Integration of the Yeast K1 Killer Toxin Gene into the Genome of Marked Wine Yeasts and Its Effect on Vinification

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The yeast K1 killer toxin gene was integrated into the genome of laboratory and commercial wine yeasts by gene replacement technology that generated recombinants containing only yeast DNA. Integration of the K1 killer gene into two K2 wine yeasts generated stable K1/K2 double killer strains which have a wider spectrum of killing and a potential competitive advantage over other sensitive and killer strains of *Saccharomyces cerevisiae* in wine fermentations. The fermentation characteristics of several killer integrant strains were examined in microvinification tests. The results indicate that wine fermentations and products from such yeasts can be similar to those obtained from fermentations by the parental strains.

KEY WORDS: wine yeasts, K1 killer toxin gene, genomic integration, stable killer phenotype, microvinification

Sophisticated procedures exist to genetically alter laboratory strains of *Saccharomyces cerevisiae,* but there remains a need to refine techniques for genetic manipulation of prototrophic industrial yeast. Transformation of industrial strains can be effected by the use of dominant selectable markers, and a range of genes conferring drug or heavy metal resistance have been used (reviewed in 16). Stability of the transformants once selected is also an important property, as it is often impractical to maintain a selection during the commercial use of the transformant. A common solution to this problem is to integrate the desired genes into the yeast genome (23,24); such integrants are usually considerably more stable than when such genes are maintained on an extrachromosomal plasmid. At a modest but still perceptible rate, integrants may also be unstable through events such as gene conversion that lead to loss of integrated genes in the polyploid backgrounds often found in industrial yeasts. Ingenious schemes have been devised to create homozygosity of integrants at chromosomal loci, to prevent the gene conversion process (23). In addition to the problem of stability, it is anticipated that a regulatory requirement for food and beverage use of recombinant yeast would be that the organism is free from *Escherichia coli* drug resistance genes and other bacterial vector DNA. We have explored the yeast killer toxin gene as a dominant selectable marker useful in genetically manipulating industrial yeast (6). The system has intrinsic value as an anticontaminant for yeasts used in wine and beer fermentations (5,21); it also has potential use as a vehicle to introduce desirable genes into such yeast. We have shown that strains containing the killer toxin/immunity gene are selectable and remain self selecting when the toxin is active (5,6). A potential problem for the plasmid-based gene was instability in the absence of selection, for example, at high or very low pH when the toxin is inactive. Here we describe the construction of killer strains through chromosomal integration of the toxin gene, demonstrate their stability in the absence of selection, and examine their fermentation characteristics in microvinification.

Materials and Methods

Yeast strains: The following strains of *Saccharomyces cerevisiae* were used: SK.S14a Mat a/Mat α ade2-5/ADE2 his4C-864/HIS4 [KIL-K1] was used as source of K1 killer toxin, H4D Mat α ade his4C-864 [KIL-K1] as a K2 tester strain (5), M471 [KIL-K2] as a K1 tester strain (5), and S6 as a wild type [KIL-O] strain sensitive to both K1 and K2 toxins. AH22 Mat a leu2-3 leu2-112 his4-519 can1 [KIL-O], T9-3c Mat a leu2-3 leu2-112 his3 ura3 [KIL-O], and an isogenic diploid, TA405 Mat a/Mat α leu2/leu2 his3/his3 can1/can1 [KIL-O] (22), were from Malcolm Whiteway. Wine strains A2DE1 (marked K1) and A47D08 (marked EC) were both [KIL-K2] prototrophs and were obtained from Lallemand Inc., Montreal. The EC and K1 strains were isolated by the Institut Oenologique de Champagne (Epernay, France) and the Institut Coopératif du Vin (La Jasse de Maurin, France) respectively. The marking consists of introducing mitochondrial-based resistance to diuron and erythromycin (A2DE1), or diuron and oligomycin (A47D08), and was conferred by the procedures of Vezhinet and Lacroix (20).

