bcy1 deletion in *Saccharomyces cerevisiae* is semi-dominant and induce autolytic phenotypes suitable for the improvement of sparkling wines

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ABSTRACT

Autolysis of *Saccharomyces cerevisiae* is the main source of molecules contributing to the quality of sparkling wines made by the traditional method. In this work the possibility of accelerating this slow process in order to improve the quality of sparkling wines, by using genetically engineered wine yeast strains, has been explored. The effect of partial or total deletions of *BCY1* (encoding for a regulatory subunit of cAMP dependent protein kinase A (PKA)), in haploid and diploid (heterozygous and homozygous) yeast strains has been studied. It is proven that heterozygous strains carrying partial or complete deletions of *BCY1*, show a semi-dominant phenotype for several of the properties studied, including autolysis under simulated second fermentation conditions, in contrast to previously published reports describing mutations in *BCY1* as recessive. Considering the degree of autolysis, ethanol tolerance, and technical feasibility, deletion of the 3' end of the ORF of a single copy of *BCY1* is proposed as a way to improve the quality of sparkling wines.

Keywords: sparkling wine, autolysis, PKA, genetic engineering, autophagy, *Saccharomyces cerevisiae*.

INTRODUCTION

Sparkling wines made by the traditional "Méthode champenoise" are the result of two main fermentative steps. First, a base wine is made by standard procedures used for the production of most high quality white wines. For the second step, called "Prise de mousse", a combination of yeasts (*Saccharomyces cerevisiae* or *Saccharomyces bayanus*), sucrose (usually 25 g/l final concentration), and eventually clarifying agents, is added to the base wine. The mixture is then bottled and allowed to ferment and age. At the end of the aging period, yeast lees are removed from the bottles by disgorging (25). Before closing the bottles for marketing, the final character of the wine is adjusted by adding wine, liquors and sugar in various proportions. The amount of sugar added will determine the sweetness of the wine.

The contribution of yeasts to the properties of sparkling wines during "Prise de mousse" also takes place in two steps. First, a secondary fermentation of the added sucrose leads to the production of ethanol, carbon dioxide and minor secondary products (25). At the end of this step, the sparkling wine has an ethanol concentration of about 9.5-11.5 % (v/v) and the pressure in the bottle can reach 5-6 atm (25). After fermentation has been completed an aging period follows this fermentative step. During aging yeast cells die and undergo autolysis, releasing intracellular compounds, mostly peptides and amino acids, to the external medium. Improvement in the sensorial quality of the wines has been correlated with the products of the hydrolytic degradation of yeast cells, including free amino acids, peptides, mannoproteins, nucleic acid derivatives or lipids (27, 30, 31, 39). However, autolysis in enological conditions

is a very slow process that leads to long aging periods involving costly storages of the wines.

Two main methods have been devised in order to accelerate the acquisition of aging-like properties during sparkling wine production: adding yeast autolysates to the wine, or increasing the temperature of aging (8, 12). Both procedures cause sensory defects in the final product, which are often described as toasty (37). A third procedure for accelerating autolysis has been proposed, based on the use of killer yeast strains, however the effect on wine quality was not evaluated (48). Finally, several authors have suggested the use of autolytic yeast strains as a way to improve the quality of sparkling wines by accelerating the acquisition of aging-like characteristics (27, 28, 46). These autolytic strains were obtained either by sporulation, random mutagenesis or genetic engineering of wine strains (7, 16, 46). The latest strategy was based in the observation that autophagy takes place during "Prise de mousse" (6), for that reason a gene inducing constitutive autophagy (43) was overexpressed in a industrial wine yeast strain (7).

Because cell death seems to precede autolysis during sparkling wine production (48), a potential alternative method to accelerate autolysis could be the construction of wine yeast strains showing a fast loss of viability under stationary phase or starvation conditions. Several *S. cerevisiae* genes whose mutation leads to reduced viability in response to nutrient limitation have been described (13), for example *bcy1* (4, 5, 18, 34, 47), *ard1*, *ubi4*, *slk1, ils1-1, ypt1, gis1*and *rvs16-1* (10, 22, 34, 50, 51). Among these, we are interested in *bcy1* defective mutants because they start losing viability during the diauxic shift (34), when glucose becomes exhausted from the medium (22, 50, 51). This is

important in order to allow the engineered strains to complete the secondary fermentation before cell death.

BCY1 encodes the regulatory subunit of the cAMP-dependent protein kinase A (PKA) (4, 5, 18, 29, 34, 45, 47, 52). The PKA pathway is activated by numerous environmental signals and plays a major role in the control of metabolism, stress resistance, proliferation and filamentous growth (33, 34, 49) (45). PKA activity is essential for glycolysis (24) and cellular proliferation (36, 44). Conversely, PKA must be inhibited for growth on non-fermentable carbon sources (24), sporulation (4), stress resistance (2, 17, 21, 44), glycogen and trehalose accumulation (20, 44), stationary phase adaptation (9, 35, 41) and autophagy (3).

The PKA holoenzyme is a tetramer consisting of a homodimer of two catalytic subunits, encoded by *TPK1*, *TPK2* and *TPK3* (18, 52) and two regulatory subunits, encoded by *BCY1* (4, 5, 18, 34, 47, 52). PKA is inactive when regulatory and catalytic subunits are associated. In the presence of glucose, cAMP levels are high and PKA is activated. Two cAMP molecules bind to the C-terminal end of each regulatory subunit, promoting a conformational change (53). This change causes the PKA holoenzyme dissociation, resulting in the release and activation of the catalytic subunits (18, 34, 47, 53).

