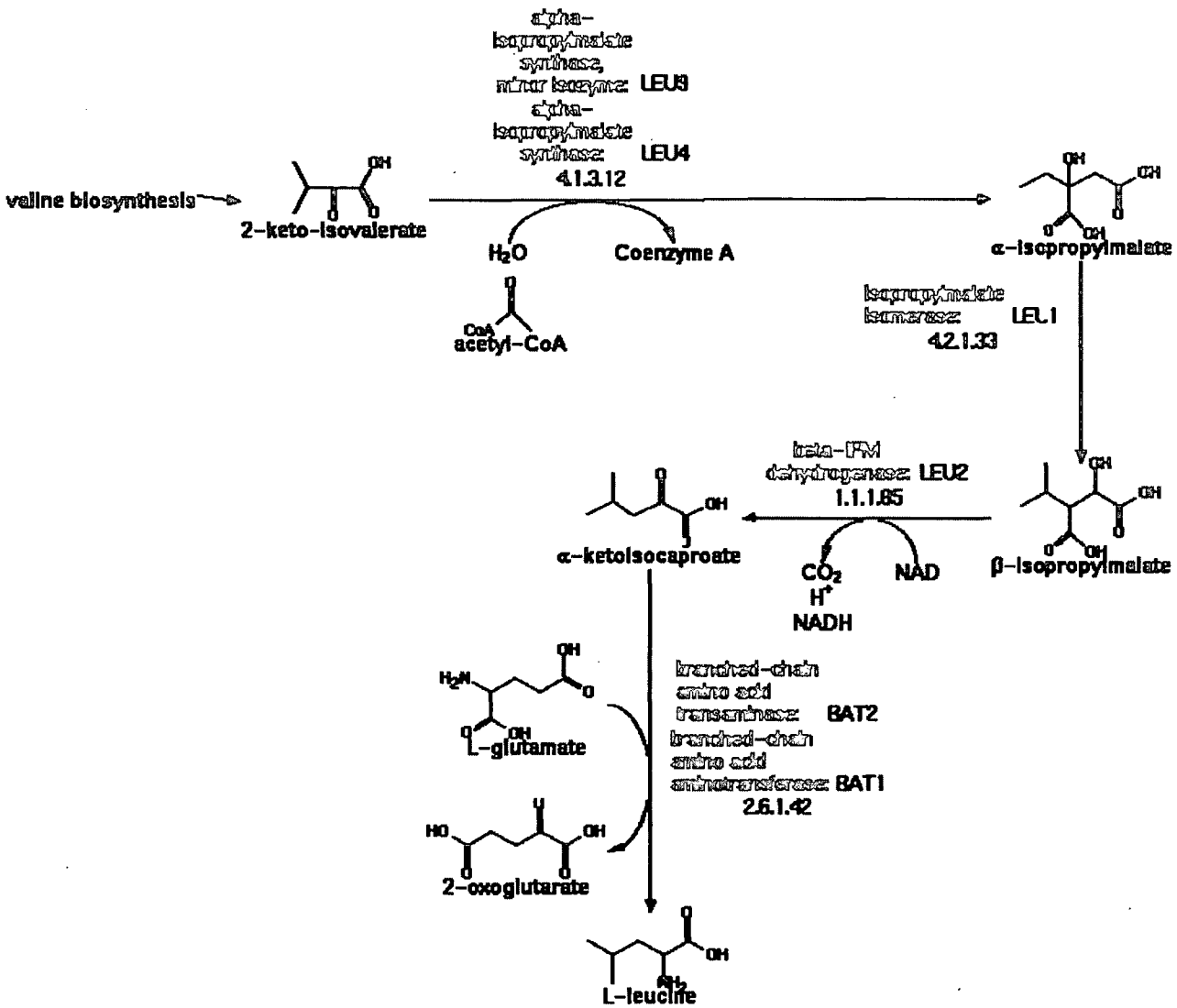


Figure 5: Leucine biosynthesis.

1.5.2.2. Leucine transport

The yeast *Saccharomyces cerevisiae* takes up amino acids from the surroundings to serve either as nitrogen source or directly in protein biosynthesis. The

transport across the plasma membrane is active, driven by the proton gradient, via amino acid permeases (Horák, 1997).

The transport of isoleucine, leucine and valine has been investigated thoroughly (Grenson et al. 1970; Tullin et al. 1991; Kotliar et al. 1994; Grauslund et al. 1995; Didion et al. 1998), and three permeases besides Gap1p (the general permease for most uncharged amino acids) have been identified as transporters of the branched-chain amino acids (Grauslund et al. 1995; Didion et al. 1998), namely Bap2p, Bap3p and Gnp1p. Bap2p and Bap3p have a less-broader specificity, only transporting isoleucine, leucine, valine, cysteine, methionine, phenylalanine, tyrosine and tryptophan.

All four permeases are transcriptionally induced by an extracellular amino acid, but differ in expression with respect to the nitrogen source. On a non-repressive nitrogen source, *GAP1* is induced while *BAP2* and *BAP3* are not. They are only induced on ammonium-based medium (Regenberg B. et al., 1999; Didion T. et al. 1996, 1998). Knowing that there are so many permeases that can transport each of the neutral amino acids, it must be noted that these systems have different affinities and different expression levels, which will determine the relative importance of a given permease.

Overall, the highest transcriptional activity for most promoters was found when Leu was present in ammonium-based medium (Regenberg B. et al., 1999). Together with Gap1p, Bap2p seems to be the most important transmembrane-transporter for branched-chain amino acids (leucine, isoleucine and valine) (Didion T. et al, 1996).

2. OBJECTIVES

Guillem Hueso from Ramón Serrano's group (Institute of Plant Molecularbiology and Cellbiology, UPV Valencia) had previously identified *GLC7´* and *LEU2* (complementing leucine auxotrophy of the strain used) as significant for better growth and exhibited extreme sensibility under acid conditions for mutants lacking *GCN2*. The role of *GLC7´*, a truncated version of the protein phosphatase type 1, was investigated by Vanessa García in the same laboratory.

For this project, the following hypothesis was established: "Acid stress inhibits leucine uptake; the resulting intracellular leucine starvation drives to a higher concentration of uncharged tRNA which activates Gcn2p. The latter confers tolerance to acid stress by induction of Gcn4p, a master regulator of gene expression during amino acid starvation in yeast (Marton J. et al., 2001)."

The present work aims at confirming this hypothesis by studying the importance of leucine for the activation of the GCN pathway and the significance of that pathway itself for weak acid stress tolerance.

During more than 5 months of laboratory work, parameters like quantification of intracellular acidification, leucine transport, intracellular leucine levels, the localization of a specific leucine transporter, the sensibility of *Δgcn*-mutants and the control of activation of Gcn2p were investigated using a range of different molecularbiological methods.

3. MATERIALS AND METHODS

3.1. YEAST STRAINS AND PLASMID DESCRIPTION:

3.1.1. Yeast strains

In the following table the yeast strains used in this project are shown.

Table 3: Yeast strains

Strain name	genotype	Source	Referred to as
BY4741	Mat a; *his*3Δ1; *leu*2Δ0; *met15*Δ0; *ura3*Δ0	Euroscarf	Wild type (wt)
YDR283c	BY4741; *gcn2*::*kan*MX4	Euroscarf	Δ*gcn2*
YEL009c	BY4741; *gcn4*::*kan*MX4	Euroscarf	Δ*gcn4*
BAP2-GFP	BY4741; BAP2-GFP	Huh W.K. et al., 2003	Bap2-GFP

3.1.2. Plasmids

pYPGE15 pHluorin: ura yeast episomal plasmid; contains the bacterial origin of replication derived from the pBR322 and the yeast 2-µm origin as well as URA3 and AmpR as marker genes. The pHluorin is inserted into the EcoRI and BamHI restriction sites. The pHluorin sequence was generated by mutating several residues of the GFP gene (Miesenbock et al., 1998)

pUN100: Derives from the base vector pUN0. It was created by a triple ligation of *Aat*II(flush)-*Eco*RI *bla* fragment of pUN0 with the *Eco*RI-*Xho*I fragment of pUN90 and the 2,2-kb *Sal*I(flush)-*Xho*I *LEU2* (Andreadis et al., 1982). pUN vectors have the ability to replicate autonomously in S. cerevisiae and are stably maintained in low copy. The restriction map of pUN100 is shown in.

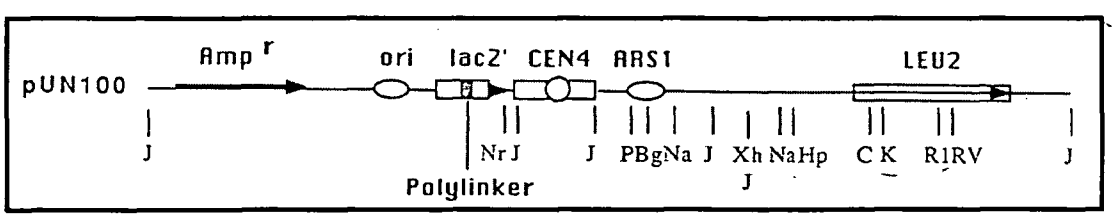


Figure 6: restriction map of pUN100. The Ampr marker is the bla gene from pUC18. The polylinker is pUC18. The symbol J is used to denote a junction between two DNA segments which was created in vitro.

YEp24: A pBR322 derivative containing the 1,1 kb Hind III fragment from the yeast URA 3+ gene, the 2,2 kb Eco RI fragment of yeast 2 μ with the origin of replication. AmpR, tetR (5 μg/ml tet). See **Figure 7**.

