UNIVERSITÀ DEGLI STUDI DI MILANO – BICOCCA

Facoltà di Scienze Matematiche, Fisiche e Naturali
Dipartimento di Biotecnologie e Bioscienze
Dottorato in Biotecnologie Industriali, XXII ciclo

MATCHING BIOTECH NEEDS AND YEASTS PHYSIOLOGY

Simone Passolunghi

Anno Accademico 2009/2010

Dottorato di Ricerca in Biotecnologie Industriali, XXII ciclo

Simone Passolunghi
Matricola: 533912

Tutor: prof. Danilo Porro

Università degli Studi di Milano-Bicocca
Piazza dell'Ateneo Nuovo 1, 20126, Milano

Dipartimento di Biotecnologie e Bioscienze
Piazza della Scienza 2, 20126, Milano

Il microbo avrà l'ultima parola

Louis Pasteur

Contents

Background: White Biotechnology

Industrial biotechnology, also known as white biotechnology, after pharmaceutical (red) and agricultural (green) applications, uses renewable raw materials as starting products and organisms (genetically modified or not) and their enzymes to make useful chemical compounds and biomaterials, most of which have been traditionally produced from crude oil. While its application in the production of fine chemicals and pharmaceuticals is already well established (e.g. insulin, interferons, erythropoietin, hepatitis B vaccine, vitamin B12, etc.), it is now increasingly being applied to produce bulk chemicals such as biofuels (e.g. ethanol) and bio-plastics. Other fields of application include food additives and supplements, colorants, vitamins, nutraceuticals, cosmeceuticals, pesticides, solvents, enzymes, bio-energy, etc..

This technology presents the inherently advantages of a clean process that has (i) reduced water consumption, (ii) reduced energy consumption, (iii) less or no waste generation and (iv) less CO_2 generation. Thus, industrial biotechnology is a key underpinning technology that might contribute to the transition of our current society, towards a more sustainable one, where renewable resources make a growing contribution to our energy and material needs.

It has been estimated that white biotechnology could be applied in the production of 10 to 20% of all chemicals sold in Europe by the year 2010. Starting with the chemical industry, white biotechnology will make inroads into a number of other industries. For example, enzymes will transform production processes in the pulp and paper industry, and new polymers will find multiple applications in the automotive and consumer industries. The greatest impact of white biotechnology may be on the fine chemicals segment, where up to 60% of products may be produced through biotechnology by 2010. A key driver here is the growth of biological pharmaceuticals such as antibodies for cancer treatment – drugs for which no traditional chemical synthesis exists. The impact on the specialty chemicals segment could vary broadly. For instance, enzymes and fermentation technologies are already used in the production of flavours and fragrances, while other markets may still be dominated by traditional chemistry through 2010 and beyond. Therefore, white biotechnology will be key to the competitiveness of many European industries that are already using biotechnology processes, including chemicals, textiles and leather, animal feed, pulp

and paper, energy, metals and minerals, as well as waste processing. It has been estimated that the chemical industry alone could generate additional added value of up to EUR 11 to 22 billion per annum by 2010, depending on whether the uptake is fast or slow. Two sources would contribute to this. One is the lower costs for raw materials and processing, combined with smaller scale investments in the fermentation of plants. The other is additional revenues from innovative, new, or performance enhanced products.

Currently, two types of feedstock can be used in the industrial biotech value chain for the production of fuels, bulk chemicals, materials and specialties. Fossil feedstock is frequently used for the bioproduction of certain compounds by enzymes and/or micro-organisms. These types of conversions are typically confined to biospecialties, such as fine chemicals, and hence are conducted on a relatively small scale compared to industrial biotech processes that use renewable resources. At present, the use of renewable feedstock, such as agricultural (by)products is gaining importance. To that end, agricultural materials, including (ligno)cellulose or starch are first converted into sugars, which are subsequently transformed into a wide range of products via fermentation. Also agricultural organic waste could be valorised in this way.

However, to address these challenges new or improved biocatalysts have to be developed, new or improved and tailored biotechnological processes have to be designed, developed and assessed. Recombinant DNA technology allows micro-organisms to be tailored to give higher yields of particular chemicals, or even to produce new ones if genes are transferred from other organisms. Increasing the efficiency of the reaction allows more and more scope for replacing established conventional processes by cleaner, lower-temperature fermentation, in a safe contained environment. The highly specific nature of individual enzymes means that chemicals can be produced in a purer form, and biological processes not only require fewer chemical inputs, but also result in smaller and more manageable waste streams. In general, most of the industrial biotech processes developed so far use the most effective and convenient biocatalytic form, which is a whole micro-organism. However this does not exclude the use of higher organisms, in particular plant, animal and human cell cultures, or the use of isolated enzymes combined with chemical catalysts.

In all cases the main driver is the cost- and eco-efficient production of the desired compounds by developing a) the best biological catalyst for a specific function or process, b) the best possible environment for

the catalyst to perform and c) the most suitable strategy for the recovery, purification and further chemical conversion of the desired products from the fermentation process.

The first aspect deals with the search for the best possible biocatalyst, with improved or totally new functionalities. Another important aspect of white biotechnology deals with the containment system or bioreactor within which the catalysts must function. Here the combined knowledge of the scientist and the bioprocess engineer interact, providing the design and instrumentation for the maintenance and control of the physiochemical environment such as temperature, aeration, pH, etc.. The third aspect, the downstream processing, can be a technically difficult and expensive procedure. Downstream processing is primarily concerned with the initial separation of the bioreactor medium into a liquid phase and a solid phase, and subsequent separation, concentration and purification of the product. In addition it includes the further chemical conversion of the fermentation product to yield the final desired compound. Chemical engineering principles play a vital role here as well in terms of designing and operation of the separation systems. Improvements in downstream processing will benefit the overall efficiency and process cost and will make the biotechnology based processes competitive with the conventional chemical ones.

Structure of the study

The research in the field of industrial biotechnology, especially regards bioproducts and bioprocesses, are aimed at developing innovative technologies that lead to obtaining compounds with the use of microorganisms, or seeking to enhance existing processes to increase yield, production and productivity, trying to ensure a higher degree of sustainability and reducing environmental impact.

To pursue these goals is possible to intervene by adopting a "technologic" approach that includes the development of systems capable of ensuring a more effective control of the parameters that govern the production processes or, with a "molecular / metabolic" approach, acting directly on the host system, namely by working on production capacity of the microorganism itself. To achieve this goal the pathways responsible for the processes of synthesis or secretion of products of interest must be identified and, if necessary, modified, or the environmental conditions in which the organism is operating during the process must be considered, to study how to improve its production capacity even in non-optimal physiological condition.

The process conditions that characterize industrial production processes often put the cells through a series of stress that inevitably act negatively on yields. It therefore becomes necessary to identify the limiting factors in relation to the host organism and, on this basis, to act in an appropriate manner. To do this it is possible to adopt different strategies, complementary and not mutually exclusive.

The most immediate is the exploration of biodiversity in order to choose a host that is intrinsically and naturally more resistant to the type of stress imposed by the process. This way is not always easy to follow, given the extent of the possible solutions and the lack of resources that allow the exploration and characterization in a reasonable time for the development of a biotechnological process. An alternative strategy is focused on the characterization of the cellular response to these stress conditions to identify the key factors involved in the mechanism. In this way, for example, through genetic manipulation on these factors it could be possible to improve the resistance of the cell itself, or by the transfer of these specific genetic traits to improve the resistance of other micro-organisms selected as host system.

The yeast *Saccharomyces cerevisiae* is one of the most widely used microorganisms for the production of compounds of biotechnological

interest because of the available knowledge on the physiology, genetics, biochemistry, and on the existence of technological and molecular tools suitable for its manipulation in order to optimize the production by fermentation. It is important to underline that *S. cerevisiae* is recognized as a GRAS organism (generally regarded as safe) by the Food & Drug Administration that has allowed the use for the production of pharmaceutical compounds for human use. While it is now increasingly clear the potential of *S. cerevisiae* as a platform for metabolic engineering, for some heterologous proteins production on a large scale this yeast is not the ideal host system. Very often the expressed proteins are hyper-glycosilated or, if retained in the periplasmic space, they suffer significant degradation. For other industrial productions, especially where the product of interest has to be cheap, the fermentation technologies needed are too complex and sophisticated (and expensive) to be implemented on a large scale. With these assumptions in recent years it has been explored the opportunity of adopting other yeasts, called "unconventional yeast" by developing new systems of expression. In this thesis project an "alternative" yeast, *Zygosaccharomyces bailii*, is considered. Although it is not well characterized from molecular and genetic point of view, it presents interesting features in view of potential biotechnological production: it allows high yields of biomass, it has a high specific growth rate and a higher resistance compared to *S. cerevisiae*, to certain types of stress, and in particular to stresses generated by an acid medium.

The cell surface, as an area of communication and exchange between the extracellular environment and the cell itself is one of the targets of this study.

In *S. cerevisiae*, thanks to the availability of molecular tools and the knowledge of the entire genome sequence, it is possible the design of in-depth studies and the engineering of the cellular metabolic network. Given the difficulties in its genetic manipulation, in the case of *Z. bailii* was first necessary to address another preliminary issue. In this diploid yeast the deletion of an essential gene was never been made. It has been developed a reproducible protocol for gene deletion by a gene-targeting approach and it was obtained the deletion of the gene *ZbLEU2*, who gave birth to the first auxotroph strain of *Z. bailii* (*leu⁻*). This represents an important step for a possible use of *Z. bailii* as host system. Thanks to this protocol a mutant strain of *Z. bailii* of potential interest for heterologous protein production was also obtained, in analogy with what reported in the literature for *S. cerevisiae*. The

deletion concerns the homologue of *ScGAS1*, coding for the enzyme *β*-1 ,3-glucanosyltransferase which catalyzes the crosslinking of cell wall glucans. *ZbGAS1*. The gene was cloned by PCR and sequenced. The deleted mutant of *Z. bailii* has morphological and phenotypic characteristics very similar to the correspondent in *S. cerevisiae*, showing an alteration of the cell wall structure, and enhanced secretive capacity than the wild type strain for some heterologous proteins that have been considered.

In parallel to these studies, populations of *Z. bailii* growing on different carbon sources were analyzed by flow cytometry. The analysis of DNA and protein content was performed to better characterize this yeast not only from molecular point of view, but also to explore its cellular features.

The characterization of this unconventional yeast confirmed once again one of the most appreciated features for yeasts used as cell factories: versatility.

This property is so strong that yeasts has been exploited for natural abilities, such as production of ethanol, and also for processes where a targeted manipulation was introduced, for example in lactic acid production, just to cite a pair of biotechnological production of industrial relevance. To make this process competitive on the market, in terms of yield, production and productivity, yeasts were pushed to their physiological limits. These limits are given by the accumulation of vast amounts of product that, in the case of ethanol, can cause damage to the lipid component of the plasma membrane, in the case of lactic acid can result in a loss of proper cellular homeostasis with the fall of the intracellular pH.

It is therefore necessary to assess whether these limitations can be overcome by acting in particular on the plasma membrane, whose role in controlling the transport and the cellular homeostasis makes it a target of interest to improve the robustness of cells in response to stress generated by the process, such as oxidative stress or generated by low pH.

The optimization of transport through the membrane plays a key role in the mechanisms of adaptation to these stresses. In particular, improving the flux of nutrients entering the cell could allow an optimal uptake of nutrient in the cytoplasm (as in the case of bioethanol production), an improvement of outflows from the cells might instead allow effective removal of compounds that may be deleterious for cell viability when present beyond a threshold value (*eg* organic acids).

By focusing on the protein fraction that characterizes the cytoplasmic membrane, we have studied the effects of modulation of the expression of H $^+$-ATPase pump of the plasma membrane (Pma1p), involved in the intracellular pH homeostasis. In particular, the gene *ScPMA1* was overexpressed in *S. cerevisiae* and this overexpression was able to confer a greater resistance to acid stress, evidenced by growth kinetics in the presence of lactic acid. The increased cell viability under restrictive conditions in respect to the wild type strain was also checked by flow cytometry. The use of this tool has enabled the development of a system able to assess quantitatively the degree of robustness of the cells in stressful conditions.

With this instrument able to assess the robustness of the cells as a function of various types of stress (oxidative, pH, ...) it is possible to design new interventions of metabolic engineering in order to provide greater resistance to yeast in restrictive process conditions, similar to conditions prevailing in the processes of production of compounds of biotech interest. It will be possible to evaluate the effectiveness of these interventions with the flow cytometer by assessing the response of the engineered cells under restrictive conditions by measuring the ability of cells to increase their robustness. The robustness remains one of the key features of yeasts as microbial cell factories, in particular to reach one of the main goal of White Biotechnology: to provide value products from renewable resources through sustainable processes with low environmental impact.

Investigating the multibudded and bi-nucleate phenotype of the yeast *Zygosaccharomyces bailii* growing on minimal medium

Laura Dato[1], Michael Sauer[2,3], Simone Passolunghi[1], Danilo Porro[1] and Paola Branduardi[1]*

[1] Dipartimento di Biotecnologie e Bioscienze - Università degli Studi di Milano-Bicocca, P.[zza] della Scienza 2, 20126 Milano, Italy

[2] School of Bioengineering, FH Campus Wien - University of Applied Sciences, Muthgasse 18, 1190 Wien, Austria

[3] Institute of Applied Microbiology, Department of Biotechnology, BOKU - University of Natural Resources and Applied Life Sciences, Muthgasse 18, 1190 Wien, Austria

Running title: multibudded and bi-nucleate *Z. bailii* cells

* Corresponding author

Tel.:+ 39 02 64483418

Fax :+ 39 02 64483565

E-mail: paola.branduardi@unimib.it

Abstract

The yeast *Zygosaccharomyces bailii,* known to have peculiar resistance to several environmental constraints, is very little known in respect to its genetics and life cycle. In addition to molecular and biochemical studies, cytofluorimetric and morphological analyses can also add information necessary to shed light on its interesting features. In the present study, DNA and protein content as well as cellular morphology of *Z. bailii* populations growing in minimal medium supplemented with different carbon sources and with the addition of different organic acids were investigated. The results show the occurrence of a multibudded phenotype and of a low, but significant percentage of bi-nucleated cells occurring in early stationary phase. These traits appear to be different in comparison to the better known laboratory yeast *Saccharomyces cerevisiae.* Experiments and speculations about these features and possible implications with *Z. bailii* main characteristics are discussed.

Keywords: *Zygosaccharomyces bailii,* flow cytometry, multibudded cells, bi-nucleate cells.

Introduction

The genus *Zygosaccharomyces* (*sensu stricto*) comprises up to now 6 different yeast species (Kurtzman, 2003) known to be responsible for much spoilage of beverages and preserved food. This is mainly due to their ability to grow in environments with high sugar and ethanol concentrations, low water activity, low pH and in the presence of preservatives, such as sorbic or benzoic acid (Thomas & Davenport, 1985; Fleet, 1992; Piper *et al.*, 2001), constraints usually not ideal for microbial growth. So, despite some species can be naturally present during grape-juice fermentation and some strains have characteristics useful for wine making (Romano & Suzzi, 1993), very often their presence constitutes an indicator of future spoilage problems. This is especially true for *Z. bailii*, which can tolerate particularly high sugar, ethanol and chemical preservatives concentrations as well as acidic environments (Thomas & Davenport, 1985; Cole & Keenan, 1986). For said reasons the interest devoted to spoilage prevention is accompanied to the industrial interest in the exploitation of its characteristics, with particular regard to stress resistance. In literature there are only some studies on the physiological aspects of *Z. bailii*'s acid tolerance (Sousa *et al.*, 1996; Ferreira *et al.*, 1997; Sousa *et al.*, 1998), and few works on its life cycle (very likely *Z. bailii* is a diploid yeast, sporulating through mitotic spores, Rodrigues *et al.*, 2003), as well as its behaviour in chemostat cultures (Rodrigues *et al.*, 2001; Merico *et al.*, 2003). Finally, very little is known about the structure/composition of a growing population of *Z. bailii*, information that could be very useful to elucidate its life cycle. Here we present a study on the physiognomy of a *Z. bailii* population growing on minimal medium added with carbon source (fermentable or a non fermentable) and eventually an organic acid, more precisely acetic or sorbic acid, both known to have an inhibitory effect on growth. For each condition we compared *Z. bailii* to the well known yeast *S. cerevisiae*. Flow cytometric and microscopic analyses were performed determining the DNA and protein content distributions in correlation with cellular morphology. The way in which *Z. bailii* reacts to conditions of nutrient limitation and enters into the stationary phase is observed and discussed. These aspects are of particular interest in light of possible biotechnological processes/applications, very often lasting till the stationary phase of growth necessary to maximize the recovery of the product of interest.

13

Materials and Methods

Microorganisms and growth conditions

The *S. cerevisiae* strain used in this study was the prototrophic GRFc (Brambilla *et al.*, 1999). *Z. bailii* strains were ATCC36947, ATCC60483 and ATCC8766. Yeasts were pre-cultured in YPD or YPF medium (1% w/v Yeast Extract, 2% w/v Peptone, 2% w/v of glucose or fructose, as carbon source), and then pre-inoculated and subsequently inoculated in minimal synthetic medium (0,67% w/v YNB Biolife without amino acids) supplied with 2% or 5% w/v of glucose or fructose or with 1% ethanol + 1% glycerol as carbon source. The organic acid limitation was realized adding 0.5 mM and 25 mM of sorbic and acetic acid, respectively, to the minimum glucose medium described above (initial pH: 3.0). In the experiments where EGTA was added, either from the time of the inoculum or after 24 hours, its final concentration was either 5 or 10 mM.

Glucose, ethanol and glycerol determinations

Glucose, ethanol and glycerol concentrations were determined using Megazyme Kits (# K-HKLGLU, K-ETOH, K-GCROL, respectively).

Flow Cytometric assays

For the simultaneous determination of cell protein and DNA content distributions, cells were collected and treated as previously described (Porro *et al.*, 2003). FITC and PI fluorescence signal intensities were acquired using a FACStar[plus] (Becton-Dickinson) equipped with an argon ion laser (excitation wavelength 488 nm, laser power 200 mW) or using a Cell Lab Quanta™ SC Flow Cytometer (Beckman Coulter) equipped with a diode laser (excitation wavelength 488 nm, laser power 22 mW). The sample flow rate during the analyses did not exceed 500-600 cells per second. Typically 25000 or 50000 cells were analysed for each sample. The average protein content per cell was

obtained determining the average channel number of the FITC fluorescence signal distribution (Alberghina & Porro, 1993).

Fluorescence microscopy

Samples of the same yeast cultures PI stained and cytofluorimetrically analysed (see previous paragraph) were subsequently observed using a Nikon Eclipse E600 fluorescence microscope (excitation wavelength 488 nm) using standard TRITC filter and dicroic lens sets. Rat monoclonal antibodies (Yol 1/34, against -tubulin, dilution 1:100) and secondary FITC-conjugated anti-rat antibodies (Jackson ImmunoResearch, dilution 1:100) were used for spindle staining, and nuclei were stained with DAPI as previously described (Surana *et al.*, 1993). Images were taken using Leika FW4000 software and processed in Adobe Photoshop 6.0.

Results

DNA distributions of S. cerevisiae and Z. bailii growing in minimal glucose medium: a comparison

Shake-flask cultures of *S. cerevisiae* (strain GRFc) and of different strains of *Z. bailii* (see Materials and Methods) were grown in glucose or fructose minimal medium, with different initial sugar concentrations, and independently repeated several times. The obtained results were very similar for all tested conditions and strains, therefore, we present here only the data concerning one *Z. bailii* strain (ATCC 60483), growing on 2 % w/v glucose minimal medium and compared with *S. cerevisiae* grown in the same conditions.
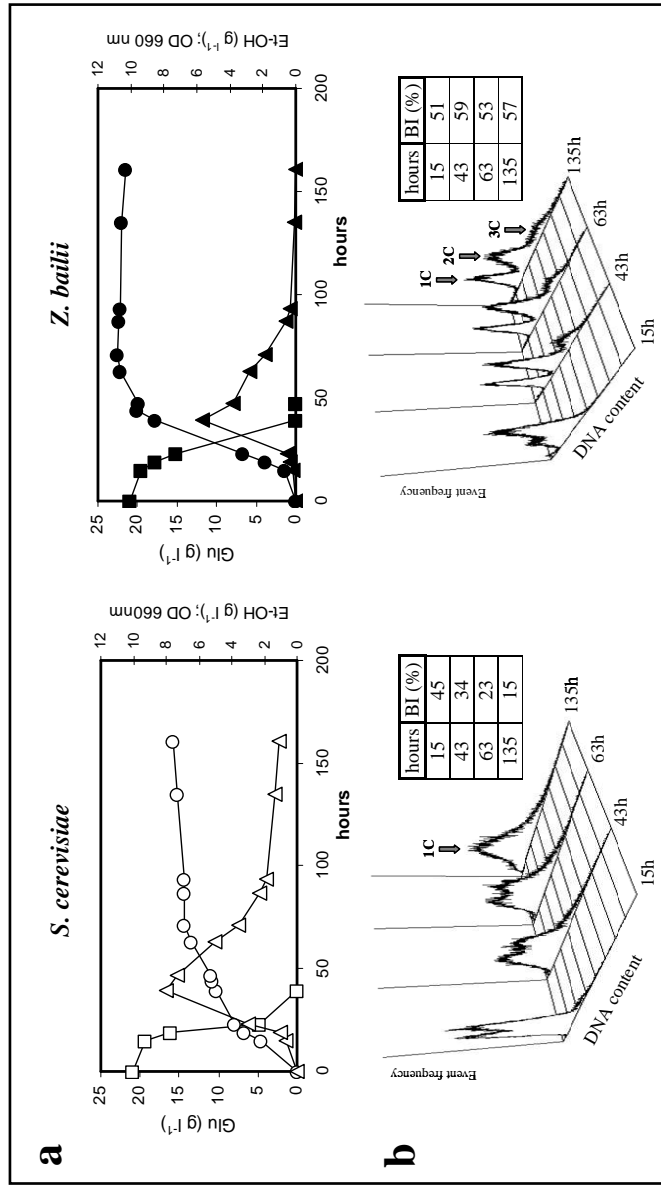
Z. bailii

hours	BI (%)
15	51
43	59
63	53
135	57

S. cerevisiae

hours	BI (%)
15	45
43	34
63	23
135	15

Figure 1. Growth kinetics and DNA content of S. cerevisiae and Z. bailii cells in glucose minimal medium
a) Growth was measured as optical density (OD 660 nm, circles) and the residual glucose (g L^{-1}, squares), together with the ethanol production (g L^{-1}, triangles), were evaluated. Comparison between *S. cerevisiae* (left, open symbols) and *Z. bailii* (right, full symbols). **b)** Flow cytometric analysis of DNA distribution of *S. cerevisiae* (on the left; amplification 8) and of *Z. bailii* (on the right; amplification 4) cells collected at indicated times and propidium iodide (PI) stained. The arrows indicate the sub-populations and their presumed genomic content in stationary phase. The budding index (BI) percentage at the time of the analyses is also given.

16

Figure 1a compares the growth properties of the two yeasts: while the glucose consumption is quite similar (square dots), the ethanol production is less pronounced in *Z. bailii* than in *S. cerevisiae* (triangles), although it follows a similar kinetic. This is in agreement with previous data (Rodrigues *et al.*, 2001; Merico *et al.*, 2003) reported for these two Crabtree-positive yeasts; the different levels of ethanol produced can at least partially justify the higher OD values reached by *Z. bailii* (circles).

The cellular DNA contents were analysed at different times by flow cytometry and the histograms compared (Figure 1b). While the *S. cerevisiae* DNA distributions reflect the already well-known trend, moving from the 2 peaks of the exponential phase (G1, 1C DNA content; G2/M, 2C DNA content) to the sole peak characteristic for the stationary phase of growth (G1, 1C DNA content), *Z. bailii* shows a more complex profile. Its DNA distribution in exponential phase is characterized by a pronounced 2C peak (also confirmed by the high budding index, see boxes in Figure 1b) and, starting from 43 hours after the inoculum, a third peak appears which corresponds to an approximately three times higher DNA content than the 1C. The area of said peak diminishes along the time, although remaining clearly visible till 135 hours after the inoculum. The simplest explanation for the higher 2C peak seen in exponential phase of *Z. bailii* cells could be a longer G2 phase (and/or shorter G1 phase) compared to *S. cerevisiae* cells. However, the persistence of the third peak is more difficult to interpret. It has to be noted that this peak is persistent even when the carbon source is exhausted and the OD is not increasing anymore. Beside this, the 3C peak, never noticed in *S. cerevisiae* cells, could reflect the presence of a subpopulation of cells with a 3C DNA content or, alternatively, it could derive from stable aggregates of cells that fail to separate despite the sonication step done prior to the flow cytometric analysis. In this respect, it has to be underlined that prolonging the sonication time up to the point it causes some cell breakage did not lead to a different distribution, thus excluding a simple problem of clogging.

Z. bailii cells growing in minimal medium: combining DNA and protein distribution with microscopic images

To better characterise the cells, samples were collected and double stained with PI and fluorescein in order to simultaneously acquire data about DNA and protein content, respectively. In addition, the cytofluorimetric data were combined with morphological observations. Figure 2 shows the dot plot of DNA and protein biparametric distributions and a fluorescent microscope image showing the representative cellular phenotype for each time point of the culture. Any "cloud" visible in the plot corresponds to a fraction of the population characterized by a certain DNA and protein content. The colour intensity is proportional to the frequency of the events that fall in a specific area.

First of all, *Z. bailii* cells show a higher medium protein content if compared to the model yeast *S. cerevisiae*. This is expected since a (probably) diploid yeast is compared with a haploid yeast. Said that, it is interesting to notice that protein content for *Z. bailii* significantly changes during growth, showing a maximum value at around 19 hours (early exponential phase), and then decreasing with time (please note the different signal amplifications used for protein data acquisition, indicated at the top of the biparametric distributions, and see the bottom-right graph for medium protein contents). This difference is usually not observed in *S. cerevisiae* laboratory strains (see Figure 2 for a comparison).

Furthermore, it can be observed that along the time there is a remarked fluctuation of events that fall in different areas of the plots, corresponding to cellular sub-populations with different protein and DNA contents. To facilitate the description we used numbers and symbols on the biparametric plots to indicate specific areas or subpopulations. From the left, in the first panel, a sub-population of cells (marked with number 1) accumulating DNA without increasing the cell mass is notable. This pattern seems to be related in some way to the exponential phase of growth. It disappears with time but re-appears if cells at the end of the exponential phase of growth are re-inoculated into fresh medium (data not shown). The morphological analyses do not help to further comment this observation; the major fraction of cells appears to be in G2/M phase, with a budded shape and their nuclei involved in mitotic events (see the image on top on the right).
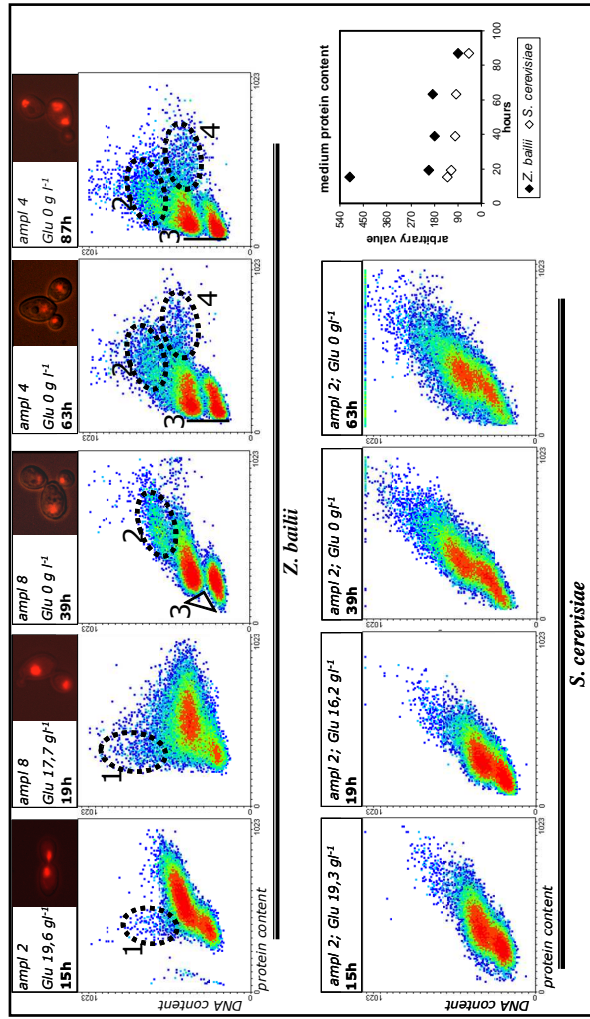
Figure 2. DNA and protein content distribution analysis of *Z. bailii* and *S. cerevisiae* cells growing on glucose

Flow cytometric analysis of DNA and protein distribution of *Z. bailii* and *S. cerevisiae* cells growing in minimal glucose medium collected at indicated times and stained with fluorescein and propidium iodide (PI). For all samples, the indication of residual glucose concentration and the amplification of protein acquisition are given. Numbers indicate specific characteristics of the structure of the DNA and protein distribution of *Z. bailii* cells: 1) a sub-population of cells accumulating DNA without increasing the cell mass; 2) a sub-population of cells bearing a 3C DNA content and a high mean protein content; 3) shift of the 2C sub-population to the left, indicating a change in its medium protein content that diminishes, becoming similar to that of the 1C sub-population; 4) a population of cells characterised by an increment of mass but not of DNA content. For *Z. bailii* cells, a fluorescent microscope image of a representative cell sample is also given. Right bottom panel: a comparison of medium protein content trend during the kinetics of growth between the two yeasts (*Z. bailii*: diamond full symbols, *S. cerevisiae*: diamond open symbols).

In the third panel from the left, number 2 marks a population of cells bearing a 3C DNA content and a higher average protein content with respect to both 1C and 2C populations: said population remains evident till the last time here analysed. The appearance of this population seems independent from the *Z. bailii* strain used, the carbon source (fermentable or not fermentable) supplied or its initial concentration (data not shown). Microscopic analysis shows that a significant fraction of the cells has two buds and most of them are in fact nucleated (as an example, see the image up on the right of Figure 2, third and fourth panel).

Another feature to be noticed is indicated with the triangle/line marked with number 3 (Figure 2, third, fourth and fifth panel from the left). The plot at 39 hours shows the expected distribution: the position of the "cloud" corresponding to the 2C cells shows a higher medium protein content in respect to the 1C cells. At 63 hours the 2C cells unexpectedly show a very similar protein content as the 1C cells, as the "cloud" of the registered events shifts to the left of the plot.

In the fourth panel, a population of cells (marked with number 4) recognizable till the latest time analysed, is characterised by an increment of mass but not of DNA content. This phenomenon is particularly evident on fructose grown cells (data not shown). At the microscope a sub-population of budded or multi-budded cells in which the mother cells show two distinct DNA masses becomes evident in the same sample (e.g. see the image up on the right of the last panel in Fig. 2, at 87 hours from the inoculum). Also these cells are present independently from the *Z. bailii* strain analysed or from the sugar supplied (data not shown), and correspond to 2.5-3.5 % of the whole population.

Summarising, a sub-population of multibudded cells appears during growth of *Z. bailii* cells on minimal medium, and a small but significant percentage of bi-nucleated cells becomes visible in the early-stationary phase. The coexistence of said populations and their progression into cycles of growth and division can be responsible for the different DNA and protein distributions observed for the spoilage compared with the baker's yeast (see again Figure 2, upper and lower panels for a comparison).

Multibudded Z. bailii phenotype can be resolved by cell wall digestion

The combined morphological and flow cytometric analyses reported above showed that the 3C population is very likely composed by apparently multi-budded cells: these cells could still have a cytoplasmic connection or, alternatively, they could be separated cells connected by some cell wall component that fails to separate after cytokinesis. To test these two hypotheses, *Z. bailii* cells were grown in minimal glucose medium until the time of appearance of the 3C population, then collected and prepared for cytofluorimetric analyses. The sample was split in two: half was PI stained as previously described; the other half was treated for cell wall digestion prior to the staining.

The histograms related to the DNA distributions obtained are shown in Figure 3. In order to avoid misinterpretations caused by possible spheroplasts clogging, BSA was added to the suspension buffer, as it is usually suggested for mammalian cell samples. The distribution obtained without BSA was nearly identical (data not shown).

The intact cell sample confirms a DNA distribution characterised by 3 distinct peaks (1C, 2C and 3C DNA content, grey line). In the DNA distribution of spheroplast cells (black line) the third peak (3C DNA content) disappears almost completely, and the 2C peak is significantly reduced, with a consequent increase of events falling in the first peak (1C DNA content). This demonstrates that the apparently multi-budded cells are separated cells with no cytoplasmic connection. Moreover, the data indicate that also some single-budded cells must already have undergone cytokinesis, but fail to separate.
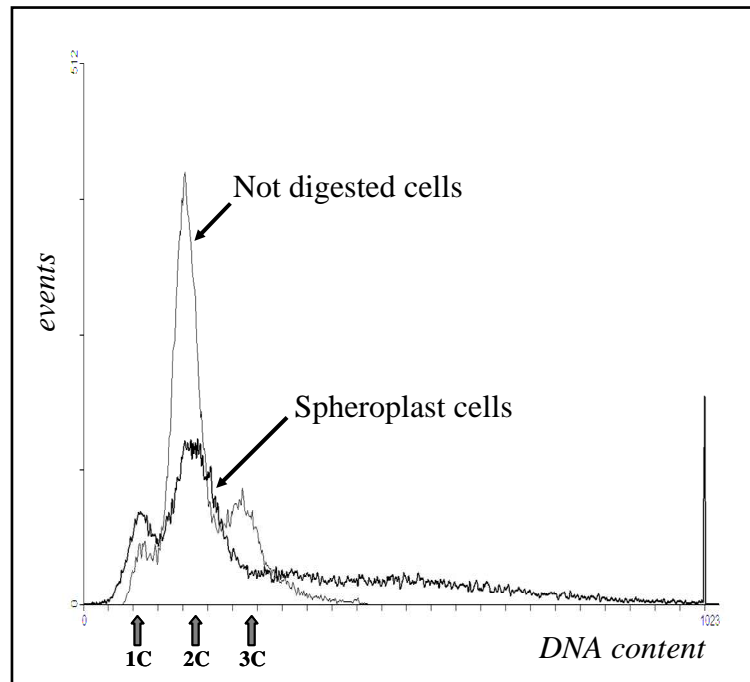
Figure 3. DNA content distribution of growing *Z. bailii* cells: a comparison between intact and spheroplast cells
Flow cytometric analysis of DNA distribution of *Z. bailii* cells growing in minimal glucose medium collected at 47 hours after the inoculum and propidium iodide (PI) stained. The grey line indicates the DNA distribution resulting from the analysis of not digested cells, while the black line indicates the DNA distribution of the same cells after enzymatic digestion (*i.e.*, without the cell wall). The arrows indicate the sub-populations and their presumed genomic content.

Spindle-nuclei staining of S. cerevisiae and Z. bailii cells during growth in different conditions

While the previous experiment clarified the source of the multi-budded phenotype, the question remains open for the presence of bi-nucleated cells in *Z. bailii* samples (Figure 2, image at 87 h). To better understand the presence of said cells, cellular samples collected during cultures were stained for spindles and nuclei.

A significant number of bi-nucleated cells shows a mitotic spindle extended between the two nuclear masses (see, as example, Figure 4a and 4b), strongly suggesting an active DNA division. In other cases, the two nuclear masses are already completely separated, each one associated to a characteristic G1 spindle (Figure 4c). Once more, these observations are independent from the *Z. bailii* strain considered, from the supplied carbon sources (fermentable or non fermentable) or their initial concentration (from 1% for ethanol and glycerol to 2 and up to 5% for glucose and fructose, data not shown).
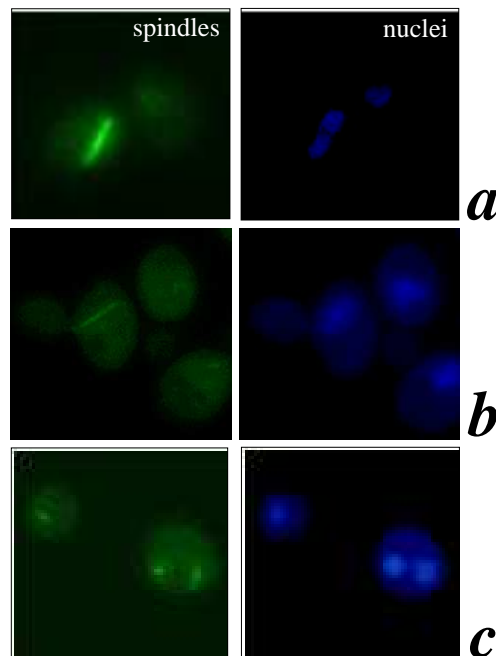

Figure 4. Spindles-nuclei fluorescent staining of (late) stationary phase *Z. bailii* cells
Z. bailii cells grown till stationary phase in minimal medium (supplied either with fermentable or non-fermentable carbon sources) were stained with a FITC conjugated secondary antibody directed against an -tubulin primary antibody and with DAPI, for spindle and nucleus visualization, respectively. **a**) upper panels: a bi-nucleated cell in the moment of nuclear division with a metaphase/ to anaphase mitotic spindle; **b**) middle panels: the budded cell at the top of the panel shows a metaphase spindle miss-oriented in respect to the bud; **c**) bottom panels: a bi-nucleated cell with two G1-aster spindles.

It can be hypothesised that the persistence of two nuclei in one cell leads a fraction of the population (here around 3%) to become polyploid. Polyploidy is generally believed to confer advantages in unfavourable growth conditions (for a review, see Comai, 2005) and can also lead to alterations in specific gene regulation (Galitsky *et al.*, 1999). For example, it was reported that in *Z. bailii* populations growing under sorbic acid stress, a fraction of stationary-phase cells, estimated around 3%, becomes super-resistant to the stress imposed. This super-resistance was not genetically inheritable, since any re-inoculum of a single resistant colony in rich medium grew showing again the same stress susceptibility as the original population (Steels *et al.*, 2000). We speculated a possible correlation between the appearance of bi-nucleate cells and the insurgence of super-resistance to sorbic (and possibly other kinds of) stress.

To address this question, *Z. bailii* and *S. cerevisiae* cells were grown in minimal glucose medium added or not with sorbic acid or acetic acid, another weak organic acid to which *Z. bailii* is typically more resistant than the model yeast. The DNA distributions and the percentages of bi-nucleated cells were monitored at different times, together with growth and budding indexes, and are reported in Figure 5. As expected and according to literature (Thomas & Davenport, 1985; Sousa *et al.*, 1998; Piper *et al.*, 2001), *Z. bailii* shows better growth levels in presence of both weak organic acids, compared to *S. cerevisiae* (Figure 5a).

Interestingly, the flow cytometric and the microscopic analysis revealed, respectively, that the appearance of multibudded and bi-nucleate cells does not occur when sorbic acid is added to the medium, but only in the presence of acetic acid (see Figure 5, b to d, histograms and side-tables).
As a consequence, it doesn't seem possible to trace an easy and direct correlation of the previously observed super-resistant phenotype with the insurgency of a polyploid genotype. It appears that also the multibudded phenotype is a transient event, since prolonging the incubation time (up to 210 hours after the inoculum in minimal medium) the DNA distribution of the whole population shifts to the left in a single 1C peak (Figure 5, b to d).
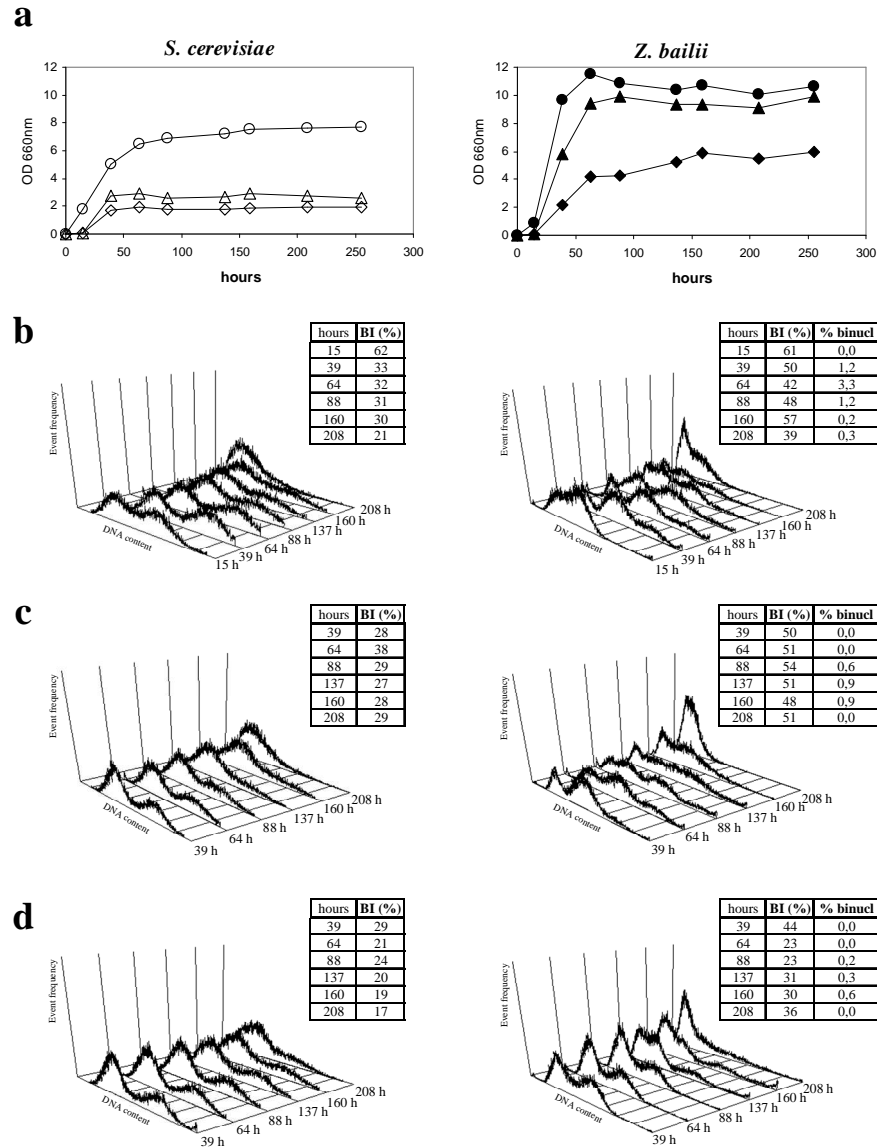
a

b

hours	BI (%)
15	62
39	33
64	32
88	31
160	30
208	21

hours	BI (%)	% binucl
15	61	0,0
39	50	1,2
64	42	3,3
88	48	1,2
160	57	0,2
208	39	0,3

c

hours	BI (%)
39	28
64	38
88	29
137	27
160	28
208	29

hours	BI (%)	% binucl
39	50	0,0
64	51	0,0
88	54	0,6
137	51	0,9
160	48	0,9
208	51	0,0

d

hours	BI (%)
39	29
64	21
88	24
137	20
160	19
208	17

hours	BI (%)	% binucl
39	44	0,0
64	23	0,0
88	23	0,2
137	31	0,3
160	30	0,6
208	36	0,0

Figure 5. Growth and DNA distributions of *S. cerevisiae* and *Z. bailii* cells in the presence of acid stress a) Cellular growth, measured as optical density at 660 nm, in minimal glucose medium with no acid added (circles), with 25 mM acetic acid added at pH 3 (triangles) and with 0.5 mM sorbic acid added at pH 3 (diamonds). Comparison between *S. cerevisiae* (left, open symbols) and *Z. bailii* (right, full symbols). **b-d**) Flow cytometric analyses of DNA distributions of *S. cerevisiae* (left panels) and of *Z. bailii* (right panels) cells grown in control medium (**b**), acetic acid medium (**c**) or sorbic acid medium (**d**), collected at the indicated times and stained with propidium iodide (PI). Adjacent boxes indicate the budding index (BI) percentage at the time of the analysis. For *Z. bailii* samples, the percentage of bi-nucleate cells is also given.

