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Determination of Killer Yeast Activity in Fermenting Grape Juice by Using a Marked *Saccharomyces* Wine Yeast Strain

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The *Escherichia coli* β-glucuronidase gene has been used as a marker gene to monitor a killer *Saccharomyces cerevisiae* strain in mixed-culture ferments. The marked killer strain was cured of its M-dsRNA genome to enable direct assessment of the efficiency of killer toxin under fermentation conditions. Killer activity was clearly evident in fermenting Rhine Riesling grape juice of pH 3.1 at 18°C, but the extent of killing depended on the proportion of killer to sensitive cells at the time of inoculation. Killer activity was detected only when the ratio of killer to sensitive cells exceeded 1:2. At the highest ratio of killer to sensitive cells tested (2:1), complete elimination of sensitive cells was not achieved.

Killer activity in yeasts was first reported for strains of *Saccharomyces cerevisiae* in 1963 by Bevan and Makower (2). Killer yeasts secrete polypeptide toxins which kill sensitive strains of the same genus and, less frequently, strains of different genera (20, 30). Previous studies indicate that the toxin of *Saccharomyces cerevisiae* is a protein which binds to a receptor on the wall of the sensitive yeast cell, disrupting the electrochemical gradient across the cell membrane and hence the intracellular ionic balance (6, 28).

Production of the toxin and immunity to it are determined by a cytoplasmically inherited double-stranded (ds) RNA plasmid, otherwise known as the M genome (4). The M-dsRNA killer plasmids are dependent satellites of L-A-dsRNA, and L-BC-dsRNA exists as a species unrelated to the first or to M. All types of dsRNA exist in virus-like particles and require a protein encoded by the L-A-dsRNA for encapsidation (4, 12, 30).

On the basis of the properties of the toxin, killer yeasts have been classified into 11 groups (K_1 through K_{11}) (18, 33). Those unique to *Saccharomyces* strains fall into the first three groups (K_1, K_2, and K_3). The *Saccharomyces* toxin is reversibly inactivated at low pH (2.0) and irreversibly inactivated at pH in excess of 5.0 (33). More specifically, the biological activity of K_1 is optimal between pH 4.6 and 4.8, while K_2 shows optimal activity between pH 4.2 and 4.7 (27). The K_2 toxin is stable over a wider pH range than the K_1 toxin (2.8 to 4.8) (23) and is therefore more relevant in wine fermentation.

Killer activity has been detected in yeasts isolated from established vineyards and wineries in various regions of the world, including Europe and Russia (1, 9, 18), South Africa (31), and Australia (14, 15). This widespread occurrence has prompted interest in the enological significance of killer wine yeasts. In theory, selected killer yeast strains could be used as the inoculated strain to suppress the growth of undesirable wild strains of *S. cerevisiae* during grape juice fermentation. In addition, as killer interactions have been reported to occur between yeasts of different genera (21, 25), the possibility of genetically engineering broad-spectrum killer strains of *S. cerevisiae* exists (3).

Studies have been conducted to assess the efficiency of killer toxin on sensitive yeast strains. However, reports on the expression of killer activity under fermentation conditions have been contradictory (5, 7, 16). Attempts to determine the population kinetics of killer and sensitive strains during wine fermentation have been restricted because of the difficulty involved in identifying the two types when they are grown in mixed cultures. Approaches used to date include (i) choice of killer and sensitive strains that can be distinguished by their growth rates (1) or their production of hydrogen sulfide (24), (ii) use of auxotrophic and respiratory-deficient mutants of killer strains and appropriate plating conditions under which they can be identified (10, 11, 26), (iii) use of killer and sensitive strains which can be distinguished by differences in colony morphology (14), and (iv) assaying colonies directly for killer activity (17). All of these methods are limited by the fact that the assays involved are laborious and time-consuming or that only killer strains with specific characteristics can be studied.