BCY1 is not an essential gene and null mutants display a variety of phenotypes: inability to grow on non fermentable carbon sources, slow growth on glucose, stress and starvation intolerance, inability to sporulate and to accumulate glycogen or trehalose (4, 5, 18, 47). *BCY1 m*utations have been classed according to the level of tolerance to starvation (4, 34). The "early acting class" of mutations (including null mutations) results in cell death quickly

after glucose exhaustion, while the "late acting class" results in cell death during the stationary phase (7 to 12 days after inoculation in rich medium) (34). Unlike null mutants, late acting mutants do not show growth defects during the exponential growth phase. In this work, we performed a more detailed phenotypic analysis of diploid heterozygous and homozygous mutants carrying partial or total deletions of *BCY1*, in order to evaluate the potential of *BCY1* defective mutants for the acceleration of autolysis during sparkling wine production. The rationale behind this approach was that strains undergoing accelerated loss of viability under starvation conditions would readily undergo autolysis under sparkling wine production conditions, once the carbon source had been completely fermented.

MATERIALS AND METHODS

Microbial strains

Escherichia coli DH5α (*supE44*, *ΔlacU169* [*Φ80 lacZΔM15*], *hsdR17*, *recA1*, *endA1*, *gyrA96*, *thi-1*, *relA1*) (19) was used for the production of the different plasmids constructed in this study. The genotypes of the *S. cerevisiae* strains used in this study are shown in Table 1.

Yeast media, culture conditions and phenotype assays

Yeast strains were grown on liquid YPD (1% yeast extract, 2% peptone, 2% glucose) or YPD plates (YPD plus 2% agar). Plates of minimal medium contained 0.67% yeast nitrogen base, 2% agar, the auxotrophic requirements

and 2% of the cognate carbon source: SD (glucose), SG (galactose), SF (fructose), SS (sucrose), SGy (glycerol), SM (maltose), SR (raffinose), SP (pyruvate), and SA (potassium acetate). Synthetic base wine contained 0.17% Yeast Nitrogen Base without amino acids and ammonium sulphate (Difco), 0.6% malic acid, 0.3% tartaric acid, 0.03% citric acid, 0.05% ammonium sulphate, the appropriate auxotrophic requirements, and ethanol from 0% to 6% (v/v), pH was adjusted to 3.5 with KOH. Sugar (glucose or sucrose) was added to a final concentration of 2%. Unless otherwise specified yeast phenotype assays were started with cells grown overnight at 30ºC and 180 rpm on YPD medium, and washed three times with saline solution (NaCl 0.87% p/v).

For carbon source utilization assays, known cell numbers as determined by microscopic cell counts were spotted in SD, SG, SF, SS, SGy, SM, SR, SP or SA plates The plates were incubated 2-5 days at 30ºC (depending on the specific medium), and photographed.

For fermentation assays 50 ml of synthetic base wine containing different amounts of ethanol was inoculated to a final concentration of 10^6 cells/ml. Cultures were maintained at 30ºC without agitation and samples were withdrawn periodically. Yeast cells were removed by centrifugation and the supernatant was stored at –20ºC. Residual reducing sugars were quantified with DNS (3,5-dinitro-salicylic acid) as described by Bernfeld (1). Before quantification, sucrose containing samples were incubated overnight at 37ºC with invertase 2.8 mg/l (Sigma-Aldrich Química, Tres Cantos, Spain) to ensure complete hydrolysis.

Survival under carbon starvation conditions was studied by inoculating a known number of cells (10^7 - 10^8 cells/ml) on 0,67% Yeast Nitrogen Base, and

the auxotrophic requirements. The suspensions were incubated at 30ºC and 180 rpm and samples were taken at different times and inoculated in the appropriate dilutions on YPD plates. The plates were incubated 2 days at 30ºC. Results were expressed as relative viabilities to the control strains for each time point.

The release of amino acids under accelerated second fermentation conditions was studied by inoculating 10^6 cells/ml in 50 ml of synthetic base wine (4% (v/v) ethanol). Cultures were maintained at 30ºC without agitation and samples were withdrawn at different times. Yeast cells were removed by centrifugation and the supernatant was stocked at –20ºC until amino acids were quantified by the modified Cd-Ninhydrin method as described by Doi, et al. (11). Residual reducing sugars were quantified with DNS (3,5-dinitro-salicylic acid) as described by Bernfeld (1).

For stress resistance assays stationary phase yeast cells were obtained by inoculating YPD medium to a final concentration of $5x10^5$ cells/ml from a fresh culture and incubating for 24 h at 30ºC and 200 rpm. Yeast cells were submitted to different stress conditions in suspensions containing 10^7 cells/ml for specific times as follows: for thermal stress, 2 h in YPD medium at 4ºC, 37ºC, 42ºC, 47ºC, and 50ºC; for oxidative stress, 2 h in YPD medium with H_2O_2 to a final concentration of 5 mM; and for osmotic stress, 3h or overnight in YPD medium with KCl to a final concentration of 3M. Known cell numbers obtained by serial dilution were spotted in YPD plates and were incubated 2-3 days at 30ºC.

Determinations were performed 3 to 6 times and, when appropriate, data analyzed by one-way ANOVA and C-Dunnett test for means comparisons.