Media: Yeast growth media were YEPD-complete medium [0.5% Yeast Extract (Difco), 1% Bacto-Peptone (Difco), Halvorson 1X salts pH 4.7 (10), 2% glucose]; and minimal medium [Halvorson 1X salts pH 4.7, 2% glucose, amino acid supplements (2.5 mg/L)] required by auxotrophic strains. Growth rates were determined following dry weight estimations and culture turbidity measurements at 600 nm in a spectrophotometer.

Bacterial growth media: 2YT [1.6% Tryptone (Difco), 1% Yeast Extract (Difco), 0.5% NaCl]. Ampi-

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cillin, when required, was used at 100 mg/L.

Microvinification and fermentation tests: A white must (Ugni) containing 163 g/L reducing sugars was used. Fermentations were carried out in fermentors with a 2-L working volume at 25°C, with constant agitation at 250 rpm. The musts were inoculated with 4 × 10^6 cells/mL from a 24-hour culture (200 mL, 100 rpm, 30°C) prepared in must diluted with water (1:2). Aliquots were taken from the fermentor twice a day, centrifuged, and the supernatant stored at -40°C until analysis. Volatile acidity and malic acid were determined according to the OIV techniques (15), while reducing sugars were analyzed by the AOAC method (2) and ethanol by the technique of Crowell and Ough (8). Fermentations were considered complete when no residual sugar was detected. One clone of A2DE1 (RW1) and two clones of A47D08 (RW2, RW3) were tested. Wines were tasted by a panel of five people including two enologists.

Yeast transformation and genetics: Yeast were transformed using the LiC1 procedure of Ito *et al.* (12). One-step gene disruption was performed as described by Rothstein *et al.* (17). Killer transformants were obtained either by selecting for the complementation of an auxotrophic requirement or via selecting for immunity to K1 toxin as described by Bussey and Meaden (6).

K1 and K2 killer phenotype testing and stability studies were carried out as described in Bussey *et al.* (5).

Recombinant DNA techniques: *Escherichia coli* MC1061 (7) was used for routine growth and maintenance of plasmids (14). Transformation of MC1061 was performed using the $CaC1_2$ procedure (14). Rapid plasmid purification was as described by Holmes and Quigley (11). DNA fragment isolation and purification was achieved using GENE CLEAN (BIO 101) according to the supplier's recommendations. DNA ligation reactions were carried out using DNA Ligase (and ligation reaction buffer) from BRL. Southern blotting hybridizations were carried out by the procedure of Southern (18).

Construction of killer LEU2 integration plasmid: Bluescript M13+ (Stratagene) was digested with Hind III, repaired by the Klenow fragment of DNA polymerase I, and ligated with the nonphosphorylated Sph I linker (5'-GGCATGCC-3', New England Biolabs), to create pL185. The 720 bp Sph I fragment containing the ADH1 promoter/terminator (separated by the MCS-7(1) multicloning site) was isolated from pVT 100-U (19), and cloned into the Sph I site of pL185. Two orientations of the ADH1 promoter/terminator fragment were obtained; the product with the promoter situated next to the Eco RI site of the bluescript polylinker was designated pL212.

The 400 bp Eco RI-Hind III fragment of pL212 containing the ADH1 promoter was replaced with a larger (promoter-containing) Eco RI-Hind III (1.5 kb) fragment isolated from pYT760 (3), to create pL19. pL19 was digested with Sma I and ligated with the nonphosphorylated Sal I linker (5´-CGTCGACG-3´, Boehringer Mannheim), the ligation product was designated pL65. Sal I digestion of pL65 allowed the isolation of a 1.9 kb fragment containing the ADH1 promoter/terminator [separated by MCS-7 (1)].