25

The microscopic analysis revealed that also the small population of bi-nucleate cells diminishes along the time (Figure 5, b to d side-tables). The possibility that bi-nucleate cells could be a prelude to sporulation was excluded by microscopic analysis since *Z. bailii* asci, whose shape is peculiar and easily recognizable, were never observed in the samples (data not shown).

Finally, it should be underlined that in rich medium (YEP + 2% glucose), either with or without organic acids added, neither the multibudded phenotype nor the presence of bi-nucleate cells were observed (data not shown).

EGTA addition to the culture medium inhibits the multibudded phenotype

In order to further investigate the basis of the cell wall linkage in (pseudo)multibudded cells, experiments were performed supplementing the minimal growth medium with 5 or 10 mM EGTA (either from the beginning of growth, or before the manifestation of the multi-budded phenotype - data not shown). EGTA complexes bivalent ions, with a special preference for Ca^{2+}. Figure 6 clearly shows that the multi-budded sub-population, evident in the plot starting at 40 hours from the inoculum, is dramatically reduced if EGTA is added to the medium, and that the reduction is somehow proportional to the EGTA concentration (Figure 6, comparison between upper and lower panels).
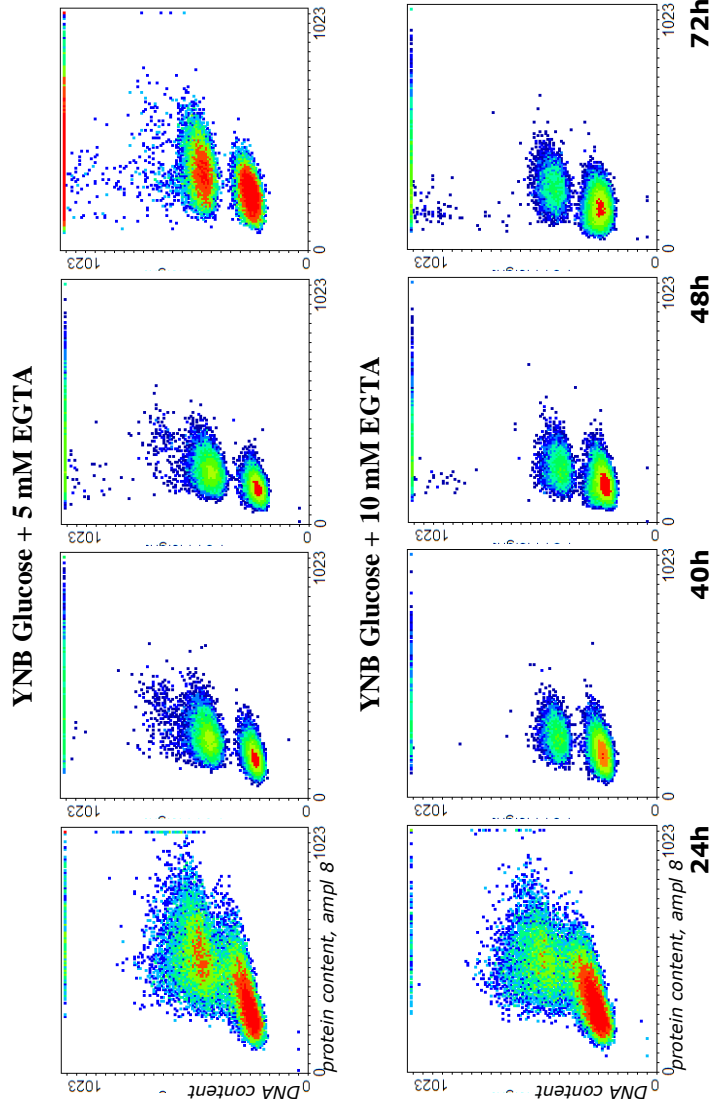
Figure 6. DNA and protein content (biparametric distribution) of *Z. bailii* cell population growing in minimal glucose medium supplemented with EGTA: a comparison Flow cytometric analysis of DNA and protein distribution of *Z. bailii* cells growing in minimal medium supplemented with 5 or 10 mM EGTA (final concentration). Samples were collected at the indicated times and stained with fluorescein and propidium iodide (PI). The protein content is on the x axes (amplification 8 for all the samples), while the DNA content is on the y axes of the dot plots.

27

Discussion

In the latest years we focused our work on the fructophilic species *Z. bailii*, developing molecular tools and protocols for heterologous protein productions (both at the cytoplasmic level or released in the growth medium) as well as for metabolic engineering applications (Branduardi 2002; Branduardi *et al.*, 2004; Sauer *et al.*, 2004; Vigentini *et al.*, 2005; Camattari *et al.*, 2007). It became natural/obvious, together with the development of new molecular tools the necessity to deepen the knowledge of the organism itself.

The study here presented is the first intended to describe the structure of a growing population of *Z. bailii* cells, particularly focusing on protein and DNA distributions. Since many aspects about *Z. bailii* capability to counteract many environmental constraints are still to be clarified, the observations here reported do add some interesting insights in this respect.

The first evidence collected is that *Z. bailii* transiently accumulates a sub-population of cells bearing a 3C DNA content (up to 20% of the population) during the early stationary phase of growth. These cells appear to have two buds connected to the mother cell. The subsequent analyses revealed that most of the multi-budded cells do not have any cytoplasmic connection, since a cell wall digestion was sufficient to separate them. This observation is a strong indication that these *Z. bailii* cells, despite forming aggregates, do not seem to have the possibility to exchange DNA material. Another relevant observation is that a little fraction of the 3C population (about 3 % of the whole population) is made up of bi-nucleate cells. The "apparatus" responsible for nuclear division is involved in the formation of these two nuclei, indicating that it is not a pathological DNA fragmentation phenomenon that originates them; moreover, the bi-nucleate cells disappear in late-stationary phase, suggesting that it could be a regulated phenomenon. We can argue that these are viable cells, and that they can count on a doubled DNA content. We thus tried to investigate if the appearance of this subpopulation could be linked to the stress resistance properties of *Z. bailii*. However, growth experiments in the presence of sorbic or acetic acid stress did not lead to higher accumulation, but rather to diminished percentages of these

cells in the whole population. Moreover, cell aggregation is inhibited, being absent or less relevant depending on the acid type and its concentration.

It is obviously interesting to understand why *Z. bailii* cells remain linked by cell wall interactions. It can be speculated that the phenomenon is related to the wildness of this yeast, especially if compared with *S. cerevisiae* laboratory strains. It was described in literature that even *S. cerevisiae* wild strains, isolated from their habitat and not laboratory selected, show the capability to form colonies in which, after several days, cells are connected by a sort of extracellular matrix (Kutan *et al.*, 2003). This supports the hypothesis that such types of aggregations are an advantage for the whole population when the environmental conditions become unfavourable (especially in respect to nutrient and water limitations). Still no detailed information about the biochemical nature of the molecules involved in these interactions is available. It is possible, nonetheless, to speculate that some glycoproteins of the cellular surface could be involved in these interactions, eventually including other (secreted) proteins. Very often the interactions occurring among these classes of proteins are dependent on bivalent ions, especially from Ca^{2+} (extensively described and reviewed for pluricellular organisms, see Timpl & Brown, 1994; Clark & Brugge, 1995; Wheelock & Johnson, 2003). The data obtained by adding EGTA to the medium support this hypothesis, but the limitation of genetic information about the yeast *Z. bailii* does not allow, at present, to further speculate in this direction.

In the end, as previously stated, the results of our study and a further progress in this direction could be very useful for a more effective biotechnological exploitation of this yeast.

References

Alberghina L & Porro D (1993) Quantitative flow cytometry: analysis of protein distributions in budding yeast. A mini-review. *Yeast* **9**(8): 815-823.

Brambilla L, Bolzani D, Compagno C, Carrera V, van Dijken JP, Pronk JT, Ranzi BM, Alberghina L & Porro D (1999) NADH reoxidation does not control glycolytic fux during exposure of respiring *Saccharomyces cerevisiae* cultures to glucose excess. *FEMS Microbiol Lett* **171**: 133-140.

Branduardi P (2002) Molecular cloning and sequence analysis of the *Zygosaccharomyces bailii HIS3* gene encoding the imidazole glycerolphosphate dehydratase. *Yeast* **19**(13): 1165-1170.

Branduardi P, Valli M, Brambilla L, Sauer M, Alberghina L & Porro D (2004) The yeast *Zygosaccharomyces bailii*: a new host for heterologous protein production, secretion and for metabolic engineering applications. *FEMS Yeast Res* **4**(4-5): 493-504.

Camattari A, Bianchi MM, Branduardi P, Porro D & Brambilla L (2007) Induction by hypoxia of heterologous-protein production with the *Kl*PDC1 promoter in yeasts. *Appl Environ Microbiol* **73**(3): 922-929.

Clark EA & Brugge JS (1995) Integrins and signal transduction pathways: the road taken. *Science* **268**(5208): 233-239.

Cole MB & Keenan MH (1986) Synergistic effects of weak-acid preservatives and pH on the growth of *Zygosaccharomyces bailii*. *Yeast* **2**: 93-100.

Comai L (2005) The advantages and disadvantages of being polyploid. *Nat Rev Genet* **6**(11): 836-846.

Esteve-Zarzoso B, Zorman T, Belloch C & Querol A (2003) Molecular characterization of the species of the genus *Zygosaccharomyces*. *System Appl Microbiol* **26**: 404-411.

Ferreira MM, Loureiro-Dias MC & Loureiro V (1997). Weak acid inhibition of fermentation by *Zygosaccharomyces bailii* and *Saccharomyces cerevisiae*. *Int J Food Microbiol* **36**(2-3): 145-153.

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

Galitski T, Saldanha AJ, Styles CA, Lander ES, Fink GR (1999) Ploidy regulation of gene expression. *Science* **285**(5425): 251-254.

Kurtzman CP (2003) Phylogenetic circumscription of *Saccharomyces*, *Kluyveromyces* and other members of the *Saccharomycetaceae*, and the proposal of the new genera *Lachancea*, *Nakaseomyces*, *Naumovia*, *Vanderwaltozyma* and *Zygotorulaspora*. *FEMS Yeast Res* **4**: 233-245.

Kuthan M, Devaux F, Janderová B, Slaninová I, Jacq C & Palková Z (2003) Domestication of wild *Saccharomyces cerevisiae* is accompanied by changes in gene expression and colony morphology. *Mol Microbiol* **47**(3): 745-54.

Martorell P, Fernández-Espinar MT & Querol A (2005) Molecular monitoring of spoilage yeasts during the production of candied fruit nougats to determine food contamination sources. *Int J Food Microbiol* **101**(3): 293-302.

Martorell P, Stratford M, Steels H, Fernández-Espinar MT & Querol A (2007) Physiological characterization of spoilage strains of *Zygosaccharomyces bailii* and *Zygosaccharomyces rouxii* isolated from high sugar environments. *Int J Food Microbiol* **114**(2): 234-242.

Merico A, Capitanio D, Vigentini I, Ranzi BM & Compagno C (2003) Aerobic sugar metabolism in the spoilage yeast *Zygosaccharomyces bailii*. *FEMS Yeast Res* **4**(3): 277-283.

Piper P, Calderon CO, Hatzixanthis K & Mollapour M (2001) Weak acid adaptation: the stress response that confers yeasts with resistance to organic acid food preservatives. *Microbiology* **147**: 2635-2642.

Porro D, Brambilla L & Alberghina L (2003) Glucose metabolism and cell size in continuous cultures of *Saccharomyces cerevisiae*. *FEMS Microbiol Lett* **229**(2): 165-171.

Rodrigues F, Côrte-Real M, Leão C, van Dijken JP & Pronk JT (2001) Oxygen requirements of the food spoilage yeast *Zygosaccharomyces bailii* in synthetic and complex media. *Appl Environ Microbiol* **67**(5): 2123-2128.

Rodrigues F, Ludovico P, Sousa MJ, Steensma HY, Côrte-Real M & Leão C (2003) The spoilage yeast *Zygosaccharomyces bailii* forms mitotic spores: a screening method for haploidization. *Appl Environ Microbiol* **69**(1): 649-653.

Romano P & Suzzi G (1993) Higher alcohol and acetoin production by *Zygosaccharomyces* wine yeast. *J Appl Bacteriol* **75**: 541- 545.

Sauer M, Branduardi P, Valli M & Porro D (2004) Production of L-ascorbic acid by metabolically engineered *Saccharomyces cerevisiae* and *Zygosaccharomyces bailii*. *Appl Environ Microbiol* **70**(10): 6086-6091.

Sousa MJ, Miranda L, Côrte-Real M & Leão C (1996) Transport of acetic acid in *Zygosaccharomyces bailii*: effects of ethanol and their implications on the resistance of the yeast to acidic environments. *Appl Environ Microbiol* **62**(9):3152-3157.

Sousa MJ, Rodrigues F, Côrte-Real M & Leão C (1998) Mechanisms underlying the transport and intracellular metabolism of acetic acid in the presence of glucose in the yeast *Zygosaccharomyces bailii*. *Microbiology* **144**: 665-670.

Steels H, James SA, Roberts IN & Stratford M (2000) Sorbic acid resistance: the inoculum effect. *Yeast* **16**(13): 1173-1183.

Surana U, Amon A, Dowzer C, McGrew J, Byers B & Nasmyth K (1993) Destruction of the *CDC28*/*CLB* mitotic kinase is not required for the metaphase to anaphase transition in budding yeast. *EMBO J* **12**(5): 1969-1978.

Thomas DS & Davenport RR (1985) *Zygosaccharomyces bailii* – a profile of characteristics and spoilage activities. *Food Microbiol* **2**: 157-169.

Timpl R & Brown JC (1994) The laminins. *Matrix Biol* **14**(4): 275-81.

Vigentini I, Brambilla L, Branduardi P, Merico A, Porro D & Compagno C (2005) Heterologous protein production in *Zygosaccharomyces bailii*: physiological effects and fermentative strategies. *FEMS Yeast Res* **5**(6-7): 647-652.

Wheelock MJ & Johnson KR (2003) Cadherins as modulators of cellular phenotype. *Annu Rev Cell Dev Biol* **19**: 207-235.

Implementing the yeast *Zygosaccharomyces bailii* as a host for biotechnological productions: from the first auxotrophic mutant to the vector for multiple integration

Paper submitted to Applied and Environmental Microbiology

Running Title
Improved molecular tools for *Zygosaccharomyces bailii*

Dato Laura[§], Passolunghi Simone [§], Riboldi Luca, Cattaneo Davide, Valli Minoska, Porro Danilo* and Branduardi Paola

[§] these authors contributed equally to the work

*corresponding author

Abstract

The nonconventional yeast *Zygosaccharomyces bailii* was recently proposed as a possible new host for biotechnological processes, due to convenient properties such as resistance to high sugar concentrations, acidic environments and relatively high temperatures. Production and secretion of heterologous proteins from different sources and with different characteristics was previously achieved in this yeast, as well as a first example of metabolic engineering. Protein production levels were shown to be higher than in *Saccharomyces cerevisiae*, probably due to the higher biomass yield of *Z. bailii*. Despite the satisfactory results, the development of molecular tools needed for the construction of suitable productive strains is still at the very beginning. Here we describe a series of new expression vectors specific for *Z. bailii* and the resulting improvements in the expression of reporter genes. The rational of plasmids design was to increase both copy number per cell and stability of the heterologous constructs. Thus, 2μm-like multicopy vectors were obtained by exploiting the sequences of the endogenous plasmid pSB2; a specific integrative vector was obtained which led to maximal stability; a vector for multiple integration was constructed, based on a portion of the rDNA unit that was cloned and sequenced for this purpose. Moreover, here we show the construction of the first stable auxotrophic mutant of *Z. bailii*, obtained by targeted gene deletion applied to *ZbLEU*2. The applicability of the new strain for heterologous productions was verified and compared to the wild type strain. Overall, the results obtained constitute an important step in view of a possible future exploitation of *Z. bailii* for industrial purposes.

Keywords

Introduction

The nonconventional yeast *Zygosaccharomyces bailii* was recently proposed as a possible new host for biotechnological processes (Branduardi 2002; Branduardi *et al.*, 2004; Sauer *et al.*, 2004; Vigentini *et al.*, 2005; Camattari *et al.*, 2007). Its ability to tolerate high sugar concentrations, acidic environments and relatively high temperatures (Thomas & Davenport, 1985; Cole & Keenan, 1986; Makdesi & Beuchat, 1996; Sousa Dias *et al.*, 1996; Sousa *et al.*, 1996; Sousa *et al.*, 1998) could, in fact, allow fermentation processes to be performed under (otherwise) restrictive conditions, simplifying the process itself and thus improving its economical value. Moreover, the high specific growth rate of *Z. bailii* and its high biomass yield make this yeast particularly attractive for heterologous proteins production, compared to the most industrially used *Saccharomyces cerevisiae*.

For the introduction of heterologous DNA sequences in *Z. bailii* cells, a set of episomal expression plasmids was previously developed, based on *Sc*ARS and *Sc*CEN sequences, allowing stable transformation of the yeast (Branduardi *et al.*, 2004). In this way, reporter proteins were successfully produced by means of different promoters (*Zb*TPI, *Sc*TPI, *Sc*ADH, *Sc*GAL and *Kl*PDC), among which the endogenous *Zb*TPI was proved to be the most effective (Branduardi *et al.*, 2004; Vigentini *et al.*, 2005; Camattari *et al.*, 2007; Branduardi P, unpublished results). Also the secretion of heterologous proteins was achieved, by in frame fusion of the respective genes with different heterologous (*Kluyveromyces lactis* killer toxin, *Sc* MFα-1, *Aa* glucoamylase, *Sd* STA2) and homologous (*Z. bailii* zygocin killer toxin) leader sequences, with the *K. lactis* and *Z. bailii* killer toxin sequences giving the best results (Branduardi *et al.*, 2004). In the same study, it was demonstrated that production levels of the recombinant human interleuchin-1β (rhIL-1β) in *Z. bailii* are higher than levels obtained with *S. cerevisiae* by the use of the same centromeric expression vector. Analysis of the relative production kinetic parameters led to the hypothesis that this effect could be due to the higher biomass yield of *Z. bailii*. In addition, secretion levels in *Z. bailii* were shown to be higher than in the model yeast, contributing to the observed higher heterologous protein production, even in comparison to the most used yeast-host *S. cerevisiae*.

Despite the satisfactory results achieved to date, still some thorny problems need to be overcome in view of a possible exploitation of *Z. bailii* for industrial purposes. First of all, the lack of stable auxotrophic

mutants, that would be needed for the construction of suitable productive strains. Then, correlated to that, the lack of a reproducible protocol for targeted gene deletion in this diploid yeast, that makes its genetic manipulation still difficult. Moreover, it would be desirable to further enhance the production and secretion capabilities of *Z. bailii*, for it to become competitive also towards other nonconventional yeasts.

The aim of this work was to optimize and further develop the molecular tools for the genetic manipulation of *Z. bailii* and for the improvement of heterologous gene expression in this yeast.

In particular, we focused our efforts on the construction of a series of new expression vectors specific for *Z. bailii* and aimed to increase both the copy number per cell and the stability of the heterologous construct. This was made by taking advantage of some already available *Z. bailii* sequences, both genomic and of one of the two endogenous plasmids, pSB2, and also by the cloning and sequencing of new genomic regions previously unknown. Moreover, we addressed the problem of the lack of *Z. bailii* auxotrophic mutants. At present, in fact, the only example of a *Z. bailii* auxotrophic strain was a "nearly-haploid" strain, auxotrophic for histidine, obtained trough chromosome loss induced by benomyl treatment (Rodrigues *et al.* 2003), but no indication about its stability and possible further manipulations were given. In the present study, a reliable strategy to obtain targeted gene deletion in this yeast was established and here applied to the disruption of *ZbLEU*2, encoding for the enzyme β-isopropylmalate dehydrogenase which is essential for the synthesis of leucine.

Materials and methods

Yeast strains, media and cultivation

The *Z. bailii* strains used were ATCC36947 (here named, for convenience, Zygo1), and ATCC60483 (here named Zygo2).

Yeast cultures were grown either in rich or in minimal medium. Rich medium was YP (1% w/v yeast extract, 2% w/v peptone) with 2% or 5% w/v glucose (D) or fructose (F) as a carbon source. Synthetic minimal medium was YNB (0.67% w/v Yeast Nitrogen Base without amino acids, Biolife, Milan, Italy) with 2% or 5% (w/v) glucose. Where required, 100 mg L^{-1} of leucine were added to the medium. For antibiotic resistance selection, G418 was added to the media at a concentration of 200 mg L^{-1}. Media for plates were solidified by the addition of 2% (w/v) agar (Biolife, Milan, Italy).

Cultivation in liquid media was performed in shake flasks using 1/5 of total flasks volume. Cultures were incubated at 30°C under agitation at 160 r.p.m. Plates were incubated at 30°C for 2 or 3 days.

PCR amplifications

Preparative PCR amplifications were made from *Z. bailii* or *S. cerevisiae* genomic DNA, extracted as described in Hoffman & Winston (1987). Reaction was performed with the Pwo DNA Polymerase (Roche) following the manufacturer's instructions. All primer sequences are listed in Table 1.

Primers used for amplifications of pSB2 fragments were as follows: for the region corresponding to IR-A and ARS, *IRA ARS_fwd* and *IRA ARS_rev*; for ORF A, *FLP_fwd* and *FLP_rev*; for the region comprising ORF C and ORF A, *FLP_fwd* and *TFC_rev*; for the IR-B region, *IRB_fwd* and *IRB_rev*.

To amplify the *Z. rouxii* STB sequence, primers *STBpSR1_fwd* and *STBpSR1_rev* were used on DNA extracted from strain LST 11.

The *ZbLEU2* gene with its promoter (165 bp upstream of the ATG) was initially amplified from Zygo1 genomic DNA using primers *ZLeu_fwd* and *ZLeu_rev* designed on the basis of the deposited sequence from strain ISA 1307 (Rodrigues *et al*., 2001a). The fragment obtained, 1261 bp long, was named LeuZ1. A second amplification comprising all the known *ZbLEU2* upstream region (1600 bp before the ATG) was made using primers *ZLeu_fwd2* and *ZLeu_rev*, obtaining a 2788 bp fragment named Leu2c (complete).

The *ScLEU*2 ORF plus 30 bp of the promoter were amplified from *S. cerevisiae* genomic DNA using primers *Scleu2d_fwd* and *Scleu2d_rev*, obtaining the *ScLEU*2d fragment.

All the DNA fragments obtained by PCR amplification were subcloned in pST-Blue1 (Novagen, Madison, WI, USA; Perfect Blunt cloning Kit, cat. No. 70191-4) following the manufacturer's instructions and sequenced to verify the correctness of amplification.

Primers used to amplify the *Z. bailii* rDNA region of interest from strain Zygo2 were *Zbr26S3'_ fwd* and *Zbr18S5'_rev*. A product of 6190 bp was obtained, which was subsequently fragmented by resctriction enzyme digestion and then sequenced upon subcloning of the fragments in pST-Blue1.

Control PCR for screening of *Z. bailii* transformants for targeted gene deletion were made using digested colonies as templates. Reactions were performed using Dynazyme II DNA Polymerase (Finnzymes) following the manufacturers' instructions. Primers used for external controls were *ZLeuEXT_fwd*, upstream to the *LEU*2 promoter, and *Zleu_rev*, while those for internal controls were *ZLeuEXT_fwd* and *hph_rev* or *kan_rev*, depending on the cassette inserted.

Control PCR on *wt* (*LEU2/LEU2*), *leu2::hph/LEU2* and *leu2::hph/leu2::kan* strains shown in Fig. 3B was performed with Pwo DNA Polymerase on extracted genomic DNA using primer *ZLeuEXT_fwd* and *Zleu_rev*.

Table 1. PCR primers

Name	Sequence 5'→3'
IRA ARS_fwd	AGA ATC AAT CAT TTA GTG TGG CAG GAG
IRA ARS_rev	TAA AAA CTG CCC GCC ATA TTT CGT C
FLP_fwd	TAG CTA CTC TTC TCC AGG TGT CAT TAG
FLP_rev	CCT ATG TCC GAG TTT AGC GAG CTT G
TFC_rev	ATT CTA TTG GGT ATG TCC CCT G
IRB_fwd	CTA ACC TCT TCC CTG GAG AAC AT
IRB_rev	TCT AAT GAC ACC TGG AGA AGA GTA
STBpSR1_fwd	ACA GGG AGC TCA TTT TCG TAC GTA G
STBpSR1_rev	TGG CTC CGC GGC TAA TTT GCA CAT ATC
ZLeu_fwd	AGA AGT TCG TTC CAC TTT CGC TTT G
ZLeu_fwd2	ATC AAA TGG CGG AAT ATA ATG ACG TC
LeuEXT_fwd	AGT AGC TCG TGC AGG CAG ATA T
ZLeu_rev	TGG TTC AAG CCA AAA TTT GCT TTA CA
LeuEXT_rev	TTG ACA AAG CGA AAG TGG AAC GAA
Leuend_fwd	ATT CTT TCT GCA GCG ATG ATG TTG AA
Leu-StuSpe_fwd	TGA GCA AGG TTT GCT AAA GAT TCG T
Leu-StuSpe_rev	AGT TCT CTA ACA ACG ATA AAA TCT GTA C
Scleu2d_fwd	TAT ATA TAT ATT TCA AGG ATA TAC CAT TCT AAT G
Scleu2d_rev	TCA TAG TTT CAT GAT TTT CTG TTA CAC CTA
Zbr26S3'_fwd	CAT TTG TAG ACG ACT AGA TG TAC ACC G
Zbr18S5'_rev	TAT GAC TAC TGG CAG GAT CAA CCA G

Real Time PCR

qPCR was performed on an Applied Biosystems 7500 realtime PCR System. SYBR Green master mix, as well as all the equipment and software, were from Applied Biosystems, CA, and were used according to the manufacturer's instructions. Relative quantification for copy number integration was performed using the comparative CT method.

Optimised reactions were performed in an ABI PRISM 96 well optical plate and covered with ABI PRISM optical adhesive covers. Each 20 µl reaction contained 1× SYBR Green master mix, 3.3 nM of each primer and 30 ng of purified genomic DNA. The reactions were run, in triplicate, for 2 min at 50°C, 10 min at 95 °C followed by 40 cycles of 95 °C for 15 s, 58 °C for 20 s and 60 °C for 60 s. The baseline and cycle threshold (CT) were calculated automatically using Sequence Detection Software version 1.4.

Melt curve analysis was performed using the equipment described for the QPCR. After the completion of a QPCR reaction, the samples were heated to 95 °C for 15 s and then cooled to 60 °C for 20 sec before ramping back to 95 °C. The melting temperature of the sample (Tm) was determined automatically using the Sequence Detection Software version 1.4.

The change in fluorescence of SYBR Green I dye in every cycle was monitored by the system software, and the threshold cycle (CT) above background for each reaction was calculated. The CT value of control DNA was subtracted from that of the gene of interest to obtain a ΔCT value. The ΔCT value of the calibrator was subtracted from the ΔCT value of each sample to obtain a ΔΔCT value. The gene expression level relative to the calibrator was expressed as $2^{-\Delta\Delta CT}$. Three different sets of primers were used, designed using the PrimerExpress software. The specificity of each primer pair for the target DNA was confirmed by the visualization of a single PCR product in agarose gel electrophoresis. The pairs of primers used to monitor the number of events of integration were, respectively *LeuEXT_fwd* / *LeuEXT_rev* in the promoter region of *LEU*2, *Leu-StuSpe_fwd* / *Leu-StuSpe_rev* between *StuI* and *SpeI* restriction sites in *LEU*2, and *Leuend_fwd* / *Zleu_rev* in the 3' region of *LEU*2 gene (see Fig. 6A). The amplicon generated by the amplification of the primers in the region between *StuI* and *SpeI* restriction sites in *LEU*2 gene in *leu2Δ* background was chosen as calibrator (single copy in *leu2Δ*, 2 copies in *LEU*2, multiple copies in transformants). The amplicon generated by the amplification

of the promoter region (primers *EXT*) was chosen as a control because its level is not affected by multiple integration (*i.e.* 2 copies in *wt*, *leu2Δ* and in multicopy transformants background). The region at 3' of *LEU*2 gene was chosen as supplementary control because its level is fixed in *wt* and *leu2* background (2 copies) and become higher in multicopy transformants (see Fig. 6A).

Construction of plasmids and deletion cassettes

The expression vector $p195Z_3$ was constructed starting from YEplac195 (Gietz & Sugino, 1998). The *URA3* marker was excised and replaced with the kan^R cassette taken from pFA6-KanMX4 (Wach *et al.*, 1994) cut with *EcoRI*/*SmaI*/blunt. The multicloning site (MCS) was substituted with the expression cassette (*Sc*TPI promoter-MCS-polyA) from pYX012 (R&D Systems). Plasmid $p195Z_3$LacZ was obtained by cloning the *LACZ* gene fragment cut *SphI*/*NheI* into the vector opened *SphI*/*NheI* (Branduardi *et al.*, 2004).

To construct the *Z. bailii* multicopy plasmid series, the pSB2 fragments subcloned in pST-Blue1 (see PCR amplifications) were excised and used as follows: the region containing IR-A and ARS was cut with *EcoRI*, blunt-ended and subcloned into $p195Z_3$LacZ opened wit *NarI*/*StuI*/blunt, obtaining p195ILacZ; ORF A (coding for FLP) was excised with *SnaBI*/*SalI*/blunt and inserted in p195ILacZ opened with *SmaI*, obtaining p195IFLacZ; the region containing ORF C (coding for TF-C) and ORF A was excised with *SnaBI*/*SalI*/blunt and inserted in p195ILacZ opened with *SmaI*, obtaining p195ITFLacZ; IR-B was excised with *EcoRI*, blunt-ended and inserted in p195ITFLacZ opened with *SalI*-blunt either in the same orientation as in the pSB2, obtaining plasmid p195ITFILacZ, or in the opposite orientation, obtaining p195ITFiLacZ.

The centromeric plasmid pZ_3bTLDH was obtained by inserting in pZ_3bT (Branduardi *et al.*, 2004), opened *XbaI*-blunt, the bacterial *LDH-A* gene with blunt ends (Branduardi *et al.*, 2006).

The backbone of the expression vectors for Z2ΔL was the commercially available *S. cerevisiae* expression plasmid YCplac111 (LGC Prochem). YCplac111*b*TLDH was constructed by inserting in YCplac111 opened *SmaI* the LDH gene cassette with the *Zb*TPI promoter, excised *BamHI*-blunt/*NaeI* from pZ_3bT. Plasmid YCplac111*Kl*IL-1β was obtained by cloning in YCplac111 cut *EcoRI*-blunt the recombinant hIL-1β expression cassette under the control of

the *Sc*TPI promoter, excised *NaeI*/*XmnI* from pZ₃*Kl*IL-1β (Branduardi *et al.*, 2004).

The *ZbLEU2* ORF plus promoter region (165 bp upstream) obtained by PCR amplification was inserted into pST-Blue1, obtaining pST-LeuZ1. Insertion of the longer ZLeu2c PCR fragment, also comprising 1600 bp upstream of the ATG, in pST-Blue1 resulted instead in pST-ZLeu2c. *LEU2* deletion cassettes were constructed as follows. For the deletion of the first copy, pST-LeuZ1 was cut StuI/SpeI/blunt and the ORF fragment excised was replaced with the *hph^R* cassette from pAG26 (Goldstein & McCusker, 1999) cut *NotI*-blunt. The cassette used to transform yeast was then prepared from the resulting plasmid by cutting *XhoI*/*BamHI*. For the deletion of the second copy, pST-ZLeu2c was cut BsteII-blunt/StuI and the ORF fragment excised was replaced with the *kan^R* cassette from pFA6-KanMX4 cut *EcoRI*/*XmaI*/blunt. From the resulting plasmid, the deletion cassette was then excised with *HincII*.

To construct the *ZbLEU*2d marker, the *LEU*2 ORF plus 30 bp of the promoter were excised from pST-ZLeu2c cutting *ScaI* /*MluI* and inserted into p212(-Nco) opened *HindIII*-blunt/*MluI*, thus obtaining p212*ZbLEU*2d in which a terminator was provided downstream of the *LEU*2 gene. The p212(-Nco) was obtained from the commercial vector pYX212 (R&D Systems) cut *EcoRI*/*BamHI*, blunted and re-circularized.

The *ScLEU*2d marker was constructed as follows: the *ScLEU*2d fragment amplified by PCR was inserted in pST-Blue1, giving pST-*ScLEU*2d. From this, it was excised back by cutting *MluI* and inserted in p212(-Nco) opened *HindIII* /*MluI*, resulting in p212*ScLEU*2d.

The *Z. bailii* rDNA fragment amplified was subcloned in pST-Blue1 giving pZbrDNA. This was digested *SacI*-blunt and the *ZbLEU*2d cassette was added, excised with *ApaI*-blunt/*PvuII* from p12ATLed (obtained by insertion of *ZbLEU*2d marker in pYX212 opened *DraIII blunt* /*PstI blunt*), thus obtaining plasmid pZbrDL2d. Insertion of the *Kl*IL-1β expression cassette from pZ₃klIL cut *BglII*/*AatII*/blunt or of the LDH expression cassette from pZ₃bT-LDH cut *BamHI*-blunt/*NaeI* in the plasmid opened *NotI*-blunt resulted, respectively, in pZbrDL2d-klIL or pZbrDL2d-LDH.

DNA manipulations, transformation and cultivation of *Escherichia coli* (DH5αF') and Novablue Competent Cells (Novagen) were performed following standard protocols (Sambrook *et al.*, 1989). All the restriction and modification enzymes used were from New

England Biolabs (Hitchin, Herts, UK) or from Roche Diagnostics (Mannheim, Germany).

Yeast transformation and determination of plasmid stability

Yeast transformation was performed using the Lithium Acetate/ssDNA method as described in Gietz & Woods (2002) and slightly modified for *Z. bailii* as previously described (Branduardi *et al.*, 2004).

For determination of plasmid stability in yeast transformants, a sample from each culture grown in the indicated medium (see individual experiment) was reinoculated both in selective and in nonselective medium until a concentration 5×10^3 cells ml^{-1} was reached. Then, 500 cells from each culture were plated both on selective and nonselective medium. Mitotic stability was expressed as the percentage of CFU found on selective versus non-selective medium. For each value, the mean of three experiments is reported. Cell number was determined using a Coulter counter (ZBI) upon sonication of the samples.

Determination of cell concentration, metabolites and enzymatic activities

Independent recombinant yeast transformants were shake-flasks cultured in minimal medium. During the cultures, followed up to stationary phase, samples were collected at regular time intervals. Cell concentration was determined by measuring the optical density at 660 nm or the cell number by a Coulter Counter determination (Vanoni *et al.*, 1983). Glucose and L(+)-lactate concentrations were determined using the Megazyme (Ireland, UK) kits (cat. # K-GLUKL, K-LATE, respectively) according to manufacturer's instructions.

For LDH activity, samples were prepared as follows: about 10^8 cells were harvested, washed in ice-cold water and resuspended in 50 mM phosphate buffer pH 7.5, 20% glycerol, 1 mM PMSF and protease inhibitors (Complete EDTA-free Protease inhibitor cocktail, Roche). Cells were broken with 5 cycles of vigorous vortexing in presence of glass beads (400-600 µm diameter, Sigma) at 4°C. After centrifugation, protein extracts concentration was determined (Bradford, Biorad). Cellular extract were incubated with 0.01 ml of 12.8 mM NADH, 0.74 ml of 50 mM acetate buffer pH 5.6 and 0.1 ml of 100 mM sodium pyruvate as previously described (Bernard *et al.*, 1991). LDH activity was assayed as micromoles of NADH oxidized per min, per mg of total protein extract, at 340 nm and 25°C.

β-galactosidase activity was determined in crude cell extracts using ortho-Nitrophenyl-β-galattoside as a substrate, as previously described (Venturini *et al.*, 1997).

Western blot

Recombinant IL-1β was analysed by Western blot. Independent transformants were cultivated in shake flasks and samples were taken at different times from the inoculum. Cells were harvested (a culture volume corresponding to 10^8 cells) by centrifugation. One volume of 2 x Laemmli buffer (Laemmli, 1970) was added to the supernatants of said samples, they were boiled for 3-5 min and stored at -20°C until loading, or loaded directly on polyacrylamide gel. For the analysis of intracellular production, crude extracts were prepared from 10^8 cells by following the trichloroacetic acid protocol (Surana *et al.*, 1993) and resuspending the final protein extract in 150 µl of Laemmli buffer. Samples were then loaded on standard polyacrylamide gels (sodium dodecyl sulfate-polyacrylamide gel electrophoresis, final concentration of the separating gel: 15%); after separation, proteins were blotted to nitrocellulose membranes and immunodecorated with an anti-IL-1β rabbit polyclonal antibody (IL-1β(H-153), Santa Cruz Biotechnology, Santa Cruz, CA, USA; cat. No. sc-7884; dilution 1 : 200). An anti-rabbit IgG (Peroxidase-conjugated AffiniPure Goat Anti-Rabbit IgG (H+L), Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA; cat. No. 111-035-003) secondary antibody was used. The protein was visualized using the ECL Western blotting system (SUPER SIGNAL® West Pico Chemiluminescent Substrate), according to the manufacturer's instructions.

Results

Development of a multicopy expression plasmid specific for Zygosaccharomyces bailii

First attempts to improve foreign protein expression in *Z. bailii* through increased plasmid copy number were made by the construction of an episomal vector based on the *S. cerevisiae* 2μm sequence. Expression plasmid p195Z$_3$ was derived from YEplac195 (Gietz & Sugino, 1998), whose *URA3* marker and MCS were substituted respectively by the kanamycin resistance cassette KanMX4 and the *S. cerevisiae* expression cassette (pTPI-MCS-polyA) from pYX012 (R&D Systems). Insertion of the *Escherichia coli LACZ* reporter gene in p195Z$_3$ allowed to test β-galactosidase activity in the supernatants of two different *Z. bailii* strains. Unfortunately, activity levels for *Z. bailii* were not improved if compared to those obtained through the centromeric plasmid pZ$_3$ (Branduardi *et al.*, 2004) (see Fig.1), whereas they were enhanced by 8-10 times for *S. cerevisiae* in the same conditions (data not shown). Moreover, the stability of the new plasmid in *Z. bailii* cells was very low compared to pZ$_3$ (see Table 2), suggesting that *S. cerevisiae* 2μm sequences are poorly recognized in *Z. bailii*.

A novel multicopy plasmid specific for *Z. bailii* was subsequently developed, exploiting the sequence of pSB2, one of the two endogenous plasmids of *Z. bailii* that were shown to be functionally and structurally related to the *S. cerevisiae* 2μm (Toh-e *et al.*, 1984; Utatsu *et al.*, 1987). The presence of pSB2 in our strains was assessed by PCR amplification and sequencing of specific fragments of the plasmid upon DNA extraction (data not shown). Hence, starting from p195Z$_3$LacZ, a series of plasmids was generated, in which the *S. cerevisiae* 2μm replication origin was replaced with the one from *Z. bailii* and, afterwards, the other pSB2 functional elements were sequentially inserted (in analogy to what was described for *S. cerevisiae*). Then, the contribution of each region to the increase in copy number per cell and stability of the recombinant plasmid was evaluated in *Z. bailii* cells transformed with the respective plasmids. In Table 2, the functional regions added to each plasmid are indicated. Relative position of the inserted elements was maintained with respect to pSB2, according to the structure described by Utatsu *et al.* (1987).

Table 2. Stability of episomal plasmids in *Z. bailii* cells

Plasmid	Functional elements	Selection	Plasmid stability (%)
pZ$_3$LacZ	*Sc*ARS/CEN	*kan*^{R}	70
p195Z$_3$LacZ	*Sc*2μm ori	*kan*^{R}	7
p195ILacZ	*Zb*2μm ori (IR-A/ARS)	*kan*^{R}	29
p195IFLacZ	*Zb*2μm ori (IR-A/ARS), FLP	*kan*^{R}	35
p195ITFLacZ	*Zb*2μm ori (IR-A/ARS), FLP, TF-C	*kan*^{R}	45
p195ITFILacZ	*Zb*2μm ori (IR-A/ARS), FLP, TF-C, IR-B(s^a)	*kan*^{R}	65
p195ITFiLacZ	*Zb*2μm ori (IR-A/ARS), FLP, TF-C, IR-B(as^b)	*kan*^{R}	10
p195ITFIStbLacZ	*Zb*2μm ori (IR-A/ARS), FLP, TF-C, IR-B(s^a), *Zr*STB	*kan*^{R}	79

^{a} *Sense*, i.e. having the same orientation as in the original pSR1.
^{b} *Antisense*, i.e. having the opposite orientation as in the original pSR1.

The β-galactosidase activity obtained from *Z. bailii* cells transformed with the new plasmids was used to compare their efficiencies of heterologous expression. As it is shown in Fig. 1, insertion of the *Z. bailii* 2μm-like replication origin (fragment IR-A/ARS) led to an increase in β-gal activity levels obtained, that were about 1.5 times if compared to both the centromeric pZ$_3$ and to the *Sc*2μm-based plasmid. Introduction of the *FLP* recombinase gene led to a doubled activity compared to the new *Z. bailii* episomic plasmid (and about 2.5 times the pZ$_3$ activity). The activity was again doubled by the addition of TF-C, that led to almost 4 times the activity of the simple *Zb*2μm-like ori based plasmid (and about 5 times the pZ$_3$ activity). The function of this gene is still unknown, but it was hypothesised to influence plasmid stability, for analogy with the *S. cerevisiae* trans-activating factors (Utatsu *et al*., 1987). Insertion of the second inverted repeat (IR-B) didn't lead to any further increase in the activity recovered, if the orientation was the same as in pSB2 (*sense*), while insertion in the opposite orientation (*antisense*) led to a fall at about half the activity levels of the centromeric plasmid. Finally, since any region of pSB2 was found sharing sequence similarity to the STB *cis-*acting sequence responsible for mitotic stability in *S. cerevisiae*

(Utatsu *et al.*, 1987), the STB sequence (locus Z) from the *Z. rouxii* 2μm-like pRS1 plasmid was inserted into the vector containing the IR-B *sense*. Insertion resulted in similar activity levels. Clones bearing the corresponding control plasmids lacking the *LACZ* sequence gave no activity (data not shown).

The stability of the new constructs in *Z. bailii* cells after 10 generations in selective medium (see Materials and Methods) was increased little by little upon substitution of the *Sc*2μm ori with the *Zb*2μm-like ori, and then with the addition of FLP and TF-C (Table 2). Remarkably, addition of the IR-B, had a significant impact on plasmid stability, which improved of a 20%, with the exception of insertion in the wrong orientation that made the stability fall down. Addition of the *Zr*STB finally raised the stability to very high levels. However, after 20 generations the stability slightly falls down, resulting lower if compared to the centromeric one.
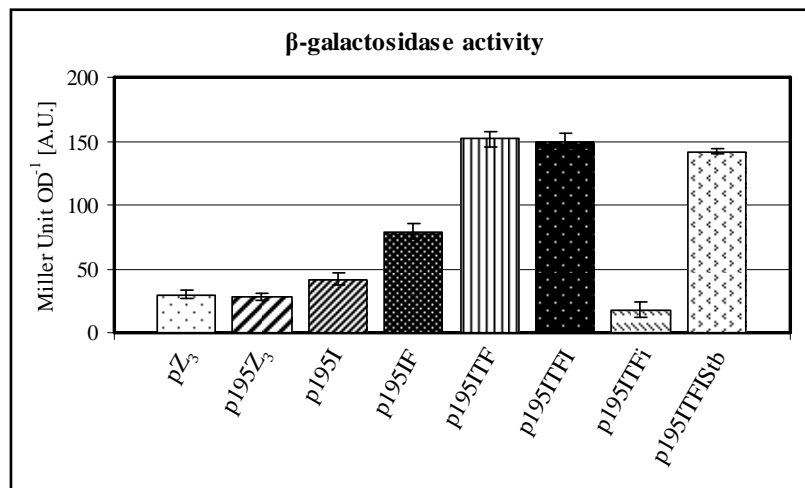

Figure 1. Heterologous β-galactosidase activity: comparison between the centromeric and the different multicopy plasmids productions.
Z. bailii (Zygo1) cells were transformed with the indicated expression vectors (a description of their characteristics is given in Table 2) bearing the *E. coli lac*Z gene, or with the corresponding empty vectors (controls). Independent clones were grown in YPD + G418 until exponential phase and then harvested. The β-galactosidase activity was essayed on culture supernatants as described in (Venturini et al 1997). Vertical bars indicate standard deviation. No activity was detected for the controls (data not shown).

