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Identification and characterization of a thermotolerant yeast strain isolated from banana leaves

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ABSTRACT: We have isolated a thermotolerant yeast strain, called TR2, from banana leaves and characterized it as *Saccharomyces cerevisiae* by sequencing about 25 kb at random, including structural genes and gene promoters. In all cases, the sequence identity was higher than 98%, and gene synteny was conserved. The strain is a homothallic diploid strain containing two sets of *S. cerevisiae* chromosomes that grows efficiently in a complete medium up to 40–41 °C. We present a method allowing the isolation of stable haploid segregants. Analysis of the thermotolerance among the segregants proved to be complex, since we recovered more thermotolerant segregants than expected if the thermoresistance would had been conferred by a single gene. We obtained stable haploid segregants that were as thermotolerant as the original diploid strains. We also obtained a few thermosensitive segregants and strains that had an intermediate phenotype. The original TR2 strain or appropriate haploid derivatives can thus be used as safe probiotics in the food industry because of their rapid growth in a variety of conditions, such as high and low temperature, and their resistance to high salt concentrations.

INTRODUCTION

With the increasing interest concerning fermentation, industrial products issued from yeast biotechnology have emerged in many commercially important sectors, for instance, food, beverages,biofuels, chemical industrial enzymes, pharmaceuticals, and agriculture. Like all microorganisms, yeasts exhibit specific characteristics when growing at minimum, optimum, and maximum growth temperatures (T_{min}, T_{opt}, and T_{max}), respectively. The maximum temperature for growth is relatively constant within a species[1]. Thermophilic yeasts have been described with T_{min} values at or above 20 °C, while thermotolerant yeasts possess T_{opt} values above 40 °C. For *S. cerevisiae* strains, T_{max} values range from 35 °C up to 40–41 °C in some cases (which is similar to *S. paradoxus*), whereas strains of *S. Bayanus* and *S. pastorianus* fail to grow above 35 °C. This has been taken as a taxonomic criterion for differentiating subgroups within *Saccharomyces sensu stricto*. Increasing interest has been given to yeasts capable of growing at high temperature (grown at temperatures above 40 °C), because they present certain advantages in industrial processes. For example, thermotolerant *Kluyveromyces marxianus* strains are capable of aerobic growth at 52 °C on lactose,whey permeate, cellobiose and xylose. These strains also ferment glucose to ethanol at 45 °C[2]. In Thailand, the chicken industry is extensively developed, and many research projects are aimed at protecting chickens from diseases but at low cost and in a safe way for humans. Therefore, the addition of yeast to the chicken diet as a probiotic is of interest, as this microorganism can be easily produced in large amounts due to its rapid growth (less than 90 minutes of doubling time) on complete media (but without addition of any drug) and at temperatures ranging from 20 °C to 40 °C. The beneficial effects of the addition of *S. cerevisiae* to chicken diets has already been reported[3], but without taking advantage of the availability of thermotolerant yeasts.

Some yeasts isolated from the wild in the northern part of Thailand could be of particular interest, since they can grow fast at temperatures as high as 41–42 °C, which is close to that of the chicken body. Such strains would be excellent probiotics, since fast growth at such temperatures would more efficiently out-compete possible contaminant organisms. However, in order to be an acceptable probiotic these yeasts should be generally recognized as safe. In this work, we show that a natural thermotolerant yeast (TR2 strain) isolated from banana leaves is genetically related to *S. cerevisiae*, and displays distinct advantageous properties that include a better growth ability at high temperature (up to 41–42°C) and higher tolerance to salts. Such a strain is suited to withstand a variety of stressful conditions more effectively than the average available *S. cerevisiae* strains. This results

from a mechanism called cross-tolerance, in which one type of stress such as heat stress gives partial protection against other stresses such as osmotic or salt stresses[4]. The natural isolate appeared to be a homothallic diploid strain that sporulated spontaneously on a YPD medium. No stable haploid segregants could be obtained after dissection of the tetrads, rendering the analysis of the thermoresistance difficult. We describe here a method allowing the isolation of stable haploid segregants, bearing either TR (thermoresistant) or ts (thermosensitive) genetic traits. TR haploids are preferable for the considered application, as they should be genetically stabilized, and well characterized. These TR and ts segregants could also be of interest for further determination of the genes important for growth at suboptimal temperatures.

