Mitotic Recombination and Genetic Changes in *Saccharomyces cerevisiae* during Wine Fermentation

SERGI PUIG,1,2* AMPARO QUEROL,1 ELADIO BARRIO,3 AND JOSÉ E. PÉREZ-ORTÍN1,2

Departamento de Biotecnología, Instituto de Agroquímica y Tecnología de Alimentos, CSIC,1 and Departamento de Bioquímica y Biología Molecular2 and Instituto ‘Cavanilles’ de Biodiversidad y Biología Evolutiva,3 Universitat de València, Valencia, Spain

Received 12 July 1999/Accepted 23 February 2000

Natural strains of *Saccharomyces cerevisiae* are prototrophic homothallic yeasts that sporulate poorly, and may be aneuploid. This genomic constitution may confer selective advantages in some environments. Different mechanisms of recombination, such as meiosis or mitotic rearrangement of chromosomes, have been proposed for wine strains. We studied the stability of the *URA3* locus of a *ura3* wine yeast in consecutive grape must fermentations. *ura3* homozygotes were detected at a rate of 1 × 10−7 to 3 × 10−9 per generation, and mitotic rearrangements for chromosomes VIII and XII appeared after 30 mitotic divisions. We used the karyotype as a meiotic marker and determined that sporulation was not involved in this process. Thus, we propose a hypothesis for the genome changes in wine yeasts during vinification. This putative mechanism involves mitotic recombination between homologous sequences and does not necessarily imply meiosis.

*Saccharomyces cerevisiae* wine strains have been selected for (i) their ability to quickly and efficiently ferment grape musts with elevated sugar concentrations, (ii) their resistance to high ethanol and sulfur dioxide concentrations, and (iii) their survival during fermentation at elevated temperatures (17). Thus, wine yeasts have unique genetic and physiological characteristics that differentiate them from other laboratory and industrial strains, such as baker’s, brewer’s, and distiller’s yeasts.

Natural yeasts are mostly prototrophic, homothallic, and heterozygous (4, 15, 17). They sporulate poorly (3), although in the case of wine yeasts, between 0 and 75% of cells sporulate, depending on the ploidy of the strain (4). In wine yeasts, spore viability also varies greatly (0 to 98%) (4) and is inversely correlated with heterozygosity (23). Wine yeasts frequently are aneuploid, with disomies, trisomies, and, less frequently, tetrasomies (3, 15). In some cases, these strains are nearly diploid or triploid. This aneuploidy may confer selective advantages by increasing the number of copies of beneficial genes or by protecting the yeast against lethal or deleterious mutations (3, 15).

The electrophoretic karyotypes of wine yeast strains differ in the number, size, and intensity of bands, allowing the identification of every strain by its chromosome pattern (37, 40). Wine strains do not have a stable and defined karyotype, like flor and distiller’s yeasts.

Chromosomal rearrangements have been described in wine yeast genomes during vegetative growth, due to recombination between homologous chromosomes (19) and to recombination between repeated or paralogous sequences (24, 39). The maintenance of these polymorphisms in a population suggests that such exchanges might be the result of an important adaptive mechanism of yeasts (1, 19).

Mortimer and coworkers (23) proposed a mechanism of evolution for natural wine yeasts, termed genome renewal. This hypothesis maintains that wine yeasts, which accumulate deleterious mutations as heterozygotes, can sporulate and, as homothallics, produce completely homozygous diploids. Some of these new homozygotes would replace the original heterozygote. However, sexual isolation in yeast populations during wine production (34), the high level of heterozygosity, and the low sporulation rates of wine yeasts (3, 4, 15) do not favor this hypothesis.

Our objective in this study was to test the genome renewal hypothesis (23). We analyzed the formation of homozygotes from a *ura3* heterozygous wine strain during consecutive wine fermentations. The chromosomal heteromorphism of this strain allowed us to determine if the formation of the homozygotes occurred as a consequence of sporulation. Chromosomal rearrangements during vinifications also were studied. We hypothesize that the mechanism of genome evolution for wine yeasts involves only mitotic recombinations.

**MATERIALS AND METHODS**

**Strains and culture conditions.** We used the diploid, homothallic *S. cerevisiae* wine yeast strain *Tn3* (Spanish Type Culture Collection reference no. CECT1894) selected in the region of Alicante, Spain (29), and commercialized by Lallemand Inc. (Montreal, Quebec, Canada). A recombinant *Tn3* strain, named *Tγ*6, was obtained by transformation with an *NdeI-StuI* fragment of plasmid pURA::KMX4, that contains the *kan* gene conferring resistance to the antibiotic G418 (28). *Tγ*6 has one allele of the *URA3* gene disrupted by the insertion of the *kanMX4* marker (38) and the wild-type allele on the homologous chromosome. It is phenotypically *Ura−* and *Kan+,* and it will be either *Ura−/Kan+* or *Ura+/Kan−* if it becomes homozygous.

