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Construction of Killer Wine Yeast Strain

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A double-stranded RNA plasmid which confers the superkiller phenotype was transferred into a wine yeast (Montrachet strain 522) and its leucine-requiring derivative (strain 694) by cytoduction, using the protoplast fusion technique. The killer wine yeast constructed completely suppressed the growth of killer-sensitive strains of *Saccharomyces cerevisiae* in yeast extract-peptone-glucose medium at pH 4.5, whereas the killer effect was somewhat decreased at pH 3.5. The wine yeast harboring the killer factor also inhibited the growth of killer-sensitive cells satisfactorily when it was grown in grape juice.

The killer strain of *Saccharomyces cerevisiae* secretes a protein toxin that kills sensitive strains (15). Bevan et al. (3) and Vodkin et al. (14) have shown that toxin production and the resistant trait are encoded in a 1.5×10^6-dalton double-stranded RNA (dsRNA) plasmid.

Often in the fermentation of alcoholic beverages, a starter culture of a specific strain is inoculated to reduce fermentation time, prevent contamination, and assure the unique quality of product. Undesirable strains of yeast sometimes spoil the fermentation, since fermentations are often carried out in an open system without sterilization. In particular, contamination by a killer yeast can be a serious problem if the starter strain of wine yeast is sensitive to the killer toxin.

To prevent contamination by undesirable yeast strains, Ouchi and Akiyama (7) constructed a killer yeast suitable for saké brewing by introducing a killer plasmid, using cytoduction and the repeated breeding method. Ouchi et al. (9) used a nuclear fusion-defective mutant, *kar1-1* (5), as the donor strain of the killer plasmid and constructed a killer yeast for saké brewing without using nuclear fusion.

The *kar1-1* mutant can form zygotes normally, but is defective in nuclear fusion during mating. The zygotes, including at least one *kar1-1* mutant, frequently segregate heteroplasmons or cytoductants having one of the haploid parental nuclei. Therefore, it is possible to transfer killer plasmids from one strain to another by cytoduction without changing the nuclear genotype. Industrial wine yeast strains have many important biochemical characteristics as nuclear genotypes, and thus it is highly undesirable to change their nuclear genotypes.

In this study, we transferred a superkiller plasmid into a wine yeast strain (the Montrachet strain) by using the protoplast fusion technique and a *kar1-1* mutant as the killer plasmid donor and tested the effect of the killer plasmid on the growth of killer-sensitive cells by using a mixed culture under wine fermentation conditions.

MATERIALS AND METHODS

Strains and media. The *S. cerevisiae* strains used in this study are listed in Table 1. A medium containing 1% yeast extract (Difco Laboratories, Detroit, Mich.), 2% peptone (Difco), and 2% glucose (YEPD medium) was used as a complete medium. Yeast nitrogen base (without amino acids; Difco) (0.67%) supplemented with 2% glucose (glucose minimal medium) was used as a minimal medium. For solid medium, 2% agar was added. Leucine (50 μg/ml) and histidine (50 μg/ml) were added to the glucose minimal medium, as needed. Killer activity was tested by determining inhibition zones on killer test medium (15), using *S. cerevisiae* 1019 as the killer-sensitive strain. For the fermentation test, cells were cultivated in either YEPD medium supplemented with 20% glucose (YEPD20 medium) or grape juice. The grape juice varieties used were California White Riesling and Traminer, and they were pretreated with pectic enzyme and sterilized by membrane filtration (0.45 μm; Millipore Corp., Bedford, Mass.) before fermentation. The contents of total phenolics in the grape juices were estimated to be less than 50 mg/liter.

