Presumed Sexual Isolation in Yeast Populations during Production of Sherrylike Wine

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The three stages in wine production in the Montilla-Moriles region of Spain are (i) fermentation, (ii) film formation in vessels, and (iii) aging, all of which selected three yeast populations. All isolates from the three stages were classified as Saccharomyces cerevisiae. The distribution of characteristics of sugar fermentation (sucrose, galactose, maltose), Li⁺ tolerance, and Cu²⁺ tolerance was different in the strains isolated in the three stages. This finding suggests that sexual isolation in the yeast populations prevents the random distribution of taxonomic characters.

In wine making, yeasts ferment the sugar of must to ethyl alcohol. In some special types of wine, film-forming yeasts ("flor yeasts") also participate in an aerobic process, the contribution of which to flavor development is not well understood (2).

In the Montilla-Moriles region (province of Córdoba, southern Spain), the production of sherrylike wine starts with a fermentation which is carried out by partial additions of new must to actively fermenting vessels (12 to 14 additions in about 15 days). Thus, in each vessel, the fermentation takes place most of the time under a high alcohol concentration. When the sugar is completely fermented, some cells rise to the surface of the liquid and form a film which can stay for several months, because the wine is left in the fermenting vessels with minor manipulations. Later, the wine is transferred from fermentation vessels to barrels, in which the wine is also covered by a characteristic film. Partial additions to and extractions from the barrels are taking place continuously through the years. This particular method of wine making in the Montilla-Moriles region gave us the opportunity to study the evolution of a yeast population when the conditions change from fermentative to aerobic growth.

MATERIALS AND METHODS

Isolation and identification. For 3 years, wine samples were taken from several wineries throughout most of the Montilla-Moriles region. Samples from three different stages, i.e., (i) alcoholic fermentation in fermentation vessels, (ii) film in fermentation vessels, and (iii) film in aging barrels, were inoculated onto yeast extract and malt extract agar (YM agar) plates (0.3% yeast extract, 0.5% peptone, 0.3% malt extract, 1% glucose [adjusted to pH 5.8 with KH₂PO₄], 2% agar) and incubated at 28°C. Cultures were maintained on YPD (1% yeast extract, 2% peptone, 2% glucose, 2% agar) agar slants. Identification tests were carried out by the method of van der Walt (5).

Li⁺ and Cu²⁺ tolerance. Li⁺ tolerance was tested by inoculating 2 × 10⁵ cells ml⁻¹ into tubes with the following medium: 0.2% yeast extract, 2% glucose, 0.5% (NH₄)₂HPO₄ (pH 5.2), with the required amount of LiCl. Growth was recorded after 15 days, but usually strains failing to grow in 7 days did not grow thereafter.

Cu²⁺ tolerance was tested on plates with the same base medium as that used for Li⁺ tolerance and 2% agar. To this medium, CuSO₄ was added in the required amount, and the pH was adjusted to 5.5. Sterilization was carried out at 115°C for 30 min.

RESULTS

Strain identification. The 1,500 isolates obtained from the three stages of wine production (i.e., fermentation, film in fermentation vessels, and film in aging barrels) were assigned to the genus Saccharomyces on the bases of vegetative reproduction by multilateral budding, spore formation (vegetative cells directly transformed into ascii, with one to four spheroidal ascospores per ascus), no nitrate assimilation, no growth in the presence of 100 ppm (100 μg/ml) of cycloheximide, and no utilization of lactose (5). A few isolates from aging film failed to sporulate, but the morphology and physiology of these isolates were identical to those of other isolates that showed normal sporulation.

According to van der Walt (5), our isolates (types I through V) could be assigned to the following five species: S. cerevisiae, S. chevalieri, S. capensis, S. aceti, and S. bayanus (Table 1), but actually, all the above-mentioned species are synonyms of S. cerevisiae (1, 6). The frequencies of each type in the isolates from the three stages of the process were constant for the 3 years during which the study was performed. Type I (S. cerevisiae) was the most abundant in fermentation, types I and II (S. cerevisiae and S. chevalieri) were the most abundant in fermentation vessel film, and type III (S. capensis) was the most abundant in aging film (Fig. 1).

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Table 1. Fermentative patterns of the isolates obtained during wine making in the Montilla-Moriles region of Spain

<table>
<thead>
<tr>
<th>Yeast type</th>
<th>Fermentation of:</th>
<th>Species*</th>
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<tbody>
<tr>
<td></td>
<td>Glucose</td>
<td>Sucrose</td>
</tr>
<tr>
<td>I</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>II</td>
<td>+</td>
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<td>III</td>
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<td>+</td>
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<tr>
<td>IV</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>V</td>
<td>+</td>
<td>+</td>
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</table>

⁴ According to reference 5.
⁵ These strains fermented trehalose and assimilated sucrose.