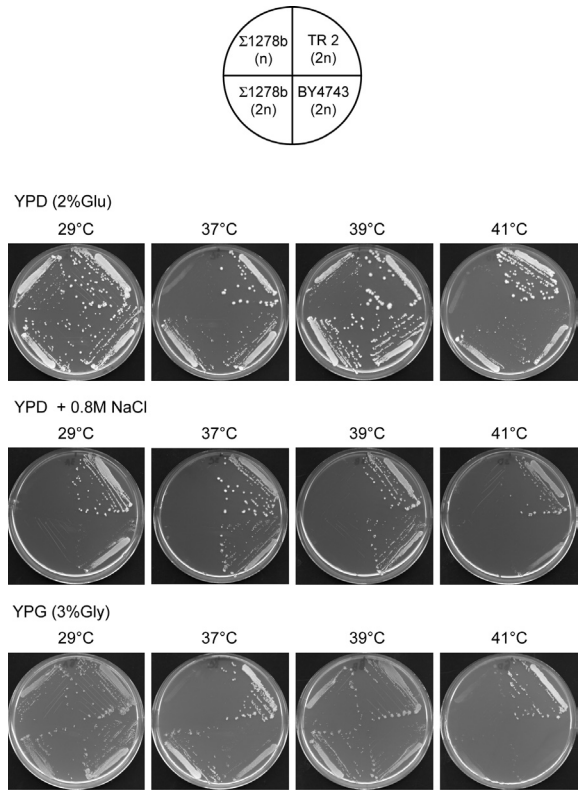

Fig. 1 Growth tests showing the growth ability of TR2 strain compared to haploid and diploid laboratory strains. Cell suspensions from each indicated strain were streaked onto agar plates [complex medium containing 2% glucose (YPD), YPD supplemented with 0.8 M NaCl (YPD+0.8 M NaCl) and complex medium containing 3% glycerol as nonfermentable carbon source (YPG)]. The plates were incubated at 37°C, 39°C or 41°C relative to 29°C for 3 days.

MATERIALS AND METHODS

Strains and medium

S. cerevisiae strains BY4709 (*MATα, ura3, ho*) and ∑1278b (*MATα, ho*) and the diploid ∑1278b/3962c (*MATα/MATa*) were used as controls in the growth tests at different temperatures. Strain BY4709Δarg80 (*MATa, ura3, ARG80::kanMX4, ho*) was used for mating with spores of the TR2 strain. The construction of the deletion of the *ARG80* gene by the *kanMX4* cassette was described in El Bakkoury *et al* [5]. All yeast strains were grown either in liquid YPD (1.0% yeast extract, 2.0% peptone, 2.0% glucose with or without 2.0% agar for plates) or on minimal medium (yeast nitrogen base) containing 2.0% glucose and 0.02 M ammonium sulphate with 2.0% agar (M.am plates). When required, 50 µg/ml of uracil was added to the plates. The concentration of geneticin used in the selection of diploids with BY4709 and TR2 was 200 µg/ml. For growth under saline stress, 0.8M NaCl was added to the YPD plates. The YPG plates contained 3.0% glycerol instead of glucose for growth tests on a non-fermentable carbon source.

Construction of a yeast genomic library with DNA from the TR2 strain

Yeast genomic DNA from the TR2 strain was prepared as described by Burke *et al*[6]. The total yeast DNA was partially digested using the *Sau*3A1 restriction enzyme to obtain fragments down to about 3 kb. After agarose gel purification, these DNA fragments were ligated with the vector pFL38 (*CEN, URA3*, *Amp*^{R}) digested with *Bam*HI enzyme and treated with shrimp alkaline phosphatase. After ligation, the DNA mixture was introduced into the electrocompetent *E. coli* cells by electroporation. AmpR transformants were selected on solid Luria Bertani medium (LB) containing 50 µg ampicillin, and collected in liquid LB + ampicillin medium. About 10,000 clones were obtained. Random minipreps were performed and their analysis indicated that about 2/3 of the plasmids contained inserts with an average size of 5 kb. For plasmid amplification, the library was spread out on solid LB+Amp and allowed to grow for 18 hours. The colonies were scraped up in liquid LB+Amp containing 20% glycerol, and aliquots were made and stored at -80 °C for long-term usage. Some of these plasmids containing DNA from the TR2 strain were isolated and sequenced and the sequences were compared to that of the SGD Database (*www.yeastgenome.org*).

DNA sequencing and analysis

DNA sequencing was based on the dideoxy-mediated chain termination method[7] using a fluores-

cent-labelled terminator[8]. The DNA fragments were sequenced by the Biovallee company (Belgium) using an automated ABI PRISM DNA sequencer (model 3100). The DNA sequences were analysed by the DNA strider and vector NTI programs.