Isolation of respiratory-deficient mutant. Cells were cultivated overnight in YEPD medium containing 10 μg of

TABLE 1. Strains of *S. cerevisiae* used in this study

Strain	Gennotype or phenotype	Source or reference[a]
522	Prototroph	Montrachet strain; Stock Culture Collection, Department of Viticulture and Enology, University of California, Davis
694	Leu⁻, homothallic diploid	Auxotrophic mutant of strain 522; isolated by Rous et al. (11)
1368	α *his4 kar1-1* [KIL-b_1]	Wickner; confers superkiller phenotype (13)
1368R	α *his4 kar1-1* [KIL-b_1] [rho^0]	Respiratory deficient; isolated in this study
1019	α *leu1 kar1-1* [KIL-o]	Wickner
KYC53	α *trp3 lys1 ura1 ura2* [KIL-o]	Oshima
5X47	Prototroph, diploid, killer sensitive	Wickner
CH23	Prototroph, [KIL-b_1]	Killer-positive derivative of strain 522; this study
CH123	Leu⁻, [KIL-b_1]	Killer-positive derivative of strain 694; this study

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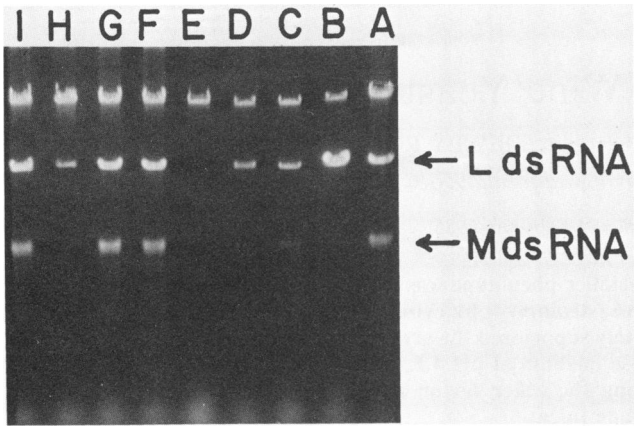

FIG. 1. Agarose (1%) gel electrophoresis patterns of dsRNA molecules. Lanes A and I, dsRNA prepared from strain 1368 (used as a killer strain); lane B, killer-sensitive strain 694; lanes C and D, killer-positive strains CH123 and CH134, respectively, derived from strain 694; lane E, strain 522; lanes F and G, killer-positive strains CH23 and CH29, respectively, derived from strain 522; lane H, killer-sensitive strain 1019.

ethidium bromide per ml and then spread onto YEPD agar plates after appropriate dilution. Triphenyltetrazolium chloride agar (0.5% glucose, 0.05% triphenyltetrazolium chloride, 1% agar) was overlaid on the colonies which grew. After being incubated for 3 h at room temperature, those colonies which were white and could not utilize glucose by respiration were selected. The respiratory deficiency of these colonies was also confirmed by a lack of growth on a minimal medium containing 2% glycerol as the sole carbon source (glycerol minimal medium) instead of glucose.

Protoplast fusion. Protoplast fusion was performed by the method of Arima and Takano (2). A 5-ml portion of an overnight culture grown in YEPD medium at 30°C was centrifuged at 3,000 rpm for 10 min, and the cells were washed twice with sterile water. The washed cells were suspended in 4 ml of a zymolyase solution (0.01% zymolyase 60000 [Kirin Beer Co., Ltd., Tokyo, Japan] in 50 mM sodium phosphate buffer containing 0.6 M KCl and 20 mM mercaptoethanol, pH 7.5) that was freshly prepared and sterilized by passage through a Millipore filter (pore size, 0.45 μm). The mixture was incubated for 2 h. Protoplasts of two parental strains were mixed and immediately centrifuged at 2,000 rpm for 10 min. The protoplasts precipitated were suspended in 4 ml of a 20% polyethylene glycol 4000 solution containing 50 mM $CaCl_2$ and allowed to stand for 15 min at room temperature. After appropriate dilution with 0.6 M KCl, the cells were embedded in an appropriate medium containing 0.6 M KCl as an osmotic stabilizer and incubated for 4 days at 30°C.

Isolation and gel electrophoresis of killer plasmid. Crude dsRNA plasmids were isolated by the method of Condo and Fink (5) and electrophoresed on 1% agarose gels in 0.4 M Tris-hydrochloride buffer (pH 8.0) containing 20 mM sodium acetate and 1 mM disodium EDTA. After being stained with an ethidium bromide solution (1 μg/ml) the dsRNA was observed with a Spectroline TR-302 UV transilluminator at 320 nm (Spectronics Corp., Westbury, N.Y.).