Bluescript M13-(LEU2) contains a unique Sal I site 3' to the Leu2 mRNA termination site which facilitated the insertion of the ADH1 promoter/terminator (1.9 kb) Sal I fragment. Bluescript M13-(LEU2) was constructed via ligation of the (2.9 kb) Xho I-Sst I (LEU 2 containing) fragment of YEp 13 (4) into Xho I-Sst I digested Bluescript M13-(Stratagene). The ADH1 promoter/terminator was cloned into bluescript M13-(LEU2) and isolated as recombinants with the promoter fragment in two different orientations, to generate pL70 and pL71. The K1 killer toxin precursor gene KIL-K1 (3) was ligated into Hind III-Bam HI digested pL70 and pL71 (Hind III, Bam HI restriction endonuclease sites are contained within the MCS-7 (1) multicloning site that separates the ADH1 promoter/terminator to create the LEU2 killer integration plasmids pm99 and pm103, respectively (see Fig. 1).

Results

Previous studies (3,5,6,13) have examined killer expression from a multicopy plasmid. We felt it was necessary to show that there was a sufficiently strong phenotype with expression of the gene from the ADH 1 promoter on a single copy centromeric plasmid YCp50, prior to attempting construction of single copy genomic integrants. A non-killer sensitive ura3 strain, T9-3c, was transformed with the killer gene on the YCp50

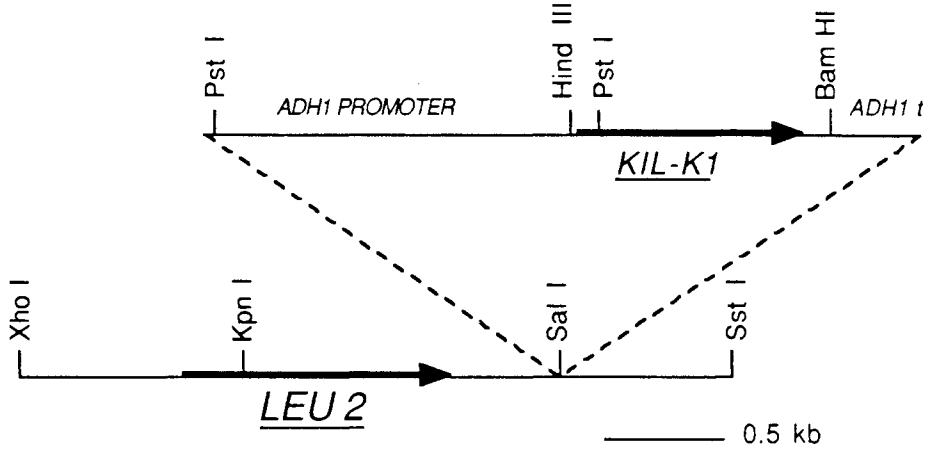

Fig. 1. Structure of yeast DNA in the LEU2 region with the inserted KIL-K1 killer gene. The structure of the wild type chromosomal LEU2 locus is shown at the bottom, with the LEU2 open reading frame and transcript direction indicated by the bold arrow. The K1 killer gene flanked by yeast ADH1 promoter and terminator elements is shown above, with the toxin precursor open reading frame and transcript direction shown by the bold arrow. This killer expression fragment is shown inserted into the Sal I site in the 3' noncoding region of the LEU2 locus, and represents the structure in plasmid pm99, with pm103 having the insert in the opposite orientation. See Materials and Methods for details of construction.