We describe here the use of a marked *S. cerevisiae* killer strain in a mixed-culture inoculum to quantify directly the effect of killer toxin on a sensitive *S. cerevisiae* strain under fermentation conditions. As a wide range of yeast strains can be readily and stably marked, this system of analysis is unlimited in application and provides a simple and unequivocal means of quantifying killer yeast strains in mixed-culture ferments.

MATERIALS AND METHODS

Strains and media. Sensitive *S. cerevisiae* strains 5A (AWRI 138) and 2A (AWRI 729) and killer (K_2) strain 11A (AWRI 92F) were obtained from the Australian Wine Research Institute. Generation of the marked killer (K_2) strain 3AM (AWRI 796) has been previously described (19). Yeast growth medium was YPD (1% yeast extract [Difco], 2% Bacto Peptone [Difco] and 2% glucose).

Curing of killer strain 3AM. A culture of strain 3AM was grown overnight in YPD at 28°C. Serial dilutions were made in 0.9% NaCl, and 0.1-ml aliquots (containing approximately 100 cells) were spread on YPD plates and incubated at 37°C.

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After 48 h of incubation, single colonies were selected at random and assayed for killer activity as described below.

Assay for cured strain. YPD (containing 1% agar) was autoclaved at 120°C for 20 min. After cooling to 49°C, the medium was buffered to pH 4.2 with a 10% tartrate solution. Methylene blue (to 0.003% [wt/vol]) and killer-sensitive strain 5A (to 10^5 cells per ml) were added to the medium before the medium was poured into the plates. Colonies isolated after heat treatment were then transferred to these assay plates and incubated at 18°C for approximately 72 h. Curing was recognized by the absence of growth inhibition (clear zones) and the lack of blue-stained cells around the colony.

dsRNA isolation. The dsRNA extraction procedure was essentially that described by Fried and Fink (8). Samples of RNA were analyzed by electrophoresis on 1.5% agarose slab gels at a constant current of 100 mA. Gels were stained with ethidium bromide and photographed on a shortwave UV light box.

Fermentation trials. Starter cultures were prepared by inoculating 10 ml of YPD medium contained in a conical flask with a loopful of yeast and incubated with vigorous aeration at 28°C. After 24 h, the cell density was determined by microscopic counts. Samples were used to inoculate Rhine Riesling must (200 ml) to a density of 5×10^6 cells per ml. The must contained 220 g of reducing sugars per liter and had a pH of 3.1. Fermentations were carried out in 250-ml conical flasks fitted with airlocks. The juice was sterilized by membrane filtration (0.45-μM pore size) prior to inoculation, and fermentations were carried out at 18°C with agitation (approximately 100 oscillations per min). Samples were removed anaerobically and aseptically during fermentation by needle and syringe through ports covered with rubber septa.

Samples were analyzed for the progress of fermentation by refractometer readings, and yeast growth was measured spectrophotometrically at 650 nm. For analysis of the proportion of marked strain in the yeast population, serial dilutions of the samples were made in sterile 0.9% NaCl, and 0.1-ml aliquots (containing 200 to 500 cells) were plated on YPD media. The plates were then assayed as described below.

β-Glucuronidase (GUS) plate assays. Yeast colonies were grown on solid YPD medium for approximately 36 h at 28°C. A solution containing 0.1 M Na_2HPO_4 (pH 7.0), 1% sarcosyl, 5-bromo-4-chloro-3-indolyl glucuronide (100 to 150 μg/ml), and 0.7% agarose was then poured as a thin overlay on the plate and allowed to set. After 4 to 6 h of incubation at 37°C, a blue precipitate could be detected in the marked colonies.

RESULTS

Curing of strain 3AM. In order to specifically analyze the effect of killer toxin in fermentations, an experiment was designed to compare two isogenic strains which differ only in the presence of the M-dsRNA genome and therefore in their ability to produce killer toxin.

