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From intracellular antioxidant availability to modulation of mRNA metabolism: two different approaches to develop industrially attractive *Saccharomyces cerevisiae* strains with improved acetic acid tolerance

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Abstract

The challenge of biorefineries is to develop processes aimed at the complete conversion of different type of biomass feedstock (wastes are preferred) to biofuels and chemicals mainly through microbial metabolism. Unfortunately, during industrial fermentations selected microorganisms, the so-called *cell factories*, meet multiple stresses, associated with either the operative conditions of the process or the presence of inhibitory compounds. This causes cell metabolism and growth impairment, leading to the reduction of yield and productivity of the process. In this context, the development of improved *cell factories* is an important issue for the attainment of the commercial requirement for yield, productivity and titer. Several rational tools, principally based on the over-expression or deletion of single gene(s), have been applied to improve microorganisms' robustness and so to minimize the consequences of the stressful conditions imposed by industrial fermentations. However, stress tolerant phenotypes are polygenic traits that require the simultaneous modification of multiple genes. In the last years, whole genome wide engineering approaches have been proposed to obtain complex phenotypes, including stress tolerance. The principle of these techniques is the creation of variability within a population and the subsequent selection of the desired phenotype through a suitable screening method.

The yeast *Saccharomyces cerevisiae* is widely employed as a *cell factory* for the production of several industrial products, and in particular for first and second generation ethanol, due to its high ethanol yield and productivity and general robustness. However, one of the major obstacles for the development of large-scale ethanol production in second generation processes is the toxic effect of compounds released during the pre-treatment of lignocellulosic biomasses, which are used as feedstock, on cell growth and metabolism.

In the present work, two different approaches to improved *S. cerevisiae* tolerance against acetic acid, one of the most toxic compounds deriving from biomass pre-treatment, are described.

The response to acetic acid exposure in a *S. cerevisiae* strain engineered to endogenously produce L-ascorbic acid (L-AA) and the parental strain was evaluated and compared. The obtained results showed that the intracellular production of L-AA increases acetic acid tolerance by partially protecting cells from the acetic acid-induced programmed cell death (AA-PCD) through the reduction of ROS accumulation. Moreover, the activation of some of the most important mechanisms that participate in cell protection from acetic acid-induced stress was found to be minor in the L-AA producing compared to parental cells, suggesting that the produced antioxidant can support a more efficient stress response by both acting as a ROS scavenger and by determining an indirect energy saving caused by the minor activation of the endogenous defenses. In addition, the effect of the intracellular L-AA production on *S. cerevisiae* chronological lifespan (CLS) was investigated. The L-AA producing strain exhibits an extended CLS and a lower apoptosis incidence compared with the parental strain. Further investigations revealed that the extended CLS of the L-AA producing strain may result from the synergic effect of a higher resistance to acetic acid, which accumulates in the culture medium as a by-product of fermentative metabolism, together with enhanced acetate consumption.

An improved resistance to acetic acid was also reached through the modulation of the mRNA metabolism. This approach was applied with the aim to unlock a complex phenotype through the modification of global gene expression at the post-transcriptional control level. To this purpose, the gene encoding for the poly(A) binding protein Pab1, that participates in the control of mRNA metabolism by interacting with several proteins, was subjected to error-prone PCR to create a mutant library that was screened in the presence of acetic acid. The screening allowed the selection of a mutant Pab1 version that increases yeast resistance to acetic acid. Furthermore, it has been demonstrated that the expression of *PAB1* in a centromeric plasmid, in addition to the chromosomal copy, increases yeast growth

performance under different stressful conditions, including acetic acid, and correlates with the formation of larger stress granules. Although further investigations will be needed to better understand the physiologic mechanisms responsible for the phenotype improvement, the ethanol production/productivity of the strains in which either Pab1 abundance or function have been modified will be tested in bioreactor.

Overall, the obtained results suggest that the engineering of both the intracellular antioxidant availability and the mRNA metabolism can represent efficient strategies to improve the robustness of a *cell factory*.

Riassunto

Il concetto di bioraffineria prevede che quasi tutti i tipi di biomassa (preferibilmente costituiti da scarti di lavorazione e produzione) possano essere utilizzati come materia prima e convertiti in differenti tipi di *biofuels* e *biochemicals* attraverso il metabolismo microbico. Sfortunatamente, durante le fermentazioni industriali i microorganismi selezionati, definiti *cell factory*, incontrano diverse condizioni stressanti associate sia alle condizioni operative del processo, sia alla presenza di composti inibitori, che possono compromettere il metabolismo e la crescita cellulare e determinare la conseguente riduzione della resa e della produttività del processo stesso. In questo contesto, risulta cruciale lo sviluppo di *cell factory* con caratteristiche migliorate per il raggiungimento delle esigenze commerciali riguardanti la resa e la produttività. A tal proposito, diversi approcci principalmente basati sulla over-espressione o delezione di singoli geni sono stati applicati per incrementare la robustezza dei microrganismi, e quindi minimizzare gli effetti dannosi dovuti alle condizioni stressanti imposte dalle fermentazioni industriali. Tuttavia, la risposta allo stress è un tratto poligenico e l'ottenimento di fenotipi più tolleranti richiede quindi la simultanea modifica di più geni o elementi molecolari. Negli ultimi anni sono stati proposti alcuni approcci di ingegneria su scala genomica per ottenere fenotipi complessi, quali la resistenza a diversi tipi di stress. Tali metodi sono essenzialmente basati sulla creazione di varianti all'interno di una popolazione e la successiva selezione del fenotipo di interesse attraverso un idoneo metodo di screening.

Il lievito *Saccharomyces cerevisiae* è ampiamente utilizzato come *cell factory* per la produzione di diversi prodotti di interesse industriale, e in particolare per la produzione di etanolo di prima e seconda generazione grazie alla elevata resa, produttività e robustezza al prodotto finale. Tuttavia, l'effetto tossico esercitato sul metabolismo e sulla crescita cellulare da parte di diversi composti, tra cui quelli liberati durante il pretrattamento delle biomasse lignocellulosiche, rappresenta uno

dei maggiori ostacoli per lo sviluppo della produzione di etanolo in larga scala in processi di seconda generazione.

In questo lavoro sono descritti due diversi approcci per ottenere ceppi di *S. cerevisiae* più tolleranti nei confronti dell'acido acetico, il quale rappresenta uno dei composti maggiormente tossici derivanti dal pretrattamento delle biomasse lignocellulosiche.

La risposta cellulare nei confronti dell'acido acetico è stata determinata e comparata tra un ceppo di lievito ingegnerizzato per produrre acido L-ascorbico e il ceppo parentale. I risultati ottenuti hanno mostrato che la produzione intracellulare di acido L-ascorbico determina un'aumentata tolleranza all'acido acetico mediante la parziale protezione nei confronti del programma di morte cellulare indotto da acido acetico e la riduzione dell'accumulo di ROS. E' stato inoltre osservato che l'attivazione di alcuni dei più importanti meccanismi coinvolti nella protezione cellulare nei confronti dello stress indotto da acido acetico è minore nel ceppo produttore di acido L-ascorbico rispetto al ceppo parentale. Nel complesso, i risultati ottenuti suggeriscono quindi che la produzione intracellulare di acido L-ascorbico possa permettere una più efficiente risposta allo stress ossidativo indotto da acido acetico sia in qualità di *ROS scavenger* sia mediante l'indiretto risparmio energetico causato dalla minore attivazione delle difese endogene. Dal momento che è noto in letteratura come alti livelli di ROS siano correlati con l'invecchiamento cellulare, è stata misurata la *chronological life span* (CLS) sia del ceppo parentale che del produttore di acido L-ascorbico, il quale ha mostrato una CLS maggiore in concomitanza con una minor percentuale di cellule apoptotiche. Ulteriori analisi hanno inoltre messo in luce che il ceppo produttore di acido L-ascorbico è più resistente all'acido acetico, il quale accumula nel terreno di coltura come sotto prodotto del metabolismo fermentativo, anche durante gli esperimenti di invecchiamento e che inoltre tende a consumarlo più rapidamente del ceppo

parentale. Quindi, l'estesa CLS mostrata potrebbe essere il risultato dell'effetto cumulativo, o sinergico, di una maggiore resistenza e di un maggior consumo.

In parallelo, sono stati ottenuti ceppi di *S. cerevisiae* con una migliorata tolleranza nei confronti dell'acido acetico mediante un approccio differente, ma comunque dall'effetto pleiotropico, che prevede la modulazione del metabolismo degli mRNA. Questo approccio è stato applicato con l'obiettivo di ottenere un fenotipo complesso attraverso l'alterazione dell'espressione genica a livello del controllo post-trascrizionale. A questo proposito, il gene codificante per la principale proteina che lega il poli(A), Pab1, la quale partecipa nel controllo del metabolismo degli mRNA interagendo con diverse proteine, è stato mutagenizzato mediante error-prone PCR. La risultante library mutata è stata successivamente sottoposta a screening in presenza di acido acetico permettendo l'identificazione di una versione mutata di Pab1 la cui espressione è in grado di determinare una maggiore tolleranza all'acido acetico. Inoltre, è stato dimostrato che l'espressione di *PAB1* in un plasmide centromerico, in aggiunta alla copia cromosomica, comporta una migliore crescita in presenza di differenti condizioni stressanti, incluso lo stress indotto da acido acetico. Questo fenotipo migliorato è stato mostrato essere correlato con la formazione di più grandi e persistenti *stress granule*. Nonostante sarà necessario comprendere più a fondo i meccanismi fisiologici responsabili del fenotipo osservato, la produzione/produttività di etanolo da parte dei ceppi ottenuti mediante l'alterazione sia della quantità che della funzione di Pab1 sarà testata in bioreattore.

Complessivamente, i risultati ottenuti suggeriscono che l'ingegnerizzazione sia della disponibilità intracellulare di un antiossidante che del metabolismo degli mRNA rappresentano delle efficaci strategie per migliorare la robustezza di una *cell factory*.

Introduction

The biorefinery and cell factory concepts

Nowadays, because of the depletion of fossil reserves and the increasing demand of energy by a continually growing world population, the production of biofuels and bio-based products deserves more and more attention. The term biofuels and bio-based products refers to fuels, such as biodiesel, ethanol, bioalcohols and biogas, alternative to fossil fuels, and chemical compounds, respectively, produced from biomasses renewable within the time of their consumption through microbial metabolism. In this context, the concept of biorefinery arises, defined by the IEA Bioenergy Task 42 ‘‘Biorefineries” as “the sustainable processing of biomass into a spectrum of marketable products and energy” (http://www.biorefinery.nl/ ieabioenergy-task42/). In a biorefinery, plant based feedstock such as sugarcane, corn, starch and wood biomasses are converted into sugars that are subsequently used by microorganisms to produce biofuels, power and chemicals through their fermentative metabolism (Cherubini, 2010; Hong and Nielsen, 2012). This concept is analogous to today’s petroleum refinery, which produces multiple fuels and products starting from petroleum.

When exploited in bio-industrial processes, microorganisms are defined as “cell factories”. The development of a cell factory is an important aspect for the establishment of a novel bioprocess for the production of fuels and chemicals. Although it is relatively quick and simple to obtain a strain able to produce the product of interest, it is generally more difficult and time-consuming the development of a strain that meets the commercial requirements for yield, productivity and titer (Hong and Nielsen, 2012). Cell factories characterized by high production rates are desirable and required to make the biorefinery-based processes competitive with the fossil fuel-based ones. Final yield is obviously dependent on the amount of product that the microorganism is able to produce, but

also strain performance during fermentation is fundamental for its attainment. One of the most relevant factors that impact the microbial metabolism, and consequently its ability to efficiently produce the product of interest, are the stressing conditions occurring during industrial bio-processes. Non optimal temperature, pH values and oxygenation levels, together with the final product toxicity, are the main determinant factors of cellular stress occurring during fermentation. Moreover, the production/productivity of the fermentative process can be impaired by the presence of inhibitory compounds that might be present in the fermentation medium depending on the substrate intended to be used. Large investments are therefore directed to create efficient cell factories characterized by high rate of production and, at the same time, improved stress resistance.

How to improve a cell factory?

Industrially relevant traits, including increase in product yield and in tolerance to products and inhibitors, as well as adaptability to process environments that are different from the natural habitats of microorganisms, have been obtained through the integration of “classic” and “modern” strain engineering approaches (Patnaik, 2008).

The “classic” strain engineering methodology is based on mutagenesis induced by X-rays, UV, or chemicals, followed by the screening of the desired phenotype. This method has been successively used to obtain different phenotypes of interest such as the improvement of secondary metabolites production. However, it is characterized by some drawbacks since it is time-consuming, it leads to significant cell damage and it is almost impossible to be applied to obtain complex phenotypes that require multiple coordinated changes at the genetic level. With the

development of genetic engineering in the 1970s, some of these limitations have been overcome by the development of several new technologies that enable targeted mutagenesis or manipulation of cellular metabolism to amplify or create the desired phenotype. The application of these technologies relies on the level of knowledge about the metabolism and the genotype-phenotype correlation of the host organism that have been achieved by using a number of experimental and mathematical tools (Patnaik, 2008). Moreover, other techniques have been developed with the scope to engineer complex polygenic phenotypes at whole genome scale. Some of the commonly used methods to improve a cell factory with relative examples will be described below.

Metabolic engineering

Metabolic engineering is the technological discipline that allows the introduction of specific modification to metabolic pathways to improve cellular properties through the manipulation of enzymatic, transport, and regulatory functions of the cell by using recombinant DNA technology (Bailey, 1991; Koffas *et al*., 1999). Generally, pathway kinetics are improved by removing flux bottlenecks, balancing precursors, and recycling cofactors to increase product formation (Bulter *et al*., 2003). Strains can also be metabolically engineered to produce not-native products through the heterologous expression of biosynthetic pathways deriving from a different organism. Metabolic engineering has been successfully employed for the production of natural and unnatural products in several organisms. To highlight a few, amino acids such as L-phenylalanine, L-tryptophan and L-tyrosine have been over-produced in *Escherichia coli* and *Corynebacterium glutamicum* (Ikeda, 2006), and *E. coli* has been engineered to produce ethanol through the introduction of two genes encoding for essential enzymes of the ethanol fermentative pathway from *Zymomonas mobilis* (Ingram *et al*., 1987). However, this approach requires an extensive biochemical and genetic knowledge about the metabolism or metabolic

pathways of interest, that, together with the complexity of cellular physiological responses and the difficulty of cloning in industrial strains, represents in some cases an obstacle for its implementation (Cakar *et al.*, 2012). Thus, the engineering of strains by metabolic engineering approach is usually confined to well-characterized hosts, such as *E. coli, S. cerevisiae* and *Bacillus subtilis* (Gong *et al.*, 2009).

Directed evolution

Directed evolution incorporates Darwinian principles of mutation and selection into experimental strategies for improving biocatalyst or cellular properties (Chatterjee and Yuan, 2006). Directed evolution experiments carried on in the laboratory comprise two steps: the creation of genetic diversity through the production of a library of genetic variants and the evaluation of the library by genetic selection and high-throughput screening to identify variants with the required properties (Chatterjee and Yuan, 2006; Jäckel *et al.*, 2008). This technique does not require structural knowledge of the protein or, generally, of the molecules of interest; however, where structural information are available, rational design and directed evolution are often combined to create "smart libraries" in which genetic variations are introduced at functional sites, such as the active site region of an enzyme *(*Leemhuis *et al.*, 2009). There are numerous studies reporting the direct evolution of single enzyme catalysts. However, as underlined in (Chatterjee and Yuan, 2006), since cellular metabolism is dependent on multiple factors, few works demonstrated to achieve improvements in metabolic pathways using this approach. As an example, Eriksen and co-workers recently reported the optimization of cellobiose consumption and ethanol productivity in *S. cerevisiae* by the simultaneous direct evolution engineering of the β-glucosidase gh1-1 and the cellodextrin transporter cdt-1 from *Neurospora crassa* (Eriksen *et al.*, 2013). Moreover, a *S. cerevisiae* strain with improved xylose consumption and ethanol

production was obtained by directed evolution of the heterologous expressed *Piromyces sp.* xylose isomerase (Lee *et al*., 2012). Despite some exceptions, directed evolution experiments or screenings of classical mutagenesis libraries resulted in the selection of strain with marginal improvements of phenotype that are mainly monogenic. However, the industrially high desirable phenotypes such as tolerance to ethanol, inhibitors or low pH and high temperature, are polygenic, and consequently require multiple simultaneous modifications at both molecular and cellular levels.

Evolutionary engineering

The main drawbacks linked to the use of "rational" metabolic engineering and direct evolution has been overcome using the evolutionary engineering approach. This methodology involves repeated batch cultivations in the presence of a selective pressure, or alternatively, a prolonged chemostat cultivation under selective conditions (Cakar *et al*., 2012). As a consequence of the applied selection pressure, spontaneous or induced mutagenesis of the initial monoclonal population results in the formation of fitter variants that can survive and grow better than the original cells under the selection conditions, and, consequently will dominate the culture (Cakar *et al.*, 2012). Both wild type and engineered strains obtained by rational design can be subjected to evolutionary engineering for further improvement (Petri and Schmidt-Dannert, 2004). Enhancements of *E. coli* lactic acid production (Fong *et al*., 2005) and stress resistance (Weikert *et al*., 1997), as well as in substrate utilization and product formation by *S. cerevisiae* (Cakar *et al*., 2012), are example of success results obtain by evolutionary engineering. Despite not impossible, improving industrial strains using this approach is more difficult, since they are usually polyploid and consequently less prone to accumulate relevant recessive mutations by laboratory evolution (Cakar *et al*., 2012).

Whole genome shuffling

Genome shuffling offers the advantage of simultaneous changes at different positions throughout the entire genome without the necessity for genome sequence data or network information (Petri and Schmidt-Dannert, 2004). This technology for strain improvement is based on protoplast fusion, in which recombination results from multiple parents deriving from a strain subjected to one or several rounds of mutagenesis using chemical or physical mutagens (Gong *et al*., 2009). Enrichment in the distribution of complex progeny is achieved by the recursive fusion of a mixed protoplast population in which protoplasts from the parental strains are mixed, fused and regenerated several times (Zhang *et al*., 2002). The desired phenotype is finally obtained from the populations resulting from recursive protoplast fusion through the screening process, that represents a crucial step to ensure the success of the whole procedure of genome shuffling (Gong *et al*., 2009). Compared with classical strain-improvement strategies and rational methods for strain engineering, genome shuffling offers more advantages:

1) the phenotypic improvement is more efficient than classical strain-improvement methods and can be achieved in a rather short period;
2) it is not limited to microorganisms whose genetic background is known;
3) it is more convenient than other molecular breeding techniques since it does not require any expensive facilities;
4) strains engineered by genome shuffling are not considered genetically modified an consequently are accepted by the public opinion (Gong *et al*., 2009).

The application of genome shuffling allowed the improvement of product yield, stress tolerance and uptake of substrates in several bacterial and fungal species (Gong *et al*., 2009). However, this method, being based on protoplast fusion, has the disadvantages of fusant instability, low fusion efficiency, and time-consuming

fusant regeneration process occurring under non-selective conditions, in which some useful mutations might be lost (Hou, 2010).

global Transcription Machinery Engineering

Global transcription machinery engineering (gTME) is an approach for reprogramming gene transcription to elicit complex phenotypes important for technological applications that cannot be achieved through common metabolic engineering techniques (Alper et al., 2006). In gTME, dominant mutant alleles of a transcription-related protein are screened for their ability to reprogram cellular metabolism and regulation, resulting in a unique and desired phenotype (Lanza and Alper, 2011). This goal is reached through the generation of a mutant library using traditional mutagenesis techniques such as error-prone PCR which is subsequently screened to select strains with the phenotype of interest. The sigma factor (σ^{70}) in *E. coli* and the RNA polymerase II transcription factor D (TFIID) component Spt15 in *S. cerevisiae* were engineered by gTME to improve product tolerance, metabolite production, and xylose utilization (Alper *et al*., 2006; Alper and Stephanopoulos, 2007; Liu *et al*., 2010). One limitation of gTME is that changes at transcriptional level may not surely correspond to remarkable modifications in the proteome composition since gene expression is controlled also at the post-transcriptional and post-translational level.

Although advances in strain engineering have been focus on metabolic engineering, the exploitation of this technique is restricted to well-defined systems where the genetic basis for a given phenotype has been established. In the absence of detailed knowledge about the biosynthetic pathways and/or metabolism of the host strain, the use of this tool is therefore limited (Patnaik, 2008). Moreover, despite metabolic engineering is almost the most powerful technique to enable the production of un-natural compounds through the insertion of genes from foreign

organisms, the engineering of polygenic traits such as stress tolerant phenotypes, which are generally dependent on multiple genes, cannot be fully reached using rational tools. On the contrary, these complex phenotypes can be successfully obtained using whole genome wide engineering approaches, including evolutionary engineering, genome shuffling and gTME. In addition, it has recently reported that chromosomal splitting technology can contribute to the expansion of strain improvement platforms (Park *et al*., 2012). However, complex phenotypes can be engineered by combining rational methods and global techniques. gTME can be per se considered as a combinatorial method since it allows the reprogramming of the whole cellular metabolism through the modulation of a single factor whose choice is imposed by rational reasons. A strain with specific properties obtained by rational design can be subjected to evolutionary engineering or genome shuffling for further improvements, and, likewise, strains generated through whole genome engineering approaches can be further optimized by rational metabolic methods. As an example, in *S. cerevisiae* a 30-fold improvement in succinate titer was obtained through the rational engineering of a mutant strain obtained by the evolutionary engineering of a multiple gene-deletion strain rationally designed thanks to the reconstruction of the genome-scale metabolic network of *S. cerevisiae* (Otero *et al*., 2013).

The exploitation of *S. cerevisiae* for second generation ethanol production

Depending on the plant-based raw materials used, biofuels are discerned in first, second or third generation biofuels. First generation fuels are mainly based on edible crops such as wheat, corn, sugarcane and sugar beet, and large scale production of first generation ethanol, biodiesel and biogas is worldwide diffused (Naik *et al*., 2010; Hong and Nielsen, 2012). However, the increasing demand for bio-based products has pushed toward second generation biofuels that are produced from biomass-based raw materials, which do not compete with food production and represent one of the most abundant and underutilized biological resources on the planet. At present, the production of such fuels is not cost-effective because there are a number of technical barriers that need to be overcome before their potential can be realized (Naik *et al*., 2010). Finally, third generation biofuels would be ideally independent from supplied substrate: it will be derived from microalgae, or generally from unicellular photosynthetic microorganisms, and mainly rely on their lipid content/accumulation (Dragone *et al*., 2010). At present, the main problem to fully exploit the potential of algae is that their productivity and photosynthetic efficiency need to be maximized under natural and artificial illumination (Abiusi, *et al*., 2013).

Non-food or second-generation ethanol has great potential as a source of bioenergy due to the abundance of plant biomass on earth. Another significant factor which adds value as well as importance to lignocellulosic ethanol is the reduction in greenhouse gas emission. Success in utilizing this plant biomass will therefore have a positive impact on the environment with benefits for the society as a whole (Laluce *et al*., 2012). Lignocellulosic biomass is mainly composed of cellulose, hemicellulose and lignin. The conversion of lignocelluloses into ethanol requires

three main steps: pre-treatment, hydrolysis and fermentation. Pre-treatment is required to alter the structure of lignocelluloses to make cellulose and hemicelluloses more accessible to acid or enzymes that converts the carbohydrate polymers into fermentable sugars. The goal is to break the lignin seal and disrupt the crystalline structure of cellulose (Mosier *et al*., 2005). Successively, during the hydrolysis process, cellulose and hemicelluloses are broken down into their corresponding monomers, so that microorganisms can utilize them (Kumar *et al*., 2009). Three major hydrolysis processes are typically used to produce a variety of sugars suitable for ethanol production: dilute acid, concentrated acid, and enzymatic hydrolysis (Kumar *et al*., 2009).

The main drawback of the pre-treatment step is the release of inhibitory compounds, such as weak acids, phenolic compounds and furan derivatives that impair cellular metabolism and, as a consequence, the yield and productivity of the fermentative process (Maiorella *et al*., 1983; Palmqvist and Hahn-Hägerdal, 2000).

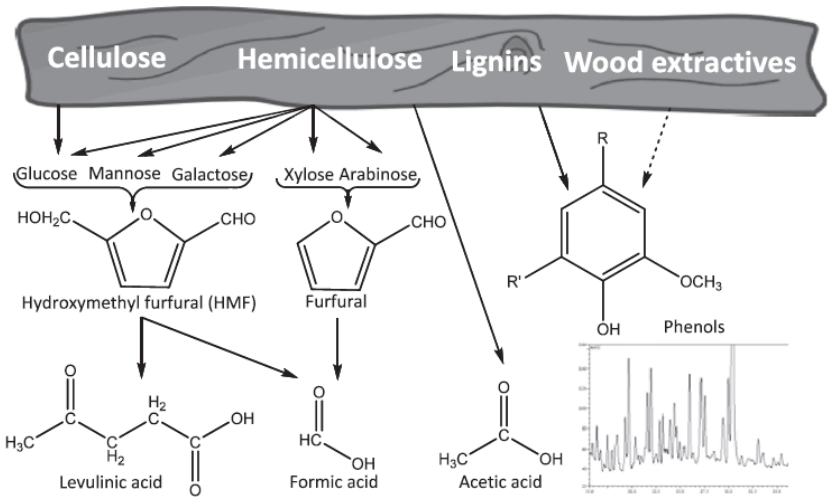

Figure 1: Scheme indicating the main routes of formation of inhibitors (Jönsson *et al*., 2013).

In fact, the pre-treatment usually degrades the hemicellulose leading to the formation of not only pentose and hexose sugars that can be fermented, but also sugar acids, aliphatic acids (primarily acetic acid, formic acid and levulinic acid), and furan aldehydes (5-hydroxymethylfurfural (HMF) and furfural) (Figure 1) (Jönsson *et al*., 2013). On the other hand, phenolic compounds are generated from partial breakdown of lignin and can be formed during acid-catalyzed hydrolysis or pre-treatment of lignocelluloses (Jönsson *et al*., 2013; Palmqvist and Hahn-Hägerdal, 2000). Phenolic compounds can also derived from sugars, although the significance of this route remains to be investigated (Jönsson *et al*., 2013).

Baker's yeast *S. cerevisiae* is the preferred microorganism for large scale ethanol production due to its high ethanol yield and productivity and general robustness (Wallace-Salinas and Gorwa-Grauslund, 2013). It is widely employed for the commercial production of bioethanol from sugars, such as the sucrose from sugar cane and the glucose or maltose from starch (Laluce *et al*., 2012). However, there are crucial limitations related to the fermentation of lignocellulosic materials by *S. cerevisiae*. Firstly, natural strains are not able to utilize xylose that represents the second most abundant sugar in lignocellulosic hydrolysates. The incomplete and inefficient fermentation of carbohydrates present in lignocellulose represents a relevant obstacle for the sustainable and economical production of second generation bioethanol. Therefore, several metabolic engineering approaches to introduce xylose metabolic pathways into *S. cerevisiae* have been practiced (Chu and Lee, 2007; Kim *et al*., 2013). Secondly, as stated before, the ethanol yield and productivity is severally affected by organic compounds released during the pre-treatment of lignocellulosic biomasses that impair *S. cerevisiae* growth and metabolism. Despite the intrinsic robustness exhibited by this yeast, a great number of approaches have been applied to increase the innate tolerance of yeast cells to inhibitors (Laluce *et al*., 2012).

The focus of this thesis is the improvement of *S. cerevisiae* tolerance to acetic acid, which represents one of the most abundant and toxic compounds released from lignocelluloses pre-treatment. The mechanisms of acetic acid-induced toxicity and cell death in *S. cerevisiae* will be briefly introduced in the next section.

Mechanisms of acetic acid-induced cell death in *S. cerevisiae*: an overview

The mechanism of acetic acid toxicity is for some aspects similar to that induced in general by weak carboxylic acids. In *S. cerevisiae*, acetic acid is not metabolized by glucose-repressed cells and, at external pH below the acetic acid pK_a value (4.75), the undissociated form of the acid predominates and may permeate plasma membrane by simple diffusion. It has been described that acetic acid can also enters the yeast cells by a process of facilitated diffusion mediated by the aquaglyceroporin Fps1 (Mollapour and Piper, 2007). Inside the cell, where the pH is near-neutral, the acid dissociates leading to the accumulation of protons (H^+) and acetate counterions (CH_3COO^-). Due to their electric charges, these ions are not able to cross the hydrophobic lipid plasma membrane bilayer and accumulate in the cell cytoplasm (Mira *et al.*, 2010b). Protons accumulation leads to intracellular acidification and to the consequent decrease of DNA and RNA synthesis, inhibition of metabolic activities and disruption of the proton gradient maintained across the plasma membrane (Mira *et al.*, 2010b). To maintain the intracellular pH within physiological levels, yeast cells activate the plasma membrane H+-ATPase Pma1, that couples ATP hydrolysis to proton extrusion (Serrano *et al.*, 1986). However, it has been reported that its activity is not enough to counteract cytosolic and vacuolar acidification (Carmelo *et al.*, 1997). On the other hand, the accumulation

of weak acid counterions may cause an increase in turgor pressure, oxidative stress, protein aggregation, lipid peroxidation, inhibition of membrane trafficking and perturbation of plasma and vacuolar membranes spatial organization (Mira *et al.*, 2010b). Several specific transporters have been implicated in yeast tolerance to different weak acids: in particular, those transporters related to the multidrug resistance (MDR). Pdr12, a plasma membrane transporter of the ATP-binding cassette (ABC) superfamily, Aqr1 and Azr1, plasma membrane transporters of the major facilitator superfamily, as well as Tpo2 and its paralog Tpo3, were found to provide protection against acetic acid (Fernandes *et al*., 2005; Piper *et al*., 1998; Tenreiro *et al*., 2002; Tenreiro *et al*., 2000).