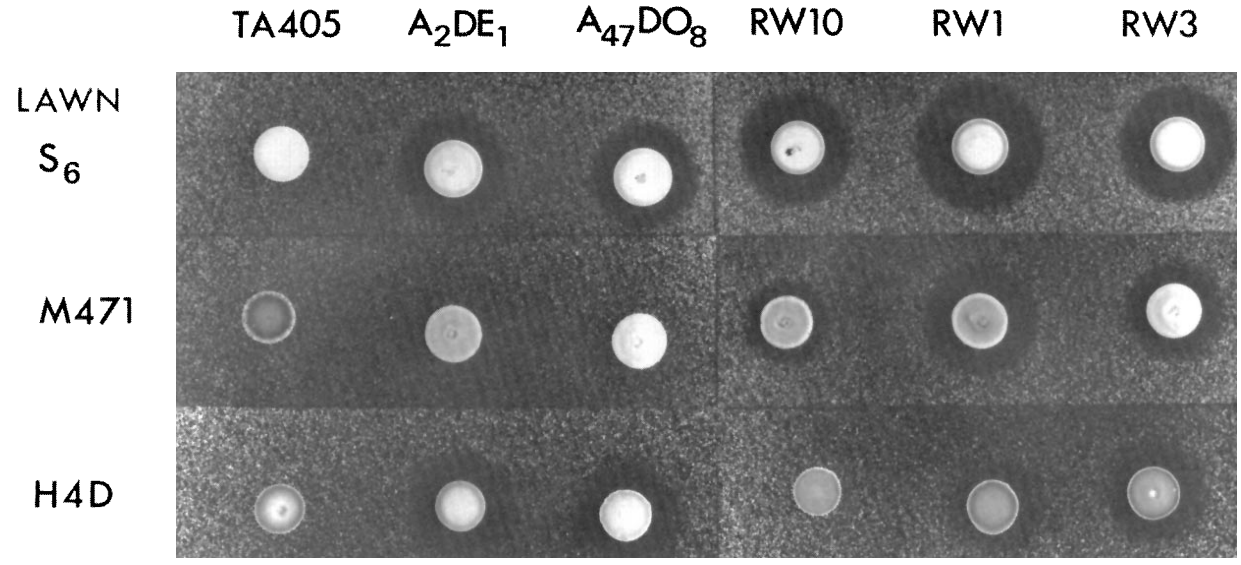

Fig. 2. Killer phenotypes of yeast strains and their K1 killer integrants. Lawns of sensitive non killer strain S6; K2 killer, M471; and K1 killer, H4D, were overlaid with 10 ml of stationary phase cells of the yeast strains indicated at the top of the figure. The plates were incubated at 18°C for 2-3 days. Killer strains form a clear (dark) halo of killing in sensitive lawns; see text for details.

plasmid, and URA+ transformants selected. These were immune killers with a phenotype similar to that seen with the two-micron-based multicopy plasmid killers and similar to that seen with a single copy integrant (Fig. 2), TA405-RW10 on S6 lawn for a phenotype. We then constructed an integrating vector, making use of the 3' noncoding region of the LEU2 gene on chromosome 3 as a site for integration of the ADH1 promoter and the killer gene (Fig. 1). This integration site, which is not transcribed (1) was chosen for ease of selection for leucine prototrophy in laboratory yeast. The actual constructions are summarized in Figure 1 and **Materials and Methods.**

Killer selections: As a test for the procedure, we transformed a laboratory haploid strain AH 22 and two wine yeasts, A2DE1 and A47D08, with Kpn1-Sst1 linearized integrating fragments containing the killer gene in one or other orientation with regard to LEU2, and selected for integrants using killer toxin immunity. Immune, killer transformants of the above strains and of laboratory diploid, TA405, obtained in this way were examined in detail. Strain TA405 is not a killer, and did not kill lawns of the sensitive non-killer strain S6, the K2 killer strain M471, or the K1 killer strain H4D. However, RW10, a K1 killer integrant of TA405 killed S6 and M471, but did not kill the K1 killer strain H4D, which is immune to the K1 toxin (Fig. 2). Wine yeasts A2DE1 and A47DO8 are natural K2 killers with K2 dsRNA containing virus-like particles. These yeasts killed S6 and the K1 killer strain H4D but not the K2 strain, M471 (Fig. 2). K1 killer integrants of A2DE1 and A47DO8, RW1 and RW3,, respectively, killed lawns of S6, M471 and H4D cells and are thus K1/K2 double killers (Fig. 2). Stability of the killer phenotype in transformants from all three strains was measured under pH conditions where the K1 toxin was inactive, preventing selection for retention of the K1 immunity gene. The transformed strains were grown for approximately 58 doublings by serial subculture in liquid YEPD medium at pH 6.2 at