Improvement of heterologous construct stability: a specific integrative vector for Z. bailii

In order to improve mitotic stability, an integrative vector specific to *Z. bailii* was developed by exploiting the *ZbLEU2* gene sequence, one of the few sequences available from this yeast. Plasmid pZ$_3$*b*TLacZ (Branduardi *et al.*, 2004), a centromeric expression plasmid analogous to pZ$_3$ but bearing an expression cassette based on the *Z. bailii* TPI promoter instead of the *S. cerevisiae* one, was used as scaffold for the new vector. The *LEU2* ORF together with its own promoter sequence, amplified by PCR from strain Zygo1, was inserted into pZ$_3$*b*TLacZ to direct the integration in the LEU2 genomic locus in *Z. bailii* cells, while the KanMX4 cassette was maintained as a selectable marker. The new integrative plasmid was named pZ$_3$I*Lb*TLacZ.

Z. bailii cells were transformed with plasmid pZ$_3$I*Lb*TLacZ linearized in *LEU2* and then selected for G418 resistance; insertion at the target locus was assessed in independent clones by qualitative control PCR on genomic DNA templates (not shown). Independent positive transformants were subsequently cultivated both in minimal (YNB-glucose) and in rich (YPD) media (see Materials and Methods) added or not with G418, and samples from exponential or stationary phase cultures were tested for β-galactosidase activity. Table 3 reports the data obtained, in comparison with β-gal activity levels determined for pZ$_3$*b*TLacZ transformants grown under the same conditions. In rich medium under selective pressure, β-gal activity levels detected for both exponential and stationary-phase integrative transformants were about the 45 and 55%, respectively, than the ones detected for the centromeric plasmid. This would be consistent with a copy number per cell of the centromeric plasmid being about two, assuming that a single copy per cell is present in integrative transformants. In rich medium without the antibiotic, relief from the selective pressure causes a fall in the activity produced from the centromeric plasmid, of about the 40 and 30% in exponential and stationary phase, respectively, while the activity levels produced by the integrative plasmid remain almost stable (90 and 100% of the selective medium ones, respectively). In minimal medium, lower activity levels were detected for both constructs in exponential phase, if compared to rich medium, although the difference was considerably lower for the integrative plasmid. In nonselective minimal medium, compared to selective medium, the centromeric-derived activity resulted lower in stationary phase but equal in exponential phase, while the integrative-derived was always

the same both in the presence and absence of G418. Overall, a considerably narrower difference was found between the activity levels determined for the two constructs in minimal media compared to rich media.

Plasmid stability was determined in the same conditions after 10 and 20 generations for each construct, and data are shown in Table 3. As expected, the integrated construct is stably maintained in all the conditions, while the centromeric plasmid is retained at most by the 60-70% of the population under selective pressure and only by the 35-40% after 20 generations in nonselective conditions.

Table 3. β-galactosidase activity and plasmid stability obtained with the centromeric and integrative vectors in *Z. bailii* cells grown in rich or minimal medium, in the presence or absence of the antibiotic selection.

Plasmid		β-gal activity (Miller U)			
		YPD		YNB-glc	
		+ G418	- G418	+ G418	- G418
pZ$_3$bTLacZ	exp^a	236	139	62	64
	stat^b	59	43	81	59
pZ$_3$I*Lb*TLacZ	exp^a	103	92	56	57
	stat^b	32	32	49	49

Plasmid		Plasmid stability (%)			
		YPD		YNB-glc	
		+ G418	- G418	+ G418	- G418
pZ$_3$bTLacZ	exp^a	69	43	66	49
	stat^b	70	36	60	39
pZ$_3$I*Lb*TLacZ	exp^a	100	100	100	100
	stat^b	100	100	100	100

^{a} Exponential phase, after 10 generations.
^{b} Stationary phase, after 20 generations.

Construction of the first isogenic auxotrophic strain of Z. bailii through targeted gene deletion

Although integration allows to circumvent long-term antibiotic use, still an initial selection will be needed for the insertion of the heterologous construct; the number of different manipulations permitted on the same strain would be therefore limited by the number of the available dominant markers for *Z. bailii*, which are only three up to date.

To broaden the perspective of strain manipulation and engineering, we therefore addressed the issue of the achievement of auxotrophic markers in *Z. bailii* through targeted gene deletion. At present, in fact, inactivation of essential genes in *Z. bailii* was never reported and, hence, no isogenic auxotrophic mutants are available (see also Introduction).

The classical strategy used to delete genes of interest is based on gene replacement. Different experimental evidences suggest that *Z. bailii* is a diploid yeast (Mollapour & Piper, 2001; Rodrigues *et al.*, 2003; Dato *et al.*, 2008). Consistent with this, the deletion of a single copy of a gene in this yeast is not sufficient to obtain the relative defective mutant. Previous attempts, carried out by different research groups, to obtain haploid strains through classical sporulation induction were ineffective; spores formation occurred, but their characteristics as well as the study of the sporulation mechanism indicated that they were vegetative (mitotic) and not meiotic spores (Mollapour & Piper, 2001; Rodrigues *et al.*, 2003). In agreement to that, germination of a single spore led to a population with a DNA content per cell that was identical if compared to the progenitor (data not shown). It follows that two alleles of the target gene have to be eliminated in consecutive transformation events to obtain a deletion mutant.

There is only one example presented by Mollapour & Piper (2001) of double gene deletion in *Z. bailii*, concerning the non-essential gene *ZbYME2*. The authors reported a very low efficiency of homologous recombination, that increased only slightly (up to a maximum of 10-15%) by lengthening the homologous flanking regions from 40 to 400 bp.
We first tried to delete the *ZbHIS3* gene by transforming the prototrophic strain Zygo2 with deletion cassettes bearing flanking homology regions of 100-150 bp, without success. Unfortunately, the

small length of the whole sequence available for this region (678 bp) did not enable us to lengthen the construct.

We then chose the essential gene *ZbLEU2* coding for the *Z. bailii* β-isopropylmalate dehydrogenase as our target, due to the sequence availability of a larger chromosomal region spanning this locus (Rodrigues *et al.*, 2001 b). Starting from the sequence PCR amplified from Zygo1 and inserted into a shuttle plasmid, a set of different deletion cassettes was constructed, with flanking homologies going from 400 to 1300 bp. (See Materials and Methods). A central portion of the coding sequence was replaced with either one of the two dominant marker genes *kan^R* and *hph^R*, conferring resistance to the antibiotics G418 and hygromycin B, respectively, that were previously demonstrated to function in *Z. bailii* (Mollapour & Piper, 2001; Branduardi *et al.*, 2004).

The *hph^R*–based deletion cassette with 400-700 bp flanking homologies (Fig. 2A, construct b) allowed us to delete the first allelic copy of *ZbLEU2*, as verified by PCR (Fig. 2B, lane 3). Homologous recombination frequency at this step was about 5%. Interestingly, the use of the corresponding *kan^R*–based deletion cassette with identical flanking regions did not bring to homologous recombination, neither in the wild type strain (i.e. deletion of the first *LEU2* copy) nor in the *leu2::hph^R/LEU2* strain (i.e. deletion of the second copy). Moreover, attempts to delete the second copy with a cassette of similar length but spanning a different sequence portion of the *ZbLEU2* gene, relative to the first integrated construct (in order to decrease the chances that recombination occurred in the mutated locus instead of in the wild type locus), were also unsuccessful. Only with the longer construct (Fig. 2A, construct c), with flanking homologies of 800-1300 bp, it was possible to replace the second copy of *ZbLEU2*, obtaining the *leu2::hph^R/leu2::kan^R* strain, as verified by PCR (Fig. 2B., lane 2). The frequency of homologous recombination at this step was even lower than in the first case (about 1%).

The mutant strain obtained is auxotrophic for leucine, being unable to grow in minimal glucose medium unless supplemented with the aminoacid, as shown in Fig. 2C. It should be noticed, however, that growth of the defective mutant in the complemented medium was reduced if compared to the wild type strain. Increasing the leucine concentration in the medium did not result in a growth improvement (not shown), indicating some limits in leucine transport.

The *leu* defective phenotype could be complemented by transforming with the YCplac111 centromeric plasmid harbouring the *ScLEU*2 gene as selectable marker (Fig. 2C). This demonstrates that the *S. cerevisiae* gene and its promoter sequence are recognized and fully functional in *Z. bailii*, as it is for *ZbLEU2* in *S. cerevisiae* (Rodrigues *et al.*, 2001 a). The *ZbLEU2* deletion resulted then in the first example of a *Z. bailii* auxotrophic mutant obtained by targeted gene deletion.

Figure 2. (on next page)Construction of the first auxotrophic mutant of *Zygosaccharomyces bailii*

(A) Schematic representation of the *Z. bailii LEU*2 locus (*a*) and of the corresponding genomic region after integration of the *hph*-based (*b*) or *kan*-based (*c*) deletion cassettes. Grey boxes represent *LEU*2 ORF; back boxes indicate promoter and terminator sequences in the antibiotic resistance cassettes. The restriction sites exploited to construct the cassettes and the primers used to control integration are indicated.

(B) Control PCR showing the amplification of the *wt* (*a*) and mutated (*b* and *c*) *LEU*2 loci (as described in A) from genomic DNA of *Z. bailii* cells: lane 3, *wt* strain (*LEU*2/*LEU*2); lane 2, strain *leu2Δ*::*hph*/*LEU*2; lane 1, strain *leu2Δ*::*hph*/*leu2Δ*::*kan*; L, DNA ladder.

(C) Growth of *Z. bailii wt* and deleted strains in minimal medium added or not with leucine, as indicated. The scheme on the left indicates positions of the different strains on the plates.
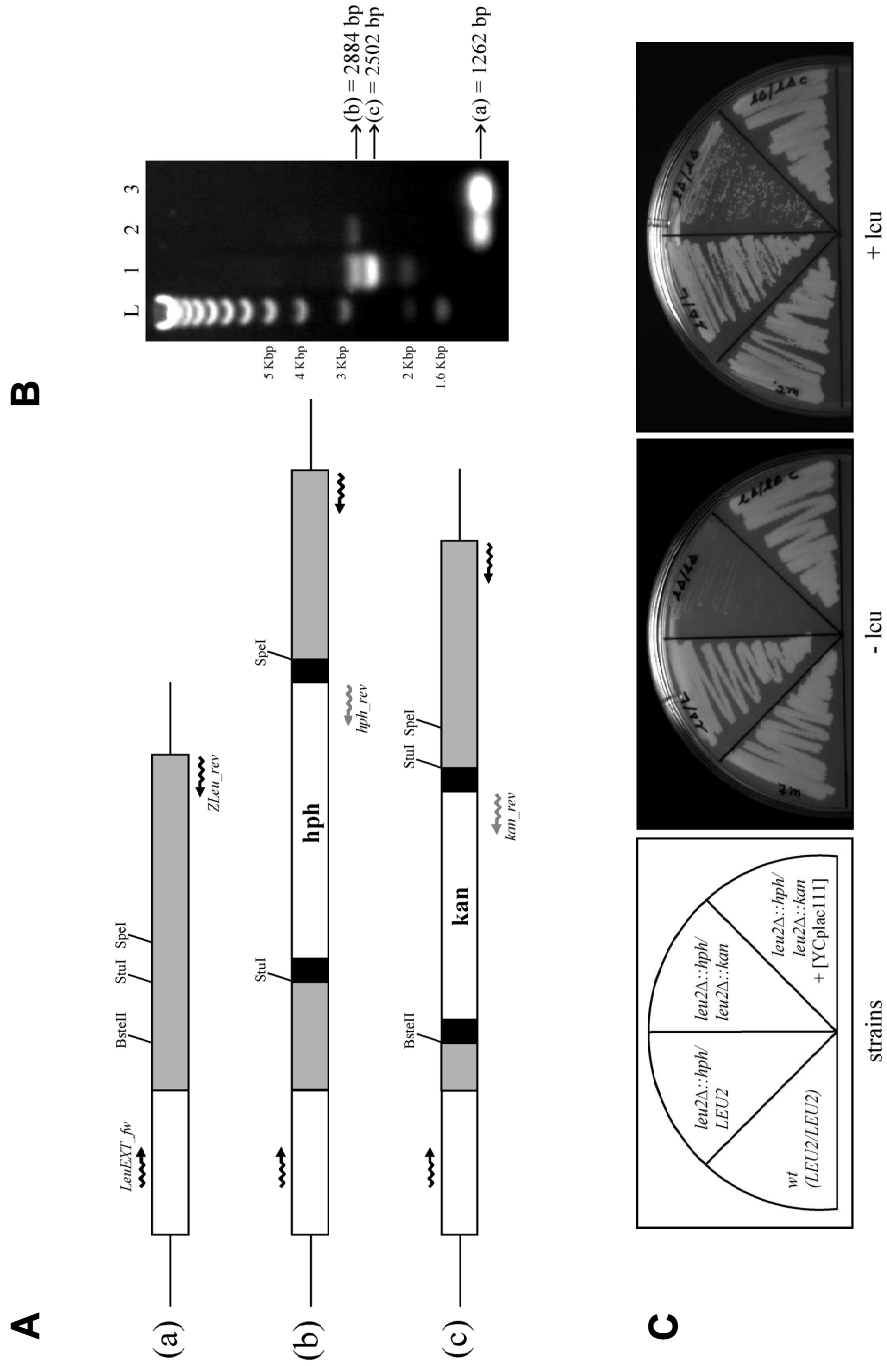
Testing the new Z2ΔL auxotrophic strain for heterologous protein production and metabolic engineering

In order to explore the production capabilities of the new *Z. bailii* auxotrophic mutant, we to tested the expression of two heterologous products having different characteristics.

The first was the human interleuchin-1β (hIL-1β), a commercially relevant protein. The sequence encoding for a recombinant form of hIL-1β (r hIL-1β), fused in frame with the leader sequence of the K1 killer toxin of *Kluyveromyces lactis* (Fleer *et al.*, 1991), and under the control the *Sc*TPI promoter, was inserted into the centromeric YCplac111 to drive synthesis and secretion of the mature product upon transformation of the Z2ΔL strain. In parallel, the *wt Z. bailii* strain was also transformed with the centromeric pZ$_3$klIL-1β (Branduardi *et al.*, 2004). To allow direct comparison of the production levels, both strains were grown in shake flasks in the same minimal medium (YNB + 5% glucose) added with G418: in fact, also the *leu2Δ* strain bears the kan^R marker in one of the two deletion cassettes, as described above. At different times from the inoculum, samples were collected to analyze the secreted and intracellular product levels, shown in the Western blot of Fig. 3.
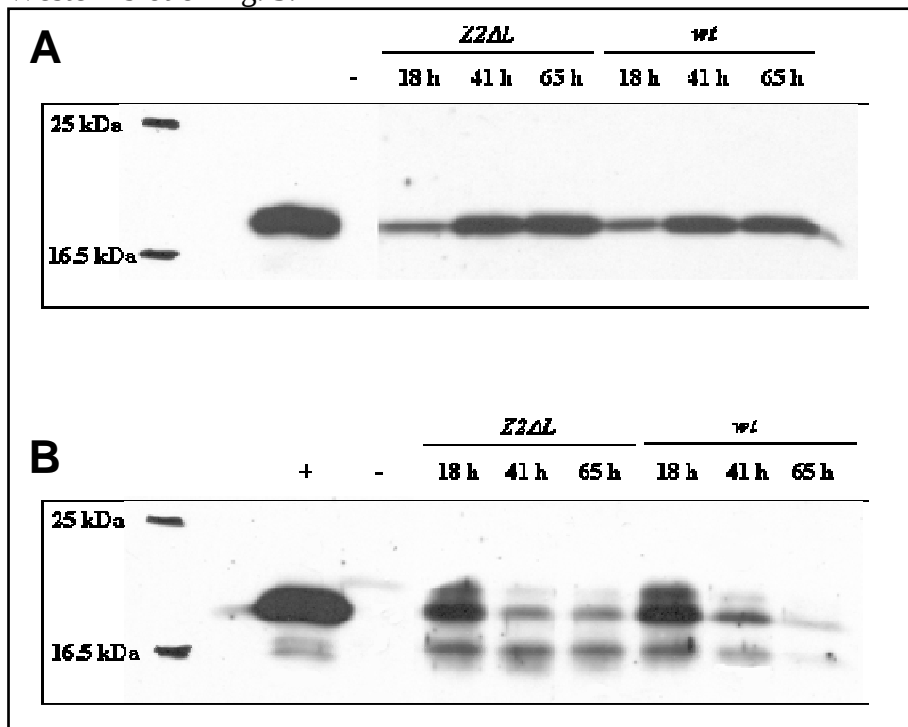
Figure 3. (previous page) Heterologous human IL-1β production and secretion in *Z. bailii wt* and Z2ΔL.
Z. bailii wt and *leu2Δ* (Z2ΔL) cells were transformed with the centromeric plasmids pZ₃*Kl*IL-1β and YCplac111*Kl*IL-1β, respectively, or with the corresponding empty plasmids. In these plasmids, the sequence encoding for the hIL-1β, under the control of the *Sc*TPI promoter, was fused in frame with the K1 leader sequence in order to drive its secretion. Transformants were grown in minimal glucose medium added with G418 and at different times from the inoculum samples were taken and analysed. Recombinant hIL-1β was detected in the supernatants (A) and in the intracellular protein extracts (B) by Western blot. Samples were normalised for OD 660nm of the culture. Commercial hIL-1β (200 ng) was loaded as a positive control (+) and samples from cells transformed with the empty plasmid as a negative control (-).

The auxotrophic mutant was found to produce higher amounts of rhIL-1β if compared to the *wt*, in particular during the last time-points analysed. The difference was mainly visible in the intracellular fractions, and especially after 65 h (Fig. 3B). Here, three different isoforms of the protein were found: the mature native form, the higher molecular weight precursor and a lower molecular weight form, probably resulting from proteolitic degradation. Analysis of the supernatants also evidenced an improvement in secretion, although the less pronounced (Fig. 3A).

Plasmid stability, shown in Table 4 in function of time, was very high with the *LEU*2 marker, with a decrease of only the 15% during the time course, while it was the 40 to 50% less with the *kan^R* marker. The overall increase in rhIL-1β production was therefore attributable to the higher stability of the auxotrophic marker-based plasmid.

Table 4. Stability of centromeric plasmids in *Z. bailii leu2Δ and wt* strains at different time-points during cultivation in minimal medium.

Plasmid	Strain	Selection	Plasmid stability (%)			
			0 h	18 h	41 h	65 h
YCPlac111*Kl*IL-1β	*Z2ΔL*	*LEU*2	100 ± 12.7	90 ± 2.7	85 ± 11.3	85 ± 8.9
pZ₃*Kl*IL-1β	*wt*	*kan*^{R}	59 ± 1.9	58 ± 15.8	58 ± 5.3	44 ± 5.5
			0 h	24 h	48 h	72 h
YCPlac111*b*TLDH	*Z2ΔL*	*LEU*2	77 ± 6	79 ± 1	65.5 ± 4.5	37 ± 3
pZ₃*b*TLDH	*wt*	*kan*^{R}	44.5 ± 0.5	24.5 ± 1.5	21 ± 2	15.4 ± 1.4

The second heterologous expression involved the bovine lactate dehydrogenase (LDH), whose catalytic activity partially redirects the glycolytic flux towards the production of lactate, as previously shown also in *Z. bailii* (Branduardi *et al.*, 2004). The *LDH* gene was inserted into YCplac111 and, under the control of the *Zb*TPI promoter. Similarly to what described before, pZ₃*b*T-LDH was also constructed and the *leu2Δ* and *wt* strains were transformed with the respective *LEU2* and *kan*^{R} plasmids. Fig. 4 shows the results obtained by shake flask cultures grown in minimal medium (YNB + 5% glucose) plus G418. The amount of lactate produced by the auxotrofic mutant was about seven-fold compared to the *wt* (Fig.4A). LDH activity, assayed in crude intracellular extracts, was about five times higher in *leu2Δ* than in *wt* at the peak (24h) and then decreased (Fig.4B). The growth kinetics of the two strains were comparable (Fig.4A).

The stability of both plasmids, reported in Table 4, was found to be lower than the stability of the rhIL-1β plasmids and to decrease over time. The different impact of the two heterologous productions could justify a different selective pressure against plasmid maintenance. Nonetheless, the auxotrophic marker-based plasmid was two to three-fold more stable than the *kan*^{R} marker-based, again correlating with the production data.
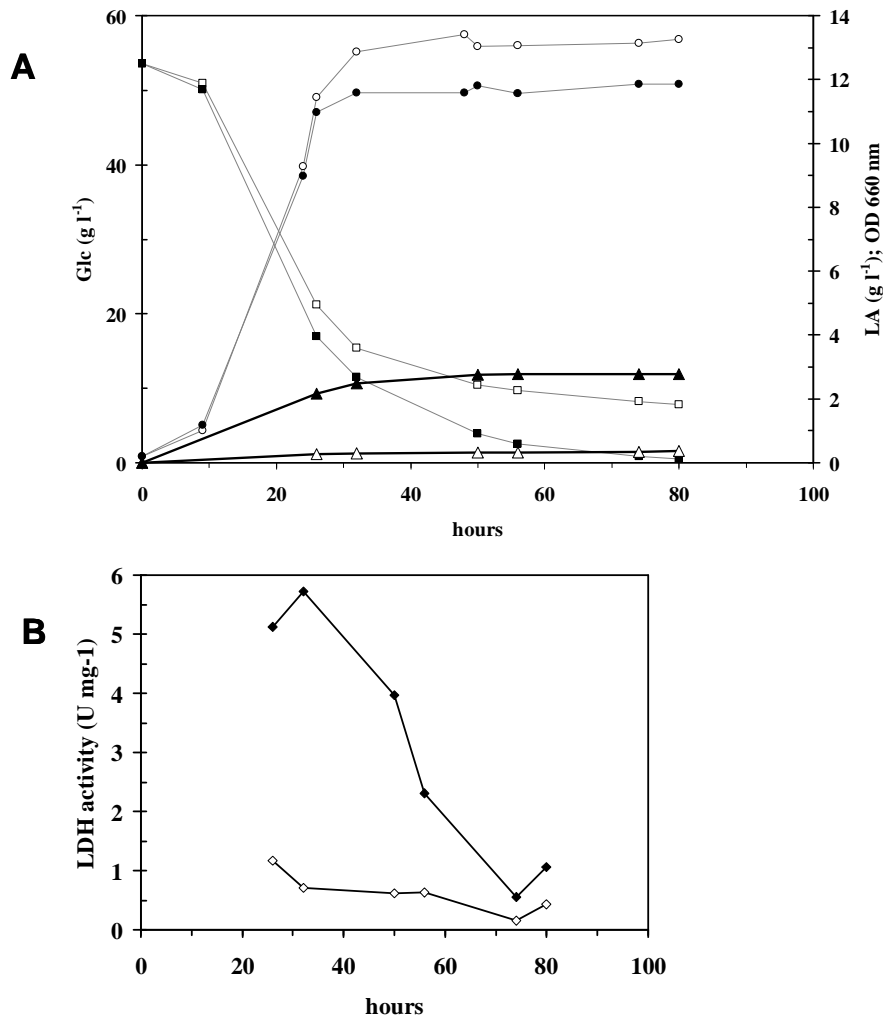
Figure 4. Production of lactic acid and heterologous LDH activity in *Z. bailii wt* and Z2ΔL.

Z. bailii wt and *leu2Δ* (Z2ΔL) cells were transformed with the centromeric plasmids pZ₃*b*TLDH and YCplac111*b*TLDH-1β, respectively, or with the corresponding empty plasmids. These plasmids bear the gene coding for the bovine LDH under the control of the *Zb*TPI promoter. Transformants were grown in shake flasks in minimal glucose medium added with G418 and their growth and production parameters were followed over time. In (A) OD 660nm (circles), lactate production (triangles) and glucose consumption (squares) are shown. Filled symbols, Z2ΔL strain. Open symbols, *wt* strain.
(B) Specific LDH activity per mg of total proteins, assayed as described in (Bernard et al, 1991). Filled symbols, Z2ΔL strain. Open symbols, *wt* strain.

Development of a multiple integration vector for Z. bailii – step 1: construction of the ZbLEU2-defective selectable marker

A strategy for maximization of recombinant protein production should couple high copy numbers of the heterologous construct, like those reachable by yeast episomal plasmids, with the high mitotic stability typical of integration into the genome. Integrative vectors satisfying these criteria were described for *S. cerevisiae* (Lopes *et al., 1989*), based on insertion into the rDNA region and selection by the *LEU2*d (defective) marker; the use such a marker was indicated by Lopes and co-workers (1991) as crucial in determining the copy number, because of the strong selective pressure created by the extremely low expression driven by the defective promoter.

In analogy with the mentioned *S. cerevisiae* vector, we aimed to construct a rDNA-based integrative vector for *Z. bailii*. The availability of the new *Z. bailii* mutant auxotrophic for leucine allowed us to develop a *LEU2*d marker specific for this yeast.

Starting from the *ZbLEU*2 sequence previously amplified, the *ZbLEU*2d selectable marker was constructed by truncating the gene promoter 30 bp before the START codon, as described for *ScLEU*2d (Erhart & Hollenberg, 1983). To test its functionality, the marker was inserted into an episomal vector (pYX212) providing also a poly-A tail. In parallel, an identical plasmid bearing the defective *S. cerevisiae* marker instead of the *Z. bailii* one was constructed as a control (see Materials and methods section for detailed description of plasmids construction), and both plasmids were used to transform yeast.

Leucine auxotrophy was complemented in *Z. bailii* by *ZbLEU*2d, with an efficiency of transformation of about 10 CFU mg_{DNA}^{-1}, but not by *ScLEU*2d. Similarly, *S. cerevisiae* was successfully transformed with the plasmid bearing its homologous defective marker, with an efficiency of about 700 CFU mg_{DNA}^{-1}, but not the *Z. bailii* one, thus indicating that the two defective markers are not functionally interchangeable.
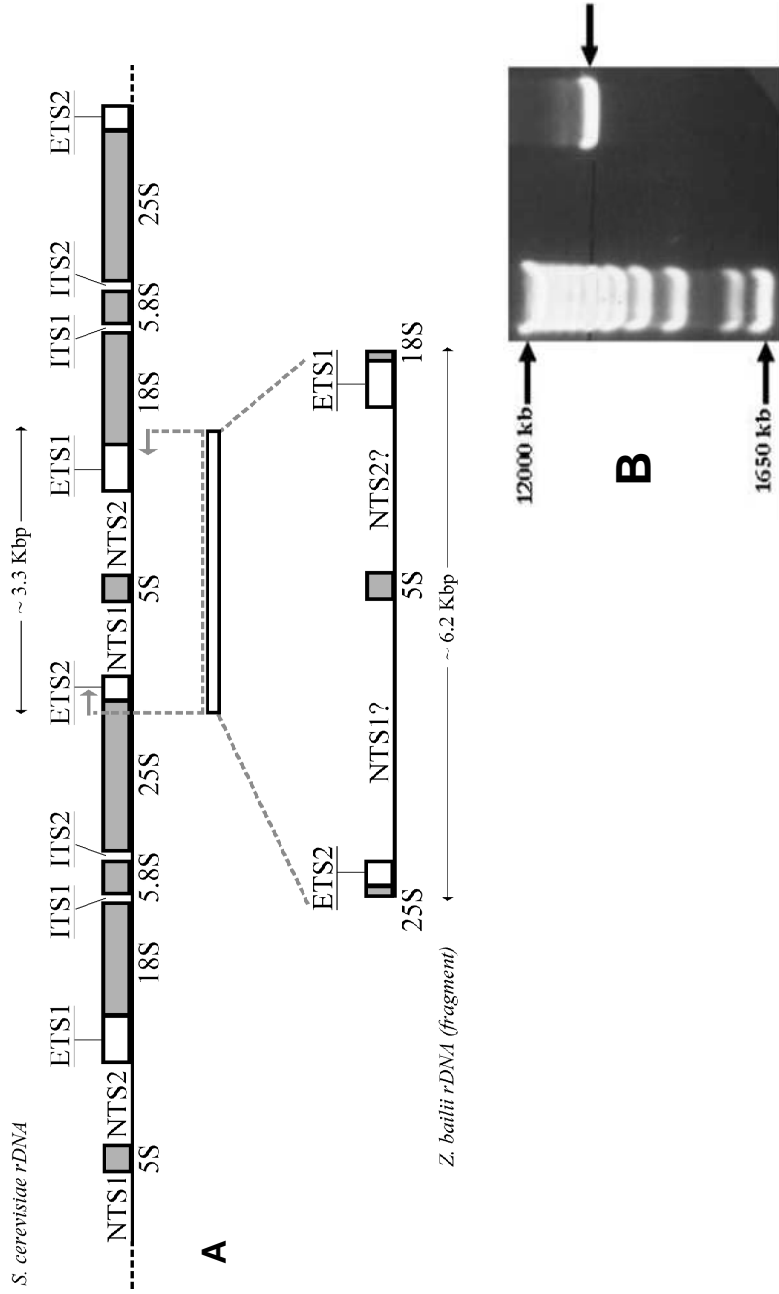
Development of a multiple integration vector for Z. bailii – step 2: cloning and sequencing of a portion of the rDNA unit of Z. bailii

Starting from some fragments of the rRNA genes available from the databases, we designed primers to amplify a portion of the rDNA unit comprised between the 3' end of the 25S and the 5' end of the 18S and encompassing the two nontrascribed spacers (NTSs). The PCR product obtained was about 6200 bp long (Fig. 5B), that is considerably longer than expected on the basis of the corresponding *S. cerevisiae* fragment length, of about 3300 bp. Sequencing of the obtained DNA fragment confirmed that it was the region of interest. Alignments with the *S. cerevisiae* rDNA unit allowed to map the external transcribed spacers (ETS), respectively downstream of the 26S and upstream of the 18S, and the 5S (see Fig. 5A). Sequence identity was very high in the fragments coding for mature rRNA (around 90%) but lower in the ETSs (around 60%). The NTS1 and NTS2 were consequently mapped in the two intergenic regions (Fig. 5A), but no correlation was found here with the *S. cerevisiae* sequences. The length of these sequences also differed, especially for the NTS1 which resulted to be about 3000 long, against about 900 bp in *S. cerevisiae*. The NTS2 resulted instead to be 2000 bp long, against about 1250 in *S. cerevisiae*. BLASTN search against all nucleotide databases scored *Z. rouxii* sequences as the most similar to the new *Z. bailii* one; here, shared sequence identity raised up to 95% in the coding fragments and 86% in the ETS. Significant similarities were also found with *Ashbya* (*Eremothecium*) *gossypii* and with sequences of *Kluyveromyces*, *Debaryomyces* and *Pichia* and *Candida* strains, among others.

Figure 5. (on next page)Cloning and sequencing of the *Z. bailii* rDNA fragment.
(A) Schematic representation of the *S. cerevisiae* rDNA units and of the newly cloned *Z. bailii* rDNA unit fragment. The positions of PCR primers annealing are shown (grey arrows) according to the *S. cerevisiae* unit structure; the amplified *Z. bailii* fragment was sequenced and, according to alignments with the *S. cerevisiae* sequence, the indicated regions were mapped. The length of the corresponding fragments in the two yeasts is indicated. NTS, nontrascribed spacer; ETS, external transcribed spacer; ITS, internal transcribed spacer.
(B) Agarose gel electrophoresis of the *Zb*rDNA 6.2 Kbp fragment obtained by PCR amplification. The length of the DNA ladder bands is shown.

S. cerevisiae rDNA

Z. bailii rDNA (fragment)

A

B

Development of a multiple integration vector for Z. bailii – step 3: plasmid design and copy number determination in transformed yeast

The *Z. bailii* rDNA fragment sequence amplified was inserted into the MCS of the commercial shuttle vector pST-Blue1. Immediately upstream of the *Zb*rDNA, the *ZbLEU*2d marker was added, thus obtaining plasmid pZbrDL2d. Two expression cassettes were alternatively added to the empty plasmid, either the *Kl*IL-1β (plus *Sc*TPI promoter and terminator) or the LDH (plus *Zb*TPI promoter and terminator), giving plasmid pZbrDL2d-*Kl*IL or pZbrDL2d-LDH, respectively.

The *Z. bailii* auxotrophic Z2ΔL strain was transformed with the empty rDNA vector and with the vectors bearing the two reporter genes, upon linearization of the plasmids within the rDNA region. A high transformation efficiency was obtained with pZbrDL2d. However, it was not possible to obtain transformants with the other two plasmids.

In order to understand if the integration of pZbrDL2d occurred in multiple copies, a quantitative real-time PCR was performed on the transformants. Three sets of primers were used for this analysis and are indicated in Fig. 6A: the first designed in the upstream promoter of *LEU*2 (not present in *ZbLEU*2d), the second within a portion of the *LEU*2 ORF which was deleted in only one of the two deletion cassettes used to obtain the *Δleu2* strain (see Materials and methods for details) and, finally, the third designed in the 3' region of *LEU*2. PCR reactions were performed on DNA extracted from *Z. bailii wt* strain, from nontransformed Z2ΔL strain and from Z2ΔL transformants bearing pZbrDL2d. The first region was chosen as the housekeeping control since it is not affected by integration or *LEU2* deletion. The amplicon obtained by second pair of primers was used to monitor the number of events of integration (calibrator), since the corresponding region is present in single copy in *Δleu2*, in two copies in *wt*, and in two or more copies in transformants. Finally, the third region was chosen as supplementary control because its copy number is fixed in *wt* and *Δleu2* background (2 copies) and should become higher in multicopy transformants.

Results are summarised in Fig. 6B, where the quantification of integrated copies relative to the control (*Δleu2* strain) is shown for eight different transformant strains. Integration of the rDNA-based plasmid resulted to occur in multiple copies, ranging from 27 to 80. In some cases, a high standard deviation was registered, indicating a heterogeneity of the population within the strain analysed.
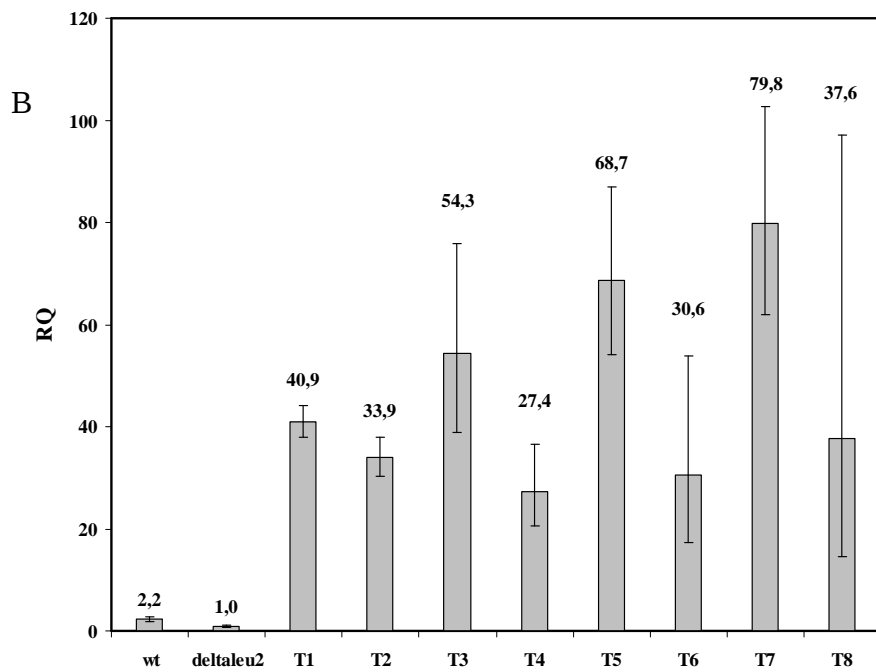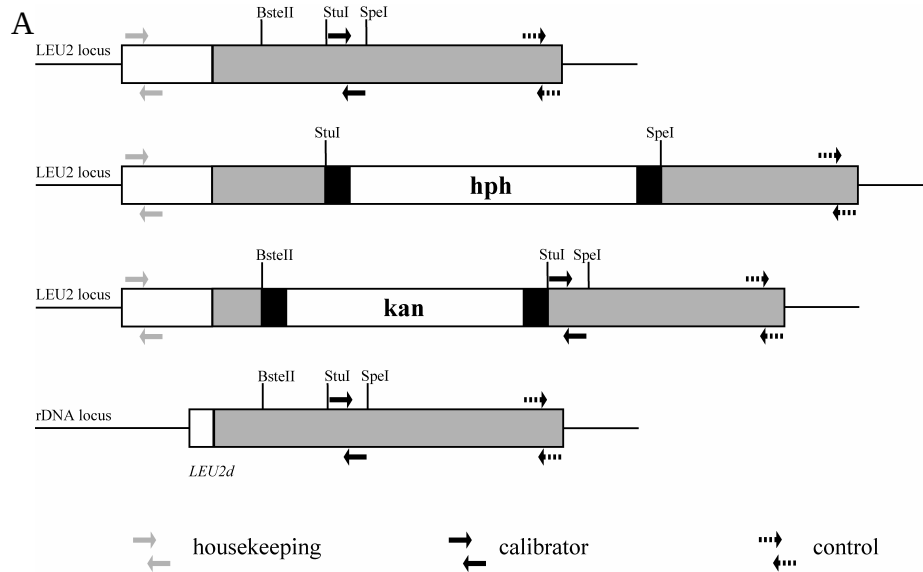
A

Figure 6. Evaluation of copy number in multiple integrative transformants of *Z. bailii* by real-time QPCR. (A) Schematic representation of the three sets of primers used for QPCR reactions and their annealing sites on the genome of *Z. bailii wt*, *leu2Δ* and *leu2Δ* transformants bearing the integrated plasmid pZbrDL2d.
(B) Results of relative quantification indicating the average number of copies of plasmid pZbrDL2d integrated in the genome of eight transformant strains (T1-T8). Error bars are indicated.

64

Discussion

One of our aims was the maximization of heterologous gene expression, that was here addressed by two different strategies: the increase of copy number per cell and the increase of stability of the heterologous construct. Satisfactory results were obtained with some of the episomal vectors described, and useful information was gained regarding the function of 2μm and 2μm-like sequences in *Z. bailii*.

The extreme instability of the *Sc*2μm ori in *Z. bailii* cells pointed out a poor recognition of the heterologous sequences responsible for amplification and maintenance of the plasmid, which correlates with the low sequence similarity shared by the natural plasmids of the two yeast species (Utatsu *et al.*, 1987). This led us to exploit the endogenous pSB2.

The results obtained with the new set of *Z. bailii* 2μm-like multicopy plasmids indicate that the replication origin contained in the pSB2 ARS fragment is functional and can be recognized by *Z. bailii* [cir$^+$] strains. In *S. cerevisiae* [cir$^+$] strains, all the trans-acting elements necessary for plasmid amplification and stable maintenance are present, so that an episomal vector must only bear the cis-acting elements, i. e. the ARS for replication, the IR for amplification and the STB for stability (Kikuchi *et al.*, 1983). The presence of only one IR, in this case, is sufficient since the second IR is provided by the endogenous plasmid, allowing the exogenous plasmid to exist as hybrids with the 2μm, due to intermolecular recombinations (Gerbaud *et al.*, 1979). The higher β-gal activity levels and the considerably lower stability of plasmid p195I (bearing the minimal sequence allowing replication) in comparison with the centromeric pZ$_3$, which was assumed to be present in 1-2 copies per cell, could indicate that the new plasmid was maintained at a slightly higher copy number, but it is not possible to assess if this was due to recombination events following recognition of IR-A or to asymmetrical segregation (see Romanos *et al.*, 1992). It appears, anyway, that a region analogous to the 2μm STB is not present proximal to the pSB2 ARS, as occurs instead in the *Sc*2μm (Futcher, 1988). This observation led us to investigate the effect of insertion of other pSB2 regions. By the data presented, it is possible to infer that insertion of the FLP recombinase gene led to higher copy numbers, probably due to a higher frequency of recombination between the IR-A and another IR of the endogenous plasmid. The highest activity levels were obtained upon introduction

of the TF-C gene, whose function is not yet clarified. This ORF had presumably an impact on copy number determination, besides the increase of stability. Finally, the *cis*-acting STB sequence of pSB2 was not yet identified; however, the sequence of *Z. rouxii* was shown to be functionally recognized in *Z. bailii*.

Improvement of expression levels is not the only parameter to take into consideration: in fact, the use of antibiotic selection would render the costs of a process prohibitive. This was overcome in this study by the specific *LEU2* integrative vector. Nonetheless, the lack of auxotrophic mutants forces to use an initial dominant selection, and this would limit the number of possible integrations. Therefore, we aimed to obtain auxotrophic mutants through targeted gene deletion.

Deletion of an essential gene in *Z. bailii* was not easy to obtain, as demonstrated by the numerous unsuccessful attempts of our as well as other laboratories (Mollapour & Piper, 2001). It appeared that the main problem consisted of the extremely low frequencies of homologous recombination. Very short sequences (20-50 bp) are usually sufficient to obtain homologous recombination with high efficiencies (up to 90 %), as for the model yeast *S. cerevisiae* (Baudin *et al.*, 1993). In some cases, however, the length of flanking regions can be crucial. For example, in the non-conventional yeast *Hansenula polymorpha* flanking regions of at least 1 Kbp seem to be a key factor to obtain satisfactory homologous recombination efficiencies (about 50 %) (Gonzales *et al.*, 1999). Similarly, for gene deletion in *Candida albicans* flanking regions of more than 1 Kbp were frequently used (Kelly *et al.*, 1986). Hence, it is possible to infer that the length of homologous recombinogenic flanking regions has to be optimized for each individual organism. The lengthening of homologous recombinogenic flanking regions to 800-1300 bp was proved here to be a successful strategy for deletion of an essential gene in *Z. bailii*, as also confirmed by deletion of another essential gene of the glycolytic pathway (Sauer M., personal communication). However, one can imagine that even longer flanking regions could be necessary to raise the frequency of the event, which in our case remained low.

The applicability of the new auxotrophic strain for heterologous productions was verified and compared to the wild type strain, showing also the advantages of using an auxotrophic marker-based selection instead of the antibiotic selection, that have a positive impact on the centromeric plasmid stability and, consequently, on production levels. The next step in this direction will be to establish a method for recycling the selection markers in *Z. bailii*, for example such as the *S.*

cerevisiae Cre-Lox system, that up to date resulted to be ineffective in the nonconventional yeast (our unpublished results). This would permit further manipulation of the same strain.

The availability of a mutant with leucine auxotrophy allowed us to design a new vector for multiple integration specific for *Z. bailii*, based on *LEU*2d marker selection and integration into rDNA. For this purpose, a rDNA region of *Z. bailii* previously unknown was cloned and sequenced. The choice of this region was based on the previously published data regarding copy numbers reached (up to 400) and stability of the plasmids, in function of the rDNA portion included, in *S. cerevisiae* as well as other yeasts (Lopes *et al.*, 1986, 1996; Klabunde *et al.*, 2003). The length of the cloned region was noticeably higher than the expected on the basis of the *S. cerevisiae* rDNA, and the sequence poorly conserved, also relative to other organisms. The high variability in length and sequence, mainly mapping on the NTSs, is anyhow not surprising: these are in fact nonhomologous sequences in eukaryotes, which can also vary among individuals in a species and even within an individual, in higher eukariotes (reviewed in Kupriyanova, 2000). Instead, the regions coding for mature rRNAs are highly conserved among all organisms, corresponding to their conserved secondary structure.

Lopes and co-workers identified the regions responsible for the maintenance of size and homogeneity of the rDNA cluster in yeast as important also for the maintenance of pMIRY-type vectors. These regions are located into the NTSs of the rDNA unit. However, another crucial factor in this sense is represented by plasmid length, influencing stability as well as initial copy number (Lopes *et al.*, 1996). Our data demonstrate that the vector constructed with *Z. bailii LEU*2d and rDNA region comprising the two NTSs effectively drives integration in multiple copies and selection of transformants with high copy numbers. Nonetheless, insertion of a foreign gene prevented the isolation of transformants. This might be due to an excessive length of the resulting plasmid. The heterogeneity of some of the strains (derived from a single clone) transformed with the empty plasmid, in respect to copy numbers, could support this hypothesis, since the instability of the integrated construct might also be caused by an excessive length. The next step will be therefore to construct pZbrDL2d derivatives comprising only shorter fragments of the rDNA. Moreover, minimization of the bacterial sequences present in the plasmid could also be helpful in this direction, since it has been previously reported that these are potentially toxic to other organisms

(Lusky & Botchian, 1981) and may decrease vector stability in yeast (Awane *et al.*, 1992).