In addition to the genes codifying for proteins involved in acetate extrusion, in *S. cerevisiae* about 650 genes were identified as required for acetic acid tolerance in a genome-wide scale study based on the screening of the EUROSCARF haploid mutant collection for susceptibility phenotypes to this weak acid (concentrations in the range 70-110 mM, pH 4.5) (Mira *et al*., 2010a). The clustering of these acetic acid-resistance genes based on their biological function indicated an enrichment of genes involved in transcription, internal pH homeostasis, carbohydrate metabolism, cell wall assembly, biogenesis of mitochondria, ribosome and vacuole, and in the sensing, signalling and uptake of various nutrients in particular iron, potassium, glucose and amino acids (Mira *et al*., 2010a).

Depending on its concentration, acetic acid added to exponentially growing *S. cerevisiae* cells at pH 3.0 was shown to induce death exhibiting either typical apoptosis-like features, or ultrastructural alterations typical of necrosis (Ludovico *et al*., 2001). The apoptotic phenotype observed in yeast cells exposed to this acid has been defined as acetic acid-induced programmed cell death (AA-PCD) (Ludovico *et al*., 2001). Oxidative stress has been demonstrated to be a determinant of AA-PCD, as well as of yeast PCD triggered by H_2O_2, in which reactive oxygen species (ROS) production and accumulation play an important role (Guaragnella *et*

al., 2011; Madeo *et al*., 1999). Whether ROS directly trigger PCD, or are generated as a result of the PCD cascade, and both the nature and the source of ROS is a matter of debate (Guaragnella *et al*., 2011). In response to oxidative stress, yeast cells activate several mechanisms involved in cellular protection against ROS, including the enzymes superoxide dismutase, catalase and peroxidase, and the synthesis of the tripeptide glutathione that acts as an antioxidant or a cofactor of enzymes such as glutathione reductase, peroxidase and transferase (Herrero *et al*., 2008). In yeast cells exposed to acetic acid, superoxide dismutase activity has been shown to be induced (Giannattasio *et al*., 2005). However, despite superoxide dismutase represent one of the first defenses, its ROS detoxifier activity is not sufficient to prevent AA-PCD. In fact, yeast cells exposed to moderately toxic concentrations of acetic acid committed to death even in the presence of high levels of superoxide dismutase (Giannattasio *et al*., 2005). On the contrary, catalase activity was found to be undetectable in cells en route to AA-PCD (Giannattasio *et al*., 2005). Whether catalase undergo enzyme inactivation and/or degradation remains to be established (Guaragnella *et al*., 2011). However, accordingly to the protective role of catalase in response to several stress conditions in *S. cerevisiae*, AA-PCD was prevented by the over-expression of the cytosolic catalase T or by the induction of catalase activity in cells exposed to extracellular acidification at pH 3.0 before acetic acid treatment (Giannattasio *et al*., 2005; Guaragnella *et al*., 2008). These findings suggest that *i*) H_2O_2 detoxification, catalyzed by catalase, may reduce AA-PCD occurrence and *ii*) H_2O_2 may have a role as a second messenger needed to start the PCD cascade triggered by acetic acid (Guaragnella *et al*., 2011).

It has been proposed that acetic acid can induce two alternative death pathways in yeast cells: the ROS-dependent and the ROS-independent AA-PCD (Figure 2) (Guaragnella *et al*., 2011; Guaragnella *et al*., 2010b).

In the ROS-dependent AA-PCD pathway, high intracellular levels of H_2O_2 are detected starting from 15 min of acetic acid exposure to 60 min, when the proteasome activation begins (Guaragnella *et al*., 2007; Valenti *et al*., 2008). Proteasome-mediated proteolysis of defined target proteins is an important regulatory mechanism, which contributes to regulate various essential pathways and programs of the eukaryotic cell, including PCD (Valenti *et al*., 2008).

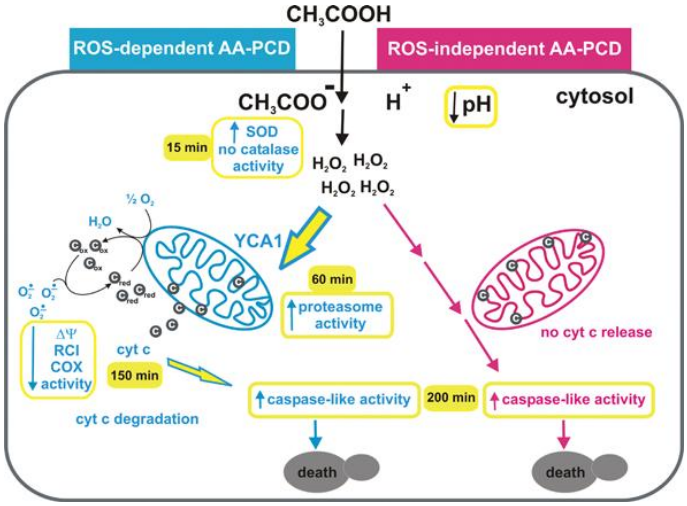

Figure 2. Schematic representation of ROS-dependent and ROS-independent AA-PCD pathways (Guaragnella *et al*., 2011).

Starting from 60 min, cytochrome *c* is released from mitochondria to the cytosol where it acts as a ROS scavenger and an electron donor (Giannattasio *et al*., 2008; Ludovico *et al*., 2002). The ROS-dependent release of cytochrome *c* is mediated by Yca1 (Guaragnella *et al*., 2010a), a protein identified as a yeast metacaspase (Madeo *et al*., 2002). Successively, at a late stage, the mitochondrial membrane potential (ΔΨ), the respiratory control index (RCI) and COX activity decrease, suggesting an impairment of mitochondrial functions, and cytochrome *c* is degraded (Giannattasio *et al*., 2008; Ludovico *et al*., 2002). Caspase-like activity

progressively increases up to 200 min and is partially ROS-dependent since cells treatment with the antioxidant N-Acetyl-L-cysteine (NAC), a H_2O_2 scavenger, resulted in 50% decrease of caspase-like activity compared to untreated cells (Guaragnella *et al*., 2010b).

The ROS-independent AA-PCD pathway was discovered through studies on AA-PCD occurrence in mutant *S. cerevisiae* cells lacking Yca1, cytochrome *c*, or both (Guaragnella *et al*., 2010a; Guaragnella *et al*., 2010b). It was indeed demonstrated that AA-PCD can occur even independently of Yca1 activity through a mechanism in which cytochrome *c* is not released, but still a late caspase-like activity increasing over time was observed which was not affected by NAC treatment (Guaragnella *et al*., 2010b). In mutant yeast cells dying through the ROS-independent AA-PCD pathway H_2O_2 accumulates as in wild type cells, but it seems to not have a direct role in the induction of AA-PCD (Guaragnella *et al*., 2010b). The death rate showed by these mutants is lower than that of wild type cells in which AA-PCD proceeds via a ROS- and Yca1-dependent pathway (Guaragnella *et al*., 2010b). The role that mitochondria play in the ROS-independent pathway in which no cytochrome *c* release takes place remains to be established.

In this thesis two different approaches to increase *S. cerevisiae* tolerance to acetic acid have been evaluated. In one case, the increased resistance to the acid of a *S. cerevisiae* strain metabolically engineered to produce L-ascorbic acid (L-AA) (Branduardi *et al*., 2007; Fossati *et al*., 2011) has been characterized. In the other, it has been demonstrated that the modulation of a key factor involved in mRNA metabolism represents a valid method for fishing *S. cerevisiae* variants with enhanced robustness. The last approach has been designed to engineer global gene expression at post-transcriptional level with the aim to unlock a complex phenotype, such as acetic acid tolerance, that requires simultaneous changes at molecular and physiological level.

Chapter 1

Different response to acetic acid stress in *Saccharomyces cerevisiae* wild type and L-ascorbic acid producing strains

Most of the results has been published in:

Martani F, Fossati T, Posteri R, Signori L, Porro D and Branduardi P. 2013. Different response to acetic acid stress in *Saccharomyces cerevisiae* wild type and L-ascorbic acid producing strains. *Yeast* 30: 365-78.

Introduction

Yeasts are nowadays regarded as effective cell factories for the production of metabolites and bio-based products of relevant economic impact as bulk and fine chemicals, protein drugs and biofuels (for recent reviews see, among others, Porro *et al*., 2011 and Hong and Nielsen, 2012).

During industrial fermentations, yeasts are subjected to different stresses, associated either with the operating parameters of the process and/or with the presence of compounds that, according to their concentrations and effects, turn out to be toxic. Yeast cells counteract these adverse conditions by triggering a stress response, with the aim to adapt to the new environment. However, under severe stress(es) the defence mechanisms may become insufficient to guarantee an efficient metabolism. This very often strongly limits the production, the productivity and the yield of the process itself (Mattanovich *et al*., 2004). Furthermore, cell death may occur.

Yeasts are also considered as important resource in fundamental research where *Saccharomyces cerevisiae* appears as one of the most used and versatile eukaryotic cell models, given its simplicity, feasibility of manipulation and genetic tractability. Indeed, *S. cerevisiae* has been the first sequenced eukaryotic organism (Goffeau, 1996). Current knowledge on cellular stress response and programmed cell death (PCD) in higher eukaryotes and yeasts confirms that, despite some differences in signalling pathways, several steps are phylogenetically conserved. Therefore, *S. cerevisiae* can be considered an ideal model system to study the molecular pathways underlying these processes (Munoz *et al*., 2012).

As a consequence of various stress conditions, different reactive oxygen species (ROS) may accumulate within the cells and are regarded as key mediators of stress response in *S. cerevisiae* (Madeo *et al*., 1999; Farrugia and Balzan, 2012). *S.*

cerevisiae counteracts ROS accumulation through several antioxidant defence mechanisms, aiming to detoxify ROS and maintaining the intracellular redox environment in a reduced state. Yeast antioxidant defences include non enzymatic compounds, like glutathione (GSH) and erythroascorbate (EAA) as well as a number of protective enzymes including superoxide dismutase, catalase, several peroxidases and thioredoxin (Huh *et al*., 1998; Herrero *et al*., 2008). Among the enzymatic systems, superoxide dismutases (SODs), which catalyze the disproportionation of superoxide anion to hydrogen peroxide, and catalases, which reduce hydrogen peroxide to water, play a relevant role (Fridovich, 1975; Switala and Loewen, 2002).

Acetic acid represents one of the most limiting by-products released from the hydrolysis of lignocelluloses, contributing to reduce ethanol yield and productivity when these waste materials are intended to be used as substrates for biofuels production (Maiorella *et al*., 1983; Palmqvist and Hahn-Hägerdal, 2000). The undissociated form of acetic acid is freely membrane permeable and therefore enters the cell by simple diffusion. Once in the cytosol, where the pH is near-neutral, it dissociates leading to the release of protons (H^+) and acetate (CH_3COO^-) (Guldfeldt and Arneborg, 1998). Protons accumulation determines the intracellular acidification, which inhibits many metabolic activities, while acetate may cause turgor pressure and free radicals production, inducing severe oxidative stress (Pampulha and Loureiro-Dias, 1990; Piper *et al*., 2001; Semchyshyn *et al*., 2011; Ullah *et al*., 2012). Acetic acid was also shown to induce programmed cell death (defined as AA-PCD) in *S. cerevisiae* cells characterized by chromatin condensation, TUNEL-positive phenotype and ROS accumulation, resulting in turn in lipid peroxidation, protein oxidation and carbonylation, and genetic damage (Ludovico *et al*., 2001; Guaragnella *et al*., 2007; Morano *et al*., 2012; Rego *et al*., 2012).

L-ascorbic acid (L-AA) is an important ROS scavenger in animals and plants and there are evidences that it is effective to exert a protective function in yeast under oxidative stress: survival of *S. cerevisiae* cells, challenged either with the oxidative agents paraquat or H_2O_2, was found to be enhanced upon treatment with exogenous L-AA from 5 up to 50 μM (Saffi *et al*., 2006; Amari *et al*., 2008). This effect was maximal when L-AA was added before the stressing agent, suggesting that prompt intracellular L-AA availability may have a key role in optimizing its antioxidant action.

On the other hand it has been reported that L-AA can also act as an oxidant, depending upon the environment where the molecule is present (Halliwell, 1999; Paolini *et al.*, 1999), and, in one case (to the best of our knowledge), it may even increase *S. cerevisiae* cell mortality, due to extracellular auto-oxidation and production of H_2O_2 (Poljšak *et al.*, 2005). The pro-oxidant effect can vary, depending on the ability of L-AA to interact with different cell culture media and to produce H_2O_2 at different rates, leading to different conflicting results obtained using ascorbate in cultured cell assays (Clément *et al.*, 2001).

We have previously developed *S. cerevisiae* strains metabolically engineered to endogenously produce L-AA, thanks to the insertion of the necessary steps of the plant biosynthetic pathway (Branduardi *et al*., 2007; Fossati *et al*., 2011). As a consequence, the L-AA producing strains became more tolerant to various stressful agents, including H_2O_2 and weak organic acids such as acetic, formic and lactic acid. Interestingly, the intracellular production of even a small quantity of L-AA (approximately 1.2 μM) was more effective in protecting cells from stress than the exogenous addition of higher amounts (up to 60 mg/l, *i.e.* 340 mM) of this antioxidant, as reported in our previous studies (Branduardi *et al*., 2007).

The aim of the present study was to better characterize the robustness exhibited by recombinant strains under acetic acid stress, with particular attention to cell

viability and ROS accumulation. The correlation between L-AA production and the main endogenous cellular defences was also investigated.

Materials and Methods

Yeast strains, media, growth conditions

The *S. cerevisiae* genetic background used in this study was GRF18U (*MATα*; *ura3*; *his3-11,15*; *leu2-3,112*; cir^{+}) (Brambilla *et al*., 1999). The strains used were GRF18Uc (*MATα*; *ura3*; *his3-11,15*; *leu2-3,112*; [pYX012; pYX022; pYX042; pZ_5]) and GRF18U L-AA producing (*MATα*; *ura3*; *his3-11,15*; *leu2-3,112*; [p012*b*T*At*ME *At*MIP; p022*At*LGDH; p042*Sc*ALO; pZ_5*At*VTC2]) (Branduardi *et al*., 2007; Fossati *et al*., 2011) and the respective strains in which *SOD1* was disrupted. To delete the *SOD1* gene, the *sod1*::hphMX4 cassette was amplified from the plasmid pAG26 (Goldstein and McCusker, 1999) using the oligonucleotides 5'-ATG GTT CAG CAG TCG CAG TGT TAA AGG GTG ATG CCG GTG TCT CT**C** GCC AGA TCT GTT TAG TAG CTT G-3' and 5'-TTA GTT GGT TAG ACC AAT GAC ACC AGG CTG GTC TTG GAC CGG CGA GCT CGT TTT CGA CAC TGG-3'. The *sod1*::hphMX4 cassette was used to replace *SOD*1 gene in both GRF18U and GRF18U L-AA producing strains. Yeast transformations were performed according to the LiAc/PEG/ss-DNA protocol (Gietz and Woods, 2002). Transformed clones were selected on YPD agar plates supplemented with the antibiotic hygromycin B at the final concentration of 2 mg/ml (Roche). Gene disruption was confirmed by PCR analyses.

Yeast cultures were grown in minimal synthetic medium (0.67% [w/v] YNB medium [catalogue no. 919-15 Difco Laboratories, Detroit, Mich.] with 2% [w/v] D-glucose as carbon source). Aminoacids histidine, valine, leucine, lysine, isoleucine and uracil (Sigma) and the antibiotic nourseotricine sulphate (cloNAT, WERNER BioAgents, Germany) were added to a final concentration of 50 mg/l. Δ*sod1* cells were grown in minimal synthetic medium supplemented with 100 mg/l of lysine and methionine. Growth curves were obtained by inoculating yeast cells

at an initial optical density of 0.1 (660 nm) and then the optical density was measured at specific time intervals over at least 78 hours from the inoculum. Each experiment was repeated at least three times. All strains were grown in shake flasks at 30 °C and 160 rpm and the ratio of flask volume/medium was 5:1.

Acetic acid treatment

Exponential phase (OD_{660}= 0.5-0.8) cells grown in minimal medium were harvested and resuspended (10^7 cells/ml) in minimal medium (pH 3.0, set with HCl) containing different concentrations of acetic acid (from 40 to 120 mM). Cells were also resuspended in minimal medium pH 3.0 without acetic acid. Cell viability was determined by measuring colony-forming units (cfu) after 2 days of growth on plate at 30°C. 100% of cell survival corresponds to cfu at time zero.

Cycloheximide (Sigma-Aldrich) dissolved in water was added at a final concentration of 50 μg/ml to yeast cells cultures grown to OD_{660} 0.5-0.7, and the cultures were then incubated for 30 minutes before acetic acid treatment. The absence of cytotoxicity by cycloheximide at this concentration was assessed after 200 min of incubation, by cfu (data not shown).

Acid adaptation was performed by incubating the cells in minimal medium pH 3.0 for 30 minutes before acetic acid treatment as described in (Giannattasio *et al*., 2005).

For spot assay, exponentially growing cells were subjected to 1:10 serial dilutions in sterile water. Five μl aliquots were spotted onto minimal medium plates at pH 3.0 in the absence or presence of increasing concentrations of acetic acid (15, 20 and 30 mM) and incubated at 30° C for 2 days. For anaerobic growth, agar plates were placed in anaerobic jars and the air was displaced with nitrogen gas.

Fluorescence microscopy analyses

Intracellular ROS production was detected with 2,7-dichlorodihydrofluorescein diacetate (H_2-DCF-DA, Sigma) and dihydroethidium (DHE). H_2-DCF-DA, dissolved in dimethylformamide (DMF) was added (as reported by Guaragnella *et al*., 2007) to yeast cell cultures at the final concentration of 10 µg/ml both 30 minutes before and during treatment with or without acetic acid. DHE, dissolved in dimethyl sulfoxide (DMSO), was added (as reported by Guaragnella *et al*., 2007) during acetic acid treatment at the concentration of 5 µg/ml 10 minutes before collecting the cells. In both cases 10^8 cells were collected and resuspended in phosphate-buffered saline (PBS: NaH_2PO_4 53 mM, Na_2HPO_4 613 mM, NaCl 75 mM). Cells were then observed in a Nikon ECLIPSE 90i fluorescence microscope (Nikon) equipped with a 100X objective. Emission fluorescence due to oxidation of H_2-DCF-DA or DHE was detected by B-2A (EX 450-490 DM 505 BA520) or G-2A (DX 510-560 DM 575 BA 590) filter (Nikon), respectively. Digital images were acquired with a CoolSnap CCD camera (Photometrics) using MetaMorph 6.3 software (Molecular Devices).

Flow cytometric analyses

Phosphatidylserine exposure was evaluated using the ApoAlert Annexin V-FITC Apoptosis Kit (Clontech Laboratories). Cells were harvested and washed with 35 mM phosphate buffer pH 6.8 containing 0.5 mM $MgCl_2$ and 1.2 M sorbitol. Cell walls were digested with 0.2 mg/ml zymolyase T100 (MP Biomedicals) in the above mentioned buffer for 20 minutes at 37°C. Protoplasts were then washed twice with binding buffer supplemented with sorbitol (10 mM Hepes/NaOH pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$, 1.2 M sorbitol). 4 µl of annexin V-FITC (20 µg/ml) or 5 µl PI (50 µg/ml) were added to 40 µl of cell suspension (10^7 cells) and incubated for 20 minutes at room temperature. Cells were then washed once and

resupendend in 1 ml of binding/sorbitol buffer containing 0.1% BSA (Sigma). Fluorescence intensity was measured in FL1 and FL3 channel for annexin V-FITC and PI signal, respectively.

Mitochondrial superoxide anion was detected using MitoSox (Molecular Probes). Briefly, cells were washed once in phosphate buffered saline (PBS, pH 6.8) and incubated in PBS with 5 µM MitoSox for 20 minutes at 30°C. Cells were then washed twice in PBS and fluorescence intensity in FL3 channel was assessed.

All measurements of fluorescence intensity were performed on a Cytomics FC 500 flow cytometer (Beckman Coulter) equipped with an argon laser (excitation wavelength 488 nm, laser power 20 mW). The fluorescence emission was measured trough a 525 band pass filter (FL1 parameter) and trough a 620 nm short pass filter (FL3 parameter). A total of 30000 cells were measured for each sample. Data analysis was performed afterwards with WinMDI 2.8 software.

Catalase and superoxide dismutase activity assay

Exponential phase (OD_{660}= 0.5-0.8) cells grown in minimal medium were harvested and resuspended (10^7 cells/ml) in minimal medium (pH 3.0, set with HCl) containing 100 mM acetic acid. At different times $3x10^8$ cells were harvested by centrifugation at 4000 rpm for 10 minutes, washed once with 1 ml of ice cold water and resuspended in 350 µl of sodium phosphate buffer 50 mM pH 7.0 for catalase assay, or Tris-HCl pH 7.5 for superoxide dismutase assay, containing 0.5 mM PMSF and protease inhibitors (complete Protease Inhibitor Cocktail-Roche). Cells were then disrupted with glass beads (425-600 µm, Sigma) in a homogenizer (BIO101/Savant FastPrep FP120) by two cycles of 20s at 6.0 speed. Lysates were collected and centrifuged 15 minutes at 4°C and the obtained supernatants were used as cell-free extracts.

Catalase activity was measured discontinuously by sampling H_2O_2 at appropriate time intervals. Briefly, 30 µl of H_2O_2 2.2 mM were added to 300 µl of cell-free

extract and rapidly mixed to give an initial H_2O_2 concentration of 200 µM. Every two minutes between 0 and 10 minutes 50 µl aliquots of the incubation mixture were removed and rapidly mixed with 950 µl of FOX reagent [ammonium ferrous sulphate (Sigma-Aldrich) 250 µM, xylenol orange (Sigma-Aldrich) 100 µM, sorbitol 100 mM, H_2SO_4 25 mM]. The mixtures were centrifuged 3 min at 12000 rpm after 30 min of incubation at room temperature. Supernatants were then read at 560 nm. The H_2O_2 concentration in the sample was calculated using a standard calibration curve made with different standard samples of known concentration of H_2O_2. Activities were calculated based on a reference curve, obtained with serial dilutions of a catalase standard (catalase from bovine liver, Sigma-Aldrich, cat. N. C30).

Superoxide dismutase activity was assayed using the Sigma SOD assay kit (19160) following the manufacturer's instructions.

Enzymatic activities were expressed as U/mg proteins. The total protein concentration was determined as described by Bradford (Bradford, 1976) by using the Bio-Rad protein assay (Bio-Rad) with bovine serum albumin as the standard.

Determination of intracellular reduced (GSH) and oxidized (GSSG) glutathione

Cell-free extracts were obtained as described for catalase and superoxide dismutase activity assays using a potassium phosphate buffer 100 mM pH 7.5. Successively, half volume of 5% (w/v) trichloroacetic acid was added to cell-free extract to precipitate the proteins. Samples were kept in ice for 20 minutes and centrifuged for 10 minutes at 14000 rpm at 4°C. The obtained supernatants were used for total glutathione (tGSH = GSH + GSSG) estimation. Samples for GSSG quantification were prepared by incubating 100 µl of the preparation above described with 2 µl of

2-vinylpyridine (Sigma) for 60 minutes at room temperature. GSH was then determined by subtracting GSSG value from tGSH.

tGSH and GSSG were determined according to Rahman *et al*., 2006. The final concentrations in the reaction mix were the following: 1 mM EDTA, 0.15 mM DTNB, 0.45 mM NADPH, 2 U/ml GSH reductase. Standard curves were constructed using GSH or GSSG (Sigma-Aldrich), respectively, ranging from 0.3125 to 10 μM. The assay was performed in 96-well plates in a microplate reader (SpectraMax 250, Molecular Devices) reading absorbance at 412 nm after the addition of GSH reductase to start the reaction. The absorbance was read every 20 s for 3 min in each reaction.

Results and discussion

Wild type and L-AA producing cells exhibit a marked different viability under acetic acid stress

Exponentially growing *S. cerevisiae* wild type and recombinant L-AA producing strains were incubated in the presence of increasing concentrations (40-120 mM) of acetic acid in minimal medium at pH 3.0. Cell viability was estimated through the determination of cfu at T=0, 50, 100, 150 and 200 minutes after acetic acid addition (Figure 1A). In the absence of acetic acid (control condition), cell viability rose to 260% during 200 minutes of growth for both strains (Figure 1A, circles; 100% corresponds to the cfu value at time T=0). At low concentration (40 mM), acetic acid exerted a cytostatic effect on both wild type and L-AA producing cells, inhibiting cell growth (Figure 1B, squares). Cell death was instead progressively induced when cells were exposed to increasing concentrations of acetic acid (100 and 120 mM, Figure 1B, triangles and diamonds). Remarkably, under these severe conditions, the L-AA producing strain exhibited a higher viability compared to the wild type. In particular, when cells were challenged with 100 mM acetic acid for 200 minutes, cell viability turned out to be 56% in L-AA producing strain (Figure 1B, closed triangles) against 5% in wild type (Figure 1B, open triangles). The same trend was highlighted when cells were treated with 120 mM acetic acid, even if this condition more severely affected cell viability of both stains (Figure 1B, diamonds). Acetic acid concentrations higher than 120 mM severely caused cell death in both stains even 30 minutes after the stressful treatment (data not shown). The kinetic of the death process induced by 100 mM acetic acid was considered suitable for further characterization of the stress response in the strains.

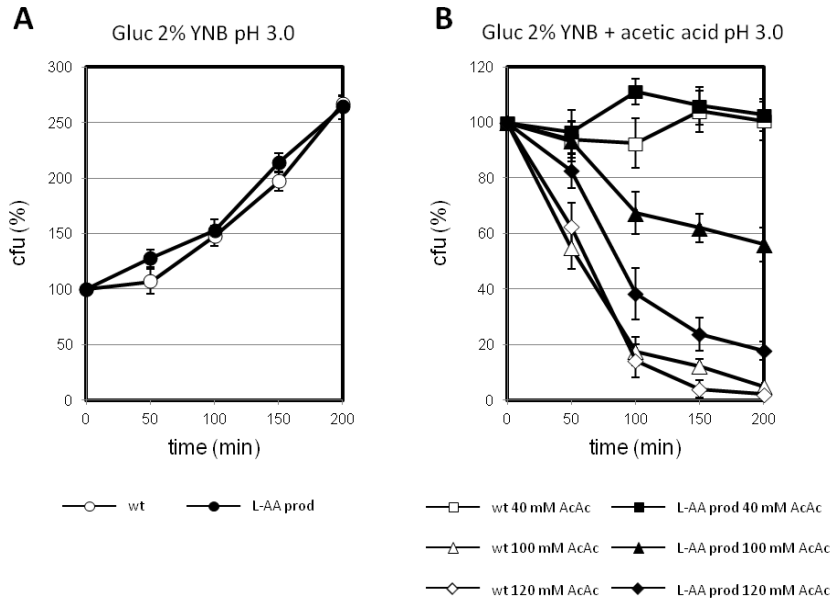

Figure 1. Viability of exponentially growing wild type and L-AA producing cells incubated in the absence (**A**) or presence (**B**) of increasing concentrations of acetic acid. Cells, grown in minimal medium up to OD_{660}=0.7-0.8, were harvested and exposed to 0, 40, 100 and 120 mM acetic acid in minimal medium pH 3.0. At indicated times, cell viability was analyzed by measuring colony-forming units (cfu) after two days of growth on plates at 30°C. 100% corresponds to cfu at time zero. Error bars correspond to SDs from three independent experiments.

It has been reported that in the yeast *S. cerevisiae* the cell death process mediated by moderately-toxic concentrations of acetic acid (up to 100 mM) is at least partially an active process dependent on *de novo* protein synthesis, denominated "acetic acid-induced programmed cell death" (AA-PCD) (Ludovico *et al.*, 2001). To address whether the higher viability in the L-AA producing strain under acid acetic stress may correlate with protection from AA-PCD, both wild type and L-AA producing strains were grown in the presence of cycloheximide, an inhibitor of protein synthesis, which is traditionally used to prevent PCD and therefore to evaluate its onset (Mattson and Furukawa, 1997; Ludovico *et al.*, 2001; Ludovico *et al.*, 2003).

As clearly shown in Figure 2, with cycloheximide cell death was partially prevented in both strains: survival was enhanced from 5 to 47% in wild type (Figure 2, open circles *vs* open squares) and from 56 to 74% in L-AA producing cells (Figure 2, closed circles *vs* closed squares) after 200 minutes of acetic acid treatment, indicating that AA-PCD occurs in both strains, but with a different degree.

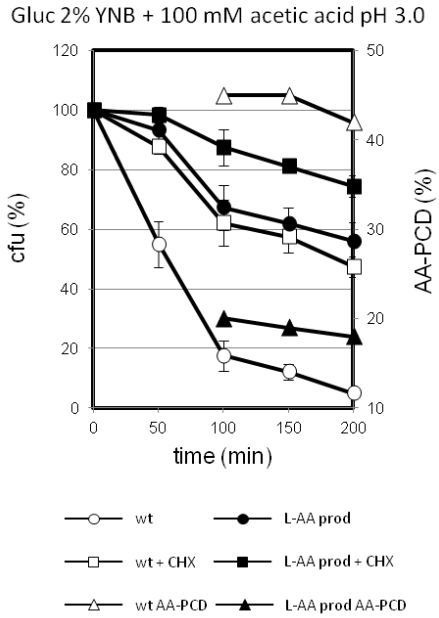

Figure 2. Viability of exponentially growing wild type and L-AA producing cells exposed to 100 mM acetic acid at pH 3.0 in the absence or presence of cycloheximide (50 mg/ml). At indicated times, cell viability was analyzed by measuring colony-forming units (cfu) after two days of growth on plates at 30°C. 100% corresponds to cfu at time zero. Error bars correspond to standard deviations from three independent experiments. Estimation of cells that undergo programmed cell death (AA-PCD, secondary axis) was calculated at 100, 150 and 200 minutes as the difference between the percentages of viable cells with and without cycloheximide treatment. SD does not exceed 7%.

