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Occurrence and Growth of Killer Yeasts during Wine Fermentation

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Sixteen wine fermentations were examined for the presence of killer yeasts. Killer property and sensitivity to killer action were found in isolates of *Saccharomyces cerevisiae* but not in isolates of *Kloeckera*, *Candida*, *Hansenula*, and *Torulaspora* spp. Several killer and killer-sensitive strains of *S. cerevisiae* were differentiated by colony morphology, and this property was used to monitor their growth kinetics in mixed cultures in grape juice. Killer-sensitive strains died off within 24 to 48 h during mixed-strain fermentation. Killer action was demonstrated at pH 3.0 and pH 3.5 and over the range of 15 to 25°C but depended on the proportion of killer to killer-sensitive cells at the commencement of fermentation. The dominance of killer strains in mixed-strain fermentations was reflected in the production of ethanol, acetic acid, and glycerol.

Certain strains of yeasts, termed killer yeasts, produce an extracellular toxin which is lethal to other strains of yeasts, termed killer-sensitive yeasts. Generally, killer interactions are restricted between strains of species within one genus, but reactions between species of different genera have been reported (4, 17, 20, 28). Some strains of *Saccharomyces cerevisiae* isolated from the well-established vineyards and wineries of European countries, as well as Russia, have exhibited the killer property and have stimulated enological interest in the significance of this property (3, 9, 16, 18, 21, 23). There are no reported studies on the occurrence of killer strains of *S. cerevisiae* in the newly developed wine regions of the United States and Australia, where a more technological approach to winemaking has been adopted.

The European studies have not provided conclusive information about the natural occurrence and growth of killer strains of *S. cerevisiae* during wine fermentation and have suggested that the killer property, although possessed by some strains, may not be expressed consistently under the conditions of wine fermentation. Killer strains of *S. cerevisiae* have been dominant at the end of fermentation in some wineries but not in others (5, 16). Consequently, the enological significance of killer yeasts remains unclear and provides the basis for further research that aims to understand those factors which govern the occurrence and behavior of these yeasts during wine fermentation. Additional impetus for such research is provided by the desire to genetically engineer specific killer strains of *S. cerevisiae* for potential use as commercial starter cultures in wine fermentation (10, 11, 14, 26, 27). It is considered that such strains could be used to prevent the growth of wild strains of *S. cerevisiae* and other closely related *Saccharomyces* spp. during and after alcoholic fermentation, thereby enabling better control over the development of unwanted wine characteristics such as excessive amounts of hydrogen sulfide, volatile acidity, and other off-flavors (G. K. Jacobsen, Eastern Grape Grower and Winery News, August/September, p. 29–31, 1985).

Progress in studying the quantitative interaction between killer and killer-sensitive strains of *S. cerevisiae* during wine fermentation has been limited by the lack of methods that permit easy recognition and differentiation of the two types of strains when grown in mixed cultures. Some researchers have approached this problem by choosing killer and killer-sensitive strains that can be distinguished by their different growth rates (3) or by their ability to produce hydrogen sulfide (24). Others (10, 11, 14, 26) have used auxotrophic and respiratory-deficient mutants of killer strains and appropriate plating media to differentiate them from killer-sensitive strains. Although these experimental strategies have proved useful, they do not provide a direct approach for quantitatively measuring the interactions between the two types of strains.

In this paper, we have noted subtle, reproducible differences in the colony morphology of several killer and killer-sensitive strains of *S. cerevisiae* and have exploited this property to quantitatively monitor the interactions of these strains during wine fermentation. In addition, we report information on the occurrence of the killer property among yeasts isolated from Australian wineries.

MATERIALS AND METHODS

Yeast strains. The yeast strains used in this study were obtained from the departmental culture collection. Strains of *Kloeckera apiculata*, *Candida* spp., *Hansenula anomala*, and *Torulaspora delbrueckii* were isolated from several Australian wineries, as were the majority of strains of *S. cerevisiae* (12, 13). Several other strains of *S. cerevisiae* were isolated from wineries in Germany (nine strains) and France (nine strains) (7, 8). The commercial strains *S. cerevisiae* (*S. bayanus*) EC-1118 and SB-1 were obtained from Lalvin Lallemond (Montreal, Quebec, Canada).