Differences were considered significant for $p<0.05$. The calculations were carried out by means of the SPSS program for Windows, release 13.0, run on a personal computer.

Molecular Biology techniques

Unless otherwise specified, all DNA manipulations were performed as described by Sambrook *et al.* (42). Polymerase chain reaction (PCR) was performed using Pfu TURBO DNA Polymerase (Stratagene, La Jolla, CA) following the instructions of the supplier. Restriction enzymes were from Roche Diagnostics SL Barcelona, Spain. Yeast genomic DNA was extracted by the method of Querol *et al.,* (40). DNA fragments resolved in agarose gels were purified with the QIAquick Gel Extraction Kit (QIAGEN GmbH, Hilden, Germany). *E. coli* was transformed by electroporation (42) and plasmids were purified from *E. coli* cells by using the High Pure Plasmid Isolation Kit (Roche). Transformation of *S. cerevisiae* followed the lithium acetate method described by Ito *et al*. (23) as modified Gietz and Woods (15). Transformants were selected in YPD plates containing 200 µg/ml geneticin (G418) (Life Technologies, Paisley, Scotland). Southern blot analyses were performed using the DIG High Prime DNA Labeling and Detection Starter Kit II (Roche, Mannheim, Germany), following the instructions of the supplier.

Western blot

Protein extracts and western blot were performed as described by Leber *et al*. (2001) (26) using a rabbit monoclonal antibody anti-acetaldehyde dehydrogenase (Rockland, Gilberstville, PA) and the ECL detection system

(Amersham Biosciences Europe, Barcelona). This antibody simultaneously reacts with Ald6p and Ald4p providing an internal control for extract concentration and gel loading (32).

Construction of *BCY1* defective strains

The cassettes for the interruption of *BCY1* in the genome of *S. cerevisiae* were constructed as follows. A selection/excision cassette containing the gene coding for *S. cerevisiae* FLP recombinase and a expression cassette for G418 resistance (KanMX), flanked by two copies of the FRT (FLP target) was PCR amplified from pHFKH using primers PromBCY1-preFRT and TermBCY1-postFRT (Table 2). Plasmid pGPCR3 was the result of cloning this 3.5 kb amplicon into pGEM-T (Promega) following the instructions of the supplier. In order to extend the *BCY1* homologous regions in pGPCR3 the following *S. cerevisiae* genomic DNA fragments were PCR amplified: tBCY1 (positions 1328 to 1855 from *BCY1* start codon), iBCY1 (positions -500 to 18 from start codon), sBCY1 (positions 653 to 1211 from *BCY1* start codon), the sequences of the primers used for these amplifications are shown in Table 2. These PCR fragments were inserted, downstream (tBCY1) or upstream (iBCY1 or sBCY1) the selection/excision cassette in pGPCR3 following the strategy described by Geiser et al. (2001) (14). The resulting plasmids were named pITGPCR3 (containing tBCY1 and iBCY1) and pSTGPCR3 (containing tBCY1 and sBCY1), (see maps in figure 1). These plasmids were digested with XbaI before yeast transformation. Correct integration of the insertion cassettes was verified by Southern Blot analysis using the DIG High Prime DNA Labeling and Detection

Starter Kit II (Roche, Mannheim, Germany), following the instructions of the supplier.

When necessary, excision of the selection/excision cassette from transformants was done as follows. Yeast cells were grown for 14 days in YPD non-selective medium at 30ºC and 180 rpm. Fresh medium was inoculated every day with 10^{-4} the volume of the previous culture, in order to avoid cultures of *BCY1* deleted strains entering the stationary phase. Appropriate cell numbers were inoculated on YPD plates and incubated for 24 h at 30ºC. The colonies were replica-plated to YPD plates containing 200 µg/ml geneticin and 40 µg/ml B-CIP (5-Bromo-4-chloro-3-indolyl phosphate). Sensitive strains were identified by poor growth and blue staining, due to the release of intracellular phosphatases. Verification of selection marker excision was done by Southern blot as described for the primary transformants.

RESULTS

Construction of strains carrying partial or complete deletions of *BCY1*

The first attempts to disrupt *BCY1* in *S. cerevisiae* were performed by transforming BY4741 or BY4743 with the 3.5 kb amplicon obtained by using pHFKH as template and primers PromBCY1-preFRT and TermBCY1-postFRT (see Materials and Methods). This PCR fragment contains 90 bp terminal extensions homologous to the *BCY1* ORF flanking regions in order to direct homologous recombination (long terminal tails were chosen because the *Saccharomyces* genome deletion project (http://www-

sequence.stanford.edu/group/yeast_deletion_project/deletions3.html) reported that systematic deletion of *BCY1* required the use of such long tails). Because no transformants were recovered, plasmid pGPCR3 was constructed and sequenced as described in Material and Methods. Sequence of the plasmid obtained indicated that the long terminal tails of the synthetic primers carried mutations and deletions that precluded their usefulness for homologous recombination. We decided to incorporate longer homologous regions to pGPCR3, as described in Materials and Methods, giving rise to plasmid pITGPR3, for total *BCY1* deletion and pSTGPR3, for partial *BCY1* deletion. The limits of this partial deletion were chosen in order to mimic mutation *bcy1-53* that has been described as “late acting class” by Peck et al. (1997) (34). The following strains were constructed by this strategy: LT11 (haploid, BY4741, total *BCY1* deletion), LT21 (diploid, BY4743, total *BCY1* deletion of one allele), LS11 (haploid, BY4741, partial *BCY1* deletion) and LS21 (diploid, BY4743, partial *BCY1* deletion of one allele). LT22 (diploid, BY4743, total *BCY1* deletion of both alleles) was constructed by excision of the selection marker cassette from LT21 and transformation of the resulting strain with pITGPCR3.