The percentage of cells that underwent AA-PCD was simply estimated by subtracting the fraction of unviable cells determined in the presence from that

measured in the absence of cycloheximide (Figure 2, triangles). Starting from 100 minutes after acetic acid exposure, the fraction of cells undergoing AA-PCD in the L-AA producing strain was about 20% (closed symbols), while it was about the double (43-45%) in the wild type strain (open symbols), suggesting that the intracellular production of L-AA in the recombinant strain effectively takes part in preventing AA-PCD.

To better elucidate this aspect, wild type and L-AA producing strains were stained with annexin V-FITC, which evidences phosphatidylserine exposure on the outer side of cell membranes, as an early marker of apoptosis. Cells were also stained with PI, which evidences severely damaged/dead cells. Flow cytometric analysis of the two strains after acetic acid and cycloheximide treatments (as described in Material and Methods) are shown in Figure 3. When stained with annexin V-FITC in the absence of cycloheximide (Figure 3A, upper panels), the fraction of annexin V-FITC positive cells progressively increased with time in both strains, but much more in the wild type one. Indeed, only a small fraction of cells in the L-AA producing strain was annexin V-FITC positive (as a clear example, see the bimodal distribution after 180 minutes of acetic acid treatment), while most of the wild type cells appeared annexin V-FITC positive.

In contrast, cycloheximide treatment (Figure 3A, bottom panels), decreased the fraction of annexin V-FITC positive cells. In this case, the difference between the two strains was even more evident. Hence the data shown support the hypothesis that L-AA at least partially prevents AA-PCD.

The same trend was observed when cells were stained with PI (Figure 3B): the L-AA producing strain displayed only a minor fraction of PI positive cells in the absence of cycloheximide (upper panels) compared to wild type cells. Cycloheximide treatment strongly prevented the accumulation of dead cells in both strains (bottom panels), as previously evidenced in Figure 2.

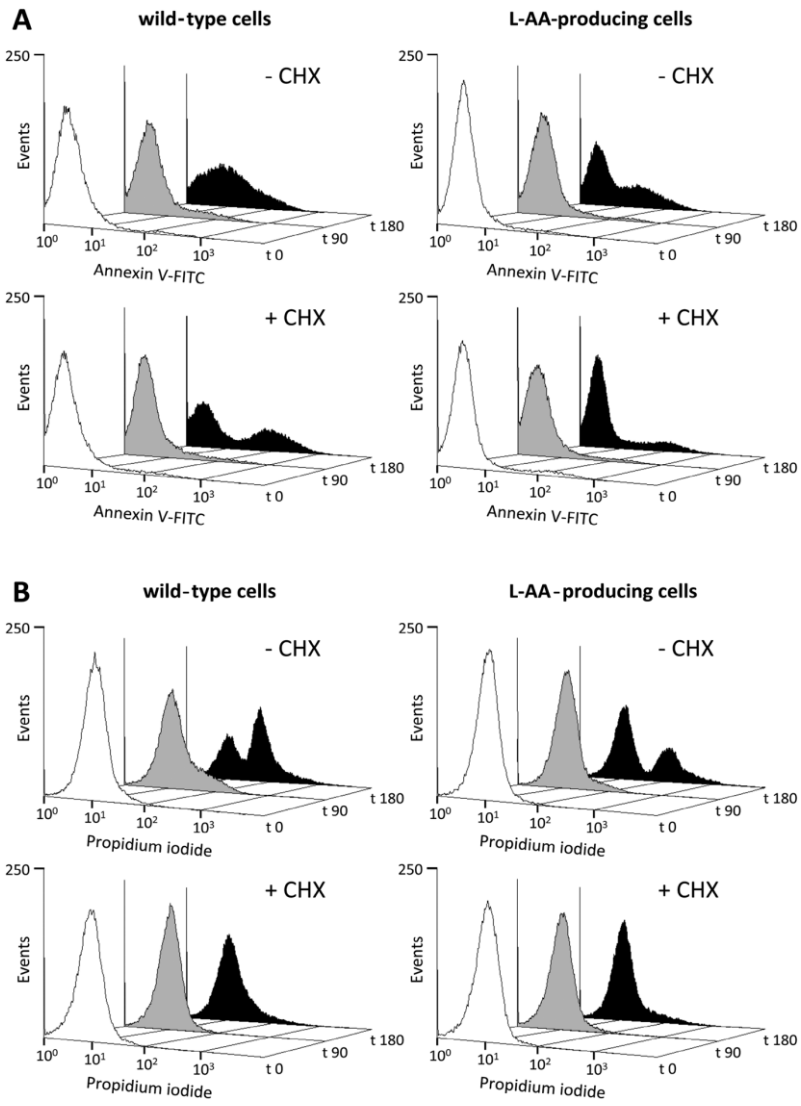

Figure 3. Flow cytometric analysis of wild type and L-AA producing cells stained with annexin V-FITC (A) and propidium iodide (B). Exponentially growing cells were incubated in the absence (-CHX) or presence (+CHX) of cycloheximide (50 µg/ml) for 30 minutes and then exposed to 100 mM acetic acid at pH 3.0. Cells were stained at 0, 90 and 180 minutes after acetic acid exposure. The fluorescence distributions of wild type (on the left) and L-AA producing (on the right) cells in the FL1 (annexin V-FITC) and FL3 (propidium iodide) channels are represented.

Wild type and L-AA producing strains exhibit different ROS content

ROS production is also a key cellular event of AA-PCD in *S. cerevisiae* (Guaragnella *et al*., 2007; Guaragnella *et al*., 2011; Farrugia and Balzan, 2012). To better understand the effect of the endogenous production of L-AA on ROS content under acetic acid stress, cells were stained with the fluorescent probes 2,7 dichlorofluorescin diacetate (H_2-DCF-DA) and dihydroethidium (DHE) during exposure to 100 mM acetic acid at pH 3.0 (Figure 4).

H_2-DCF-DA permeates into the cell, where it is hydrolyzed by cellular esterases to DCFH, which is then oxidised primarily by H_2O_2, but also by other reactive species such as $HO^{\bullet}$, $ROO^{\bullet}$ and by reactive nitrogen species (RNS), such as $^{\bullet}NO$ and $ONOO^-$, to the green fluorescent DCF (Gomes *et al*., 2005). DHE has been used to detect superoxide anion ($O_2^{-\bullet}$) due to its reported relative specificity for this ROS, which has been described as associated to cells committed to death (Gomes *et al*., 2005; Guaragnella *et al*., 2007).

No fluorescence signal associated with both H_2-DCF-DA and DHE was detected when cells were incubated in minimal medium at pH 3.0 (data not shown).

Consistently with the literature data (Guaragnella *et al*., 2007), fluorescence signal of H_2-DCF-DA was detected during the first 45 minutes of acetic acid treatment and not at later times (Figure 4A). DHE signal was instead detected after 60 minutes of acetic acid treatment and not before (Figure 4B). The L-AA producing strain exhibited a lower percentage of both H_2-DCF-DA and DHE positive cells respect to the wild type at all the tested times (Figures 4A and 4B). The lower intracellular ROS accumulation correlates with the higher viability of the L-AA producing strain compared with the wild type strain.

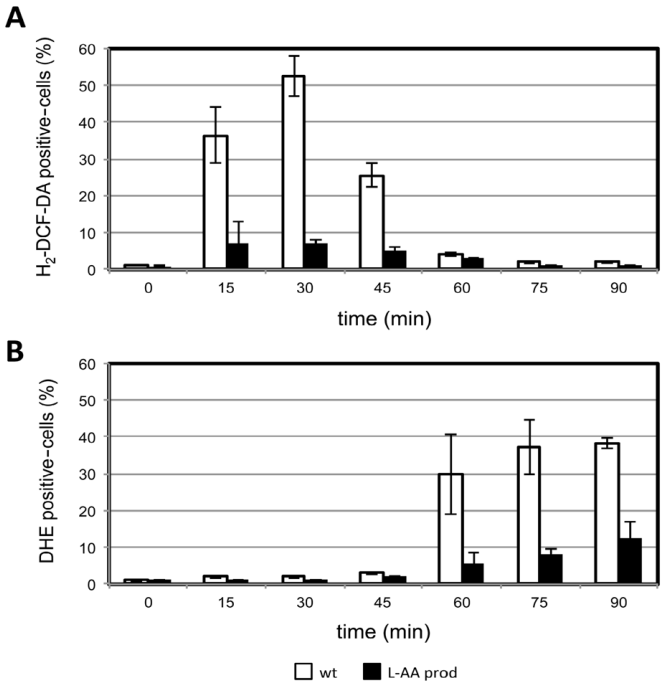

Figure 4. A) H_2-DCF-DA and B) DHE positive cells (%) during acetic acid treatment in wild type and L-AA producing cells. To determine the percentage of cells positive to the dyes, at least 400 cells were evaluated for each sample and for each time and analysed in three independent experiments. Error bars correspond to SDs from three independent experiments.

To further support this conclusion, the MitoSox probe was used to monitor mitochondrial superoxide anion. The data obtained showed that the percentage of cells not accumulating $O_2^{-\bullet}$ in the mitochondrial matrix after 100 minutes of acetic acid exposure was higher in the L-AA producing compared to the wild type strain (45% *vs* 36%), confirming the better detoxification occurring in the engineered strain.

Low pH pre-treatment affect cell survival under acetic acid stress of both wild type and L-AA producing strains

Many organisms become more tolerant to severe doses of a stress agent as a consequence of an initial exposure to a mild dose of the same or of another type of stress. This phenomenon, termed "acquired stress resistance", has been observed in microbes such as bacteria and yeasts as well as in multicellular organisms including plants, mammals, and even humans (Davies *et al*., 1995; Durrant and Dong, 2004; Hecker *et al*., 2007; Kensler *et al*., 2007; Matsumoto *et al*., 2007). This adaptive response relays upon the *de novo* protein synthesis occurring during the initial mild pre-treatment, but not during the severe stress treatment (Berry and Gasch, 2008; Lewis *et al*., 2010). It has been previously demonstrated that acid stress adaptation of *S. cerevisiae* (pre-incubation of cells in minimal medium at pH 3.0 for 30 minutes prior to the exposure to acetic acid) protects cells from subsequent AA-PCD, and this correlates with a high catalase activity (Giannattasio *et al*., 2005). Consistently, over-expression of *CTT1*, encoding for the cytosolic form of catalase, leads to reduced oxidative stress and increased cell resistance to various stress conditions (Guaragnella *et al*., 2008; Abbott *et al*., 2009; Collinson *et al*., 2011).

To investigate the effect of the simultaneous intracellular L-AA production and low pH pre-treatment on yeast viability under acetic acid stress, cell survival of wild type and L-AA producing strains was assayed after pre-incubation in minimal medium at pH 3.0 for 30 minutes prior to acetic acid exposure (Figure 5).

Acid stress adaptation significantly enhanced cell viability, and in particular from 5 to 41% in wild type (Figure 5, open circles *vs* open squares) and from 45 to 69% in L-AA producing cells (Figure 5, closed circles *vs* closed squares) after 200 minutes of acetic acid exposure. Hence, acquisition of acetic acid tolerance through pre-adaptation at low pH occurs more evidently in wild type cells, but plays a relevant

role also in the L-AA producing strain, suggesting that the endogenous defences may usefully cooperate with L-AA to further increase cell robustness.

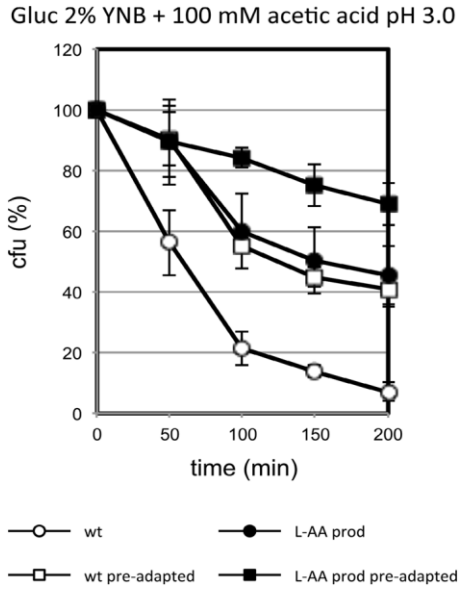

Figure 5. Viability of exponentially growing wild type and L-AA producing cells exposed to 100 mM acetic acid at pH 3.0 with or without pre-treatment in minimal medium at pH 3.0. At indicated times, cell viability was analysed by measuring colony-forming units (cfu) after two days of growth on plates at 30°C. 100% corresponds to cfu at time zero. Error bars correspond to SDs from three independent experiments.

Acetic acid stress induces high SOD activity in wild type, but not in L-AA producing strain

As stated in the Introduction, the yeast antioxidant defences include, among others, GSH, EAA and enzymes like SODs and catalases, contributing to modulate ROS levels (Fridovich, 1975; Switala and Loewen, 2002). H_2O_2 and $O_2^{-\bullet}$ levels in any single cell are therefore the result of their generation counteracted by the efficiency of their detoxification. To further characterize the difference in ROS accumulation observed between the wild type and L-AA producing strains, the SOD activity was

determined. We focused our attention on the first 60 minutes of stress, where a good fraction (at least 50%) of cells is still viable in both strains (Figure 1B, triangles).

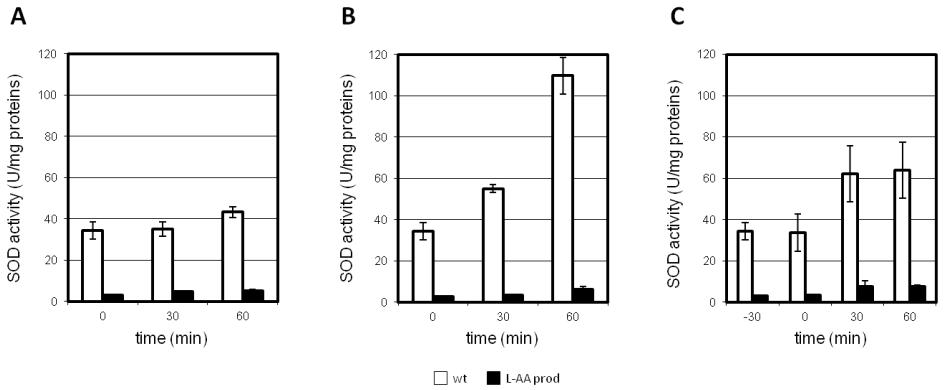

Figure 6. SOD activity in wild type and L-AA producing strains. Both strains were grown in minimal medium till the exponential phase (OD_{660}=0.7-0.8) and then inoculated in minimal medium pH 3.0 in the absence (A) or presence (B) of 100 mM acetic acid. SOD activity was assayed at 0, 30 and 60 minutes. (C) Exponentially growing cells were incubated in minimal medium at pH 3.0 for 30 minutes and then exposed to 100 mM acetic acid at pH 3.0. SOD activity was assayed at -30 (corresponding to the time 0 of incubation in minimal medium at pH 3.0), 0, 30 and 60 minutes. Error bars correspond to SDs from three independent experiments.

In the absence of acetic acid, a "basal" SOD activity was detected in the wild type strain that remained essentially constant over time. Also in the L-AA producing strain the SOD activity remained essentially constant, but at a significantly lower (8-fold) level (Figure 6A).

After exposure to acetic acid, the SOD activity increased significantly in the wild type strain with time, while, interestingly, it remained substantially negligible in the L-AA producing strain (Figure 6B). This data indicates that L-AA may possibly avoid the triggering of SOD, in both the absence and the presence of acetic acid, conferring a pronounced difference between the two strains. SOD activity was also measured in cells exposed to 100 mM acetic acid (pH 3.0) after

pre-adaptation at pH 3.0 for 30 minutes. SOD activity was substantially not influenced by the low pH treatment (Figure 6C), as it remained constant in both strains for 30 minutes of pre-treatment. After 30 minutes of acetic acid exposure, it was subsequently induced in both strains, even though in the L-AA producing strain it remained very low, resembling the trend obtained in the absence of acetic acid treatment (Figure 6C). Moreover, under acetic acid stress, in the wild type strain the low pH pre-treatment causes a minor SOD activation respect to that observed in cells directly exposed to the acid (compare time 60 min between Figures 6B and C).

It is important to underline once more that SOD generally represents the first line of defence in the wild type strain in the early phases of oxidative stress exposure. Indeed, it has been shown that *sod1* and *sod2* yeast mutants, defective in the cytosolic and mitochondrial SOD respectively, turned out to be hypersensitive to oxidative stress induced by various pro-oxidant agents (Outten *et al*., 2005; Auesukaree *et al*., 2009; Kwolek-Mirek *et al*., 2011). However, literature already suggested that a triggering of the sole SOD activity is, in any case, not enough to overcome a severe stress (Harris *et al*., 2005; Guaragnella *et al*., 2008), but it should at least be coupled with the overexpression of *CCS1*, which encodes for a chaperone responsible of Sod1 apoprotein activation (Brown *et al*., 2004; Harris *et al*., 2005). Indeed, a high SOD activity in the wild type strain after 60 minutes (Figure 6B) is not enough to prevent cell death, a process that is much avoided in the L-AA producing strain (Figure 1B).

To better elucidate the role of SOD, we deleted the *SOD1* gene in the wild type and L-AA producing strains. Deleted strains were grown in liquid medium either in the absence or in the presence of acetic acid 40 mM (Figure 7). Methionine and lysine (100 mg/l) were added to the medium, as *SOD*1 deletion has been reported to cause auxotrophy for both amino acids when cells are grown aerobically (Liu *et al*., 1992).

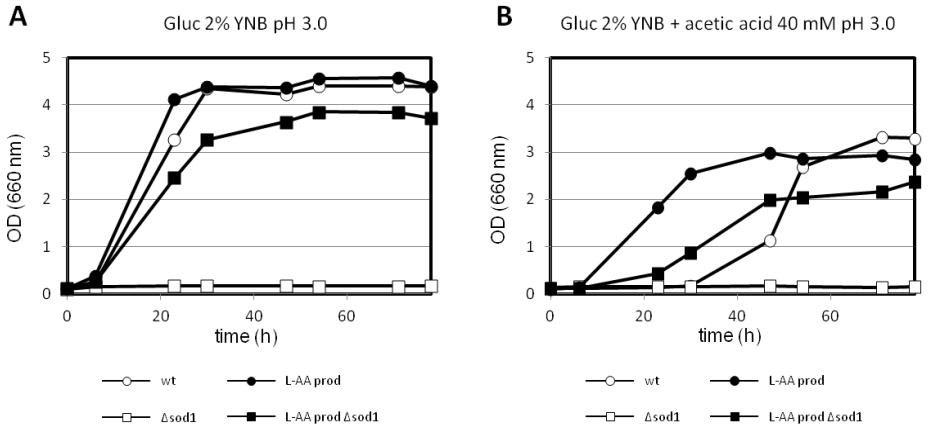

Figure 7. Growth curves of wild type, Δ*sod1*, L-AA producing and L-AA producing Δ*sod1* cells inoculated in minimal medium supplemented with lysine and metionine (100 mg/l) in the absence (A) or presence (B) of 40 mM acetic acid at pH 3.0.

In the absence of acetic acid, the *sod1*-deleted wild type strain did not start growing up to 78 hours (Figure 7A, open squares), and, on the contrary, the growth of L-AA producing *sod1*-deleted strain (Figure 7A, closed squares) was only slightly affected compared to wild type and L-AA producing strains (Figure 7A, open and closed circles). When cells were challenged with 40 mM acetic acid, growth was completely inhibited in the *sod1*-deleted wild type strain (Figure 7B, open squares), while the L-AA producing *sod1*-deleted strain was still able to resume growth, showing a shorter lag phase compared with the wild type strain (Figure 7B, closed squares). These results confirm, once more, that Sod1 activity appears to be strictly necessary in wild type strain, even in the absence of stress, and less essential in the L-AA producing one. Nevertheless, the L-AA producing strain (Figure 7B, closed circles) was still the best performing strain under these stress conditions, suggesting that also in this strain the activity of Sod1, even if low, is not completely dispensable. Deeper investigations will be necessary to elucidate this aspect, with a particular glance to the other known functions of Sod1, as reported by Culotta *et al.*, 1995.

Under acetic acid stress, the catalase response is similar in wild type and L-AA producing strains, but differs when cell are pre-adapted at low pH

Catalase activity was measured in minimal medium at pH 3.0 in the absence or presence of 100 mM acetic acid. Catalase activity increased over time in both strains reaching, after 60 minutes, a specific activity of 1 U/mg proteins for the wild type and of 1.3 U/mg proteins for the L-AA producing strain (Figure 8A). This observation confirms that the simple exposure to low pH values modulates this activity, as previously described by Giannattasio *et al*., 2005. When cells were pre-treated with cycloheximide for 30 minutes before being exposed at pH 3.0, we did not observe any increase of the catalase activity (data not shown), indicating that the observed increase shown in Figure 8A cannot be due to a generic catalytic activation by post-translational mechanisms.

In the presence of acetic acid, the catalase activity was hardly detectable in both wild type and L-AA producing cells (measured activity of about 0.2 U/mg proteins) and did not changed over time (Figure 8B), suggesting that under the tested conditions the enzyme may not be activated or remain inactive, as previously proposed by Giannattasio and co-workers (2005).

Catalase activity was also measured in cells exposed to 100 mM acetic acid (pH 3.0) after pre-adaptation at pH 3.0 for 30 minutes. Differently from what observed for SOD activity, catalase activity was influenced by the low pH treatment, as it was found to be increased in both strains upon pre-incubation for 30 minutes at pH 3.0 (Figure 8C, time points -30 and 0 minutes), consistently with the literature data (Giannattasio *et al*., 2005). Moreover, the activity went on to increase up to 30 minutes of acetic acid treatment in the wild type strain, while it remained essentially constant and significantly lower in the L-AA producing strain (Figure 8C, time points 30 and 60), reaching after 60 minutes a specific value of 1.7 U/mg proteins in the first and of 1.1 U/mg proteins in the second. We can therefore argue

that catalase action is at least in part responsible for the increased survival under acetic acid after low pH pre-treatment in both strains (see again Figure 5, closed symbols), while SOD activity seems required only in the wild type strain.

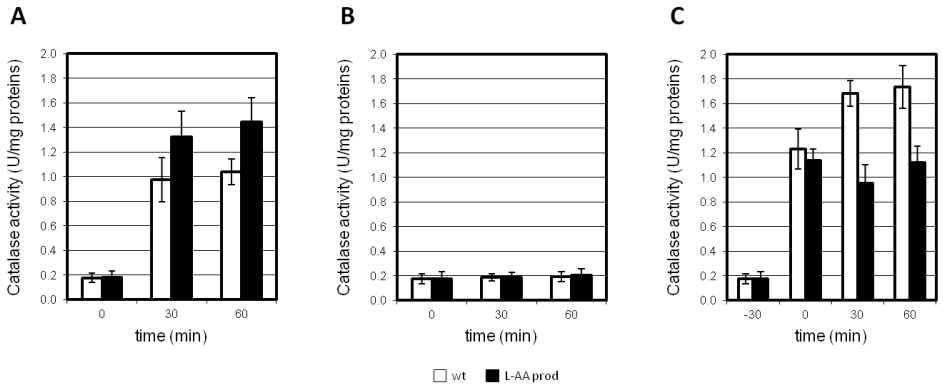

Figure 8. Catalase activity in wild type and L-AA producing strains. Both strains were grown in minimal medium till the exponential phase (OD_{660}=0.7-0.8) and then inoculated in minimal medium pH 3.0 in absence (A) or presence (B) of 100 mM acetic acid. Catalase activity was assayed at 0, 30 and 60 minutes. (C) Exponentially growing cells were incubated in minimal medium at pH 3.0 for 30 minutes and then exposed to 100 mM acetic acid at pH 3.0. Catalase activity was assayed at -30 (corresponding to the time 0 of incubation in minimal medium at pH 3.0), 0, 30 and 60 minutes. Error bars correspond to SDs from three independent experiments.

It has been previously shown that the addition of exogenous L-AA to yeast cells challenged with paraquat enhanced cell viability and reduced catalase activity compared with cells treated with the sole stressing agent (Saffi *et al.*, 2006). Our results seem to confirm this trend, as catalase, even if induced, remained significantly lower in the L-AA producing strain, suggesting that also this activity (as previously shown in the case of SOD) can be "economized", without affecting the better performance of said strain under acetic stress conditions.

On the other hand, catalase induction in the pre-adapted L-AA producing strain, and the consequent further increase of stress tolerance, demonstrate that L-AA and

catalase effects are only partially overlapping and that the stress response is a complex trait.

Moreover, as a second consideration, it turns out that SOD and catalase enzymes do not necessary act in sequence, as it may be expected. We could measure catalase activity even in the absence of SOD activity, as in the case of pre-adapted L-AA producing strain. In this sense our results corroborate the hypothesis of catalase involvement in the acquisition of tolerance and in the adaptation response to the stress, as previously suggested (Izawa *et al.*, 1996). Further investigations are still necessary to fully explore the effective role of catalase in the stress response and its relations with the GSH system as suggested by previous studies (Grant *et al.*, 1998).

Reduced and oxidized glutathione accumulate more in the wild type compared with the L-AA producing strain during acetic acid stress

Glutathione is a tripeptide with antioxidant properties composed of cysteine, glutamate and glycine. Studied on *gsh1*-deleted yeast strains, defective in one of the two enzymes responsible for glutathione synthesis, revealed that this molecule is indispensable for an efficient oxidative stress response (Izawa *et al.*, 1995; Stephen and Jamieson, 1996). Consistently, yeast strains over-expressing *GSH1* have been shown to be more tolerant to H_2O_2-induced oxidative stress compared with wild type strains (Collinson *et al.*, 2011). Glutathione can exist in either its reduced (GSH) or oxidized (GSSG) form, the last occurring when two GSH molecules are interlinked with a disulfide bond. In fact, the utilization of GSH results in the oxidation to its disulfide form, from which GSH can be regenerated by the action of the NADPH-dependent glutathione reductase (Grant, 2001).

The intracellular content of GSH and GSSG has been measured in both strains every 30 minutes during 120 minutes of exposure to 100 mM acetic acid at pH 3.0 (Figure 9).

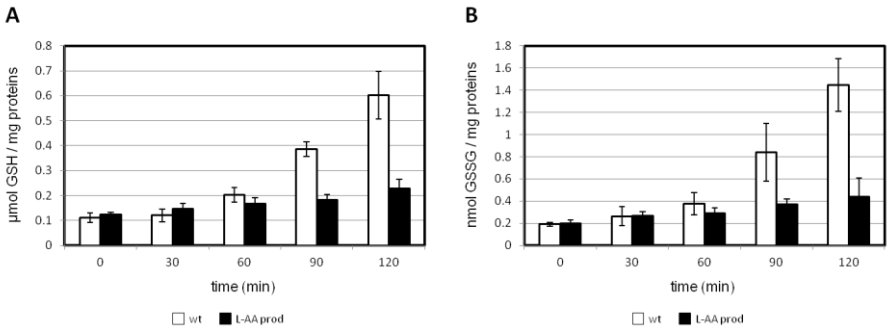

Figure 9. Intracellular GSH (A) and GSSG (B) content in wild type and L-AA producing strains. Both strains were grown in minimal medium till the exponential phase (OD_{660}=0.7-0.8) and then inoculated in minimal medium pH 3.0 in the presence of 100 mM acetic acid. GSH and GSSG were measured at time 0, 30, 60, 90 and 120 minutes. Error bars correspond to SDs from three independent experiments.

As shown in Figure 9, the intracellular GSH and GSSG content increased with time in both strains during acetic acid stress. However, starting from 90 minutes of acetic acid exposure, GSH accumulated proportionally to GSSG more in the wild type than the L-AA producing strain. The higher level of GSSG measured in wild type cells (Figure 9B) suggests that GSH is more consumed compared with L-AA producing cells. On the other hand, the more pronounced GSH accumulation observed in the wild type strain (Figure 9A) can be attribute to a greater activation of those mechanisms involved in the maintenance of the GSH/GSSG ratio within cell, such as GSH synthesis and GSH recycling catalyzed by glutathione reductase. Overall, these results demonstrate that in response to acetic acid stress GSH is less utilized and, consequently, the GSH system is less activated in the L-AA producing compared with the wild type strain. Since 2 ATP molecules or 1 NADPH molecule are required to synthesize or recycle 1 GSH molecule, respectively, these data further support the hypothesis that the intracellular L-AA production may determine a lower energy requirement.

We therefore suggest that the L-AA producing strain is favored under stress conditions both by the direct intracellular availability of L-AA and by the indirect

energy saving of endogenous defences. This consideration is additionally supported by the observation that L-AA producing cells directly exposed to acetic acid (negligible SOD and catalase activities) showed a viability comparable to that of adapted wild type cells (high SOD and catalase activities) (Figure 5, closed circles *vs* open squares).

The L-AA producing strain is more resistant to acetic acid than the wild type strain also in anaerobic conditions

The improved tolerance to acetic acid showed by the L-AA producing strain makes it has an attractive cell factory to be exploited in industrial bio-processes, such as ethanol production. However, the ethanol production is normally carried out in anaerobic or micro-aerobic conditions, while all the experiments here presented were performed in aerobic conditions. We therefore analyzed the tolerance to acetic acid of wild type and L-AA producing cells in anaerobic conditions. To this purpose, the growth phenotypes of both strains were examined by spot assay on minimal medium plates containing increasing concentrations of acetic acid (0, 15, 20 and 30 mM) at pH 3.0. The plates were placed in anaerobic jars in which the air was displaced with nitrogen gas.