Screening of strains for killer activity and killer sensitivity. The seeded-agar-plate technique was used with malt extract agar (MEA) (Oxoid Ltd., London, England) buffered at pH 4.5 with 0.5 M phosphate-citrate buffer (23). Approximately 10^5 cells of the sensitive strain per ml were suspended in 15 ml of presterilized, buffered MEA (45°C), and the suspension was poured into a sterile petri dish. The killer strain was streak inoculated onto the surface of the seeded agar, which was then incubated at 20°C for 48 h. Killer activity was recognized by the inhibition of growth (zones of clearing) of the seeded strain surrounding the growth of the killer strain.

Wine fermentations and yeast enumeration. Juice was extracted from Semillon grapes, sterilized by filtration through 0.45-μm-pore membranes, pH adjusted to 3.5, and suspended in 300-ml volumes in sterile flasks. The juice was inoculated with approximately 10^5 cells of the yeast strains

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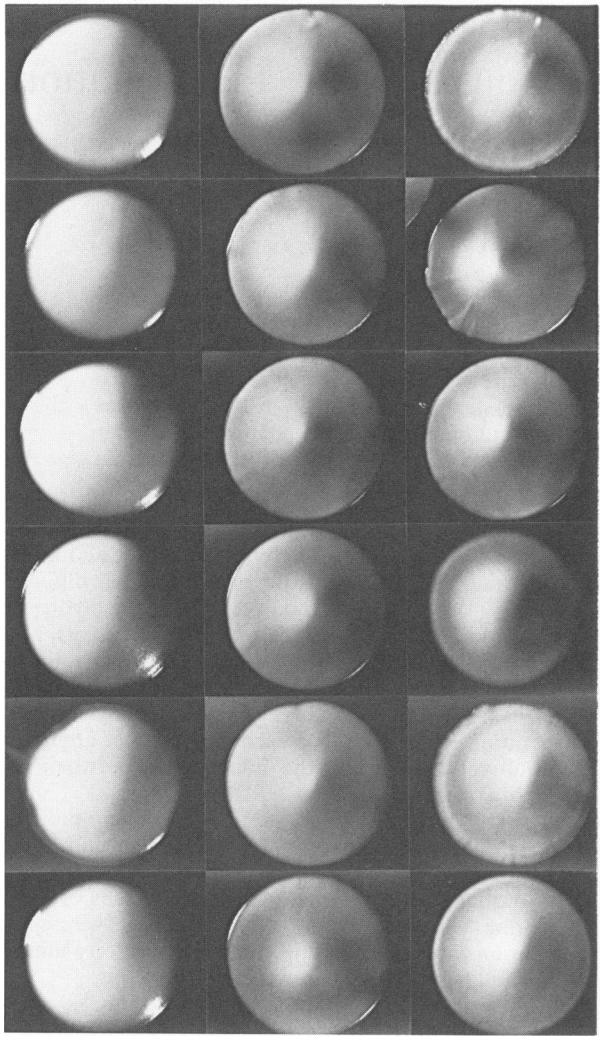

FIG. 1. Morphological appearance of colonies of killer strains (K) and killer-sensitive strains (S) of *S. cerevisiae* on MEA after incubation at 20°C for 4, 12, and 16 days (left to right). From top to bottom the strains are K_A, S_A, K_B, S_B, K_C, and S_C. Colony color at 16 days varied from white through cream to brown.

to be tested per ml, and fermentations were conducted at 20°C. Samples of the fermenting juice were removed daily and spread inoculated onto plates of MEA for the determination of yeast counts. The plates were incubated at 20°C and examined after 4, 12, and 16 days for colony morphology and counts of each morphological type.