BCY1 deletion behaves as a semi-dominant mutation

As a first step towards the technological characterization of the *BCY1* deleted strains, all the strains generated during this work (LS11, LT11, LS21, LT21 and LT22), as well as Y27300 were assayed for known phenotypes of *BCY1* defective strains. These included survival to heat shock, osmotic stress and oxidative stress, as well as growth in different fermentable and non-fermentable carbon sources. The results of this analysis are shown in tables 3 and 4. As

expected, the stress resistance of haploid strains carrying a complete deletion of *BCY1* (LT11), or diploid strains homozygous for the same deletion (LT22), were hypersensitive to all the stress conditions analyzed (table 3). Even though *BCY1* loss of function mutations have been described as recessive (47), results obtained for strain LT21 clearly suggest that *BCY1* deletion behaves as a semi-dominant mutation, showing an intermediate response to heat shock and osmotic stress and similar to LT22 for oxidative stress. Surprisingly, Y27300, an heterozygous strain also carrying a complete deletion of the *BCY1* ORF in the same genetic background (BY4743), showed a slightly better response to heat shock stress than LT21.

The effect of partial deletion of *BCY1* was quite less pronounced as compared to total deletion of the ORF. The stress tolerance observed for the haploid strain carrying the partial deletion, LS11, paralleled that of the heterozygous LT21 strain, while the stress tolerance of the strain heterozygous for the partial deletion, LS21, was indistinguishable from the wild type BY4743 strain, with the only exception of a somewhat reduced tolerance to oxidative stress.

The phenotypic differences observed between the strains were confirmed by testing growth in different carbon sources (table 4). LT11 and LT22 showed the strongest dependence on easily fermentable carbon sources. The unexpected differences previously found between Y27300 and LT21 were confirmed and the parallelism between LS11 and LT21 was again observed.

All these results challenged the accepted view of *BCY1* mutations as recessive (47) and prompted us to continue the technological characterization

of *BCY1* mutants by also including heterozygous strains. This would have practical consequences that will be discussed later.

Ethanol tolerance under simulated second fermentation conditions.

Tolerance to ethanol of the different *BCY1* defective strains was tested under conditions that resemble those of second fermentation experiments. For this purpose synthetic base wines containing different amounts of ethanol and 2% glucose or sucrose were prepared. The fermentative capacity of each strain was studied by inoculating these media and recording sugar consumption at least until it was exhausted in all cultures with the control strains. Strain LT11 was impaired for the fermentation of sugar added to the base wine already in the absence of ethanol, and it was completely unable to do so in the presence of 2% or 4% ethanol (almost 1.5% of residual sugar at the end of the experiment). Results for sucrose consumption in the presence of 4% ethanol are shown in figure 2. In contrast, LT21, a diploid strain heterozygous for the same deletion, completely fermented glucose in the absence of ethanol (data not shown) and left less than 0.5% residual sugar in the presence of 2%, 4% or 6% ethanol, showing again a semi-dominant effect of *BCY1* deletion. Results for sucrose consumption in the presence of 6% ethanol are shown in figure 3. No apparent reduction in fermentative power was observed for the strains partially deleted for *BCY1*, LS11 and LS21. These results are relevant for the practical application of the findings described in this work, as will be discussed below.

Loss of viability under carbon starvation conditions.

Loss of viability under carbon starvation conditions was chosen in order to further assess the differences between the deletion strains under study. This test revealed to be more sensitive than the plate assays used in the first step of this characterization. It has also the advantage of focusing on the actual objective of this work, identifying genetic modifications potentially leading to accelerated autolysis in *S. cerevisiae*. The results of the analysis are shown in figure 4. There was a clear detrimental effect on cell survival of both mutations tested (complete or partial *BCY1* deletion) either on haploid strains or in diploid heterozygous strains. After one day under starvation conditions the viability drop was noticeably higher for all the mutant strains tested as compared to the cognate controls. As expected, the ability to survive under starvation conditions was specially reduced in LT11 (viability was almost completely lost after two days). Again, the behaviour of LS11 and LT21 was more or less similar, with relative viability stabilising at 20-30 % of the wild type after four days. This confirmed the semi-dominant character of *BCY1* deletion. Interestingly, this test also served to reveal a semi-dominant behaviour of the partial deletion of *BCY1*, even though LS21 was almost indistinguishable from the wild type strain in all the previous assays.

Release of amino acids to the external medium

The release of amino acids under accelerated second fermentation conditions (figure 5) was tested for a number of mutant strains as described in Materials and Methods. Because of its low ethanol tolerance, LT11 was excluded from this analysis. The results of this assay confirmed the picture deduced from the previous experiments under starvation conditions. All the mutant strains

experienced accelerated autolysis as compared to their wild type counterparts (BY4741 and BY4743). After three days, before sugar was completely exhausted, LS11 had already released more amino acids to the medium than all the other strains tested, the difference with the BY4741 control strain reaching a maximum after ten days of incubation. The heterozygous strain LS21 carrying the same partial deletion in one of the alleles of *BCY1* showed a similar kinetics of amino acid release, although the amounts released were intermediary between the control and, LT21. The disparities are less pronounced with prolonged incubation, especially for the haploid strains. All the differences discussed above were confirmed by ANOVA analysis ($p<0.05$). This experiment confirms that strains partially or totally deleted for *BCY1* undergo accelerated autolysis. The co-dominant character of both the partial and total deletion of *BCY1* is also confirmed by this experiment.