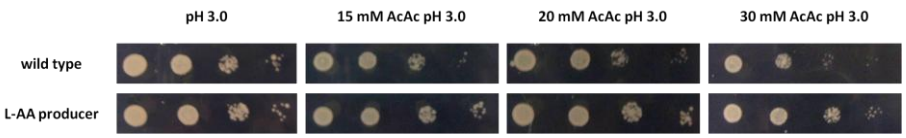

Figure 10. Acetic acid susceptibility of wild type and L-AA producing cells grown in anaerobic condition. Five µl of 1:10 serial dilutions were spotted onto minimal medium agar plates at pH 3.0 in the absence (control) or presence of increasing concentrations of acetic acid (15, 20 and 30 mM). Growth differences were recorded following incubation of the plates in jars in the absence of oxygen for 48 h at 30°C.

As shown in Figure 10, under anaerobic conditions the L-AA producing strain exhibited a lower growth inhibition compared with the wild type strain at all the tested acetic acid concentrations.

Conclusions

In conclusion, here we show that the higher viability of the L-AA producing strain exposed to acetic acid in comparison with the wild type strain correlates with a minor to null activation of SOD, with a lower GSH requirement and, following the pre-treatment at pH 3.0, with a minor induction of catalase. Moreover, in the engineered strain there is a significantly lower fraction of cells undergoing an active mechanism of death, correlating with a lower accumulation of ROS, suggesting a protective effect of L-AA against the ROS-dependent AA-PCD pathway. To better elucidate this aspect, it would be interesting to study the effect of the intracellular L-AA production on acetic acid response in mutant strains in which AA-PCD occurs in a ROS-independent way (Guaragnella *et al*., 2010b). Last but not least, a relevant difference in SOD activation between the two strains is also detected in the absence of stress conditions, and a consequence on growth is visible also at non-lethal concentration of acetic acid (40 mM, Figure 7).

Furthermore, the demonstration that the L-AA producing strain is more tolerant to acetic acid than wild type strain also under anaerobic conditions suggests that it is worth to assay the ethanol production of the engineered strain in bioreactors.

Finally, we could speculate that the effects derived from the endogenous vitamin C production allows the cells to save energy, and these features may be desirable for a cell factory which requires energy to be devoted to growth and biomass/metabolites accumulation/production.

For these said reasons, the engineered yeast is an interesting model for further studying how, and to which extent, intracellular antioxidants can prevent cellular damages and their consequences, and at the same time for further suggesting how to improve a cell factory.

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Chapter 2

Effects of the intracellular production of vitamin C on *Saccharomyces cerevisiae* chronological life span

Manuscript in preparation for *FEMS Yeast Research*

Introduction

Ascorbic acid, or Vitamin C, is a water-soluble vitamin with antioxidant properties. It is produced by several organisms such as plants and most animals except some primates, including humans, and other species such as guinea pigs and bats, being therefore an essential nutraceutical/nutrient. By acting as an anti-oxidant and as a cofactor for a number of enzymes, several physiological functions of Vitamin C for regular cell metabolism and functions have been described (as reviewed in Traber and Stevens, 2011). Because of its highly effective antioxidant properties and because reactive oxygen species (ROS) have been recognized as responsible of cellular aging (Golden *et al*., 2002; Linnane *et al*., 2007), the effect of Vitamin C supplementation on lifespan in higher eukaryotes has been extensively investigated, but it remains quite controversial (Pallauf *et al*., 2013). Some studies on model organisms reported a beneficial effect of Vitamin C on longevity (see, as example, Bahadorani *et al*., 2008; Massie *et al*., 1984), while others were unable to recognize a role of Vitamin C to prolong lifespan (see, as example, Selman *et al*., 2006; Tappel *et al*., 1973) or even a reduction in longevity was observed (see, as example, Massie *et al*., 1976; Sohal *et al*., 1985). Moreover, an inverse correlation between the amount of Vitamin C supplemented and the effect on lifespan was noted (Pallauf *et al*., 2013), possibly due to pro-oxidant properties of Vitamin C (Podmore *et al*., 1998). It has been indeed demonstrated *in vitro* that Vitamin C maintains iron and other transition metals in a reduced state leading to the generation of hydroxyl radicals ($HO^{\bullet}$) or lipid alkoxyl radicals ($LO^{\bullet}$) through the Fenton reaction (Buettner and Jurkiewicz, 1996; Halliwell, 1996).

The yeast *Saccharomyces cerevisiae* has been widely exploited as a model system to provide significant insights in the molecular mechanisms involved in the regulation of aging, allowing the identification of pathways characterized by

similarities with those regulating the lifespan in higher eukaryotes (Fabrizio and Longo, 2003; Fabrizio and Longo, 2007). It is generally accepted that, in yeast, as well as in higher eukaryotes, oxidative stress, induced by the production of ROS by mitochondrial metabolism, plays an important role in aging processes (Longo *et al*., 2012). Consistently, several genetic factors involved in stress response have been identified to modulate lifespan, pointing out a link between stress resistance and longevity (Fabrizio *et al*., 2003; Longo *et al*., 2012). In particular, an important role in long-term survival has been attributed to cytoplasmic (Sod1) and mitochondrial (Sod2) superoxide dismutases, whose activity is essential for oxygen radicals' detoxification. In fact, yeast mutants lacking either *SOD1* or *SOD2*, or both, showed a short-life phenotype compared to wild type strains (Longo *et al*., 1996). Coherently, the single over-expression of *SOD1* or *SOD2* increased *S. cerevisiae* lifespan, and a more pronounced effect on cell survival was observed when *SOD1* and *SOD*2 were together over-expressed (Fabrizio *et al*., 2003). As humans, *S. cerevisiae* does not produce Vitamin C, but it has been demonstrated that the reduced lifespan due to the lack of Sod1 can be ameliorate by the exogenous addition of ascorbate (Krzepiłko *et al*., 2004). However, the effect of Vitamin C on longevity in wild type yeast strains has never been studied.

One of the classic approaches used to measure lifespan in *S. cerevisiae* is based on the chronological longevity of a population of non-dividing yeast cells, named chronological life span (CLS) (Fabrizio *et al*., 2003; Longo *et al*., 2012). CLS is generally determined by growing cells till stationary phase and monitoring cell survival over time. Viability is measured as cell ability to growth upon return to rich-nutrient conditions. It has been suggested that the effect of ascorbic acid cannot be accurately detected *in vivo* in cells treated with exogenous ascorbic acid, since it leads to H_2O_2 production as a consequence of its auto-oxidation in cell culture medium (Clément *et al*., 2001). The employment of a yeast strain able to produce L-ascorbic acid by itself should therefore be preferred, especially in

studies requiring a long-time cultivation, such as those regarding CLS measurements. Therefore, here we studied CLS in a *S. cerevisiae* wild type strain compared to the same strain metabolically engineered to produce L-ascorbic acid (L-AA) (Branduardi *et al*., 2007; Fossati *et al*., 2011). This strain has been previously shown to be characterized by an increased stress resistance toward different kinds of stress (Branduardi *et al*., 2007; Fossati *et al*., 2011), and in particular to acetic acid (Martani *et al*., 2013), which represents a chronological aging-inducer factor in yeast (Burtner *et al*., 2009).

Our data demonstrate that under standard conditions, *i.e.* growth in minimal medium containing 2% glucose as initial carbon and energy source, the L-AA producing strain exhibited an extended CLS compared with the wild type strain, most likely linked to higher acetic acid tolerance and acetate metabolism.

Materials and methods

Yeast strain, media and growth conditions

The *S. cerevisiae* genetic background used in this study was GRF18U (*MATα*; *ura3*; *his3-11,15*; *leu2-3,112*; cir^+) (Brambilla *et al*., 1999). The strains used were GRF18Uc [pZ5] (*MATα*; *ura3*; *his3-11,15*; *leu2-3,112*; [pYX012; pYX022; pYX042; pZ_5]) (Branduardi *et al*., 2007) and GRF18U L-AA producing (*MATα*; *ura3*; *his3-11,15*; *leu2-3,112*; [p012*b*T*At*ME *At*MIP; p022*At*LGDH; p042*Sc*ALO; pZ_5*At*VTC2] (Fossati *et al*., 2011).

Yeast cultures were grown in minimal synthetic medium (0.67% [w/v] YNB medium [catalogue no. 919-15 Difco Laboratories, Detroit, Mich.] with 2% [w/v] D-glucose as carbon source). Histidine, uracil, leucine, lysine, isoleucine and valine (Sigma) and the antibiotic nourseotricine sulphate (cloNAT, WERNER BioAgents, Germany) were added to a final concentration of 50 mg/L. Each experiment was repeated at least three times. All strains were grown in shake flasks at 30 °C and 160 rpm and the ratio of flask volume/medium was 5:1.

CLS determination

Cells were inoculated at OD_{660}= 0.25 in minimal medium containing 2% glucose supplemented with histidine, leucine, isoleucine, lysine, valine, and uracil and the antibiotic nourseotricine sulphate. Starting from 72 h from the inoculum (Day 3, first age point), when cells were in stationary phase, cells were plated every 2-3 days onto rich YPD medium (2% glucose, 1% yeast extract, 2% tryptone) agar plates containing 50 mg/L of the antibiotic nourseotricine sulphate. Viability was determined by measuring colony-forming units (cfu) after 2 days of growth on plate at 30°C. 100% of cell survival corresponds to cfu at Day 3. CLS experiments in buffered medium were performed by growing cells in minimal medium

containing 2% glucose added with a citrate phosphate buffer (64.2 mM Na_2HPO_4 and 17.9 mM citric acid, pH 6.0) prior to the inoculum.

Acetic acid treatment

Acetic acid treatment was performed essentially as described in (Burtner *et al*., 2009). 500 µL of 5 days old cells, grown in minimal medium containing 2% glucose, were transferred into round bottom tubes and 55.5 µL of 1, 2 or 3 M concentrated acetic acid were added to the culture to obtain the final concentration of 100, 200 or 300 mM, respectively. Cells were incubated for 200 min at 30°C at 160 rpm and successively a 1000 fold culture dilution was plated onto rich YPD medium agar plates. Cell viability was determined by measuring cfu after 2 days of growth on plate at 30°C. 100% of cell survival corresponds to cfu measured in culture incubated with 55.5 µL of water (0 mM acetic acid treatment).

Annexin V-FITC staining

Phosphatidylserine exposure was evaluated using the ApoAlert Annexin V-FITC Apoptosis Kit (Clontech Laboratories). Cells were harvested and washed with 35 mM phosphate buffer pH 6.8 containing 0.5 mM $MgCl_2$ and 1.2 M sorbitol. Cell walls were digested with 0.2 mg/mL zymolyase T100 (MP Biomedicals) in the above mentioned buffer for 20 minutes at 37°C. Protoplasts were then washed twice with binding buffer supplemented with sorbitol (10 mM Hepes/NaOH pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$, 1.2 M sorbitol). 4 µL of annexin V-FITC (20 µg/ml) was added to 40 µl of cell suspension ($1x10^7$ cells) and incubated for 20 minutes at room temperature. Cells were then washed once and resuspendend in 1 mL of binding/sorbitol buffer containing 0.1% BSA (Sigma) and analyzed by flow cytometry. The measurement of fluorescence intensity was performed on a Cytomics FC 500 flow cytometer (Beckman Coulter) equipped with an argon laser

(excitation wavelength 488 nm, laser power 20 mW). The fluorescence emission was measured trough a 525 band pass filter (FL1 parameter). A total of 30000 cells were measured for each sample. Data analysis was performed afterwards with Cyflogic software.

Metabolites determination

At indicated time-points, 1 ml was sampled from cell culture and centrifuged at 14000 rpm for 15 min; the supernatant was collected and stored at -20 °C until analysis. The amount of extracellular glucose, ethanol and acetic acid were determined by HPLC based method using H_2SO_4 5 mM as mobile phase and Aminex HPX-87P column, 300 × 7.8 mm with a polystyrene divinylbenzene-based matrix (BioRad). The analyses were performed at 35°C.

Results

Chronological life span increases and aging-induced apoptosis incidence decreases in a S. cerevisiae strain engineered for L-ascorbic acid production

As stated in the introduction, chronological life span (CLS) is the length of time that a non-dividing cell survives. Cell survival of wild type and L-AA producing strains was analyzed and compared after they had reached the maximal cell density value. To this purpose, wild type and L-AA producing cells were grown in minimal medium in the presence of 2% glucose till the stationary phase, and cell viability was successively estimated every 2-3 days. The number of cfu obtained at the first age-point (Day 3) corresponds to cell viability at 72 h after the inoculum and was considered as the initial survival (100%).

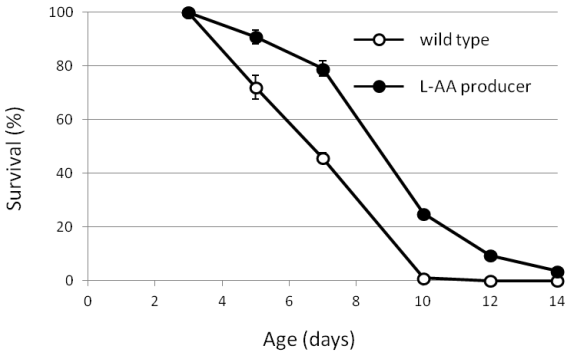

Figure 1: Relative survival of aging wild type and L-AA producing cells grown in minimal medium containing 2% glucose. At indicated age-points, cell viability was analyzed by measuring colony-forming units (cfu) after two days of growth on plates at 30°C. 100% corresponds to cfu at day 3. Error bars correspond to standard deviations from two independent experiments.

As shown in Figure 1, the progressively aging-induced cell death in the long-term cultivation observed for the wild type strain resulted delayed in the L-AA producing strain, which exhibited 25% of cell viability after 10 days of aging, when wild type cells were already completely dead. Thus, in the tested growth conditions the intracellular L-AA production increases the *S. cerevisiae* CLS.

It has been reported that chronologically aged yeast cells display typical markers of apoptosis, such as ROS accumulation, cleavage of chromosomal DNA and phosphatidylserine exposure, suggesting that apoptosis induction is a determinant of aging-cells mortality (Fabrizio and Longo, 2008; Herker *et al*., 2004). Therefore, 3, 5 and 10 days old wild type and L-AA producing cells were stained with annexin V-FITC to detect phophatidylserine exposure on the outer side of cell membranes as a marker of apoptosis, and analyzed by flow cytometry. For both aging wild type and L-AA producing strains, Figure 2 shows the overlay histograms representing the fluorescence distribution in the FL1 channel of a define population of not stained (black) and annexin V-FITC stained (green) cells at time 3, 5 and 10 days. Cells were considered positive to annexin V-FITC staining when exhibited a higher fluorescence intensity compared to that of not stained cells. At day 3, no cells were annexin V positive in both strains, since the distributions of stained and not-stained cells were completely overlapping. After 5 days, a subpopulation characterized by higher fluorescence values appeared in the wild type strain, but not in the L-AA producing one, corresponding to the fraction of annexin V-FITC labeled cells. In the wild type strain this subpopulation became more pronounced after 10 days; on the contrary, a negligible fraction of 10 days old L-AA producing cells showed higher fluorescence levels. These results suggest that in the wild type strain apoptosis is progressively induced over time, whereas in the L-AA producing strain it occurs to a minor extent. Consistently, we have previously demonstrated that the intracellular L-AA production protects at a certain extent of degree *S. cerevisiae* cells from acetic acid-induced programmed cell death (AA-PCD) (Martani *et al*.,

2013), which is characterized by several hallmarks of apoptosis (Ludovico *et al*., 2001).

We therefore speculate that the increased CLS displayed by the producing strain likely correlates with the protective effect of L-ascorbic acid toward apoptosis incidence/occurrence.

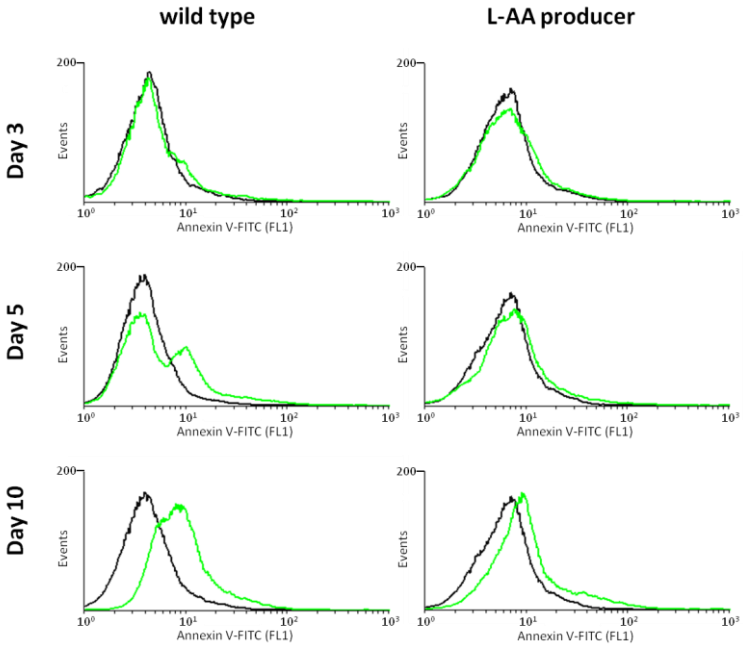

Figure 2: FACS analyses of aging wild type and L-AA producing unstained (black histograms) or annexin V-FITC stained (green histograms) cells at day 3, 5 and 10. The fluorescence distributions of wild type (on the left) and L-AA producing cells (on the right) are represented. One representative experiment is shown.

Acetate metabolism is enhanced in the L-AA producing compared with wild type strain

When *S. cerevisiae* cells are grown in a synthetic defined media containing 2% glucose, aerobic respiration is limited and glucose is partially fermented to ethanol and carbon dioxide. Upon glucose depletion, cells shift from a fermentative to a respiratory metabolism in which ethanol, produced during fermentation, is oxidized to acetaldehyde and subsequently converted to acetate. Acetate is initially excreted in the medium culture and, during the post-diauxic phase, it is metabolized to acetil-CoA which is used as a substrate for the glyoxylate and TCA cycles. In yeast, a molecular mechanism of chronological aging has been suggested in which acetic acid, produced as a by-product of glucose fermentation, is the main determinant of cellular aging (Burtner *et al*., 2009; Orlandi *et al*., 2013). Therefore, ethanol contributes to yeast chronological aging since it is metabolized to acetate (Burtner *et al*., 2009). The pro-aging effect of these two by-products has been supported by several studies which brought to light the existence of an inverse correlation between the amount of extracellular acetic acid or ethanol and CLS (Orlandi *et al*., 2013).

Ethanol and acetate accumulation in the medium, as well as glucose consumption, were quantified by high performance liquid chromatography (HPLC) during the exponential growth on glucose and after the diauxic shift, in wild type and L-AA producing cells cultures. In both strains glucose was already completely consumed after 24 hours from the inoculums, and acetate and ethanol were present in the medium at similar concentrations. However, the L-AA producing cells showed a higher glucose consumption rate, as measured during the first 8 hours of growth, accordingly with a slightly higher ethanol production rate. During the post-diauxic phase, ethanol was consumed with a similar rate by both strains, but, whereas acetic acid concentration decreased in the L-AA producing culture, it continued to

increase in the wild type one, reaching, after 72 hours, an accumulation 6-fold higher than in the engineered strain culture. These results demonstrate that acetate metabolism is different between wild type and the L-AA producing cells and that the enhanced acetate consumption showed by the L-AA producing strain leads to a reduced cell exposure to this toxic compound.

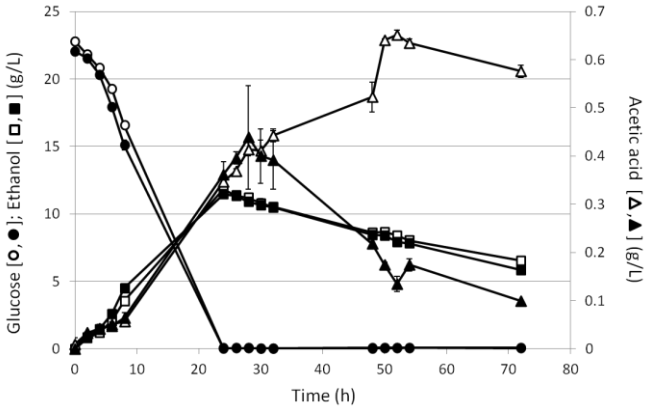

Figure 3: Determination of glucose, ethanol and acetic acid concentration (g/L) in the culture medium of wild type (open symbols) and L-AA producing (closed symbols) strains during the first 72 h after the inoculum. Cells were inoculated at the initial optical density of 0.25 (660 nm) in minimal medium containing 2% glucose and grown at 30 °C and 160 rpm. Extracellular glucose, ethanol and acetic acid concentrations were determined at the indicated time-points by HPLC analysis. Errors bars correspond to standard deviations from two independent experiments.

Aged L-AA producing cells show an improved acetic acid tolerance compared to wild type cells

The intracellular production of L-ascorbic acid has been previously demonstrated to increase acetic acid tolerance of exponentially growing *S. cerevisiae* cells (Martani *et al*., 2013). The extended CLS observed in the yeast strain engineered to produce this antioxidant may therefore also correlate with its intrinsic resistance to acetic acid. To test this possibility, 5-days old wild type and L-AA producing cells

grown in 2% glucose were challenged with increasing concentrations of acetic acid (100, 200 and 300 mM) or with water, as a control. The pH value of extracellular cultures was 2.5 for all the acid concentrations in both strains. After 200 minutes of incubation at 30°C, cells were plated onto rich agar medium and cell viability was measured after 2 days of growth. In all the tested conditions, the L-AA producing strain exhibited a higher viability compared to the wild type strain (Figure 3). These results demonstrated on one hand that the intracellular L-AA production confers protection toward acetic acid also in quiescent stationary-phase cells, and, on the other, that the extended CLS showed by the L-AA producing strain correlates with its enhanced tolerance to acetic acid. The correlation between extended CLS and acetic acid tolerance is supported by the observation that some longevity-enhancing interventions such as growth in high osmolarity medium (Burtner *et al*., 2009) and the deletion of *SCH9* or *RAS2* genes (Fabrizio *et al*., 2001; Fabrizio *et al*., 2003), are all characterized by an increased cellular resistance to acetic acid (Burtner *et al*., 2009).

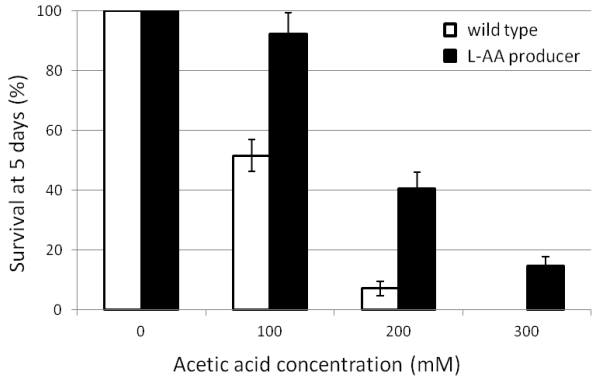

Figure 4: Relative survival of 5 days old wild type and L-AA producing cells exposed to different acetic acid concentrations. Cells, grown in minimal medium containing 2% glucose up to 120 h, were harvested and exposed to 0, 100, 200 and 300 mM acetic acid. After 200 min at 30 °C, cell viability was analyzed by measuring colony-forming units (cfu) after two days of growth on plates at 30°C. 100% corresponds to cfu of not treated cells. Error bars correspond to standard deviations from three independent experiments.

The L-AA producing strain showed a reduced CLS compared with the wild type strain when grown in buffered culture medium

The toxic effect of acetic acid on chronological aging cells has been demonstrated to be promoted by the extracellular acidification occurring during fermentation (Burtner *et al*., 2009; Orlandi *et al*., 2013). Acetic acid can permeate plasma membrane only when present in the undissociated form which, once in the cytoplasm, dissociates leading to cytotoxicity. Medium acidification, which is dependent on the starting glucose concentration (Burtner *et al*., 2009), correlates inversely with CLS possibly by influencing the predominance of the harmful undissociated form over the relatively not dangerous dissociated form of the organic acid. The toxic effect of acetic acid can be therefore attenuated by buffering the growing medium at pH higher than the pKa of the acid (pKa = 4.75). To elucidate the effect of the intracellular L-AA production on yeast chronological aging in conditions in which acetic acid toxicity is strongly reduced, we compared CLS of wild type and L-AA producing cells grown in synthetic defined medium containing 2% glucose buffered at pH 6.0 using a citrate phosphate buffer. Buffering the pH of 2% glucose medium to 6.0 has been shown to increase CLS in several different laboratory yeast strains (Burtner *et al*., 2009; Longo *et al*., 2012). Consistently, as depicted in Figure 5, for both strains, the CLS was extended when cells were grown in buffered medium compared to the CLS measured in medium whose pH drastically dropped to pH 2.5 during the first 24 h of growth (Figure 1). However, differently to what observed in the CLS experiment performed in an acidic environment, when grown at pH 6.0 the L-AA producing strain exhibited a short-lived phenotype compared to the wild type strain (Figure 5). These opposite results supported the hypothesis that the intracellular L-AA production increases *S. cerevisiae* CLS in 2% glucose by conferring an increased cell tolerance against acetic acid, which is, as stated before, an extrinsic factor promoting chronological

aging. Consequently, in conditions in which acetic acid is relatively not dangerous, *i.e.* when mainly present in its dissociated form, the advantage conferred by L-AA to aging cells failed and, on the contrary, led to a reduced CLS compared to wild type cells.

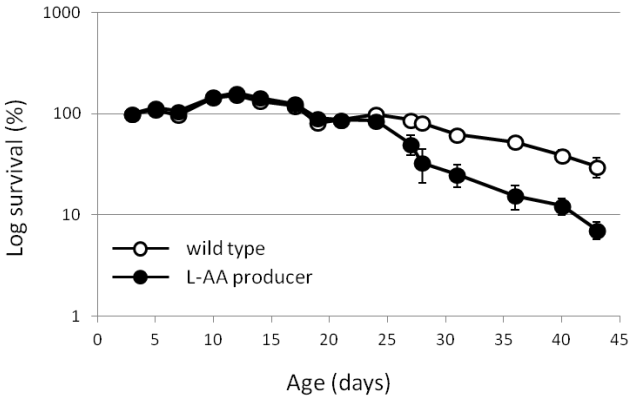

Figure 5: Relative survival of aging wild type and L-AA producing cells grown in minimal medium containing 2% glucose buffered at pH 6.0. At indicated age-points, cell viability was analyzed by measuring colony-forming units (cfu) after two days of growth on plates at 30°C. 100% corresponds to cfu at day 3. Error bars correspond to standard deviations from two independent experiments.

Discussion

The here presented results demonstrate that the growth conditions used to determine *S. cerevisiae* CLS strongly influence the effect of Vitamin C on yeast longevity. In fact, the intracellular L-AA production increased or decreased *S. cerevisiae* CLS, respect to wild type strain, when cells were grown in an acidic or near-basic environment, respectively. An explanation of these conflicting results can be deduced by taking into account the molecular mechanism of yeast chronological aging, in which acetic acid has been suggested to be the main aging promoting factor and ROS play an important role (Burtner *et al*., 2009; Pan, 2011). The acidification of the culture medium, as the result of the accumulation of organic acids during fermentative metabolism, correlates inversely with CLS by likely enhancing cellular acetic acid uptake, and consequently its toxicity (Burtner *et al.*, 2009). It is therefore reasonable to suppose that under normal growth conditions, in which extracellular acidification occurs, the intracellular L-AA production extended CLS by increasing yeast cells resistance to acetic acid. In fact, aging cells engineered to produce this antioxidant exhibited a higher tolerance to this stressing factor when challenged with high acetic acid concentrations (Figure 4). Moreover, phosphatidylserine exposure (as a marker of apoptosis-induced cell death), occurring in chronologically aged yeast cells, has been shown to be prevented in the L-AA producing strain by annexin V-FITC staining (Figure 2). Since it has been speculated that acetic acid may contribute to induce this kind of death process in aging cells (Burtner *et al*., 2009), this evidence further supports the existence of a correlation between increased CLS and acetic acid resistance exhibited by the L-AA producing strain. Additionally, we cannot exclude that the enhanced acetate utilization of the L-AA producing strain can determine an extended lifespan by reducing the extracellular amount of acetic acid and

consequently cell exposure to this toxic compound. The short and long-lived phenotypes that have been associated to yeast mutants characterized by an impaired or improved acetate metabolism, respectively, strongly support this hypothesis (Casatta *et al*., 2013; Orlandi *et al*., 2012).

On the contrary, when the culture medium was buffered at pH 6.0, and consequently acetic acid uptake was reduced, a detrimental effect of L-AA production on CLS was observed (Figure 5). Recently, it has been demonstrated that H_2O_2 promotes longevity in *S. cerevisiae* under caloric restriction or as consequence of catalase inactivation by activating stress response mechanisms, such a superoxide dismutases activity, which protect cells against oxidative stress occurring in aging cells (Mesquita *et al*., 2010). These findings agree with the hormetic effects of ROS, described as beneficial or detrimental depending on the amount they are produced (Ristow and Schmeisser, 2011). In fact, it has been assumed that ROS, produced by mitochondria during respiratory metabolism, represent in yeast, as well as in other eukaryotic organisms, signaling molecules involved in the regulation of several physiological processes, including the triggering of antioxidant defences enzymes, essential for longevity (Linnane *et al*., 2007; Mesquita *et al*., 2010; Schulz *et al*., 2007). Vitamin C, by scavenging free radicals, could therefore interfere with this mechanism and consequently affect longevity. Accordingly, several prospective clinical intervention studies did not find a positive correlation between antioxidants supplementation and health-beneficial effects (Ristow and Schmeisser, 2011). We have previously shown that the intracellular L-AA production induces a lower ROS accumulation in response to H_2O_2 or acetic acid induced stress, suggesting a protective role of L-AA against ROS-induced oxidative stress (Branduardi *et al*., 2007; Martani *et al*., 2013). Moreover, the reduced ROS production was accompanied with a negligible superoxide dismutase activation, corroborating the hypothesis that L-AA negatively affects the activation of cellular antioxidant defences (Martani *et al*.,

2013). We therefore argument that in conditions in which yeast CLS is not affected by acetic acid and low extracellular pH, the endogenous L-AA production may reduce the activation of the antioxidant defences and, as a consequent, cell survival in stationary phase.