Fermentation test. Mixed fermentation cultures containing the killer strain and the killer-sensitive strain were grown in 500 ml of YEPD20 medium (initial pH, 4.5 or 3.5) or grape juice (pH 3.5) by using 700-ml minijar fermentors (Wheaton Instrument, Millville, N.J.) at 20°C without aeration. Cell density was measured by determining turbidity at 660 nm with a Spectronic 710 instrument (Bausch & Lomb, Inc., Rochester, N.Y.), and cell number was determined by the colony counting method on an appropriate agar medium. Glucose concentration was measured enzymatically by using a model 23A glucose analyzer (Yellow Springs Instrument Co., Inc., Yellow Springs, Ohio), and total sugar concentration was determined by the Somogyi-Nelson method (12). Ethanol concentration was determined enzymatically by using an Alcohol Kit (Sigma Chemical Co., St. Louis, Mo.).

RESULTS AND DISCUSSION

Cytoductive transfer of killer plasmid into wine yeast by protoplast fusion. The *S. cerevisiae* strain that produces k_1 killer toxin harbors two kinds of dsRNA plasmids (*L* dsRNA, 3×10^6 daltons; *M* dsRNA, 1.5×10^6 daltons). Plasmid [KIL-k_1] is one of the dsRNA plasmids which codes for production of killer toxins in *S. cerevisiae* (4), and plasmid [KIL-b_1] was derived from this plasmid (13). Strain 1368 (α *his4 kar1-1* [KIL-b_1]), which was used as a killer plasmid donor, harbored plasmid [KIL-b_1]. This strain was isolated as a mutant to overcome the chromosomal mutation of genes that control the maintenance of dsRNA plasmids. The strain harboring plasmid [KIL-b_1] produced much more killer toxin (about three times more) than the parental strain that harbored plasmid [KIL-k_1] due to a higher copy number of the plasmid (13). Strain 1368 is also a nuclear fusion-defective mutant after mating (5). To avoid the migration of mitochondrial DNA in cytoduction, a respiratory-deficient strain, strain 1368R, was isolated by ethidium bromide treatment.

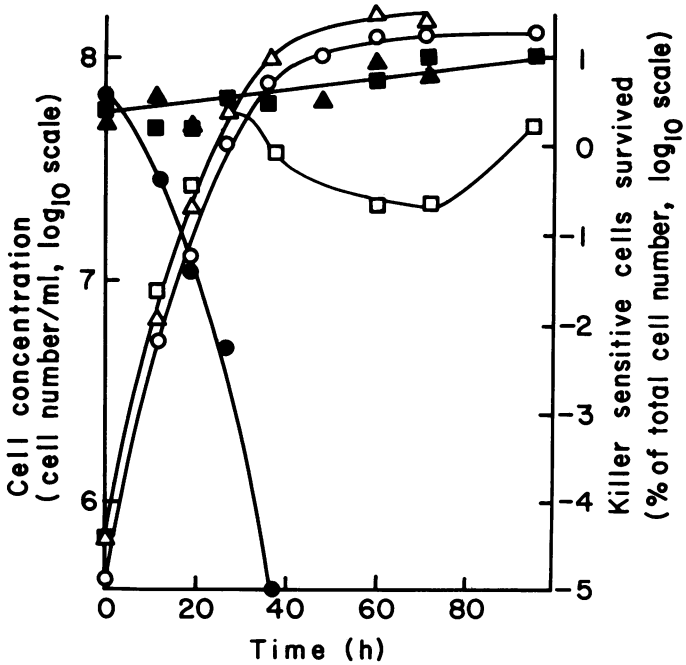

FIG. 2. Total cell number and fraction of killer-sensitive cells during mixed cultivation in YEPD20 medium at pH 4.5. Symbols: ○, △, and □, total cell numbers per milliliter; ●, ▲, and ■, percentages of killer-sensitive cells; ○ and ●, mixed culture of killer strain CH123 and killer-sensitive strain 5X47; △ and ▲, mixed culture of parental strain 694 and strain 5X47; □ and ■, mixed culture of strain 694 and killer strain 1368.