Autophagy.

It has been described that Ald6p is preferentially degraded during autophagy, in contrast to Ald4 (32). The availability of antibodies simultaneously recognising both enzymes provides an excellent experimental tool for monitoring autophagy. In order to strengthen the observations described above, the effect of *BCY1* mutations on yeast autophagy was tested for the diploid strains LS21, LT21, and LT22 compared to the wild type strain BY4743. The results are shown in figure 6. As expected, a clear reduction in the relative Ald6p levels was observed for the control BY4743 strain after 10 days of incubation under nitrogen starvation conditions. The total lack of *BCY1* activity in LT22 results in clearly higher relative Ald6p levels under the same conditions (figure 6). Again

the intermediate relative levels of Ald6p in LT21 prove the semi-dominant behaviour of BCY1 deletion (figure 6). In this assay Ald6p levels were very similar for BY4743 and LS21 (figure 6).

DISCUSSION

Two main observations can be highlighted from the results presented above. First, neither a total deletion of the ORF nor a partial deletion involving the last 13 codons are completely recessive, as previously described, but they are codominant. Second, heterozygous strains carrying one of the mutant *BCY1* versions mentioned above, undergo accelerated autolysis under conditions resembling those of second fermentation during sparkling wine production. Both findings have practical implications for the genetic improvement of second fermentation wine yeast strains. The second one because it suggest a genetic engineering strategy for industrial strains aimed to accelerate autolysis, using *BCY1* as a target. The former one because *in situ* deletion of one single copy of *BCY1* in industrial strains would be technically much simpler than the consecutive deletion of two or more copies of the gene. In addition, heterozygous strains would have the advantage of an intermediary phenotype, especially in the case of the partial deletion, showing little or no increase in ethanol susceptibility but still undergoing accelerated autolysis as compared to the wild type strains.

One of the main expected consequences of PKA constitutive activity of *BCY1* defective strains is delayed or reduced autophagy, and this has been confirmed for several of the strains analyzed in this work. The results we have obtained are hence in contrast with a previous observation that strains

specifically impaired in autophagy do not show accelerated autolysis (7). This is probably due to the pleiotropic effects of *BCY1* mutations, impaired autophagy being just one of them.

We have not found a convincing explanation to the different behavior of Y27300 and LT21. Both strains have been constructed in the same genetic background, BY4743, and both carry a complete deletion of the ORF of *BCY1* and in both cases the interruption cassettes carry the gene conferring resistance to geneticin. The only appreciable difference consists in the fact that the cassette used for the construction of the strain LT21 carries additional sequences aimed to facilitate the excision of the cassette for marker recycling. Perusal of the genomic context in order to find essential genes whose expression could be impaired by changes in the *BCY1 locus* failed to identify any essential gene adjacent to it.

The information obtained in this work has been used to design two alternative genetic engineering strategies for the improvement of industrial second fermentation yeast strains. In one of them we are trying to sequentially delete the 3' end of all the copies of *BCY1* in one or two of the commercial strains available at our collection. Indeed, preliminary results with an industrial strain heterozygous for this deletion indicate that it is able to complete second fermentation while undergoing accelerated autolysis (data not shown). The second strategy, based on the observations by Portela *et al*. (38) would consist in making phenocopies of heterozygous strains partially deleted in *BCY1*, by non-directed insertion of a one or a few copies of the truncated version of the gene in the genome of the industrial strains.

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Table 1. *S. cerevisiae* strains used in this work.

Strains	Genotype	Origin
BY4741	*MATa his3Δ leu2Δ0 met15Δ0 ura3Δ0*	EUROSCARF[a]
LS11	*MATa his3Δ leu2Δ0 met15Δ0 ura3Δ0 bcy1-53::Kan-FLP-FRT*	This study
LT11	*MATa his3Δ leu2Δ0 met15Δ0 ura3Δ0 bcy1::Kan-FLP-FRT*	This study
BY4743	*MATa/α his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 lys2Δ0/LYS2 MET15/met15Δ0 ura3Δ0/ura3Δ0*	EUROSCARF[a]
Y27300	*MATa/α his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 lys2Δ0/LYS2 MET15/met15Δ0 ura3Δ0/ura3Δ0 BCY1/bcy1::KanMX4*	EUROSCARF[a]
LS21	*MATa/α his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 lys2Δ0/LYS2 MET15/met15Δ0 ura3Δ0/ura3Δ0 BCY1/bcy1-53::Kan-FLP-FRT*	This study
LT21	*MATa/α his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 lys2Δ0/LYS2 MET15/met15Δ0 ura3Δ0/ura3Δ0 BCY1/bcy1::Kan-FLP-FRT*	This study
LT22	*MATa/α his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 lys2Δ0/LYS2 MET15/met15Δ0 ura3Δ0/ura3Δ0 bcy1::Kan-FLP-FRT /bcy1::Kan-FLP-FRT*	This study

[a] European *Saccharomyces cerevisiae* Archive for Functional Analysis

Table 2. Primers used in this work.