Conclusions

We have demonstrated that, under standard conditions, the intracellular production of Vitamin C can extend CLS in *S. cerevisiae*. Burtner and co-workers proposed that acetic acid is the main cause of chronological aging in yeast and suggest that CLS can be extended by either increasing cellular resistance to acetic acid or by reducing the amount of acetic acid produced via a shift toward respiratory metabolism (Burtner *et al*., 2009). In this study, we showed that the extended CLS of the L-AA producing strain is likely the result of the synergic effect of *i)* higher resistance to acetic acid and *ii)* enhanced acetate consumption that leads to acetic acid removal from the culture medium. Therefore, although the role of acetic acid in CLS is controversial (Longo *et al*., 2012), our results further supports the hypothesis that acetic acid, which accumulates in the culture medium as a by-product of fermentative metabolism, is at least in part responsible for chronological aging in yeast. Moreover, the different results obtained when cells were aging in an acidic or pH 6.0 buffered medium, demonstrate the importance of the experimental conditions used to assess the effect of Vitamin C, or even other antioxidants, on yeast chronological lifespan.

The increased CLS showed by the L-AA producing strain under growth conditions in which the pH values drop to low levels suggest a possible its utilization as a cell factory, in particular for industrial fermentations carry out mostly by non-dividing cells and/or at very low pH to keep bacterial contaminations restrained. This conclusion is further supported by the demonstration that the production profile of metabolites of industrial relevance is influenced by genetic manipulation that modulate yeast life span during its biotechnological use (Orozco *et al*., 2013).

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Chapter 3

mRNA metabolism engineering to improve *Saccharomyces cerevisiae* stress tolerance

Manuscript in preparation for *Metabolic Engineering*

Introduction

The yeast *Saccharomyces cerevisiae* is widely employed for the production of several industrial products, such as fine and bulk chemicals, pharmaceutics, and biofuels (Hong and Nielsen, 2012). In particular, this microorganism is extensively used for the production of ethanol due to its high ethanol yield and productivity and robustness (Wallace-Salinas and Gorwa-Grauslund, 2013). Nowadays, bioethanol is mainly produced by *S. cerevisiae* from sugars deriving from sugar cane and starch, but the increasing demand for bio-based products has pushed toward the utilization of lignocellulosic biomasses for second generation ethanol production. One of the major obstacles for the development of large-scale ethanol production from lignocellulosic feedstock is the toxic effect of compounds such as weak acids, furan derivatives and phenolic compounds, released during biomasses pre-treatment (Palmqvist and Hahn-Hägerdal, 2000). These inhibitors impair strain performance and, consequently, reduce the ethanol yield and productivity of the fermentative process (Almeida *et al*., 2007). Moreover, during industrial fermentations, yeasts meet multiple stresses associated with the operative parameters of the process that, together with the final product toxicity, can contribute to the reduction of cell metabolism and growth. The evolution of robust cell factories is therefore desirable to attain a higher production/productivity. Several approaches have been applied to improve *S. cerevisiae* robustness and so to minimize the effect of both the stressful conditions imposed by industrial fermentations and the toxic products generated in second generation processes. Strains with increased stress tolerance have been obtained mainly by genetic engineering through the deletion or over-expression of single genes (for some recent examples see Hong *et al*., 2010; Lewis *et al*., 2010; Zhang *et al*., 2011; Avrahami-Moyal *et al*., 2012). However, the evolution of a robust phenotype is

hardly obtained through the alteration of a molecular element with finite action since the cellular stress response is a complex trait resulting from coordinated changes at molecular and cellular level. Ideally, a simultaneous multiple gene modification might evoke a cellular reprogramming aimed at unlocking a complex phenotype, which is likely unreachable by sequential multigene modifications (Alper *et al*., 2006; Alper and Stephanopoulos, 2007). In the last years, the engineering of complex phenotypes have been reached by using novel genome wide engineering approaches such as whole genome shuffling (WGS), evolutionary engineering and global transcription machinery engineering (gTME), that enable the simultaneous modulation and manipulation of multiple phenotype conferring loci's (Patnaik, 2008). Whereas WGS and evolutionary engineering mainly follow the nature's 'engineering' principle of variation and selection (Gong *et al*., 2009), gTME allows to elicit cellular phenotypes relevant for industrial applications from the remodeling of whole gene expression obtained through the alteration of a protein involved in the control of global gene transcription (Alper *et al*., 2006; Alper and Stephanopoulos, 2007; Lanza and Alper, 2011). In addition to gene transcription regulation, post-transcriptional events, including the processing, export, localization, turnover and translation of mRNAs, play an important role in the control of gene expression, and, consequently, influence the proteome composition (Keene and Lager, 2005; Mata *et al*., 2005). In response to stressful conditions, the regulation of mRNA stability is, for instance, an important factor involved in the determination of gene expression, which allows, together with transcriptional regulation, cells to rapidly change their level of transcripts (Fan *et al*., 2002; Talarek *et al*., 2013). Alterations in mRNA metabolism, as well as in gene transcription, can therefore potentially reorganize gene expression leading to the desired phenotype.

mRNA metabolism is controlled by RNA-binding proteins (RBPs) which establish the fate of transcripts by binding to specific RNA-sequence elements (Mata *et al*.,

2005; Glisovic *et al*., 2008). In *S. cerevisiae*, Pab1 is the major poly(A) binding protein which regulates mRNA metabolism trough its binding to the mRNAs poly(A) tails and interaction with several proteins whose activity is relevant in different aspects of the post-transcriptional control (Sachs *et al*., 1986; Caponigro and Parker, 1996; Richardson *et al*., 2012)

In this work, *S. cerevisiae* strains with improved stress tolerance were obtained by altering mRNA metabolism through the modulation of Pab1 abundance and/or function. To this purpose, *i)* parental strain were transformed with a centromeric plasmid harboring an additional copy of Pab1 promoter and coding sequence gene and *ii)* a library of the same plasmid but containing randomly mutagenized copies of Pab1 promoter and coding sequence was screened to select dominant mutations leading to increased *S. cerevisiae* robustness toward acetic acid, one of the most toxic compound resulting from the hydrolysis of lignocelluloses.

Matherials and methods

Yeast strains, media, growth conditions

The *S. cerevisiae* genetic backgrounds used in this study were BY4741 (*MATa*; *his3Δ0; leu2Δ0; met15Δ0; ura3Δ0*) (Brachmann *et al.*, 1998), CEN.PK113-5D (*MATa*; *MAL2-8c*; *SUC2*; *ura3-52*) and CEN.PK113-11C (*MATa*; *MAL2-8c*; *SUC2*; *ura3-52*; *his3Δ1*) (Entian and Kötter, 1998). Yeast transformations were performed according to the LiAc/PEG/ss-DNA protocol (Gietz and Woods, 2002). The *S. cerevisiae* strains constructed in this study are listed in Table 1, with their respective genotypes.

To delete the *EDC3* gene, the *edc3*::hphMX4 cassette was amplified from the plasmid pAG26 (Goldstein and McCusker, 1999), using the oligonucleotides 5'-ATG TCA CAA TTT GTT GGT TTC GGA GTA CAA GTG GAG CTA AAA GAT CGC CAG ATC TGT TTA GCT TG-3' and 5'-TTA CAA ATC TAA TAG CAG GGA CCC GTC AGT GAC GAA AAG ATC ACA GAG CTC GTT TTC GAC ACT GG-3'. The *edc3*::hphMX4 cassette was used to replace *EDC3* gene in CEN.PK113-11C strain. Transformed clones were selected on YPD agar plates supplemented with the antibiotic hygromycin B (Roche) to the final concentration of 2 mg/ml. Gene disruption was confirmed by PCR analysis.

The chromosomally integrated GFP-tagged version of Pab1 was created using the cassette *PAB1-GFP*::His3MX6 amplified from the plasmid pFA6a-GFP(S65T)-His3MX6 (Longtine *et al.*, 1998), using the oligonucleotides 5'-AGT CTT TCA AAA AGG AGC AAG AAC AAC AAA CTG AGC AAG CTC GGA TCC CCG GGT TAA TTA A-3' and 5'-GTT TGT TGA GTA GGG AAG TAG GTG ATT ACA TAG AGC ATT AGA ATT CGA GCT CGT TTA AAC TGG-3'. The PCR-amplified fragment was used directly for yeast transformation.

Yeast cultures were grown in minimal synthetic medium (0.67% YNB Biolife without amino acids) with 2% w/v D-glucose as carbon source. When required, supplements such as histidine, leucine, metionine and uracil (Sigma) were added to the final concentration of 50 mg/l.

Growth curves were obtained by inoculating yeast cells at an initial optical density of 0.1 (OD_{660}) and then the OD was measured at specific time intervals over at least 78 hours from the inoculums. Each experiment was repeated at least three times. All strains were grown in shake flasks at 30 °C, unless otherwise stated, and 160 rpm. The ratio of flask volume:medium was 5:1.

Strain	Genotype	Source
BY4741-HULM	*MATa; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0*	Euroscarf
BY4741c	*MATa; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0* [*pYX012; pYX022; pYX052; YCplac33*]	This study
BY4741 Pab1(+)	*MATa; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0* [*pYX012; pYX022; pYX052; YCplac33PAB1*]	This study
BY4741-HLM	*MATa; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0* [*YCplac33*]	This study
BY4741 *pan2Δ*	*MATa; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0; YGL094c::kanMX4* [*YCplac33*]	This study
BY4741 *pan3Δ*	*MATa; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0; YKL025c::kanMX4* [*YCplac33*]	This study
CEN.PK113-5D	*MATa; MAL2-8c; SUC2; ura3-52*	Entian and Kötter, 1998
CEN.PK113-5Dc	*MATa; MAL2-8c; SUC2; ura3-52* [*YCplac33*]	This study
CEN.PK113-5D Pab1(+)	*MATa; MAL2-8c; SUC2; ura3-52* [*YCplac33PAB1*]	This study
CEN.PK113-5D Pab1_A60-9	*MATa; MAL2-8c; SUC2; ura3-52* [*YCplac33PAB1_A60-9*]	This study
CEN.PK113-11C	*MATa; MAL2-8c; SUC2; ura3-52; his3Δ1*	Entian and Kötter, 1998
PAB1-GFP EDC3-mCh	*MATa; MAL2-8c; SUC2; ura3-52; his3Δ1; edc3::hphMX4; PAB1-GFP::His3MX6* [pRP1574]	This study
PAB1-GFP (+) EDC3-mCh	*MATa; MAL2-8c; SUC2; ura3-52; his3Δ1; edc3::hphMX4; PAB1-GFP::His3MX6* [pRP1657]	This study

Table 1 : List of yeast strains constructed and used in this study.

Gene amplification and expression plasmid construction

The *PAB1* endogenous ORF and the native promoter region were PCR amplified from genomic DNA, isolated from S288c *S. cerevisiae* strain, on a GeneAmp PCR System 9700 (PE Appl. Biosystems, Inc.) using Q5® High-Fidelity DNA Polymerase (New England Biolabs) and following the manufacturer's instructions. The amplification was performed using the oligonucleotides PAB1_PstI_fw 5'-

GAT TTA CTG CAG GTA TAT ATA TTT GCG TGT AAG TGT GTG T-3' and PAB1_SalI_rev 5'-ATT ATA GTC GAC TAG AGC ATT AAG CTT GCT CAG TTT GT-3'. The PCR product was *Pst*I and *Sal*I cut and sub-cloned in the low copy number expression vector pYF8 digested with the same restriction enzymes to excide the TPI promoter. To create the plasmid pYF8, the DNA sequence corresponding to the TPI promoter-multi cloning site-poly(A) terminator was amplified from the integrative plasmid pYX042(-ATG), previously obtained from the pYX042 integrative expression vector (R&D Systems) (*Eco*RI and *Bam*HI cut, blunt-ended and re-legated), using the oligonucleotides TPIp_PstI_fw 5'-GAT TTA CTG CAG CCT GAC GTC TAA GAA ACC ATT A-3' and MCS_EcoRI_rev 5'-CTT AGA GAA TTC TCT TCG CTA TTA CGC CAG CT-3'. The PCR-product was cut with *Pst*I and *Eco*RI and sub-cloned in the plasmid YCplac33 (centromeric, *URA3* auxotrophic marker) (Gietz and Sugino, 1988). All the restriction enzymes used were from New England Biolabs (Hitchin, Herts, UK). Standard procedures were employed for all cloning purposes (Sambrook *et al*., 1989).

The plasmids pRP1574 (Edc3-mCh, centromeric, *URA3* auxotrophic marker) and pRP1657 (Pab1-GFP, Edc3-mCh; centromeric, *URA3* auxotrophic marker) were kindly provided by Prof. Parker (Buchan *et al*., 2008).

Mutant library construction

The library was created using the GeneMorph II EZClone Domain Mutagenesis Kit (Agilent Technologies) following the manufacturer's instructions. Briefly, mutations in the *PAB1* promoter and coding sequence were introduced trough error-prone PCR using the Mutazyme II DNA polymerase provided by the kit. The oligonucleotides used for this amplification were 5'-AAG CTT GCA TGC CTG CAG GTA TAT A-3' and 5'-GGG TCG ACT AGA GCA TTA AGC TT-3'. 50, 250 or 750 ng of the plasmid YCplac33*PAB1* were used as DNA template in order

to produce a low (0-4.5 mutations/kb), medium (4.5-9 mutations/kb) or high (9-16 mutations/kb) mutational frequency, respectively, according to manufacturer's instructions. Following purification, the resulting mutated PCR products were used as "megaprimers" for the EZClone reaction, during which they were denaturated and annealed to the original donor plasmid and extended with a specialized enzyme mix containing a high fidelity DNA polymerase. The plasmid library was transformed into *E. coli* XL10-Gold ultracompetent cells that were plated onto LB-ampicillin agar plates (antibiotic concentration of 100 mg/l). The library size was approximately $6x10^3$. Plasmid DNA was isolated from *E. coli* using the alkaline lysis method (Brinboim and Doly, 1979) and transformed into CEN.PK yeast background. Yeast cells were scraped off the plates, inoculated in minimal medium and incubated for about 6 hours at 30 °C at 160 rpm. This liquid culture was used to create frozen glycerol stocks (80% yeast culture, 20% glycerol) that were conserved at -80 °C.

Phenotype selection

The yeast glycerol stocks were inoculated in minimal medium and incubated for about 4-6 hours at 30 °C at 160 rpm. Successively, cell cultures were diluted to the final OD (660 nm) of 0.001 and 250 µl were plated onto minimal medium agar plates containing increasing concentrations of acetic acid at pH 3 (20, 50, 60, 70 and 80 mM), in quadruple. Cells were also plated on minimal medium agar plates in the absence of stress in order to verify that an equal amount of cells transformed with the control plasmid and the mutant library were plated. For anaerobic growth, agar plates were placed in anaerobic jars and the air was displaced with nitrogen gas. Plates were incubated at 30 °C for 3 days and the colony forming units (CFU) were counted.

Fluorescence microscopy analysis

Stress granules and P bodies were analyzed in cells grown in minimal medium till the exponential phase (OD_{660} = 0.4-0.6) and exposed at 46 °C for 2 hours. Cell cultures were then shifted at 30 °C for 20 minutes to rescue them from heat stress. During the whole experiment, about 10^8 cells were collected, resuspended in PBS (NaH_2PO_4 53 mM, Na_2HPO_4 613 mM, NaCl 75 mM) and then observed in a Nikon ECLIPSE 90i fluorescence microscope (Nikon) equipped with a 100X objective. Emission fluorescence due to Pab1-GFP or Edc3-mCh was detected by B-2A (EX 450-490 DM 505 BA520) or G-2A (DX 510-560 DM 575 BA 590) filter (Nikon), respectively. Digital images were acquired with a CoolSnap CCD camera (Photometrics) using MetaMorph 6.3 software (Molecular Devices). The images were merged using Adobe Photoshop CC.

Protein extraction and western blot analysis

The expression level of Pab1-GFP in control and Pab1(+) strains was detected by western blot analysis. Exponential growing cells were collected by centrifugation at 4000 rpm for 10 min, washed once in ice-cold water and resuspended in 25 mM Tris-HCl pH 8.0 containing 0.5 mM EDTA and protease inhibitors (complete Protease Inhibitor Cocktail, Roche). Cells were disrupted with glass beads (425-600 µm, Sigma) in an homogenizer (FastPrep® FP120, MP Biomedicals) by three cycles of 20 s at speed 6.0. Lysates were collected and centrifuged for 15 min at 4 °C and the obtained supernatants were used as cell-free extracts. Protein concentration was determined using the Bio-Rad protein assay (Bio-Rad) with bovine serum albumine as the standard. Equal amounts of total protein (15 µg) were resolved in a SDS-polyacrylamide gel electrophoresis and successively transferred on a nitrocellulose membrane. The nitrocellulose was blocked with 5% milk in TBS+Tween overnight at 4 °C. Pab1-GFP was then detected using an

monoclonal anti-GFP antibody (Living Colors A.v JL-8, Diatech labline) diluted 1:1000 in TBS+Tween+5% milk for 2 hours at RT followed, after membrane washing, by incubation with Rabbit anti-Mouse IgG (FC) secondary antibody, AP (alkaline phosphatase) conjugate diluted 1:15000 in TBS+Tween+5% milk for 1 hour at RT. The nitrocellulose membrane was incubated with CDP-Star Chemiluminescent Substrate (Sigma) for 5 min at RT under gentle agitation and then exposed to Pierce CL-Xposure film to reveal Pab1-GFP signal.

Results

Effect of the additive expression of PAB1 on S. cerevisiae growth in the presence of stress

The objective of this work was the identification of dominant mutations in *PAB1* able to improve *S. cerevisiae* tolerance to acetic acid in the presence of the unaltered chromosomal copy of the gene. To allow the selection of the desired phenotype, the expression of the gene copy harbored by the plasmid does not have to negatively affect cell growth. *S. cerevisiae* cells in which *PAB1* was over-expressed under the control of the strong constitutive TPI promoter were unviable (data not shown), consistently with the reported evidences that the galactose-inducible *PAB1* over-expression causes severe growth inhibition (Swisher and Parker, 2010). This evidence suggests that cells cannot tolerate high expression levels of this gene. With the aim to not severely affect *PAB1* gene dosage, we planned to create a plasmid library of mutants in which *PAB1* was expressed under the control of its endogenous promoter in the low copy number plasmid YCplac33. The *in vivo* impact of the additional expression of *PAB1* was assessed by analyzing the cell growth of *S. cerevisiae* strains transformed with a centromeric plasmid (YCplac33) containing the wild type coding sequence of *PAB1* downstream to 500 bp corresponding to a part of the promoter sequence of *PAB1* identified with the Promoter Database of *Saccharomyces cerevisiae* (http://rulai.cshl.edu/SCPD/). This portion of the promoter was demonstrated to be sufficient to drive *PAB1* expression through the fluorescence analysis of yeast cells containing a GFP-tagged version of Pab1 whose expression was under its control (data not shown). The strains expressing the additional copy of *PAB1* in the plasmid were denominated Pab1(+). BY4741 and CEN.PK yeast strains were used and analyzed in parallel to eventually confirm the data in different genetic backgrounds. In

minimal medium at pH 5.5 cell growth was not affected by the additional expression of *PAB1* in both yeast strains (data not shown). Successively, the response to acetic acid stress at low pH was investigated. Cells were inoculated at an initial optical density (OD 660) of 0.1 in minimal medium at pH 3 in the absence or in the presence of acetic acid and cell growth was monitored at specific intervals of times over about 70 hours (Figure 1). Different concentrations of the stressing agent were used for the two yeast genetic backgrounds because of their different intrinsic resistance.

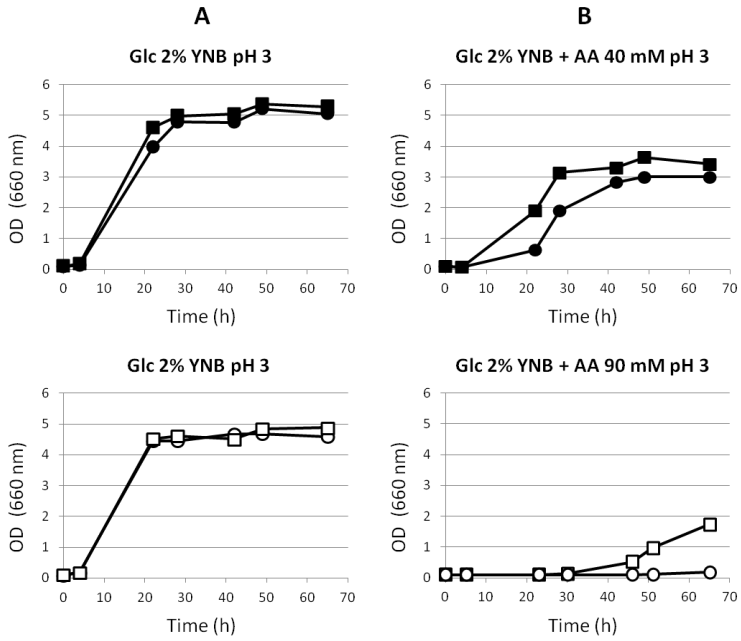


Figure 1: Growth curves of wild type (circle) and Pab1(+) (square) strains of BY4741 (closed symbol, upper panels) and CEN.PK (open symbol, lower pannels) background grown in minimal medium at pH 3 in the absence (A) or presence (B) of acetic acid. The results of one representative experiment of three are shown.

In the absence of acetic acid, at low pH, both control and Pab1(+) strains grew with a similar trend (Figure 1A). However, when cells were exposed to acetic acid, the

additional expression of *PAB1* led to growth advantage in both genetic backgrounds (Figure 1B).

We investigated whether the improved strain robustness could be extended to other stresses that typically occur during industrial fermentations, such as oxidative and heat stress. Yeast cells were then grown in minimal medium in the presence of hydrogen peroxide or high temperature. Figure 2 shows that Pab1(+) strains were more tolerant to the stress conditions compared to the control strains, confirming the robust phenotype observed in the presence of acetic acid.

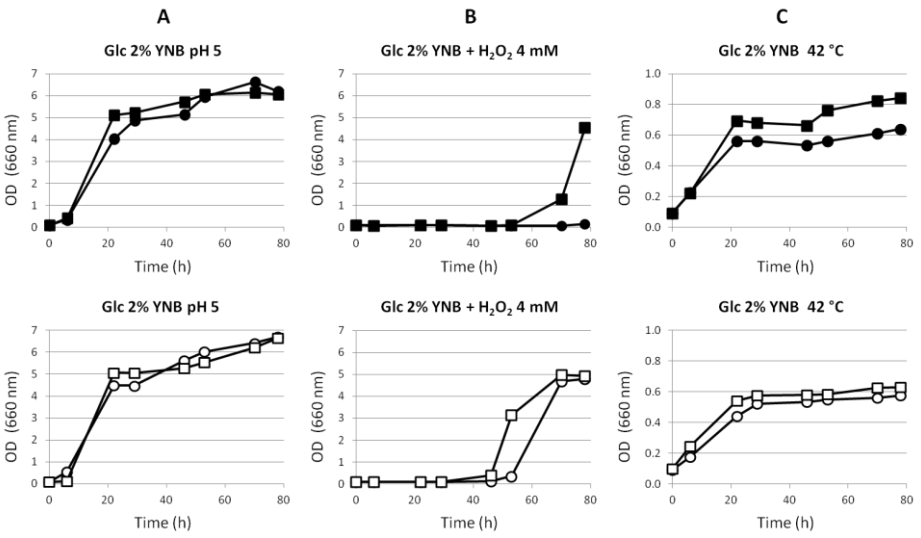


Figure 2: Growth curves of wild type (circle) and Pab1(+) (square) strains of BY4741 (closed symbol, upper panels) and CEN.PK (open symbol, lower panels) background grown in minimal medium at 30 °C in the absence of stress (A) or in the presence of 4 mM H_2O_2 (B) or at 42 °C (C). Note the different scale of the ordinate axes of growth curves performed at 42 °C. The results of one representative experiment of three are shown.

All together, these data showed that the additional expression of *PAB1* at a low gene dosage led to an increased stress tolerance rather than negatively affect cell growth, differently from what occurred when *PAB1* was over-expressed under the control of a strong promoter (our unpublished data and Swisher and Parker, 2010).

The additional expression of PAB1 alters stress granules and P bodies formation during heat stress

In eukaryotic cells, when translation initiation is impaired during a stress response, non-translating mRNAs accumulate in mRNA-protein complexes denominated stress granules and processing bodies (P bodies) (Hoyle *et al.*, 2007; Buchan and Parker, 2009). These aggregates differ in composition, and consequently in their function: P bodies generally contain the mRNA decay machinery and therefore mRNAs addressed to be degraded, whereas stress granules are mainly composed of translational initiation factors bound to untranslated mRNAs and are therefore believed to represent sites of translation reinitiation (Anderson and Kedersha, 2009; Buchan and Parker, 2009). Moreover, P bodies have been demonstrated to promote stress granules assembly in *S. cerevisiae*, suggesting the existence of a mRNA cycle in which mRNPs are exchanged between these cytoplasmic granules (Buchan *et al.*, 2008).

The protein Pab1 is a specific component of stress granules, and it is therefore commonly used a stress granule marker (Buchan *et al.*, 2008; Buchan *et al.*, 2011). Studies on a *pab1Δ spb2Δ S. cerevisiae* strain, where defects in 60S subunit biogenesis lead to suppression of the lethal phenotype induced by *pab1*-deletion (Sachs and Davis, 1990), demonstrated that Pab1 promotes stress granules formation, although it is not absolutely required (Swisher and Parker, 2010). On the other hand, differently from what observed for the over-expression of other stress granules related factors such as Dhh1 and Pbp1, the growth inhibition caused by the over-expression of *PAB1* via a galactose-inducible promoter was reported not to be associated with stress granules formation in the absence of stress, suggesting that the lethality caused by an excess of Pab1 is not due to premature stress granules or protein aggregation (Swisher and Parker, 2010).

To compare stress granules between wild type and Pab1(+) strains, we used a strain containing the chromosomally integrated GFP-tagged version of Pab1 as the control, and the strain PAB1(+)-GFP in which both *PAB1* copies present in the genome and in the plasmid were fused with GFP. Moreover, Edc3-mCherry was used as a P bodies marker (see Materials and Methods) (Buchan *et al*., 2008). Cells grown in minimal medium till the exponential phase were then exposed for two hours at 46 °C to induce stress granules and P bodies formation as reported by (Grousl *et al*., 2009). Cells were analyzed under the fluorescence microscope before, during, and after heat stress. In the absence of stress, the fluorescence associated to both Pab1 and Edc3 was diffused in the cytoplasm in both strains and no stress granules and P bodies were present (Figure 3A, CEN.PK strain, similar data were obtained in the BY4741 background, data not shown), according to their accumulation and function restricted to the stress (Buchan *et al*., 2008; Shah *et al*., 2013). Moreover, the intensity of Pab1-GFP fluorescence seemed not to be different between the two strains. The relative quantification made by western blot analysis of Pab1-GFP fusion protein levels using an anti-GFP antibody showed as expected a higher abundance of Pab1 around 20-40 % in the Pab1(+)-GFP strain respect to the control strain (supplementary Figure S1).

After 2 hours of incubation at 46 °C stress granules and P bodies were clearly visible in both strains (Figure 3B). Whereas for P bodies no differences between strains were detectable, stress granules seemed to be brighter and larger in the Pab1(+)-GFP strain.

Successively, cell cultures were shifted at 30 °C and analyzed after 20 minutes. In the control strain stress granules and P bodies were almost completely dissolved; on the contrary, in Pab1(+)-GFP strain both aggregates were still visible (Figure 3C).

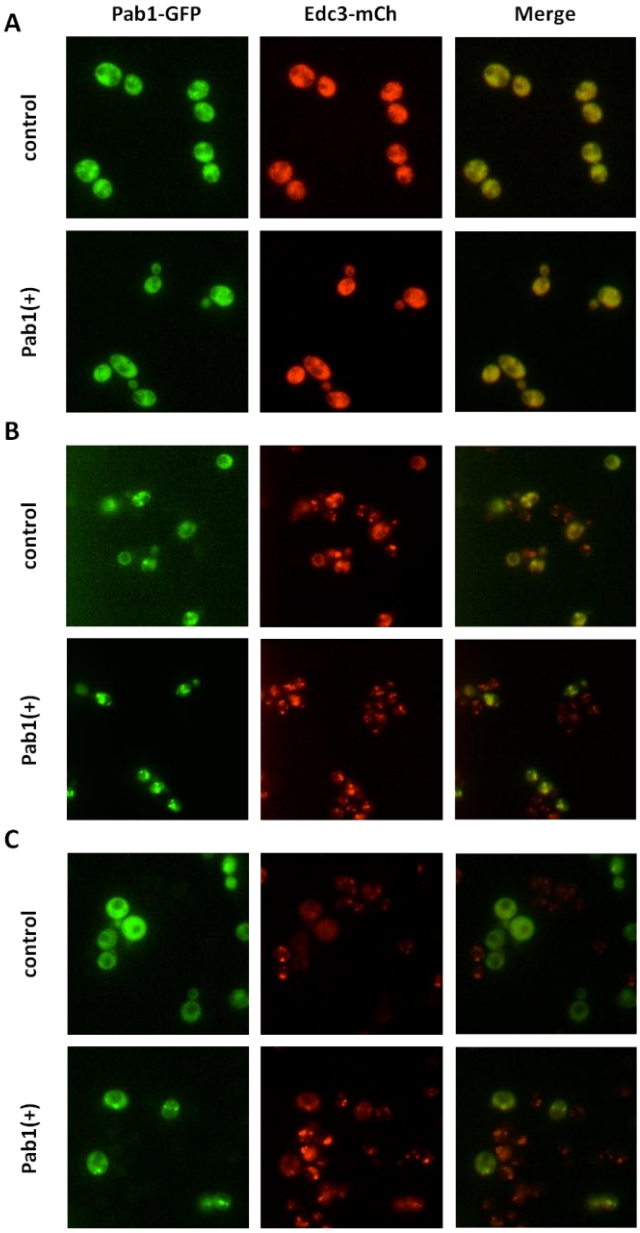


Figure 3: Pab1 and Edc3 associated fluorescence of wild type (control) and Pab1(+) strain in the absence of stress (A) or after incubation at 46 °C for 2 h (B) or after 20 min of incubation at 30 °C following 2 h of heat stress at 46 °C (C). Cells, grown in minimal medium till the exponential growth phase, were incubated at 46 °C for 2 h and successively shifted to 30 °C.