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Film formation. Once sugar has been fermented, the only way for yeasts to continue growing is by rising to the surface of the liquid. Tests with our isolates proved that all strains isolated from after-fermentation stages (isolated from film) formed film in test tubes with sugar-free wine, but only a small proportion (15%) of isolates from the fermentation stage was able to form film. In all cases, strains unable to form film grew in sugar-free wine under aeration. In these conditions, film-forming and non-film-forming strains did not show growth differences.

Li⁺ tolerance. By testing the growth of isolates in media with increasing Li⁺ concentrations, we found that the distribution of Li⁺ tolerance in fermentation isolates differed from the tolerance distribution in isolates from the other two stages (Fig. 2). With the exception of type IV, fermentation strains showed a broad variation in Li⁺ tolerance (0 to 145 mM Li⁺), with a small proportion of isolates unable to grow at 25 mM Li⁺. On the other hand, after-fermentation isolates showed a much narrower range of Li⁺ tolerance (0 to 85 mM Li⁺), with a large proportion of isolates unable to grow at 25 mM Li⁺. In type IV, the distribution of Li⁺ tolerance was the same in isolates from all three stages, with a large proportion of low-tolerance strains.

Cu²⁺ tolerance. In sherry yeasts, Cu²⁺ tolerance was a useful characteristic for distinguishing strains isolated from different stages of the production process (J. Conde, Cruz Campo, Sevilla; personal communication). In a sample of 440 strains, with representatives of all isolates from all stages and types, we found remarkable differences in Cu²⁺ tolerance between fermentation and after-fermentation strains. As with Li⁺ tolerance, fermentation strains varied widely in Cu²⁺ tolerance, with a large proportion of strains growing at 1,200 μM Cu²⁺ (60%), while most after-fermentation strains did not grow at 600 μM Cu²⁺ (70%). Figure 3 shows the distribution of strains able to grow at 800 μM Cu²⁺. More than 55% of the fermentation strains, of any type, grew at 800 μM Cu²⁺. In types I, II, and III, 77% of the fermentation strains grew at 800 μM Cu²⁺, while only 7% of the after-fermentation strains grew at this Cu²⁺ concentration. In types IV and V, the differences in Cu²⁺ tolerance between
fermentation and after-fermentation strains were lower than in types I, II, and III, but in any case, Cu\(^{2+}\) tolerance was higher in fermentation strains.

**DISCUSSION**

In the present work we studied three populations of wine yeasts, corresponding to the three stages of wine making in the region studied. The most important condition in the selection of these three yeast populations was the depletion of sugar and oxygen in the fermentation vessels. This condition determines that, once fermentation has finished, only strains able to rise to the surface of the liquid can continue growing. Of these strains, aging strains are the result of selection, for hundreds of years, of those better adapted to grow on the surface of wine. Film formation capacity and other characteristics which play a role in the selection of after-fermentation strains are probably unrelated to those characteristics tested in strain characterization (i.e., sugar fermentation and Li\(^+\) and Cu\(^{2+}\) tolerance). However, each of the three populations has a different pattern of frequencies of the test characteristics (Fig. 1, 2, and 3), a fact inconsistent with a common genetic background for all the strains. The only explanation for this observation is linkage between the genes involved in adaptation and those of the tested characteristics or sexual isolation of the strains, which prevents the random distribution of genes in the population. The first possibility is not the case. The gene for film formation is not linked to sucrose fermentation (4), and it is very unlikely that it will be linked to other sugar fermentation genes or to genes for quantitative characteristics such as Li\(^+\) and Cu\(^{2+}\) tolerance. Therefore, sexual isolation is a more probable explanation. Obviously, sexual isolation would take place between members of different species, but sexual isolation may also occur when sexual reproduction is uncommon. Genetic (3) and taxonomic (1) reasons do not support the existence of three species, i.e., one for each of the three populations. For this reason, it can be concluded that sexual reproduction is uncommon in wine yeasts and perhaps in other natural yeast populations. Sexual isolation between strains of *S. cerevisiae* would explain the great number of species proposed in the past and also the way simple characteristics, such as sugar fermentation, could be successfully used for the classification of apparently different species.

Sexual isolation in wine yeast strains suggests that it may be difficult to find a strain having all the characteristics required for the wine-making industry. These characteristics may exist in different strains. To obtain an industrial strain, genetic manipulation would be required.

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**LITERATURE CITED**