Primer	Sequence 5´→ 3´	Insert
PromBCY1--preFRT	5´- CCATTACACGCATTTCACGCATACATAAATAGGAAGATCATTTACA - - ACAAGCAGATTATTTTCAAAAGACAACAGTAAGAATAAACGATG - TATCACGAGGCCCTTTCG - 3´	KAN-FLP-FRT
TermBCY1--postFRT	5´- GAAGGAAGAAGAAAGAAAAAGGGAACAGAAGAAGAAGAAAAAG - - AGAAAGGAAATTCATGTGGATTTAAGATCGCTTCCCCTTTTTACTTA - GATAAGCTGTCAAACATGAG - 3´	KAN-FLP-FRT
PreFRT´-IBCY3´	5´- CGAAAGGGCCTCGTGATAGGGCAAAGAAGATACCATCG - 3´	iBCY1
5´GEM – iBCY5´	5´- CGACTCACTATAGGGCGAAT*CTAG*AAGAGGAGCATACGACTTCG - 3´	iBCY1
PostFRT´-TBCY5´	5´- CTCATGTTTGACAGCTTATCTTCTTCCTTCAACGTCTACG - 3´	tBCY1
3´GEM – tBCY3´	5´- GGTGACACTATAGAATACTC*TAGA*GCTGTCTTGTAGATCCTTTGG - 3´	tBCY1
PreFRT´-StopsBCY3´	5´- CGAAAGGGCCTCGTGATATTATCATGCAGGACCCAGTAAACGTTG -3´	sBCY1
5´GEM – sBCY5´	5´- CGACTCACTATAGGGCGAAT*CTAGA*GGACTACTTCTATGTCGTCG – 3´	sBCY1

pHFKH plasmid hybridization sites are underlined. pGPCR3 plasmid hybridization sites are double underlined. Italics indicate added nucleotides to create restriction site XbaI. Shaded region indicates added stop codons.

Table 3. Stress responses.

Strain	Temperature						Osmotic (KCl 3M)		Oxidative (H_2O_2 5mM)
	4ºC	30ºC	37ºC	42ºC	47ºC	50ºC	3 h	ON	
BY4741	++	++	++	+	+	+	++	++	+/-
LS11	++	++	++	+	-	-	+/-	+	-
LT11	+/-	+/-	+/-	-	-	-	-	-	-
BY4743	++	++	++	++	+	+	++	++	+
LS21	++	++	++	++	+	+	++	++	+/-
LT21	+	++	++	+	+/-	-	+	+	-
LT22	+/-	+/-	+/-	-	-	-	-	-	-
Y27300	++	++	++	++	+/-	+/-	+	+	-

++: normal growth; +: reduced growth; +/-: very reduced growth; -: no growth

Table 4. Growth on different carbon sources.

	Glucose	Sucrose	Galactose	Raffinose	Fructose	Maltose	Glycerol	AcK	Pyruvate
BY4741	+ +	+ +	+ +	+ +	+ +	+ +	+	+	+
LS11	+ +	+ +	+/-	+ +	+ +	+/-	-	+	+/-
LT11	+	+	-	-	+	-	-	-	-
BY4743	+ +	+ +	+ +	+ +	+ +	+ +	+	+	+ +
LS21	+ +	+ +	+ +	+ +	+ +	+ +	+	+	+ +
LT21	+ +	+ +	+	+ +	+ +	+/-	+/-	+	+
LT22	+	+	-	-	+	-	-	-	-
Y27300	+ +	+ +	+	+ +	+ +	+	+/-	+	+ +

++: normal growth; +: reduced growth; +/-: very reduced growth; -: no growth

FIGURE LEGENDS

FIG. 1. (A) pITGPCR3 plasmid containing iBCY1 (*BCY1* initial region from -500 to 18 respect first ATG) and tBCY1 (*BCY1* terminal region from 1328 to 1855 respect first ATG); (B) pSTGPCR3 plasmid containing sBCY1 (*BCY1* region from 653 to 1211 respect first ATG) and tBCY1. FLP, *FLP* gene that encodes FLP recombinase; TEFp, *TEF* gene promoter; Kan, gene of transposon Tn903 coding for aminoglycoside phosphotransferase; TEFt, *TEF* gene terminator; FRT, FLP recombinase recognition sequences; LacZα, *E. coli* beta-galactosidase gene alpha peptide; ORI, *E. coli* pMB1 replication origin; AmpR, *E. coli* beta-lactamase gene; F1ORI, F1 phage replication origin.