Killer strain 3AM has previously been marked with the *Escherichia coli* GUS gene (19). This system allows the marked strain to be readily identified in a mixed population by a simple plate assay, which results in the formation of a blue precipitate in marked colonies. Strain 3AM was cured of its M-dsRNA plasmid by heat treatment (32), the cured or sensitive colonies being identified by killer activity plate assays. Figure 1 shows the response of strain 3AM and an isolated cured derivative (designated 3AMC) to the killer activity plate assay. The zone of inhibition evident around strain 3AM is absent around 3AMC, indicating that strain 3AMC is not producing killer toxin. GUS activity was detected in strain 3AMC by the agar plate method (results not shown), indicating that the cured strain is an authentic derivative of strain 3AM.

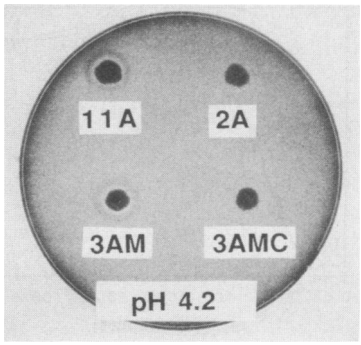

FIG. 1. Agar plate assay for killer activity. The agar (pH 4.2, 0.003% methylene blue) is seeded with an overnight culture of strain 3AMC, and strains to be tested for killer activity are patched onto the solid media. 11A is a known killer strain, and 2A is a known sensitive strain. Strain 3AM displays a response identical to that of killer 11A, with a clear zone and methylene blue-stained border around the patch of growth.

Finally, dsRNA species were isolated from strains 3AM and 3AMC and analyzed by standard electrophoresis techniques (Fig. 2). A band representing the M-dsRNA genome is present in strain 3AM and absent in strain 3AMC.

Fermentation trials were then performed with strains 3AM and 3AMC to determine the effect of the curing procedure on yeast growth and fermentation rates. Starter cultures of each strain were inoculated in triplicate into flasks of Rhine

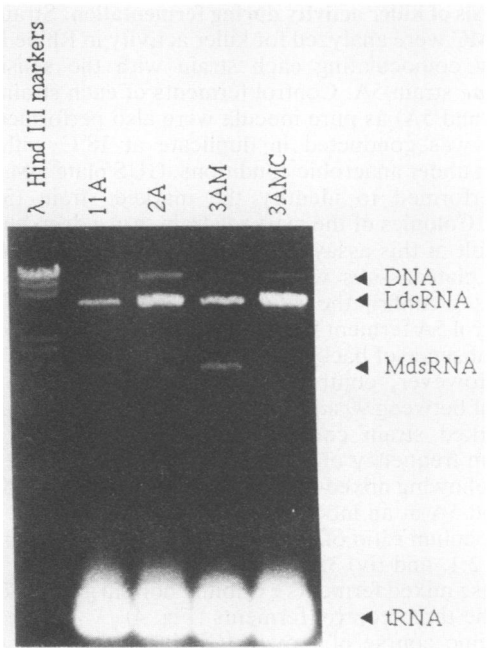

FIG. 2. Electrophoresis of dsRNA species from killer strains 11A and 3AM and sensitive strains 2A and 3AMC. Contaminating DNA and tRNA species are also present.

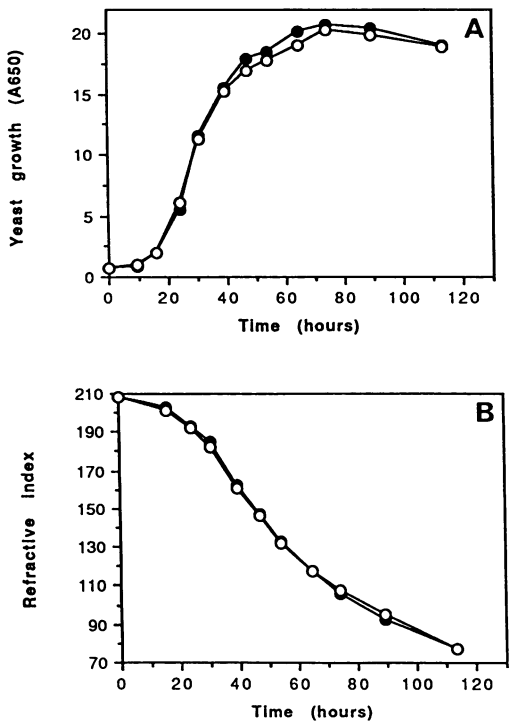

FIG. 3. Yeast growth (A) and sugar utilization (B) curves of strains 3AM (●) and 3AMC (○).