Overall, in this study significant advances were reported in the development of the tools for the manipulation of *Z. bailii* and its application to heterologous productions. Having in mind the potential advantages offered by the stress resistance abilities of this yeast, it is possible to state that we are on the road towards a new cell factory.

References

Awane, K., Naito, A., Araki, H. and Oshima, Y. (1992). Automatic elimination of unnecessary bacterial sequences from yeast vectors. *Gene* **121**, 161-165.

Baudin, A., Ozier-Kalogeropoulos, O., Denouel, A., Lacroute, F., Cullin, C. (1993). A simple and efficient method for direct gene deletion in *Saccharomyces cerevisiae*. *Nucl Ac Res* **21**(14), 3329-30.

Bernard, N., Ferain, T., Garmyn, D., Hols, P., Delcour, J. (1991). Cloning of the D-lactate dehydrogenase gene from *Lactobacillus delbrueckii* subsp. *bulgaricus* by complementation in *Escherichia coli*. *FEBS Lett* **290** (1-2), 61-4.

Branduardi, P. (2002). Molecular cloning and sequence analysis of the *Zygosaccharomyces bailii* HIS3 gene encoding the imidazole glycerolphosphate dehydratase.*Yeast* **19**(13),1165-70.

Branduardi, P., Valli, M., Brambilla, L., Sauer, M., Alberghina, L., Porro, D. (2004). The yeast *Zygosaccharomyces bailii*: a new host for heterologous protein production, secretion and for metabolic engineering applications. *FEMS Yeast Res* **4**, 493-504.

Branduardi, P., Sauer, M., De Gioia, L., Zampella, G., Valli, M., Mattanovich, D., Porro, D. (2006) Lactate production yield from engineered yeasts is dependent from the host background, the lactate dehydrogenase source and the lactate export. *Microb Cell Fact* **5**, 4

Camattari, A., Bianchi, M.M., Branduardi, P., Porro, D., Brambilla, L. (2007). Induction by hypoxia of heterologous-protein

production with the KlPDC1 promoter in yeasts. *Appl Environ Microbiol* **73**(3), 922-9.

Cole, M. B. & Keenan, M. H. J. (1987). Effects of weak acids and external pH on the intracellular pH of *Zygosaccharomyces bailii*, and its implication in weak-acid resistance. *Yeast* **3**, 23-32.

Dato, L., Sauer, M., Passolunghi, S., Porro, D., Branduardi, P. (2008). Investigating the multibudded and binucleate phenotype of the yeast *Zygosaccharomyces bailii* growing on minimal medium. *FEMS Yeast Res* **8**(6), 906-15.

Erhart, E. & Hollenberg, C.P. (1983). The presence of a defective LEU2 gene on 2 mu DNA recombinant plasmids of *Saccharomyces cerevisiae* is responsible for curing and high copy number. *J Bacteriol* **156**(2), 625-35.

Fleer, R., **Xin, J. C.**, **Amellal, N.**, **Yeh, P.**, **Fournier, A.**, **Guinet, F.**, **Gault N.**, **Faucher, D.**, **Folliard, F.**, **Fukuhara, H.**, **Mayaux, J-F. (1991).** High-level secretion of correctly processed recombinant human interleukin-1 in *Kluyveromyces lactis*. *Gene* **107**, 285-295.

Futcher, A.B. (1988). The 2 micron circle plasmid of *Saccharomyces cerevisiae*. *Yeast* **4**(1), 27-40.

Gerbaud, C., Fournier, P., Blanc, H., Aigle, M., Heslot, H., Guerineau, M. (1979). High frequency of yeast transformation by plasmids carrying part or entire 2-micron yeast plasmid. *Gene* **5**(3), 233-53.

Gietz, R.D. & Woods, R.A. (2006). Yeast transformation by the LiAc/SS Carrier DNA/PEG method. *Methods Mol Biol* **313**, 107-120.

Goldstein, A.L. & McCusker, J.H. (1999). Three new dominant drug resistance cassette for gene disruption in *Saccharomyces cerevisiae*. *Yeast* **15(**14), 1541-1553.

Gonzales, C., **Perdono, G.**, **Teyera, P.**, **Brito, N.**, **Siverio, J.M. (1999).** One step, PCR mediated, gene disruption in the yeast *Hansenula polymorpha*. *Yeast* **15**(13), 1323-9.

Hoffman, C.S. & Winston, F. (1987). A ten-minute DNA preparation from yeast efficiently releases autonomous plasmids for transformation of Escherichia coli. *Gene* **57**(2-3), 267-72.

Kelly, R., **Miller, S.M.**, **Kurtz, M.B.**, **Kirsch, D.R. (1987).** Directed mutagenesis in *Candida albicans*: one-step gene disruption to isolate ura3 mutants. *Mol Cell Biol* **7**(1), 199-208.

Kikuchi, Y. (1983). Yeast plasmid requires a cis-acting locus and two plasmid proteins for its stable maintenance. *Cell* **35**(2 Pt 1), 487-93.

Klabunde, J., Diesel, A., Waschl, D., Gellissen, G., Hollenberg, C.P., Suckow, M. (2002). Single-step co-integration of multiple expressible heterologous genes into the ribosomal DNA of the methylotrophic yeast *Hansenula polymorpha*. *Appl Environ Microbiol* **58**(6), 797-805.

Kupriyanova, N. S. (2000). Conservation and variation of ribosomal DNA in eukaryotes *Mol Biol* 34(5), 753-765.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685.

Lopes, T.S., **Klootwijk, J.**, **Veenstra, A.E.**, **van de Aar, P.C.**, **van Heerikhuizen, H.**, **Raue, H.A.**, **Planta, R.J. (1989).** High-copy-number integration into the ribosomal DNA of *Saccharomyces cerevisiae:* a new vector for high-level expression. *Gene* **79**, 199-206.

Lopes, T.S., **Hakkaart, G-J.A.J.**, **Koerts, B.L.**, **Raue, H.A.**, **Planta, R.J. (1991).** Mechanism of high copy-number integration of pMIRY-type vectors into the ribosomal DNA of *Saccharornyces cerevisiae*. *Gene* **105**, 83-90.

Lopes, T.S., **De Wijs, I.J.**, **Steenhauer, S.I.**, **Verbakel, J.**, **Planta, R.J**. (1996). Factors affecting mitotic stability of high-copy-number integration into ribosomal DNA of *Saccharomyces cerevisiae*. *Yeast* **12**, 467-477.

Lusky, M. & Botchian, M. (1981). Inhibition of SV40 replication in simian cells by specific pBR322 DNA sequences. *Nature* **293**, 79-81.

Mollapour, M. & Piper, P. (2001). Targeted gene deletion in *Zygosaccharomyces bailii*. *Yeast* **18**, 173-186.

Rodrigues, F., Corte-Real, M., Leao, C., van Dijken, J. P. & Pronk, J. T. (2001a). Oxygen requirements of the food spoilage yeast *Zygosaccharomyces bailii* in synthetic and complex media. *Appl Environ Microbiol* **67**, 2123-2128.

Rodrigues, F., Zeeman, A. M., Alves, C., Sousa, M. J., Steensma, H. Y., Corte-Real, M. & Leao, C. (2001b). Construction of a genomic library of the food spoilage yeast *Zygosaccharomyces bailii* and isolation of the beta-isopropylmalate dehydrogenase gene (ZbLEU2). *FEMS Yeast Res* **1**, 67-71.

Rodrigues, F., Ludovico, P., Sousa, M. J., Steensma, H. Y., Corte-Real, M. & Leao, C. (2003). The spoilage yeast *Zygosaccharomyces bailii* forms mitotic spores: a screening method for haploidization. *Appl Environ Microbiol* **69**, 649-653.

Romanos, M.A., Carol, A., Scorer, A., Clare, J.J. (1992). Foreign gene expression in yeast: a review. *Yeast* **8**, 423-488.

Sambrook, J., Maniatis, T., Fritsch, E.F. (1989). Molecular cloning : a laboratory manual, 2nd edn. Cold Spring Harbor, N.Y.: Cold Spring Harbor Laboratory.

Sauer, M., Branduardi, P., Valli, M., Porro, D. (2004). Production of L-ascorbic acid by metabolically engineered *Saccharomyces cerevisiae* and *Zygosaccharomyces bailii*. *Appl Environ Microbiol* **70**(10), 6086-91.

Sousa, M. J., Miranda, L., Corte-Real, M. & Leao, C. (1996). Transport of acetic acid in *Zygosaccharomyces bailii*: effects of ethanol and their implications on the resistance of the yeast to acidic environments. *Appl Environ Microbiol* **62**, 3152-3157.

Sousa, M. J., Rodrigues, F., Corte-Real, M. & Leao, C. (1998). Mechanisms underlying the transport and intracellular metabolism of acetic acid in the presence of glucose in the yeast *Zygosaccharomyces bailii*. *Microbiology* **144**(3), 665-670.

Sousa-Dias, S., Goncalves, T., Leyva, J. S., Peinado, J. M. & Loureiro-Dias, M. C. (1996). Kinetics and regulation of fructose and glucose transport systems are responsible for fructophily in *Zygosaccharomyces bailii*. *Microbiology* **142**, 1733-1738.

Surana, U., Amon, A., Dowzer, C., McGrew, J., Byers, B. & Nasmyth, K. (1993). Destruction of the CDC28/CLB mitotic kinase is not required for the metaphase to anaphase transition in budding yeast. *EMBO J* **12**, 1969-1978.

Thomas, D. S. & Davenport, R. R. (1985). *Zygosaccharomyces bailii* - a profile of characteristics and spoilage activities. *Food Microbiology* **2**, 157-169.

Toh-e, A., Araki, H., Utatsu, I., Oshima, Y. (1984). Plasmids resembling 2-micrometers DNA in the osmotolerant yeasts *Saccharomyces bailii* and *Saccharomyces bisporus*. *J Gen Microbiol* **130**(10), 2527-34.

Utatsu, I., Sakamoto, S., Imura, T., Toh-e, A. (1987). Yeast plasmids resembling 2µm DNA: regional similarities and diversities at the molecular level. *J Bacteriol* 5537-5545.

Vanoni, M., Vai, M., Popolo, L., Alberghina, L. (1983). Structural heterogeneity in populations of the budding yeast *Saccharomyces cerevisiae*. *J. Bacteriol* **156**, 1282-1291.

Venturini, M., Morrione, A., Pisarra, P., Martegani, E., Vanoni, M. (1997). In *Saccharomyces cerevisiae* a short amino acid sequence facilitates excretion in the growth medium of periplasmic proteins. *Mol Microbiol* **23**, 997-1007.

Vigentini, I., Brambilla, L., Branduardi, P., Merico, A., Porro, D., Compagno, C. (2005). Heterologous protein production in *Zygosaccharomyces bailii*: physiological effects and fermentative strategies. *FEMS Yeast Res* **5**(6-7), 647-52.

Wach, A., Brachat, A., Pohlmann, R. and Philippsen, P. (1994). New heterologous modules for classical or PCR-based gene disruptions in *Saccharomyces cerevisiae*. *Yeast* **10**, 1793-1808.

Cloning of the *Zygosaccharomyces bailii GAS*1 homologue and effect of cell wall engineering on protein secretory phenotype

Paper submitted to Microbial Cell Factories

Simone Passolunghi[1]*, Luca Riboldi [12]*, Laura Dato[1], Danilo Porro[1§], Paola Branduardi[1]

[1]University of Milano-Bicocca, Department of Biotechnology and Bioscience, Milan, Italy
[2] current address: CPC Biotech S.r.l., Naples, Italy

*These authors contributed equally to this work
§Corresponding author

Email addresses:
    SP: simone.passolunghi@unimib.it
    LR: luca.riboldi@cpcbiotech.it
    LD: laura.dato@unimib.it
    DP: danilo.porro@unimib.it
    PB: paola.branduardi@unimib.it

Abstract

Background

Zygosaccharomyces bailii is a diploid budding yeast still poorly characterized, but widely recognised as tolerant to several stresses, most of which related to industrial processes of production. Because of that, it would be very interesting to develop its ability as a cell factory. Gas1p is a -1,3-glucanosyltransglycosylase which plays an important role in cell wall construction and in determining its permeability. Cell wall defective mutants of *Saccharomyces cerevisiae* and *Pichia pastoris*, deleted in the *GAS*1 gene, were reported as super-secretive. The aim of this study was the cloning and deletion of the *GAS*1 homologue of *Z. bailii* and the evaluation of its deletion on recombinant protein secretion.

Results

The *GAS*1 homologue of *Z. bailii* was cloned by PCR, and when expressed in a *S. cerevisiae GAS*1 null mutant was able to restore the parental phenotype. The respective *Z. bailii gas*1 deleted strain was obtained by targeted deletion of both alleles of the *ZbGAS*1 gene with deletion cassettes having flanking regions of ~400bp. The morphological and physiological characterization of the *Z. bailii* null mutant resulted very similar to that of the corresponding *S. cerevisiae* mutant. As for *S. cerevisiae*, in the *Z. bailii gas*1 the total amount of protein released in the medium was significantly higher. Moreover, three different heterologous proteins were expressed and secreted in said mutant. The amount of enzymatic activity found in the medium was almost doubled in the case of the *Candida rugosa* lipase CRL1 and of the *Yarrowia lipolytica* protease XPR2, while for human IL-1 secretion disruption had no relevant effect.

Conclusions

The data presented confirm that the engineering of the cell wall is an effective way to improve protein secretion in yeast. They also confirmed that *Z. bailii* is an interesting candidate, despite the knowledge of its genome and the tools for its manipulation still need to be improved. However, as already widely reported in literature, our data confirmed that an "always working" solution to the problems related to recombinant protein production can be hardly, if never, found; instead, manipulations have to be finely tuned for each specific product and/or combination of host cell and product.

Background

The "non-conventional" yeast *Zygosaccharomyces bailii* has been proposed as a possible new player in biotech processes arena [1]. Its tolerance to high sugar concentrations, low pH environments caused by organic acids, relatively high temperatures [2] could allow its growth under restrictive conditions. These features can be very useful for industrial fermentations because they can simplify the process, improving the economical value. In addition, the high specific growth rate of *Z. bailii* and its high biomass yield render this yeast particularly attractive for those applications were the biomass itself, or a specific protein or metabolite, is the desired product. In respect to recombinant protein production, it has been demonstrated that production and secretion levels of the recombinant human interleuchin-1β (rhIL-1β) and of fungal glucoamylases in *Z. bailii* are higher than the levels obtained with *S. cerevisiae* by the use of the same centromeric expression vector [3].

From an industrial point of view, production and secretion capabilities of the host systems currently in use are still insufficient. In particular, secretion of the heterologous protein often represents one of the main bottlenecks. Different strategies can be utilized in this respect, and among them are: improvement of the heterologous gene expression levels, signal sequence optimization, co-expression of chaperones and foldases, introduction of mutations which improve secretion

capabilities and reduction of the proteolytic activity in secretion vesicles [4].

Since the yeast cell wall constitutes a physical barrier to large molecules transit, it represents one of the main limits for heterologous protein secretion. Thus, mutations which directly or indirectly alter the cell wall organization can lead to better secretion capabilities of a yeast host [5].

Mutations in genes involved in the construction and in the maintenance of the cell wall, such as *PMR1*, *SEC14*, *ERD1*, *MNN9* and *MNN10*, have in some cases been demonstrated to lead to supersecretive mutants in *S. cerevisiae* and other yeasts [5]. The results evidence a tight correlation between glycosylation processes and protein secretion [6]. It was hypothesized that this correlation is not directly linked to the heterologous secreted proteins, but rather to an altered structure of the glycosidic residues added to the cell wall (glycol)proteins [5], which in turn leads to an altered permeability of the cell wall itself.

Among the already mentioned target, also the inactivation of the *GAS*1 gene, whose product is directly involved in the synthesis of the cell wall, led to a hypersecretive phenotype in *S. cerevisiae* [7]. The *Sc*Gas1p is an extracellular glycoprotein, anchored to the cell membrane through a GPI tail, which has a β-1,3-glucanosyltransferase activity. This enzyme plays a central role in the formation of cell wall glucans: it catalyzes the crosslinking of the glucans resulting in the β-1,3-glucan elongation [8]. The identification of Gas1p homologues in other yeast species and some pathogenic fungi led to the definition of a new glycosyl-hydrolases family. Recently, the hypothetical 3D structure of the catalytic domain of the protein was built by homology modelling [9]. The *GAS*1 gene disruption leads to peculiar morphological and physiological phenotypes due to an altered cell wall structure and composition. In particular, it was reported an altered distribution of the percentages of β-1,3- and β-1,6-glucans, the latter being higher than in wild type strain [10]. It was also reported an increase in the chitin and mannoproteins levels, together with altered linkages between the different components [11]. As a direct or indirect result of these cell wall structure modifications, the *gas1* mutant shows higher secretion levels if compared to the wild type, both for total and for the heterologous recombinant proteins, as the human insuline-like growth factor (rhIGF1), [7]. More recently, the *Pichia pastoris GAS*1 homologous gene was cloned and then deleted, also resulting in a yeast mutant showing a supersecretive phenotype for

some heterologous proteins, like a yeast lipase, but not for other, like the human trypsinogen [12].

Here we demonstrate that the deletion of the *Z. bailii GAS*1 homologue leads to an improved heterologous protein secretion in this new promising host. For reaching this goal, the *ZbGAS1* gene was firstly cloned and then deleted thanks to the setting up of a protocol for gene deletion in this non-conventional diploid yeast. We describe here the morphological and physiological characterization of the *Zb∆gas*1, which, in analogy to the *Sc∆gas*1 and *Pp∆gas*1mutants, resulted to have a supersecretive phenotype.

Results

PCR cloning for *Z. bailii GAS*1 homologue

Because of the high phylogenetic correlation between *Z. bailii* and *S. cerevisiae* [13] and because of the high percentage of identity among the few known *Z. bailii* and the corresponding *S. cerevisiae* gene sequences, our first attempt was to clone the gene homologous to *GAS*1 in *Z. bailii* by PCR amplification, using primers designed on the 3' and 5' ends of the *ScGAS*1 coding sequence, but with no success.

It was therefore necessary to design internal primers choosing the most conserved regions of the gene, based on multiple sequence alignments. The *Candida glabrata* and the *Schyzosaccharomyces pombe* homologous genes (overall sequence identity to *ScGAS*1: 71% and 62% respectively), in addition to the *S. cerevisiae* one, were chosen for comparison. Two amplification primers were designed on *ScGAS1* in (sub)regions sharing nearly 100% sequence identity with the two homologues, at about 200 bp and 300 bp from the ATG and the STOP codon, respectively. A 1137 bp fragment was amplified, using *Z. bailii* genomic DNA as a template, sharing 72% sequence identity with the corresponding internal region of *ScGAS*1. Translation into the putative aminoacidic sequence led to the identification of a region with 74% identity to the *Sc*Gas1p protein fragment spanning from the catalytic domain to the beginning of the Cys Box (WU-Blast2 alignment results, Figure 1).

```
              10        20        30        40        50        60        70        80        90
ZbGAS1 MLFQAFSTLALGALYAASGAVAASSSSATKLPAIEVAGNKFFYSNNGSQFYIKGVAYQADTANSSSDDSIDDPLADYSKCSRDIPYLQKLQTN  93
ScGAS1 MLFKSLSKLATAAAFFAG------VATADDVPAIEVVGNKFFYSNNGSQFYIRGVAYQADTANETSGSTVNDPLANYESCSRDIPYLKKLNTN  87

             100       110       120       130       140       150       160       170       180
ZbGAS1 VVRVYAVNTTLDHSKCMEALADAGIYVIADLSTPADSVNRNDPTWDIALYQRYTGVVDAFANYTNVLGFFAGNEVTNNASNTDASAFVKAAVR 186
ScGAS1 VIRVYAINTTLDHSECMKALNDADIYVIADLAAPATSINRDDPTWTVDLFNSYKTVVDTFANYTNVLGFFAGNEVTNNYTNTDASAFVKAAIR 180

             190       200       210       220       230       240       250       260       270
ZbGAS1 DVKQYIKDKKYRTIPVGYSSNDDEDTRVAMADYFACGDEDQKADFYGINMYEWCGDSTYQKSGYQDRTNDFKNLSIPIFFSEYGCNAVTPRKF 279
ScGAS1 DVRQYISDKINYRKIPVGYSSNDDEDTRVKMTDYFACGDDDVKADFYGINMYEWCGKSDFKTSGYADRTAEFKNLSIPVFFSEYGCNEVTPRLF 273

             280       290       300       310       320       330       340       350       360       370
ZbGAS1 TEVQTLYGDQMTDVWSGGIVYMYFEETNKYGLVSIDDDQVKTLTDFDNLSSQMAKISPSAAKNSSYTAKSTSLSCPPATGKYWKANTKLPPTPS 372
ScGAS1 TEVEALYGSNMTDVWSGGIVYMYFEETNKYGLVSIDGNDVKTLDDFNNYSSEINKISPTSANTKSYSATTSDVACPATGKYWSAATELPPTPN 366

             380       390       400       410       420       430       440       450       460
ZbGAS1 KDLCTCMEDSLSCVVDDKVDEDDYSDLFSYICSKVDCSGITANGTSGKYGSYSFCSAKEQLSFVMNLYYEKNGGSKSDCSFSGSASLKSATTK 465
ScGAS1 GGLCSCMNAANSCVVSDDVDSDDYETLFNWICNEVDCGISANGTAGKYGAYSFCTPKEQLSFVMNLYYEKSGGSKSDCSFSGSATLQTATTQ 459

             470       480       490       500       510       520       530       540       550
ZbGAS1 SACSSALKQIGSSGLGSASKSVT-GSASGSSSGGSSSASGSSESSSGKGSSSSKGGDSSSSSKKSSSTASTKEKNFAAASKASLSQVILSFVAT 557
ScGAS1 ASCSSALKEIGSMGTNSASGSVDLGSGTESSTASSNASGSS-SKSNSGSS----GSSSSSSSASSSSSKKNAATNVKANLAQVFTSIIS 547

             560
ZbGAS1 LGVVAGAGFALA 569
ScGAS1 LSIAAGVGFALV 559
```

Figure 1 – **Aminoacidic sequence of ZbGAS1p**
Putative aminoacidic sequence of the *ZbGas1*p protein and alignment with the homologue *Sc*Gas1p. Grey scale coloring represents aminoacid similarity based on Blosum 62 scores. Alignment was performed with ClustalW software (http://www.ebi.ac.uk/clustalw) and graphical editing was made using Jalview software [30].

This confirms data reported in literature which indicate the catalytic domain and the Serine and Cysteine Boxes as the most conserved regions of the *GAS*1 homologues in different yeast species [8].

The 5' and 3' regions of the *ZbGAS1* homologue were subsequently amplified from a Genome Walker library constructed from the *Z. bailii* genomic DNA using the Universal GenomeWalker® Kit (Clontech). Gene-specific primers used were *ZbGAS1*intREV and *ZbGAS1*intFW for the 5' and 3' ends respectively (see Methods). The complete ORF of *ZbGAS*1 was reconstructed in this way, and additional 262 bp upstream to the ATG and 177 bp after the STOP codon were also sequenced (GeneBank Accession N° GU136594).

Based on the new sequence, it was possible to design specific primers for the *ZbGAS1* gene (see Methods) and a DNA fragment of the expected length was amplified from the *Z. bailii* genomic DNA.

The complete *ZbGAS*1 ORF resulted to be 1710 bp long (+ 30 bp *vs ScGAS*1), sharing a 67 % sequence identity with the *S. cerevisiae* homologue (file 1 "clustalw_ntd.pdf" of additional file section). This sequence encodes for a putative aminoacidic sequence of 569 aa (+10 aminoacids *vs Sc*Gas1p) having 68 % identity with the *Sc*Gas1p protein (Figure 1). Sequence alignments were also performed between the newly isolated *Z. bailii* ORF and sequences of other homologous *GAS* genes in *S. cerevisiae* (*GAS*1-5): the lower identity scores obtained for sequences other than *ScGAS*1 (not shown) further indicated that we cloned the *GAS*1 homologue of *Z. bailii*.

Hence, considering the sequence similarities between the *ScGAS*1 gene and their homologues in other yeasts, the *GAS*1 gene resulted to be highly conserved also in *Z. bailii*. The overall deduced protein structure seems to be also conserved.

The *ZbGAS*1 gene is one of the few *Z. bailii* gene sequences isolated to date, hence representing a further little step towards the knowledge of this still poorly characterized yeast.

ZbGAS1 gene disruption

Two different *ZbGAS*1 deletion cassettes were constructed based on the *kan*[R] and *hph*[R] markers. Differently from the case of the *ZbYME*2 gene deletion [14], where flanking regions of about 60 to 80 bp were described to be sufficient for obtaining homologous recombination, long homologous flanking regions were necessary to obtain gene replacement. In particular, we constructed both the deletion cassettes, represented in Figure 2a, with flanking regions of about 400 bp, which resulted to be sufficient to disrupt the first and as well the second allelic copy of *ZbGAS*1, despite with quite low efficiency (about only 10% of the transformants resulted to effectively have both the deleted alleles, confirming what previously described [14]). The two cassettes (see Methods) were therefore used to transform *Z. bailii* cells, resulting in the *gas1* null mutant. The insertion of both deletion cassettes in the homologous loci was confirmed by PCR (Figure 2b). We applied the same strategy for the deletion of the first essential *Z. bailii* gene, leading to the first auxotrophic mutant of this yeast, and in this case we had to further extend the length of the homologous region for specific recombination, and despite that the percentage of positive events was even lower (manuscript in preparation). These results could lead to hypothesize that for targeted gene deletion in *Z. bailii* the length of the flanking regions could be crucial according to the specific role that a specific protein plays, thus explaining results obtained by other researchers [14].
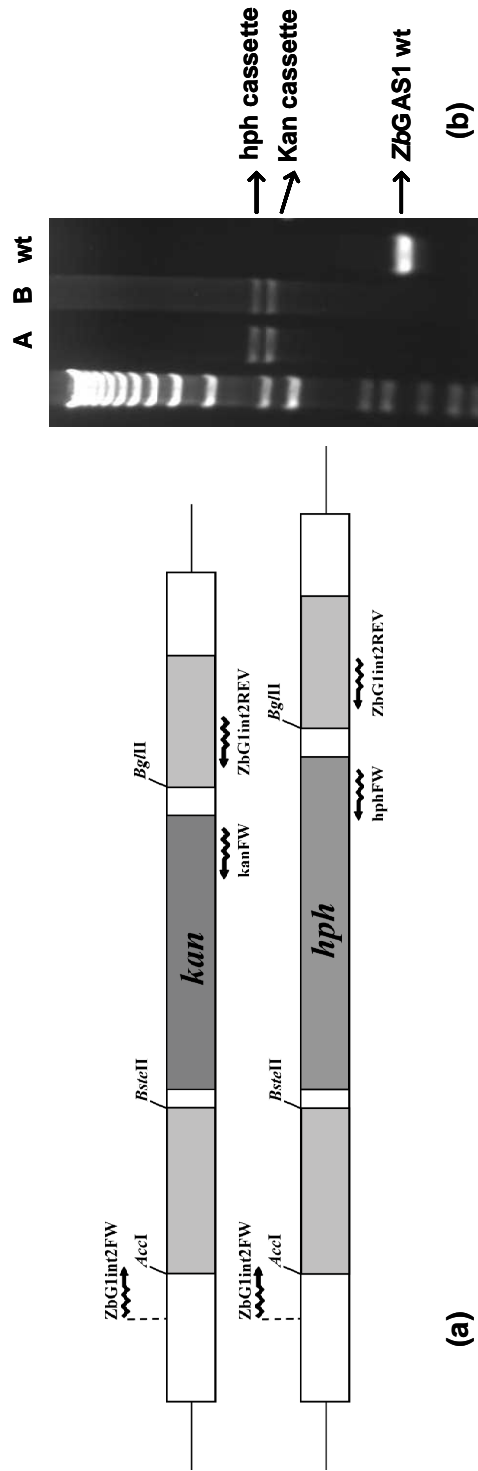
*Figure 2 - **Deletion of ZbGAS1***
(a) Schematic representation of the *ZbGAS1* deletion cassettes. Restriction enzymes used to obtain the constructs and PCR primers used to verify recombination are indicated, light grey bars represents *ZbGAS1* sequence. (b) Control PCR performed to verify the deletion of both the allelic copies of *ZbGAS1*. Two independent mutant clones (A and B) were tested. In both clones it is possible to see the presence of bands corresponding to the two deletion cassettes and the absence of the band corresponding to the wild type gene copy.

Morphological and physiological characterization of the null mutant

The *GAS*1 gene inactivation leads to different peculiar phenotypes in *S. cerevisiae* that were widely characterized [10]. We therefore performed a morphological and physiological characterization of the obtained *Z. bailii* Δ*gas*1 mutant in analogy to what reported in literature for the model yeast. First, some morphological traits of the *Sc* Δ*gas*1 mutant are visible both by light and by fluorescence microscopy after Calcofluor White staining. It was reported for *S. cerevisiae* (both haploid and diploid) mutants a rounded shape and higher cell diameter if compared to wild type cells, especially in the stationary phase [10]. We performed the analysis of the *Zb*Δ*gas*1 and wild type strains in parallel to the *S. cerevisiae* BY4741 Δ*gas*1 and wt strains. Microscopy images are shown in Figure 3. It is possible to see the typical rounded phenotype also for the *Zb*Δ*gas*1 (panels C and G). Moreover, like in *S. cerevisiae*, intracellular granules and altered vacuoles are also visible (indicated by arrows, panels C and G). The altered shape is also reflected by cellular volumes, being higher in the mutants, as assessed by coulter counter measurements in cell populations growing in minimal or in rich medium; like for the model yeast, differences are more pronounced in stationary phase, were the mutant cell volume is 1.6 fold larger in respect to the wild type cells (data not shown). Another feature described for the *Sc*Δ*gas*1 mutant is the appearance of large cellular aggregates, still permanent after repeated sonication cycles [10]. This feature is also conserved in the *Z. bailii* mutant, as it is visible from the microscopy images of sonicated cells (Figure 3, panels C, D, G and H). The morphological alterations described are likely a consequence of the altered structure and composition of the mutant cell wall: it was reported for *S. cerevisiae* a lowered glucans content together with a marked increase in the chitin and mannoproteins content [15]. Moreover, an altered distribution of the cell wall components in the mother and daughter cell have been reported [10]. Our analyses confirmed these alterations also for the *Z. bailii* mutant: overall Calcofluor staining, in fact, was more intense in Δ*gas*1 than in wild type strain (Figure 3, panels D and H), indicating a higher chitin content in the mutant. The chitin seems to be delocalized throughout the entire cell wall in the mutant, while in the wild type it is more concentrated at the budding ring and at the bud scars.
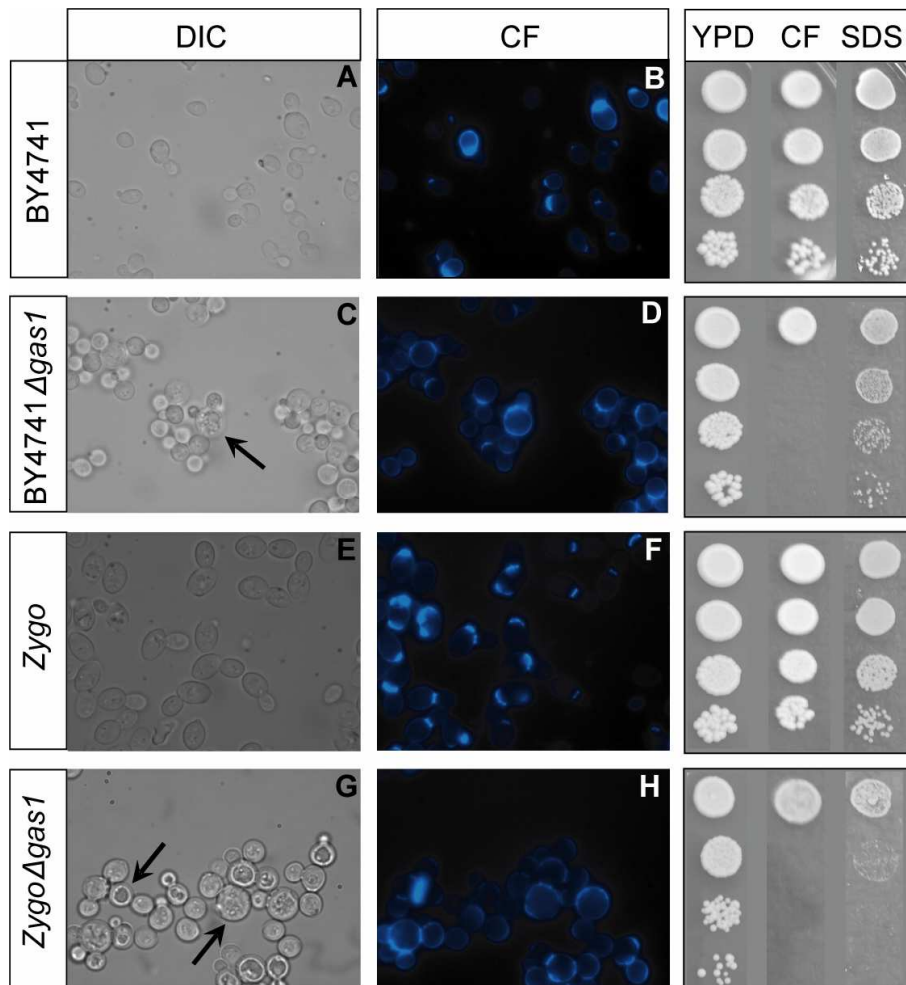
82

Figure 3 - **Morphology of *Z. bailii* and *S. cerevisiae* wild type and *gas1* null mutant strains and their sensitivity to Calcofluor White and SDS**

Cellular morphology: from A to H: dicroic and fluorescence microscope images of *Z. bailii* and *S. cerevisiae* wild type and *gas*1 null mutant strains. Cells were grown in minimal medium and harvested in stationary phase of growth. Arrows indicate granules and enlarged vacuoles in mutant cells (panels C and G).

Calcofluor White and SDS sensitivity of S. cerevisiae (BY4741) and Z. bailii (Zygo) gas1 null mutants: 4 μl of cultures of the different strains (BY4741, BY4741 Δ*gas*1, *Zygo* and *Zygo* Δ*gas*1) were spotted starting with OD (660 nm) = 1 and then in 3 serial solution 1:10 (2,3,4) on rich solid medium (YPD) and on the same medium added with 12.5 μg ml^{-1} of CFW or 50 μg ml^{-1} SDS. Pictures were captured after 5 days of incubation at 30°C.

Moreover, in the mutant the cellular buds are also Calcofluor-stained, in contrast to the wild type. It was proposed that the increase in chitin levels and cross-links with β-1,6-glucosyl mannoproteins may be the effect of the activation of a compensatory mechanism in mutants characterized by defects in the cell wall architecture, like the Δ*gas*1 mutants. The aim of this response would be to prevent excessive loss of mannoproteins as a consequence of the inability to cross-link the β-1,6-glucosyl mannoproteins and the β-1,3-glucans, a process in which Gas1p plays a central role [15]. It was also hypothesized that the loss of Gas1p may cause a weakening of the cell wall of the emerging bud. The yeast cell could, in response to this weakening, target the accumulation of chitin at the bud surface in order to protect it from lyses [16]. Alteration of the processes directly or indirectly linked to budding leads to the appearance of a relative high fraction, in the cell population, of multibudded cells (the so-called "Mickey Mouse" phenotype) (fig. 3). A similar phenotype was also visible in *Zb*Δ*gas*1 mutants (Figure 3).

The altered cell wall structure renders the deleted bakers' yeast strain more sensitive to sodium dodecyl sulphate (SDS) and to Calcofluor White (CFW), as reported in literature for the W303 background [17]. Here we confirm by spot assays the sensitivity to both agents of the *Sc*Δ*gas*1 mutant, background BY4741 (Figure 3, upper right panels), and as well of the *Zb*Δ*gas*1 mutant (Figure 3, lower right panels).

Finally, to verify the functionality of the *GAS*1 homologue of *Z. bailii* in *S. cerevisiae* and the similarity of its biological role, the *ZbGAS*1 gene was expressed into the *GAS1*-deficient *S. cerevisiae* strain, resulting in wild type phenotype restoration either in respect to the morphological traits as well as for the chitin distribution (data not shown). Moreover, to verify the functionality of the *S. cerevisiae GAS*1 in *Z. bailii*, the *ScGAS*1 gene was expressed into the *GAS1*-deficient *Z. bailii* strain, also in this case restoring the native phenotype (data not shown).

Overall morphological and physiological data strongly indicate that the *ZbGAS*1 gene may have a similar role, with respect to the *ScGAS*1 homologous, in the assessment of the cell wall structure and, hence, their inactivation might have similar effects in the two yeasts in respect to secretion abilities.

Supersecretive phenotype of Δ*gas*1 strains

As already mentioned (see Introduction), the most interesting phenotype of Δ*gas1* mutants reported in literature is the supersecretive property. Besides the increased secretion of heterologous proteins, which appears to be dependent on the individual protein [12], the levels of total secreted proteins in late-stationary phase cultures of *S. cerevisiae* mutants were two fold higher with respect to the wild-type [7]. We therefore started our evaluation of secretive properties of the *Z. bailii* mutant, in the perspective of a possible application in industrial processes, by determining the amount of total proteins secreted in the growth medium during batch cultivation with respect to the wild type strain. *Z. bailii* and *S. cerevisiae* wild type and Δ*gas1* respective strains were grown in shake flasks in minimal YNB-based medium containing 5% glucose. Cells were harvested at different times of cultivation and the fermentation broths were assayed. At least triplicate experiments were performed on three independent transformants, and Figure 4 shows the mean data obtained reported as mg of total proteins secreted per litre of the culture broth, normalised for OD values. The values refers to 72h of cultivation, but it was possible to observe a trend leading to this result starting at the end of exponential phase and becoming more pronounced in stationary phase of growth. The data clearly underline the overall higher secretive capacity of *Z. bailii* if compared to *S. cerevisiae*, in accordance to heterologous proteins secretion data already reported [3]. Moreover, they indicate a significant improvement in the secretive capability of the *Z. bailii* mutant, being nearly 80% more than the wild type in terms of total endogenous protein, similarly to the *S. cerevisiae* mutant.
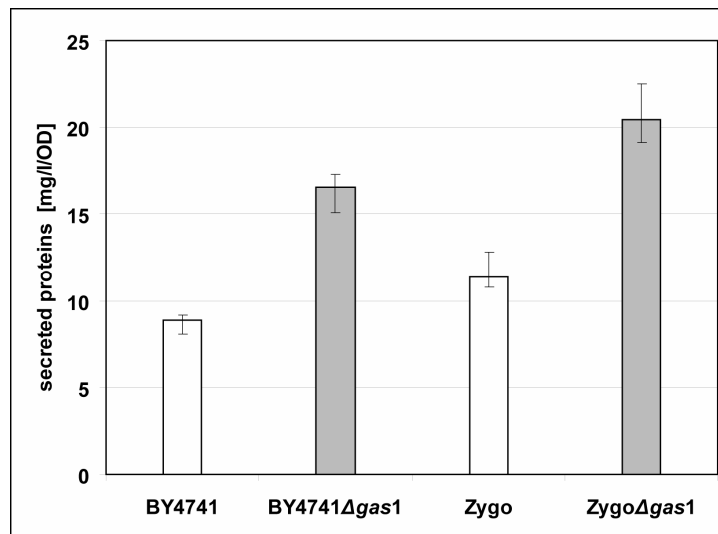
Figure 4 - **Total proteins secretion levels**
Total proteins secretion levels in the culture medium of batch cultures of the indicated wild type and Δgas1 strains of *S. cerevisiae* and *Z. bailii*. Yeasts were grown in shake flasks in minimal YNB-based medium with 5% glucose. Cells were inoculated at 0.1 OD and harvested after 72 hours of cultivation. Amount of secreted proteins (in mg l^{-1} of culture broth) were normalized for OD values.

Overexpression of CRL lipase, XPR2 protease and hIL-1β in *Z. bailii* Δgas1 strains

With the intention to evaluate the potential effects of cell wall engineering and to test the secretive performance in the presence of an heterologous protein, three model proteins were tested: a fungal lipase from *Candida rugosa* (CRL1), a fungal protease from *Yarrowia lipolytica* (XPR2) and the human interleukin 1β (hIL-1β).
Four clones of *Z. bailii* Δgas1 [pZLN022XPR2] and four clones of the parental strain *Z. bailii* [pZLN022XPR2], together with control clones harbouring the respective empty plasmid were pre-cultivated for 16 hours in shake flasks using YPD medium, and then transferred in shake flasks in minimal YNB-based medium containing 5 % glucose. The amount of secreted recombinant XPR2 in culture supernatants was tested in terms of enzymatic activity after 72, 96, 120 and 148 h with the Azocoll method (see methods for detail). The *Zb*Δgas1 transformants showed a relevant increase in specific activity levels

since 72 h (+ 25% at 72 h, more than two-fold at 148 h, see Figure 5a), in respect to wild type transformants.

Similarly, three clones of *Z. bailii* Δ*gas*1 [pZLN022LIP1] and three clones of the parental strain *Z. bailii* [pZLN022LIP1], together with control clones harbouring the respective empty plasmid were pre-cultivated for 16 hours in shake flasks using YPD medium, and then transferred in shake flasks in minimal YNB-based medium containing 5 % glucose. The amount of secreted recombinant LIP1 in culture supernatants was tested in terms of enzymatic activity after 24, 48, 72 and 120 h using the hydrolysis of *p*-nitrophenylesters method (see methods for detail). Also for this second heterologous product the Δ*gas*1 transformants showed a relevant increase in specific activity levels since 24 h (+35% at 24 h, more than two-fold at 120 h, see Figure 5b), in respect to wild type transformants.

Finally, three clones of *Z. bailii* Δ*gas*1 [pZ$_5$klIL-1β] and three clones of the parental strain *Z. bailii* [pZ$_5$klIL-1β], together with control clones harbouring the respective empty plasmid were tested for secretion level of the human interleukin, since the coding sequence was functionally linked to the signal peptide of the K1 killer toxin from *K. lactis*. The independent transformants were pre-cultivated and then cultured in shake flasks containing rich medium (YPD, 5% w/v glucose). Samples were collected at the indicated times (see Figure 5c), cells were harvested and both the supernatants and the cell protein extracts of said samples were loaded on a polyacrylamide gel. The data obtained by Western blot (see Figure 5c) performed on the supernatant showed a well represented band corresponding to the recombinant protein for both *Z. bailii* and *Z. bailii* Δ*gas*1 samples, but differently from the case of recombinant fungal enzymes, no relevant differences in favour of the deleted yeast are appreciable. Remarkably, the signal corresponding to the protein retained intracellularly is more intense in *gas1* null mutant than the signal obtained from *Z. bailii* parental strain, indicating an incomplete secretion of this protein.
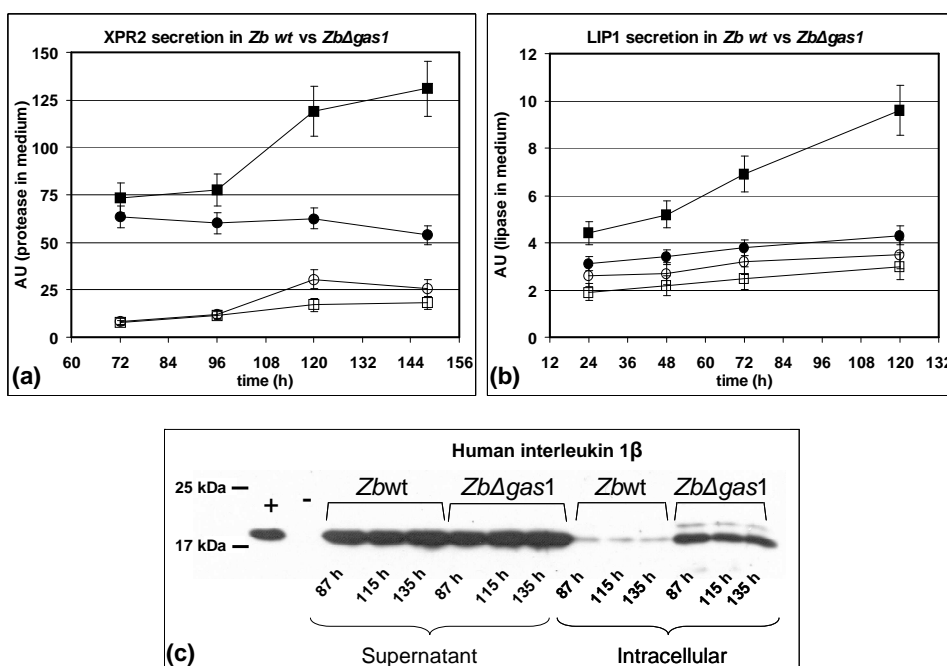
Figure 5 - **Secretion levels of yeast protease, lipase and rhIL-1β in *Z. bailii* and *ZbΔgas*1**

(a) Secretion levels of protease in *Z. bailii* and *ZbΔgas*1 grown in min media with 5% glucose. Open circles and open squares indicate wild type and Δ*gas*1 mutant transformed with control plasmid. Solid circles and solid squares indicate wild type and Δ*gas*1 mutant transformed with plasmid with *XPR2* gene.