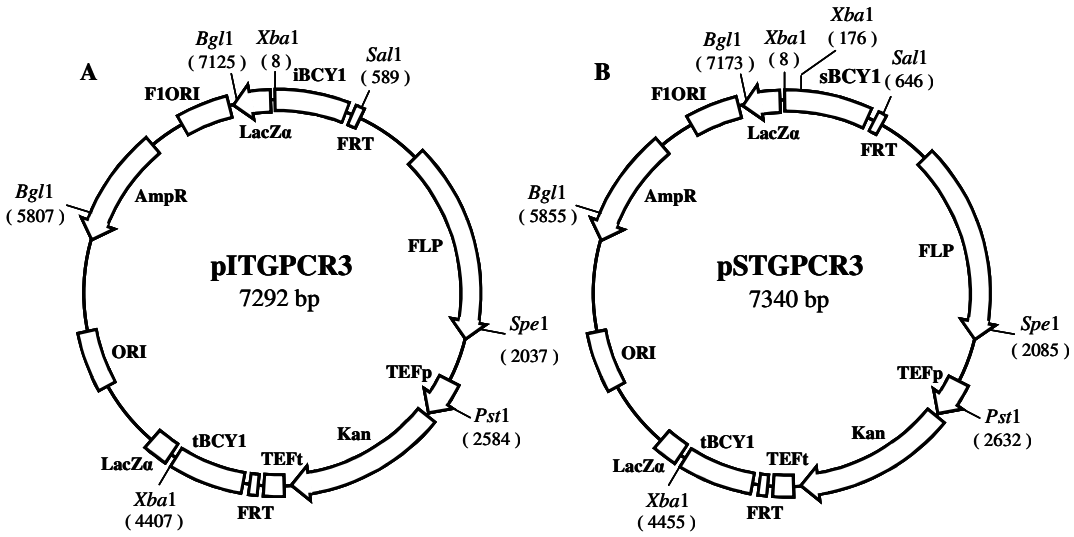
FIG. 2. Fermentative capacity of haploid strains on synthetic base wines containing 4% ethanol and 2% sucrose, as described in Materials and Methods. Residual reducing sugars were quantified at several time points, as described in Materials and Methods. BY4741 (circles); LS11 (squares); LT11 (triangles). Values are expressed as mean ± standard deviation (vertical bars).

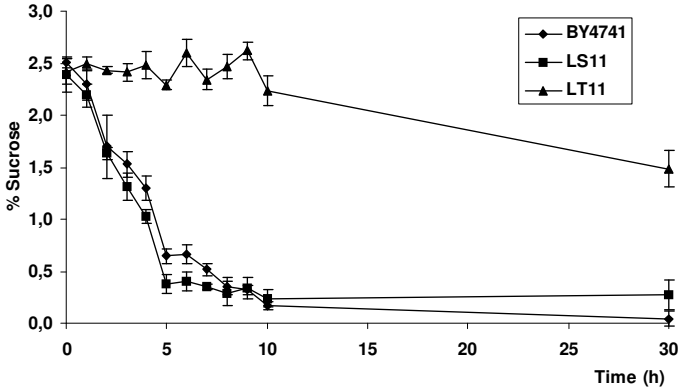
FIG. 3. Fermentative capacity of diploid strains on synthetic base wines containing 6% ethanol and 2% sucrose as described in Materials and Methods. Residual reducing sugar were quantified at several time points, as described in Materials and Methods. BY4743 (circles); LS21 (squares); LT21 (triangles). Values are expressed as mean ± standard deviation (vertical bars).

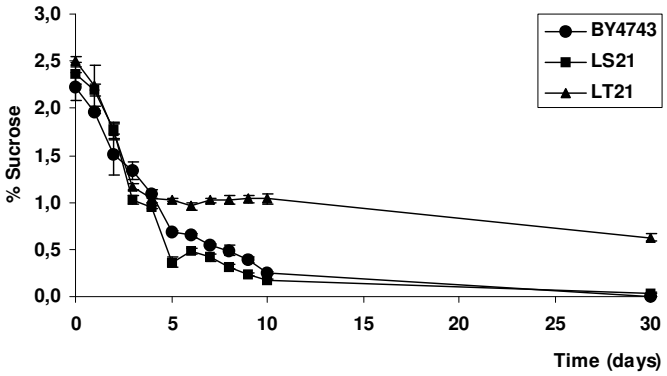
FIG. 4. Relative loss of viability of haploid and diploid strains under carbon starvation conditions. Cells were inoculated in S- medium as described in Materials and Methods. The number of viables was calculated by platting in YPD medium. (A) Haploid cells: BY4741 (circles); LS11 (squares); LT11 (triangles). (B) Diploid cells: BY4743 (circles); LS21 (squares); LT21 (triangles). Values are expressed as mean ± standard deviation (vertical bars).

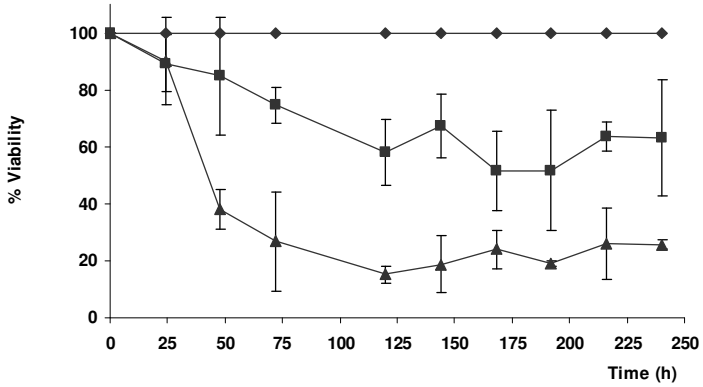
FIG. 5. Release of amino acids by haploid and diploid strains under accelerated second fermentation conditions. Results are expressed as milligrams of leucine equivalents per ml. BY4741 (white bar), LS11 (striped bar), BY4743 (dotted bar), LS21 (squared bar), LT21 (black bar). Values are expressed as mean ± standard deviation (vertical bars).