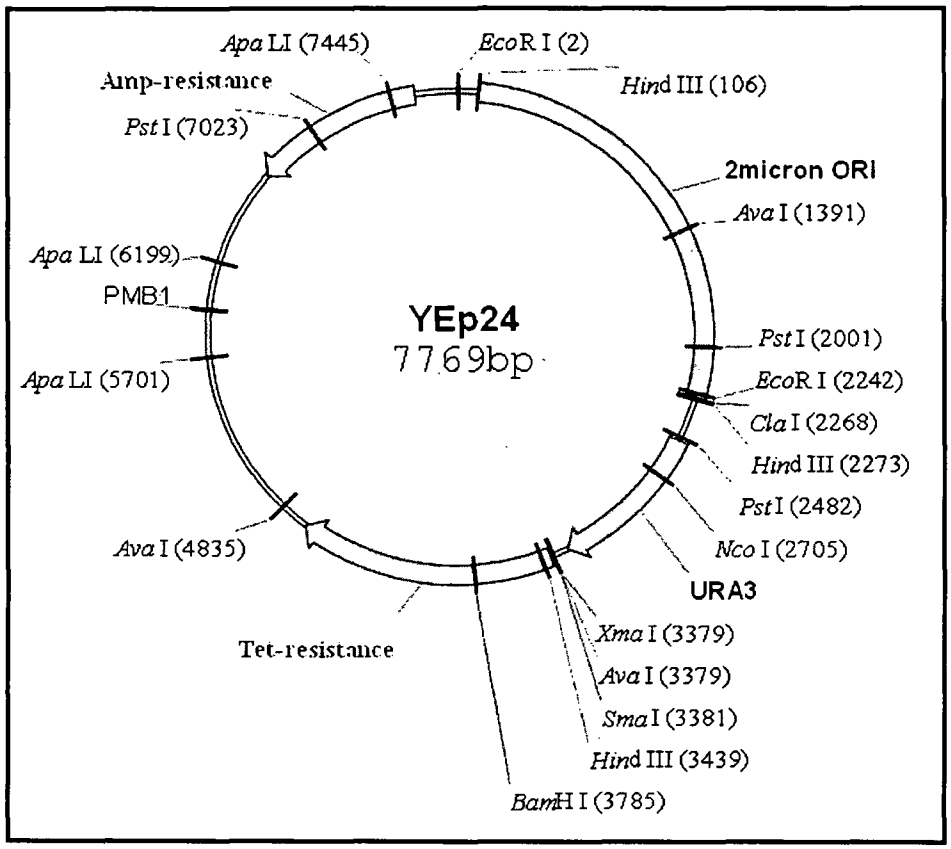


Figure 7: Plasmid YEp24. Used empty or with GLC7' for transformation of BY4741.

3.2. YEAST CULTIVATION

Yeast strains were cultivated at 28° C in either minimal synthetic glucose medium (SD) or rich medium (YPD). SD medium contained 2 % glucose, 0,7 % yeast nitrogen base without amino acids (Pronadisa) and 50 mM succinic acid adjusted to pH 5,5 with Tris (Tris-(hydroxymethyl)-aminomethane), plus the required amino acids (100 μg/ml leucine, 100 μg/ml methionine, 30 μg/ml histidine and 30 μg/ml uracile) as indicated. YPD medium contained 1 % yeast extract (Pronadisa), 2 % Bacto peptone (Pronadisa) and 2 % glucose. When used for

plates, YPD was supplemented with 2 % agar (Pronadisa). Media were supplemented with the additives and acids indicated in the Figure legends. To assure reproducible results at constant pHs, 50 mM succinic acid was added for buffering the media at pH 5,5 or pH 3,15. Yeast was only handled under sterile conditions.

3.3. PLASMID TRANSFORMATION "LAZY BONES" OF YEAST COLONIES

150 µl of saturated yeast culture grown overnight in liquid YPD media were centrifuged during 2 minutes at 800 rpm (Eppendorf Centrifuge 5415D) and the supernatant was discarded. The pellet was resuspended in 50 µl DNA carrier (ssDNA, 0,1 %) and 5 µl transforming plasmidic DNA. After vortexing, 0,5 ml PLATE solution (40 % polyethilenglycol 4000 (PEG, w/v); 100 mM lithium acetate (LiAc); 10 mM Tris, pH 7,5; 0,4 mM EDTA) were added and the solution was vortexed again.

The mix was incubated from 1 - 4 days at room temperature and heat shocked at 42° C during 15 minutes. After the subsequent centrifugation step (10 seconds at 8000 rpm), 300 µl supernatant were discarded and the pellet was resuspended in the rest approx. (250 µl). This mix was directly plated on selection media (solid SD containing required amino acids) and incubated at 28° C until the appearance of colonies.

3.4. ^{14}C-LEUCINE TRANSPORT

The use of radioactive labelled leucine (^{14}C-leucine) enables the measurement of leucine inside the cells. Sample taking over a time course allows the analysis of leucine uptake in yeast cells. One part of a yeast culture was treated with acetic acid in order to compare the leucine transport into the cell of stressed and unstressed cells.

For this experiment 50 ml of yeast culture in exponential growth phase (approx. 0,5*107 cells/ml) were splitted into two. One part was treated with 30 mM AcH and the other part was brought to a stable pH by adding 50 mM succinic acid pH 5,5.

After 1,5 hours incubation at 28° C, cell pellets were collected by centrifugation. The media was discarded and the pellets were washed with 25 ml MilliQ water. Pellets were resuspended in 3 ml starvation medium ("SuGlu"; 50 mM Succinic acid, 2 % Glucose) and incubated for 20 minutes at room temperature. 1,2 ml samples were taken from each cell culture and transferred to an eppendorf tube on ice. Duplicate 100 µl samples were taken from the four aliquots and mixed with 10 µl formaldehyde. They were later used for background counting and cell number measurement.

Then, the leucine mix (280 µl leucine (300 µM), 20 µl 14C LEU (0,1 mCi/ml) with a final concentration of 10 µM was added to all four culture aliquots. Samples were taken at Time 0; 0,5; 1; 1,5; 3; 6; 12 and 24 minutes and reaction was stopped putting samples on ice and adding 10 µl formaldehyde. Samples were transferred to a filtering cylinder containing 10 ml of cold water. The nitrocellulose millipore filter papers were placed in 6 ml vials and dried over night at 60° C. The next day, 2 ml of scintillation cocktail were added to each filter and measurement of radioactivity was effected with a scintillation counter. The results presented are averages of at least two independent experiments.

3.5. DETERMINATION OF INTERNAL CONTENT OF AMINO ACIDS

This experiment allows tracing the changes of cell internal amino acid levels within a time course. It was conducted using a 90 mM acetic acid treated cell culture and an untreated cell culture in order to evaluate the cell response to weak acid stress.

Cells were grown in YPD until an absorbance at 660 nm of 0,4-0,8, harvested by centrifugation and transferred to fresh YPD medium or to fresh YPD medium containing 90 mM acetic acid for 120 minutes. The cells were then washed with ice-cold water and extracted by heating 12 minutes at 95° C. The supernatant was collected by centrifugation during 1 minute at max. rpm and sent to the CNB laboratory in Madrid for the analysis of the internal amino acid content.

3.6. PROTEIN EXTRACTION

3.6.1. Simple extraction

Cell pellets were collected by centrifugation and stored at -80° C.

3.6.2. Fractionation

In order to compensate the low specificity of the Mouse α GFP antibody, sample purification was performed by separating the soluble and the insoluble fraction. The protein of interest, the Bap2 membrane transporter, was located in the insoluble fraction of the yeast cells. The fractionation was conducted as follows. 20 ml of a Bap2 transformed cell culture in mid-log phase was centrifuged during 4 minutes at 2000 rpm (Mixtasel) in order to obtain the pellet which was then washed once in 1 ml MilliQ water by transferring the solution to an eppendorf tube and spinning 20 seconds at maximum speed (Eppendorf Centrifuge 5415D). The supernatant was discarded and the pellet was stored at -80° C.

The next day, pellets were resuspended in 300 µl fractionation buffer (50 mM Tris-HCl pH 7,6; 0,1 M KCl, 5 mM EDTA, 5 mM DTE, 20 % sucrose, 1 tablet protease inhibitors/10 mls of buffer). After the addition of glass beads (500 µl) the solution was vortexed 3 times during 1 minute at 4° C. 200 µl more fractionation buffer was added and centrifuged 5 minutes at 2000 rpm. Subsequently, the supernatant (≈ 300 µl) was carefully transferred to a new tube and spinned 30 minutes at 14000 rpm at 4° C (Eppendorf Centrifuge 5415R). The supernatant was discarded; the pellet represented the insoluble fraction and was resuspended in 30 µl 2x LSB buffer. It could be directly used for SDS-PAGE.

3.7. WESTERN BLOT

3.7.1. SDS-PAGE electrophoresis

Protein samples were loaded on an 8 % acrylamide/bis-acrylamide gel. The SDS-PAGE electrophoresis was run at 50 volts until samples entered the re-solving gel and then the voltage was increased to 100-120 volts during 1 hour.

3.7.2. Protein transfer

The protein transfer from gel to membrane was performed by using the tank transfer method. 2 pieces of whatman paper, a nitrocellulose membrane (Pro-tran; Whatman), 2 sponges and the resolving gel were wetted with transfer buffer [100 ml Towbin buffer 10x (144 g/l glycine, 5,3 g Tris-base, 20 ml SDS 10 %, 1 L MilliQ water), 700 ml MilliQ water, 200 ml Methanol]. A sandwich of a sponge, a filter paper, the gel, the membrane, a filter paper and a sponge was prepared and air bubbles were removed. It was closed, the plastic gel cassette was secured and the gel cassette placed in the transfer system so that the membrane is on the same side as the anode. After adding an ice cassette, the transfer apparatus was filled to the top of the tank with 1x transfer buffer and run at 100 V for two hours at 4° C.

3.7.3. Detection

The membrane was removed from the transfer system and immersed in Pon-ceau S stain (0,1 % Ponceau S, 1 % Acetic acid) for 2-3 minutes. Then the membrane was washed by rinsing with distilled water until bands are visible. After scanning, the membrane was placed into blocking solution.

For western blots with the GFP-marked membrane transporter Bap2, blocking was done during 1 hour in TBS (150 mM NaCl, 20 mM Tris-HCl (pH 7,6)), 0,1 % Tween and 2 % nonfat milk while western blots with P-eIF-2α were blocked in TBS + 0,05 % Tween + 0,5 % nonfat milk during 2 hours, changing blocking solution after the first 15 minutes. Next, membranes were incubated overnight on a rocker at 4° C with the primary antibody. Mouse anti-GFP was diluted 1:10.000 in TBS, 0,1 % Tween and 2 % nonfat milk while rabbit anti-P-eIF-2α was diluted 1:2.000 in TBS, 0,05 % Tween and 0,5 % nonfat milk.