(b) Secretion levels of lipase in *Z. bailii* and *ZbΔgas*1 grown in min media with 5% glucose. Open circles and open squares indicate wild type and Δ*gas*1 mutant transformed with control plasmid. Solid circles and solid squares indicate wild type and Δ*gas*1 mutant transformed with plasmid with *LIP1* gene.

(c) Western blots for rhIL-1β of protein samples from supernatants and cell extracts of *Z. bailii* and *ZbΔgas*1 transformants grown in YPD media with 5% glucose, inoculated at OD660 = 0,1. From left to right: (A) rhIL-1β; (B) negative control *Z. bailii*[pZ5]; (C) (D) (E) (I) (L) (M) *Z. bailii* [pZ5*Kl*rhIL-1β]; (F) (G) (H) (N) (O) (P) *ZbΔgas*1 [pZ5*Kl*rhIL-1β]. Time and sample types are indicated in the picture.

88

Discussions

Cell wall engineering can improve the release of heterologous proteins from yeasts [18]. Generally speaking, *GAS*1, which mediates the crosslinking of cell wall components, is directly involved in defining the permeability of the cell. Cloning of the unknown *Z. bailii GAS*1 homologue by PCR was only possible starting from highly conserved internal regions, identified upon alignment of the *GAS1* homologues of three yeast species. The 5' and 3' ends were subsequently amplified using a Genome Walker library. With this approach, the full-length *Z. bailii GAS*1 homologue could be amplified and sequenced. The cloned gene was expressed in the *GAS*1-deficient *S. cerevisiae* strain and reversion of the phenotype proved that this homologue is not only structurally but also functionally similar to the *S. cerevisiae* gene. Moreover, also the *ScGAS*1 gene complemented the *Z. bailii* mutant. Disruption of *GAS*1 in *Z. bailii* revealed its influence on the cell wall morphology. The *GAS*1 null mutants of *Z. bailii* showed the same phenotype reported for *S. cerevisiae* [10], with enlarged cells often showing granular cytoplasm, big vacuoles and two buds (Mickey Mouse-like appearance). Finally, also the sensitivity to SDS and CFW appeared very similar to what observed in the *S. cerevisiae* mutant. These results suggest a similarity of the cell wall structures of the two yeasts. Moreover, also the *ScGAS*1 gene restored the defect of the *Z. bailii* mutant. Disruption of *GAS*1 in *Z. bailii* revealed the influence of the gene on the cell wall morphology. The *GAS*1 null mutants of *Z. bailii* showed the same phenotype as reported for *S. cerevisiae* [10], where enlarged and cells very often show granular cytoplasm, big vacuoles and where budded cells frequently appear with two buds (Mickey Mouse-like appearance). Finally, also the sensitivity to SDS and CFW appears very similar to what observed in the *S. cerevisiae* mutant. A comparison of the different *GAS*1 null phenotype mutants and the functionality of the *Z. bailii GAS*1 homologue in *S. cerevisiae* suggest a similarity of the cell wall structure of *Z. bailii* compared with the cell wall structure of *S. cerevisiae*. Considering the secretory pathway of a secreted protein, it was speculated whether the protein becomes retarded in the cell wall after release from the plasma membrane. The disruption of *GAS*1 in *Z. bailii* first of all confirmed a general phenomenon of protein release in respect to the wild type strain, as demonstrated by the higher accumulation of total protein in the growing medium of the deleted strain when compared to the

parental strain. Moreover, production strains for XPR2 and LIP1 showed a clear improvement in the secretion of the respective proteins. In the case of h IL-1β the enhancement of product secretion was not so evident, and difficult to interpret in respect to the higher accumulation of intracellular product. It could be speculated that this can be related to the nature or to the source of the protein, but too few information are available at the moment to further comment in this direction. What seems to be true is unfortunately what we very often experience for recombinant protein production, which is the protein and protein-host dependence. In fact, also in *P pastoris* two out of three heterologous proteins resulted more secreted in the mutant, as for *Z. bailii*, and when we tested the same protein, in particular the IL-1 secretion in the *S. cerevisiae gas*1 mutant (data not shown), we did not register any improvement.

Methods

Yeast strains, media and transformation

The *S. cerevisiae* strains used in this study were the auxotrophic haploid BY4741 (*Mat a, his3Δ1, leu2Δ0, met5Δ0, ura3Δ0*) and the relative null mutant in *GAS*1 sequence, called in this study, BY4741 Δ*gas*1 (*Mat a, his3Δ1, leu2Δ0, met5Δ0, ura3Δ0,* YMR307*::kanMX4*). Both strains are commercially available from Euroscarf collection (http://web.uni-frankfurt.de/fb15/mikro/euroscarf/col_index.html).The *Z. bailii* wild type strain is ATCC60483. Yeast cultures were grown in YP medium (10 g l^{-1} yeast extract, 20 g l^{-1} peptone) with 2% or 5% w/v glucose (D) or fructose (F) as carbon source. Where needed, NAT (80 mg l^{-1}) or G418 (500 mg l^{-1} for *S. cerevisiae* and 200 mg l^{-1} for *Z. bailii*) or hygromicine (100 mg l^{-1} for *Z. bailii*) were added to the media. Alternatively, yeast cultures were grown in minimal synthetic medium (0.67 % w v^{-1} YNB Biolife without amino acids) with 2% or 5% w v^{-1} glucose as carbon source. When required, supplements such as histidine, leucine, methionine and uracil were added to a final concentration of 50 mg l^{-1}. Media for plates were solidified by addition of agar (Biolife, Milan, Italy) to 1.5% w v^{-1}. Shake flask experiments were carried out with 50 ml of medium in 250 ml shake

flasks on a shaker at 30°C with 160 rpm. For sensitivity tests in Petri dishes 12.5 µg ml^{-1} of CFW or 50 µg ml^{-1} SDS were added to solid media. Yeast transformation was performed using the Lithium Acetate/ssDNA method as described [19] and slightly adapted for *Z. bailii*, as described [3].

Genes amplifications, deletion cassettes and expression plasmid construction

PCR amplifications were made from yeasts genomic DNA, prepared as described in [20]. Amplifications were performed by the Pwo DNA Polymerase (Roche).

The amplification of the *ZbGAS*1 gene was as follows: the primers *GAS*1int_fw: 5'-AGT TGT TCC AGA GAT ATT CCA TAC CTC AA-3' and *GAS*1int_rev: 5'-GCA GAA CCG CTG AAG CTA CAG T-3' designed on internal conserved region of the available sequences from other yeasts were firstly used to amplify an internal region of 1137 bp of the *Z. bailii* gene with the following program: 94° C 5 min; (94°C 15 s, 57°C 30 s, 72°C 1 min) x25; 72°C 7 min; 4°C ∞. After that, the designed primers *ZbGAS*1int_fw: 5'-GGA CGA TAA GGT TGA CGA AGA-3' and *ZbGAS*1int_rev 5'-TGC TGT GGT CCA AAG TTG TG-3' were used for the amplification (program: 7 cycles: 94°C 2 min, 72°C 3 min; 32 cycles: 94°C 2 min, 67°C 3 min; 67°C 4 min; 4°C ∞) of the terminal ends of *ZbGAS*1 utilizing a *Z. bailii* Genome Walker library constructed using the Universal Genome Walker kit (Clontech) following the manufacturers instructions. From the obtained and reconstructed sequence the final primers were established and used for the *ZbGAS*1 1710 bp full length coding sequence amplification: *ZbGAS*1_fw: 5'-ACT AAT GTT ATT CCA GGC GTT TTC G-3' and *ZbGAS*1real_rev: 5'-AAA TCA AGC CAA AGC AAA TCC AGC A-3' (program: 94° C 5 min; (94°C 20 s, 58°C 30 s, 72°C 2 min) x30; 72°C 10 min; 4°C ∞). The DNA sequence of *ScGAS*1 was PCR amplified with the following primers: *ScGAS*1fw: 5'-ACA ATG TTG TTT AAA TCC TTT CAA GTT GCA A-3' and *ScGAS*1rev: 5'-TTT TTA AAC CAA AGC AAA ACC GAC ACC AG-3' (program: 94° C 5 min; (94°C 20 s, 60.5°C 30 s, 72°C 2 min) x30; 72°C 10 min; 4°C ∞), and the DNA sequence of *YlXPR*2 was PCR amplified with the following primers: *XPR*2_fwd: 5'-ACA ATG AAG CTC GCT ACC GCC TTT A-3' and *XPR*2_rev: 5'-TGC CTA AAT GCC AAC ACC GTT GTA G-3' (program: 94° C 4 min;

(94°C 15 s, 62.5°C 30 s, 72°C 2 min) x30; 72°C 7 min; 4°C ∞). All the unique fragments obtained were sub-cloned in the vector pSTblue-1 utilising the Perfectly Blunt® Cloning Kit (Novagene) and sequenced. The resulting respective plasmids utilised in this work were named as follows: pSTb*Zb*GAS1int, pSTb*Zb*GAS1, pSTb*Sc*GAS1 and pSTb*Yl*XPR2.

For the construction of the *ZbGAS*1 deletion cassettes, the Kan[R] or the hph[R] cassette, derived from plasmid pFA6-KanMX4 [21] and pAG26 [22] and conferring resistance to G418 and hygromycin respectively, were bluntended excised and inserted in the pSTb*Zb*GAS1int opened *Bst*EII *Bgl*II, bluntended and dephosphorylated, resulting in the plasmids pSTb*Zb*GAS1 K and pSTb*Zb*GAS1 H. The resistance cassettes resulted in both cases counter-clockwise inserted. The *Z. bailii* wild type cells were transformed with the respective deletion cassettes obtained by cutting the two described plasmids in the *Acc*I and *Sna*BI sites. The effective deletion was PCR checked by using the following couples of primers: ZbG1int2_fw 5'- TGC AGA AGT TAC AGA CCA ATG TTG T -3' and and ZbG1int2_rev 5'-AAG ATA GAG AAG TGC TCT TGG CA -3' (see Figure 2) or ZbG1int2_fw and Hph_fw 5'- ATA TGA AAA AGC CTG AAC TCA CCG AC -3' or Kan_fw 5'- ATG GGT AAG GAA AAG ACT CAC GTT -3' (not shown) in order to verify the presence of the cassettes in the desired locus.

For complementing the yeasts *GAS*1 deletion, pZ$_5$(-Nco)*ScGAS1* and pYX022*ZbGAS1* were constructed. pZ$_5$(-Nco)*ScGAS1* was obtained from pZ5(-Nco) opened *Sac*I/*Eco*RV with the insertion of *ScGAS1*, from pSTb*Sc*GAS1 cut with *Sac*I/*Sna*BI. The pZ$_5$(-Nco) derives from pZ$_3$ [3] where at the *Kpn*I site the *Kan*[R] cassette was substituted from the NAT[R] cassette obtained from vector pAG25 [22] by cutting *Pvu*II/*Sac*I/blunt (obtaining the pZ$_5$ plasmid): finally this plasmid was *Eco*RI/*Bam*HI cut and bluntended and reclosed. pYX022*ZbGAS1* was obtained with the insertion of *ZbGAS*1 from pSTb*Zb*GAS1 into pYX022 (R&D Systems, Wiesbaden, Germany) both *Eco*RI cut.

For heterologous protein expression the plasmids pZLN022*XPR2*, pZLN022*LIP1* and pZ$_5$*Kl*hIL-1β were constructed. The first two integrative plasmids derives from pZLN022 (manuscript in preparation), obtained from pYX022 opened *Dra*III blunt/*Spe*I blunt (to exclude *HIS*3) with the insertion of *ZbLEU2* cut from pSTb*ZbLEU2* with *Eco*RI blunt; in *Kpn*I blunt site the NAT[R] cassette was inserted, obtained from vector pAG25 [22] by cutting *Pvu*II/*Sac*I/blunt. The pSTb*ZbLEU*2 was obtained by sub-cloning

*ZbLEU*2 amplified by PCR from *Z.bailii* genomic DNA (manuscript in preparation) in pSTBlue (Novagen) cut by *Eco*RV. For pZLN022*XPR2* construction, the pZLN022 was *Aat*II/*Nhe*I cut, and the *XPR2* from pSTb*XPR2* was inserted. For pZLN022*LIP1* construction, the pZLN022 was *Aat*II blunt/*Nhe*I blunt opened and the LIP1 was inserted by *Bam*HI blunt/*Nsi*I blunt excision from the pGAP*sLIP* [23]. For rh interleukin the pZ_5KlhIL-1β was built: it was obtained from pZ_5 opened with *Eco*RI-blunt, with the insertion of hIL-1β in frame with *Kl* signal sequence from vector pKSSPI/3 [24] cut *Eco*RI blunt/*Xba*I blunt.

DNA manipulations, transformation and cultivation of *Escherichia coli* (DH5 F' (80dlacZ M15, (lacZYA-argF), U169, deo, rec1, end1, sup44, , THI-1, gyrA96, relA1) and Novablue Competent Cells (Novagene) were performed following standard protocols [25]. All the restriction and modification enzymes used were from New England Biolabs (Hitchin, Herts, UK) or from Roche Diagnostics (Mannheim, Germany).

Calcofluor staining and fluorescence microscopy

Cells were harvested, washed in PBS and resuspended in 1 mg ml^{-1} Calcofluor White solution, incubated 10-30 min at room temperature, washed again twice in PBS. Fluorescence microscope images were taken by a Nikon Eclipse 90i microscope using the Metamorph software version 2.2 (Nikon), using emission filter UV-1A.

Quantification of total secreted proteins

Cultures were harvested and centrifuged at maximal speed, then total proteins in the supernatants were ethanol-precipitated. The amount of proteins was determined by the dye-binding method of Bradford (Quick Start Bradford Dye Reagent - BIORAD), using BSA as standard. All the data were normalized by OD, after verification of the linear correlation between OD and dry weight as a measure of biomass.

Quantification of secreted protease

Cultures were harvested and centrifuged at maximal speed, then total proteins in the supernatants were separated and stored at -20°C until analysis. The amount of protease secreted in the medium was determined by the Azocoll assay (Azocoll - Calbiochem), using commercial protease as positive control. Azocoll assays were

performed according to [26] with some modifications. Reaction mixtures contained 15 mg ml^{-1} Azocoll , 50 mM Tris/HCl (pH 7.6), 150 mM NaCl and 5 mM CaCl$_2$. Proteolytic activities were measured by absorbance changes at 490 nm (Shimadzu UV 1601) after incubation for 18 h at 30°C.

Quantification of secreted lipase

Cultures were harvested and centrifuged at maximal speed, then the supernatants were separated and stored at -20°C until analysis. The amount of lipase secreted in the medium was determined according to [27] with some modifications. Reaction mixtures contained: (A) 60 mg of *p*-nitrophenyl palmitate (Sigma Aldrich) in 20 ml of isopropanol (Sigma Aldrich), (B) 1 g Triton X-100 (Sigma Aldrich) and 0.2 g of Arabic gum (Sigma Aldrich) in 200 ml Tris/HCl 0.1 M (pH 7.5). To obtain the mixture (C) add slowly 1 part of A solution to 9 part of B solution under continuous agitation. Add 9 part of (C) solution to one part of the enzyme containing solution (*i.e.* the supernatant). Lipase activities were measured by absorbance changes at 410 nm (Shimadzu UV 1601) and the amount of enzyme is proportional to OD according to the relation U/m = 0.667 x OD$_{410nm}$ (if OD$_{410nm}$<0.5).

Quantification of interleukin

Human IL-1β were analysed by Western blot. Independent transformants were cultured in shake flasks in minimal or rich medium and during the kinetics samples were collected. Cells were harvested (a culture volume corresponding to 10^8 cells) by centrifugation. One volume of 2×Laemmli buffer [28] was added to the supernatants of said samples, they were boiled for 3–5 min and stored at −20°C until loading, or loaded directly on a polyacrylamide gel. For the analysis of intracellular production, crude extracts were prepared from 10^8 cells by following the trichloroacetic acid protocol [29] and resuspending the final protein extract in 150 µl of Laemmli buffer. Samples were then loaded on standard polyacrylamide gels (sodium dodecyl sulfate–polyacrylamide gel electrophoresis, final concentration of the separating gel: 15%); after separation, proteins were blotted to nitrocellulose membranes and immunodecorated. An anti-IL-1β rabbit polyclonal antibody (IL-1β(H-153), Santa Cruz Biotechnology, USA; cat. no. sc-7884; dilution 1:200) was used for the interleukin immunodecoration. An anti-rabbit IgG horseradish

peroxidase-conjugated (Amersham Pharmacia Biotech, UK cat no. NA934; dilution 1:10.000) secondary antibody was used. For the interleukin detection, as a positive control, the human recombinant IL-1β from *E. coli* (Roche cat. no. 1 457 756) was always loaded in parallel on the polyacrylamide gels. The proteins were visualised using the ECL Western blotting system (Amersham Biosciences, UK), according to the manufacturer's instructions.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

PB and DP initiated and coordinated the project. LD was responsible for *Zb*GAS1 gene identification by PCR and its deletion. LR analyzed the mutant phenotype with CFW and SDS. LR and SP performed the batch cultivation, analyzed the secretory phenotype in null mutant and performed the experiment with heterologous proteins. All authors wrote the paper and approved the final version of the manuscript.

Acknowledgements

Additional file 1 – clustalw_ntd.pdf

Nucleotidic sequence of the *ZbGas1* gene and alignment with the homologue *ScGas1* (entry: YMR307W). Grey coloring represent nucleotidic identity. Alignment was performed with ClustalW software (http://www.ebi.ac.uk/clustalw) and graphical editing was made using Jalview software [30].

```
ZbGAS1 ATGTTATTCCAGGCGTTTTCGACCCTCGCACTTGGTGCACTATACGCTGCCTCCGGTGC 59
ScGAS1 ATGTTGTTTAAATCCCTTTCAAAGTTAGCAACCGCTGC------TGCTTTTTTTGCTGG 53

ZbGAS1 AGTTGCAGCCAGTAGCAGCAGTGCTACCAAACTTCCAGCAATTGAAGTTGCTGGTAACA 118
ScGAS1 CGTCGCAAC------------TGCGGACGATGTTCCAGCGATTGAAGTTGTTGGTAATA 100

ZbGAS1 AGTTCTTCTACTCGAACAACGGTTCTCAATTTTACATCAAAGGTGTTGCATACCAGGCC 177
ScGAS1 AGTTTTTCTACTCCAACAACGGTAGTCAGTTCTACATAAGAGGTGTTGCTTATCAGGCT 159

ZbGAS1 GACACGGCTAACTCATCTTCTGATGACAGCATCGACGATCCATTGGCCGACTACTCCAA 236
ScGAS1 GATACCGCTAATGAAACTAGCGGATCTACTGTCAACGATCCTTTGGCCAATTATGAGAG 218

ZbGAS1 ATGTTCGAGAGATATTCCATATTTGCAGAAGTTACAGACCAATGTTGTTCGTGTCTACG 295
ScGAS1 TTGTTCCAGAGATATTCCATACCTCAAAAAATTGAACACAAATGTTATCCGTGTCTACG 277

ZbGAS1 CAGTTAACACAACTTTGGACCACAGCAAGTGTATGGAAGCTCTAGCTGACGCTGGTATT 354
ScGAS1 CTATCAATACCACTCTAGATCACTCCGAATGTATGAAGGCTTTGAATGATGCTGACATC 336

ZbGAS1 TACGTCATTGCAGACTTGTCCACCCCAGCTGACTCTGTTAACAGAAATGACCCTACTTG 413
ScGAS1 TATGTCATCGCTGATTTAGCAGCTCCAGCCACCTCTATCAATAGAGACGATCCAACTTG 395

ZbGAS1 GGATATCGCACTATACCAGCGTTACACTGGCGTGGTGGACGCTTTTGCTAACTACACAA 472
ScGAS1 GACTGTTGACTTGTTCAACAGCTACAAAACCGTTGTTGACACTTTTGCTAATTACACCA 454

ZbGAS1 ACGTGTTGGGTTTCTTTGCCGGTAACGAAGTGACCAACAATGCTAGTAACACTGATGCA 531
ScGAS1 ACGTTTTGGGTTTCTTCGCCGGTAATGAAGTTACTAACAATTACACCAACACAGATGCA 513

ZbGAS1 TCTGCCTTTGTGAAGGCTGCCGTTAGAGACGTTAAGCAGTACATCAAGGACAAGAAATA 590
ScGAS1 TCTGCTTTCGTGAAGGCAGCTATTAGAGACGTCAGACAATACATCAGCGACAAGAACTA 572

ZbGAS1 CAGAACAATTCCTGTTGGTTACTCCTCCAATGATGACGAGGACACCAGAGTTGCTATGG 649
ScGAS1 CAGAAAAATTCCAGTTGGCTACTCTTCCAATGATGACGAAGATACCAGAGTTAAGATGA 631

ZbGAS1 CTGACTACTTTGCTTGCGGTGACGAAGACCAAAAGGCTGATTTCTACGGTATTAACATG 708
ScGAS1 CTGATTATTTCGCTTGTGGTGATGATGATGTTAAGGCTGATTTTTACGGTATTAATATG 690

ZbGAS1 TACGAATGGTGTGGTGACTCAACCTACCAGAAATCTGGTTACCAGGACAGAACCAATGA 767
ScGAS1 TATGAATGGTGTGGTAAATCTGACTTCAAAACTTCTGGTTATGCTGATAGAACTGCAGA 749

ZbGAS1 CTTCAAGAATTTGTCGATCCCTATCTTCTTCTCTGAGTACGGTTGCAACGCTGTCACTC 826
ScGAS1 ATTCAAAAACTTATCTATTCCTGTTTTCTTCTCTGAATACGGTTGTAACGAAGTAACAC 808

ZbGAS1 CAAGAAAGTTTACCGAGGTTCAGACTCTTTACGGTGATCAGATGACTGATGTGTGGTCC 885
ScGAS1 CAAGACTATTTACTGAGGTTGAAGCCTTGTACGGTTCTAATATGACAGATGTCTGGTCT 867

ZbGAS1 GGTGGTATTGTTTACATGTATTTCGAAGAGACCAACAAGTACGGTTTGGTCAGTATTGA 944
ScGAS1 GGTGGTATCGTATACATGTACTTCGAAGAGACTAACAAATACGGTTTAGTTAGCATCGA 926

ZbGAS1 TGATGATCAGGTCAAGACTTTGACTGACTTCGACAACTTGTCGAGCCAGATGGCCAAGA 1003
ScGAS1 TGGTAATGATGTTAAAACTTTGGATGACTTCAACAACTATTCTTCTGAAATCAACAAAA 985

ZbGAS1 TCTCTCCAAGTGCTGCCAAGAACTCTTCTTACACTGCCAAGAGCACTTCTCTATCTTGC 1062
ScGAS1 TATCACCAACTTCCGCCAACACAAAGTCTTACAGTGCAACAACAAGTGATGTTGCTTGT 1044

ZbGAS1 CCAGCCACCGGCAAGTACTGGAAAGCTAACACCAAATTGCCACCTACCCCAAGCAAGGA 1121
ScGAS1 CCAGCTACTGGTAAGTACTGGTCCGCTGCAACAGAATTACCACCAACTCCAAACGGAGG 1103

ZbGAS1 CCTCTGTACCTGTATGGAAGACTCCTTGTCCTGTGTTGTGGACGATAAGGTTGACGAAG 1180
ScGAS1 CTTGTGTTCATGTATGAATGCAGCCAATAGTTGTGTTGTTTCCGATGACGTTGATTCTG 1162

ZbGAS1 ATGACTACAGTGATTTGTTCAGTTACATCTGCTCGAAGGTGGACTGTTCTGGTATTACC 1239
ScGAS1 ATGATTACGAAACCTTATTTAACTGGATCTGTAATGAAGTCGACTGTAGCGGTATTTCA 1221

ZbGAS1 GCCAACGGTACTAGTGGTAAATATGGTTCTTACTCTTTCTGCTCTGCCAAGGAGCAGCT 1298
ScGAS1 GCAAACGGTACCGCCGGTAAGTATGGTGCTTACTCTTTCTGTACACCAAAGGAACAGCT 1280

ZbGAS1 ATCCTTTGTCATGAACTTGTATTACGAGAAGAACGGTGGTAGCAAGTCTGACTGTAGCT 1357
ScGAS1 ATCTTTCGTTATGAATTTGTACTACGAGAAGAGTGGTGGTAGCAAATCTGACTGTAGCT 1339

ZbGAS1 TCAGTGGTTCTGCCAGCTTGAAGTCTGCCACCACCAAGTCTGCCTGTTCCTCTGCTTTG 1416
ScGAS1 TCAGCGGTTCTGCCACTCTACAAACTGCCACCACGCAAGCTAGTTGCTCCTCCGCTTTG 1398

ZbGAS1 AAGCAGATTGGTAGCTCCGGTCTAGGCTCTGCCAGCAAAAGTGTCACTGGTTCCGCCTC 1475
ScGAS1 AAAGAGATTGGTAGTATGGGTACCAACTCTGCATCAGGTAGTGT---TGATTTGGGTTC 1454

ZbGAS1 TGGCTCCTCCTCTGGCTCATCTTCTGCTTCTGGTTCTTCTGAATCTTCCTCTGGCAAGG 1534
ScGAS1 CGGAACTGAATCCAGTAC---TGCCTCTTCTAACGCTTCGGGGTCTTCTTCCAAGTCTA 1510

ZbGAS1 GTTCTAGCTCCAGTAAGGGTGGTGACTCTTCCTCTAGTTCCAAGAAGAGCTCAAGTACA 1593
ScGAS1 ACTCCGGCTCTTCTGGTTCTTCCAGTTCTTCTTCTTCTTCTTCTTCAG---CTTCATCTTCA 1566

ZbGAS1 GCTAGCACCAAGGAAAAGAACTTTGCTGCCGCTTCCAAGGCTAGTCTGTCTCAGGTAAT 1652
ScGAS1 TCTTCTAGCAAG---AAGAATGCTGCCACCAACGTTAAAGCTAACTTAGCACAAGTGGT 1622

ZbGAS1 CCTATCCTTCGTGGCCACTTTGGGTGTGGTCGCCGGTGCTGGATTTGCTTTGGCTTGA 1710
ScGAS1 CTTTACCTCCATCATTTCCTTATCCATTGCCGCTGGTGTCGGTTTTGCTTTGGTTTAA 1680
```

96

References

1. Branduardi P: **Molecular cloning and sequence analysis of the** *Zygosaccharomyces bailii HIS3* **gene encoding the imidazole glycerolphosphate dehydratase.** *Yeast* 2002, **19**(13):1165-1170.

2. Thomas DS, Davenport RR: *Zygosaccharomyces bailii* - **a profile of characteristics and spoilage activities**. *Food Microbiology* 1985, **2**(2):157-169.

3. Branduardi P, Valli M, Brambilla L, Sauer M, Alberghina L, Porro D: **The yeast** *Zygosaccharomyces bailii***: a new host for heterologous protein production, secretion and for metabolic engineering applications.** *FEMS Yeast Res* 2004, **4**(4-5):493-504.

4. Chow T, Ash J, Dignard D, Thomas D: **Screening and identification of a gene, PSE-1, that affects protein secretion in** *Saccharomyces cerevisiae.* *J Cell Sci* 1992, **101 (Pt 3)**:709-719.

5. Bartkeviciūte D, Sasnauskas K: **Disruption of the** *MNN10* **gene enhances protein secretion in** *Kluyveromyces lactis* **and** *Saccharomyces cerevisiae.* *FEMS Yeast Res* 2004, **4**(8):833-840.

6. Bankaitis V, Aitken J, Cleves A, Dowhan W: **An essential role for a phospholipid transfer protein in yeast Golgi function.** *Nature* 1990, **347**(6293):561-562.

7. Vai M, Brambilla L, Orlandi I, Rota N, Ranzi B, Alberghina L, Porro D: **Improved secretion of native human insulin-like growth factor 1 from gas1 mutant** *Saccharomyces cerevisiae* **cells.** *Appl Environ Microbiol* 2000, **66**(12):5477-5479.

8. Popolo L, Vai M: **The Gas1 glycoprotein, a putative wall polymer cross-linker.** *Biochim Biophys Acta* 1999, **1426**(2):385-400.

9. Papaleo E, Fantucci P, Vai M, De Gioia L: **Three-dimensional structure of the catalytic domain of the yeast beta-(1,3)-glucan transferase Gas1: a molecular modeling investigation.** *J Mol Model* 2006, **12**(2):237-248.

10. Popolo L, Vai M, Gatti E, Porello S, Bonfante P, Balestrini R, Alberghina L: **Physiological analysis of mutants indicates involvement of the** *Saccharomyces cerevisiae* **GPI-anchored**

protein gp115 in morphogenesis and cell separation. *J Bacteriol* 1993, **175**(7):1879-1885.

11. Kapteyn J, Ram A, Groos E, Kollar R, Montijn R, Van Den Ende H, Llobell A, Cabib E, Klis F: **Altered extent of cross-linking of beta1,6-glucosylated mannoproteins to chitin in** *Saccharomyces cerevisiae* **mutants with reduced cell wall beta1,3-glucan content.** *J Bacteriol* 1997, **179**(20):6279-6284.

12. Marx H, Sauer M, Resina D, Vai M, Porro D, Valero F, Ferrer P, Mattanovich D: **Cloning, disruption and protein secretory phenotype of the** *GAS1* **homologue of** *Pichia pastoris*. *FEMS Microbiol Lett* 2006, **264**(1):40-47.

13. James S, Collins M, Roberts I: **Genetic interrelationship among species of the genus** *Zygosaccharomyces* **as revealed by small-subunit rRNA gene sequences.** *Yeast* 1994, **10**(7):871-881.

14. Mollapour M, Piper P: **Targeted gene deletion in** *Zygosaccharomyces bailii*. *Yeast* 2001, **18**(2):173-186.

15. Popolo L, Gilardelli D, Bonfante P, Vai M: **Increase in chitin as an essential response to defects in assembly of cell wall polymers in the ggp1delta mutant of** *Saccharomyces cerevisiae*. *J Bacteriol* 1997, **179**(2):463-469.

16. Carotti C, Ferrario L, Roncero C, Valdivieso M, Duran A, Popolo L: **Maintenance of cell integrity in the gas1 mutant of** *Saccharomyces cerevisiae* **requires the Chs3p-targeting and activation pathway and involves an unusual Chs3p localization.** *Yeast* 2002, **19**(13):1113-1124.

17. Turchini A, Ferrario L, Popolo L: **Increase of external osmolarity reduces morphogenetic defects and accumulation of chitin in a gas1 mutant of** *Saccharomyces cerevisiae*. *J Bacteriol* 2000, **182**(4):1167-1171.

18. Ferrer P, Diers I, Asenjo J, Andrews B: **Yeast cell permeabilizing beta-1,3-glucanases: A tool for the integration of downstream processes and metabolic engineering applications to yeast.** *Biotechnol Bioeng*, **58**(2-3):321-324.

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

20. Hoffman C, Winston F: **A ten-minute DNA preparation from yeast efficiently releases autonomous plasmids for**

transformation of *Escherichia coli*. *Gene* 1987, **57(2-3):267-272.

21. Wach A, Brachat A, Pöhlmann R, Philippsen P: **New heterologous modules for classical or PCR-based gene disruptions in *Saccharomyces cerevisiae*. *Yeast* 1994, **10**(13):1793-1808.

22. Goldstein A, McCusker J: **Three new dominant drug resistance cassettes for gene disruption in *Saccharomyces cerevisiae*. *Yeast* 1999, **15**(14):1541-1553.

23. Brocca S, Schmidt-Dannert C, Lotti M, Alberghina L, Schmid R: **Design, total synthesis, and functional overexpression of the *Candida rugosa* lip1 gene coding for a major industrial lipase. *Protein Sci* 1998, **7**(6):1415-1422.

24. Camattari A, Bianchi M, Branduardi P, Porro D, Brambilla L: **Induction by hypoxia of heterologous-protein production with the KlPDC1 promoter in yeasts. *Appl Environ Microbiol* 2007, **73**(3):922-929.

25. Sambrook J, Maniatis T, Fritsch EF: **Molecular cloning : a laboratory manual**, 2nd edn. Cold Spring Harbor, N.Y.: Cold Spring Harbor Laboratory; 1989.

26. Chavira RJ, Burnett T, Hageman J: **Assaying proteinases with azocoll. *Anal Biochem* 1984, **136**(2):446-450.

27. Brocca S, Persson M, Wehtje E, Adlercreutz P, Alberghina L, Lotti M: **Mutants provide evidence of the importance of glycosydic chains in the activation of lipase 1 from *Candida rugosa*. *Protein Sci* 2000, **9**(5):985-990.

28. Laemmli U: **Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970, **227**(5259):680-685.

29. Surana U, Amon A, Dowzer C, McGrew J, Byers B, Nasmyth K: **Destruction of the CDC28/CLB mitotic kinase is not required for the metaphase to anaphase transition in budding yeast. *EMBO J* 1993, **12**(5):1969-1978.

30. Waterhouse A, Procter J, Martin D, Clamp M, Barton G: **Jalview Version 2--a multiple sequence alignment editor and analysis workbench. *Bioinformatics* 2009, **25**(9):1189-1191.

Overexpression of the *Saccharomyces cerevisiae* and of the newly cloned *Zygosaccharomyces bailii* plasma membrane H$^+$-ATPase *PMA1* gene improves lactic acid stress resistance in said yeasts

Laura Dato, Simone Passolunghi, Paola Paganoni, Danilo Porro and Paola Branduardi*

Dipartimento di Biotecnologie e Bioscienze - Università degli Studi di Milano-Bicocca, P.^{zza} della Scienza 2, 20126 Milano, Italy

Keywords:
Saccharomyces cerevisiae; *Zygosaccharomyces bailii*; lactic acid; PMA1; plasma membrane H$^+$-ATPase.

Running title:
PMA1 overexpression and lactic acid stress

* Corresponding author
Dr. Paola Branduardi
Dipartimento di Biotecnologie e Bioscienze
P.^{zza} della Scienza 2, 20126 Milano, Italy
Tel.:+ 39 02 64483418
Fax:+ 39 02 64483565
E-mail: paola.branduardi@unimib.it

ABSTRACT

Most of the known yeast species are naturally able to survive in acidic environments by partially counteracting the toxic effects caused by the acids. This feature may be advantageous if exploited for biotechnological processes, where yeasts can be suitable candidates for industrial weak organic acids productions. In this view, it would be ideal to further increase the host stress resistance in order to improve growth and, consequently, production levels.

The plasma membrane H^+-ATPase Pma1p was selected for this study due to its key role in the acid response. Its coding gene was (over)expressed in the baker's yeast as well as in the more acid tolerant yeast *Zygosaccharomyces bailii*, in the latter case after cloning of the previously unknown sequence. Here it is shown that the *ScPMA*1 and *ZbPMA*1 (over)expression in the respective yeasts confers a growth advantage to cell populations subjected to lactic acid stress, opening promising application in this as well as in similar fields of yeast exploitation. Flow cytometric analyses showed that lactic acid stress caused the death of a major fraction of the cell population in *S. cerevisiae*, but not in *Z. bailii*; however, the overexpression of *PMA*1 significantly increased cell viability in *S. cerevisiae*.

INTRODUCTION

The maintenance of physiological intracellular pH (pH_i) values is one of the most important needs for cellular homeostasis and survival (Booth & Kroll, 1989; Booth & Stratford, 2003). In this respect, acidic environments constitute a big challenge for microbial cells. In fact, since protons tend to cross the plasma membrane following their concentration gradient, cells have to spend energy to maintain a correct pH_i, and this can be detrimental for growth (Beales, 2003).

Organic acids are even more challenging than inorganic acids, since they can passively diffuse through the plasma membrane in their undissociated form. Once inside the cells, because of the relatively high pH_i, organic acids dissociate causing cytoplasm acidification due to proton release (Krebs et al., 1983; Warth 1985) and accumulation of the acid anion to potentially toxic levels. Therefore, in this case even more energy will be required, to maintain the physiological pH_i and to extrude and/or metabolize the anion.

This phenomenon was particularly examined in yeasts (Piper *et al.*, 1998; Brul & Coote, 1999), either from a physiological, but also from a biotechnological point of view, being these microorganisms particularly appealing for organic acids production (reviewed in Branduardi *et al.*, 2008 and Sauer *et al.*, 2008). Yeasts in general, and some species in particular, are indeed able to survive in acidic environments by partially counteracting the toxic effects of inorganic and/or organic acids (Serrano *et al.*, 1986; Warth 1989; Verduyn *et al.*, 1992; Piper *et al.*, 2001; Macpherson *et al.*, 2005).

In particular, the main interest in the production of lactic acid comes from its applications in the synthesis of biodegradable polymer materials (Benninga, 1990). It is important in this context to underline the difference between lactic acid and lactate production. Since the desired product is lactic acid, isolation of this compound requires the pH of the broth to be much lower than its pKa (3,78).

Lactic acid bacteria, which are the traditional producers, are inhibited by the product itself and especially by the low pH; for this reason, many studies in the last decade were devoted to engineering yeasts for the redirection of the ethanol to lactate fermentation, at low pH. In this respect, it would be ideal to further increase the host acid stress

resistance capabilities, since the desired yield of said productions generate environmental conditions that becomes inhibiting even for yeasts (Valli *et al.*, 2006). This type of manipulation could improve the host growth abilities and, hopefully, the organic acids production and/or productivity levels.

The plasma membrane H^+-ATPase Pma1p was selected for this study because it plays a key role not only in the pH_i homeostasis, but also in the acid response (Goffeau & Slayman, 1981; Serrano R, 1984).

The main aim of this work was to investigate if the overexpression of this proton pump could help yeast cells when subjected to acid stress, in particular to weak organic acids and especially to lactic acid stress. We tested the overexpression of said protein both in the well known baker's yeast *Saccharomyces cerevisiae* as well as in the more acid tolerant - but less characterised - *Zygosaccharomyces bailii*; both yeasts were already proven to be able to produce lactic acid after engineering of the natural fermentative pathway (Branduardi *et al.* 2006, Branduardi *et al.* 2004) and starting from batch cultures at very high glucose concentration (*Valli et al.,* 2006 and data not published), as it is very often required for the industrial production of bulk chemicals. While the *Sc*Pma1p, encoded by the *PMA*1 gene, is well described in literature (Serrano, 1978; Serrano, 1983; Serrano *et al.,* 1986; Eraso & Gancedo, 1987; Capieaux *et al.,* 1989; Morsomme *et al.,* 2000; Lecchi *et al.,* 2005), we primarily had to identify and clone the homologous gene of *Z. bailii,* previously unknown.

The main relevance of the data here reported is mainly represented by the evidence that lactic acid stress can be (at least partially) relieved in yeast by modulating the expression of a single gene. Such improvement is also possible in the yeast *Z. bailii*, whose tolerance to this kind of stress is already highly pronounced.

MATERIALS AND METHODS

Yeast strains

The *S. cerevisiae* strains developed for this work were obtained from the parental strain CEN.PK 102-5B (*Mat a, ura3-52, his3Δ1, leu2-3,112*) (Dr. P. Kötter, Institute of Microbiology, Johann Wolfgang Goethe-University, Frankfurt, Germany), initially transformed with the pYX012 integrative plasmid (R&D Systems) to complement the *ura3* auxotrophy. The resultant strain was then transformed either with the integrative pYX042-*ScPMA1* or with the multicopy pYX242-*ScPMA1* expression plasmid to obtain the *PMA1*-overexpressing strains, or with the corresponding empty plasmids, pYX042 or pYX242 (R&D Systems), to obtain the respective control strains.

The *Z. bailii* strains used for the *PMA1* overexpression experiments were obtained from strain Z₂ΔL (diploid, *leu2Δ::Kan/leu2Δ::hph*), derived from the parental strain ATCC60483; construction of this auxotrophic strain is described in a separate study (Dato L *et al.*, manuscript in preparation). Z₂ΔL was transformed either with the integrative pYX042-*hph-ZbPMA1* or with the multicopy pYX242-*ZbPMA1* expression plasmid to obtain the *PMA1*-overexpressing strains, or with the corresponding empty plasmids, pYX042-*hph* or pYX242, to obtain the respective control strains. For the *ZbPMA1* gene cloning, the *Z. bailii* strain ATCC36947 was used as the source of genomic DNA.

*ScPMA*1 amplification, *ZbPMA*1 cloning and sequence alignment

The *ScPMA*1 gene was amplified by PCR from CEN.PK genomic DNA, prepared as described in Hoffman & Winston (1987), using *ScPMA1*_fw (5'-ATC AAT ATG ACT GAT ACA TCA TCC TC-3') and *ScPMA1*_rev (5'-ACA GGA TTA GGT TTC CTT TTC GTG-3'). Reaction was performed with Pwo DNA Polymerase Super Yield (Roche) in the "GC-rich" buffer supplied, following the manufacturer's instructions.

Cloning of the *ZbPMA1* gene was achieved in two separate steps. Amplification of bases 420 to 2775 was done with primer Sc*PMA1*_rev and primer *PMA1*_fw (5'-GAT TGG GTC GAT TTC GGT GTT ATC TGT-3'), the latter designed on a region highly conserved between *S. cerevisiae* ([Z72530](#)) and *Z. rouxii* ([D10764](#)) sequences. Bases 1 to 480 were amplified using primer Zr*PMA1*_fw

(5'-TTA GCA ATG TCT GAC GAG CGT ATT AC-3') and primer *Z1PMA1i*_rev (5'-CCG GCG TTA AGC ATC AAA AGA C-3'), the latter specific for *ZbPMA1*.

Both reactions were performed with Pwo DNA Polymerase (Roche), with annealing temperature of 58 ˚C. Once the *ZbPMA1* sequence was reconstructed, the whole CDS was amplified using new specific primers: *ZbPMA1*_fw (5'-AAT ATG TCT GAC GAG CGT ATC ACT GAG AAG CAT C-3') and *ZbPMA1*_rev (5'-ACA GGA TTA GGT TTC CTT TTC ATG TTG AGT AGA AAC TC-3'). Reaction was performed with Pwo DNA Polymerase Super Yield in the "GC-rich" buffer. All the amplified DNA fragments were sub-cloned into the shuttle plasmid vector pSTBlue-1 by using the Perfectly Blunt Cloning Kit (Novagen) and subsequently sequenced to verify the accuracy of amplification or to determine the unknown sequences.

Sequence alignments were performed with ClustalW software version 1.83 (European Bioinformatics Institute, Cambridge, UK [http://www.ebi.ac.uk/Tools/clustalw/]).