RESULTS AND DISCUSSION

Characteristics of the selected yeast TR2 strain

Three hundred and four yeast strains were isolated from different sources (longan, banana, custard apple, grape, coconut, green plum, pineapple, papaya, pumpkin, and sweet potato) and were grown in liquid complete medium (YPD) at 40 °C and 42 °C and the turbidity of the cultures was measured after 15 h of growth. Twenty eight strains were then chosen for further analysis and nine of those strains presented a doubling time in YPD culture at 42 °C ranging from 255 to 355 minutes. The TR2 strain was finally selected on the basis of having the best growth rates (as estimated from the size of single isolated colonies) at high temperature (40 °C to 42 °C) in different media supplemented with 2% agar (complex medium supplemented with glucose (YPD) or glycerol (YPG) as carbon sources, minimal medium (M.am)). The TR2 strain also showed the highest resistance to various "extreme" conditions (*e.g.* low temperature growth at 16 °C, saline media, osmotic stress) (see Materials and Methods for media definitions).

The growth ability of the TR2 strain on YPD (2% glucose), YPD + 0.8M NaCl; and YPG (3% glycerol) at 29 °C, 37 °C, 39 °C, and 41 °C is shown in Fig. 1. As the yeast strains isolated in nature were diploid (2n), TR2 was compared to different laboratory strains, namely, the haploid ∑1278b (n), the diploid ∑1278b/3962c (2n) and the diploid BY4743 (2n). These tests showed the high temperature growth capacity (HTG), even on a nonfermentable carbon source such as glycerol, and the salt tolerance of the TR2 strain. It is worth noting that the optimal growth rate of these laboratory strains is between 28 °C and 32 °C, and that the control strain ∑1278b is particularly temperature sensitive compared to other laboratory strains such the haploid BY 4709.

Identification of the TR2 strain

As stated in the introduction, to feed chickens safely with a TR2-like strain as a probiotic, it is of great importance to determine the yeast species it belongs to. The Genolevures consortium analysed the genome of 13 yeast species by sequencing random genomic libraries. To analyse genome evolution in yeasts among the hemiascomycete phylum, they compared the full genome of *Candida glabrata, Kluyveromyces lactis, Debaryomyces hansenii, Yarrowia lipolytica, and Saccharomyces cerevisiae*[9,10]. For each species, a random genomic library was prepared to generate fragments ranging in size from 3 to 5 kb. This size was chosen based on the average length of the *S. cerevisiae* ORFs and intergenic regions. Single-pass sequencing (up to 1 kb) of both ends of each insert led to the characterization of each insert by 2 Random Sequence Tags (RST). Each set of RST was compared to the *S. cerevisiae* genome and annotated accordingly; DNA sequence identity and gene synteny (order and orientation maintenance of neighbouring genes). The extent of divergence among species of ascomycetous yeasts is usually based on the DNA sequence maintenance in the variable D1/D2 domain of large subunit (26S) ribosomal DNA. Divergence in this domain is generally sufficient to resolve individual species[11,12]. However, to discriminate among *S. cerevisiae* strains, several groups have reported that there was no single PCR-mediated typing technique able to discriminate at the strain level[13-15]. To clearly identify our strain as a *S.cerevisiae* strain we adopted a similar strategy to the Genolevures consortium (large scale DNA sequence identity and synteny conservation) to analyse the genome of the diploid TR2 strain, allowing us also to determine whether or not this strain isolated from the wild is a hybrid between two closely related species. A genomic library was constructed with DNA from the TR2 strain inserted in the yeast centromeric plasmid pFL38 (*ARS, CEN6, URA3*) (for details see Materials and Methods). A total of 30 clones from this library were sequenced. The inserts ranged from 1.5 kb to 10 kb, and about 100 bp to 900 bp were sequenced from each insert. Thirty clones appeared to be dispersed on the 16 *S. cerevisiae* chromosomes (Fig. 2), and the average sequence identity to the SGD data base was higher than 98%, even in promoter regions which are known to diverge more than ORFs. In addition, fragments from seven other genes were synthesized by PCR using *S. cerevisiae* DNA primers (256 bp from the *CAR1* promoter, 440 bp from the CDC5 gene, 412 bp from the PHO11 gene, and 413 bp from the *STE2* gene) and were then sequenced. All these sequences showed about 99% identity to the *S. cerevisiae* genome sequence deposited in the SGD database. Moreover, the entire DNA sequences of two genes encoding protein phosphatases (*PTC1*, about 2,000 nt and *SSD1*, 3,751 nt) were also determined, showing the same high degree of sequence identity. A total of 25,095 nucleotides were thus sequenced. Fourteen

fragments presented synteny for two genes, indicating that the gene order was maintained in the TR2 strain (*MYO4* and *FRT2, YBR094w* and *RXT2, YDL010w* and *PTC1, GCN2* and *ZIP1, HNT2* and *SUM1, BCK2p* and *CCA1, MAD1* and *SCY1, RHO3p* and *SDP1, SPC1p* and *ESS1, UGP1* and *YKL033w, RDN5-1p* and *RDN37-2, IKI3* and *SNR34, SOK2p* and *SPO2p, NRD1* and *RAD50*). Moreover, the gene orientation was also conserved in all cases.