After incubation, the membrane was washed several times with TBS 1x-Tween 20 and incubated with the HRP labelled secondary antibody at room temperature. Anti-mouse (Bap2-GFP) was diluted 1:20.000 in TBS, 0,1 % Tween and 2 % nonfat milk and incubated during 1 hour while anti-rabbit (P-eIF-2 α) was diluted 1:10.000 in TBS, 0,05 % Tween and 0,5 % nonfat milk and incubated during 30 minutes.

For the chemiluminescence detection of the proteins, the "ECL Plus Western Blotting Detection Reagents Kit" Protocol was used. Membrane was covered with substrate working solution and incubated for 5 minutes, wrapped in transparent film and exposed from 5 seconds to 10 minutes. Film (Fuji medical XR-film) was developed using developing solution and fixative.

3.8. CYTOPLASMIC PH MEASUREMENTS

The relevant strains were transformed with the plasmid pYPGE15 containing the Phluorin coding region. Phluorin is a mutated version of wild type green fluorescent protein (GFP) which displays a reversible excitation ratio change between pH 7,5 and 5,5 and attached to the inner surface of a membrane, will reflect a pH change in its excitation spectrum (Miesenböck G. et al., 1998) (see **Figure 8**).

Cultures were grown in SD medium without succinic acid until exponential growth phase. All cultures were proved to contain the same cell number. Treatments were started by adding different dilutions of either acetic or sorbic acid. One part was used as untreated control. Various samples were taken within a time course and fluorescence was immediately measured in the Luminescence Spectrometer LS50B (Perkin Elmer). Samples for the tracking of cell culture growth were taken as well and analyzed with the Z2™ Coulter counter® (Beckman). For each employed yeast strain, a calibration curve was prepared by measuring samples at 8 different pHs adjusted with a buffer (50 mM MES, 50 mM HEPES, 50 mM KCl, 0,2 M ammonium acetate, 10 mM NaN_3 (Azide), 10 mM 2-deoxyglucose, 75 µM Monensin, 10 µM Nigericin) from pH 4,5 to 8.

To estimate the cytoplasmic pH, resulting experimental I405/I485 values from a given strain were compared to the calibration curve generated from this strain. All experiments were performed at 28° C.

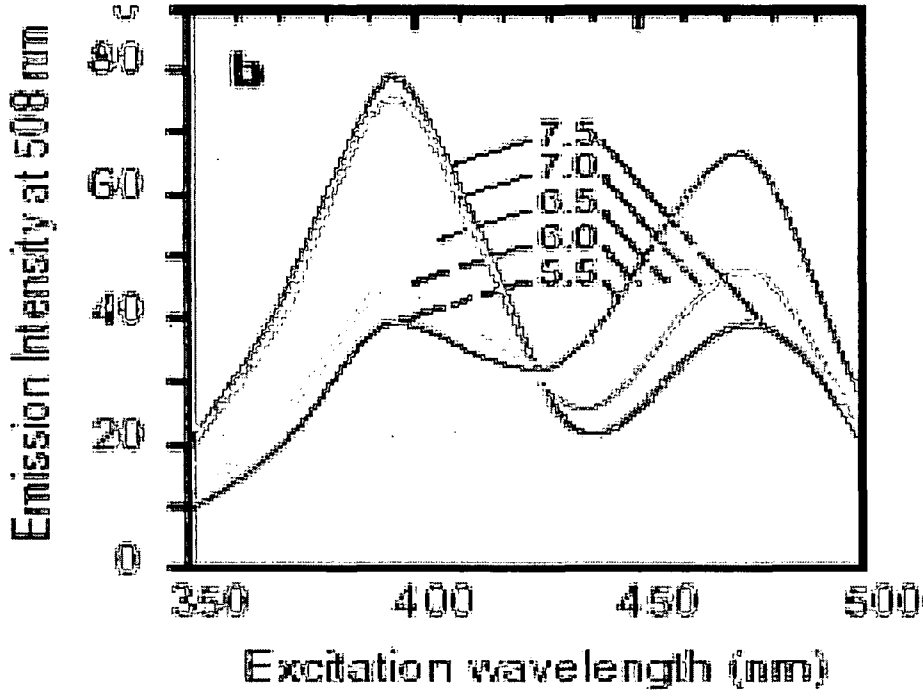


Figure 8: Fluorescente excitation spectra of ratiometric pHluorin. Samples contained 27,5 µM chromophore in 50 mM sodium cacodylate plus 50 mM sodium acetate, adjusted to the indicated pH values; and 100 mM NaCl, 1 mM CaCl2, 1 mM MgCl2. The ordinate scales reflect normalized differences in emitted fluorescence intensity (Miesenböck et al., 1998).

3.9. BIOSCREEN

The growth of cell cultures can be precisely analyzed with the performance of a bioscreen. It is based on the simultaneous measurement of the optical density of numerous cell cultures in small intervals during a certain time course. For this project, the experiment was conducted with various mutants of the wild type strain BY4741, with our without media acidification.

The starting point of the experiment were saturated cell cultures which were each diluted 1:200 in YPD media containing different acid concentrations: 0,8; 1,2; 1,5 and 1,8 mM sorbic acid and 15, 30, 60, 70 and 80 mM acetic acid were

used for treatments. Triplicates of each 350 µl were loaded on a multiwell plate which was then introduced into Bioscreen C (Termo Labsystems) for measurement which was done every 20 minutes after previous shaking (30 seconds). The total duration of the experiment was 3 days. Data were analyzed using MS Excel.

3.10. CONFOCAL MICROSCOPY

This technique enables visualization deep within both living and fixed cells and tissues and affords the ability to collect sharply defined optical sections from which three-dimensional renderings can be created (www.olympus-confocal.com). In this project, confocal microscopy was used to visualize the localization of the branched chain amino acid transporter Bap2. Yeast cells transformed with green fluorescent protein (GFP) labelled Gap2 were grown in YPD until mid-log phase and various samples of 2 µl were analyzed microscopically.

4. RESULTS

4.1. ACID STRESS CAUSES A DECREASE OF THE INTRACELLULAR PH IN YEAST.

The first assay was targeted on controlling changes of the cytoplasmic pH of yeast when media is supplemented with acids.

G. Miesenböck et al. had developed a pH sensitive green fluorescent protein (GFP)-version in 1998 by structure-directed combinatorial mutagenesis of GFP. Wild-type GFP has a bimodal excitation spectrum with peaks at 395 and 475 nm (Ward, W.W. et al., 1982 and Ward, W.W. 1981). Underlying the two maxima are protonated and deprotonated states of Tyr 66, which forms part of the chromophore (Heim, R. et al., 1994; Chattoraj et al., 1996; Ormö, M. et al., 1996; Yang, F. et al., 1996 and Brejc, K. et al., 1997). In order to converting GFP to a reliable pH sensor, histidines were introduced at 7 key positions. The obtained protein, called pHluorin, displays a reversible excitation ratio change between pH 7,5 and 5,5.

In this experiment, cell internal acidification was measured by addition of low doses of AcH or sorbic acid to the cultivation media. To avoid effects due to the toxic effect of anions and not to acidification, two different acids were used. To assure that the cultivation media does not contain auto fluorescent components, cells were grown in minimal synthetic media (SD) supplemented with the required amino acids (leucine, methionine, histidine at indicated concentrations).

Yeast cultures responded as expected with a rapid decline of intracellular pH and a cease of cell growth (data not shown) to the acidification of the media. 30 mM AcH provoke a decrease from pH 7,2 to 5,4 while addition of 0,4 mM sorbic acid leads to a lighter decrease from pH 7,2 to 6,3 after 15 minutes treatment. After about 15 to 20 minutes cells start getting adapted to the acid conditions as can be seen by the recovery of the pH (**Figure 9**) and the resuming of cell growth.

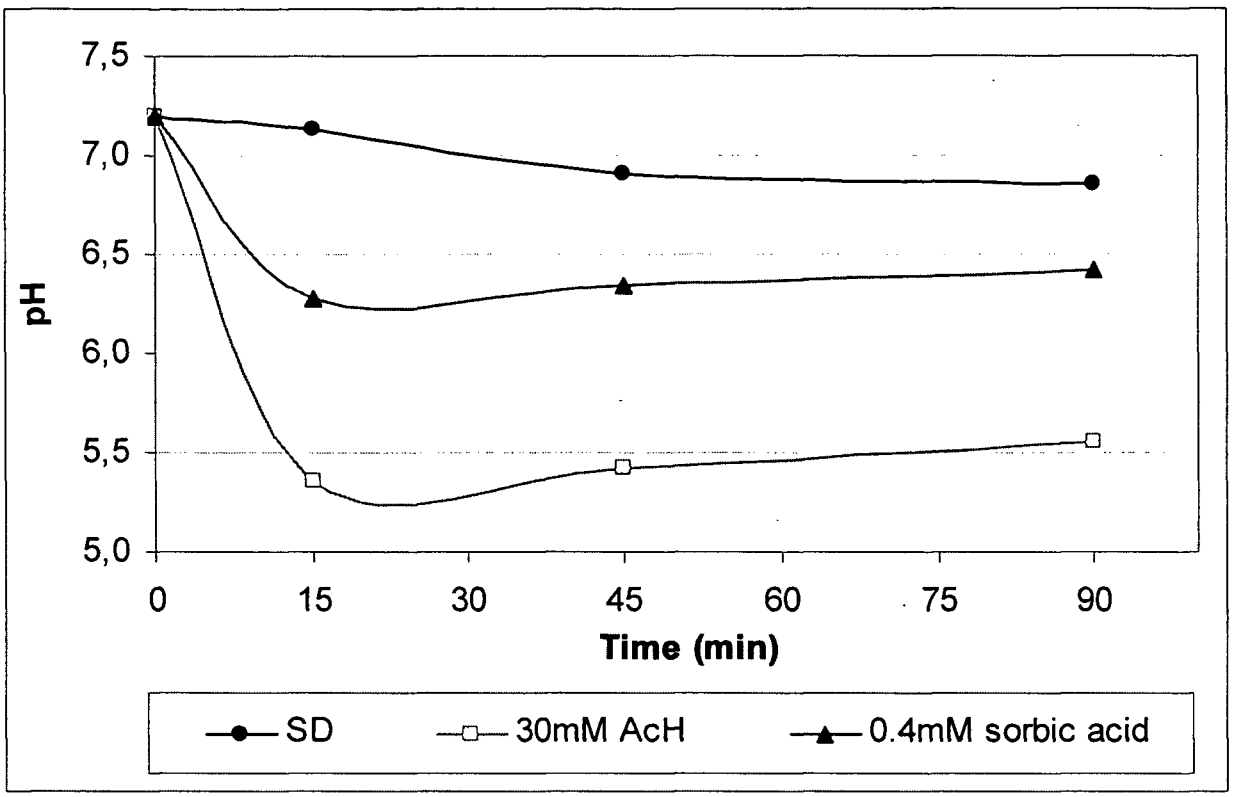


Figure 9: Intracellular pH. In SD grown mid-log phase BY4741 cells transformed with pYPGE15 pHluorin were supplemented with 30 mM AcH (red squares) or 0,4 mM sorbic acid (blue triangles); a control culture was grown only in SD media (black circles). Fluorimetric analysis was done at excitation spectrum λex = 380-500 nm, emission at λem = 508 nm.