Plasmids construction and yeast transformation

Plasmid pSTBlue-1-*ScPMA*1 was cut *Apa*I-*Mlu*I and the *ScPMA1* CDS excised was ligated into pYX042(-ATG) and pYX242(-ATG), both also cut *Apa*I-*Mlu*I, obtaining the pYX042-*ScPMA1* and pYX242-*ScPMA1* expression vectors, respectively. The plasmids pYX042(-ATG) and pYX242(-ATG) were previously obtained from the pYX042 integrative and pYX242 multicopy expression vectors (R&D Systems) *Eco*RI and *Bam*HI cut, blunt-ended and re-ligated.

For integrative *S. cerevisiae* transformation, plasmids pYX042 and pYX042-*ScPMA1* were linearized in the *Hpa*I and *Nar*I sites, respectively, and used for *leu2* complementation. Plasmid pYX012 was linearized in the *Pst*I site and used for *ura3* complementation.

Plasmid pSTBlue-1-*ZbPMA*1 was cut *Eco*RI and the *ZbPMA1* CDS obtained was inserted into pYX042 and pYX242, both also cut *Eco*RI, obtaining the pYX042-*ZbPMA1* and pYX242-*ZbPMA1* expression vectors, respectively. Plasmid pYX042-*ZbPMA1* was then linearized with *Aat*II, blunt-ended and ligated to the *hph*[R] cassette taken from pAG26 (Goldstein & McCusker, 1999) cut *Eco*RV-*Sma*I, obtaining plasmid pYX042-*hph*-*ZbPMA1*. From the latter, the *ZbPMA1* gene fragment was excised by cutting *Sph*I/blunt and the plasmid was re-circularized, obtaining plasmid pYX042-*hph*. Integrative transformation of strain $Z_2\Delta L$ with plasmids pYX042-*hph* and

pYX042-*hph-ZbPMA1* was performed after linearization in the *Sac*II site to direct insertion at the *hph* locus. All $Z_2\Delta L$ transformants were selected for leucine prototrophy.

Yeast transformation was performed using the Lithium Acetate/ssDNA method as described (Gietz & Woods, 2002).

Growth media and conditions

All experiments were performed in YNB-based medium (Yeast Nitrogen Base without amino acids, DIFCO) with 2% glucose (w/v), supplemented or not with the indicated concentrations of lactic acid (pKa = 3.86). The initial pH of the medium without organic acid was around 5. When lactic acid was added, the initial pH of the medium was adjusted to 3 by adding NaOH. In this way a buffered medium is generated that maintains a pH around 3 till the last points considered in the kinetics. For CEN.PK cultures, histidine (50 mg l^{-1}) was also added to the media.

Yeast cells were pre-cultivated till the exponential phase in liquid glucose YNB-based medium and then re-inoculated in the indicated media at initial $OD_{660 \text{ nm}}$ 0.02 or 0.03 for shake flasks and 0.003 for microtiter plate cultures. Shake flasks were incubated at 30 ˚C under shaking at 160 r.p.m. Growth in multiwells was performed in 96-well U-shaped plates with 150 μl single culture volume; incubation was at 30 ˚C with shaking at 1200 r.p.m. in a TITRAMAX 1000 shaker (Heidolph); plates were read by a Spectramax 250 spectrophotometer (Molecular Devices).

Crude membrane preparation and Western blot

Immunodetection of the Pma1p was performed on crude membrane extracts. Cells were harvested in mid-log phase of growth, briefly washed with ice-cold water, resuspended in 25 mM Tris-HCl pH 8.5, 5 mM EDTA pH 8, 0.5 mM phenylmethylsulfonyl fluoride and 1 x Complete Protease Inhibitor Cocktail (Roche) and homogenized by shaking with glass beads (600 μm diameter) in a FastPrep® FP120 oscillating system (MP Biomedicals). Afterward, the homogenates were first centrifuged for 10 min at 700 *g* to remove debris and the supernatants further centrifuged for 40 min at 20000 *g*. The obtained total membranes pellets were resuspended in TED buffer (10 mM Tris-HCl pH 7.5, 0.2 mM EDTA and 0.2 mM dithiothreitol)

containing 20% glycerol (v/v) and stored at -20 ˚C. All the steps were performed on ice or at 4 ˚C and using ice-cold solutions.

Samples were then normalized for total protein content, determined by the dye-binding method of Bradford (Quick Start Bradford Dye Reagent - BIORAD) using bovine serum albumine as standard, separated by SDS-PAGE (8% acrylamide) and blotted on a nitrocellulose membrane for 2.5 h at 250 mA. Membranes were incubated with a primary anti-Pma1 antibody (mouse monoclonal, Abcam ab4645) (1 : 1500) for 2 h at room temperature. A secondary anti-mouse antibody (Amersham NA931V) was used (1 : 10000) for the detection of immunoreactive protein bands using the Super Signal West Pico Western blotting system (Pierce), according to the manufacturer's instruction.

H^+-ATPase activity assay

Assays were performed on crude membranes samples, obtained by differential centrifugation as described above, following the protocol described by Serrano (1988) and slightly modified.

For each sample, 1 ml of incubation mix was prepared in 100 mM MES buffered to pH 6 with Tris, containing 5 mM $MgCl_2$, 50 mM KNO_3, 5 mM NaN_3, 0.2 mM ammonium molybdate and a volume of membranes corresponding to 25 µg of proteins. The reaction was started by adding 60 µl of 0.1 M ATP. After 30 min of incubation at 30 ˚C, the reaction was stopped by adding 2 ml of stop solution (2% v/v H_2SO_4, 0.5% w/v SDS and 0.5% w/v ammonium molybdate). Then 20 µl of 10% (w/v) ascorbic acid were added and, after 10 min, the $A_{750\ nm}$ was read. The resulting specific activity was expressed as units per mg of total proteins in the sample.

Flow cytometry

For the determination of cell viability, yeast cells were harvested at $OD_{660\ nm}$ = 0.5-1, washed twice with phosphate-buffered saline, and resuspended in 0.46 mM propidium iodide. Labelled cells were sonicated and analysed using a Cell Lab Quanta™ SC Flow Cytometer (Beckman Coulter) equipped with a diode laser (excitation wavelength 488 nm, laser power 22 mW). Fluorescence emission was measured through a 670 nm long pass filter (FL3 parameter). At least total 20000 events were recorded for each sample. The sample flow rate during analysis did not exceed 600-700 cells s^{-1}. Data analysis

was performed with WinMDI 2.9 software (Purdue Univ., Cytometry Labs [http://facs.scripps.edu/software.html]).

RESULTS

Effect of *ScPMA*1 overexpression on growth of *S. cerevisiae* cells in control and lactic acid stressing conditions: a microtiter screening

The *S. cerevisiae* CEN.PK strain (that bears its own wild type copy of the *PMA*1 gene) was transformed either with an integrative or with an episomal 2μm plasmid carrying the *ScPMA*1 gene under the control of the glycolytic TPI promoter. The corresponding empty plasmids were used to obtain the control strains. Independent clones were tested for growth in YNB-based medium at initial pH 5 (control medium), YNB-based medium at low pH (different values) and YNB-based medium containing different concentrations of lactic acid. The initial pH of lactic acid media was adjusted to 3, a value lower than the dissociation constant of the acid (see Methods) in order to have a higher fraction of the relative undissociated form. Screening of the growth abilities was performed in microtiter plates under vigorous shaking. Relevant data are shown in Fig. 1, where mean values are reported for growth levels reached by independent clones in one representative experiment (at least two independent clones were tested in at least triplicate experiments).

The growth abilities of the *PMA1*-overexpressing strains in the control medium (Fig. 1a and b, upper panels) and in media at low pH (not shown) were not distinguishable from those of the respective control strains. When lactic acid was added, growth of all strains was delayed and biomass accumulation decreased (Fig. 1a and b, bottom panels), proportionally to the lactic acid concentration (data not shown). In particular, in these experimental conditions, about 30 g l^{-1} of lactic acid became inhibiting for the *S. cerevisiae* control strains (open symbols). Remarkably, *PMA*1 overexpression (full symbols) gave a growth advantage, although a distinction has to be pointed out: when one additional copy of the gene was integrated (Fig. 1a, bottom), a positive effect was clearly evident; instead, the multicopy expression did not give a clear-cut answer (Fig. 1b, bottom), since a high clonal variability was registered.
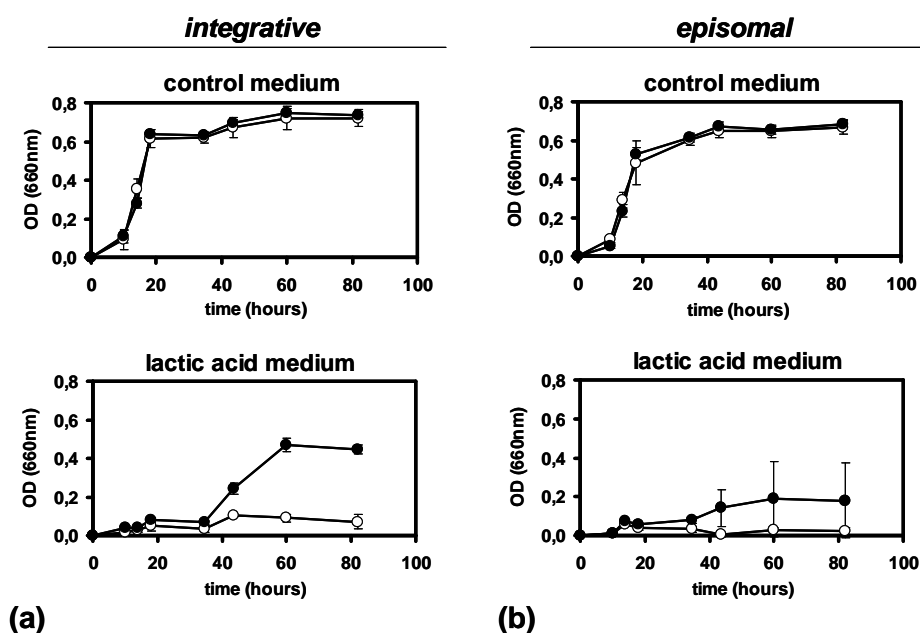
Figure 1. Effect of *ScPMA*1 overexpression on the ability of *S. cerevisiae* cells to grow in minimal glucose medium added or not with lactic acid, tested in microtiter plates *S. cerevisiae* CEN.PK cells were transformed either with an integrative (**a**) or with an episomal (**b**) plasmid bearing the *ScPMA*1 gene under the control of the *ScTPI* promoter. Transformants were grown in 96-well microtiter plates in minimal glucose medium (initial pH 5, upper panels) or in minimal glucose medium added with 30 g l^{-1} of lactic acid (initial pH 3, lower panels). Inoculum was at $OD_{660\ nm}$ = 0.003 and incubation was at 30 °C under agitation. Growth is indicated as $OD_{660\ nm}$. The values reported represent the mean of 2 independent clones for the control strains (empty plasmids, empty symbols) and of 3 independent clones for the *PMA1*-overexpressing strains (full symbols). Vertical bars represent standard deviations

This last aspect, might be related to a possible metabolic burden caused by an overloading of the expression system. Eraso *et al.* (1987) reported the Pma1p overexpression to be somehow toxic for *S. cerevisiae* RS-16 cells, maybe due to structural constraints of the membrane systems: expression on a 2μm plasmid led to slowed growth and, consequently, to selection of cells with a low plasmid

copy number. Although in that case expression was driven by the *PMA1* promoter (i.e. transcription levels may differ in the two cases) one can speculate that a similar selection also takes place in our multicopy transformants, leading to different gene dosage and therefore to the clonal difference observed in respect to growth. Moreover, Supply *et al.* (1993) showed accumulation of Pma1p in intracellular ER membranes occurring in *PMA*1-multicopy-overexpressing cells, but not (or at least in very few cases) in integrative-overexpressing cells. We speculated that a similar effect might have been responsible for the multicopy phenotype observed. However, fluorescence microscopy of Pma1p-immunostained cells did not reveal a significant difference between integrative and multicopy transformants, as intracellular accumulation of Pma1p-containing membranes was visible in all lactic acid samples, regardless of the expression system (data not shown).

Based to these results, the next experiments were focused on integrative transformants.

*ScPMA*1 overexpression in *S. cerevisiae* cells: protein levels and H$^+$-ATPase activity determined during batch growth under lactic acid stress

To get deeper insights into the microtiter screening indications, the control and the *PMA1*-overexpressing strains were cultivated in shake flasks in YNB-based medium with or without 35 g l^{-1} of lactic acid. At least two independent clones for each strain were tested in at least triplicate experiments, giving similar results. Fig. 2a shows representative growth curves obtained for single clones. The growth kinetic of the two strains in YNB-based medium (Fig. 2a, upper panel) was nearly identical. The presence of lactic acid at initial pH 3 (lower panel) determined a growth impairment that considerably delayed growth of the wild type control, while the *PMA1*-overexpressing strain was able to grow more rapidly, thus confirming the microtiter plate data. The final OD reached in these conditions were very similar for the two strains (as up to 120 h it remained essentially the same as at 75 h, data not shown) and lower than in the control medium. Instead, the growth kinetics of the two strains in lactic acid added medium at initial pH 5 were indistinguishable (data not shown).

(b)

(a)

(c)

(b)
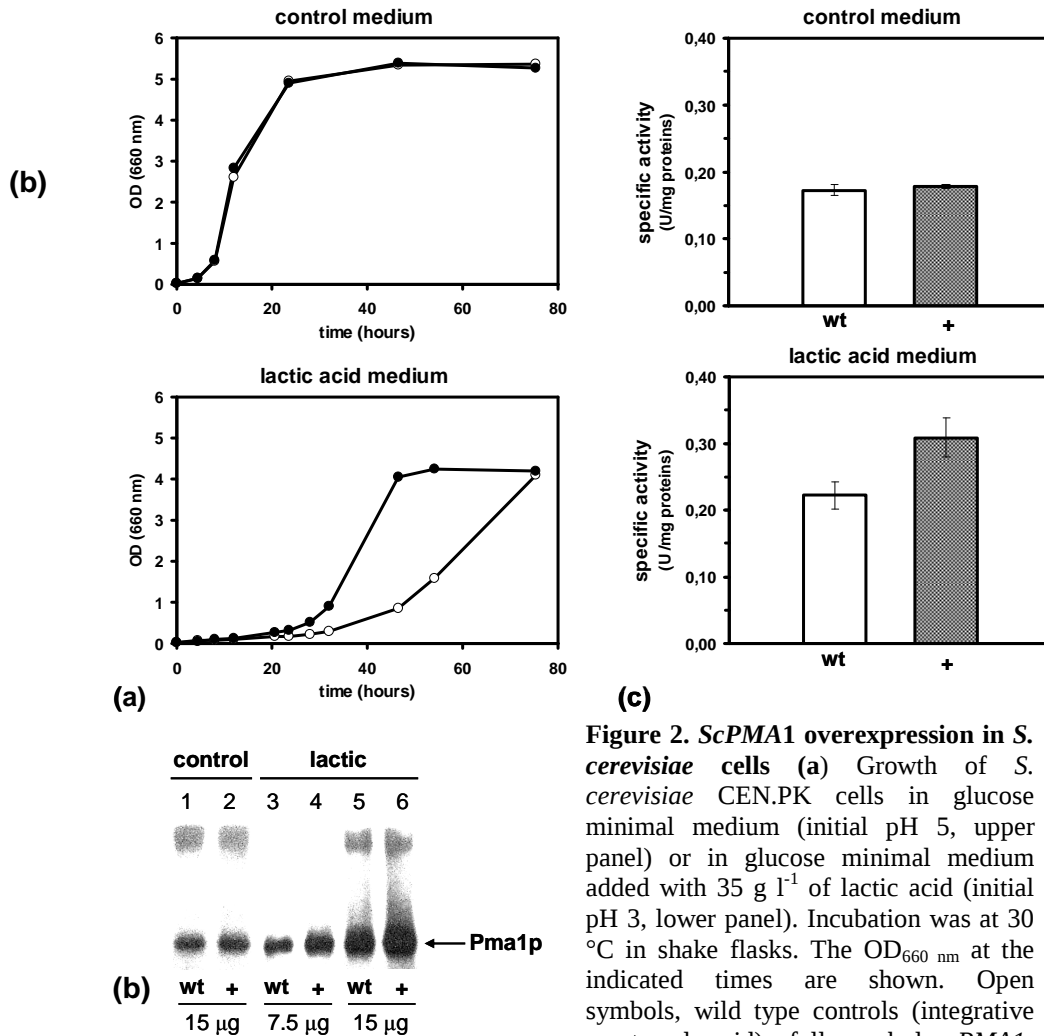
Figure 2. *ScPMA*1 overexpression in *S. cerevisiae* cells (a) Growth of *S. cerevisiae* CEN.PK cells in glucose minimal medium (initial pH 5, upper panel) or in glucose minimal medium added with 35 g l^{-1} of lactic acid (initial pH 3, lower panel). Incubation was at 30 °C in shake flasks. The $OD_{660\ nm}$ at the indicated times are shown. Open symbols, wild type controls (integrative empty plasmid); full symbols, *PMA1*-overexpressing cells (integrative *ScPMA*1 plasmid). (**b**) Western Blot analysis of the Pma1p levels: immunostaining visualization of Pma1p in crude protein extracts obtained from wt (lanes 1, 3 and 5) and *PMA1*-overexpressing (lanes 2, 4 and 6) cells grown under lactic acid stress (lanes 3-6) and in control medium (lanes 1 and 2), as described above, and collected when they reached the mid-log phase (about $OD_{660\ nm}$ = 2). Respectively, 7.5 μg of total proteins were loaded in lanes 3 and 4 and 15 μg in lanes 1, 2, 5 and 6. M = protein markers. (**c**) Pma1p specific activity detected on crude membrane extracts. Cells were grown as described, either in the control medium (upper panel) or under lactic acid stress (lower panel), and collected when they reached the mid-log phase (about $OD_{660\ nm}$ = 2). Open columns, wild type; filled columns, *PMA1*-overexpressing. Vertical bars are standard deviations for three independent assays on samples from a representative experiment.

112

In order to investigate the correlation between these behaviors and the overexpression of the proton pump, analyses at protein level were performed. In fact, owing to its crucial importance, this proton pump is finely regulated, both at the transcriptional and at the post-translational level (Goossens *et al.*, 2000; Portillo, 2000). Regulatory elements located in the Pma1 transcriptional promoting region determine that the gene expression can be triggered by glucose metabolism, in addition to a cell cycle-dependent regulation. However, since the *PMA*1 gene, which is under the control of the general transcription factor TUF/RAP1, is highly expressed (Serrano *et al.*, 1987; Capieaux *et al.,* 1989) and the half-life of the enzyme is relatively long (Benito *et al.,* 1991), the amount of protein results to be rather constant during yeast growth (Mason *et al.,* 1998). In addition, there are evidences that in growth conditions determining a reduction of *PMA*1 mRNA by 50%, the related activity is only modestly reduced (Carmelo *et al.,* 1996). Moreover, a previous study demonstrated that the addition of a single *PMA*1 copy determines a 60% increase of the mRNA levels, but only a 20% increase of the activity (Eraso *et al.,* 1987). Therefore, it has been already concluded that the regulatory mechanisms operating on Pma1p are mainly post-translational.

A Western blot analysis with an anti-Pma1p specific antibody was performed on crude membrane samples separated by SDS-PAGE. Cells were grown as just described and collected in the mid-exponential phase ($OD_{660 \text{ nm}}$ around 2). Results are shown in Fig. 2b, where respectively 15 μg proteins of samples from control cultures (lanes 1 and 2) and 7.5 μg (lanes 3 and 4) or 15 μg proteins (lanes 5 and 6) from lactic acid stressed cultures were loaded. It can be primarily noted that a higher Pma1p accumulation was visible in lactic samples compared to control medium samples. Furthermore, *PMA*1 overexpression led to a further higher protein accumulation in lactic acid medium (more clearly visible at the higher dilution: lane 4 versus 3). Slightly higher Pma1p amounts were also observed in *PMA*1-overexpressing cells grown in control medium (lane 2 versus 1).

The same crude membrane samples used for Western blot were also assayed for H^+-ATPase specific activity. At least triplicate experiments were performed giving similar results and Fig. 2c shows data from a representative experiment. Similarly to what observed for protein levels, the Pma1p specific activities of both strains were higher in lactic acid stressed samples than in unstressed ones (lower

versus upper panel). Furthermore, under lactic acid stress the activity of *PMA*1-overexpressing cells was higher than in the wild type (about 37% more).

Although differences found in the measured activity appear lower than differences in the protein levels, an incorrect folding or final targeting of the overexpressed protein could be responsible for this discrepancy. Another possible explanation takes into account other elements that might become limiting for a correct functionality of the H$^+$-ATPase. One of the most important factors in this respect is Pmp1p, a small proteolipid ancillary to Pma1p (Navarre *et al.*, 1992) whose expression was found to be enhanced under lactic acid stress in *S. cerevisiae* (our unpublished results). However, in all the cases tested, a combined overexpression of the *PMA*1 with the *PMP*1 gene did not confer any growth advantage (data not shown) and, consequently, its eventual effect on Pma1p activity was not tested.

Overall, and according to the literature (Viegas & Sa-Correia 1991; Alexandre *et al.*, 1996; Holyoak *et al.*, 1996; Carmelo *et al.*, 1997; Viegas *et al.*, 1998; Macpherson *et al.*, 2005), our data clearly indicate increased Pma1p levels and specific activity determined by the lactic acid stressing condition. Moreover, in the *PMA*1-overexpressing strain, both higher protein levels and activity, compared to the control under the same stressing conditions, correlate with the observed growth advantage.

Cloning of the *PMA*1 gene from *Zygosaccharomyces bailii*

Z. bailii is very well known for its ability to grow in acidic environments: its acids - and especially organic acids - resistance far exceeds that of the model yeast (Thomas & Davenport, 1985; Cheng *et al.*, 1999; Mollapour & Piper, 2001; Piper *et al.*, 2001 and our unpublished results). Like just described for *S. cerevisiae*, we wanted to test the possibility to further increase the resistance of *Z. bailii* through overexpression of its own plasma membrane H$^+$-ATPase. In order to do that, it was previously necessary to clone the corresponding gene, never isolated so far.

Although sequence conservation within the whole P-type ATPase family, to which Pma1p belongs, is poor (Morsomme *et al.*, 2000), fungal ATPases are highly similar, with nucleotide identities going from 70%, between *S. cerevisiae* and *Schizosaccharomyces pombe*, to even 82% between *S. cerevisiae* and *Z. rouxii* (data not shown), a species closely related to *Z. bailii* (Kurtzman, 1990). Therefore, first

attempts to clone the *ZbPMA*1 gene by PCR amplification were carried out using 5' and 3' primers designed on both sequences of *S. cerevisiae* and *Z. rouxii*, however with no success. We then searched for regions of high sequence identity in the *S. cerevisiae-Z. rouxii* alignment (not shown); based on this, an internal forward primer was designed (corresponding to the second transmembrane domain of the protein) that, together with the *S. cerevisiae* 3' primer, led to the amplification of the central plus C-terminal region of the gene. The N-terminal region was amplified using the *Z. rouxii* 5' primer, and a specific internal reverse primer designed near the 5' end of the *ZbPMA1* fragment previously sequenced. On the basis of the sequencing results, a final PCR reaction was performed to obtain the 2775 bp full length *ZbPMA*1 gene (see Methods), that shared a 79% sequence identity with *ScPMA*1 and a 83% with *ZrPMA*1 (not shown).

The deduced aminoacidic sequence of the newly cloned gene was compared with the corresponding *Sc*Pma1 and *Zr*Pma1 sequences (Fig. 3). Identity at this level was 89% between *Zb*Pma1 and *Zr*Pma1 and 85% between *Zb*Pma1 and *Sc*Pma1. Hence, as it was expected, *Z. bailii* proton pump is more similar to the *Z. rouxii* than to the *S. cerevisiae* one. Interestingly, it can be noted that while in the N-terminal portion *Zb*Pma1 resembles more to *Zr*Pma1 than to *Sc*Pma1, the C-terminal portion is more similar to *Sc*Pma1 than to *Zr*Pma1. In fact, in the region 30-290, 31 aminoacids are identical in *Zb*Pma1 and *Zr*Pma1, but different in *Sc*Pma1, while 14 are identical in *Sc*Pma1 and *Zb*Pma1 and different in *Zr*Pma1; instead, in the region 290-925 there are 14 aminoacids identical between *Zr*Pma1 and *Zb*Pma1 and different in *Sc*Pma1 versus 20 identical in *Sc*Pma1 and *Zb*Pma1, but different in *Zr*Pma1.

A lot of information is available on the role of specific domains and residues especially in *S. cerevisiae* H$^+$-ATPase (reviewed in Morsomme *et al.*, 2000), which has ten transmembrane domains (M1-M10), two major cytoplasmic loops and cytoplasmic N- and C-termini. Here, the major difference between the sequences was ascribable to the N-terminal domain of the protein (Fig. 3). In particular, the Ser cluster present in *S. cerevisiae* was not found in *Z. bailii*; since this portion was described as involved in the regulation of protein turnover (Hasper *et al.*, 1999), one can imagine that regulation at this level might differ for the two proteins. On the other hand, *Z. rouxii* and other yeasts also show an N-terminal aminoacidic pattern

that is more similar to *Z. bailii* (Fig. 3 and not shown), so *S. cerevisiae* could represent a special case. The other domains of the protein show fewer differences. The small cytoplasmic loop, comprised between M2 and M3, contains residues crucial for the catalytic activity, and most of them are conserved in *Z. bailii*. One exception is residue 212, where Thr in *S. cerevisiae* corresponds to Ala in *Z. bailii* (and *Z. rouxii*). It was described that such substitution in *S. cerevisiae* leads to a lethal phenotype (Morsomme *et al.*, 2000). The large cytoplasmic loop, comprised between M4 and M5, is mainly devoted to the ATP binding and contains the site where the phospho-intermediate is formed. The phosphorylation site and the crucial residues for the catalysis resulted conserved between the two yeasts (see Fig. 3).

Figure 3. (next page) Pma1 amino acid sequence alignment
The deduced primary structure of the newly cloned *Z. bailii* H+-ATPase proton pump Pma1p (**FJ948858**) is compared with the deduced homologous sequences from *S. cerevisiae* (*Sc*Pma1) and *Z. rouxii* (*Zr*Pma1). Grey scale coloring represent aminoacid similarity based on Blosum 62 scores. Alignment was performed with ClustalW software and graphical editing was made using Jalview software (Waterhouse et *al.*, 2009). Based on *Sc*Pma1, vertical lines indicate, respectively, the N-terminus and the C-terminus; full-line boxes indicate the essential regions of the small cytoplasmic loop; dashed-line box indicates the essential region of the large cytoplasmic loop

```
                 10        20        30        40        50
ScPma1- MTDTSSSSSSSASSVSAHQP-------TQEKPAKTYDDAASE-SSDDDDIDALIEELQS 52
ZbPma1- MSDERITEKHPHQQSDSEEEEDVPQSQSEEEVEEETEEEVTDESSSEDD-IDALIDELQS 59
ZrPma1- MSDERITEKPPHQQPESEGEP-VP----EEEVEEETEEEVPDEQSSEDDDIDGLIDELQS 55

                 70        80        90       100       110
ScPma1- NHGVDDEDSDNDGPVAAGEARPVPEEYLQTDPSYGLTSDEVLKRRKKYGLNQMADEKESL 112
ZbPma1- NPGEDEEEED-DGPAAAGEARKIPEELLQTDPSYGLTEEEVVHRRKKYGLNQMSEESENL 118
ZrPma1- QEAHEEAEED-DGPAAAGEARKIPEELLQTDPSVGLSSDEVVNRRKKYGLNQMREESENL 114

                130       140       150       160       170
ScPma1- VVKFVMFFVGPIQFVMEAAAILAAGLSDWVDFGVICGLLMLNAGVGFVQEFQAGSIVDEL 172
ZbPma1- FVKFLMFFIGPIQFVMEAAAILAAGLEDWVDFGVICGLLMLNAGVGFIQEYQAGSIVDEL 178
ZrPma1- LVKFLMFFIGPIQFVMEAAAVLAAGLEDWVDFGVICGLLFLNAGVGFIQEFQAGSIVEEL 174

                190       200       210       220       230
ScPma1- KKTLANTAVVIRDGQLVEIPANEVVPGDILQLEDGTVIPTDGRIVTEDCFLQIDQSAITG 232
ZbPma1- KKTLANSAMVIRDGQLQEIPVNEVVPGEIMQLEDGTVISADGRLVTEDCFLQVDQSSITG 238
ZrPma1- KKTLANTATVIRDGSVQEAPANEIVPGDILKLEDGTVIPADGRLVTEECFLQVDQSSITG 234

                250       260       270       280       290
ScPma1- ESLAVDKHYGDQTFSSSTVKRGEGFMVVTATGDNTFVGRAAALVNKAAGGQGHFTEVLNG 292
ZbPma1- ESLAVDKHYGDTVFSSSTVKRGEGFMIVTATGDNTFVGRAASLVGSASGGQGHFTEVLNG 298
ZrPma1- ESLAVDKHYGDEVFSSSTVKRGEGFMIVTATGDNTFVGRAASLVNAAAGGQGHFTEVLNG 294

                310       320       330       340       350
ScPma1- IGIILLVLVIATLLLVWTACFYRTNGIVRILRYTLGITIIGVPVGLPAVVTTTMAVGAAY 352
ZbPma1- IGIILLILVIITLLLIWTACFYRTDRIVPILRYTLGITIVGVPVGLPAVVTTTMAVGAAY 358
ZrPma1- IGVILLVLVVITLLLIWTACFYRTVRIVPILRYTLGITIVGVPVGLPAVVTTTMAGGAAY 354

                370       380       390       400       410
ScPma1- LAKKQAIVQKLSAIESLAGVEILCSDKTGTLITKNKLSLHEPYTVEGVSPDDLMLTACLAA 412
ZbPma1- LAKKQAIVQKLSAIESLAGVEILCSDKTGTLITKNKLSLHEPYTVDGVSDDDLMLTACLAA 418
ZrPma1- LAKKQAIVQKLSAIESLAGVEILCSDKTGTLITKNKLSLHEPYTVEGVSSDDLMLTACLAA 414

                430       440       450       460       470
ScPma1- SRKKKGLDAIDKAFLKSLKQYPKAKDALTKYKVLEFHPFDPVSKKVTAVVESPEGERIVC 472
ZbPma1- SRKRKGLDAIDKAFLKSLINYPKAKNALTKYKVLEFHPFDPVSKKVTAIVESPEGERIIC 478
ZrPma1- SRKKKGLDAIDKAFLKSLAQYPKAKGALTKYKVLEFHPFDPVSKKVTAVVESPEGERIIC 474

                490       500       510       520       530
ScPma1- VKGAPLFVLKTVEEDHPIPEDVHENYENKVAELASRGFRALGVARKRGEGHWEILGVMPC 532
ZbPma1- VKGAPLFVLKTVEEDHPIPEDVHENYENKVAELASRGFRALGVARKRGEGHWEILGVMPC 538
ZrPma1- VKGAPLFVLKTVEEDHPIPEDVHENYENKVAELASRGFRALGVARKRGEGHWEILGVMPC 534

                550       560       570       580       590
ScPma1- MDPPRDDTAQTVSEARHLGLRVKMLTGDAVGIAKETCRQLGLGTNIYNAERLGLGGGGDM 592
ZbPma1- MDPPRDDTAATIAEAKYLGLRIKMLTGDAVGIAKETCRQLGLGTNIYNAERLGLGGGSTM 598
ZrPma1- MDPPRDDTAATVNEAKRLGLSVKMLTGDAVGIAKETCRQLGLGTNIYDAERLGLGGGGSM 594

                610       620       630       640       650
ScPma1- PGSELADFVENADGFAEVFPQHKYRVVEILQNRGYLVAMTGDGVNDAPSLKKADTGIAVE 652
ZbPma1- PGSELFDFVENADGFAEVFPQHKYAVVDILQKRGYLVAMTGDGVNDAPSLKKADTGIAVE 658
ZrPma1- PGSEMYDFVENADGFAEVFPQHKFAVVDILQQRGYLVAMTGDGVNDAPSLKKADTGIAVE 654

                670       680       690       700       710
ScPma1- GATDAARSAADIVFLAPGLSAIIDALKTSRQIFHRMYSYVVYRIALSLHLEIFLGLWIAI 712
ZbPma1- GATDAARSAADIVFLAPGLHAIIDALKTSRQIFHRMYSYVVYRIALSLHLEIFLGLWIAI 718
ZrPma1- GATDAARSAADIVFLAPGLSAIIDALKTSRQIFHRMYAYVVYRIALSLHLEIFLGLWIAI 714

                730       740       750       760       770
ScPma1- LDNSLDIDLIVFIAIFADVATLAIAYDNAPYSPKPVKWNLPRLWGMSIILGIVLAIGSWI 772
ZbPma1- LNHSLDIDLIVFIAIFADVATLAIAYDNAPYSPKPVKWNLPRLWGMSIIMGCILAVGTWI 778
ZrPma1- LNHSLDIDLIVFIAIFADVATLAIAYDNAPFSPSPVKWNLPRLWGMSIMMGIILAAGTWI 774

                790       800       810       820       830
ScPma1- TLTTMFLPKGGIIQNFGAMNGIMFLQISLTENWLIFITRAAGPFWSSIPSWQLAGAVFAV 832
ZbPma1- TLTTMFLPRGGIIQNFGSIDGVLFLEISLTENWLIFVTRAAGPFWSSIPSWQLAGAVAAV 838
ZrPma1- TLTTMFLPKGGIIQNFGSIDGILFLEISLTENWLIFITRAVGPFWSSIPSWQLAGAVFVV 834

                850       860       870       880       890
ScPma1- DIIATMFTLFGWWSENWTDIVTVVRVWIWSIGIFCVLGGFYYEMSTSEAFDRLMNGKPMK 892
ZbPma1- DVIATMFTLFGWWSQNWSDMVTVVRVYIWSIGVFCVLGGAYYLMSESVAFDRLMNGKPMK 898
ZrPma1- DVVATMFTLFGWWSQNWTDIVTVVRIYIWSIGIFCCLGGAYYLMSESETFDRLMNGKPLK 894

                910       920
ScPma1- EKKSTRSVEDFMAAMQRVSTQHEKET 918
ZbPma1- ENKSSRSVEDFLAAMQRVSTQHEKET 924
ZrPma1- ENKSTRSVEDFLASMRRVSTQHEKGN 920
```

Effect of *ZbPMA*1 overexpression in *Z. bailii* cells under lactic acid stress: microtiter screening, batch growth and H$^+$-ATPase activity determination

The newly cloned gene was exploited to generate two expression plasmids - an integrative and an episomal - based on the same vectors used for *S. cerevisiae* (see Methods). The *Sc*TPI promoter and the *ScLEU*2 marker, as well as the *S. cerevisiae* 2μm origin, were previously demonstrated to be functional also in *Z. bailii* (Branduardi *et al.*, 2004 and Dato L *et al.*, manuscript in preparation). The *Z. bailii* auxotrophic strain Z$_2$ΔL, previously obtained in our laboratory (Dato L *et al.*, manuscript in preparation), was transformed with the resulting plasmids. Transformants were first screened for growth in microtiter plates under different conditions and relevant results are shown in Fig. 4.

First of all, the inhibitory concentrations observed (Fig. 4, versus Fig. 1 and data not shown) proved the higher resistance of *Z. bailii*, compared to *S. cerevisiae*, also with respect to lactic acid stress.
Integrative *PMA*1-overexpressing transformants grown in minimal medium were indistinguishable from the controls. Addition of lactic acid at a concentration of 40 g l^{-1} caused a growth delay that was comparable in both strains (Fig. 4a, bottom), with just a slight advantage observed for the *PMA*1-overexpressing. At higher lactic acid concentrations the growth defect became more pronounced in both strains, with no significant advantage for the transformed one (data not shown). Instead, independent transformants bearing the episomal construct resulted significantly inhibited in growth already in minimal medium (Fig. 4a and b, upper panels). Under lactic acid stress, the multicopy overexpression caused a severe growth defect (Fig. 4b, bottom) at a concentration of lactic acid (35 g l^{-1}) that was not inhibiting for the control strain. An excessive metabolic burden caused by the multicopy expression, similarly to what hypothesized for *S. cerevisiae*, could justify the growth impairment observed for episomal transformants.
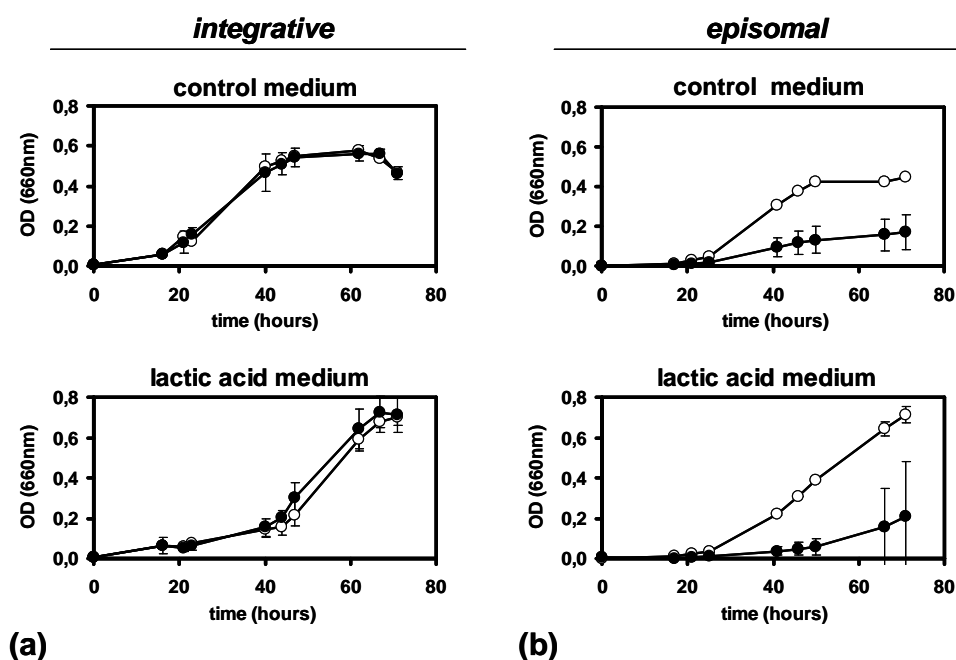
Figure 4. Effect of *ZbPMA*1 overexpression on the ability of *Z. bailii* cells to grow in minimal glucose medium added or not with lactic acid, tested in microtiter plates

Z.bailii $Z_2\Delta L$ cells were transformed either with an integrative (**a**) or with an episomal (**b**) plasmid bearing the *ZbPMA*1 gene under the control of the *ScTPI* promoter. Transformants were grown in 96-well microtiter plates in minimal glucose medium (initial pH 5, upper panels) or in minimal glucose medium added with 40 g l^{-1} (for integrative) or 35 g l^{-1} (for episomal) of lactic acid (initial pH 3, lower panels). Inoculum was at $OD_{660 nm}$ = 0.003 and incubation was at 30 °C under agitation. Growth is indicated as $OD_{660 nm}$. The values reported represent the mean of 2 independent clones for the control strains (empty plasmids, empty symbols) and of 3 independent clones for the *PMA1* overexpressing strains (full symbols). Vertical bars represent standard deviations.

Our previous data on the use of the *S. cerevisiae* 2μm-based plasmid in *Z. bailii* cells pointed out a high instability of the heterologous construct (manuscript in preparation). We therefore speculated that transformants of Fig. 4b still retained several copies of the episomal plasmid that might have been lost after many generations.

For said reason, transformants were restreaked several times and growth kinetics was repeated to compare their behavior at different generations; Fig. 5a shows the results obtained. In lactic acid medium, newly transformed cells (bottom panel, dotted lines), like previously shown, were strongly inhibited in growth; surprisingly, transformants grown for four (dashed lines) or six (full lines) serial restreakings (approximately 100 and 150 generations, respectively) showed a clear advantage in growth if compared to the control strains.

The same observations can be made starting from glycerol stock cultures prepared at the different generations, confirming the stability of the advantage exhibited by the restreaked transformants. An advantage became visible also in the control medium (Fig. 5a, upper panel).

A further observation may be made in respect to the final OD reached, higher in lactic acid added medium if compared to the control medium (Fig. 4a and 5a). We do not have any direct evidence to better explain that, but we can speculate that lactic acid could be used as carbon and energy source (as recently proven by Dang *et al.*, 2009)

Therefore, Pma1 specific activity was determined in episomal transformants grown after six restreakings. At least triplicate experiments were performed giving similar results and Fig. 5b shows data from one representative experiment. First of all, activation occurred in lactic acid medium for both control and overexpressing strains (bottom versus upper panel), similarly to what seen in *S. cerevisiae*. For *PMA*1-overexpressing cells, although activity levels in minimal medium resulted lower if compared to the control strain, they were far higher in the stressed condition, correlating to the growth levels observed.
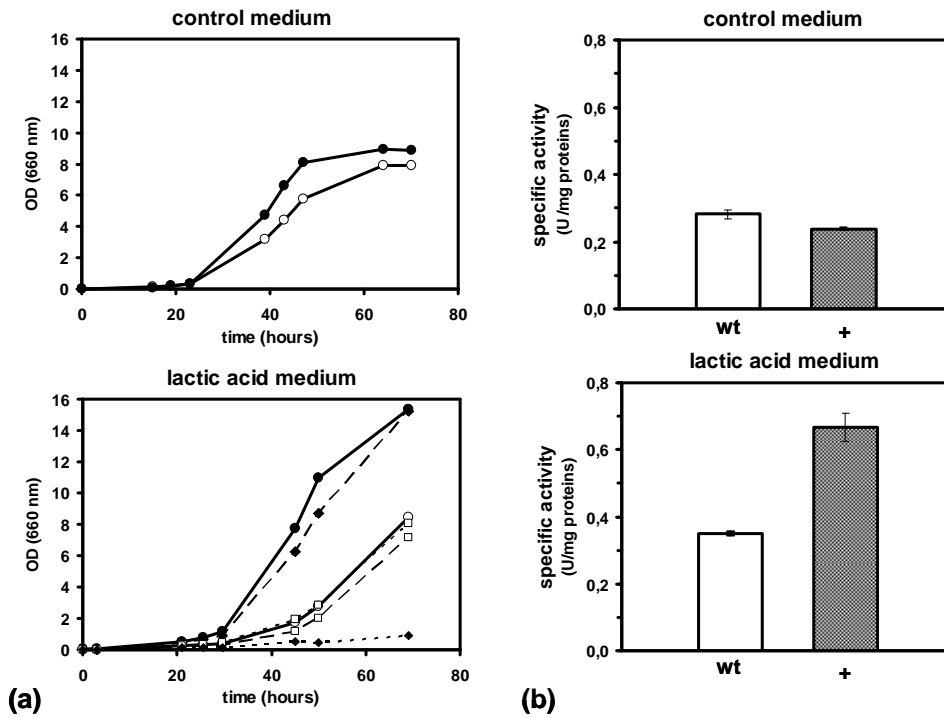
Figure 5. *ZbPMA*1 overexpression in *Z. bailii* cells

(**a**) Growth of *Z. bailii* $Z_2\Delta L$ cells in glucose minimal medium (initial pH 5, upper panel) or in glucose minimal medium added with 35 g l^{-1} of lactic acid (initial pH 3, lower panel). Incubation was at 30 °C in shake flasks under standard agitation. The $OD_{660\ nm}$ at the indicated times are shown. Open symbols, wild type controls (episomal empty plasmid); full symbols, *PMA1*-overexpressing (episomal *ZbPMA*1 plasmid). Full lines, transformants after 6 serial restreakings; dashed lines, transformants after 4 serial restreakings; dotted lines, newly transformed cells.

(**b**) Pma1p specific activity detected on crude membranes extracts. Cells were grown as described, either in the control medium (upper panel) or under lactic acid stress (lower panel), and collected when they reached the mid-log phase (about $OD_{660\ nm}$ = 1.5). Open columns, wild type after 6 streaking cycles; filled columns, *PMA1*-overexpressing after 6 streaking cycles. Vertical bars represent standard deviations for three independent assays on samples from a representative one.