Chemical analyses. Acetic acid was measured by high-performance liquid chromatography as reported previously (6). Glycerol and ethanol concentrations were determined by using enzymatic kits (Boehringer GmbH, Mannheim, Federal Republic of Germany).

RESULTS AND DISCUSSION

Occurrence of killer and killer-sensitive properties in wine yeasts. The presence of killer activity and sensitivity to killer action was examined in 61 strains of *S. cerevisiae*, 10 strains of *K. apiculata*, 8 strains of *C. colliculosa*, 5 strains of *C. krusei*, 5 strains of *C. stellata*, 3 strains of *C. valida*, 2 strains of *C. famata*, 2 strains of *C. sake*, and 1 strain each of *C. pulcherrima*, *H. anomala*, and *T. delbrueckii*. The non-*Saccharomyces* yeasts were examined because they are important wild or indigenous species that grow during the early stages of many wine fermentations (8, 12, 13). Each strain was tested for killer activity against all other strains, and each strain was tested for killer sensitivity against all other strains. Killer activity and sensitivity to killer action were not observed in any of the non-*Saccharomyces* yeasts, although other researchers have noted positive results for these properties in some species. Some strains of *H. anomala* are able to kill *S. cerevisiae* (19, 25). Such strains would be undesirable during wine fermentation, as they have the potential to destroy *S. cerevisiae* and arrest the fermentation. However, strains of *H. anomala* that are sensitive to the killer toxins of *S. cerevisiae* have not been reported (25). Some strains of *C. colliculosa* appear to be sensitive to the killer toxins of *S. cerevisiae* (27); this is enologically significant, since this species frequently occurs during the early stages of wine fermentation (12, 13). Consistent with our data, other studies have shown that strains of *K. apiculata* and *C. stellata* do not produce killer toxins and are not sensitive to killer toxins produced by other yeasts (23). *Hanseniaspora uvarum*, the sporogenous form of *K. apiculata*, has been reported to produce killer toxins (22). It may be concluded from existing data that there is considerable heterogeneity among non-*Saccharomyces* wine yeasts with respect to killer activity and sensitivity to killer action and that further study of a greater number of strains is still needed.

A total of 9 killer and 15 killer-sensitive strains were identified among the isolates of *S. cerevisiae* which were obtained from four different Australian wineries (12, 13). Both types of strains were obtained from red and white wines and from the early and late stages of alcoholic fermentation. Of the 16 wine fermentations examined, 3 showed the presence of both killer and killer-sensitive strains. These findings are consistent with those reported elsewhere (1, 2, 5) and confirm the natural occurrence of both killer and killer-sensitive strains of *S. cerevisiae* during wine fermentation.

A further 3 killer strains and 16 killer-sensitive strains of *S. cerevisiae* were detected among isolates from French (8) and German (7) wines. In addition, the commercial strains *S. cerevisiae* EC-1118 and SB-1 were confirmed to be killer and killer sensitive, respectively.

The killer and killer-sensitive strains of *S. cerevisiae* examined in this study were indistinguishable by standard morphological and biochemical tests (15). However, it was noted that some strains exhibited distinct differences in colony topography and color on MEA plates when the incubation period at 20°C was extended beyond the usual 4 to 7 days. Such differences were very apparent after 12 days and were reproducible upon continued subculturing of the strains. On the basis of these properties, we were able to consistently distinguish between three killer (K_A, K_B, and K_C) and three killer-sensitive (S_A, S_B, and S_C) strains of *S. cerevisiae* (Fig. 1). Strains K_A and S_B were isolated from Australian wines, strains K_C and S_A were obtained from German wines, and strains K_B and S_C were commercial strains from Lalvin. However, it was not possible to use colony characteristics as a criterion to distinguish between all isolates of killer and killer-sensitive strains.