4.2. YEAST'S SENSIBILITY TO ACID STRESS IS DOSE DEPENDENT

The next step of the investigation was to determine whether sensibility to acid stress is dose dependent. A bioscreen analysis was performed in order to control culture growth by measuring simultaneously and with high frequency the optical density (OD) of up to 200 samples (Bioscreen C, Termo Labsystems) at an optimal temperature (28° C). The possibility of tracing the development of various cell cultures coevally is a big advantage of this analyzing system.

Starting from a saturated preculture, a wild type strain was grown in YPD and treated with different kinds of acids at varying acid concentrations. Yeast growth

was measured every 20 minutes during 3 days. **Figure 10** shows that BY4741 starts its exponential growth after a lag-phase of 7-10 hours while acid treated cells show lag-phases of between 17 and more than 30 hours. In any case acid treated cell cultures reach optical densities higher than 2,4. Wild type cells enter stationary phase at an optical density of about 2,7. It can be seen that sensibility to acid stress is dose dependent.

As this sensibility can be observed for different kinds of acids, it can be deduced that it is a consequence of simple acidification and not of any possible toxic effect produced by a specific acid.

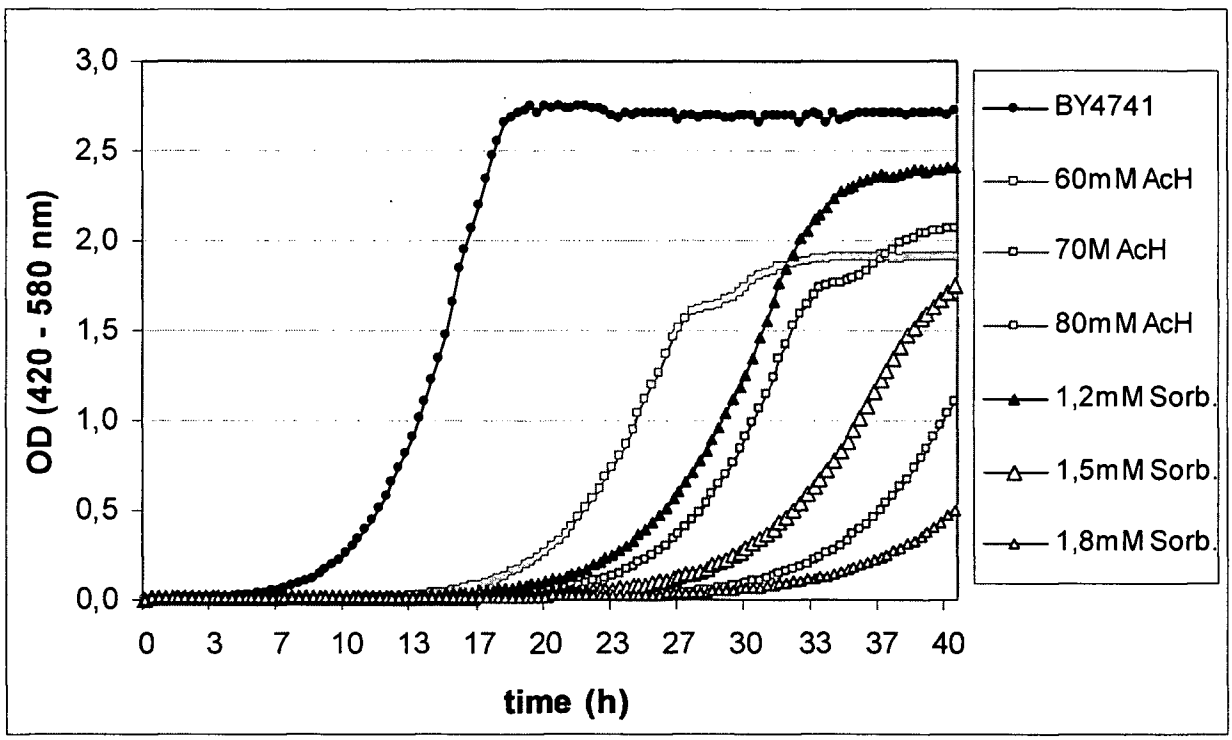


Figure 10: Sensibility to acid stress is dose dependent. Cultivation media of in YPD grown mid-log phase wt cells was supplemented with acetic acid or sorbic acid at the indicated concentrations.

4.3. LEUCINE IMPROVES GROWTH IN ACID MEDIA

As Guillem Hueso had identified leucine as an important factor for acid stress tolerance in previous investigations, growth of wild type cells and LEU-

prototroph mutants was compared performing a bioscreen analysis. Cell cultures were analyzed under normal and acid conditions.

It was expected to see a clear improvement of growth under acid conditions by both, leucine supplementation and the LEU-prototroph strain. As seen in **Figure 11**, growth of wild type and LEU-prototroph mutant in YPD media is comparable. In YPD media supplemented with 80mM AcH, the LEU-prototroph mutant shows a considerably better growth than the wild type in only YPD and with leucine supplemented YPD media.

Lag-phase of the LEU-prototroph in acid media lasts around 8 hours while leucine supplemented wild type starts to grow after about 25 hours lag-phase. Wild type in 80 mM AcH media starts to recover after 30 hours. Transformation or supplementation with other auxotrophic requirements of the strain such as histidine and uracil had a lower effect on acid tolerance (data not shown).

The obtained data indicate that leucine does improve growth in acid media, but better when the amino acid is synthesized by the cell itself. This leads to the presumption that leucine uptake from the media may be inhibited under acid stress conditions. As leucine seems to play an important role in acid stress tolerance, the next step was to find out whether leucine starvation activates the general amino acid control (GCN) pathway which is responsible for cells adaptation to stress conditions.

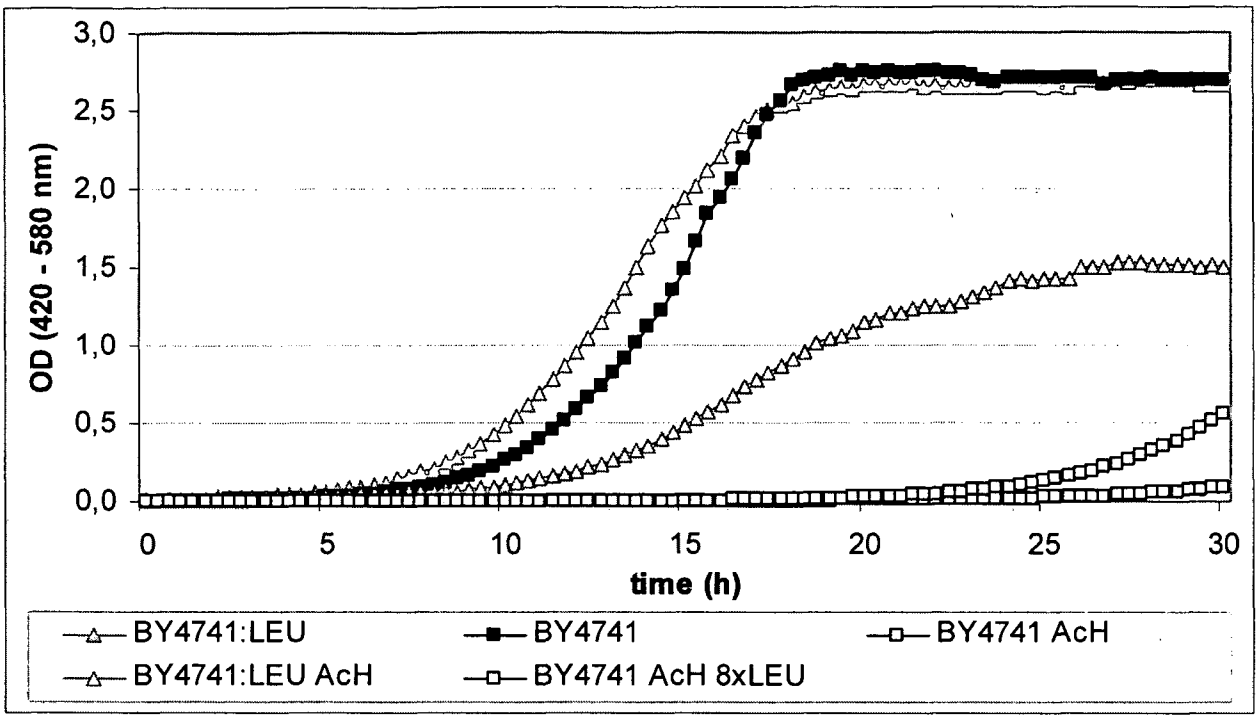


Figure 11: Leucine improves tolerance to acid stress. Growth in YPD (close symbols), in YPD supplemented with 80 mM AcH (open symbols) and in YPD supplemented with 80 mM AcH and 800 µg/ml leucine (black/grey squares).

4.4. IMPLICATION OF THE GCN PATHWAY

The capacity of adaptation to changes of intra and extracellular conditions is a universal requirement for the survival and evolution of an organism (Rodrigues-Pousada et al., 2005).