121

Cellular viability during batch growth under lactic acid stress for wild type and *PMA*1-overexpressing *S. cerevisiae* and *Z. bailii* cells

The impaired growth of the wild type strains of both *S. cerevisiae* and *Z. bailii* in lactic acid-stressed cultures at low pH could be ascribed to a longer lag time (needed to adapt to the acid) and/or to cell death. We therefore determined the viability of wild type and transformed cells through flow cytometric analyses. Yeasts were grown as described above, harvested in exponential phase and labelled with propidium iodide to detect severely damaged and/or dead cells. Fluorescence distributions are shown in Fig. 6.

All the cells grown in minimal medium were viable (panels a-d) independently from the species considered and from *PMA*1 overexpression. However, in lactic acid-stressed cultures (panels e-f), significant differences can be noticed. Wild type *S. cerevisiae* cells resulted severely damaged by lactic acid addition, as evidenced by the high fraction of dead cells (above 56%). Said fraction was almost half when *ScPMA*1 was overexpressed (about 34%). It can be argued that thanks to the higher H^+-ATPase activity a higher number of cells can counteract the negative effect of the organic acid, becoming able to grow and duplicate.

Remarkably, a quite different picture appears for *Z. bailii*. Both wild type and transformed populations were viable, despite the presence of the lactic acid at high concentration (only a small fraction of dead cells was detectable, but equal between wild type and transformed cells). So, it can be said that wild type cells delay their growth without loosing viability, being maybe in a quiescent state, and overexpression of *ZbPMA*1 leads to a faster growth.
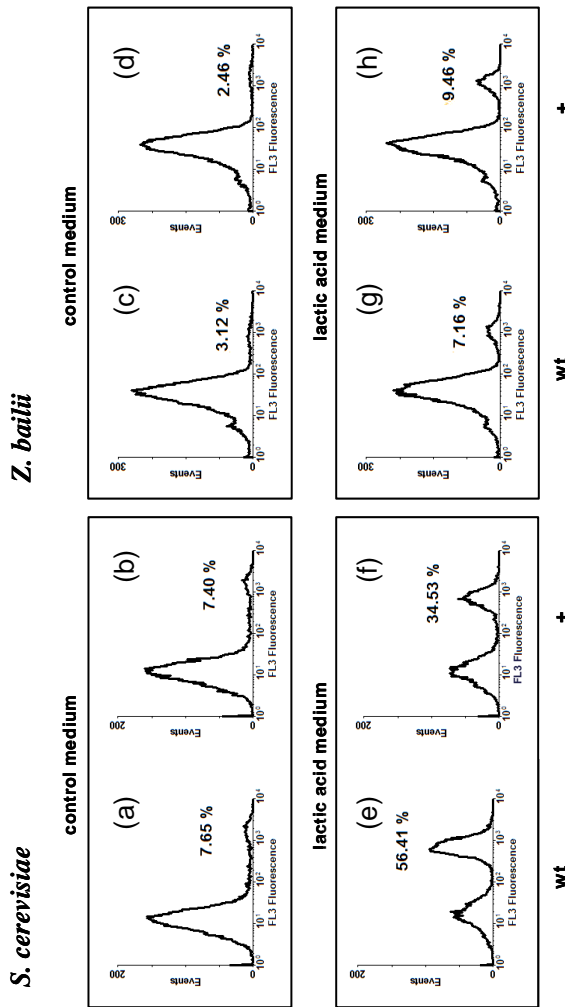
Figure 6. Determination of cell viability by flow cytometric analysis in *S. cerevisiae* and *Z. bailii PMA1*-overexpressing and control strains Yeast cells were grown in shake flasks in YNB-glucose medium added (e - h) or not (a - d) with 35 g l⁻¹ (for *S. cerevisiae*) or 50 g l⁻¹ (for *Z. bailii*) of lactic acid until they reached the exponential phase, then harvested and labelled with 0.46 mM propidium iodide. FL3 fluorescence emission distributions are represented by histograms; a high propidium iodide content (right peaks) corresponds to severely damaged and/or dead cells. The frequency of such events on the total population is indicated by percent values.

(a), (e): *S. cerevisiae* CEN.PK transformed with the integrative empty plasmid pYX042 (wt control).

(b), (f): CEN.PK bearing the integrative plasmid pYX042-*ScPMA1* (+ transformant).

(c), (g): *Z. bailii* Z₂ΔL transformed with the episomal empty plasmid pYX242 (wt control).

(d), (h): Z₂ΔL bearing the episomal plasmid pYX242-*ZbPMA1* (+ transformant).

DISCUSSION

A positive regulation of the endogenous plasma membrane H^+-ATPase activity was previously demonstrated for *S. cerevisiae* cells exposed to octanoic (Viegas & Sa-Correia, 1991, Viegas *et al.*, 1994, Viegas *et al.*, 1998), sorbic (Holyoak *et al.*, 1996), benzoic (Macpherson *et al.*, 2005), decanoic (Alexandre *et al.*, 1996), acetic and succinic acid (Carmelo *et al.*, 1997). The data here presented showed for the first time that a Pma1p stimulation is also triggered by lactic acid, concerning both protein and activity levels.
Moreover, Pma1p activity induction in response to lactic acid also occurs in *Z. bailii* cells, further supporting its role in the organic acid response of this highly acid-resistant yeast, in agreement to what previously reported (Macpherson *et al.*, 2005). We still have no information about protein levels in *Z. bailii*, but with the availability of the corresponding gene here reported, further studies on this subject will be possible and useful.

Further, overall higher plasma membrane ATPase activity levels were found in *Z. bailii* cells compared to *S. cerevisiae* cells, in all the conditions tested (Fig. 5b versus Fig. 2c and data not shown). This could partially explain the different ability of the two yeasts to resist lactic acid stress: we may speculate, in fact, that the *Z. bailii* ATPase is already in a more activated state in wild type cells and it can more readily counteract a cytoplasm acidification, if compared to *S. cerevisiae*. Both the cell viability data and the extent of Pma1p activity stimulation by lactic acid in wild type cells (that was lower for *Z. bailii* than for *S. cerevisiae*) could support this hypothesis.

Despite the key role of Pma1p in acid resistance, a positive effect of *PMA1* gene overexpression was not predictable, on the basis of data reported for overexpression of membrane proteins in general (Österberg *et al.*, 2006) and Pma1p in particular (Eraso *et al.*, 1987; Supply *et al.*, 1993). Also, it was not so straightforward: for both yeasts it was necessary to test different gene dosages. In the conditions and for the strains tested, the best combinations resulted in the integration of a single copy for *S. cerevisiae* and an episomal low-copy expression for *Z. bailii*.

More in detail, in *S. cerevisiae*, one additional copy of the gene under the control of the TPI promoter is sufficient to improve resistance to

lactic acid stress, whereas a further increase in gene copy number does not lead to a proportional improvement of robustness. The fact that the gene copy number appears particularly critical in the balance between positive and detrimental effect on growth might be correlated not only with the metabolic burden caused by the overexpression of a membrane protein, as already mentioned in the Results section, but also with the well described multiple and tight regulation that acts at different levels on Pma1p production and activation (Goossens *et al.*, 2000; Portillo, 2000). Such regulation appears to act also in the *PMA*1-overexpressing cells in both yeasts, as evidenced from the specific activity measured that were either equal or even slightly lower in non-limiting conditions, if compared to the wild type cells, but significantly higher in the presence of lactic acid stress.

In *Z. bailii*, the addition of a single copy of *ZbPMA*1 is not sufficient to significantly improve resistance to lactic acid; we propose that a high-copy expression is detrimental while a low-copy expression (that we speculate might be about 2-4 copies per cell, according to our unpublished results) is advantageous for cells under lactic acid stress. The higher basal Pma1p activity levels and/or the higher viability of the cell population could explain the dosage difference seen, compared to *S. cerevisiae*. Alternatively, a simple ploidy-effect could account for this difference.

It is not possible to generalize the positive effect of *PMA1* overexpression in respect to other organic acids, at least in our cellular systems. In fact, effects in the presence of other organic acids were tested, but only sorbic acid stress was slightly relieved by *PMA*1 overexpression (data not shown). Toxicity of weak organic acids has been mainly attributed to intracellular acidification and anion accumulation, the second having different effects in function of the different characteristics of the acyl chain. A first distinction was made in respect to the lipophilicity of the anion, the more lipophilic ones causing membrane disruption (Holyoak *et al.*, 1999; Krebs *et al.*, 1983); recently, however, it was shown that transcriptional responses to different weak acids in *S. cerevisiae* do not exhibit extensive similarities, in spite of a similar solubility, as is the case for sorbic and benzoic acids (Abbott *et al.*, 2007), suggesting that molecular responses could be unique for individual acids. Moreover, since a charge balance is needed, which also involves anion extrusion, it is possible to speculate that different efficiencies/capacities of different

anion-export mechanisms require different rates of proton removal. This could explain why a higher H^+-ATPase activity can help in resistance to certain acids and not to others.

Overall, our data demonstrated for the first time the possibility to increase yeast tolerance to lactic acid stress through the overexpression of the *PMA*1 gene. This was confirmed in two different yeast species, *S. cerevisiae* and *Z. bailii*, the latter being already particularly resistant to acidic stress. It should be underlined that the effect(s) of this manipulation could be either direct or indirect. In the first case, a higher rate of proton extrusion could lead to both a more efficient modulation of pH_i value, as well as to a faster export of the lactate anion, thus limiting these toxic accumulations.

Moreover, other indirect effects of such overexpression could influence the physiological state of the cells and, consequently, their responses to stresses. As sake of example, accumulation of the Pma1p in the plasma membrane above the physiological levels could lead to a decreased fluidity (i.e., or increased rigidity); this, in turn, could result in a decreased permeability of undissociated lactic or other organic acids. This indirect effect could be both stress and cell dependent.

In conclusion, the results here presented might open promising applications, for example in the development of new host strains for lactic acid production: *S. cerevisiae* has been already engineered for lactic acid production (Dequin & Barre, 1994; Porro *et al.*, 1995); since toxicity of the product is the main bottleneck for this kind of production, an improvement in tolerance could increase productivity. Similarly, the concept and possible applications of such manipulation might be extended to *Z. bailii* and other microorganisms.

ACKNOWLEDGEMENTS

REFERENCES

Abbott DA, Knijnenburg TA, de Poorter LM, Reinders MJ, Pronk JT & van Maris AJ (2007) Generic and specific transcriptional responses to different weak organic acids in anaerobic chemostat cultures of *Saccharomyces cerevisiae*. FEMS Yeast Res 7: 819-833.

Alexandre H, Mathieu B & Charpentier C (1996) Alteration in membrane fluidity and lipid composition, and modulation of H(+)-ATPase activity in *Saccharomyces cerevisiae* caused by decanoic acid. Microbiology 142(3): 469-475.

Beales N (2004) Adaptation of microorganisms to cold temperatures, weak acid preservatives, low pH, and osmotic stress: a review. Compr Rev Food Sci Food Saf 3(1): 1-20.

Benninga HA (1990) *A history of lactic acid making*. Kluyver Academic Publisher, Dordrecht, The Netherlands.

Booth IR & Kroll RG (1989) The preservation of foods by low pH. *Mechanisms of action of food preservation procedures* (Gould GW, ed), pp. 119-160. Elsevier Applied Science, London.

Booth IR & Stratford M (2003) Acidulants and low pH. *Food preservatives* (Russel NJ & Gould GW, eds), pp. 25-42. Blackie, Glasgow.

Branduardi P, Valli M, Brambilla L, Sauer M, Alberghina L & Porro D (2004) The yeast *Zygosaccharomyces bailii*: a new host for heterologous protein production, secretion and for metabolic engineering applications. FEMS Yeast Res 4: 493-504.

Branduardi P, Sauer M, De Gioia L, Zampella G, Valli M, Mattanovich D & Porro D (2006) Lactate production yield from engineered yeasts is dependent from the host background, the lactate dehydrogenase source and the lactate export. Microb Cell Fact 30(5): 4-15.

Branduardi P, Smeraldi C & Porro D (2008) Metabolically engineered yeasts: 'potential' industrial applications. J Mol Microbiol Biotechnol 15: 31-40.

Brul S & Coote P (1999) Preservative agents in foods. Mode of action and microbial resistance mechanisms. Int J Food Microbiol 50: 1-17.

Capieaux E, Vignais ML, Sentenac A & Goffeau A (1989) The yeast H+-ATPase gene is controlled by the promoter binding factor TUF. J Biol Chem 264: 7437-7446.

Carmelo V, Bogaerts I & Sa-Correia I (1996) Activity of plasma membrane ATPase and expression of *PMA*1 and *PMA*2 genes in *Saccharomyces cerevisiae cells* grown at optimal and low pH. Arch. Microbiol 166: 315-320.

Carmelo V, Santos H & Sa-Correia I (1997) Effect of extracellular acidification on the activity of plasma membrane ATPase and on the cytosolic and vacuolar pH of *Saccharomyces cerevisiae*. Biochim Biophys Acta 1325: 63-70.

Cheng L, Moghraby J & Piper PW (1999) Weak organic acid treatment causes a trehalose accumulation in low-pH cultures of *Saccharomyces cerevisiae*, not displayed by the more preservative-resistant *Zygosaccharomyces bailii*. FEMS Microbiol Lett 170: 89-95.

Dang TD, Vermeulen A, Ragaert P & Devlieghere F (2009) A peculiar stimulatory effect of acetic and lactic acid on growth and fermentative metabolism of *Zygosaccharomyces bailii*. Food Microbiol 26: 320-327.

Dequin S & Barre P (1994) Mixed lactic acid-alcoholic fermentation by *Saccharomyces cerevisiae* expressing the *Lactobacillus casei* L(+)-LDH. Biotechnology (N Y) 12: 173-177.

Eraso P & Gancedo C (1987) Activation of yeast plasma membrane ATPase by acid pH during growth. *FEBS Lett* **224**: 187-192.

Eraso P, Cid A & Serrano R (1987) Tight control of the amount of yeast plasma membrane ATPase during changes in growth conditions and gene dosage. FEBS Lett 224: 193-197.

Gietz RD & Woods RA (2002) Transformation of yeast by lithium acetate/single-stranded carrier DNA/polyethylene glycol method. Methods Enzymol 350: 87-96.

Goffeau A & Slayman CW (1981) The proton-translocating ATPase of the fungal plasma membrane. Biochim Biophys Acta 639: 197-223.

Goldstein AL & McCusker JH (1999) Three new dominant drug resistance cassettes for gene disruption in *Saccharomyces cerevisiae*. Yeast 15: 1541-1553.

Goossens A, de La Fuente N, Forment J, Serrano R & Portillo F (2000) Regulation of yeast H(+)-ATPase by protein kinases belonging to a family dedicated to activation of plasma membrane transporters. Mol Cell Biol 20: 7654-7661.

Hasper A, Soteropoulos P & Perlin DS (1999) Modification of the N-terminal polyserine cluster alters stability of the plasma membrane H(+)-ATPase from *Saccharomyces cerevisiae*. Biochim Biophys Acta 1420: 214-222.

Hoffman CS & Winston F (1987) A ten-minute DNA preparation from yeast efficiently releases autonomous plasmids for transformation of *Escherichia coli*. Gene 57: 267-272.

Holyoak CD, Stratford M, McMullin Z, Cole MB, Crimmins K, Brown AJ & Coote PJ (1996) Activity of the plasma membrane H(+)-ATPase and optimal glycolytic flux are required for rapid adaptation and growth of *Saccharomyces cerevisiae* in the presence of the weak-acid preservative sorbic acid. Appl Environ Microbiol 62: 3158-3164.

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

Kurtzman CP (1990) DNA relatedness among species of the genus *Zygosaccharomyces*. Yeast 6: 213-219.

Lecchi S, Allen KE, Pardo JP, Mason AB & Slayman CW (2005) Conformational changes of yeast plasma membrane H(+)-ATPase during activation by glucose: role of threonine-912 in the carboxy-terminal tail. Biochemistry 44: 16624-16632.

Macpherson N, Shabala L, Rooney H, Jarman MG & Davies JM (2005) Plasma membrane H+ and K+ transporters are involved in the weak-acid preservative response of disparate food spoilage yeasts. Microbiology 151: 1995-2003.

Mollapour M & Piper PW (2001) The *ZbYME*2 gene from the food spoilage yeast *Zygosaccharomyces bailii* confers not only *YME*2 functions in *Saccharomyces cerevisiae*, but also the capacity for catabolism of sorbate and benzoate, two major weak organic acid preservatives. Mol Microbiol 42: 919-930.

Morsomme P, Slayman CW & Goffeau A (2000) Mutagenic study of the structure, function and biogenesis of the yeast plasma membrane H(+)-ATPase. Biochim Biophys Acta 1469: 133-157.

Navarre C, Ferroud C, Ghislain M & Goffeau A (1992) A proteolipid associated with the plasma membrane H(+)-ATPase of fungi. Ann N Y Acad Sci 671: 189-193.

Osterberg M, Kim H, Warringer J, Melen K, Blomberg A & von Heijne G (2006) Phenotypic effects of membrane protein overexpression in *Saccharomyces cerevisiae*. Proc Natl Acad Sci U S A 103: 11148-11153.

Piper P, Mahe Y, Thompson S, Pandjaitan R, Holyoak C, Egner R, Muhlbauer M, Coote P & Kuchler K (1998) The pdr12 ABC transporter is required for the development of weak organic acid resistance in yeast. EMBO J 17: 4257-4265.

Piper P, Calderon CO, Hatzixanthis K & Mollapour M (2001) Weak acid adaptation: the stress response that confers yeasts with resistance to organic acid food preservatives. Microbiology 147: 2635-2642.

130

Porro D, Brambilla L, Ranzi BM, Martegani E & Alberghina L (1995) Development of metabolically engineered *Saccharomyces cerevisiae* cells for the production of lactic acid. Biotechnol Prog 11: 294-298.

Portillo F (2000) Regulation of plasma membrane H(+)-ATPase in fungi and plants. Biochim Biophys Acta 1469: 31-42.

Sauer M, Porro D, Mattanovich D & Branduardi P (2008) Microbial production of organic acids: expanding the markets. Trends Biotechnol 26: 100-108.

Serrano R (1978) Characterization of the plasma membrane ATPase of *Saccharomyces cerevisiae*. Mol Cell Biochem 22: 51-63.

Serrano R (1983) In vivo glucose activation of the yeast plasma membrane ATPase. FEBS Lett 156: 11-14.

Serrano R (1984) Plasma membrane ATPase of fungi and plants as a novel type of proton pump. Curr Top Cell Regul 23: 87-126.

Serrano R, Kielland-Brandt MC & Fink GR (1986) Yeast plasma membrane ATPase is essential for growth and has homology with (Na+ + K+), K+- and Ca2+-ATPases. Nature 319: 689-693.

Serrano R (1988) H+-ATPase from plasma membranes of *Saccharomyces cerevisiae* and *Avena sativa* roots: purification and reconstitution. Methods Enzymol 157: 533-544.

Supply P, Wach A, Thines-Sempoux D & Goffeau A (1993) Proliferation of intracellular structures upon overexpression of the *PMA*2 ATPase in *Saccharomyces cerevisiae*. J Biol Chem 268: 19744-19752.

Thomas DS & Davenport RR (1985) *Zygosaccharomyces bailii* – a profile of characteristics and spoilage activities. Food Microbiol 2: 157-169.

Valli M, Sauer M, Branduardi P, Borth N, Porro D & Mattanovich D (2006) Improvement of lactic acid production in *Saccharomyces*

cerevisiae by cell sorting for high intracellular pH. Appl Environ Microbiol 72: 5492-5499.

Verduyn C, Postma E, Scheffers WA & Van Dijken JP (1992) Effect of benzoic acid on metabolic fluxes in yeasts: a continuous-culture study on the regulation of respiration and alcoholic fermentation. Yeast 8: 501-517.

Viegas CA & Sa-Correia I (1991) Activation of plasma membrane ATPase of *Saccharomyces cerevisiae* by octanoic acid. J Gen Microbiol 137 : 645-651.

Viegas CA, Supply P, Capieaux E, Van Dyck L, Goffeau A & Sa-Correia I (1994) Regulation of the expression of the H(+)-ATPase genes *PMA*1 and *PMA*2 during growth and effects of octanoic acid in *Saccharomyces cerevisiae*. Biochim Biophys Acta 1217: 74-80.

Viegas CA, Almeida PF, Cavaco M & Sa-Correia I (1998) The H(+)-ATPase in the plasma membrane of *Saccharomyces cerevisiae* is activated during growth latency in octanoic acid-supplemented medium accompanying the decrease in intracellular pH and cell viability. Appl Environ Microbiol 64: 779-783.

Warth AD (1985) Resistance of yeast species to benzoic and sorbic acids and to sulphur dioxide. J Food Prot 48(7): 564-569.

Warth AD (1989) Relationships among Cell Size, Membrane Permeability, and Preservative Resistance in Yeast Species. Appl Environ Microbiol 55: 2995-2999.

Waterhouse AM, Procter JB, Martin DM, Clamp M & Barton GJ (2009) Jalview Version 2--a multiple sequence alignment editor and analysis workbench. Bioinformatics 25(9): 1189-1191.

Monitoring oxidative and acidic stress at single cell level

Manuscript in preparation

Authors in alphabetical order:

Paola Branduardi[1]*§
Tiziana Fossati [1]*
Simone Passolunghi[1]*
Danilo Porro[1]*§

[1]University of Milano-Bicocca, Department of Biotechnology and Bioscience, Milan, Italy

*These authors contributed equally to this work
§Corresponding author

Email addresses:
PB: paola.branduardi@unimib.it
TF: tiziana.fossati@unimib.it
SP: simone.passolunghi@unimib.it
DP: danilo.porro@unimib.it

Abstract

The ideal cell factory performs a bioprocess with high production, productivity and product yield. To fully get this goal, the cell factory and/or the cellular environment have to be often engineered or modified. Furthermore, the cell factory has also to be robust enough to overcome the physical/chemical/biochemical constraints often encountered during industrial processes.

Looking for a robust host, we have recently developed a *S. cerevisiae* strain able to convert D-glucose in L-Ascorbic Acid (L-AA). This goal has been obtained by implementing endogenous enzymatic activities with heterologous ones, fished from plant kingdom.

Thanks to the endogenous production of L-AA, which is widely recognised to be among the best powerful oxygen scavenger, the recombinant strain becomes more tolerant to various stresses, including those driven by oxidative conditions, low inorganic or organic pH values. Aimed to shed more light on this better fitness, we challenged control and L-AA producing cells with different stresses caused by various organic acids and by hydrogen peroxide. Cells have been analysed by flow cytometry. We determined the amount of reactive oxygen species and the viability degree of any single cell. Stationary and dynamic growth conditions have been analysed to track the stressful pathway going from the appearance of the stress to the death or to the recovery of the cell.

Background

Organic acids constitute a key group among the building-block chemicals that can be produced by microbial processes. Because of their functional groups, organic acids could be useful starting materials for the chemical industry [1]. Most of these acids are natural products of microorganisms, or natural intermediates in major metabolic pathways.

Despite the "natural" origin, the majority of new microbial organic acid production processes are not well established and not industrially viable due to the high cost of operation at large scale. This can be due to many different reasons: i) natural organic acid producers are often not well characterized and/or difficult to cultivate, ii) natural and recombinant strain are forced to produce to high and non-physiological concentration of acid, iii) this "unnatural" concentration of a natural product together with harsh process condition generate a stressful environment where cells seldom survive.

By increasing stress resistance of the microorganisms, the process costs could be reduced appropriately, so strain robustness inevitably has to be considered a crucial key point in order to obtain a viable and feasible process even in industrial relevant scale.

Microbial cells will almost inevitably encounter stresses during parts of their growth and reproductive cycles but also during production processes. As a result, signatures of stress response mechanisms are widespread in the genome, cytoplasm, and plasma membrane of cells. During the process of biomass propagation, yeast cells are dynamically exposed to a mixed and interrelated group of known stresses such as osmotic, oxidative, acidic, thermal and/or starvation.

Moreover, a range of environmental factors (such as exposure to radiation, heavy metal ions, and free radical-generating compounds) can lead to reactive oxygen species (ROS) that can damage many of the cellular components, including DNA, proteins and lipids [2]. Yeast are particularly suitable as microbial cell factories of recombinant pharmaceutical compound [3], fine chemicals [4] and especially of building blocks [1], [5].Yeast cells have a range of responses to ROS that depend on the dose. At very low doses the cells can adapt to become more resistant to a subsequent lethal exposure. At higher

doses the cells activate various antioxidant functions including a program of gene expression mediated mainly by the Yap1p and Msn2,4p transcription factors and cell-division cycle delay [6]. At even higher doses, death of a proportion of cells in the population occurs, initially by apoptosis, but at extreme doses by necrosis. It has been observed that yeast cells during ageing accumulate ROS and undergo apoptotic death, indicating that oxidative stress defence plays a major role in governing ageing-induced apoptosis. In particular it has been demonstrated that low concentration of acetic acid and formic acid induces apoptosis-like cell death in *S. cerevisiae*, and this active process is accompanied by ROS burst [7,8], [8].

Acidic environments constitute a big challenge for yeast, but in general for microbial, cells for another reason. Since protons tend to cross the plasma membrane following their concentration gradient, cells have to spend energy to maintain the correct physiological intracellular pH (pHi), crucial for cellular homeostasis and survival and this energy demand process can be detrimental for growth [9].

Organic acids can passively diffuse through the plasma membrane in their undissociated form: once inside the cells, because of the relatively high pHi, organic acids dissociate causing cytoplasm acidification due to proton release [10], [11] and accumulation of the acid anion to potentially toxic levels. Therefore, in this case even more energy will be required, to maintain the physiological pHi and to extrude and/or metabolize the anion. This phenomenon was particularly examined in yeasts [12], either from a physiological, but also from a biotechnological point of view, being these microorganisms particularly appealing for organic acids production [13].

In fact, yeasts in general, and some species in particular, are able to survive in acidic environments by partially counteracting the toxic effects of inorganic and/or organic acids [14] [15] [16] [17,18]. In this paper a new method to detect and measure ROS accumulation in yeast after exposition to oxidative and acidic stress is evaluated. This is just a first step in setting up a screening tool able to select cells with increased robustness to low pH environment, condition where cells has often to operate as cell factories.

Results

The ideal cell factory performs a sustainable process with high production, productivity and product yield. To fully get this goal, the cell factory and/or the cellular environment have to be often engineered or modified. Furthermore, the cell factory has also to be robust enough to overcome the physical/chemical and biochemical constraints often encountered during industrial processes.

Looking for a robust host, we recently developed a *S. cerevisiae* strain able to convert D-glucose in L-Ascorbic Acid (L-AA, naturally not produced by yeasts) [19, and further developed as described in Methods section]. Such goal has been obtained by implementing endogenous enzymatic activities with heterologous ones, fished from plant kingdom.

The *A. thaliana* enzymes *AtLGDH*, *AtME, AtVTC2* and *AtMIP* together with the endogenous *ScALO* were cloned and expressed in yeasts (different *S. cerevisiae* backgrounds).
We have already demonstrated that engineered yeast strains able to endogenously produce L-ascorbic acid (L-AA) become significantly more tolerant to different limiting environmental conditions. Specifically, this "strain robustness" correlates with a lower accumulation of ROS and with improved cell viability.
In order to better understand this phenomenon we challenged the control and L-AA producing cells with different stresses and their response was measured by flow cytometry.

In the presence of different kind of stresses (oxidative as well as acidic due to inorganic or organic acids) the growth of wild type strain is negatively affected proportionally to the concentration of the stress agent (figure 1 and data not shown). Significantly, under these conditions, the recombinant strains producing L-AA are still able to grow, showing strong robustness and enhanced resistance [figure 1]. Interestingly, while a positive correlation between oxidative stress determined by hydrogen peroxide and higher antioxidant levels could be anticipated, the data regarding the low pH and the different organic acids at low pH need further characterization and investigation.

Figure 1. Kinetics of growth of GRF18U wild (blue symbols) and engineered strains (red symbols) cultivated in minimal medium (insert A), in minimal medium with H_2O_2 3.5 mM (B), in minimal medium lowered to pH 2.2 (C), in minimal medium with formic acid 15 mM (D), in minimal medium with acetic acid
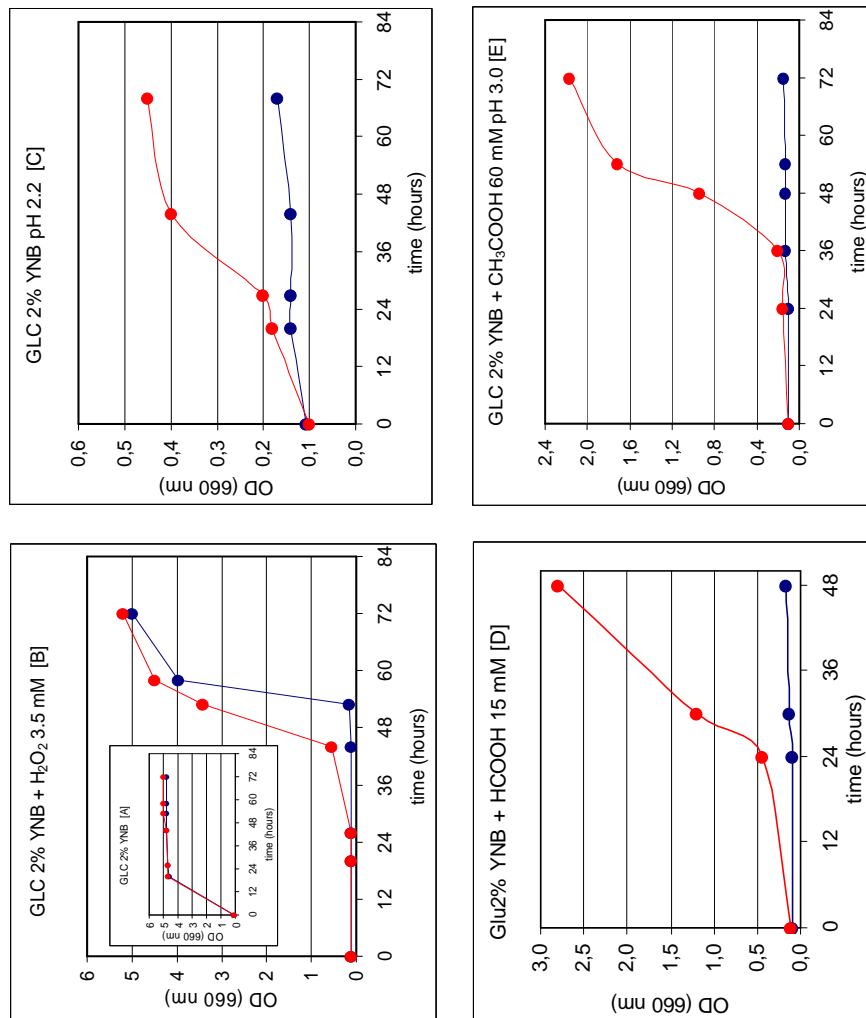
In order to study the correlation between L-AA production and cell robustness, wild type and engineered strains were cultivated in presence of H_2O_2 and subsequently stained with DHR123, (which detects the intracellular ROS accumulation) and with PI (which identifies severely damaged/dead cells). In the engineered strain (figure 2, panel C) the flow cytometric diagram clearly shows both a reduction of ROS accumulation and of the portion of dead cells (low DHR-FL1, high PI-FL3) compared to the wild type strain (high DHR and low PI, figure 2, panel B). Thus, endogenous L-AA production increases cell viability, and scavenges from ROS accumulation, enhancing tolerance to oxidative stress. The showed samples were taken at 24 hours of growth, when both strains started to grow.

To better characterize the robustness of the engineered strain versus wild type in presence of organic acids we applied the same staining protocol (implying an incubation time with the dye on cells still exposed to the stressing agent) to both strains cultivated in presence of formic acid 50 mM, succinic acid 508 mM, acetic acid 100 mM and we didn't identify neither relevant ROS production nor differences between strains.

Following the approach adopted in [7] with some modifications (see methods for details) we set up a panel of growth and stain test where L-AA producing strain and wild type strain were cultivated until exponential phase and then exposed to a "pulse" of a stressor agent (i.e.: formic acid 50 mM, succinic acid 508 mM, acetic acid 100 mM and hydrogen peroxide 4 mM).
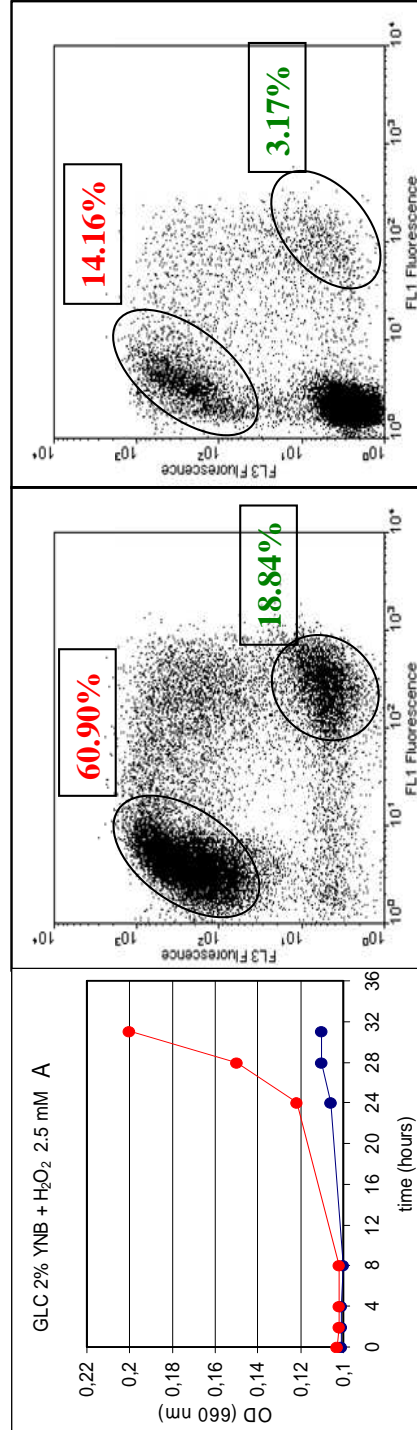
Figure 2. Kinetics of growth in minimal medium with H2O2 2.5 mM (A), cytometric analysis (B, C) in wild type GRF18U and engineered GRF18U [ALO LGDH ME MIP VTC2]. In the producing strain the most common situation is well represented by panel C, where almost no signal for ROS is detectable.

The fluorescence distributions obtained (figure 3) shown that all the organic acids evoke a cellular answer characterized by ROS accumulation. The differential ROS accumulation is very likely related to the nature of stressor agent and still very likely indicates only one of the cellular responses. Surprisingly, no significative differences were observed between engineered and wild type strain, while we demonstrated the higher robustness of the first in growing conditions.
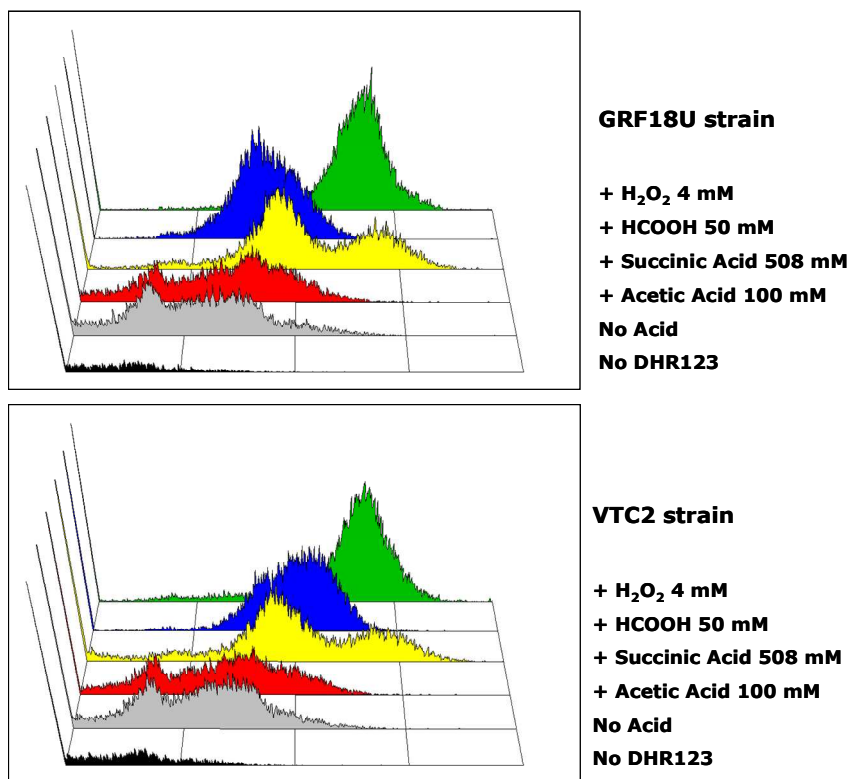

Figure 3. Cytometric analysis of ROS accumulation in wild type GRF18U (upper panel) and engineered GRF18U [ALO LGDH ME MIP VTC2] (lower panel) after stress exposure. Type and concentration of stressor agent are indicated

It has to be underlined that with this approach we can have an instant snapshot of ROS accumulation caused by acidic or peroxide treatment at single cell level. To investigate about the kinetics of ROS scavenging and about the path from stress appearance to cells death , samples were taken at different time points during early phase of incubation in limiting condition. Because of the problems with the staining procedure mentioned before, we went back to examine what happens when cells are inoculated in medium containing H_2O_2. Samples at different time starting from time 0 were collected and subsequently stained with DHR123 and PI, as previously described. Flow cytometric diagrams show the different accumulation kinetic between wild type (figure 4, upper panel) and L-AA producing strain during time (figure 4, lower panel).

It is clearly possible to observe that initially both strains suffer because of the stress imposed, resembling the situation described in Figure 3 and occurring with H_2O_2 as well as with all the organic acids, but very soon in the plot of the engineered strain a subpopulation of healthy cells appears. It is also possible to observe that in less than 24 hours in this sample the ROS subpopulation disappears, suggesting a complete detoxification. The PI staining indicates that after a dramatic increase in the percentage of the severely damaged/dead cells in the first eight hours, the viable subpopulations not stained with PI and with low content of ROS promptly increase after 24 hours. Even for wild type cells a mechanism of detoxification starts after 24 hours of cultivation but the kinetic of ROS scavenging is significative slower than observed for the L-AA producing strain.
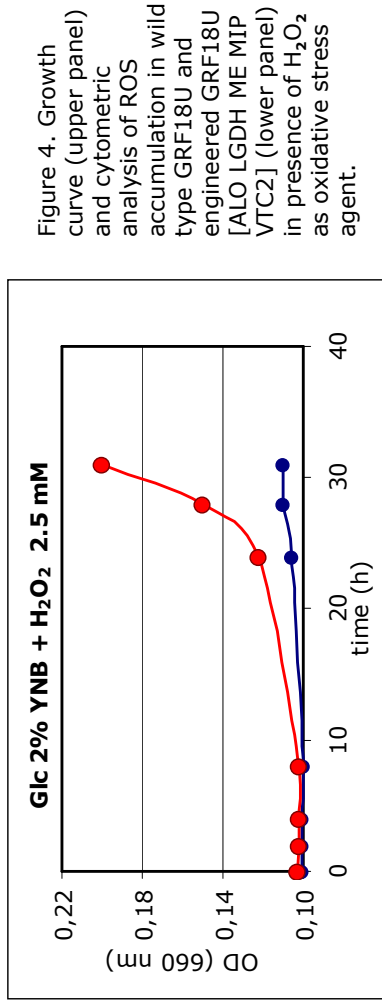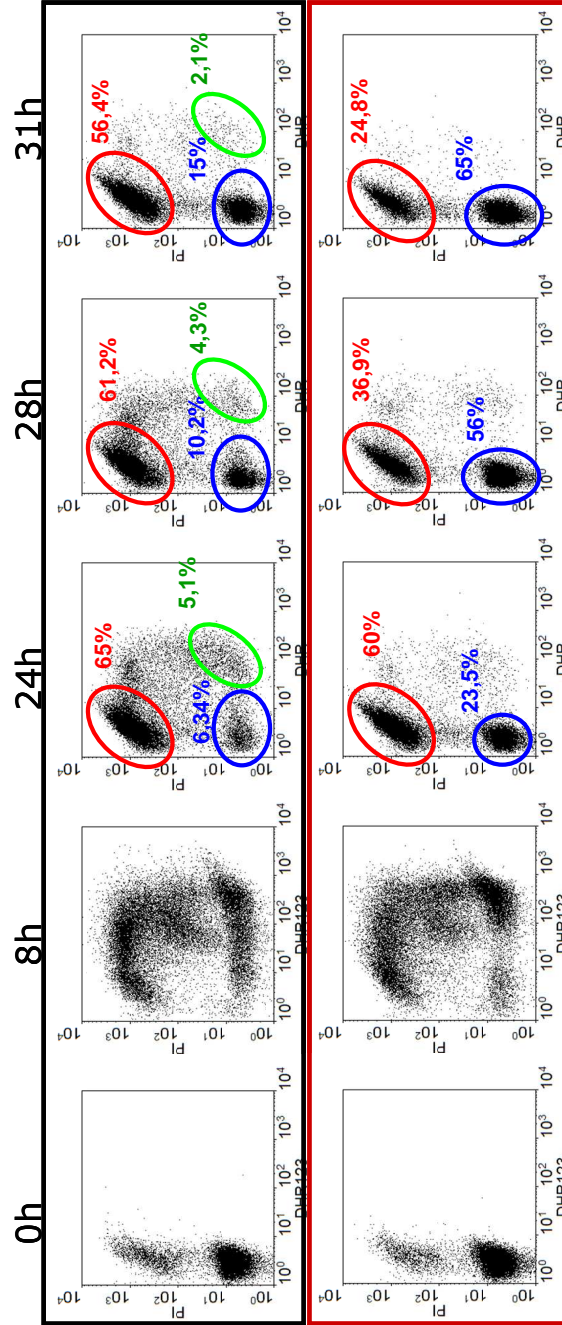
Figure 4. Growth curve (upper panel) and cytometric analysis of ROS accumulation in wild type GRF18U and engineered GRF18U [ALO LGDH ME MIP VTC2] (lower panel) in presence of H_2O_2 as oxidative stress agent.

Discussion

The data reported in this paper shown a ROS accumulation in yeast after acid stress exposure and that the extent of ROS generation is correlated with the intensity of the stressor agent used and its chemical nature. This is the first example of a direct relation between different organic acids and ROS generation measured at single cell level.

Moreover, while it is clear that L-AA producing yeast is able to accumulate a lower amount of ROS than the wild type parental strain, some questions still remain open. It is still not possible to argue if the engineered strain grows better than the control in presence of the stressor agent or if the treatment with H_2O_2 strongly affects the viability and in this way a small group of resistant will have been selected. It is not yet clear if this resistant subpopulation was selected from a small group of severely damaged cells or if they were newborn.

In addition, the overall kinetic of ROS scavenging between the strains is still not clearly understood. In order to answer to these questions further analyses are necessary to address the mechanism of detoxification. The above reported observations will be helped by sampling and analysing the cells also between 8 and 24 hours and, if possible according to the stain procedure, between 0 and 2 hours, where the major differences in terms of subpopulation appearance/disappearance occur.

Moreover, we are optimizing methods for the ROS staining even in the presence of organic acids to extend the panel of observation and to evaluate if the different stresses could cause different responses that can be visualized over time also at the level of cellular subpopulations. At the best of our knowledge there are no experimental data concerning ROS accumulation with persistent organic acid treatment in yeast measured by flow cytometry.

The data obtained under the acidic stresses are of particular relevance since these conditions can often be met during industrial fermentations by cell factories as already explained in the introduction. Detailing the mechanisms of acid stress may be helpful to shed light on the network of tolerance and is crucial, from applicative point of view, for increasing yeast robustness to obtain cell factories able to guarantee competitive performance during large scale acid production.

Methods

The *S. cerevisiae* parental strains used in this study were GRF18U [20] (MATα; *ura3*; *leu2-3,112*; *his3-11,15*; cir$^+$), and BY4742 (MATa; *ura3Δ0*; *his3Δ1*; *leu2Δ0*; *lys2Δ0*; cir$^+$), EuroScarf Accession No Y10000-http://www.rz.uni-frankfurt.de/FB/fb16/mikro/euroscarf). The *S. cerevisiae* engineered L-AA producing strains were described in [19] and it was further developed by introduction of VTC2 gene from *A. thaliana* (as described in [21]) and transferred in pZ5 centromeric vector according to {Fossati *et al*, manuscript in preparation}.