Therefore, the additional expression of *PAB1* induced the formation of larger and more persistent stress granules which might correlate with the increased robustness against stress showed by the Pab1(+) strains (see again, Figures 1 and 2).

The persistence of P bodies observed 20 minutes after removal from the stress in the Pab1(+) strain could be linked to the enduring presence of stress granules. It has been observed, indeed, that P bodies disassemble slower than stress granules when cells were enabled to reenter growth from stationary phase (Brengues and Parker, 2007). Moreover, since stress granules and P bodies are dynamically linked sites of mRNP remodeling (Kedersha *et al.*, 2005), it is not surprising that the modulation of *PAB1* expression might also affect P bodies assembly and disassembly kinetics.

Overall, such evidences supported the importance of Pab1 function in the stress response and therefore reinforced the idea to obtain *S. cerevisiae* strains with improved stress tolerance through the modulation of its function.

Selection of PAB1 variants conferring an improved acetic acid phenotype to yeast transformants

The proof that the additional *PAB1* expression in the plasmid does not impair cell growth allowed the feasibility to screen dominant mutant alleles of this gene possibly leading to an improved phenotype toward acetic acid. Both the *PAB1* promoter and coding sequence were subjected to random mutagenesis by error-prone PCR in order to create a mutant plasmid library (see Materials and Methods). Three mutant plasmid libraries were obtained with an average frequency of 4 (low), 7.43 (medium) and 9.3 (high) mutations/kb, respectively. It was decided to use that with the medium mutation rate for the screening protocol. The yeast screening and selection was performed in the *S. cerevisiae* CEN.PK background strain containing the unmutated chromosomal copy of *PAB1*. Cells were

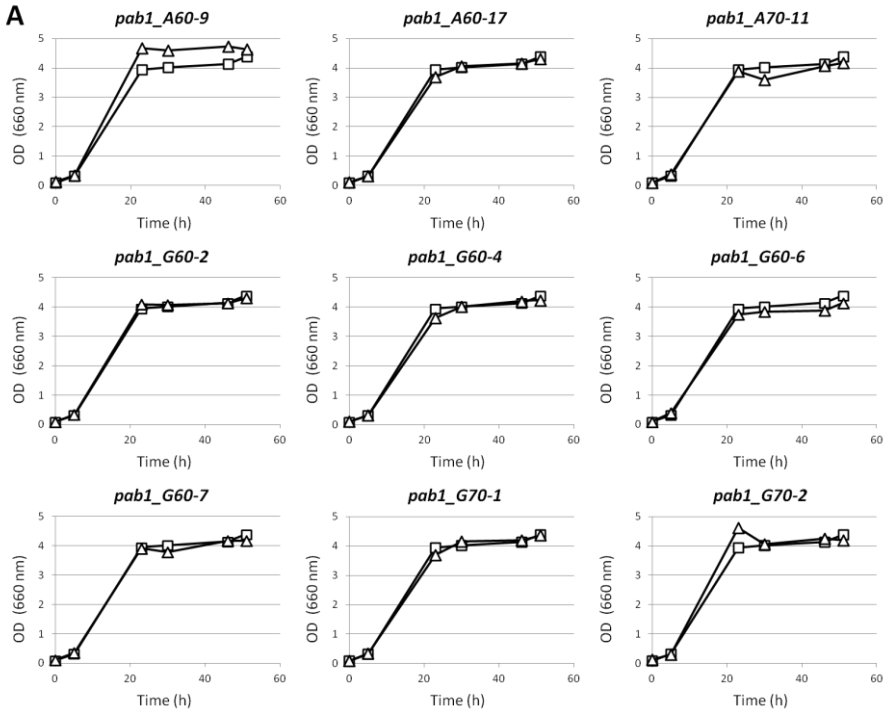
transformed with the mutant library and the resulting yeast library was selected in the presence of increasing concentrations of acetic acid at pH 3 (20, 50, 60, 70 and 80 mM) on solid minimal medium in aerobic conditions. In parallel, the selection was also performed on acetic acid containing agar plates incubated under anaerobic conditions in order to screen mutants for the acetic acid resistance and for the ethanol production, simultaneously. In such a way, it should be possible to select mutant clones more resistant to acetic acid that, at the same time, have not surely lost the ability to produce ethanol. Genetic manipulations aimed to create industrial relevant strains with increased robustness are indeed appropriate only when the ethanol production/productivity is not impaired or reduced. The number of colonies (CFU) obtained from the yeast strain transformed with the mutant plasmid library was compared to that of a control strain transformed with the plasmid containing the wild type promoter and coding sequence of *PAB1* (Pab1(+) strain). The numbers of CFU obtained for both strains and both growth conditions in the presence of different concentrations of acetic acid are reported in Table 2.

		AEROBIC GROWTH		ANAEROBIC GROWTH	
		control	mutant library	control	mutant library
ACETIC ACID pH 3	20 mM	1621 CFU	2412 CFU	1558 CFU	3252 CFU
	50 mM	592 CFU	1165 CFU	705 CFU	1715 CFU
	60 mM	18 CFU	32 CFU	0 CFU	7 CFU
	70 mM	0 CFU	11 CFU	0 CFU	2 CFU
	80 mM	0 CFU	0 CFU	0 CFU	0 CFU

Table 2: Numbers of colony forming units (CFU) obtained by the screening in the presence of acetic acid. CEN.PK cells transformed with the control plasmid (YCplac33*PAB1*) or the mutant library were plated onto minimal medium agar-plates containing increasing concentrations of acetic acid at pH 3 (20, 50, 60, 70 and 80 mM). CFUs were obtained after 3 days of growth on plate at 30 °C in aerobic or anaerobic conditions.

In all the tested conditions, up to 80 mM acetic acid that was not permissive for growth, a higher number of CFU was obtained from the screening of the yeast library compared with the control strain. Since growth was mainly inhibited in the presence of 60 and 70 mM acetic acid, we considered the 52 mutant clones obtained in the presence of these acid concentrations as the most promising.

These mutants were analyzed in multi-well plates for growth in minimal medium at pH 3 in the absence or presence of 60 and 80 mM acetic acid and compared to that of Pab1(+) strain, here used as a control. In such conditions, nine mutants grew better in the presence of the acid respect to the control (data not shown). The nucleotide sequence of *PAB1* promoter and coding sequence of these mutants were sequenced and the resulting amino acid sequence was compared to the wild type one to identify the amino acid substitutions (supplementary Figure S2 and S3).



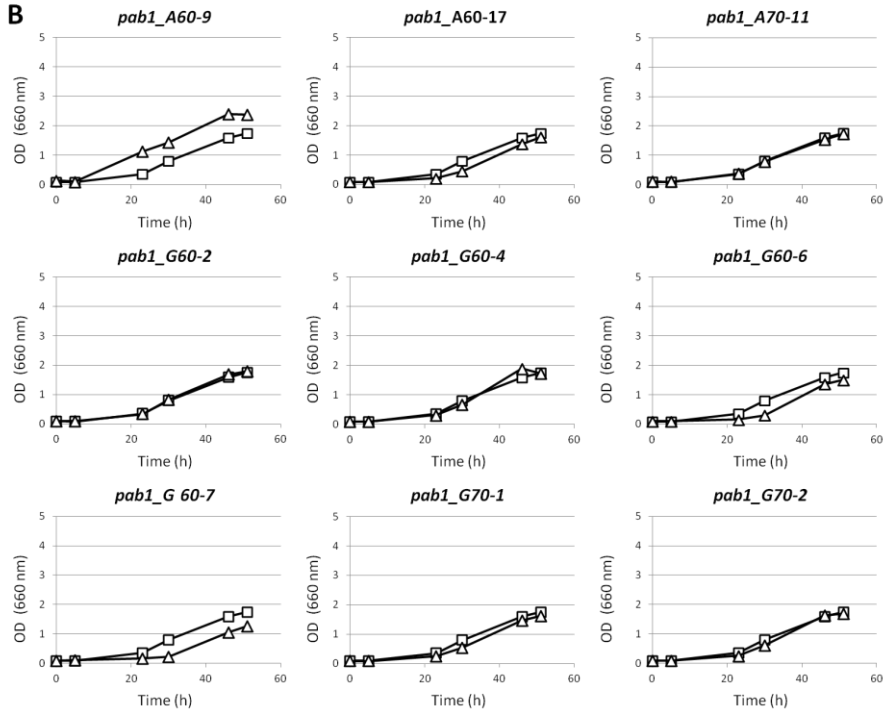


Figure 4: Growth curves of the Pab1(+) strain (square) and mutant clones (triangle) grown in minimal medium at pH 3 in the absence (A) or presence (B) of 80 mM acetic acid. The letter and numbers reported on the top of the graphics correspond to the codex use to identify the mutants selected during the screening. A: clones selected under aerobic conditions; G: clones selected under anaerobic conditions; 60: clones selected in the presence of 60 mM acetic acid pH 3; 70: clones selected in the presence of 70 mM acetic acid pH 3; the last is the sequential number of clones selected under the same condition.
The results of one representative experiment of three are shown.

Successively, these mutants were grown in minimal medium at pH 3 in the absence or presence of 80 mM acetic acid in shake flasks to more accurately outline their growth (Figure 4). Only the mutant *pab1_A60-9* showed a better growth both in the absence and in the presence of the acid compared to Pab1(+) strain. On the contrary, mutants *pab1_G60-6* and *pab1_G60-7*, selected under anaerobic conditions, resulted inferior if compared with the control.

To confirm that the improved phenotype of the mutant *pab1_A60-9* was conferred by mutations in the *PAB1* promoter and/or coding sequence, *S. cerevisiae* CEN.PK background strain was newly transformed with the plasmid extracted from the *pab1_A60-9* mutant. As clearly visible in figure 5, the growth of this strain was similar to that of the *pab1_A60-9* mutant selected during the screening.

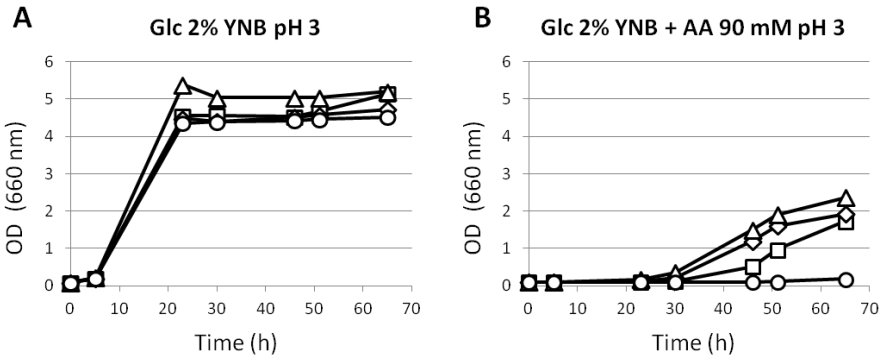


Figure 5: Growth curves of wild type (circle) and Pab1(+) strain (square), mutant *pab1_A60-9* (triangle), and the strain transformed with the plasmid A60-9 (diamonds) grown in minimal medium at pH 3 in the absence (A) or presence (B) of 90 mM acetic acid. The results of one representative experiment of three are shown.

Characterization of the mutant pab1_A60 -9

The nucleotide sequence of *PAB1* promoter and the deduced amino acid sequence of the mutant protein are shown in Figure 6.

The promoter sequence contained two mutations localized at position -112 and -240 before the starting codon ATG (Figure 6A). Both mutations were in two different binding sites of the putative transcription factor RC2 predicted *in silico* by using Alggen PROMO software V 3.0.2 (Messeguer *et al.*, 2002; Farré *et al.*, 2003). Five point mutations were found in the *PAB1* coding sequence: one silent mutation and four missense mutations, resulting in the alteration of the amino acid sequence of Pab1 in correspondence of the residues F168, V322, R492 and Y514

(Figure 6B). Interestingly, the amino acid substitutions were in four different functional domains of the protein.

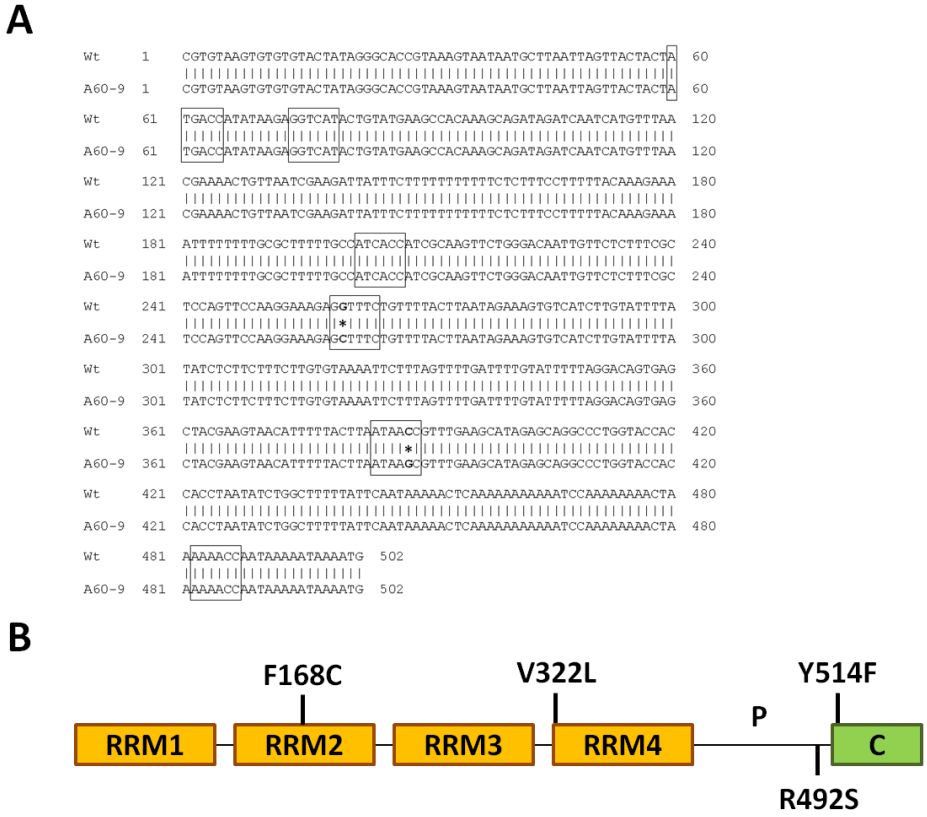


Figure 6: Characterization of the mutant *pab1_A60-9*. (A) Sequence alignment of the promoter region of *pab1_A60-9* mutant. The mutated nucleotides are indicated with stars. Rectangular forms indicate the binding sites of the putative transcription factor RC2. (B) Amino acid substitutions in the *pab1_A60-9* mutant are shown on a schematic representation of Pab1 protein composed of six different functional domains. RRM: RNA recognition motif; P: proline-rich linker; C: C-terminal region.

Mutations in the F168 RNA-binding site, and in particular F168C, were indicated as deleterious for Pab1 function by a deep mutational scanning of the RRM2 domain (Melamed *et al.*, 2013). However, our results seem in contradiction with

that. This discordance can be ascribed to the different approaches and genetic backgrounds used to characterize or select this mutation. The deep mutational scanning of RRM2 was performed in strains lacking the chromosomal wild type *PAB1* gene, whereas in our strategy mutations leading to the desired phenotype were selected in the presence of the endogenous unmutated version of Pab1, which could compensate the deleterious effect of F168C present in the additional mutated copy.

Moreover, the association of F168C with growth impairment was determined using a truncation form of Pab1 composed of the RRM1-RRM2-RRM3 and the N-terminal 25 amino acids of RRM4, instead of the full-length protein (Melamed *et al*., 2013). The effect of this mutation on full-length Pab1 functionality may be therefore different. This hypothesis is supported by the evidence that F170V, which drastically reduce the protein binding to poly(A), was identified in the same study as a deleterious mutation for Pab1 function injuring cell growth when present in the truncated form of the protein, but not in the full-length one (Deardorff and Sachs, 1997; Melamed *et al*., 2013).

V322L is localized at the beginning of the RRM4 domain, which seems to be implied in mRNA transport from the nucleus to the cytoplasm, and R492S is in correspondence of the P domain, which is critical for Pab1 self-association and poly(A) tail deadenylation mediated by Ccr4-NOT deadenylase complex (Brune *et al*., 2005; Yao *et al*., 2007). In literature there are not data about the role of V322 and R492 in Pab1 function.

Interestingly, the mutation in the C-terminal region was in correspondence to an amino acid (Y514) which participates in the interaction of Pab1 with Pan3, an essential subunit of the Pan2p-Pan3p poly(A)-ribonuclease complex (PAN) which controls poly(A) tail length (Brown *et al*., 1996; Mangus *et al*., 2004). Mangus and co-workers reported that the substitution of the tyrosine 514 to cysteine determines

a reduction of Pab1 interaction with Pan3, suggesting that mutations in this residue can affect the Pab1-dependent PAN activity (Mangus *et al.*, 2004).

To elucidate the effect of the lack of PAN activity on the cellular stress response, we analyzed cell growth of *Δpan2* and *Δpan3 S. cerevisiae* BY4741 strains in minimal medium at pH 3 in the absence or presence of 40 mM acetic acid (Figure 7). In the absence of the organic acid, *pan*-deleted mutants grew similarly to the wild type strain. In the presence of acetic acid, however, both *Δpan2* and *Δpan3* strains showed a better growth compared to the wild type strain, which did not start growing. It is therefore evident that the lack of PAN activity increased yeast tolerance to acetic acid.

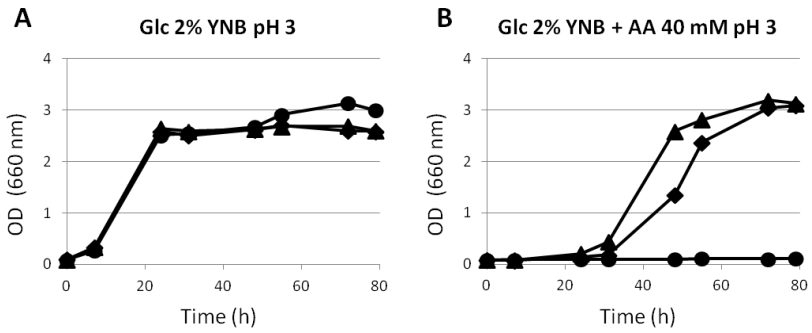


Figure 7: Growth curves of wild type (circle), *Δpan2* (rombus) and *Δpan3* (triangle) BY4741 strains grown in minimal medium at pH 3 in the absence (A) or presence (B) of 40 mM acetic acid. The amino acids histidine, leucine and metionine (50 mg/l) were supplemented to compensate strains auxotrophies. The results of one representative experiment of three are shown.

Discussion

We proved that the engineering of mRNA metabolism through the modulation of Pab1 abundance and/or function represents an innovative tool to improved *S. cerevisiae* robustness. On one hand, the expression of *PAB1* in a low copy number plasmid, in addition to the chromosomal copy, increased yeast growth performance under stressful conditions that often occur during industrial large scale fermentations, such as heat, oxidative and acetic acid stress (Figures 1 and 2). On the other, *S. cerevisiae* resistance to acetic acid was further improved through the selection and identification of a mutant Pab1 protein (Figure 5). Both approaches demonstrate the feasibility to enhanced yeast performance under stressful conditions through either the alteration of the abundance or function of a factor that orchestrates several aspects of mRNA metabolism. Two different hypotheses were elaborated to explain the reason of the observed phenotypes of each approach.

The increased stress tolerance showed by Pab1(+) strains might correlate with the presence of larger stress granules

Pab1 is a multifunctional poly(A)-binding protein which determines, together with its interacting proteins, the fate of the transcripts, having a fundamental role in the protection of mRNAs from deadenylation-dependent degradation (Coller and Parker, 2004). It has been suggested that Pab1 may play its protective function by promoting the transit of poly(A) mRNA from P bodies to stress granules (Swisher and Parker, 2010). We observed that Pab1(+) strains, containing a *PAB1* copy on a centromeric plasmid in addition to the genomic one, were more tolerant to stress (Figures 1 and 2) and, at the same time, accumulated under heat stress larger stress granules as judged by Pab1 localization (Figure 3) compared with wild type strains. One possibility is that, during a stress response, the presence of more Pab1 proteins

than normal allows transcripts to be more efficiently stored and so protected from degradation in stress granules, that as a consequence appear bigger. The presence of more stable mRNAs in Pab1(+) strains need to be demonstrated, for example by real time-PCR, to confirm this hypothesis. However, the direct comparison of mRNA stability between the control and Pab1(+) strains is not so trivial to be performed since the housekeeping gene necessary to normalize the mRNA levels could be also stabilized in Pab1(+) strains. Moreover, since stress granules have been proposed to be sites of translation reinitiation containing translationally silenced mRNAs prone to be rapidly translated when the stress is overcome (Anderson and Kedersha, 2009; Buchan *et al*., 2008), larger stress granules observed in Pab1(+) cells may be the consequence of the accumulation of a greater mRNAs pool addressed to be translated, thus allowing cells to more rapidly recover from the stress-induced damage in respect to wild type cells. The importance of stress granules for stress recovery has been proved by the evidence that *S. cerevisiae* null mutants *pbp1Δ*, *pub1Δ* and *TIF4632Δ*, impaired in the constitution of stress granules, didn't resume growth after removal from ethanol stress *(*Buchan *et al*., 2008; Kato *et al*., 2011).

Mutation Y514F might be responsible for the improved phenotype showed by the A60_9 mutant

The screening of the mutant library in the presence of acetic acid revealed a mutant Pab1 protein which confers to yeast cells an improved phenotype, further improving the positive effect conferred by the additional Pab1 wild type copy. Although the phenotype associated with each mutation identified in the mutant protein has not been established yet, literature data suggest that mutations in tyrosine 514 can impair Pab1-Pan3 interaction and, consequently, PAN activity (Mangus *et al*., 2004). In *S. cerevisiae*, point mutations in other residues next to

Y514 within the C-terminal region, which completely eliminate Pab1-Pan3 interaction, as well as a C-terminal truncation of Pab1 and the deletion of *PAN3* gene, resulted in the accumulation of mRNAs with longer poly(A) tails both *in vitro* and *in vivo* that, as a consequence, are supposed to be stabilized (Brown and Sachs, 1998; Mangus *et al*., 2004). Since tyrosine 514 has been demonstrated to participate in Pab1-Pan3 association (Mangus *et al*., 2004), we speculate that Y514F may abrogate or reduce PAN activity and, consequently, affect the poly(A) tail control. The improved phenotype associated with *pab1_a609* mutant may be therefore related to the accumulation of mRNAs with longer poly(A) tails than normal resulting from an altered Pab1-Pan3 interaction and, as a consequence, PAN activity.

Moreover, the positive effect of the lacking of PAN activity on stress tolerance has been proved by the evidence that Pan2 or Pan3-deficient strains are more tolerant to acetic acid compared to wild type strain (Figure 7). The presumed PAN activity reduction, as well as the presence of mRNAs with longer poly(A) tail, will be verified in the *pab1_A60-9* mutant. Moreover, future studies on *S. cerevisiae* strains containing Pan3-noninteracting *pab1* alleles will help to shed light on the effect of *pab1* mutations abrogating PAN activity on stress tolerance.

Beyond the possible effect of Y514F on the improvement of yeast acetic acid tolerance, we cannot exclude that the other three mutations (F168C, V322L and R492S) identified in the *pab1_A60-9* mutant have a role in the phenotype improvement. In addition, two point mutations were found in the promoter region of the same mutant in correspondence of two binding sites of the putative factor RC2. It may therefore possible that the transcriptional regulation of the mutant *pab1* allele harbored by the *pab1_A60-9* mutant has changed. Recombinant constructs containing the single mutations and their combination are currently under evaluation. Finally, we cannot exclude that also in this case the effect will be

dependent on the efficiency and the persistence of stress granules. This aspect is currently under investigation by fluorescence microscope observations.

Conclusions

We have demonstrated that both the alteration of Pab1 abundance and function increase *S. cerevisiae* stress tolerance. The data obtained in shake flasks are promising for testing the strains in bioreactors under conditions as much as possible similar to those occurring during industrial fermentations. The ethanol production will be tested in operative condition resembling those of lignocellulosic hydrolysate fermentation, to verify the robustness of the obtained cellular design. In this perspective, the here reported results suggest that the engineering of mRNA metabolism may represent an innovative approach to obtain strains with an improved phenotype against stressing factors.

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Supplementary figures

Figure S1. Western blot analysis of Pab1-GFP level in control and Pab1(+)-GFP strains. Anti-GFP was used as the primary antibody and AP conjugated rabbit anti-Mouse IgG (FC) as the secondary antibody.