S. cerevisiae Montrachet strain 522 is used in industrial winemaking, and strain 694 was isolated as a leucine-requiring mutant of strain 522 by Rous et al. (11). To transfer the killer plasmid of strain 1368 into strain 694, protoplasts of both strains were fused in the presence of polyethylene glycol 4000 and $CaCl_2$, and the fusants were regenerated on YEPD medium containing 0.6 M KCl. Regenerated colonies were transferred into the following four kinds of media: glucose minimal medium, glucose minimal medium supplemented with histidine, glucose minimal medium supplemented with leucine, and killer test medium. Of the 123 regenerated colonies tested, 107 were the same as parental strain 694, because they could not grow on glucose minimal medium without leucine and did not show killer activity. Of the 16 strains that were killer positive against killer-sensitive strain 1019, 5 were found to be the same as the other parental strain, strain 1368, since they required histidine for growth on glucose minimal medium. However, the other 10 colonies required leucine for growth on glucose minimal medium, as strain 694 did. We concluded that the killer plasmids of strain 1368 were transferred by cytoduction to strain 694. Although the *kar1-1* mutation prevents nuclear fusion after mating (5), this mutation appears to be effective during the protoplast fusion process. Only one colony was found to be a true hybrid between strains 694 and 1368R, since it was able to grow on glucose minimal medium without any amino acid supplement. Although strain 1368R was defective in nuclear fusion, nucleus hybridization reportedly has been observed at a low frequency (5).

The killer plasmid was also transferred to wild-type strain 522. Strains 522 and 1368R were fused and regenerated on glycerol minimal medium, on which parental strain 1368R was unable to grow because of its histidine auxotrophy and respiratory deficiency. Of 79 colonies isolated, 8 were killer positive and showed wild-type characteristics. The other colonies were the same as parental strain 522, since they did not show killer activity and were prototrophic. The fact that

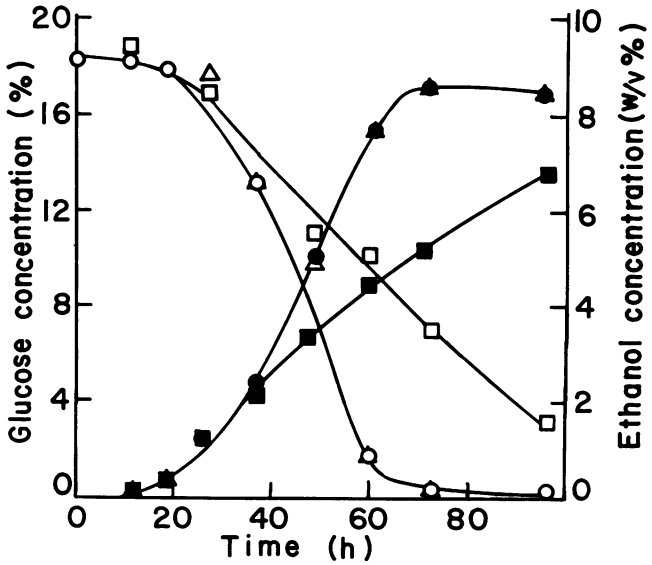

FIG. 3. Concentrations of glucose and ethanol during mixed culture in YEPD20 medium (pH 4.5). Symbols: ○, △, and □, glucose concentration; ●, ▲, and ■, ethanol concentration; ○ and ●, mixed culture of killer strain CH123 and killer-sensitive strain 5X47; △ and ▲, mixed culture of parental strain 694 and strain 5X47; □ and ■, mixed culture of strain 694 and killer strain 1368.