Expression of the killer effect during wine fermentation. Colony morphology was used to differentiate between killer and killer-sensitive strains of *S. cerevisiae* during controlled fermentations of grape juice. Fig. 2a to f shows the growth of

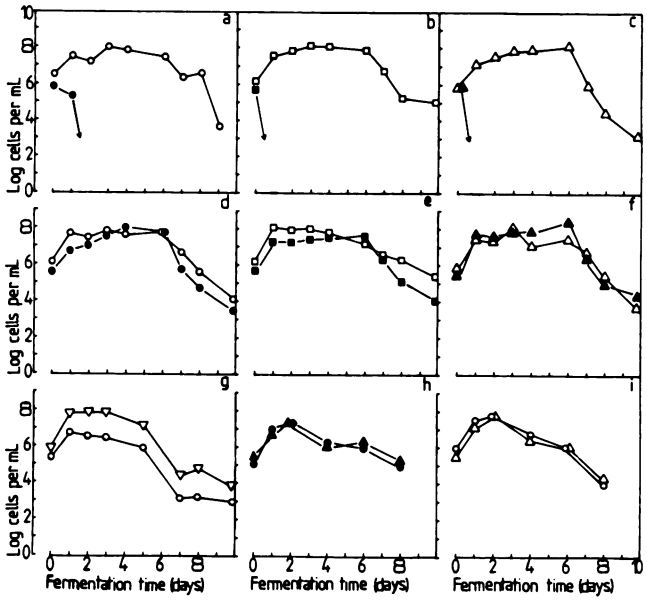

FIG. 2. Growth of killer, killer-sensitive, and neutral strains of *S. cerevisiae* during fermentation of grape juice at pH 3.5 and 20°C. (a, b, and c) Mixed cultures of killer and sensitive strains; (d, e, and f) separate cultures of killer and sensitive strains (data for each strain shown in the subfigure); (g) mixed culture of a killer strain and a neutral strain; (h) mixed culture of two sensitive strains; (i) mixed culture of two killer strains. Symbols: ○, K_A; □, K_B; △, K_C; ●, S_A; ■, S_B; ▲, S_C; ▽, neutral.

killer and killer-sensitive strains of *S. cerevisiae* in grape juice inoculated with a mixture of the two types of strains and in grape juice inoculated singly with each type of strain. Killer action was evident in the mixed-strain fermentations (Fig. 2a, b, and c), as indicated by the rapid disappearance of the sensitive strains and the dominance of the killer strains. The sensitive strains exhibited normal growth kinetics when cultured in the absence of the killer strains (Fig. 2d, e, and f). In control experiments (Fig. 2g, h, and i), the killer effect was not observed when the fermentation was conducted with (i) a mixture of a killer strain and a neutral strain, (ii) a mixture of two sensitive strains, or (iii) a mixture of two killer strains. In addition, killer strains of *S. cerevisiae* did not affect the growth of *K. apiculata*, *C. krusei*, *C. pulcherrima*, or *H. anomala* in mixed cultures in grape juice. This result confirms the specificity of the killer action and eliminates the possibility of using killer strains of *S. cerevisiae* to control the growth of indigenous non-*Saccharomyces* yeasts during the early stages of wine fermentation.

Thus, by using colony morphology to differentiate strains, we have been able to demonstrate more directly than previously (3, 10, 11, 24, 26) that the killer property is very effectively expressed under the conditions of wine fermentation. Most notably, the populations of killer-sensitive cells were reduced from initial values of 10^6 cells per ml to nondetectable values ($<10^3$ to $<10^4$ cells per ml) within 24 to 48 h.

The dominance of the killer strains was also evident after measurement of the concentrations of some key products at the end of fermentation (8 days; Fig. 2). Juice fermented by K_C alone contained 101 g of ethanol, 0.42 g of acetic acid, and 4.24 g of glycerol per liter, whereas juice fermented by S_C alone contained 128 g of ethanol, 0.47 g of acetic acid, and 4.84 g of glycerol per liter. Juice fermented by a mixture of K_C and S_C exhibited properties similar to that fermented by K_C alone, namely, 105 g of ethanol, 0.41 g of acetic acid, and 4.17 g of glycerol per liter. Strains K_B and S_B alone produced fermented juices with similar concentrations of those key products and, consequently, it was not possible to see differences in mixed fermentations. This was the case also for strains K_A and S_A, except for the production of acetic acid. The concentrations of acetic acid produced in single-strain fermentations by K_A and S_A were, respectively, 0.43 and 0.61 g/liter. In mixed-strain fermentations the final concentration of acetic acid was that produced by killer strain K_A alone, namely, 0.43 g/liter.