Yeast cultures were grown in minimal synthetic medium (0.67% w/v YNB Biolife without amino acids) with 2% w/v of D-glucose as carbon source. When required, supplements such as leucine, uracil, lysine and histidine were added to a final concentration of 50 mg/l, while the antibiotic nourseotricine sulphate (cloNAT, WERNER BioAgents, Germany) was added to a final concentration of 100 mg/l. In the case of growth under oxidative or acidic stress, the desired stress condition was imposed by adding different concentrations of H_2O_2 (specifically 2.5 and 4 mM), of formic acid (50 mM), succinic acid (508 mM), acetic acid (100 mM) directly to the medium or by lowering the medium pH to 2.2 with HCl 6N. In the case of acid addition, the pH value was adjusted to 3 with NaOH 4N. Yeast cells were inoculated at an initial optical density of 0.1 (always 660 nm where not differently specified) and then optical density was measured at specific intervals of time over at least 55 hours from the inoculum for "live" experiment. 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 of 5/1.

ROS staining for "pulse" experiments
To detect ROS production 5 µg/mL dihydrorhodamine 123 (DHR123, Sigma-Aldrich) was added to exponentially growing yeast cells (OD$_{660}$ = 0.4-0.6) and incubated at 30 °C for 20 min in darkness. The cells were then rinsed once with fresh medium and resuspended in minimal medium containing various concentrations of acids. After incubation at 30 °C for at least 75 min, Samples were harvested at different time points, the cells were washed once with PBS and analyzed immediately by FACS.

ROS staining for "live" experiments

Reactive oxygen species (ROS) were detected by Dihydrorhodamine 123 as described in [2]. Cells were incubated with Dihydrorhodamine 123 (DHR 123, Sigma Chemical Co., St. Louis, MO, USA) for 2 h, washed twice with PBS buffer and subsequently resuspended in propidium iodide solution 0.46 mM for the identification of dead or severely compromised cells.

Samples were then analyzed using a Cell Lab Quanta^{TM} SC flow cytometer (Beckman Coulter, Fullerton, CA, USA) equipped with a diode laser (excitation wavelength 488 nm, laser power 22 mW). The fluorescence emission was measured through a 525–550 nm band pass filter (FL1 parameter) for DHR signal and through a 670 nm long pass filter (FL3 parameter) for PI signal. The sample flow rate during analysis did not exceed 600–700 cells/s. A total of 20000 cells was measured for each sample. Data analysis was performed afterwards with WinMDI 2.8 software, build#13 01-19-2000 (Purdue University, Cytometry Laboratories [http://facs.scripps.edu/software.html]).

References

1. Sauer M, Porro D, Mattanovich D, Branduardi P (2008) Microbial production of organic acids: expanding the markets. Trends Biotechnol 26: 100-108.

2. Madeo F, Fröhlich E, Ligr M, Grey M, Sigrist S, et al. (1999) Oxygen stress: a regulator of apoptosis in yeast. J Cell Biol 145: 757-767.

3. Ferrer-Miralles N, Domingo-Espín J, Corchero J, Vázquez E, Villaverde A (2009) Microbial factories for recombinant pharmaceuticals. Microb Cell Fact 8: 17.

4. Pscheidt B, Glieder A (2008) Yeast cell factories for fine chemical and API production. Microb Cell Fact 7: 25.

5. Rude M, Schirmer A (2009) New microbial fuels: a biotech perspective. Curr Opin Microbiol 12: 274-281.

6. Beckhouse A, Grant C, Rogers P, Dawes I, Higgins V (2008) The adaptive response of anaerobically grown *Saccharomyces cerevisiae* to hydrogen peroxide is mediated by the Yap1 and Skn7 transcription factors. FEMS Yeast Res 8: 1214-1222.

7. Du L, Su Y, Sun D, Zhu W, Wang J, et al. (2008) Formic acid induces Yca1p-independent apoptosis-like cell death in the yeast *Saccharomyces cerevisiae*. FEMS Yeast Res 8: 531-539.

8. Mollapour M, Shepherd A, Piper P (2008) Novel stress responses facilitate *Saccharomyces cerevisiae* growth in the presence of the monocarboxylate preservatives. Yeast 25: 169-177.

9. Beales N (2004) Adaptation of Microorganisms to Cold Temperatures, Weak Acid Preservatives, Low pH, and Osmotic Stress: A Review %J Comprehensive Reviews in Food Science and Food Safety. 3: 1-20.

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

11. Warth A (1989) Relationships between the resistance of yeasts to acetic, propanoic and benzoic acids and to methyl paraben and pH. Int J Food Microbiol 8: 343-349.

12. Piper P, Mahé Y, Thompson S, Pandjaitan R, Holyoak C, et al. (1998) The pdr12 ABC transporter is required for the development of weak organic acid resistance in yeast. EMBO J 17: 4257-4265.

13. Branduardi P, Smeraldi C, Porro D (2008) Metabolically engineered yeasts: 'potential' industrial applications. J Mol Microbiol Biotechnol 15: 31-40.

14. Serrano R, Kielland-Brandt M, Fink G Yeast plasma membrane ATPase is essential for growth and has homology with (Na+ + K+), K+- and Ca2+-ATPases. Nature 319: 689-693.

15. Warth A (1991) Mechanism of action of benzoic acid on *Zygosaccharomyces bailii*: effects on glycolytic metabolite levels,

energy production, and intracellular pH. Appl Environ Microbiol 57: 3410-3414.

16. Verduyn C, Postma E, Scheffers W, Van Dijken J (1992) Effect of benzoic acid on metabolic fluxes in yeasts: a continuous-culture study on the regulation of respiration and alcoholic fermentation. Yeast 8: 501-517.

17. Piper P, Calderon C, Hatzixanthis K, Mollapour M (2001) Weak acid adaptation: the stress response that confers yeasts with resistance to organic acid food preservatives. Microbiology 147: 2635-2642.

18. Macpherson N, Shabala L, Rooney H, Jarman M, Davies J (2005) Plasma membrane H+ and K+ transporters are involved in the weak-acid preservative response of disparate food spoilage yeasts. Microbiology 151: 1995-2003.

19. Branduardi P, Fossati T, Sauer M, Pagani R, Mattanovich D, et al. (2007) Biosynthesis of vitamin C by yeast leads to increased stress resistance. PLoS One 2: e1092.

20. Brambilla L, Bolzani D, Compagno C, Carrera V, van Dijken J, et al. (1999) NADH reoxidation does not control glycolytic flux during exposure of respiring *Saccharomyces cerevisiae* cultures to glucose excess. FEMS Microbiol Lett 171: 133-140.

21. Linster C, Gomez T, Christensen K, Adler L, Young B, et al. (2007) *Arabidopsis* VTC2 encodes a GDP-L-galactose phosphorylase, the last unknown enzyme in the Smirnoff-Wheeler pathway to ascorbic acid in plants. J Biol Chem 282: 18879-18885.

Processes for a sustainable economy

Simone Passolunghi, Carla Smeraldi, Danilo Porro, Paola Branduardi
Dipartimento di Biotecnologie e Bioscienze
Università degli Studi di Milano-Bicocca
Corresponding Author: danilo.porro@unimib.it

The challenge for the industrial biotechnology is to develop sustainable and environmental friendly processes based on renewable sources and resulting in biodegradable products. Innovative approaches to solving environmental and energy problems for the realization of a sustainable bio-based economy are required. Examples and applications are presented in the following article.

On the agenda of every conscious government, humanitarian organization, of every economic entity and analyst there are major issues or concerns regarding the entire humanity that we can not avoid facing anymore. An economic growth to be sustainable and socially responsible needs to take into account resource availability and effects on the environment. Fossil fuels, besides their use to produce energy, act as building blocks or reagents for fabricating several materials. There are many nonfuel uses for petroleum, including various specialized products for use in the textile, metallurgical, electrical, and other industries. With regards to fossil reserves, we are now faced with the paradoxical situation that while crude oil (petroleum) is being consumed faster than ever, the "proven oil reserves" have remained at about the same level of thirty years ago as a consequence of new oil findings. Nevertheless, these "proven oil reserves" are located in increasingly difficult to reach places. Therefore, the cost for extracting the crude oil rises continuously. More important in this respect, the world's crude oil reserves will not last forever.

What can be done? The innovative approach of Industrial Biotechnology

Industrial Biotechnology (IB) uses renewable raw materials as starting product and micro-organisms (genetically modified or not) and their enzymes to make useful chemical compounds and biomaterials. While their application in the production of fine chemicals and pharmaceuticals is already well established (e.g. insulin, interferons, erythropoietin, hepatitis B vaccine, vitamin B12, etc.), it is now increasingly being applied to produce bulk chemicals such as biofuels (e.g. ethanol) and bio-plastics. These technologies present the inherently advantages of a clean process that has reduced water and energy consumption, less or no waste generation and less CO2 generation. Additionally, the costs of new biotechnological processes could be 40% lower than those of conventional processes and could imply up to 70% savings on capital equipment (www.suschem.org/) .

The biorefinery concept

To explain the process by which biomasses are transformed into energy and products, the concept of "biorefinery" has been introduced. A biorefinery (Fig. 1) is a fully integrated manufacturing facility that integrates biomass conversion processes and equipment to produce fuels, power, and chemicals from biomass, with minimal waste and emissions. A biorefinery is analogous to today's petroleum refineries, which produce multiple fuels and products from petroleum. Industrial biorefineries have been identified as the most promising route to the creation of a new biobased industry. The starting products used in a biorefinery, all renewable, are starches, lignocellulosic materials, oils and proteins, urban and agro-industrial wastes.
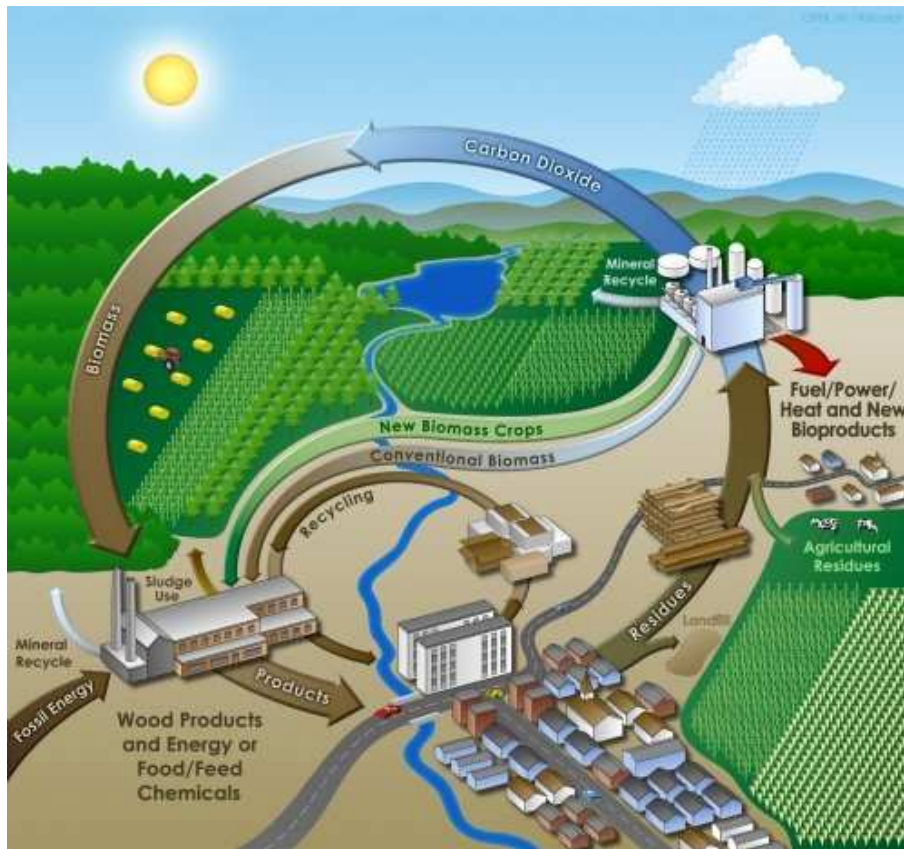
Fig. 1 - Idealized biorefinery concept (Adapted from ORNL Review www.ornl.gov/info/ornlreview/v33_2_00/bioenergy.htm)

Industrial biotechnology and metabolic engineering

Evolution has produced a huge variety of organisms living in radically different environments. These organisms make biochemicals through a series of enzyme catalyzed reactions referred to as pathways. In particular, some of these organisms have evolved metabolic pathways leading to the synthesis of potentially useful compounds that are difficult to produce by chemical industry or that are environmentally harmful to manufacture. It has to be reminded that the fundamental basis of evolution is the need to survive and reproduce, not to produce potentially important and commercially valuable products. Indeed, interesting metabolites are very often produced by wild type organisms in such low concentrations that biotechnological

151

exploitation is, at least today, impractical. Metabolic pathway manipulation for improving the properties and productivity of microorganisms is an old concept. Cell metabolism can be manipulated changing the external or the internal environment of the cell. Engineeringmanipulations of cell performance under changing the external environment has long been practiced by choosing operating conditions during batch processes to improve growth and productivity. Traditionally internal changes have been achieved by random mutagenesis and selection, searching for metabolites that render the cells more active for the desired metabolic process. This approach made use of chemical mutagens and creative selection techniques to identify superior strains. There are many examples of this strategy in the area of antibiotics, amino acids, vitamins, alcohol, solvents and others. However, despite widespread acceptance of many successes, mutagenesis remains essentially a random process. Thanks to recombinant DNA-technology, one can now specifically intervene into the genetic material of these microorganisms. The metabolism of microorganisms can be modified or even completely changed (so-called "metabolic engineering") or genes from higher organisms (plants and animals) or other microorganisms (yeast, bacteria, virus, algae) can be inserted into microorganisms and brought to expression. Thus, new direct gene products can be made or new metabolic pathways can be created to produce chemical substances with high efficiency via industrial fermentation processes. The ability to select mutants or to develop organisms by means of rDNA technologies to enable fast and environmental friendly productions of these products has the obvious potential to revolutionize the biotechnological industry. The research team of Industrial Microbiology of Milano-Bicocca is involved in the above mentioned research activities and applications related to industrial biotechnology (Fig. 2). In the last years, the team has developed, patented and licensed the technology for the production of lactic and ascorbic acids from recombinant yeast hosts.
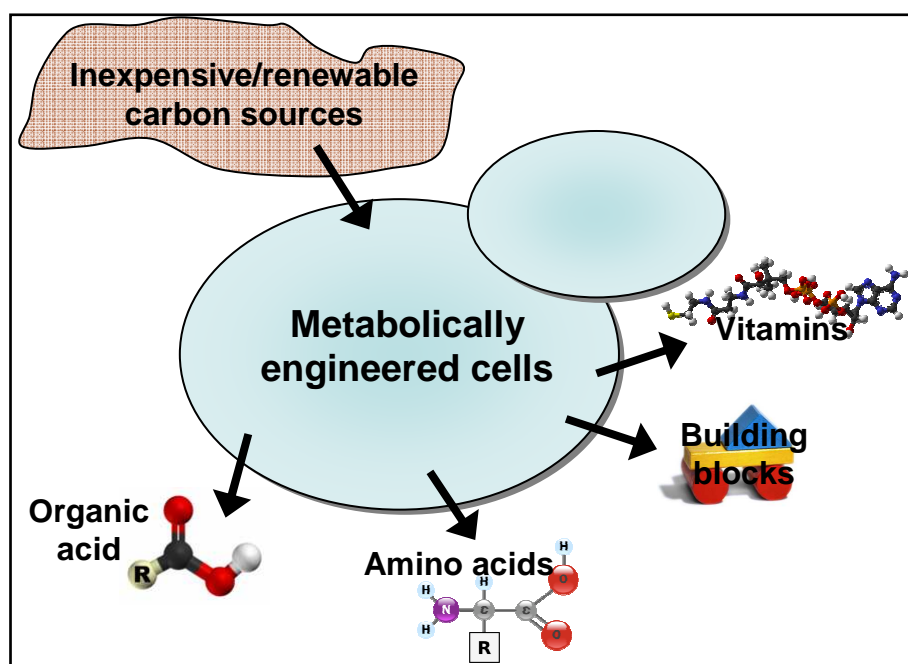
Fig. 2 - Schematic representation of the development of cellular-based bioprocesses: biomasses are transformed in chemical compounds of interest through metabolic engineering of (well-known) microorganisms

Lactic acid production from recombinant yeasts

L-Lactic acid, first discovered by the Swedish chemist Scheele (1780), has been traditionally used as a food preservative and food flavouring compound [1]. It also finds applications in cosmetics and pharmaceuticals. The world-wide production of lactic acid (currently an estimated 250,000 t/year) [2] is rapidly increasing, mainly as a result of the growing market for polylactic acid. It is expected that this biodegradable polymer, produced from renewable resources, will replace various petrochemical-based polymers in applications ranging from packaging to fibers. This carboxylic acid is currently mainly produced using lactic-acid bacteria, such as various *Lactobacillus* species, via an anaerobic fermentation that operates optimally at pH values where the salt of the organic acid rather than the free acid is formed, although free lactic acid is preferred for most industrial processes [1]. Different research teams have been involved in the production of lactate from metabolic engineered yeasts such as *Saccharomyces cerevisiae* [2-9], *Kluyveromyces lactis* [10a,b, 11], *Torulaspora delbrueckii* [10b] and *Zygosaccharomyces bailii* [12].

The use of microorganisms like yeasts, that are more tolerant to low pH values than the current production organisms, could strongly decrease the amount of neutralizing agents required and lower the cost of down-stream processing. Pyruvate is the end product of glycolysis; it can be further metabolized either by the pyruvate dehydrogenase complex (Pdh, EC 1.2.4.1) to acetylcoenzyme A or by pyruvate decarboxylase (Pdc, EC4.1.1.1) to acetaldehyde and subsequently to ethanol. We analysed the lactate production from metabolic engineered *Saccharomyces cerevisiae* cells expressing a heterologous lactate dehydrogenase (*LDH*) gene. The *LDH* gene expression in a budding yeast cell introduces a novel and alternative pathway for the NAD+ regeneration, allowing a direct reduction of the intracellular pyruvate to lactate, leading to a simultaneous accumulation of lactate and ethanol [4]. Four different *S. cerevisiae* strains were transformed with six different wild type and one mutagenised *LDH* genes, in combination or not with the over-expression of a lactate transporter [13]. Fig. 3 summarises some of the obtained data. The resulting yield values varied from as low as 0.0008 to as high as 0.52 (grams of lactate produced per grams of glucose consumed).

Different recombinant *S. cerevisiae* GRF18U cells were shake-flask grown in glucose 2% wv^{-1} minimal selective medium till stationary phase. Samples were collected at indicated times (h) for lactate (g l^{-1}) and yield (gram of lactate produced per gram of glucose consumed; values are here below reported) determinations.
▲: *B. taurus LDH, yield 4%±0.2%;*
◇: *L. casei LDH, yield 21%±0.4%;* ■: *L. plantarum LDH, yield 31%±0.5%;* ● : *Mutated L. plantarum LDH, yield 52%±0.7%*
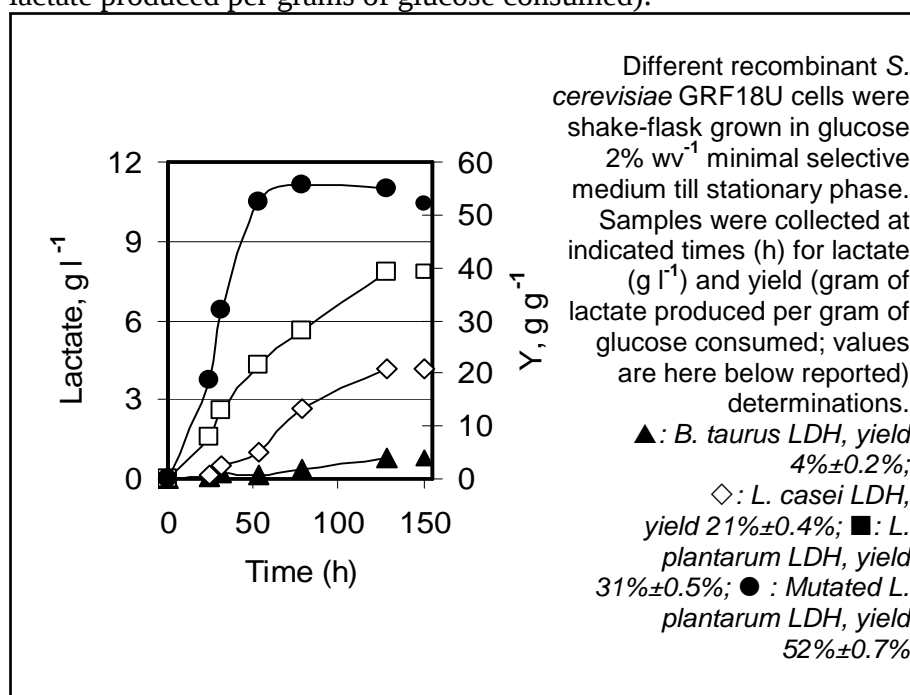
Fig. 3 - Lactate production and yield obtained from recombinant GRF18U host cells

In this respect, and to the best of our knowledge, higher redirections of the glycolysis flux have never been obtained before without any disruption and/or limitation of the competing biochemical pathways. This was also implemented by disrupting the *PCD* gene and/or the *PDH* gene in other well knownyeast, *K. lactis* [10a,b, 11].Finally, the physiological consequences of such production were evaluated. Despite yeasts can grow at low pH, a high production of lacticacid reflects on a high accumulation of lactate and H+ into thecells, that have to spend energy to keep the internal pH at physiologicallevels. A flow cytometric method to determine internal pH wassettled, and subsequently coupled with a sorting system aimed toenrich in subpopulation with the higher internal pH. From these cellssingle clones were isolated and it could be possible to further increase the production [14]. In conclusion, we proved that the redirection of the pathway towards the lactate production can be strongly modulated by the genetic background of the host cell, by the source of the heterologous Ldh enzyme, by improving its biochemical properties, by modulating the export of lactate in the culture media as well as by improving physiological properties. The results obtained have been patented and partially licensed, offering, in this way, a clear example of technology transfer [15].

Ascorbic acid production from recombinant yeasts

Vitamin C or L-ascorbic acid is an indispensable important metabolite for different physiological functions and an essential nutrient for animals lacking its biosynthetic pathway like humans. L-ascorbic acid acts as a scavenger of reactive oxygen species, protecting tissues from harmful oxidative products. This capacity leads to an increasing demand as a food additive [16]. L-ascorbic acid is conventionally synthesized by a variety of chemical methods, which are generally variations of the Reichstein process, which utilize glucose as starting material [17, 18]. Novel biotechnological processes, which convert glucose into vitamin C in one step, would be desirable and yeasts, such as *S. cerevisiae,* offer themselves as biocatalysts due to their GRAS (Generally Recognized As Safe) status. However, yeasts lack the ability to produce L ascorbic acid naturally, producing instead erythroascorbic acid, a structurally but not biologically related compound.
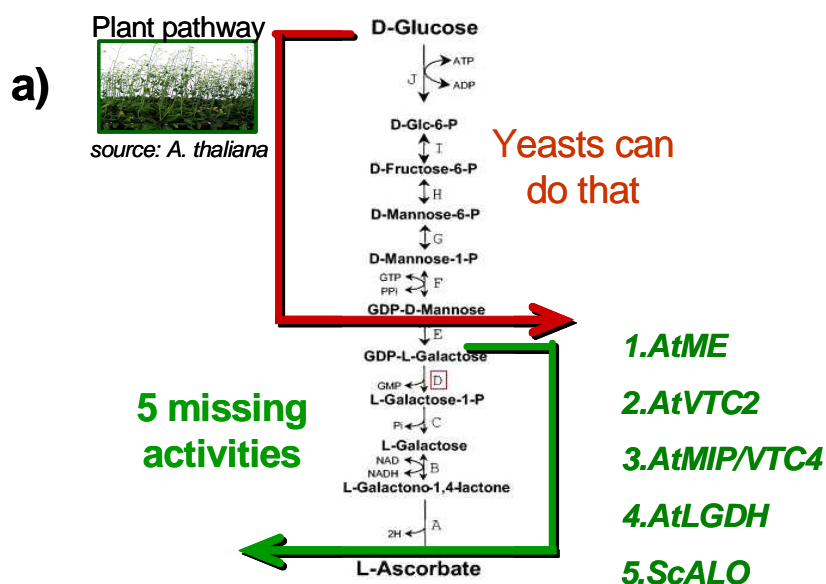
a) Schematic representation of the biosynthetic pathways leading from D-glucose to L-ascorbic acid in plants, underlying activities that are shared with yeasts. The following enzymes are involved: A. Lgalactono-1,4-lactone dehydrogenase (1.3.2.3); B. L-galactose dehydrogenase; C. sugar phosphatise (3.1.3.23); D. hydrolase; E. GDP-mannose-3,5-epimerase (5.1.3.18); F. mannose-1-phosphate guanylyltransferase (2.7.7.22); G. phosphomannomutase (5.4.2.8); H. mannose-6-phosphate isomerase (5.3.1.8); I. glucose-6-phosphate isomerase (5.3.1.9); J. hexokinase (2.7.1.1)
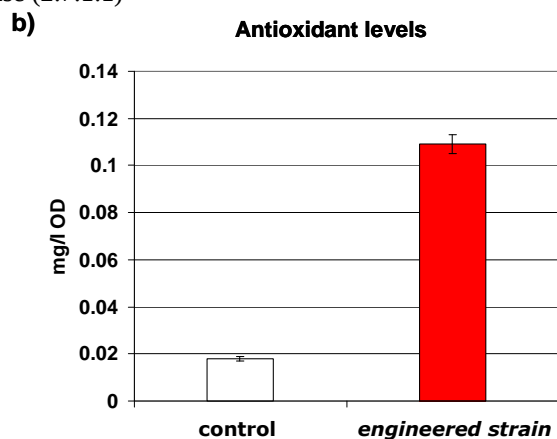
b) Comparison of intracellular antioxidant levels measured by spectrophotometric analyses in the control yeast strain (open bar) compared to the engineered yeast strain (full bar). Cells were grown in minimal synthetic medium with 2% Glucose as carbon source and samples were collected after 24 hours from the inoculum

Fig. 4 - Development of a yeast strain capable to convert D-Glucose into L-Ascorbic acid

It is possible to take advantage of existing enzymatic activities already present in yeast and complement them with heterologous ones taken from naturally occurring biosynthetic pathways, in order to create a yeast strain capable of producing ascorbic acid. The project presents different challenges since the naturally occurring synthetic pathways see at least 14 steps. The engineered yeast complemented with 5 genes cloned from *A. thaliana* (*AtME*, *AtVTC2*, *AtMIP/VTC4*, *AtLGDH*, *AtAGD*) and then further optimized by substitution with one endogenous activity (*ScALO*, Fig. 4a) is able to produce ascorbic acid by direct conversion of D-glucose (Fig. 4b). An interesting characteristic acquired by the host yeast strain during ascorbic acid production is an increment of its robustness. Stress can damage subcellular components and can induce apoptosis (programmed cell death), cell necrosis and cell lyses. These effects are often mediated by the generation of Reactive Oxygen Species (ROS) (for recent reviews see: [19, 20]). Experiments were performed that clearly correlated the ascorbic acid production with both a reduction of ROS generation and an improvement in the viability of the producing strain in respect to the non-producing one, when an oxidative stress was imposed (Fig. 5) [21]. This method can be used for further investigations either on strains with improved ascorbic acid production as well as strains growing under different stressing conditions [22]. These results are important in prospective of an industrial production process, wherein stress on the organism used as means for production, typically leads to lower or zero production of the product, productivity, yield of the product, or two or more thereof.

Wild type (GRF18U, left picture) and recombinant strains ([AtME, AtVTC2, AtMIP/VTC4, AtLGD, AtAGD, ScALO], right picture) grown in minimal glucose medium added with H_2O_2 3.0 mM were analyzed after DHR123 and PI staining (rodamine signal is reported in the abscissa and PI signal on the ordinate axes). The numbers in the circle represent the percentage of dead or severely damaged cells in the analysed population
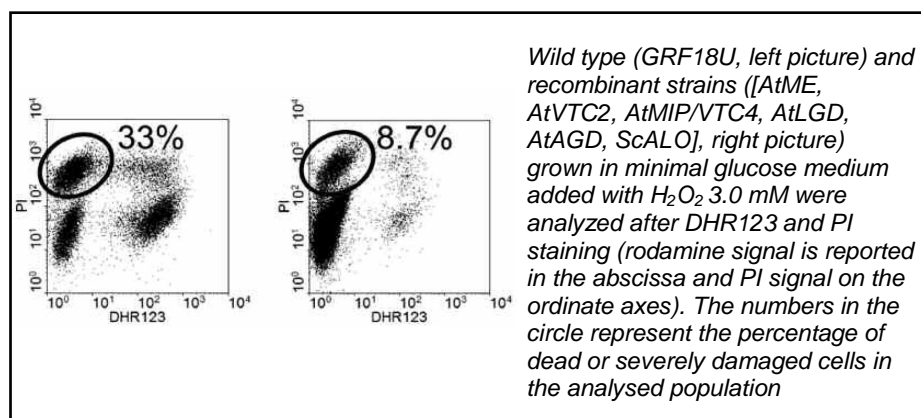
Fig. 5 - Flow cytometric analysis of wild type and vitamin C producing yeasts under oxidative stress

Conclusions

The ability to produce numerous compounds and chemicals using renewable resources and micro-organisms will offer alternatives to the use of petroleum, with clear environmental benefits. The task at stakes is huge and requires a sincere collaboration between public and private research facilities, universities, and governments. Knowledge in genetics, biochemistry, molecular biology, fermentation technologies, organic chemistry and process engineering need to be integrated. Public education policies that will help the commercialization of industrial biotechnology products will have to be designed. It is therefore evident the need of adequate funding to promote R&D and collaborative research of selected laboratories and policy initiatives to realize a sustainable biobased economy. Together with the benefit of reducing emissions of carbon dioxide and airborne pollutants, soil erosion, and protecting water supplies and quality, the implementation of Industrial Biotechnology processes will open up new technologies, industries, and export opportunities and it will stimulate growth, especially in rural, farm and forest economies.

References

[1] H. Benninga, A history of lactic acid making, Kluwer Academic Publishers, Dordrecht, The Netherlands, 1990.
[2] A.J. van Maris et al., Appl Environ Microbiol., 2004, 70(5), 2898.
[3] S. Dequin, P. Barre, Biotechnology (NY), 1994, 12(2), 173.
[4] D. Porro et al., Biotechnol Prog, 1995, 11(3), 294.
[5] V. Ansanay et al., Yeast, 1996, 12(3), 215.
[6] C.D. Skory. J. Ind. Microbiol. Biotechnol., 2003, 30(1), 22.
[7] S. Colombie, J.M. Sablayrolles, J. Ind. Microbiol. Biotechnol., 2004, 31(5), 209.
[8] N. Ishida et al., Appl. Environ. Microbiol., 2005, 71(4), 1964.
[9] S. Saitoh et al., Appl. Environ. Microbiol., 2005, 71(5), 2789.
[10] a) D. Porro et al., Appl. Environ. Microbiol., 1999, 65(9), 4211; b) D. Porro et al., Yeast strains for the production of lactic acid, 1999, PCT WO 99/14335.
[11] M.M. Bianchi et al., Appl. Environ. Microbiol., 2001, 67(12), 5621.
[12] P. Branduardi et al., FEMS Yeast Res., 2004, 4(4-5), 493.
[13] P. Branduardi et al., Microb. Cell. Fact., 2006, Jan 30; 5(1), 4.

[14] M. Valli et al., Appl. Environ. Microbiol., 2006, 72(8), 5492.

[15] D. Porro et al., Yeast strains for the production of lactic acid, 2002, Pat. Appl. U.S. No. 6,429,006.

[16] J.M. May, H. Asard, Vitamin C: Function and Biochemistry in Animals and Plants, H. Asard et al. (Eds.), Bios Scientific Publishers, Oxford, 2004, 139.

[17] T. Reichstein, 2-Keto-levo-gulonic acid and process for the manufacture of same, 1942, U.S. Pat. No 2,301,811.

[18] J.F. Roland et al., Fermentation production of ascorbic acid from L-galactonic substrate, 1986, U.S. Pat. No 4,595,659.

[19] R. Kim et al., J. Pathol., 2006, 208(3), 319.

[20] V.P. Skulachev, Apoptosis, 2006, 11(4), 473.

[21] P. Branduardi et al., PLoS ONE, 2007, 2(10), e1092.

[22] P. Branduardi et al., Ascorbic acid production from D-glucose in yeast, Application for United States Letters Patent, 2005, Serial number No. EV 512294478 US

Concluding remarks

Concluding, one key to the success of genetic engineering for enhancing product formation is to individualize all the possible limiting steps involved in product biosynthesis. The production of a product of biotech interest mainly combines genetic engineering and microbial physiology with the objective of increasing the production rate of a desired product. Such approach is often hampered by the lack of knowledge of the production pathway and its dynamic profile in producing cells.

Therefore, for a successful production detailed physiological studies are required, studies devoted to the identification of the different physiological determinants that could maximize the potential of the genetic determinants. We presented here results which open promising perspectives in relation to the use of the two yeasts *Saccharomyces cerevisiae* and *Zygosaccharomyces bailii* in biotech industrial processes.

First, we gained an increased knowledge of *Z. bailii* physiology through flow cytometric analysis, where the DNA and protein content were investigated as well as the cellular morphology of populations growing in medium supplemented with different carbon sources and with the addition of different organic acids. Second, we solved a critical problem related to the manipulation of *Z. bailii*, by developing a targeted gene deletion protocol and by constructing the first auxotrophic mutant ever reported for this yeast. Third, we cloned a *Z. bailii* novel gene, *ZbGAS*1, and constructed the relative null mutant, which has a supersecreting phenotype and we tested the secretion capability with three different heterologous proteins. Fourth, we proved that the overexpression of the plasma membrane proton pump Pma1p can improve resistance to lactic acid stress in *S. cerevisiae* and in *Z. bailii* as well. Fifth, we had shown the direct link between oxidative stress and acidic stress and how it is possible to track them at single cell level. Finally, we described examples and applications on how the innovative industrial biotechnology approach is able to develop sustainable and environmental friendly processes based on renewable sources and resulting in biodegradable products.

In this thesis work we showed the potential of microrganisms to address the development of a biobased economy, this potential will be completely unlocked only if we will able to match our understanding in microbial physiology with biotech needs.

Riassunto

Le ricerche nel settore delle biotecnologie industriali sono volte allo sviluppo di processi produttivi innovativi che portano all'ottenimento di *fine* o *bulk chemicals* o ancora di *building blocks* mediante l'uso di microrganismi, oppure si prefiggono di migliorare processi già esistenti per aumentarne la resa, produzione e produttività, cercando di garantire un più alto grado di sostenibilità e riducendo l'impatto ambientale.

Per perseguire questi scopi è possibile intervenire adottando un approccio di tipo "tecnologico" che prevede lo sviluppo di sistemi in grado di garantire un controllo più efficace dei parametri che regolano i processi di produzione oppure, con un approccio "molecolare/metabolico", agendo direttamente sul sistema ospite, ossia intervenendo sulle capacità produttive del microrganismo stesso. Per raggiungere questo obiettivo occorre individuare ed eventualmente modificare i *pathways* responsabili dei processi di sintesi o di secrezione dei prodotti d'interesse, oppure considerare le condizioni ambientali in cui il microrganismo si trova ad operare durante il processo, per poter studiare come migliorarne le capacità produttive anche in condizioni "non ottimali" dal punto di vista fisiologico.

Le condizioni di processo che spesso caratterizzano i processi di produzione industriale, infatti, sottopongono le cellule ad una serie di stress che inevitabilmente agiscono in maniera negativa sulle rese, limitandole. Diventa pertanto necessario individuare i fattori limitanti in relazione all'organismo ospite e, sulla base di ciò, intervenire in maniera opportuna. A questo scopo è possibile adottare differenti strategie, tra loro complementari e non mutuamente esclusive.

La più immediata prevede l'esplorazione della biodiversità e consiste nella scelta di un ospite che sia intrinsecamente e naturalmente più resistente al tipo di stress imposto dal processo. Questa strada non è sempre facilmente percorribile, data la vastità delle possibili soluzioni e la scarsità di mezzi che ne consentono l'esplorazione e la caratterizzazione in un tempo ragionevole per lo sviluppo di un processo biotecnologico.

Una strategia alternativa consiste invece nel caratterizzare la risposta cellulare a tali condizioni di stress ed individuarne i fattori chiave, per intervenire successivamente, ad esempio tramite manipolazioni genetiche su tali fattori, nel tentativo di migliorare le capacità di

resistenza della cellula stessa, oppure trasferendo i tratti genetici coinvolti verso altri microrganismi scelti come sistema ospite.

Il lievito *Saccharomyces cerevisiae* è uno tra i microrganismi più utilizzati per la produzione di composti d'interesse biotecnologico, per le conoscenze disponibili sulla fisiologia, sulla genetica, sulla biochimica e per l'esistenza di strumenti tecnologici e molecolari atti alla sua manipolazione allo scopo di ottimizzare le produzioni per via fermentativa. Occorre ricordare che *S. cerevisiae* è riconosciuto come microrganismo GRAS (*generally regarded as safe*) dalla *Food & Drug Administration* americana che ne ha consentito l'utilizzo per la produzione di composti farmaceutici ad uso umano. Mentre è oggi sempre più evidente il potenziale di *S. cerevisiae* negli interventi di ingegneria metabolica, per alcune produzioni di proteine eterologhe su larga scala tale lievito non è il sistema ospite ideale: spesso le proteine espresse sono iperglicosilate o, se trattenute nello spazio periplasmico, subiscono significative degradazioni. Per altre produzioni industriali, specialmente dove il prodotto d'interesse non ha grande valore aggiunto, le tecnologie fermentative necessarie a *S. cerevisiae* risultano essere troppo complesse e sofisticate (e quindi costose) per essere implementate su larga scala. Con queste premesse negli ultimi anni si è esplorata la possibilità di adottare altri lieviti, definiti "non convenzionali", sviluppando nuovi sistemi di espressione. In questo progetto di tesi è stato preso in considerazione un lievito "alternativo", *Zygosaccharomyces bailii*, poco caratterizzato dal punto di vista genetico-molecolare, che presenta caratteristiche interessanti nell'ottica di potenziali produzioni biotecnologiche: consente infatti di ottenere elevate rese di biomassa, presenta un'alta velocità di crescita specifica ed una maggior resistenza, rispetto a *S. cerevisiae*, ad alcuni tipi di stress, ed in particolare a quello generato da ambienti acidi.

La superficie cellulare, in quanto area di comunicazione e scambio tra la cellula e l'ambiente extracellulare costituisce uno dei target di questo studio.

In *S. cerevisiae*, grazie alla disponibilità di *tools* molecolari e alla conoscenza dell'intera sequenza del genoma, è possibile progettare studi approfonditi e interventi di ingegnerizzazione della cellula.

Nel caso di *Z. bailii* è stato necessario affrontare dapprima un problema a monte, dato dalle difficoltà nella sua manipolazione genetica. Non era infatti mai stata ottenuta la delezione di un gene essenziale in questo lievito, che risulta essere diploide. E' stato messo a punto un protocollo riproducibile di delezione genica tramite *gene-targeting* e con esso è stata effettuata la delezione del gene *ZbLEU2*,

che ha dato origine al primo ceppo auxotrofico di *Z. bailii* (*leu⁻*). Ciò rappresenta uno passaggio importante per un possibile utilizzo di *Z. bailii* come sistema ospite. Grazie a questo protocollo è stato inoltre ottenuto un ceppo mutante di *Z. bailii* di potenziale interesse per la produzione di proteine eterologhe, in analogia con quanto riportato in letteratura per *S. cerevisiae*. La delezione riguarda in questo caso l'omologo di *ScGAS1*, codificante per l'enzima *β*-1,3-glucanosiltransferasi che catalizza il *crosslinking* dei glucani della parete cellulare. Il gene *ZbGAS1* è stato clonato per PCR e sequenziato. Il mutante deleto di *Z. bailii* presenta caratteristiche morfologiche e fenotipiche del tutto simili al corrispondente in *S. cerevisiae*, date da un'alterazione della struttura della parete, e risulta avere maggiori capacità secretive rispetto al ceppo *wild type* anche per alcune proteine eterologhe prese in considerazione.

Parallelamente a questi studi, popolazioni di *Z. bailii* in crescita su differenti fonti di carbonio sono state analizzate attraverso tecniche di citofluorimetria a flusso. L'analisi del contenuto proteico e di DNA rappresenta uno strumento per meglio caratterizzare e conoscere questo lievito non solo dal punto di vista molecolare, ma anche cellulare.

La caratterizzazione di questo lievito non convenzionale ha confermato ancora una volta una delle caratteristiche più apprezzate per i lieviti utilizzati come *cell factories*: la versatilità.

Questa proprietà è così marcata che ha consentito che i lieviti vengano sfruttati sia per capacità naturali, quali la produzione di etanolo, sia per processi indotti da manipolazioni mirate, quali la produzione di acido lattico, per citare ad esempio due produzioni biotecnologiche di attuale rilevanza industriale. Per rendere questi processi competitivi sul mercato, dal punto di vista della resa, della produzione e della produttività, i lieviti sono stati spinti ai loro limiti fisiologici. Questi limiti sono dati dall'accumulo di enormi quantità di prodotto che, nel caso dell'etanolo determinano danni a livello della componente lipidica della membrana plasmatica, nel caso dell'acido lattico una perdita della corretta omeostasi cellulare con conseguente caduta del pH intracellulare.

Si rende quindi necessario valutare se questi limiti possano essere superati agendo in particolare sulla membrana plasmatica, il cui ruolo fondamentale nel controllo del trasporto e dell'omeostasi cellulare la rende un target d'interesse per interventi volti a migliorare la robustezza delle cellule in risposta agli stress generati dal processo, ad esempio stress di tipo ossidativo o generato da bassi valori di pH.

L'ottimizzazione del trasporto attraverso la membrana gioca un ruolo fondamentale nei meccanismi di adattamento a questi stress. In particolare, il miglioramento del flusso dei nutrienti in ingresso nella cellula potrebbe consentire un uptake ottimale dei nutrienti verso il citoplasma (come nel caso della produzione di bioetanolo), un miglioramento dei flussi in uscita dalle cellule potrebbe invece consentire un'efficace eliminazione di composti che, se presenti oltre un valore limite, potrebbero essere deleteri per la vitalità cellulare (ad esempio acidi organici).

Focalizzando l'attenzione sulla frazione proteica che caratterizza la membrana citoplasmatica, sono stati studiati gli effetti della modulazione dell'espressione della pompa H^+-ATPasi della membrana plasmatica (Pma1p), coinvolta nell'omeostasi del pH intracellulare. In particolare, il gene *ScPMA1* è stato overespresso in *S. cerevisiae* e l'overespressione è risultata in grado di conferire una maggiore resistenza allo stress acido, evidenziata mediante cinetiche di crescita in presenza di acido lattico. La maggiore vitalità cellulare in condizioni restrittive rispetto al ceppo wild type è stata verificata anche mediante citofluorimetria a flusso. L'uso di questo strumento ha consentito la messa a punto di un sistema in grado di valutare in modo quantitativo il grado di robustezza delle cellule in presenza di condizioni che generano stress.

Disponendo di uno strumento in grado di valutare la robustezza delle cellule in funzione di vari tipi di stress (ossidativo, dovuto al pH, …) è possibile progettare nuovi interventi di ingegneria metabolica allo scopo di conferire maggiore resistenza al lievito in condizioni di processo restrittive, simili alle condizioni che caratterizzano i processi di produzione di composti biotecnologici. Sarà possibile valutare l'efficacia di tali interventi di ingegneria metabolica misurando con il citometro di flusso la risposta delle cellule ingegnerizzate nelle condizioni restrittive previste, misurando la capacità delle cellule di aumentare la propria robustezza. La robustezza rimane una delle caratteristiche chiave dei lieviti nel ruolo di *cell factories*, soprattutto per perseguire gli obiettivi futuri delle *white biotechnologies*: fornire prodotti di valore a partire da risorse rinnovabili attraverso processi sostenibili e a basso impatto ambientale