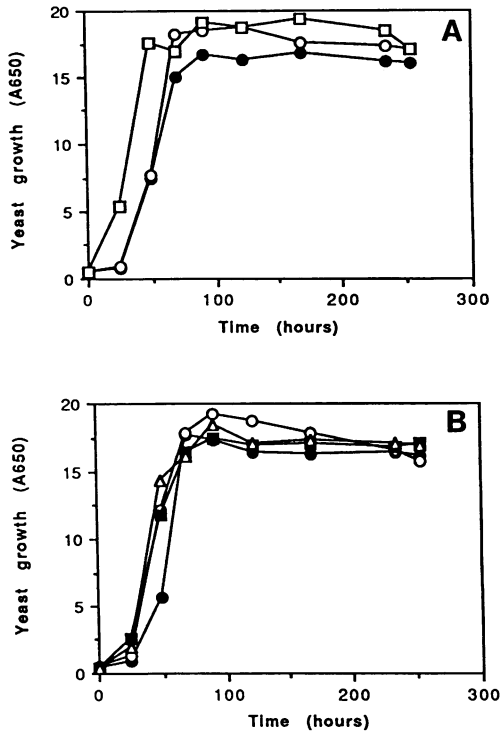

FIG. 4. (A) Growth curves of control single monoculture ferments. Symbols: ●, 3AM; ○, 3AMC; □, 5A. (B) Growth curves of mixed-culture ferments. Symbols: ●, 3AM and 5A at an inoculum ratio of 2:1; ○, 3AMC and 5A at an inoculum ratio of 2:1; ■, 3AM and 5A at an inoculum ratio of 1:1; △, 3AMC and 5A at an inoculum ratio of 1:1.

Riesling grape juice at a concentration of 5×10^6 cells per ml. Samples were taken at regular intervals and assayed for yeast growth and progress of fermentation. The average readings for each strain were plotted over time (Fig. 3). There are no significant differences in the growth or fermentation rates between strains 3AM and 3AMC.

Analysis of killer activity during fermentation. Strains 3AM and 3AMC were analyzed for killer activity in Rhine Riesling juice by coinoculating each strain with the sensitive *S. cerevisiae* strain 5A. Control ferments of each strain (3AM, 3AMC, and 5A) as pure inocula were also performed. Each ferment was conducted in duplicate at 18°C with gentle agitation under anaerobic conditions. GUS plate assays were then performed to identify the marked strain (3AM or 3AMC). Colonies of the marked strain turn a deep blue color as a result of this assay, allowing simple identification.

GUS plate assays were also performed on the control ferments to confirm the validity of the assay. Plate assays on the control 5A ferment were consistently negative, highlighting the absence of background GUS activity in natural yeast cells. However, control 3AM and 3AMC ferments gave values of between 99 and 100% of total colonies per plate for the marked strain count. This observation represents a reversion frequency of less than 1% for the GUS gene.

The following mixed-culture ferments were carried out: (i) 3AM and 5A at an inoculum ratio of 1:1, (ii) 3AMC and 5A at an inoculum ratio of 1:1, (iii) 3AM and 5A at an inoculum ratio of 2:1, and (iv) 3AMC and 5A at an inoculum ratio of 2:1. These mixed ferments exhibited normal growth kinetics, as did the three control ferments (Fig. 4).