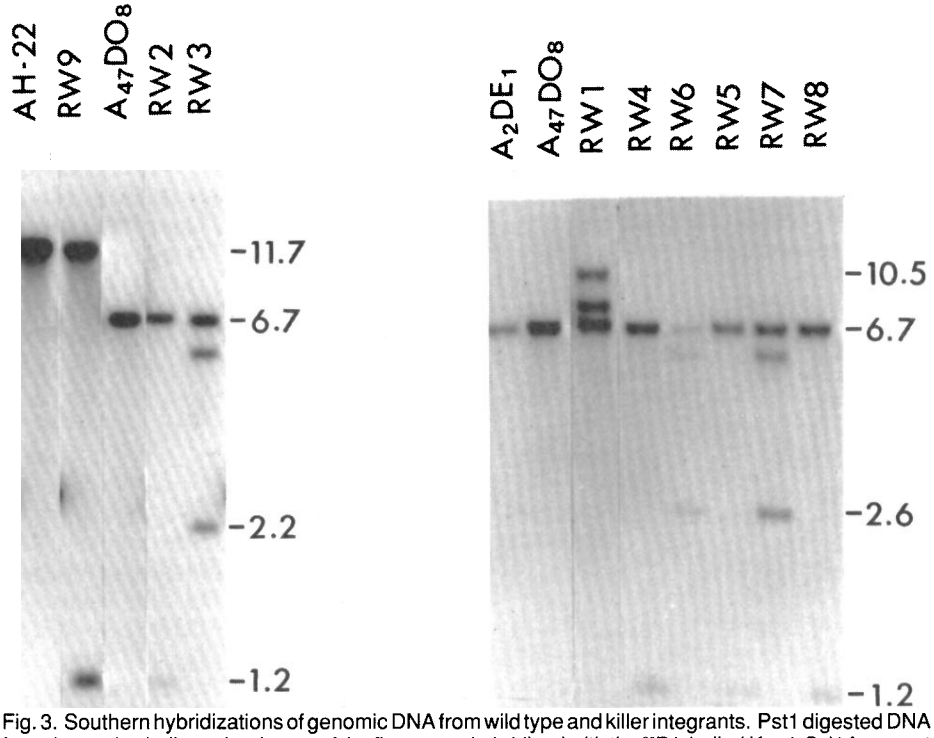

Fig. 3. Southern hybridizations of genomic DNA from wild type and killer integrants. Pst1 digested DNA from the strains indicated at the top of the figure was hybridized with the ^{32}P labelled Kpn1-Ssl1 fragment of LEU2 in the PBSK vector. The size of fragments in kilobases is indicated on the left (see text for details).

30°C. At least 99.6% of the cells retained the killer phenotype following such a growth regime.

The location of the integration of the killer gene in the transformants was examined by Southern blotting of their genomic DNA. Probing of Pst1 digested genomic DNA of transformants with a LEU2 fragment resulted in the appearance of new LEU2-containing DNA fragments absent from the parent strain (Fig. 3). In the case of transformants RW2 and RW4 through 8, the size of the new LEU2-containing fragments was consistent with the interpretation that both pm99 and pm103 directed integration of the ADH1/KIL-K1 DNA to the LEU2 locus. Both parent strains, A47D08 and A2DE1, appear to have similar LEU2 alleles and produce 6.7 kb Pst1 fragments detectable in the Southern blots. Integration of ADH1/KIL-K1 DNA using vector pm103 consistently produced a new 1.2 kb LEU2-containing fragment. In this case, assuming that integration took place at the LEU2 locus, then Pst1 sites would be situated approximately 5.5 kb 5', and 1.2 kb 3' of the Sal1 site found in the 3' noncoding region of LEU2 (see Fig. 1, and note that the LEU2 transcript defines the strand orientation we refer to). Consistent with this analysis, the Southern blots of RW6 and RW7 showed two new LEU2-containing fragments of 5.5 and 2.6 kb, as expected if pm99 directed integration of ADH1/KIL-K1 DNA to the LEU2 locus. Vector pm99 also directed integration at two other unknown loci, as seen in the Southern blots of RW1 and RW3.