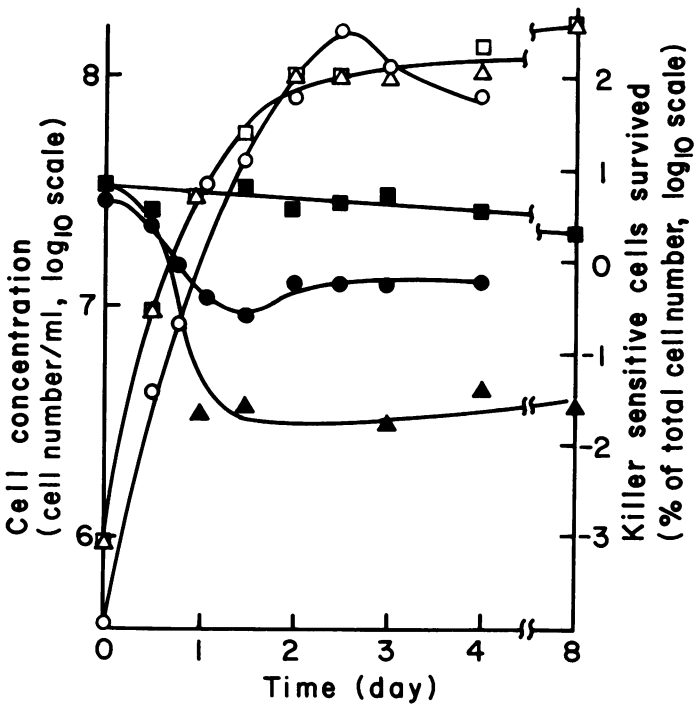

FIG. 4. Total cell number and fraction of killer-sensitive cells during mixed cultivation in YEPD20 medium (pH 3.5) and in grape juice. Symbols: ○, △, and □, total cell numbers per milliliter; ●, ▲, and ■, percentages of killer-sensitive cells; ○ and ●, mixed culture of killer strain CH123 and killer-sensitive strain 5X47 in YEPD20 medium (pH 3.5); △ and ▲, mixed culture of killer strain CH123 and strain 5X47 in grape juice (pH 3.5); □ and ■, mixed culture of strains 694 and 5X47 in grape juice.

no segregant was observed during successive cultivation of the eight killer-positive clones suggested that the killer plasmid was transferred from strain 1368R by cytoduction, as observed in the transfer of killer plasmid into strain 694.

To confirm that the killer-positive strains which we constructed had the same dsRNA plasmids as strain 1368, the plasmids were extracted from 8 strains obtained from the cross between strains 1368 and 522 and 10 strains obtained from the cross between strains 1368 and 694 and electrophoresed on 1% agarose gels. All killer-positive derivatives of strains 522 and 694 showed two plasmid bands corresponding to the *L* and *M* dsRNAs harbored in strain 1368, whereas parental strains 522 and 694 showed no *M* dsRNA band (Fig. 1).

Growth inhibition of killer-sensitive strain. To test the growth inhibition of a killer-sensitive contaminant, strain CH123, one of the killer strains constructed from strain 694, was cultivated in YEPD20 medium with a killer-sensitive strain, strain 5X47. Since the optimal pH range for killer toxin protein has been reported to be 4.5 to 4.8 (8, 14), the initial pH of the medium was adjusted to 4.5. Strain CH123 was inoculated at a density of about 5×10^6 cells per ml, whereas the cells of killer-sensitive strain 5X47 were inoculated at a density which was about 5% of the killer cell inoculum. The total cell number was monitered by determining colony counts on YEPD medium and counts of killer-sensitive strain 5X47 on glucose minimal medium, which did not allow the growth of strain CH123 because of its leucine requirement. Although the total cell number increased up to 60 h, the growth of killer-sensitive strain 5X47 was sup-