The time course of growth (CFU per milliliter) of each strain in the mixed-culture ferments is plotted in Fig. 5. At an inoculum ratio of 1:1, there was a notable increase in the proportion of killer strain 3AM, whereas the cured strain 3AMC failed to exert any dominance over the sensitive strain under otherwise identical conditions. Statistical analysis was used to test the null hypothesis that the ratio of killer to sensitive cells remains 1:1 throughout the ferment. A goodness of fit test (normal test) rejected the null hypothesis, with $P \ll 0.001$. However, identical analysis of the cured to sensitive strain ferment ratio accepted the null hypothesis that the ratio of the two strains remains at 1:1 throughout the ferment. With an increased proportion of strain 3AM in the inoculum (ratio 2:1), the dominating effect of strain 3AM was more pronounced. It is important to note that strain 5A persisted, albeit at low levels, throughout the ferments.

Experiments were conducted to determine the lowest inoculum ratio of killer to sensitive cells at which significant killer activity can be observed. Mixed ferments of strain 3AM and 5A at inoculum ratios of 1:2 and 1:4, respectively, were carried out under the conditions described above. No change from the initial proportion of strain 3AM was detected in either of these ferments. The results of all mixed-culture ferments involving strain 3AM are summarized in Fig. 6.

DISCUSSION

Previous studies have indicated 100% stability of the GUS marker gene in strain 3A throughout fermentation (19). However, analysis of a larger sample of colonies in these experiments has revealed an instability of the construct. This instability was detected in the control fermentations which were inoculated with monocultures of either the marked

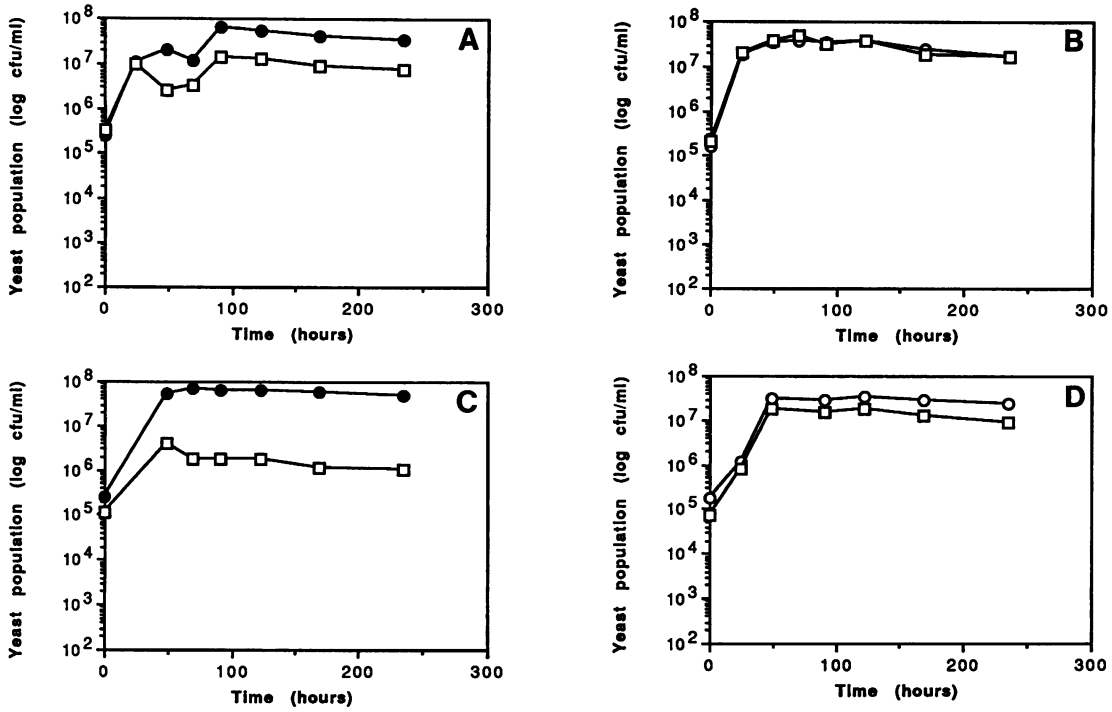

FIG. 5. Growth curves of each strain in mixed-culture ferments expressed as CFU per milliliter. (A) Mixed ferment of 3AM (●) and 5A (□) at an inoculum ratio of 1:1. (B) Mixed ferment of 3AMC (○) and 5A (□) at an inoculum ratio of 1:1. (C) Mixed ferment of 3AM (●) and 5A (□) at an inoculum ratio of 2:1. (D) Mixed ferment of 3AMC (○) and 5A (□) at an inoculum ratio of 2:1.