Protein phosphorylation is a reversible mechanism which plays an essential role in the release of responses to extracellular signals and in the subsequent regulation of its metabolic pathways. (Ballou and Fischer, 1986; Cohen, 1989). The phosphorylation state of a certain substrate is determined by the relative activities of the protein kinases and the protein phosphatases (Edelman et al., 1987; Hanks et al., 1988; Cohen, 1989).

The activity of Gcn2p is induced by high levels of uncharged tRNA, implying that uncharged tRNA is a critical upstream signal for derepression of GCN4

translation under starvation conditions (Hinnebusch A.G., 1996). Assuming that leucine (among other amino acids) uptake is partly inhibited under acid stress conditions, the activation of the general amino acid control (GCN) pathway was investigated performing a bioscreen experiment and a drop test with wild type cells and mutants lacking important GCN pathway genes, namely *GCN2* and *GCN4*. These mutants are further called *Δgcn2* and *Δgcn4*.

Figure 12 shows that culture development in YPD media is comparable for all three cell cultures, for wild type as well as for the mutants. In 60 mM acetic acid media, growth rates observed are generally inferior but especially for *Δgcn2*. After 20 hours cultivation, *Δgcn2* has reached an OD of 0,375 while wild type cells are at an OD of 0,996 and *Δgcn4* even shows OD 1,224 which is more than three times higher than the OD of *Δgcn2*.

The high sensibility of *Δgcn2* to acid stress was also demonstrated complementing the obtained results by performing a drop test. These tests are also used to analyze growth of yeast under different conditions, but have some limitations and disadvantages. Since yeast cultures are stamped on solid media in Petri dishes, no data can be obtained during cell growth, like growth speed or duration of the lag phase; the result shows only saturated colonies. Using solid media can also lead to a slightly contorted result when colonies metabolize all the acid of the media around them and subsequently improve their growth in "acid free" media. However, it serves as a good complement to other types of analysis since it serves to clearly show different phenotypes.

The results of the drop test are displayed in Figure 13. *Δgcn4* shows again the same growth pattern as wild type cells, while *Δgcn2* does not present growth in 30 mM acetic acid media and just little growth in 1,5 mM sorbic acid media at the lowest dilution (1:10 of a saturated cell culture). These findings indicate that GCN2 is crucial for cell adaptation to acid stress but surprisingly not GCN4, as had been previously assumed.

Subsequently it has to be considered that the GCN pathway does become activated (the mutant *Δgcn2* is sensible to acid stress and p-eIF-2α levels augment,

results shown later) but maybe this activation is not "complete" because GCN4 does not seem to be necessary for the response to weak acid stress. *Δgcn4* mutants do not show sensibility what means that this gene which is an important translation initiator is not required for adapting to weak acid stress.

As a consequence of this result, the importance of Gcn2p was subsequently confirmed by proving its activation via Western Blot analysis and the role of leucine was thoroughly investigated.

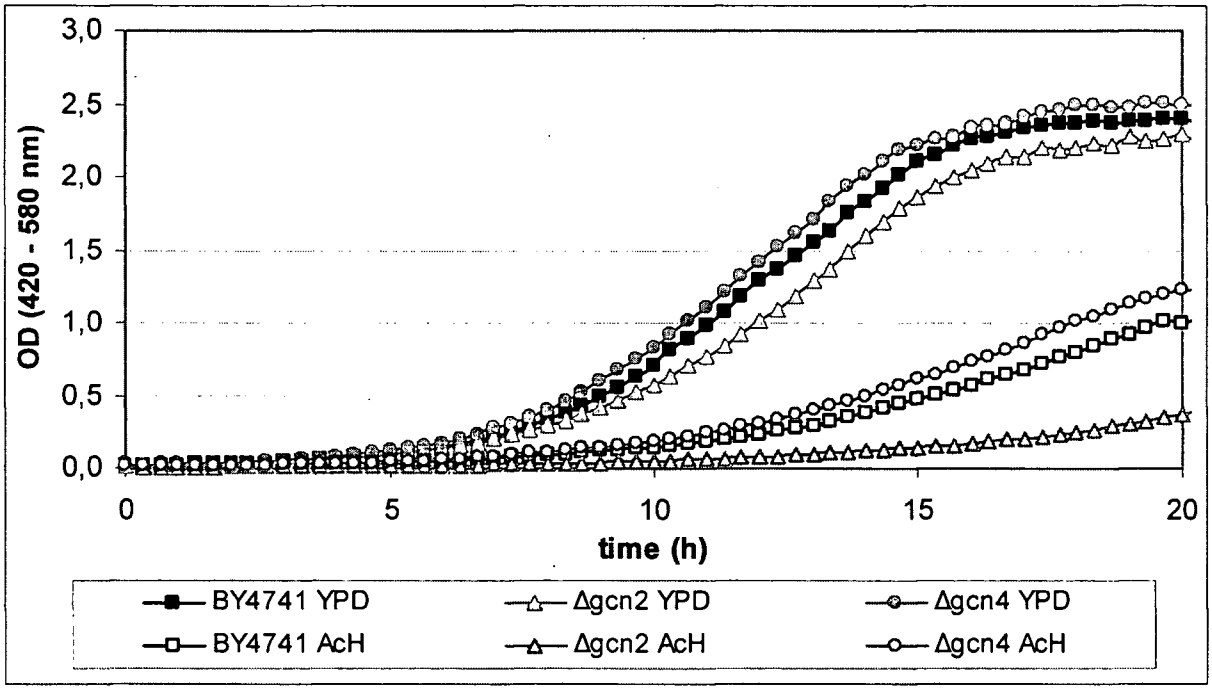


Figure 12: Growth of GCN pathway mutants. Growth in YPD (close symbols) and YPD supplemented with 60 mM acetic acid (open symbols). Wild type, Δgcn2 and Δgcn4 mutants are compared.

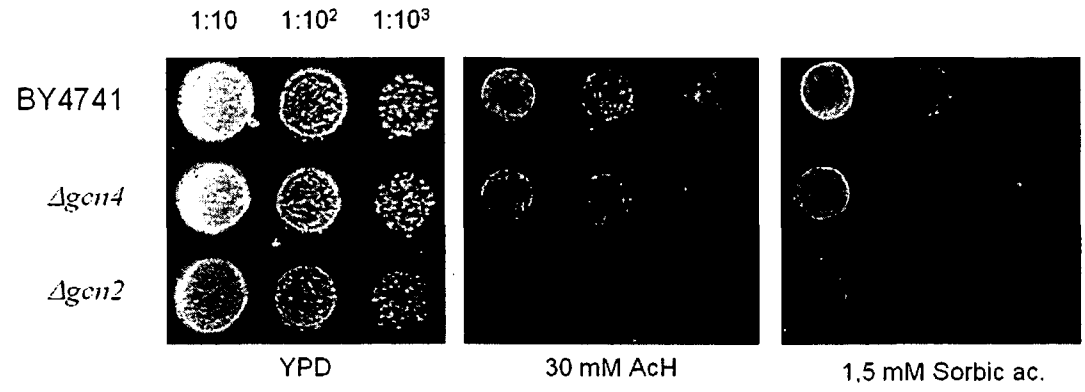


Figure 13: Growth of gcn pathway mutants. Droptest in YPD and YPD supplemented with either 30 mM acetic acid or 1,5 mM sorbic acid. Wt, Δgcn2 and Δgcn4 cells were applied in three different dilutions.

4.5. ACTIVATION OF GCN2

As mentioned above, Gcn2 is a protein kinase which, apart from other functions, activates Gcn4 in the GCN pathway by phosphorylating the eukaryotic Initiation factor 2α (eIF-2α). This phosphorylation can be reversed by a protein phosphatase, called GLC7. It was shown that yeast lacking the GCN2 gene has a considerably higher sensibility to acid stress than the wild type. In order to confirm that acid stress indeed activates Gcn2, phosphorylation of eIF-2α was visualized via Western Blot analysis.

Wild type cells containing the empty plasmid YEp24 and wild type cells transformed with the plasmid YEp24 containing *GLC7'*, a truncated version of GLC7, were used for this experiment. The truncated version of *GLC7* inhibits the dephosphorylation of eIF-2α. As a consequence, phosphorylation levels should augment during the experiment with *GLC7'* containing cells under acid conditions. Acid stressed (30 mM AcH) wild type cells in mid-log growth phase should reflect real phosphorylation levels at determined points in time. Looking at the results, activation can indeed be seen in both wild type and *GLC7'* cells at different points in time.

Phosphorylation of eIF-2α in wild type cells is most evident after 60 minutes acid treatment, decreasing afterwards. This decrease may coincide with the cellular adaptation to acid stress. The *GLC7´* mutant shows first increasing and then stable phosphorylation levels, as expected (**Figure 14**). This result stresses again the importance of Gcn2p in acid stress.

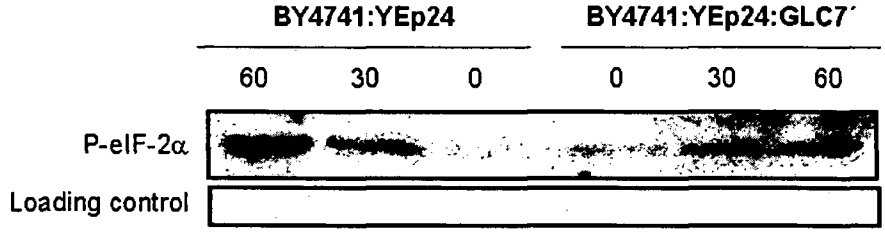


Figure 14: Activation of Gcn2 under acid stress conditions. In YPD grown mid-log phase cells were treated with 30 mM AcH during 120 minutes. Harvested cells were loaded at equal concentrations (1,5*10^6 cells/ml).

4.6. LEUCINE UPTAKE IS AFFECTED UNDER ACID STRESS CONDITIONS

All results obtained until now indicated Gcn2p activation caused by amino acid starvation, as explained before, most relevant leucine starvation. In order to verify impairment in leucine transport under acid conditions, an uptake experiment was performed with radioactive labelled ^{14}C leucine. In general, acids produce a membrane depolarization what leads to an overall alteration of the membrane permeases. A positive result of the uptake experiment would confirm the degradation of the leucine membrane transporters under acid conditions.