Figure S2. Multiple sequences alignment of *PAB1* promoter and coding sequence of mutants *pab1_G60-4*, *pab1_G70-1*, *pab1_G60-2*, *pab1_A60-9*, *pab1_G60-6*, *pab1_G60-7* and *pab1_A60-17*. The sequence of mutants *pab1_A70-11* and *pab1_G70-2* are in the process of sequencing. The starting codon ATG and nucleotide substitutions are highlighted in red and yellow, respectively.

```
PAB1_wt          CGTGTAAGTGTGTGTACTATAGGGCACCGTAAAGTAATAATGCTTAATTAGTTACTACTA 60
PAB1_G60-4       CGTGTAAGTGTGTGTACTATAGGGCACCGTAAAGTAATAATGCTTAATTAGTTACTACTA 60
PAB1_G70-1       CGTGTAAGTGTGTGTACTATAGGGCACCGTAAAGTAATAATGCTTAATTAGTTACTACTA 60
PAB1_G60-2       CGTGTAAGTGTGTATACTATAGGGCACCGTAAAGTAATAATGCTTAATTAGTTACTACTA 60
PAB1_A60-9       CGTGTAAGTGTGTGTACTATAGGGCACCGTAAAGTAATAATGCTTAATTAGTTACTACTA 60
PAB1_G60-6       CGTGTAAGTGTGTGTACTATAGGGCACCGTAAAGTAATAATGCTTAATTAGTTACTACTA 60
PAB1_G60-7       CGTGTAAGTGTGTGTACTATAGGGCACCGTAAAGTAATAATGCTTAATTAGTTACTACTA 60
PAB1_A60-17      CGTGTAAGTGTGTGTACTATAGGGCACCGTAAAGTAATAATGCTTAATTAGTTACTACTA 60
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PAB1_wt          TGACCATATAAGAGGTCATACTGTATGAAGCCACAAAGCAGATAGATCAATCATGTTTAA 120
PAB1_G60-4       TGACCATATAAGAGGTCATACTGTATGAAGCCACAAAGCAGATAGATCAATCATGTTTAA 120
PAB1_G70-1       TGACCATATAAGAGGTCATACTGTATGAAGCCACAAAGCAGATAGATCAATCATGTTTAA 120
PAB1_G60-2       TGACCATATAAGAGGTCATACTGTATGAAGCCACAAAGCAGATAGATCAATCATGTTTAA 120
PAB1_A60-9       TGACCATATAAGAGGTCATACTGTATGAAGCCACAAAGCAGATAGATCAATCATGTTTAA 120
PAB1_G60-6       TGACCATATAAGAGGTCATACTGTATGAAGCCACAAAGCAGATAGATCAATCATGTTTAA 120
PAB1_G60-7       TGACCACATAAGAGGTCATACTGTATGAAGCCACAAAGCAGATAGATCAATCATGTTTAA 120
PAB1_A60-17      TGACCATATAAGAGGTCATACTGTATGAAGCCACAAAGCAGATAGATCAATCATGTTTAA 120
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PAB1_wt          CGAAAACTGTTAATCGAAGATTATTTCTTTTTTTTTTTCTCTTTCCTTTTTACAAAGAAA 180
PAB1_G60-4       CGAAAACTGTTAATCGAAGATTATTTCTTTTTTTTTTTCTCTTTCCTTTTTACAAAGAAA 180
PAB1_G70-1       CGAAAACTGTTAATCGAAGATTATTTCTTTTTTTTTTTCTCTTTCCTTTTTACAAAGAAA 180
PAB1_G60-2       CGAAAACTGTTAATCGAAGATTATTTCTTTTTTTTTTTCTCTTTCCTTTTTACAAAGAAA 180
PAB1_A60-9       CGAAAACTGTTAATCGAAGATTATTTCTTTTTTTTTTTCTCTTTCCTTTTTACAAAGAAA 180
PAB1_G60-6       CGAAAACTGTTAATCGAAGATTATTTCTTTTTTTTTTTCTCTTTCCTTTTTACAAAGAAA 180
PAB1_G60-7       CGAAAACTGTTAATCGAAGATTATTTCTTTTTTTTTTTCTCTTTCCTTTTTACAAAGAAA 180
PAB1_A60-17      CGAAAACTGTTAATCGAAGATTATTTCTTTTTTTTTTTCTCTTTCCTTTTTACAAAGAAA 180
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PAB1_wt          ATTTTTTTTTGCGCTTTTTGCCATCACCATCGCAAGTTCTGGGACAATTGTTCTCTTTCGC 240
PAB1_G60-4       ATTTTTTTTTGCGCTTTTTGCCATCACCATCGCAAGTTCTGGGACAATTGTTCTCTTTCGC 240
PAB1_G70-1       ATTTTTTTTTGCGCTTTTTGCCATCACCATCGCAAGTTCTGGGACAATTGTTCTCTTTCGC 240
PAB1_G60-2       ATTTTTTTTTGCGCTTTTTGCCATCACCATCGCAAGTTCTGGGACAATTGTTCTCTTTCGC 240
PAB1_A60-9       ATTTTTTTTTGCGCTTTTTGCCATCACCATCGCAAGTTCTGGGACAATTGTTCTCTTTCGC 240
PAB1_G60-6       ATTTTTTTTTGCGCTTTTTGCCATCACCATCGCAAGTTCTGGGACAATTGTTCTCTTTCGC 240
PAB1_G60-7       ATTTTTTTTTGTGCTTTTTGCCATCACCATCGCAAGTTCTGGGACAATTGTACTCTTTCGC 240
PAB1_A60-17      AATTTTTTTTGCGCTTTTTGCCATCACCATCGCAAGTTCTGGGACAATTGTTCTCTTTCGC 240
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PAB1_wt          TCCAGTTCCAAGGAAAGAGGTTTCTGTTTTACTTAATAGAAAGTGTCATCTTGTATTTTA 300
PAB1_G60-4       TCCAGTTCCAAGGAAAGAGGTTTCTGTTTTACTTAATAGAAAGTGTCATCTTGTATTTTA 300
PAB1_G70-1       TCCAGTTCCAAGGAAAGAGGTTTCTGTTTTACTTAATAGAAAGTGTCATCTTGTATTTTA 300
PAB1_G60-2       TCCAGTTCCAAGGAAAGAGGTTTCTGTTTTACTTAATAGAAAGTGTCATCTTGTATTTTA 300
PAB1_A60-9       TCCAGTTCCAAGGAAAGAGCTTTCTGTTTTACTTAATAGAAAGTGTCATCTTGTATTTTA 300
PAB1_G60-6       TCCAGTTCCAAGGAAAGAGGTTTCTGTTTTACTTAATAGAAAGTGTCATCTTGTATTTTA 300
PAB1_G60-7       TCCAGTTCCAAGGAAAGAGGTTTCTGTTTTACTTAATAGAAAGTGTCATCTTGTATTTTA 300
PAB1_A60-17      TCCAGTTCCAAGGAAAGAGGTTTCTGTTTTACTTAATAGAAAGTGTCATCTTGCATTTTA 300
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PAB1_wt          TATCTCTTCTTTCTTGTGTAAAATTCTTTAGTTTTGATTTTGTATTTTTAGGACAGTGAG 360
PAB1_G60-4       TATCTCTTCTTTCTTGTGTAAAATTCTTTAGTTTTGATTTTGTATTTTTAGGACAGTGAG 360
PAB1_G70-1       TATCTCTTCTTTCTTGTGTAAAATTCTTTAGTTTTGATTTTGTATTTTTAGGACAGTGAG 360
PAB1_G60-2       TATCTCTTCTTTCTTGTGTAAAATTCATTAGTTTTGATTTTGTATTTTTAGGACAGTGAG 360
PAB1_A60-9       TATCTCTTCTTTCTTGTGTAAAATTCTTTAGTTTTGATTTTGTATTTTTAGGACAGTGAG 360
PAB1_G60-6       TATCTCTTCTTTCTTGTGTAAAATTCTTTAGTTGTGATTTTGTATTTTTAGGACAGTGAC 360
PAB1_G60-7       TATCTCTTCTTTCTTGTGTAAAATTCTTTAGTTGTGATTTTGTATTTTTAGGACAGTGAG 360
PAB1_A60-17      TATCTCTTCTTTCTTGTGTAAAATTCTTTAGTTTTGATTTTGTATTTTTAGGACAGTGAA 360
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PAB1_wt          CTACGAAGTAACATTTTTACTTAATAACCGTTTGAAGCATAGAGCAGGCCCTGGTACCAC 420
PAB1_G60-4       CTACGAAGTAACATTTTTATTTAATAACCGTTTGAAGCATAGAGCAGGCCCTGGTACCAC 420
PAB1_G70-1       CTACGAAGTAACATTTTTACTTAATAACCGTTTGAAGCATAGAGCAGGCCCTGGTACCAC 420
PAB1_G60-2       CTACGAAGTAACATTTTTACTTAATAACCGTTTGAAGCATAGAGCAGGCCCTGGTACCAC 420
PAB1_A60-9       CTACGAAGTAACATTTTTACTTAATAAGCGTTTGAAGCATAGAGCAGGCCCTGGTACCAC 420
PAB1_G60-6       CTACGAAGTAACATTTTTACTTAATAACCGTTTGAAGCATAGAGCAGGCCCTGGTACCAC 420
PAB1_G60-7       CTACGAAGTAACATTTTTACTTAATAACCGTTTGAAGCATAGAGCAGGCCCTGGTACCAC 420
PAB1_A60-17      CTACGAAGTAACATTTTTACTTAATAACCGTTTGAAGCATAGAGCAGACCCTGGTACCAC 420
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PAB1_wt          CACCTAATATCTGGCTTTTTATTCAATAAAAACTCAAAAAAAAAAAATCCAAAAAAAACTA 480
PAB1_G60-4       CACCTAATATCTGGCTTTTTATTCAATAAAAACTCAAAAAAAAAAAATCCAAAAAAAACTA 480
PAB1_G70-1       CACCTAATATCTGGCTTTTTATTCAATAAAAACTCAAAAAAAAAAGTCCAAAAAAAACTA 480
PAB1_G60-2       CACCTAATATCTGGCTTTTTATTCAATAAAAACTCAAAAAAAAAAATCCAAAAAAAACTA 480
PAB1_A60-9       CACCTAATATCTGGCTTTTTATTCAATAAAAACTCAAAAAAAAAAATCCAAAAAAAACTA 480
PAB1_G60-6       CACCTAATATCTGGCTTTTTATTCAATAAAAACTCAAAAAAAAAAATCCAAAAAAAACTA 480
PAB1_G60-7       CACCTAATATCTGGCTTTTTATTCAATAAAAACTCAAAAAAAAAAATCCAAAAAAAACTA 480
PAB1_A60-17      CACCTAATATCTGGCTTTTTATTCAATAAAAACTCAAAAAAAAAAATCCAAAAAAAACTA 480
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PAB1_wt          AAAAACCAATAAAAATAAAATGGCTGATATTACTGATAAGACAGCTGAACAATTGGAAAA 540
PAB1_G60-4       AAAAACCAATAAAAATAAAATGGCTGATATTACTGATAAGACAGCTGAACAATTGGAAAA 540
PAB1_G70-1       AAAAACCAATAAAAATAAAATGGCTGATATTACTGATAAGACAGCTGAACAATTGGAAAA 540
PAB1_G60-2       AAAAACCAATAAAAATAAAATGGCTGATATTACTGATAAGACAGCTGAACAATTGGAAAA 540
PAB1_A60-9       AAAAACCAATAAAAATAAAATGGCTGATATTACTGATAAGACAGCTGAACAATTGGAAAA 540
PAB1_G60-6       AAAAACCAATAAAAATAAAATGGCTGATATTACTGATAAGACAGCTGAACAATTGGAAAA 540
PAB1_G60-7       AAAAACCAATAAAAATAAAATGGCTGATATTACTGATAAGACAGCTGAACAATTGGAAAA 540
PAB1_A60-17      AAAAACCAATAAAAATAAAATGGCTGATATTACTGATAAGACAGCTGAACAATTGGAAAA 540
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PAB1_wt          CTTGAATATTCAAGATGACCAAAAGCAAGCCGCCACTGGTTCAGAAAGCCAATCTGTTGA 600
PAB1_G60-4       CTTGAATATTCAAGATGACCAAAAGCAAGCCGCCACTGGTTCAGAAAGCCAATCTGTTGA 600
PAB1_G70-1       CTTGAATATTCAAGATGACCAAAAGCAAGCCGCCACTGGTTCAGAAAGCCATTCTGTTGA 600
PAB1_G60-2       CTTGAATATTCAAGATGACCAAAAGCAAGCCGCCACTGGTTCAGAAAGCCAATCTGTTGA 600
PAB1_A60-9       CTTGAATATTCAAGATGACCAAAAGCAAGCCGCCACTGGTTCAGAAAGCCAATCTGTTGA 600
PAB1_G60-6       CTTGAATATTCAAGATGACCAAAAGCAAGCCGCCACTGGTTCAGAAAGCCAATCTGTTGA 600
PAB1_G60-7       CTTGAATATTCAAGATGACCAAAAGCAAGCCGCCACTGGTTCAGAAAGCCAATCTGTTGA 600
PAB1_A60-17      CTTGAATATTCAAGATGACCAAAAGCAAGCCGCCACTGGTTCAGAAAGCCAATCTGTTGA 600
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PAB1_wt          AAACTCTTCTGCATCATTATATGTTGGTGACTTAGAACCTTCTGTTTCCGAAGCCCACTT 660
PAB1_G60-4       AAACTCTTCTGCATCATTATATGTTGGTGACTTAGAACCTTCTGTTTCCGAAGCCCACTT 660
PAB1_G70-1       AAACTCTTCTGCATCATTATATGTTGGTGACTTAGAACCTTCTGTGTCCGAAGCCCACTT 660
PAB1_G60-2       AAACTCTTCTGCATCATTATATGTTGGTGACTTAGAACCTTCTGTTTCCGAAGCCCACTT 660
PAB1_A60-9       AAACTCTTCTGCATCATTATATGTTGGTGACTTAGAACCTTCTGTTTCCGAAGCCCACTT 660
PAB1_G60-6       AAACTCTTCTGCACCATTATATGTTGGTGACTTAGAACCTTCTGTTTCCGAAGCCCACTT 660
PAB1_G60-7       AAACTCTTCTGCATCATTATATGTTGGTGACTTAGAACCTTCTGTTTCCGAAGCCCACTT 660
PAB1_A60-17      AAACTCTTCTGCATCATTATATGTTGGTGACTTAGAACCTTCTGTTTCCGAAGCCCACTT 660
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PAB1_wt          ATATGATATCTTCTCTCCAATCGGTTCAGTCTCCTCCATTCGTGTCTGTCGTGATGCCAT 720
PAB1_G60-4       ATATGATATCTTCTCTCCAATCGGTTCAGTCTCCTCCATTCGTGTCTGTCGTGATGCCAT 720
PAB1_G70-1       ATATGATATCTTCTCTCCAATCGGTTCAGTCTCCTCCATTCGTGTCTGTCGTGATGCCAT 720
PAB1_G60-2       ATATGATATCTTCTCTCCAATCGGTTCAGTCTCCTCCATTCGTGTCTGTCGTGATGCCAT 720
PAB1_A60-9       ATATGATATCTTCTCTCCAATCGGTTCAGTCTCCTCCATTCGTGTCTGTCGTGATGCCAT 720
PAB1_G60-6       ATATGATATCTTCTCTCCAATCGGTTCAGTCTCCTCCATTCGTGTCTGTCGTGATGCCAT 720
PAB1_G60-7       ATATGATATCTTCTCTCCAATCGGTTCAGTCTCCTCCATTCGTGTCTGTCGTGATGCCAT 720
PAB1_A60-17      ATATGATATCTTCTCTCCAATCGGTTCAGTCTCCTCCATTCGTGTCTGTCGTGATGCCAT 720
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PAB1_wt          CACTAAGACCTCTTTGGGCTATGCTTATGTTAACTTTAACGACCATGAAGCCGGCAGAAA 780
PAB1_G60-4       CACTAAGACCTCTTTGGGCTATGCTTATGTTAACTTTAACGACCATGAAGCCGGCAGAAA 780
PAB1_G70-1       CACTAAGACCTCTTTGGGCTATGCTTATGTTAACTTTAACGACCATGAAGCCGGCAGAAA 780
PAB1_G60-2       CACTAAGACCTCTTTGGGCTATGCTTATGTTAACTTTAACGACCATGAAGCCGGCAGAAA 780
PAB1_A60-9       CACTAAGACCTCTTTGGGCTATGCTTATGTTAACTTTAACGACCATGAAGCCGGCAGAAA 780
PAB1_G60-6       CACTAAGACCTCTTTGGGCTATGCTTATGTTAACTTTAACGACCATGAAGCCGGCAGAAA 780
PAB1_G60-7       CACTAAGACCTCTTTGGGCTATGCTTATGTTAACTTTAACGACCATGAAGCCGGCAGAAA 780
PAB1_A60-17      CACTAAGACCTCTTTGGGCTATGCTTATGTTAACTTTAACGACCATGAAGCCGGCAGAAA 780
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PAB1_wt          AGCAATTGAGCAATTGAACTACACTCCAATCAAGGGTAGATTATGCCGTATTATGTGGTC 840
PAB1_G60-4       AGCAATTGAGCAATTGAACTACACTCCAATCAAGGGTAGATTATGCCGTATTATGTGGTC 840
PAB1_G70-1       AGCAATTGAGCAATTGAACTACACTCCAATCAAGGGTAGATTATGCCGTATTATGTGGTC 840
PAB1_G60-2       AGCAATTGAGCAATTGAACTACACTCCAATCAAGGGTAGATTATGCCGTATTATGTGGTC 840
PAB1_A60-9       AGCAATTGAGCAATTGAACTACACTCCAATCAAGGGTAGATTATGCCGTATTATGTGGTC 840
PAB1_G60-6       AGCAATTGAGCAATTGAACTACACTCCAATCAAGGGTAGATTATGCCGTATTATGTGGTC 840
PAB1_G60-7       AGCAATTGAGCAATTAAACTACACTCCAATCAAGGGTAGATTATGCCGTATTATGTGGTC 840
PAB1_A60-17      AGCAATTGAGCAATTGAACTACACTCCAATCAAGGGTAGATTATGCCGTATTATGTGGTC 840
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PAB1_wt          TCAACGTGACCCATCATTGAGAAAGAAGGGTTCTGGTAACATCTTTATCAAGAACTTGCA 900
PAB1_G60-4       TCAACGTGACCCATCATTGAGAAAGAAGGGTTCTGGTAACATCTTTATCAAGAACTTGCA 900
PAB1_G70-1       TCAACGTGACCCATCATTGAGAAAGAAGGGTTCTGGTAACATCTTTATCAAGAACTTGCA 900
PAB1_G60-2       TCAACGTGACCCATCATTGAGAAAGAAGGGTTCTGGTAACATCTTTATCAAGAACTTGTA 900
PAB1_A60-9       TCAACGTGACCCATCATTGAGAAAGAAGGGTTCTGGTAACATCTTTATCAAGAACTTGCA 900
PAB1_G60-6       TCAACGTGACCCATCATTGAGAAAGAAGGGTTCTGGTAACACCTTTATCAAGAACTTGCA 900
PAB1_G60-7       TCAACGTGACCCATCATTGAGAAAGAAGGGTTCTGGTAACATCTTTATCAAGAACTTGCA 900
PAB1_A60-17      TCAACGTGACCCATCATTGAGAAAGAAGGGTTCTGGTAACATCTTTATCAAGAACTTGCA 900
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PAB1_wt          CCCTGATATTGACAACAAGGCTTTGTATGACACTTTCTCTGTGTTTGGTGACATCTTGTC 960
PAB1_G60-4       CCCTGATATTGACAACAAGGCTTTGTACGACACTTTCTCTGTGTTTGGTGACATCTTGTC 960
PAB1_G70-1       CCCTGATATTGACAACAAGGCTTTGTATGACACTTTCTCTGTGTTTGGTGACATCTTGTC 960
PAB1_G60-2       TCCTGATATTGACAACAAGGCTTTGTATGACACTTTCTCTGTGTTTGGTGACATCTTGTC 960
PAB1_A60-9       CCCTGATATTGACAACAAGGCTTTGTATGACACTTTCTCTGTGTTTGGTGACATCTTGTC 960
PAB1_G60-6       CCCTGATATTGACAACAAGGCTTTGTATGACACTTTCTCTGTGTTTGGTGACATCTTGTC 960
PAB1_G60-7       CCATGATATTGACAACAAGGCTTTGTATGACACTTTCTCTGTGTTTGGTGACATCTTGTC 960
PAB1_A60-17      CCCTGATATTGACAACAAGGCTTTGTATGACACTTTCTCTGTGTTTGGTGACATCTTGTC 960
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PAB1_wt          CAGCAAGATTGCCACCGACGAAAACGGAAAATCCAAGGGTTTTGGGTTTGTTCACTTCGA 1020
PAB1_G60-4       CAGCAAGATTGCCACCGACGAAAACGGAAAATCCAAGGGTTTTGGGTTTGTTCACTTCGA 1020
PAB1_G70-1       CAGCAAGATTGCCACCGACGAAAACGGAAAATCCAAGGGTTTTGGGTTTGTTCACTTCGA 1020
PAB1_G60-2       CAGCAAGATTGCCACCGACGAAAACGGAAAATCCAAGGGTTTTGGGTTTGTTCACTTCGA 1020
PAB1_A60-9       CAGCAAGATTGCCACCGACGAAAACGGAAAATCCAAGGGTTGTGGGTTTGTTCACTTCGA 1020
PAB1_G60-6       CAGCAAGATTGCCACCGACGAAAACGGAAAATCCAAGGGTTTTGGGTTTGTTCACTTCGA 1020
PAB1_G60-7       CAACAAGATTGCCACCGACGAAAACGGAAAATCCAAGGGTTTTGGGTTTGTTCACTTCGA 1020
PAB1_A60-17      CAGCAAGATTGCCACCGACGAAAACGGAAAATCCAAGGGTTTTGGGTTTGTTCACTTCGA 1020
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PAB1_wt          AGAAGAAGGTGCTGCCAAGGAAGCTATTGATGCTTTGAATGGTATGCTGTTGAACGGTCA 1080
PAB1_G60-4       AGAAGAAGGTGCTGCCAAGGAAGCTATTGATGCTTTGAATGGTATGCTGTTGAACGGTCA 1080
PAB1_G70-1       AGAAGAAGGTGCTGCCAAGGAAGCTATTGATGCTTTGAATGGTATGCTGTTGAACGGTCA 1080
PAB1_G60-2       AGAAGAAGGTGCTGCCAAGGAAGCTATTGATGCTTTGAATGGTATGCTGTTGAACGGTCA 1080
PAB1_A60-9       AGAAGAAGGTGCTGCCAAGGAAGCTATTGATGCTTTGAATGGTATGCTGTTGAACGGTCA 1080
PAB1_G60-6       AGAAGAAGGTGCTGCCAAGGAAGCTATTGATGCTTTGAATGGTATGCTGTTGAACGGTCA 1080
PAB1_G60-7       AGAAGAAGGTGCTGCTAAGGAAGCTATTGATGCTTTGAATGGTATGCTGTTGAACGGTCA 1080
PAB1_A60-17      AGAAGAAGGTGCTGCCAAGGAAGCTATTGATGCTTTGAATGGTATGCTGTTGAACGGTCA 1080
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PAB1_wt          AGAAATTTATGTTGCTCCTCACTTGTCCAGAAAGGAACGTGACTCTCAATTGGAAGAGAC 1140
PAB1_G60-4       AGAAATTTATGTTGCTCCTCACTTGTCCAGAAAGGAACGTGACTCTCAATTGGAAGAGAC 1140
PAB1_G70-1       AGAAATTTATGTTGCTCCTCACTTGTCCAGAAAGGAACGTGACTCTCAATTGGAAGAGAC 1140
PAB1_G60-2       AGAAATTTATGTTGCTCCTCACTTGACCAGAAAGGAACGTGACTCTCAATTGGAAGAGAC 1140
PAB1_A60-9       AGAAATTTATGTTGCTCCTCACTTGTCCAGAAAGGAACGTGACTCTCAATTGGAAGAGAC 1140
PAB1_G60-6       AGAAATTTATGTTGCTCCTCACTTGTCCAGAAAGGAACGTGACTCTCAATTGGAAGAGAC 1140
PAB1_G60-7       AGAAATTTATGTTGCTCCTCACTTGTCCAGAAAGGAACGTGACTCTCAATTGGAAGAGAC 1140
PAB1_A60-17      AGAAATTTATGTTGCTCCTCACTTGTCCAGAAAGGAACGTGACTCTCAATTGGAAGAGAC 1140
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PAB1_wt          TAAGGCACATTACACTAACCTTTATGTGAAAAACATCAACTCCGAAACTACTGACGAACA 1200
PAB1_G60-4       TAAGGCATATTACACTAACCTTTATGTGAAAAACATCAACTCCGAAACTACTGACGAACA 1200
PAB1_G70-1       TAAGGCACATTACACTAACCTTTATGTGAAAAACATCAACTCCGAAACTACTGACGAACA 1200
PAB1_G60-2       TAAGGCACATTACACTAACCTTTATGTGAAAAACATCAACTCCGAAACTACTGACGAACA 1200
PAB1_A60-9       TAAGGCACATTACACTAACCTTTATGTGAAAAACATCAACTCCGAAACTACTGACGAACA 1200
PAB1_G60-6       TAAGGCACATTACACTAACCTTTATGTGAAAAACATCAACTCCGAAACTACTGACGAACA 1200
PAB1_G60-7       TAAGGCACATTACACTAACCTTTATGTGAAAAACATCAACTCCGAAACTACTGACGAACA 1200
PAB1_A60-17      TAAGGCACATTACACTAACCTTTATGTGAAAAACATCAACTCCGAAACTACTGACGAACA 1200
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PAB1_wt          ATTCCAAGAATTGTTTGCCAAATTTGGTCCAATTGTTTCTGCCTCTTTGGAAAAGGATGC 1260
PAB1_G60-4       ATTCCAAGAATTGTTTGCCAAATTTGGTCCAATTGTTTCTGCCTCTTTGGAATAGGATGC 1260
PAB1_G70-1       AATCCAAGAATTGTTTGCCAAATTTGGTCCAATTGTTTCTGCCTCTTTGGAAAAGGATGC 1260
PAB1_G60-2       ATTCCAAGAATTGTTTGCCAAATTTGGTCCAATTGTTTCTGCCTCTTTGGAAAAGGATGC 1260
PAB1_A60-9       ATTCCAAGAATTGTTTGCCAAATTTGGTCCAATTGTTTCTGCCTCTTTGGAAAAGGATGC 1260
PAB1_G60-6       ATTCCAAGAATTGTTTGCCAAATTTGGTCCAATTGTTTCTGCCTCTTTGGAAAAGGATGC 1260
PAB1_G60-7       ATTCCAAGAATTGTTTGCCAAATTTGGTCCAATTGTTTCTGCCTCTTTGGAAAAGGATGC 1260
PAB1_A60-17      ATTCCAAGAATTCTTTGCCAAATTTGGTCCAATTGTTTCTGCCTCTTTGGAAAAGGATGC 1260
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PAB1_wt          TGATGGAAAATTGAAGGGTTTCGGGTTTGTTAACTACGAAAAGCATGAAGACGCTGTGAA 1320
PAB1_G60-4       TGATGGAAAATTGAAGGGTTTCGGGTTTGTTAACTACGAAAAGCATGAAGACGCTGTGAA 1320
PAB1_G70-1       TGATGGAAAATTGAAGGGTTTCGGGTTTGTTAACTACGAAAAGCATGAAGACGCTGTGAA 1320
PAB1_G60-2       TGATGGAAAATTGAAGGGTTTCGGGTTTGTTAACTACGAAAAGCATGAAGACGCTGTGAA 1320
PAB1_A60-9       TGATGGAAAATTGAAGGGTTTCGGGTTTGTTAACTACGAAAAGCATGAAGACGCTGTGAA 1320
PAB1_G60-6       TGATGGAAAATTGAAGGGTTTCGGGTTTGTTAACTACGAAAAGCATGAAGACGCTGTGAA 1320
PAB1_G60-7       TGATGGAAAATTGAAGGGTTTCGGGTTTGTTAACTACGAAAAGCATGAAGACGCTGTGAA 1320
PAB1_A60-17      TGATGGAAAATTGAAGGGTTTCGGGTTTGTTAACTACGAAAAGCATGAAGACGCTGTGAA 1320
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PAB1_wt          AGCTGTTGAAGCTTTGAATGACTCTGAACTAAATGGAGAAAAGTTATACGTTGGTCGTGC 1380
PAB1_G60-4       AGCTGTTGTAGCTTTGAATGACTCTGAACTAAATGGAGAAAAGTTATACGTTGGTCGTGC 1380
PAB1_G70-1       AGCTGTTGAAGCTTTGAATGACTCTGAACTAAATGGAGAAAAGTTATACGTTGGTCGTGC 1380
PAB1_G60-2       AGCTGTTGAAGCTTTGAATGACTCTGAACTAAATGGAGAAAAGTTATACGTTGGTCGTGC 1380
PAB1_A60-9       AGCTGTTGAAGCTTTGAATGACTCTGAACTAAATGGAGAAAAGTTATACGTTGGTCGTGC 1380
PAB1_G60-6       AGCTGTTGAAGCTTTGAATGACTCTGAACTAAATGGAGAAAAGTTATACGTTGGTCGTGC 1380
PAB1_G60-7       AGCTGTTGAAGCTTTGAATGACTCTGAACTAAATGGAGAAAAGTTATACGTTGGTCGTGC 1380
PAB1_A60-17      AGCTGTTGAAGCTTTGAATGACTCTGAACTAAATGGAGAAAAGTTATACGTTGGTCGTGC 1380
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PAB1_wt          CCAAAAGAAGAATGAACGTATGCATGTCTTGAAGAAGCAATACGAAGCTTACAGATTGGA 1440
PAB1_G60-4       CCAAAAGAAGAATGAACGTATGCATGTCTTGAAGAAGCAATACGAAGCTTACAGATTGGA 1440
PAB1_G70-1       CCAAAAGAAGAATGAACGTATGCATGTCTTGAAGAAGCAATACGAAGCTTACAGATTGGA 1440
PAB1_G60-2       CCAAAAGAAGAATGAACGTATGCATGTCTTGAAGAAGCAATACGAAGCTTACAGATTGGA 1440
PAB1_A60-9       CCAAAAGAAGAATGAACGTATGCATGTCTTGAAGAAGCAATACGAAGCTTACAGATTGGA 1440
PAB1_G60-6       CCAAAAGAAGAATGAACGTATGCATGTCTTGAAGAAGCAATACGAAGCTTACAGATTGGA 1440
PAB1_G60-7       CCAAAAGAAGAATGAACGTATGCATGTCTTGAAGAAGCAATACGAAGCTTACAGATTGGA 1440
PAB1_A60-17      CCAAAAGAAGAATGAACGTATGCATGTCTTGAAGAAGCAATACGAAGCTTACAGATTGGA 1440
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PAB1_wt          AAAAATGGCCAAGTACCAAGGTGTTAATTTGTTTGTTAAGAACTTAGATGACAGCGTTGA 1500
PAB1_G60-4       AAAAATGGCCAAGTACCAAGGTGTTAATTTGTTTGTTAAGAACTTAGATGACAGCGTTGA 1500
PAB1_G70-1       AAAAATGGCCAAGTACCAAGGTGTTAATTTGTTTGTTAAGAACTTAGATGACAGCGTTGA 1500
PAB1_G60-2       AAAAATGGCCAAGTACCAAGGTGTTAATTTGTTTGTTAAGAACTTAGATGACAGCGTTGA 1500
PAB1_A60-9       AAAAATGGCCAAGTACCAAGGTCTTAATTTGTTTGTTAAGAACTTAGATGACAGCGTTGA 1500
PAB1_G60-6       AAAAATGGCCAAGTACCAAGGTGTTAATTTGTTTGTTAAGAACTTAGATGACAGCGTTGA 1500
PAB1_G60-7       AAAAATGGCCAAATACCAAGGTGTTAATTTGTTTGTTAAGAACTTAGATGACAGCGTTGA 1500
PAB1_A60-17      AAAAATGGCCAAGTACCAAGGTGTTAATTTGTTTGTTAAGAACTTAGATGTCAGCATTGA 1500
                 ************ ********* ************************** **** ****

PAB1_wt          TGACGAAAAGTTGGAAGAAGAATTTGCTCCATATGGTACTATCACTTCTGCAAAGGTTAT 1560
PAB1_G60-4       TGAGGAAAAGTTGGAAGAAGAATTTGCTCCATATGGTACTATCACTTCTGCAAAGGTTAT 1560
PAB1_G70-1       TGACGAAAAGTTGGAAGAAGAATTTGCTCCATATGGTACTATCACTTCTGCAAAGGTTAT 1560
PAB1_G60-2       TGACGAAAAGTTGGGAGAAGAATTTGCTCCATATGGTACTATCACTTCTGCAAAGGTTAT 1560
PAB1_A60-9       TGACGAAAAGTTGGAAGAAGAATTTGCTCCATATGGTACTATCACTTCTGCAAAGGTTAT 1560
PAB1_G60-6       TGACGAAAAGTTGGAAGAAGAATTTGCTCCATATGGTACTATCACTTCTGCAAAGGTTAT 1560
PAB1_G60-7       TGACGAAAAGTTGGAAGAAGAATTTGCTCCATATGGTACTATCACTTCTGCAAAGGTTAT 1560
PAB1_A60-17      TGACGAAAAGTTGGAAGAAGAATTTGCTCCATATGGTACTATCACTTCTGCAAAGGTTAT 1560
                 *** ********** ************************************************

PAB1_wt          GAGAACCGAAAACGGTAAGTCTAAGGGTTTTGGTTTTGTTTGTTTCTCAACTCCAGAGGA 1620
PAB1_G60-4       GAGAACCGAAAACGGTAAGTCTAAGGGTTTTGGTTTTGTTTGTTTCTCAACTCCAGAGGA 1620
PAB1_G70-1       GAGGACCGAAAACGGTAAGTCTAAGGGTTTTGGTTTTGTTTGTTTCTCAACTCCAGAGGA 1620
PAB1_G60-2       GAGAACCGAAAACGGTAAGTCTAAGGGTTTTGGTTTTGTTTGTTTCTCAACTCCAGAGGA 1620
PAB1_A60-9       GAGAACCGAAAACGGTAAGTCTAAGGGTTTTGGTTTTGTTTGTTTCTCAACTCCAGAGGA 1620
PAB1_G60-6       GAGAACCGAAAACGGTAAGTCTAAGGGTTTTGGTTTTGTTTGTTTCTCAACTCCAGAGGA 1620
PAB1_G60-7       GAGAACCGAAAACGGTAAGTCTAAGGGTTTTGGTTTTGTTTGTTTCTCAACTCCAGAGGA 1620
PAB1_A60-17      GAGAACCGAAAACGGTAAGTCTAAGGGTTTTGGTTTTGTTTGTTTCTCAACTCCAGAGGA 1620
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PAB1_wt          AGCTACTAAGGCCATTACAGAAAAGAACCAACAAATTGTTGCTGGTAAGCCATTATACGT 1680
PAB1_G60-4       AGCTACTAAGGCCATTACAGAAAAGAACCAACAAATTGTTGCTGGTAAGCCATTATACGT 1680
PAB1_G70-1       AGCTACTAAGGCCATTACAGAAAAGAACCAACAAATTGTTGCTGGTAAGCCATTATACGT 1680
PAB1_G60-2       AGCTACTAAGGCCATTACAGAAAAGAACCAACAAATTGTTGCTGGTAAGCCATTATACGT 1680
PAB1_A60-9       AGCTACTAAGGCCATTACAGAAAAGAACCAACAAATTGTTGCTGGTAAGCCATTATACGT 1680
PAB1_G60-6       AGCTACTAAGGCCATTACAGAAAAGAACCAACAAATTGTTGCTGGTAAGCCATTATACGT 1680
PAB1_G60-7       AGCTACTAAGGCCATTACAGAAAAGAACCAACAAATTGTTGCTGGTAAGCCATTATACGT 1680
PAB1_A60-17      AGCTACTAAGGCCATTACAGAAAAGAACCAACAAATTGTTGCTGGTAAGCCATTATACGT 1680
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PAB1_wt          TGCCATTGCTCAAAGAAAAGACGTAAGACGTTCTCAATTGGCTCAACAAATCCAAGCCAG 1740
PAB1_G60-4       TGCCATTGCTCAAAGAAAAGACGTAAGACGTTCTCAATTGGCTCAACAAATCCAAGCCAG 1740
PAB1_G70-1       TGCCATTGCTCAAAGAAAAGACGTAAGACGTTCTCAATTGGCTCAACAAATCCAAGCCAG 1740
PAB1_G60-2       TGCCATTGCTCAAAGAAAAGACGTAAGACGTTCTCAATTGGCTCAACAAATCCAAGCCAG 1740
PAB1_A60-9       TGCCATTGCTCAAAGAAAAGACGTAAGACGTTCTCAATTGGCTCAACAAATCCAAGCCAG 1740
PAB1_G60-6       TGCCATTGCTCAAAGAAAAGACGTAAGACGTTCTCAATTGGCTCAACAAATCCAAGCCAG 1740
PAB1_G60-7       TGCCATTGCTCAAAGAAAAGACGTAAGACGTTCTCAATTGGCTCAACAAATCCAAGCCAG 1740
PAB1_A60-17      TGCCATTGCTCAAAGAAAATACGTAAGACGTTCTCAATTGGCTCAACAAATCCAAGCCAG 1740
                 ******************* ****************************************

PAB1_wt          AAATCAAATGAGATACCAGCAAGCTACTGCTGCCGCTGCCGCCCGCCGCTGCCGGTATGCC 1800
PAB1_G60-4       AAATCAAATGAGATACCAGCAAGCTACTGCTGCCGCTGCCGCCCGCCGCTGCCGGTATGCC 1800
PAB1_G70-1       AAATCAAATGAGATACCAGCAAGCTACTGCTGCCGCTGCCGCCCGCCGCTGCCGGTATGCC 1800
PAB1_G60-2       AAATCAAATGAGATACCAGCAAGCTACTGCTGCCGCTGCCGCCCGCCGCTGCCGGTATGCC 1800
PAB1_A60-9       AAATCAAATGAGATACCAGCAAGCTACTGCTGCCGCTGCCGCCCGCCGCTGCCGGTATGCC 1800
PAB1_G60-6       AAATCAAATGAGATACCAGCAAGCTACTGCTGCCGCTGCCGCCCGCCGCTGCCGGTATGCC 1800
PAB1_G60-7       AAATCAAATGAGATACCAGCAAGCTACTGCTGCCGCTGCCGCCCGCCGCTGCCGGTATGCC 1800
PAB1_A60-17      AAATCAAATGAGATACCAGCAAGCTACTGCTGCCGCTGCCGCCCGCCGCTGCCGGTATGCC 1800
                 ************************************************************

PAB1_wt          AGGTCAATTCATGCCTCCAATGTTCTATGGTGTTATGCCACCAAGAGGTGTTCCATTCAA 1860
PAB1_G60-4       AGGTCAATTCATGCCTCCAATGTTCTATGGTGTTATGCCACCAAGAGGTGTTCCATTCAA 1860
PAB1_G70-1       AGGTCAATTCATGCCTCCAATGTTCTATGGTGTTATGCCACCAAGAGGTGTTCCGTTCAA 1860
PAB1_G60-2       AGGTCAATTCATGCCTCCAATGTTCTATGGTGTTATGCCACCAAGAGGTGTTCCATTCAA 1860
PAB1_A60-9       AGGTCAATTCATGCCTCCAATGTTCTATGGTGTTATGCCACCAAGAGGTGTTCCATTCAA 1860
PAB1_G60-6       AGGTCAATTCATGCCTCCAATGTTCTATGGTGTTATGCCACCAAGAGGTGTTCCATTCAA 1860
PAB1_G60-7       AGGTCAATTCATGCCTCCAATGTTCTATGGTGTTATGCCACCAAGAGGTGTTCCATTCAA 1860
PAB1_A60-17      AGGTCAATTCATGCCTCCAATGTTCTATGGTGTTATGCCACCAAGAGGTGTTCCATTCAA 1860
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PAB1_wt          CGGTCCAAACCCACAACAAATGAACCCAATGGGCGGTATGCCAAAGAACGGCATGCCACC 1920
PAB1_G60-4       CGGTCCAAACCCACAACAAATGAACCCAATGGGCGGTATGCCAAAGAACGGCATGCCACC 1920
PAB1_G70-1       CGGTCCAAACCCACAACAAATGAACCCAATGGGCGGTATGCCAAAGAACGGCATGCCACC 1920
PAB1_G60-2       CGGTCCAAACCCACAACAAATGAACCCAATGGGCGGTATGCCAAAGAACGGCATGCCACC 1920
PAB1_A60-9       CGGTCCAAACCCACAACAAATGAACCCAATGGGCGGTATGCCAAAGAACGGCATGCCACC 1920
PAB1_G60-6       CGGTCCAAACCCACAACAAATGAACCCAATGGGCGGTATGCCAAAGAACGGCATGCCACC 1920
PAB1_G60-7       CGGTCCAAACCCACAACAAATGAACCCAATGGGCGGTATGCCAAAGAACGGCATGCCACC 1920
PAB1_A60-17      CGGTCCAAACCCACAACAAATGAACCCAATGGGCGGTATGCCAAAGAACGGCATGCCACC 1920
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PAB1_wt          TCAATTTAGAAATGGTCCGGTTTACGGCGTCCCCCCACAAGGTGGTTTCCCAAGAAATGC 1980
PAB1_G60-4       TCAATTTAGAAATGGTCCGGTTTACGGCGTCCCCCCACAAGGTGGTTTCCCAAGAAATGC 1980
PAB1_G70-1       TCAATTTAGAAATGGTCCGGTTTACGGCGTCCCCCCACAAGGTGGTTTCCCAAGAAATGC 1980
PAB1_G60-2       TCAATTTAGAAATGGTCCGGTTTACGGCGTCCCCCCACAAGGTGGTTTCCCAAGAAATGC 1980
PAB1_A60-9       TCAATTTAGAAATGGTCCGGTTTACGGCGTCCCCCCACAAGGTGGTTTCCCAAGTAATGC 1980
PAB1_G60-6       TCAATTTAGAAATGGTCCGGTTTACGGCGTCCCCCCACAAGGTGGTTTCCCAAGAAATGC 1980
PAB1_G60-7       TCAATTTAGAAATGGTCCGGTTTACGGCGTCCCCCCACAAGGTGGTTTCCCAAGAAATGC 1980
PAB1_A60-17      TCAATTTAGAAATGGTCCGGTTTACGGCGTCCCCCCACAAGGTGGTTTCCCAAGAAATGC 1980
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PAB1_wt          CAACGATAACAACCAATTTTATCAACAAAAGCAAAGACAAGCTTTGGGTGAACAATTATA 2040
PAB1_G60-4       CAACGATAACAACCAATTTTATCAACAAAAGCAAAGACAAGCTTTGGGTGAACAATTATA 2040
PAB1_G70-1       CAACGATAACAACCAATTTTATCAACAAAAGCAAAGACAAGCTTTGGGTGAACAATTATA 2040
PAB1_G60-2       CAACGATAACAACCAATTTTATCAACAAAAGCAAAGACAAGCTTTGGGTGAACAATTATA 2040
PAB1_A60-9       CAACGATAACAACCAATTTTATCAACAAAAGCAAAGACAAGCTTTGGGTGAACAATTATT 2040
PAB1_G60-6       CAACGATAACAACCAATTTTATCAACAAAAGCAAAGACAAGCTTTGGGTGAACAATTATA 2040
PAB1_G60-7       CAACGATAACAACCAATTTTATCAACAAAAGCAAAGACAAGCTTTGGGTGAACAATTATA 2040
PAB1_A60-17      CAACGATAACAACCAATTTTATCAACAAAAGCAAAGACAAGCTTTGGGTGAACAATTATA 2040
                 ************************************************************

PAB1_wt          CAAGAAGGTTTCTGCTAAGACTTCAAATGAAGAAGCAGCTGGTAAAATTACTGGTATGAT 2100
PAB1_G60-4       CAAGAAGGTTTCTGCTAAGACTTCAAATGAAGAAGCAGCTGGTAAAATTACTGGTATGAT 2100
PAB1_G70-1       CAAGAAGGTTTCTGCTAAGACTTCAAATGAAGAAGCAGCTGGTAAAATTACTGGTATGAT 2100
PAB1_G60-2       CAAGAAGGTTTCTGCTAAGACTTCAAATGAAGAAGCAGCTGGTAAAATTACTGGTATGAT 2100
PAB1_A60-9       CAAGAAGGTTTCTGCTAAGACTTCAAATGAAGAAGCAGCTGGTAAAATTACTGGTATGAT 2100
PAB1_G60-6       CAAGAAGGTTTCTGCTAAGACTTCAAATGAAGAAGCAGCTGGTAAAATTACTGGTATGAT 2100
PAB1_G60-7       CAAGAAGGTTTCTGCTAAGACTTCAAATGAAGAAGCAGCTGGTAAAATTACTGGTATGAT 2100
PAB1_A60-17      CAAGAAGGTTTCTGCTAAGACTTCAAATGAAGAAGCAGCTGGTAAAATTACTGGTATGAT 2100
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PAB1_wt          TTTGGATTTGCCACCTCAAGAGGTCTTCCCATTGTTGGAAAGTGATGAATTGTTCGAACA 2160
PAB1_G60-4       TTTGGATTTGCCACCTCAAGAGGTCTTCCCATTGTTGGAAAGTGATGAATTGTTCGAACA 2160
PAB1_G70-1       TTTGGATTTGCCACCTCAAGAGGTCTTCCCATTGTTGGAAAGTGATGAATTGTTCGAACA 2160
PAB1_G60-2       TTTGGATTTGCCACCTCAAGAGGTCTTCCCATTGTTGGAAAGTGATGAATTGTTCGAACA 2160
PAB1_A60-9       TTTGGATTTGCCACCTCAAGAAGTCTTCCCATTGTTGGAAAGTGATGAATTGTTCGAACA 2160
PAB1_G60-6       TTTGGATTTGCCACCTCAAGAGGTCTTCCCATTGTTGGAAAGTGATGAATTGTTCGAACA 2160
PAB1_G60-7       TTTGGATTTGCCACCTCAAGAGGTCTTCCCATTGTTGGAAAGTGATGAATTGTTCGAACA 2160
PAB1_A60-17      TTTGGATTTGCCACCTCAAGAGGTCTTCCCATTGTTGGAAAGTGATGAATTGTTCGAACA 2160
                 ********************* ************************************

PAB1_wt          ACACTACAAAGAAGCTTCTGCTGCCTATGAGTCTTTCAAAAAGGAGCAAGAACAACAAAC 2220
PAB1_G60-4       ACACTACAAAGAAGCTTCTGCTGCCTATGAGTCTTTCAAAAAGGAGCAAGAACAACAAAC 2220
PAB1_G70-1       ACACTACAAAGAAGCTTCTGCTGCCTATGAGTCTTTCAAAAAGGAGCAAGAACAACAAAC 2220
PAB1_G60-2       ACACTACAAAGAAGCTTCTGCTGCCTATGAGTCTTTCAAAAAGGAGCAAGAACAACAAAC 2220
PAB1_A60-9       ACACTACAAAGAAGCTTCTGCTGCCTATGAGTCTTTCAAAAAGGAGCAAGAACAACAAAC 2220
PAB1_G60-6       ACACTACAAAGAAGCTTCTGCTGCCTATGAGTCTTTCAAAAAGGAGCAAGAACAACAAAC 2220
PAB1_G60-7       ACACTACAAAGAAGCTTCTGCTGCCTATGAGTCTTTCAAAAAGGAGCAAGAACAACAAAC 2220
PAB1_A60-17      ACACTACAAAGAAGCTTCTGCTGCCTATGAGTCTTTCAAAAAGGAGCAAGAACAACAAAC 2220
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PAB1_wt          TGAGCAAGCTTAA 2233
PAB1_G60-4       TGAGCAAGCTTAA 2233
PAB1_G70-1       TGAGCAAGCTTAA 2233
PAB1_G60-2       TGAGCAAGCTTAA 2233
PAB1_A60-9       TGAGCAAGCTTAA 2233
PAB1_G60-6       TGAGCAAGCTTAA 2233
PAB1_G60-7       TGAGCAAGCTTAA 2233
PAB1_A60-17      TGAGCAAGCTTAA 2233
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Figure S2. Multiple sequences alignment of amino acid sequence of mutants *pab1_G60-4*, *pab1_G70-1*, *pab1_G60-2*, *pab1_A60-9*, *pab1_G60-6*, *pab1_G60-7* and *pab1_A60-17*. The amino acid substitutions are highlighted in yellow. In the mutant *pab1_G60-4* the substitution of K252 with a stop codon, highlighted in red, resulted in the formation of a truncated protein.