Factors affecting killer activity during wine fermentation. The pH and temperature of wine fermentation may affect the rate of growth of killer cells in relation to sensitive cells as well as the production and activity of killer toxin. Fig. 3a and b show the action of killer strain K_A on sensitive strain S_A at pH 3.0 and at temperatures of 15 and 25°C. These data can be compared with those in Fig. 2a, in which fermentation was conducted at pH 3.5 and 20°C. Killer strain K_A was active over the range of 15 to 25°C and at pHs 3.0 and 3.5, conditions applicable to most white wine fermentations. The slower effect of killer strain K_A at 15°C probably reflects the slower growth of the strain at this lower temperature. Other studies have shown that the killer toxins of several wine strains of *S. cerevisiae* are active over a pH range of 3.5 to 4.5 and at temperatures under 30°C (20, 26), but they have not emphasized the effect of these parameters on the growth rates of the killer strains. Assuming that toxin production correlates with growth, a fermentation condition that decreases the rate of growth of a killer strain in comparison to a sensitive strain will, of course, reflect itself in decreased killer action.

The killer effect was not observed when the proportion of killer to killer-sensitive cells at the commencement of fermentation was 1:10 (Fig. 3c). In another experiment we did not observe any killer effect when the proportion of killer to killer-sensitive cells was approximately 1:7. The data in Fig.

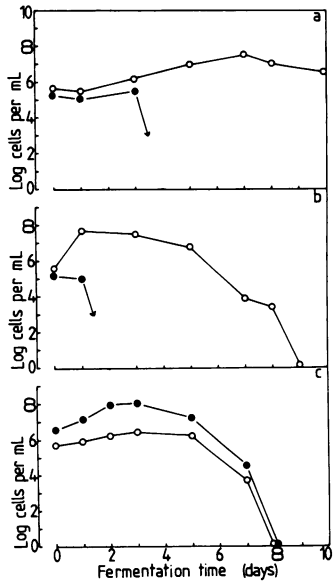

FIG. 3. Mixed cultures of killer and killer-sensitive strains of *S. cerevisiae* during grape juice fermentation at pH 3.0 and 15°C (a), pH 3.0 and 25°C (b), and with a killer/killer-sensitive inoculum ratio of 1:10 at pH 3.5 and 20°C. Symbols: ○, K_A; ●, S_A.

2a, b, and c, in which pronounced killer action was observed, were obtained when the killer and killer-sensitive cells were inoculated in approximately equal proportions (1:1). Rosini (24) has reported similar results, but it is noteworthy that the killer effects reported by Shimizu et al. (27) and Seki et al. (26) were obtained with very high killer cell/sensitive cell ratios of approximately 100:1 and 25:1, respectively. Nevertheless, others have observed effective killing action with killer cell/sensitive cell ratios of 1:10 and lower (3, 10, 11). Thus, there appears to be considerable heterogeneity between strains in the extent to which they produce killer toxins and perhaps in the specific activities of these toxins. These properties as well as the proportion of killer cells needed for effective killing action require further study because of their economic significance at the practical level of winemaking.

The concept of killer yeasts is relatively new to the wine industry, and research into the possibility of exploiting these yeasts during commercial wine fermentation is still at an early stage of development (26; Jacobsen, Eastern Grape Grower and Winery News, August/September, p. 29–31, 1985). In this study, we have shown how direct morphological observation can be used to differentiate between several killer and killer-sensitive strains of *S. cerevisiae* and to monitor the growth of the two types of strains during a laboratory-scale wine fermentation. This relatively simple procedure could form the basis of further fundamental research that is needed to understand the behavior of these yeasts under the diverse conditions of commercial winemaking.

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