It is noteworthy that the library constructed with the DNA from the diploid strain TR2 arose from two sets of chromosomes. All the cloned fragments selected randomly showed very high DNA sequence identity to the genome of *S. cerevisiae* as well as gene synteny, indicating that the two sets of chromosomes from the TR2 strain are highly homologous, and that this strain isolated from the wild is not a hybrid between two closely related species. All these results are in favour of the TR2 strain being *S. cerevisiae*. Further genetic approaches will give additional support to this statement.

Isolation of stable haploid segregants from the diploid TR2 strain

Diploid *S. cerevisiae* laboratory strains sporulate under conditions of nutrient deficiency (nitrogen starvation and acetate as a carbon source), but TR2 strain sporulated spontaneously even on a rich medium (YPD). Moreover, when the asci were dissected and the spores were germinated on YPD medium, no stable α or a cells could be obtained, as shown by the absence of conjugation of cells from the colonies with a or α haploid tester strains, indicating that the TR2 strain is homothallic (*HO*). This behaviour impaired classical genetic analysis of the TR2 strain. In such cases, one could envisage disrupting the *HO* gene to produce stable haploid TR cells. Deletion of the *HO* gene could be performed by using the *kanMX4* deletion cassette conferring resistance to geneticin, but the *ho* segregants of interest should then all contain an antibiotic resistance gene. In order to be able to eliminate the *kanMX4* cassette after having generated *ho* segregants, we adopted another approach, consisting in mating haploid spores of TR2 strain obtained after digestion of sporulated asci, with a stable *ho* haploid derived from the laboratory strain BY4709. The genotype of the TR2 strain is *TR, HO, URA3*, kan^s, whereas the genotype of the BY4709Δ*arg80* strain is *ts, ho, ura3, arg80::kanMX4* (carrying a dominant selective marker in replacement of gene ORF *ARG80*). This gene is located on chromosome XIII and functions as a specific regulator of the metabolism of arginine, with no effect on growth except on arginine as a sole nitrogen source. In this case, the presence of the *kanMX4* gene among the segregants would not cause a problem since we can easily recover TR and ts segregants that will be *ho* but also *ARG80*. Thus the digested asci from TR2 were mated en masse with strain BY4709Δ*arg80*. Diploid cells were selected on the basis of growth in the absence of uracil (*URA3* coming from the TR2 spores) and resistance to geneticin (*kanMX4* coming from BY4709Δ*arg80*) at 29 °C. All these diploid strains were able to grow at 41 °C, indicating that the TR trait coming from the TR2 strain is dominant. After sporulation of three different diploids, tetrads were dissected and the spores were analysed for the genetic characters, *URA3, HO, kanMX4,* and TR. The segregation of *URA3/ura3* was tested by growth on minimal medium in the absence or presence of uracil. *HO/ho* was tested by the spontaneous sporulation capacity of the newly formed diploids or the mating type capacity of stable haploids. *ARG80/arg80::kanMX4* was tested by the resistance to geneticin. The TR/ts segregation was assayed by growth at 29 °C, 39 °C and 40 °C on YPD plates. All the characters segregated 2/2, with the exception of the *TR/ts* phenotype that varied from 4TR/0ts, 3TR/1ts or 2TR/2ts (Fig. 3). These experiments showed that we recovered a much higher number of TR spores than ts spores, but that the thermotolerance varied among the segregants, some being as thermotolerant as the original TR2 diploid strain (named TR), and some still growing at high temperature but less efficiently

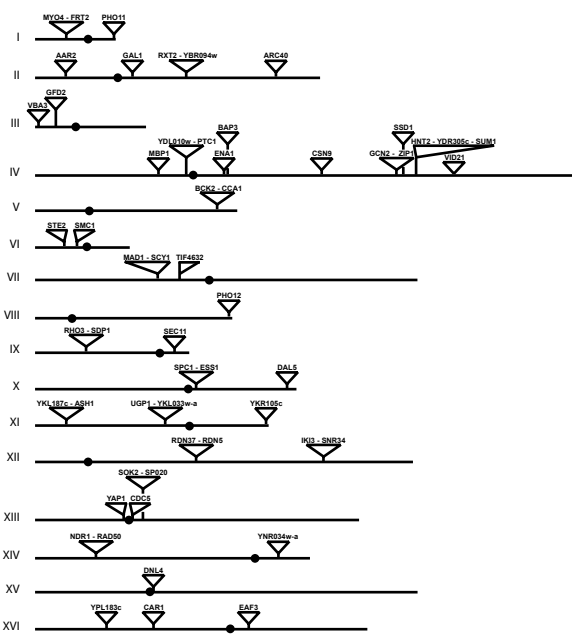

Fig. 2 Positioning of the sequenced DNA fragments from the genomic library of TR2 strain on the *S. cerevisiae* chromosomal map.