For laboratory cultures, yeast cells were grown at 30°C in YPD (1% yeast extract, 2% bacteriological peptone, 2% glucose) or in SD (0.67% yeast nitrogen base without amino acids [Difco Laboratories, Detroit, Mich.], 2% glucose). For *ura 1* screening, 107 cells were spread on a plate of 5-fluoro-orotic acid (FOA) medium (22) containing 1 mg of FOA (Toronto Research Chemicals, Ontario, Canada) per ml.

*Escherichia coli* DH5α was used for the construction of plasmids. It was grown at 37°C in LBA medium (1% tryptone, 0.5% yeast extract, 30 mg of ampicillin per ml). Media were solidified with 2% agar.

**DNA manipulations.** Standard protocols were followed (33).

**Yeast transformation protocol.** In order to transform *S. cerevisiae*, cells were washed with 0.9% NaCl, and then resuspended at 1 × 107 cells/ml in sterile water.transformants were selected by applying 0.4 mg/ml of 5-fluoro-orotic acid.

* Corresponding author. Present address: Department of Biological Chemistry, University of Michigan Medical School, Medical Science I, 1301 Catherine Road, Ann Arbor, MI 48109-0606. Phone: (734) 764-7514. Fax: (734) 763-7799. E-mail: spuig@neptune.biochem.med.umich.edu.
their resistance to the antibiotic G418 sulfate (Geneticin; GIBCO-BRL, Rockville, Md.) (28, 38).

Sporulation and tetrad analysis. Sporulation was induced (12), and aseii were dissected with a micromanipulator (35), as previously described.

Microvinification experiments. Four consecutive microvinifications with strain T 73-6 were performed at 22°C, using 1 liter of red grape Bobal must (27). The initial yeast inoculum was 2.5 × 10⁵ cells/ml from overnight cultures. At the end of each fermentation, wine was removed and residual yeast cells were maintained for 2 weeks in the original bottles at 22°C until fresh grape must, sterilized with dimethyl dicarbonate (Velcorin; Bayer, Leverkusen, Germany), was added. Thus, material from the previous fermentation was used as inoculum for the next one. This procedure simulates the seasonal rebreeding that occurs in wine cellars. During each microvinification, samples of cells were spread on YPD and FOA plates, to determine the total number of viable and Ura⁻ homozygous cells, respectively. We used reducing sugar concentration to indicate fermentation progress.

Chromosomal DNA preparations and pulsed-field gel electrophoresis. Karyotypes were determined by the contour-clamped homogeneous electric field technique with a CHEF-DRH apparatus (Bio-Rad Laboratories, Hercules, Calif.). Chromosomal DNA was prepared in agarose plugs (7) and washed three times in TE buffer (10 mM Tris-HCl, 1 mM EDTA [pH 8.0]) at 50°C for 30 min and then twice in the same buffer at room temperature for 30 min. Plugs were loaded into 1% agarose gels in 0.5× TBE buffer (44.5 mM Tris-borate, 1.25 mM EDTA [pH 8.0]); migration was at 14°C and 6 V/cm for 13 h with 60 s between field changes, and then 9 h with 90 s between field changes.

Southern blot analysis. The chromosomal DNA separated by CHEF gel electrophoresis was transferred to nylon filters (Hybond-N; Amersham-Pharmacia Biotech, Buckinghamshire, United Kingdom) as suggested by the manufacturer. Karyotype filters were hybridized with 3²P-labeled probes corresponding to rDNA (chromosome XII), HSP42 (chromosome IV), CAR1 (chromosome XVI), YML128w (chromosome XIII), URA3 (chromosome V), CUP1 (chromosome VIII, right arm), and SNF6 (chromosome VIII, left arm) (33).

RESULTS

Characterization of T 73 wine yeast strain. Strain T 73 is approximately diploid, homothallic, and prototrophic for most common requirements (data not shown). Sixty percent of T 73 cells sporulated, and spore viability was 70% (168 out of 240). Most of the tetrads analyzed had two or three viable spores. The colony sizes (diameters) of the meiotic derivatives varied widely (between one- and fourfold), suggesting that this strain is highly heterozygous.

The CHEF gel karyotype of T 73 has 14 different bands (Fig. 1), some of which have a lower intensity, suggesting aneuploidy or the presence of homologous chromosomes of different sizes.

![FIG. 1. Electrophoretic karyotypes of wine yeast strain T 73 and two complete meiotic derivatives (2A to 2D; 3A to 3D). Putative chromosomes corresponding to every band according to the pattern obtained for laboratory strain S288c are indicated.](image)

We used two tetrads, each with four viable spores, to analyze the karyotype following meiosis (Fig. 1). Small differences were detected for chromosomes XIII and I (data not shown). More extensive changes were observed for chromosomes XII, XVI, and VIII, which are represented by two bands of different sizes that segregate 2:2 in these tetrads (Fig. 2). To demonstrate that chromosome VIII was dimorphic, with the usual band of 580 kb and a second of approximately 1,000 kb, we hybridized with probes from both arms of the chromosome with the same result. Thus, we conclude that T 73 has at least five pairs of heteromorphic chromosomes.