```
PAB1_wt          MADITDKTAEQLENLNIQDDQKQAATGSESQSVENSSASLYVGDLEPSVSEAHLYDIFSP 60
PAB1_G60-4       MADITDKTAEQLENLNIQDDQKQAATGSESQSVENSSASLYVGDLEPSVSEAHLYDIFSP 60
PAB1_G70-1       MADITDKTAEQLENLNIQDDQKQAATGSESHSVENSSASLYVGDLEPSVSEAHLYDIFSP 60
PAB1_G60-2       MADITDKTAEQLENLNIQDDQKQAATGSESQSVENSSASLYVGDLEPSVSEAHLYDIFSP 60
PAB1_A60-9       MADITDKTAEQLENLNIQDDQKQAATGSESQSVENSSASLYVGDLEPSVSEAHLYDIFSP 60
PAB1_G60-6       MADITDKTAEQLENLNIQDDQKQAATGSESQSVENSSAPLYVGDLEPSVSEAHLYDIFSP 60
PAB1_G60-7       MADITDKTAEQLENLNIQDDQKQAATGSESQSVENSSASLYVGDLEPSVSEAHLYDIFSP 60
PAB1_A60-17      MADITDKTAEQLENLNIQDDQKQAATGSESQSVENSSASLYVGDLEPSVSEAHLYDIFSP 60
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PAB1_wt          IGSVSSIRVCRDAITKTSLGYAYVNFNDHEAGRKAIEQLNYTPIKGRLCRIMWSQRDPSL 120
PAB1_G60-4       IGSVSSIRVCRDAITKTSLGYAYVNFNDHEAGRKAIEQLNYTPIKGRLCRIMWSQRDPSL 120
PAB1_G70-1       IGSVSSIRVCRDAITKTSLGYAYVNFNDHEAGRKAIEQLNYTPIKGRLCRIMWSQRDPSL 120
PAB1_G60-2       IGSVSSIRVCRDAITKTSLGYAYVNFNDHEAGRKAIEQLNYTPIKGRLCRIMWSQRDPSL 120
PAB1_A60-9       IGSVSSIRVCRDAITKTSLGYAYVNFNDHEAGRKAIEQLNYTPIKGRLCRIMWSQRDPSL 120
PAB1_G60-6       IGSVSSIRVCRDAITKTSLGYAYVNFNDHEAGRKAIEQLNYTPIKGRLCRIMWSQRDPSL 120
PAB1_G60-7       IGSVSSIRVCRDAITKTSLGYAYVNFNDHEAGRKAIEQLNYTPIKGRLCRIMWSQRDPSL 120
PAB1_A60-17      IGSVSSIRVCRDAITKTSLGYAYVNFNDHEAGRKAIEQLNYTPIKGRLCRIMWSQRDPSL 120
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PAB1_wt          RKKGSGNIFIKNLHPDIDNKALYDTFSVFGDILSSKIATDENGKSKGFGFVHFEEEGAAK 180
PAB1_G60-4       RKKGSGNIFIKNLHPDIDNKALYDTFSVFGDILSSKIATDENGKSKGFGFVHFEEEGAAK 180
PAB1_G70-1       RKKGSGNIFIKNLHPDIDNKALYDTFSVFGDILSSKIATDENGKSKGFGFVHFEEEGAAK 180
PAB1_G60-2       RKKGSGNIFIKNLYPDIDNKALYDTFSVFGDILSSKIATDENGKSKGFGFVHFEEEGAAK 180
PAB1_A60-9       RKKGSGNIFIKNLHPDIDNKALYDTFSVFGDILSSKIATDENGKSKGCGFVHFEEEGAAK 180
PAB1_G60-6       RKKGSGNTFIKNLHPDIDNKALYDTFSVFGDILSSKIATDENGKSKGFGFVHFEEEGAAK 180
PAB1_G60-7       RKKGSGNIFIKNLHHDIDNKALYDTFSVFGDILSNKIATDENGKSKGFGFVHFEEEGAAK 180
PAB1_A60-17      RKKGSGNIFIKNLHPDIDNKALYDTFSVFGDILSSKIATDENGKSKGFGFVHFEEEGAAK 180
                 ******* *****: ******************.************ ************

PAB1_wt          EAIDALNGMLLNGQEIYVAPHLSRKERDSQLEETKAHYTNLYVKNINSETTDEQFQELFA 240
PAB1_G60-4       EAIDALNGMLLNGQEIYVAPHLSRKERDSQLEETKAYYTNLYVKNINSETTDEQFQELFA 240
PAB1_G70-1       EAIDALNGMLLNGQEIYVAPHLSRKERDSQLEETKAHYTNLYVKNINSETTDEQIQELFA 240
PAB1_G60-2       EAIDALNGMLLNGQEIYVAPHLTRKERDSQLEETKAHYTNLYVKNINSETTDEQFQELFA 240
PAB1_A60-9       EAIDALNGMLLNGQEIYVAPHLSRKERDSQLEETKAHYTNLYVKNINSETTDEQFQELFA 240
PAB1_G60-6       EAIDALNGMLLNGQEIYVAPHLSRKERDSQLEETKAHYTNLYVKNINSETTDEQFQELFA 240
PAB1_G60-7       EAIDALNGMLLNGQEIYVAPHLSRKERDSQLEETKAHYTNLYVKNINSETTDEQFQELFA 240
PAB1_A60-17      EAIDALNGMLLNGQEIYVAPHLSRKERDSQLEETKAHYTNLYVKNINSETTDEQFQEFFA 240
                 **********************:*************:*****************:**:**

PAB1_wt          KFGPIVSASLEKDADGKLKGFGFVNYEKHEDAVKAVEALNDSELNGEKLYVGRAQKKNER 300
PAB1_G60-4       KFGPIVSASLEStop---------------------------------------------- 300
PAB1_G70-1       KFGPIVSASLEKDADGKLKGFGFVNYEKHEDAVKAVEALNDSELNGEKLYVGRAQKKNER 300
PAB1_G60-2       KFGPIVSASLEKDADGKLKGFGFVNYEKHEDAVKAVEALNDSELNGEKLYVGRAQKKNER 300
PAB1_A60-9       KFGPIVSASLEKDADGKLKGFGFVNYEKHEDAVKAVEALNDSELNGEKLYVGRAQKKNER 300
PAB1_G60-6       KFGPIVSASLEKDADGKLKGFGFVNYEKHEDAVKAVEALNDSELNGEKLYVGRAQKKNER 300
PAB1_G60-7       KFGPIVSASLEKDADGKLKGFGFVNYEKHEDAVKAVEALNDSELNGEKLYVGRAQKKNER 300
PAB1_A60-17      KFGPIVSASLEKDADGKLKGFGFVNYEKHEDAVKAVEALNDSELNGEKLYVGRAQKKNER 300
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PAB1_wt          MHVLKKQYEAYRLEKMAKYQGVNLFVKNLDDSVDDEKLEEEFAPYGTITSAKVMRTENGK 360
PAB1_G60-4       ------------------------------------------------------------ 360
PAB1_G70-1       MHVLKKQYEAYRLEKMAKYQGVNLFVKNLDDSVDDEKLEEEFAPYGTITSAKVMRTENGK 360
PAB1_G60-2       MHVLKKQYEAYRLEKMAKYQGVNLFVKNLDDSVDDEKLGEEFAPYGTITSAKVMRTENGK 360
PAB1_A60-9       MHVLKKQYEAYRLEKMAKYQGLNLFVKNLDDSVDDEKLEEEFAPYGTITSAKVMRTENGK 360
PAB1_G60-6       MHVLKKQYEAYRLEKMAKYQGVNLFVKNLDDSVDDEKLEEEFAPYGTITSAKVMRTENGK 360
PAB1_G60-7       MHVLKKQYEAYRLEKMAKYQGVNLFVKNLDDSVDDEKLEEEFAPYGTITSAKVMRTENGK 360
PAB1_A60-17      MHVLKKQYEAYRLEKMAKYQGVNLFVKNLDVSIDDEKLEEEFAPYGTITSAKVMRTENGK 360
                 **********************:******** *:*:*** **********************

PAB1_wt          SKGFGFVCFSTPEEATKAITEKNQQIVAGKPLYVAIAQRKDVRRSQLAQQIQARNQMRYQ 420
PAB1_G60-4       ------------------------------------------------------------ 420
PAB1_G70-1       SKGFGFVCFSTPEEATKAITEKNQQIVAGKPLYVAIAQRKDVRRSQLAQQIQARNQMRYQ 420
PAB1_G60-2       SKGFGFVCFSTPEEATKAITEKNQQIVAGKPLYVAIAQRKDVRRSQLAQQIQARNQMRYQ 420
PAB1_A60-9       SKGFGFVCFSTPEEATKAITEKNQQIVAGKPLYVAIAQRKDVRRSQLAQQIQARNQMRYQ 420
PAB1_G60-6       SKGFGFVCFSTPEEATKAITEKNQQIVAGKPLYVAIAQRKDVRRSQLAQQIQARNQMRYQ 420
PAB1_G60-7       SKGFGFVCFSTPEEATKAITEKNQQIVAGKPLYVAIAQRKDVRRSQLAQQIQARNQMRYQ 420
PAB1_A60-17      SKGFGFVCFSTPEEATKAITEKNQQIVAGKPLYVAIAQRKYVRRSQLAQQIQARNQMRYQ 420
                 ************************************** ******************

PAB1_wt          QATAAAAAAAAGMPGQFMPPMFYGVMPPRGVPFNGPNPQQMNPMGGMPKNGMPPQFRNGP 480
PAB1_G60-4       ------------------------------------------------------------ 480
PAB1_G70-1       QATAAAAAAAAGMPGQFMPPMFYGVMPPRGVPFNGPNPQQMNPMGGMPKNGMPPQFRNGP 480
PAB1_G60-2       QATAAAAAAAAGMPGQFMPPMFYGVMPPRGVPFNGPNPQQMNPMGGMPKNGMPPQFRNGP 480
PAB1_A60-9       QATAAAAAAAAGMPGQFMPPMFYGVMPPRGVPFNGPNPQQMNPMGGMPKNGMPPQFRNGP 480
PAB1_G60-6       QATAAAAAAAAGMPGQFMPPMFYGVMPPRGVPFNGPNPQQMNPMGGMPKNGMPPQFRNGP 480
PAB1_G60-7       QATAAAAAAAAGMPGQFMPPMFYGVMPPRGVPFNGPNPQQMNPMGGMPKNGMPPQFRNGP 480
PAB1_A60-17      QATAAAAAAAAGMPGQFMPPMFYGVMPPRGVPFNGPNPQQMNPMGGMPKNGMPPQFRNGP 480
                 ************************************************************

PAB1_wt          VYGVPPQGGFPRNANDNNQFYQQKQRQALGEQLYKKVSAKTSNEEAAGKITGMILDLPPQ 540
PAB1_G60-4       ------------------------------------------------------------ 540
PAB1_G70-1       VYGVPPQGGFPRNANDNNQFYQQKQRQALGEQLYKKVSAKTSNEEAAGKITGMILDLPPQ 540
PAB1_G60-2       VYGVPPQGGFPRNANDNNQFYQQKQRQALGEQLYKKVSAKTSNEEAAGKITGMILDLPPQ 540
PAB1_A60-9       VYGVPPQGGFPSNANDNNQFYQQKQRQALGEQLFKKVSAKTSNEEAAGKITGMILDLPPQ 540
PAB1_G60-6       VYGVPPQGGFPRNANDNNQFYQQKQRQALGEQLYKKVSAKTSNEEAAGKITGMILDLPPQ 540
PAB1_G60-7       VYGVPPQGGFPRNANDNNQFYQQKQRQALGEQLYKKVSAKTSNEEAAGKITGMILDLPPQ 540
PAB1_A60-17      VYGVPPQGGFPRNANDNNQFYQQKQRQALGEQLYKKVSAKTSNEEAAGKITGMILDLPPQ 540
                 *********** ********************:************************

PAB1_wt          EVFPLLESDELFEQHYKEASAAYESFKKEQEQQTEQA 577
PAB1_G60-4       ------------------------------------- 577
PAB1_G70-1       EVFPLLESDELFEQHYKEASAAYESFKKEQEQQTEQA 577
PAB1_G60-2       EVFPLLESDELFEQHYKEASAAYESFKKEQEQQTEQA 577
PAB1_A60-9       EVFPLLESDELFEQHYKEASAAYESFKKEQEQQTEQA 577
PAB1_G60-6       EVFPLLESDELFEQHYKEASAAYESFKKEQEQQTEQA 577
PAB1_G60-7       EVFPLLESDELFEQHYKEASAAYESFKKEQEQQTEQA 577
PAB1_A60-17      EVFPLLESDELFEQHYKEASAAYESFKKEQEQQTEQA 577
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Conclusions

The results presented in this work demonstrated that the engineering of both the intracellular antioxidant availability and the mRNA metabolism are efficient strategies to improve the robustness of a *cell factory*.

In one case, the goal was obtained through the metabolic engineering of a *S. cerevisiae* strain for the intracellular production of the highly effective antioxidant L-ascorbic acid. This strain was shown to be more resistant to acetic acid principally because of a lower ROS accumulation compared with the parental strain. Furthermore, the discovery that in the L-AA producing strain some endogenous stress response mechanisms were reduced suggests that the production of this antioxidant can reorganize the stress response at different levels. Moreover, an effect on lifespan and on acetate consumption during CLS experiments was observed. Despite the mechanisms of these phenomena have not been established yet, it is therefore possible to speculate that the phenotype of the engineered strain may be also the result of some interactions of L-AA with the endogenous cellular mechanisms. Remarkably, the extended survival in stationary phase (CLS) exhibited by the L-AA producing strain is an additional important trait that might be exploited in industrial processes where re-pitching is used, or in those requiring the production during stationary phase, such as the production of secondary metabolites.

An increased resistance to acetic acid was also obtained through the modulation of abundance or with variants of the poly(A)-binding protein Pab1, one of the key molecule involved in the control of mRNA metabolism. This approach was applied with the aim to obtain an improved phenotype by modifying global gene expression at the post-transcriptional level. Although the effect of this engineering approach on mRNA stability, storage, and translation has yet to be verified at the molecular level, the preliminary results clearly show an improvement of acetic acid tolerance in growth experiments and an effect on stress granule dimension and persistence. Future experiments will be run in bioreactor, in conditions approaching

industrial production, to verify the robustness of the obtained results. Furthermore, additional experiments on mutant strains will help in clarifying the effect of the Pab1 variants, providing useful information about its role in cells.

The results reported in this thesis strongly suggest that the engineering of vitamin C production (or, more generally, of the antioxidant level) or of the mRNA metabolism may represent effective approaches to improve a *cell factory*. We suggest that, similarly to whole genome wide engineering approaches, these methods can allow the obtainment of complex phenotypes. Remarkably, by altering the redox balance or the post-transcriptional control, the results of said modifications are more directly connected to protein translation/levels and to their function.

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