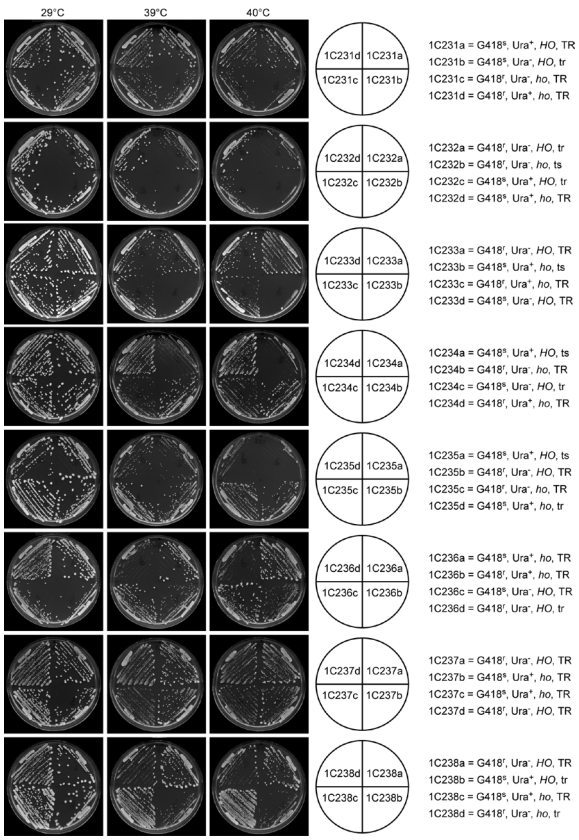

Fig. 3 Phenotypic analysis of tetrads generated from the cross between a TR2 haploid spore and BY4709-Δ*arg80* strain. The genotypes of the four spores of each tetrad are listed at the right of the plates. TR, tr and ts refer to the growth capacity of each spore to grow at 40 °C (TR), 39°C (tr) or only at 29°C (ts) on YPD medium. Ura^- refers to the absence of growth of the cells on M.am plates, thus to the presence of a mutation in the *URA3* gene (data not shown). $G418^r$ and $G418^s$ refer to the presence of the *kanMX4::arg80* (*ARG80* gene disruption cassette leading to geneticin resistance) or of the *ARG80* allele (geneticin sensitivity) (data not shown). *HO* and *ho* refer to the presence or absence of a functional *HO* gene (allowing mating type switching and diploid formation leading to sporulation) and absence of mating with *Mat a* and *Mat α* cells (data not shown).

(named tr), but in contrast with ts strains they are able to form single colonies at 40 °C, although these were very small. In a total of 18 tetrads, 9 were 4TR or tr/0ts, 6 were 3TR or tr/1ts, 1 was 2TR/2ts and 1 was 1TR/3ts. These results indicated that the thermotolerance of the original TR2 strain was associated with more than one gene and/or that TR trait(s) was (were) extrachromosomal. Further analysis of high thermotolerance can now be undertaken, since we have at hand haploid strains that are *TR, URA3, ARG80*, *ho* such as spore 1C232d or *ts, URA3, ARG80, ho* such as spore 1C232b. Further crosses provided ts, *ura3*, *ho* spores which could be recipients for cloning gene(s) capable of allowing thermosensitive strains to grow at higher temperatures. It is worth noting that the diploids from the cross between isolated haploid spores from strain TR2 and haploid cells from BY4709Δ*arg80* did generate 4 spore asci. Morever, all the spores germinated, giving additional support to the fact that the TR2 strain belongs to the *S. cerevisiae* species.

In view of the considered probiotic application, TR ho haploids devoid of *kanMX4* marker should be selected, as the presence of exogenous DNA (and particularly genes conferring antibiotic resistance) is not desirable. However, the presence of appropriate genetic marker(s) having no impact on biomass production (such as an *ura3* mutation), should allow one to easily characterize yeast batches. Finally, in view of selecting an appropriate strain, the stability of the TR trait of the candidate strains will need to be evaluated after extensive growth.

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