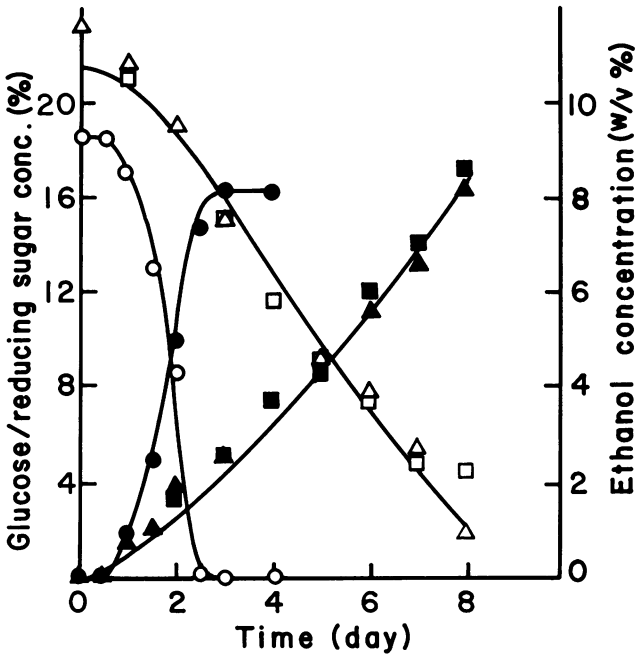

FIG. 5. Concentrations of glucose and reducing sugar in YEPD20 medium and grape juice, respectively, and concentrations of ethanol produced during mixed cultivation in YEPD20 medium (pH 3.5) and in grape juice. Symbols: ○, △, and □, glucose concentration; ●, ▲, and ■, ethanol concentration; ○ and ●, mixed culture of killer strain CH123 and killer-sensitive strain 5X47 in YEPD20 medium (pH 3.5); △ and ▲, mixed culture of killer strain CH123 and strain 5X47 in grape juice (pH 3.5); □ and ■, mixed culture of strains 694 and 5X47 in grape juice.

pressed completely, and practically no cells were detected after 36 h of cultivation (Fig. 2). In the mixed culture, 8.5% ethanol was produced after 72 h of cultivation (Fig. 3). Similar results were found with pure cultures of strain CH123 and parental strain 694. When the inoculum size for killer-sensitive cells was increased to 30% of the killer cell inoculum, the killer cells still inhibited the growth of killer-sensitive cells at pH 4.5. The growth of killer-sensitive cells was suppressed by the killer toxin produced, not by the difference in growth rates of these strains. The growth rates of both strain CH123 and strain 5X47 were about 0.21 h^{-1} when the individual pure cultures were incubated in YEPD20 medium.

When parental strain 694 was cultivated in YEPD20 medium with strain 5X47 under the same mixed culture conditions, the fraction of strain 5X47 cells increased from 2.1% at zero time to 9.6% at 60 h, as shown in Fig. 2, but the ethanol production was the same as it was in the mixed culture of strains CH123 and 5X47. When strain 694 was cultured with killer strain 1368, the growth of strain 694 stopped at 30 h, and ethanol production was also inhibited (Fig. 3).

Since the killer toxin protein was unstable both at high and low pH values (8, 10), the effect of the killer strain was tested in YEPD medium and grape juice adjusted to pH 3.5, a pH similar to the pH of some wine broth (1). As shown in Fig. 4, the population of the killer-sensitive strain was reduced from 4% of the total cell number at zero time to 0.5% of the total cell number at the end of cultivation (96 h) in YEPD20 medium (initial pH, 3.5), and the killer toxin at pH 3.5 was found to be less active than the killer toxin at pH 4.5. In grape juice, however, the fraction of killer-sensitive contaminant strain 5X47 decreased markedly from 6.5% of the total cell number at zero time to 0.022% at 92 h (Fig. 4). When parental strain 694 was cultivated with strain 5X47 in grape juice, the fraction of strain 5X47 was almost constant. The marked effect of the killer-positive strain found in grape juice compared with the effect found in YEPD20 medium was due to the slow growth rate of this strain (0.12 h^{-1}) in grape juice. However, the levels of ethanol production with and without contaminant cells in grape juice were the same, and about 42% of the reducing sugar in the grape juice was converted to ethanol (1) (Fig. 5).

Killer-positive strain CH23 derived from wild-type strain 522 effectively suppressed the growth of killer-sensitive yeast strain KYC53 in YEPD20 medium at pH 4.5 and also in grape juice (data not shown). Killer toxin production by strain CH23 was found to be stable.

There appears to be a good possibility that the wine yeast strains harboring one or more (multiple) killer factors could prevent or eliminate contamination problems caused by undesirable yeast strains in industrial wine fermentation systems. More work needs be done to confirm this hypothesis. Also warranted is research to develop industrial wine yeast strains having high killer activities, as well as multiple killer factors that are active against more than one undesirable yeast strain.

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