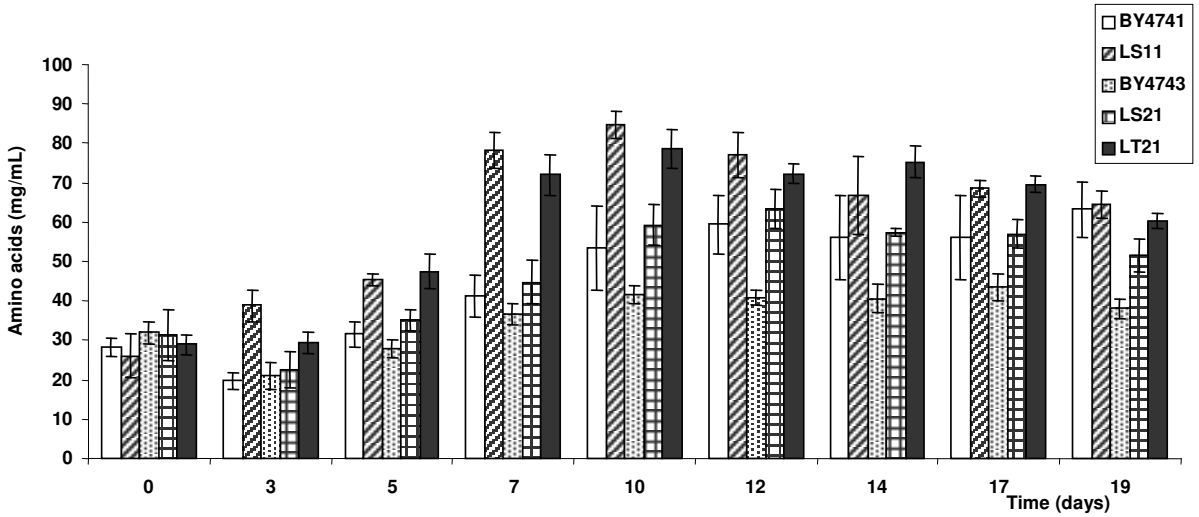
FIG. 6. Ald6p and Ald4p levels of diploid strains (BY4743, LS21, LT21 and LT22) under nitrogen starvation conditions, detected by wester blot as described in Materials and Methods.

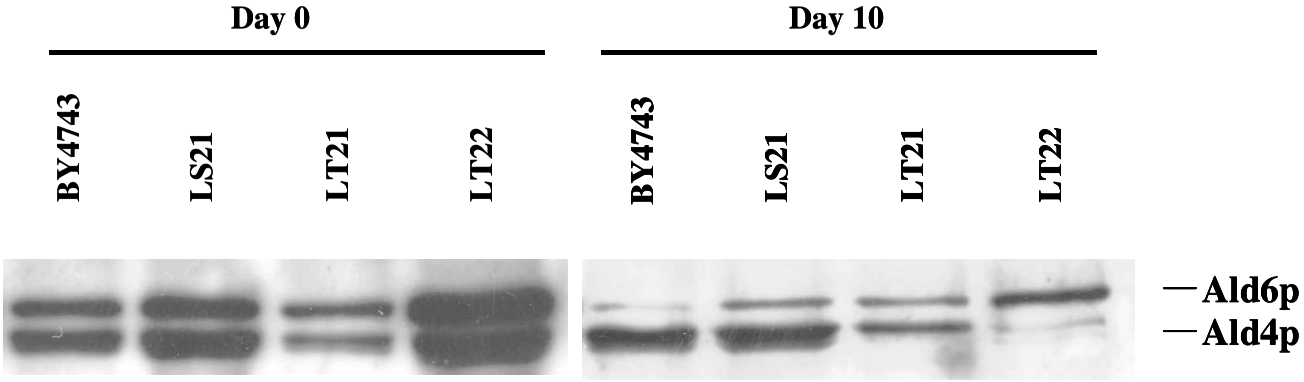

A
Bgl1
(7125)
Xba1
(8)
iBCY1
Sal1
(589)
F1ORI
LacZα
FRT
Bgl1
(5807)
AmpR
FLP
pITGPCR3
7292 bp
Spe1
(2037)
ORI
TEFp
Pst1
(2584)
tBCY1
Kan
LacZα
TEFt
Xba1
(4407)
FRT
B
Xba1
(176)
Bgl1
(7173)
Xba1
(8)
sBCY1
Sal1
(646)
F1ORI
LacZα
FRT
Bgl1
(5855)
AmpR
FLP
pSTGPCR3
7340 bp
Spe1
(2085)
ORI
TEFp
Pst1
(2632)
tBCY1
Kan
LacZα
TEFt
Xba1
(4455)
FRT


3,0
2,5
2,0
1,5
1,0
0,5
0,0
% Sucrose
0
5
10
15
20
25
30
Time (h)
BY4741
LS11
LT11


3,0
2,5
2,0
1,5
1,0
0,5
0,0
% Sucrose
0
5
10
15
20
25
30
Time (days)
BY4743
LS21
LT21


% Viability
100
80
60
40
20
0
0
25
50
75
100
125
150
175
200
225
250
Time (h)


BY4741
LS11
BY4743
LS21
LT21
Amino acids (mg/mL)
100
90
80
70
60
50
40
30
20
10
0
0
3
5
7
10
12
14
17
19
Time (days)


Day 0
Day 10
BY4743
LS21
LT21
LT22
BY4743
LS21
LT21
LT22
Ald6p
Ald4p