strain 3AM or 3AMC. Samples from these fermentations gave rise to colonies which responded negatively to the GUS plate assay at a frequency of less than 1% of the total plate count. Occasionally, a colony which was sectored in its response to the assay was detected, suggesting either excision of the gene by homologous recombination (29) or loss of the gene after mitotic crossing-over (22). The frequency of instability did not increase over time during fermentation and could be directly quantified in the control 3AM and 3AMC ferments.

This marking system has enabled a direct comparison to be made between the inoculation efficiency of a killer strain (3AM) and an isogenic cured derivative (strain 3AMC) in fermenting grape juice. At a ratio of killer to sensitive cells of 1:1, the cured strain, 3AMC, remained at 50% of the total population, while the killer strain increased to 80%. The

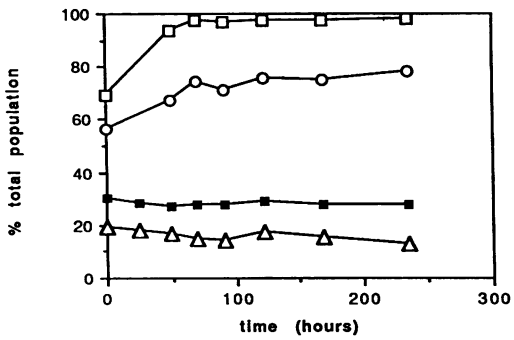

FIG. 6. Time course in the proportion of killer strain 3AM to strain 5A in the total population of a mixed-culture ferment for inoculum ratios of 2:1 (□), 1:1 (○), 1:2 (■), and 1:4 (△).

ability of strain 3AM to dominate strain 5A during fermentation is likely to be due to the production of killer toxin by strain 3AM and not to a difference in respective growth rates favoring the killer strain. We can conclude, therefore, that the killer toxin has displayed significant activity under these fermentation conditions. This result is of particular interest to the enologist, since the K_2 toxin produced by strain 3A is reported to show maximum activity at pH 4.2 (23), which is 0.5 to 1 pH unit higher than generally found in grape musts.

In cases in which killer activity in fermenting grape juice has been reported, a discrepancy as to whether effective killing action occurs when the proportion of killer cells is less than 50% of a mixed-culture ferment exists. Heard and Fleet (14) did not observe killer action when the ratio of killer to sensitive cells was approximately 1:7, whereas others have reported killer activity with killer-to-sensitive-cell ratios of 1:10 and lower (1, 10, 11). Our results showed that an increase in the ratio of killer to sensitive cells to approximately 2:1 resulted in a pronounced dominance of the fermentation by strain 3AM to 97% of the total mixed population by the end of the fermentation. However, with killer-to-sensitive-cell ratios of 1:2 or 1:4, no effective killer action was evident. It is possible that differences in either composition of medium, fermentation conditions, or strain sensitivity may account for discrepancies in reports of killer toxin efficiency.

The relevance of killer strains in wine making has been the focus of attention in countries where selected yeast cultures are inoculated into musts to induce fermentation. This focus has intensified since the observation that yeasts which are naturally present in the must also play significant roles in supposedly "pure" culture fermentations (13, 16). These natural yeasts include species from the genera *Kloeckera*, *Candida*, *Hansenula*, and *Saccharomyces*. Killer *Saccharo-*

myces wine yeast strains may be effective in suppressing natural *Saccharomyces* yeast strains during fermentation, and the possibility of engineering broad-range killer yeast strains to control strains from other genera exists. For these reasons, further study is needed to determine appropriate fermentation conditions for effective killer activity.

The GUS marking system provides a method which allows a broad range of killer strains to be rapidly and unequivocally identified in a mixed culture. This system can be employed to gain a better understanding of killer activity during fermentation.

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