The transport of isoleucine, leucine and valine has been investigated thoroughly (Grenson et al. 1970; Tullin et al. 1991; Kotliar et al. 1994; Grauslund et al. 1995; Didion et al. 1998), and three permeases besides Gap1p (the general permease for most uncharged amino acids) have been identified as transporters of the branched-chain amino acids (Grauslund et al. 1995; Didion et al. 1998), namely Bap2p, Bap3p and Gnp1p. Bap2p and Bap3p have a less-broader specificity, only transporting isoleucine, leucine, valine, cysteine, me-

thionine, phenylalanine, tyrosine and tryptophan. Together with Gap1p, Bap2p seems to be the most important transmembrane-transporter for branched-chain amino acids (Didion T. et al, 1996).

Results show that acid treated samples effectively have a decreased leucine uptake, the difference after 25 minutes treatment is about 2,85 % of the total CPM measured what corresponds to a reduction in leucine uptake of about 59 % (**Figure 15**). This discrepancy is very likely to be due to delocalization or inhibition of the specific leucine membrane permease Bap2.

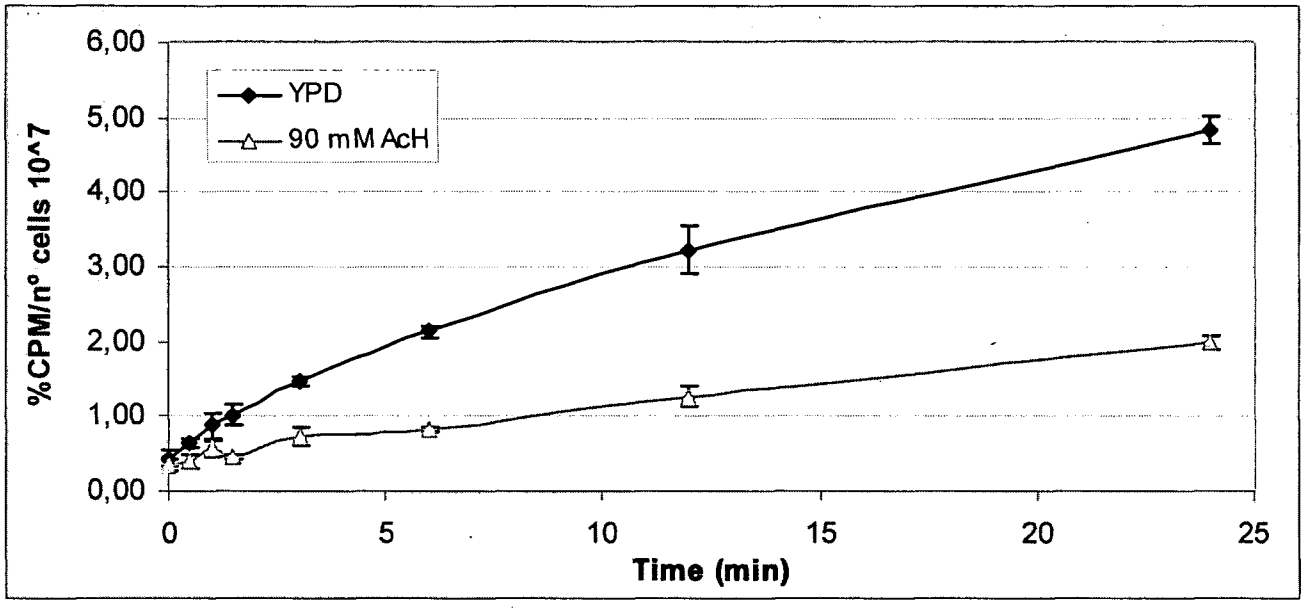


Figure 15: Leucine transport. Wt cells were grown in YPD media until mid-log phase and then partly supplemented with 90 mM AcH, as indicated. Values are referred to 10^7 cells and shown in % counts per minute (CPM) of total CPM.

4.7. LOCALIZATION OF THE GFP LABELLED SPECIFIC LEUCINE PERMEASE BAP2

With the aim of confirming the theory that delocalization of Bap2 could be the reason for the decreased leucine uptake, membrane localization of Bap2 was studied performing a western blot analysis. As no primary antibody for Bap2 is commercially available the investigation was carried out using GFP labelled

Bap2 to facilitate its immunological detection. The localization of Bap2-GFP in the cell was visualized with confocal microscopy (**Figure 16**). It can be seen that under non starvation conditions, Bap2 is localized both in the cell membrane and in the vacuoles.

In order to trace the delocalization of Bap2 within a time course under acid conditions, a part of an in SD grown mid-log phase cell culture was treated with 80 mM AcH, harvested at different points in time and fractionated in order to purify the membrane proteins. Samples were loaded on a gel at equal concentrations. SDS PAGE and Western Blot analysis were performed as indicated.

Surprisingly, Bap2 shows even stronger signals in acid treated samples than in SD grown cells (see **Figure 17**) what means that there are higher amounts of the permease in the cell membrane than under normal conditions. Contrary to our expectations, the highly specific membrane transporter does not seem to become degraded due to acid stress. As a previous assay indicated problems in leucine uptake, the next experiment was targeted on measuring the intracellular amino acid pools of yeast under acid stress conditions.

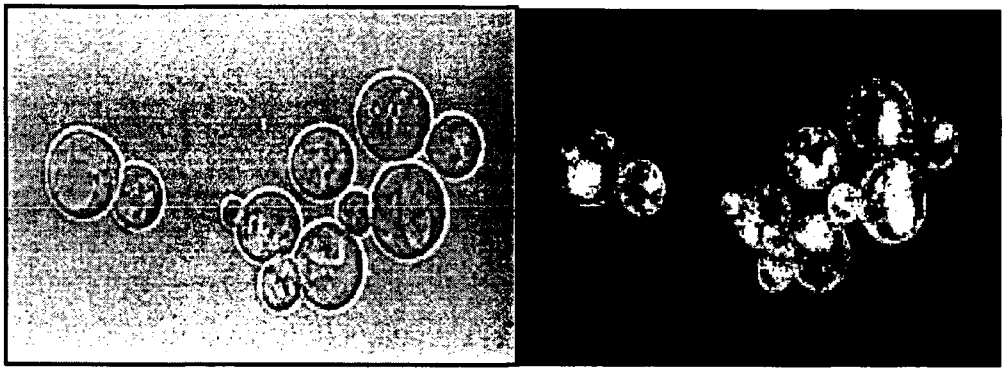


Figure 16: Localization of the GFP labelled branched chain amino acid transporter Bap2.

Figure 17: Immunological detection of membrane bound Bap2

4.8. INTRACELLULAR AMINO ACID POOLS

To complement and understand the obtained data concerning amino acid uptake and the localization of the specific leucine membrane transporter, intracellular amino acid pools during acid treatment were assayed. The intracellular pools of 16 different amino acids were analyzed as previously described. Contrary to the expectations, although leucine uptake is affected, acid treated cells show a higher intracellular leucine concentration than untreated cells.

Figure 18 shows a representative selection of three amino acids, proving that no general tendency could be detected concerning increase or decrease of amino acid levels during acid treatment. One possible explication for the higher leucine level in acid stressed cells is that due to that stress conditions less leucine may be used for protein synthesis. Therefore the membrane permeases are apparently partly inhibited under acid stress conditions, although they may stay in the cell membrane.

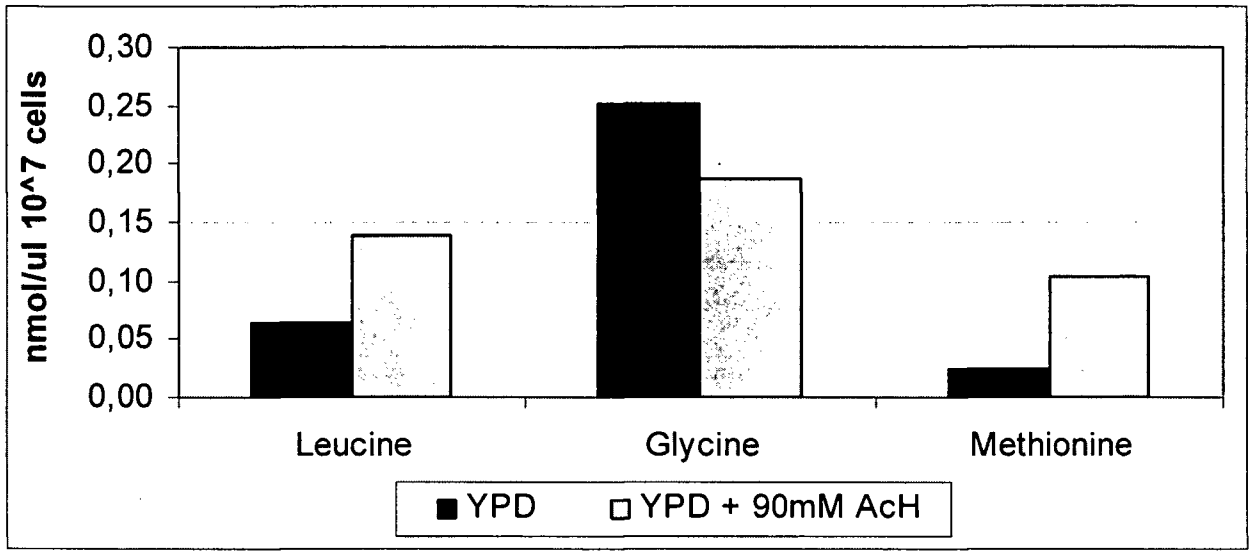


Figure 18: Amino acid pools. Wt cells were grown in YPD (black bars) and YPD supplemented with 90 mM AcH (red bars). Differences in amino acid pools are shown after 60 minutes acid treatment.

5. DISCUSSION

Studying yeasts response to weak acid stress is an ambitious aim. Cell mechanisms are very complex and cross-linked and the amount of proteins, processes and pathways influenced by only one variable, the pH, is remarkable. A lot of research has already been done; many targets and stress responses are known and studied with different yeasts presenting different genetic backgrounds.