Genetic changes during consecutive wine fermentations. Homozygous Ura⁻ cells were generated from the URA3/ura3 heterozygote T 73-6 during consecutive microvinifications (Table 1). The relative frequency of Ura⁻ cells increases with each microvinification. A reduction in residual cells occurred between the end of one microvinification and the beginning of the following (Table 1). This fact could be explained by the lower viability of Ura⁻ cells than of Ura⁺ cells in these conditions.

We estimate that Ura⁻ cells appear at a rate of 1 × 10⁻⁵ to 3 × 10⁻⁵ cells per generation (Table 1). This result was lower in the first fermentation. In our calculations, we assume that the growth rate (fitness) is the same for both heterozygous and...
TABLE 1. Determination of the formation rate of Ura<sup>-</sup> strains during four consecutive microvinifications with T<sub>73-6</sub> strain<sup>a</sup>

<table>
<thead>
<tr>
<th>Days of fermentation</th>
<th>Reducing sugars (g/liter)</th>
<th>Total viable cells/ml (T)</th>
<th>Ura&lt;sup&gt;-&lt;/sup&gt; cells/ml (U)</th>
<th>No. of generations (n)</th>
<th>U rate</th>
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<tr>
<td>1st vinification</td>
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<tr>
<td>0</td>
<td>160</td>
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<tr>
<td>2</td>
<td>140</td>
<td>4.9 x 10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>840</td>
<td>7.6</td>
<td>0.2 x 10&lt;sup&gt;-5&lt;/sup&gt;</td>
</tr>
<tr>
<td>15</td>
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<td>1.5 x 10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>9.6</td>
<td>0.7 x 10&lt;sup&gt;-5&lt;/sup&gt;</td>
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<tr>
<td>2nd vinification</td>
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<td>170</td>
<td>3.3 x 10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>16</td>
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<td>5.0 x 10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>4,600</td>
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<sup>a</sup>The number of cell generations during the first fermentation was higher than in the second one, due to a lower inoculum. The cell densities reached in the third and fourth fermentations were lower because of the addition of SO<sub>2</sub>. To calculate the U rate (rate of Ura<sup>-</sup> formation per generation), we applied the following equation: U rate = 1 - (1 - q<sup>n</sup>)/(1 - q<sup>1</sup>), where q = U/T. This expression can be simplified to (q<sup>n</sup> - q<sup>1</sup>)/(q<sup>1</sup> - 1) in the case of q<sup>1</sup> ≪ 1.

**DISCUSSION**

**Chromosomal features of wine yeast T<sub>73</sub>.** S. cerevisiae industrial yeasts commonly are aneuploid (3, 15). In wine yeasts, strains with approximately diploid DNA contents, such as T<sub>73</sub>, are well known (11, 15, 21, 24). This result does not imply that such strains are strictly diploid. Indeed, preliminary results with the strain T<sub>73</sub> suggest that chromosome IV may be aneuploid (J. V. Gimeno-Alcañiz and E. Matallana, personal communication). Other wine strains are near diploid or triploid (3, 15). The tolerance of wine yeasts to these DNA levels suggests that meiosis is not a common occurrence in their life cycles (3).

Strain T<sub>73</sub> carries several homologous chromosomes of different sizes. Thus, this strain possesses two chromosomes XII of unequal size, probably due to differences in the number of rDNA repeats (Fig. 2A, lane 1), as has been demonstrated for other strains (9, 24, 25, 31, 32). T<sub>73</sub> also has two different-sized rDNA repeats (Fig. 2A, lane 1), as has been demonstrated for other strains (9, 24, 25, 31, 32). T<sub>73</sub> also has two different-sized rDNA repeats (Fig. 2A, lane 1), as has been demonstrated for other strains (9, 24, 25, 31, 32). T<sub>73</sub> also has two different-sized rDNA repeats (Fig. 2A, lane 1), as has been demonstrated for other strains (9, 24, 25, 31, 32).
microinfections, but we have no evidence for meiosis or sporulation. Therefore, we interpret these data to mean that mitotic gene conversion or mitotic crossing over is the most likely mechanism for their formation.

We propose a process of gradual adaptation to vinification conditions, as chromosomal rearrangements and aneuploidies acquired following numerous mitotic divisions are maintained under these conditions, as chromosomal rearrangements and aneuploidies likely mechanism for their formation.

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ACKNOWLEDGMENTS

We thank Tahía Benítez, Benjamín Piña, Emilia Matallana, and Daniel Ramón for helpful discussions and critical reading of the manuscript, and we thank P. Philippson for providing kanMX plasmids.

This work was supported by grants ALI95-0566 and ALI98-1041 (to I.E.P.-G.) from Comisión Interministerial de Ciencia y Tecnología of the Spanish Government. S.P. was a recipient of an FPI fellowship from the Ministerio de Educación y Cultura.

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