My tutor Guillem Hueso started his doctoral thesis performing an overexpression screening in order to discover acid stress-resistant phenotypes. He identified leucine as a key-gene. A subsequent working hypothesis established that acid stress inhibits leucine uptake, among other amino acids, what drives to a higher concentration of uncharged tRNA which then activates Gcn2p, part of the GCN pathway. Gcn2p confers tolerance to acid stress by induction of Gcn4p.

The present project was aimed at verifying this hypothesis. First we proved that acid stress indeed lowers the cytosolic pH in yeast. The results were clear and confirmed our assumption. Another important step was to determine whether there is a correlation between acid concentrations and growth inhibition in yeast cells, so we performed bioscreen analysis with yeast subjected to different concentrations of acid and sorbic acid. We also used leucine supplementation and leucine-prototroph strains in order to analyse whether leucine could improve weak acid stress resistance or not.

Our results show that yeasts sensitivity indeed rises with increasing acid supplementation of the media and leucine supplementation improves resistance to weak acid stress, but not as much as leucine prototrophy does. This finding confirms the importance of this amino acid for adaptation to acid stress.

Leucine is an upstream signal for the activation of Gcn2p and subsequently the GCN pathway. The implication of the latter was determined by a series of bioscreen experiments and drop tests which were conducted using different deletion mutants of the GCN pathway (see **chapter 1.4**). Results showed that mutants lacking *GCN2* are extremely sensible to weak acid stress confirming the importance of this gene for acid stress resistance. Surprisingly, mutants lacking *GCN4* did not show higher sensibility to acid stress than wild type cells. This

result was contrary to our hypothesis, since we suggested the complete activation of the GCN pathway. Nonetheless, we wanted to confirm the importance of Gcn2p by measuring its activation under acid stress conditions. As explained in **chapter 1.4.5**, Gcn2p is activated by uncharged tRNA and phosphorylates as a consequence eIF-2α. As our western blot analysis shows, this phosphorylation takes also place under weak acid stress conditions.

Back to the finding that leucine prototrophy confers better acid resistance than leucine supplementation; we thought that leucine transport could be inhibited under acid stress conditions. In order to prove this hypothesis, we measured uptake of radioactive labelled leucine and found a partial inhibition of leucine transport into the cell, in media supplemented with 30 mM as well as with 90 mM AcH. This inhibition is probably due to the intracellular acid pH acting on the leucine-H^+ cotransporters and is not caused by degradation of the transporters. Amino acids are cotransported with protons whose uptake is driven by a proton gradient. A low cell internal pH due to the presence of acid may disturb this gradient and impede proton and leucine transport into the cell.

The next interesting point to clear up was, whether this uptake inhibition is due to the degradation or the inhibition of the leucine membrane transporters. Leucine uptake is conducted by 4 different permeases, being Bap2 the most important (and the specific one). We focused on visualizing possible degradation and performed a western blot analysis in order to determine possible changes of the Bap2's presence in the plasma membrane during weak acid treatment.

According to our results, Bap2 even increases its presence. Consequently, the leucine transporter is not degraded under acid stress conditions but strongly inhibited.

Finally we measured the intracellular amino acid pools. According to our initial working hypothesis, leucine uptake inhibition and cell internal leucine consumption should lead to a higher concentration of uncharged tRNA which activates Gcn2p. Surprisingly acid stressed cells contain even higher leucine-concentrations than unstressed cells. The other analyzed amino acids show

different profiles, increased as well as decreased internal concentrations are found. We could not observe any tendency.

Our final working hypothesis, presently being tested in the laboratory of Ramón Serrano, is that leucine transport is inhibited by intracellular acidification but that the leucine-$tRNA^{leu}$ synthetase is even more inhibited, resulting in accumulation of leucine and of uncharged $tRNA^{leu}$. The latter causes the activation of Gcn2p which increases the expression of leucine transporters (see **Figure 17**) by a mechanism independent of Gcn4.

6. CONCLUSIONS

- Intracellular pH decreases under weak acid stress conditions, in a dose dependent manner

- Leucine uptake and cell internal leucine consumption are at least partially inhibited. There is no evidence for leucine starvation.

- The GCN pathway is partly activated under acid stress conditions; Gcn2p plays a key role in weak acid stress resistance while Gcn4p does not seem to be implicated.

- New working hypothesis: Leucine transport is inhibited by intracellular acidification but the leucine-tRNA^{leu} synthetase is even more inhibited, resulting in accumulation of leucine and of uncharged tRNA^{leu}. The latter causes the activation of Gcn2p which increases the expression of leucine transporters by a mechanism independent of GCN4.

7. BIBLIOGRAPHY

1. **Andreadis, A., Hsu, Y.-P., Kohlhaw, G.B. and Schimmel, P.** (1982). Nucleotide sequence of yeast LEU2 shows 5'-noncoding region has sequences cognate to leucine. Cell 31, 319-325.

2. **Bauer B.E., Rossington D., Mollapour M., Mamnun Y., Kuchler K. and Piper P.W.** (2003). Weak organic acid stress inhibits aromatic amino acid uptake by yeast, causing a strong influence of amino acid auxotrophies on the phenotypes of membrane transporter mutants. Eur. J. Biochem. 270, 3189–3195.

3. **Bracey, D., Holyoak, C.D. & Coote, P.J.** (1998) Comparison of the inhibitory effect of sorbic acid and amphotericin B on Saccharomyces cerevisiae: is growth inhibition dependent on reduced intracellular pH? J. Appl. Microbiol. 85, 1056–1066.

4. **Brejc, K. et al.** (1997). Structural basis for dual excitation and photoisomerization of the Aequorea Victoria green fluorescent protein. Proc. Natl Acad. Sci. USA 94, 2306-2311.

5. **Chattoraj, M., King, B.A., Bublitz, G.U., Boxer, S.G.** (1996). Ultra-fast excited state dynamics in green fluorescent protein: Multiple states and proton transfer. Proc, Natl Acad. Sci. USA 93, 8362-8367.

6. **Dawes, I.W.** (2004). Stress responses. In: The metabolism and molecular physiology of Saccharomyces cerevisiae. Vol II (Dickinson J.R. and Schweizer M. eds), CRC press. P 376.

7. **Deak, T.** (1991). Food borne yeasts. Adv. Appl. Microbiol. 36:179–278.

8. **Didion T., Grauslund M., Kielland-Brandt M.C., Andersen H.A.** (1996). Amino acids induce expression of BAP2, a branched-chain amino-acid permease gene in Saccharomyces cerevisiae. J Bacteriol 178, 2025-2029.

9. **Didion T., Regenberg B., Jørgensen M.U., Kielland-Brandt M.C., Andersen H.A.** (1998). The permease homologue Ssy1p controls the expression of amino-aid and peptide transporter genes in Saccharomyces cerevisiae. Mol Microbiol 27, 643-650.

10. **Didion, T., Grauslund, M., Kielland-Brandt, M. C., and H. A. Andersen**. (1996). Amino acids induce expression of BAP2, a branched-chain amino acid permease gene in Saccharomyces cerevisiae. J. Bact. Apr. 1996: 2025-2029

11. **Epel, D. and Dubé, F.** (1987). Intracellular pH and cell proliferation. In: Control of Animal Cell Proliferation. Vol. II (Boynton, A.L. and Leffert, H.L. eds.), Academic Press. Pp. 363-393.

12. **Epstein, C.B., Waddle, J.A., Hale, W.T., Dave, V., Thornton, J., Macatee, T.L., Garner, H.R. & Butow, R. A**. (2001) Genome-wide responses to mitochondrial dysfunction. Mol. Biol. Cell. 12, 297–308.

13. **Fleet, G.** (1992). Spoilage yeasts. Crit. Rev. Biotechnol. 12:1–44.

14. **Flores, C.L., Rodriguez, C., Petit, T. y Gancedo, C**. (2000). Carbohydrate and energy-yielding metabolism in non conventional yeast. FEMS Microbiol Rev. 24, 507-529.

15. **Garcia-Barrio M, et al.** (2000) Association of GCN1-GCN20 regulatory complex with the N-terminus of eIF2alpha kinase GCN2 is required for GCN2 activation. EMBO J 19(8):1887-99.

16. **Gietz, R.D. and Woods, R.A.**. (2002). Transformation of yeast by lithium acetate/single-stranded carrier DNA/polyethylene glycol method. Methods Enzymol. 350: 87-96.

17. **Gillies, R.J., Ugurbil, K., Hollander, J.A. and Shulman, R.G**. (1981) 31P NMR studies on intracellular pH and phosphate metabolism during

cell division cycle of Saccharomyces cerevisiae. Proc. Natl. Acad. Sci. USA 78, 2125-2129.

18. **Goffeau, B. G. Barrell, H. Bussey, R. W. Davis, B. Dujon, H. Feldmann, F. Galibert, J. D. Hoheisel, C. Jacq, M. Johnston, E. J. Louis, H. W. Mewes, Y. Murakami, P. Philippsen, H. Tettelin, and S. G. Oliver.** (1996). Life with 6000 genes. Science 1996 Oct 25; 274(5287):546, 563-7. Review.

19. **Gottlieb, R.A., Nordberg, J., Skowronski, E. and Babior, B.M**. (1996) Apoptosis induced in Jurkat cells by several agents is preceded by intracellular acidification. Proc. Natl. Acad. Sci. USA 93, 654-658.

20. **Grauslund M., Didion T., Kielland-Brandt M.C., Andersen H.A.** (1995). BAP2, a gene encoding a permease for ranched-chain amino acids in Saccharomyces cerevisiae. Biochim Biophys Acta 1269:275-280.

21. **Grenson M., Hou C., Crabeel M.** (1970). Multiplicity of the amino-acid permeases in Saccharomyces cerevisiae. IV. Evidence for a general amino –acid permease. J Bacteriol 103:770-777.

22. **Heim, R., Prasher, D.C., Tsien, R.Y.** (1994) Wavelength mutations and posttranslational autoxidation of green fluorescent protein. Proc. Natl Acad. Sci. USA 91, 12501-12504.

23. **Hinnebusch A.G., Natarajan K., Meyer M.R., Jackson B.M., Slade D., Roberts C., Marton M.J.** (2001). Transcriptional profiling shows that Gcn4p is a master regulator of gene expression during amino acid starvation in yeast. MCB, july 2001, p. 4347-4368.

24. **Hinnebusch, A.G.** (1992). "General and pathway-specific regulatory mechanisms controlling the synthesis of amino acid biosynthetic enzymes in Saccharomyces cerevisiae." The Molecular and Cellular Biology of the yeast Saccharomyces: Gene Expression, Vol 2, pp. 319-414.

25. Hinnebusch, A.G. (1984) Evidence for translational regulation of the activator of general amino acid control in yeast. Proc Natl Acad Sci U S A 81(20):6442-6

26. Hinnebusch, A.G. (1996). Translational control of GCN4: gene-specific regulation by phosphorylation of eIF2, p.199-244. In J.W.B. Herschey, M.B. Mathews, and N. Sonenberg (ed.), Translational control. Cold Spring Harbor Laboratory Press, Plainview, N.Y.

27. Hinnebusch, A.G. (1997) Translational regulation of yeast GCN4. A window on factors that control initiator-trna binding to the ribosome. J Biol Chem 272(35):21661-4

28. Hinnebusch, A.G. and Fink, G.R. (1983) Positive regulation in the general amino acid control of Saccharomyces cerevisiae. Proc Natl Acad Sci U S A 80(17):5374-8

29. Holyoak, C.D., Bracey, D., Piper, P.W., Kuchler, K. & Coote, P.J. (1999) The Saccharomyces cerevisiae weak acid-inducible ABC transporter Pdr12 transports fluorescein and preservative anions from the cytosol by an energy-dependent mechanism. J. Bacteriol. 181,4 644–4652.

30. Holyoak, C.D., Thompson, S., Ortiz Calderon, C., Hatzixanthis, K., Bauer, B., Kuchler, K., Piper, P.W. & Coote, P.J. (2000) Loss of Cmk1 Ca2+/calmodulin-dependent protein kinase in yeast results in constitutive weak organic acid resistance, associated with a posttranscriptional activation of the Pdr12 ATP-binding cassette transporter. Mol. Microbiol. 37,595 –605.

31. Horak, J. (1997) Yeast nutrient transporters. Biochim Biophys Acta 1331:31-79

32. Imai, T. and Ohno, T. (1995). The relationship between viability and intracellular pH in the yeast Saccharomyces cerevisiae. Applied and Environmental Microbiology. 61, 3604-3608.

33. Izumi et al. (2003). Cellular pH regulators: potentially molecular targets for cancer chemotherapy. Cancer Treta. Rev. 29, 541-549.

34. Jauniaux, J.-C., and M. Grenson. (1990). GAP1, the general amino aid permease gene of Saccharomyces cerevisiae. Nucleotide sequence, protein similarity with the other bakers yeast amino acid permeases, and nitrogen catabolite repression. Eur. J. Biochem. 190:39-44

35. Kohlhaw, G.B. (2003). "Leucine biosynthesis in fungi: entering metabolism through the back door." Microbiol Mol Biol Rev 67(1);1-15, table of contents. PMID: 12626680

36. Kornitzer D., et al. (1994) Regulated degradation of the transcription factor Gcn4. EMBO J 13(24):6021-30

37. Kotliar N., Stella C.A., Ramos E.H., Mattoon J.R. (1994). L-leucine transport systems I Saccharomyces cerevisiae. Participation of Gap1, S1 and S2 transport systems. Cell Mol Biol 40:833-842.

38. Krebs, H.A., Wiggins, D., Stubbs, M., Sols, A. and Bedoya, F. (1983). Studies on the mechanism of the antifungal action of benzoate. Biochem. J. 214, 657-663.

39. Kren, A., Mamnun, Y.M., Bauer, B.E., Schüller, C., Wolfger, H., Hatzixanthis, K., Mollapour, M., Gregori, C., Piper, P. & Kuchler, K. (2003) War1p, a novel transcription regulator controlling weak acid stress response in yeast. Mol. Cell. Biol. 23, 1775–1785.

40. Kubota H., et al. (2001) Budding yeast GCN1 binds the GI domain to activate the eIF2alpha kinase GCN2. J Biol Chem 276(20):17591-6.

41. **Kumar and Snyder, M.** (2001). Emerging technologies in yeast genomics. Nat. Rev. Genet. 2001 Apr;2(4):302-12. Review.

42. **Lambert, R.J., and Stratford, M.** (1999). Weak-acid preservatives: modelling microbial inhibition and response. J. Appl. Microbiol. 86:157–164.

43. **Ludovico, P., Sousa, M.J., Silva, M.T., Leao, C. and Corte-Real, M.** (2001). Saccharomyces cerevisiae commits to a programmed cell death process in response to acetic acid. Microbiol. 147, 2409-2415.

44. **Marton J., Natarajan K., Meyer M.R., Jackson B.M., Slade D., Roberts C. and Hinnebusch A.G.** (2001) Transcriptional profiling shows that Gcn4p Is a Master Regulator of Gene Expression during Amino Acid Starvation in Yeast. Molecular and cellular Biology, July 2001, p.4347-4368.

45. **Meimoun A., et al.** (2000) Degradation of the transcription factor Gcn4 requires the kinase Pho85 and the SCF(CDC4) ubiquitin-ligase complex. Mol Biol Cell 11(3):915-27

46. **Miesenböck G., De Angelis D.A., Rothman J.E.** (1998). Visualizing secretion and synaptic transmission with pH-sensitive green fluorescent proteins. Nature 394. 192-195

47. **Natarajan K, et al.** (2001) Transcriptional profiling shows that Gcn4p is a master regulator of gene expression during amino acid starvation in yeast. Mol Cell Biol 21(13):4347-68

48. **Oberhaensli et al.** (1986). Biochemical investigation of human tumours in vivo with phosphorus-32 magnetic resonance spectroscopy. The Lancet, july 5, pp.8-11.

49. Orberá T. (2004). Acción perjudicial de las levaduras sobre los alimentos. Rev Cubana Salud Pública 30(3)

50. Ormö, M. et al. (1996) Crystal structure of the Aequorea Victoria green fluorescent protein. Science 273, 1392-1395.

51. Paiva, S., Devaux, F., Barbosa, S., Jacq, C. y Casal, M. (2004). Adyp2p is essential for the acetate permease activity in the yeast Saccharomyces cerevisiae. Yeast. 21, 201-210.

52. Perona, R. and Serrano, R. (1988). Increased pH and tumorigenicity of fibroblasts expressing a yeast proton pump. Nature 334, 438-440.

53. Piper, P., Mahé, Y., Thompson, S., Pandjaitan, R., Holyoak,C., Egner, R., Mühlbauer, M., Coote, P. & Kuchler, K. (1998) The Pdr12 ABC transporter is required for the development of weak organic acid resistance in yeast. EMBO J. 17, 4257 –4265.

54. Piper, P., Ortiz Calderon, C., Hatzixanthis, K. & Mollapour, M. (2001) Weak acid adaptation: The stress response that confers yeasts with resistance to organic acid food preservatives. Microbiol. 147,2635 –2642.

55. Ploger R., Zhang J., Bassett D., Reeves R., Hieter P., Boguski M., and Spencer F. (2000). XREF db: cross-referencing the genetics and genes of mammals and model organisms. Nucleic Acids Res. 28: 120-122.

56. Portillo, F. and Serrano, R. (1989). Growth control strength and active site of yeast plasma membrana ATPase studied by site-directed mutagenesis. Eur. J. Biochem. 186, 501-507.

57. Pretorius, I.S. (2000) Tailoring wine yeast for the new millennium: novel approaches to the ancient art of winemaking. Yeast 16, 675–729.

58. Primo Yúfera, E. (1997). Química de los Alimentos. Editorial Síntesis, Madrid. Páginas 426-429.

59. Regenberg B., Düring-Olsen L., Kielland-Brandt M.C., Holmberg S. (1999). Substrate specificity and gene expression of the amino-acid permeases in Saccharomyces cerevisiae. Curr Genet 36:317-328.

60. Salmond, C.V., Kroll, R.G. and Booth, I.R. (1984) The effect of food preservatives on pH homeostasis in Escherichia coli. J. Gen. Microbiol. 130, 2845-2850.

61. Sherman. (2002). Getting started with yeast, Methods Enzymol. 350, 3-41.

62. Stratford, M. & Anslow, P.A. (1996) Comparison of the inhibitory action on Saccharomyces cerevisiae of weak-acid preservatives, uncouplers, and medium-chain fatty acids. FEMS Microbiol. Lett. 142, 53–58.

63. Stratford, M. & Anslow, P.A. (1998) Evidence that sorbic acid does not inhibit yeast as a classic "weak acid preservative". Lett. Appl. Microbiol. 27, 203–206.

64. Thomas K.C., Hynes S.H., Ingledew W.M., (2002). Influence of medium buffering capacity on inhibition of Saccharomyces cerevisiae growth by acetic and lactic acids. Appl Environ Microbiol 68(4):1616-23

65. Tullin S., Gjermansen C., Kielland-Brandt M.C. (1991). A high-affinity uptake system for branched-chain amino acids in Saccharomyces cerevisiae. Yeast 7:933-941.

66. Ward, W.W. in Bioluminescence and Chemiluminescence (eds DeLuca, M.A. & McElroy, W.D.) 235-242 (Academic, New York, 1981).

67. Ward, W.W., Prentice, H.J., Roth, A.F., Cody, C.W., Reeves, S.C. (1982). Spectral perturbations of the Aequorea green-fluorescent protein. Photochem. Photobiol. 35, 803-808.

68. Wek R.C., Cannon J.F., Dever T.E., Hinnebusch A.G. (1992). Truncated protein phosphatase GLC7 restores translational activation of GCN4 expression in yeast mutants defective for the eIF-2 alpha kinase GCN2. Mol Cel Biol, 12: 5700-10

69. Yang, F., Moss, L.G., Phillips, G.N.Jr. (1996). The molecular structure of green fluorescent protein. Nature Biotechnol. 14, 1246-1251.

70. http://www.olympusconfocal.com/theory/index.html (reviewed: 26.9.2007)