In all cases examined, integration of the killer led to a reduced growth rate compared to the parent strains (Table 1). In the most extreme case, A2DE1 derived RW6, this was a 30% increase in generation time.

Site of integration: While the LEU2 integration site enables stable killer strains to be constructed, we have some evidence that it is haploid lethal. Diploid strain TA405 was transformed with the killer integration plasmid pm103 linearized with Xho1 and Sst1 and selected for complementation of the leu2 allele. Southern blot analysis revealed that the integration event had taken place at the leu2 locus (data not shown). The transformants were stable for the killer phenotype as diploids; however, when two such integrants were sporulated, most tetrads gave only two meiotic progeny (16 of 19 tetrads), and these spores were all phenotypically killer- leu-, suggesting that integrants at this locus were deleterious in the haploid spores. Only three LEU +killer+ were recovered from these tetrads; two of these were very slow growing. A dissection of 16 tetrads from the parent TA405 strain, done as a control, gave 12 complete tetrads; and of the other four, three gave three viable spores and only one tetrad gave two viable spores. Attempts to obtain killer integrants with a functional LEU2 gene at the leu2 locus on chromosome 3 in the haploid AH22 were unsuccessful. All transformants recovered were at sites not closely linked to the leu2 locus on chromosome 3. In five transformants examined, the LEU+ integrants were non-allelic to the leu2 locus; of 33 tetrads examined, only seven were parental ditype for the leu2 markers. In addition, segregation of killer and his4 in these integrants showed no linkage, 7 PD:5 NPD:17 TT, although his4 is 15cM from leu2 on chromosome 3. These observations suggest that haploid yeast strains cannot tolerate integrants at the site we have used in the 3' noncoding region of the LEU2 gene.

Table 1. Growth rates of wine yeasts and killer integrants.

Strain	Generation* Time (h)	% Increase	Comments
A47D08	1.24	—	Parent wine strain
A47D08-RW3	1.40	13	K1+ integrant not at LEU2 locus
A47D08-RW2	1.45	17	K1+ integrant at LEU2 locus
A2DE1	1.15	-	Parent wine strain
A2DE1-RW6	1.5	30	K1+ integrant at LEU2 locus
A2DE1-RW1	1.4	22	K1+ integrant not at LEU2 locus

* In YEPD medium at 30°C

Microvinification using killer integrants: To examine the fermentation characteristics of some killer integrants, microvinifications were made using a white Ugni must (see **Materials and Methods** for detailed procedures). For strain A2DE1, the non-LEU2 integrant RW1 had a lag phase intermediate between the K1 parent and the A2DE1 marked strain (Fig. 4A). Integrants of killer in strain A47D08, RW2, and RW3 both had a longer lag phase, approximately 40 hours, compared to a 20-hour lag phase for the parent strain (Fig. 4B). In all case,s the rate of sugar utilization during the active phase of fermentation as well as the ethanol yield (0.47 - 0.49 g ethanol/g sugar) were similar among integrants and parent strains. Analysis of the wines for volatile acidity, pH, and malic acid degradation also showed no differences between killer integrants and their parents. Preliminary wine tasting of the samples suggested that wine made from integrants could be similar to that made from parent strains, but more detailed sensory analyses would be necessary to substantiate this conclusion. In a further series of fermentation tests using a concentrated must supplemented with 1 g yeast extract per liter, all clones showed fermentation kinetics similar to that of the K1 strain in white Ugni and no latency was observed (data not shown).

Discussion

The use of the dominant property of the killer phenotype allows for the selection of chromosomally based integrants of this gene. Such integrants have a number of advantages over plasmid-based killers that have been used previously. The integrants are considerably more stable than 2-micron-based killers under non-selective conditions at pH 6.2 (5), yet retain the ability to select against the rare cell losing the gene by killing with toxin under appropriate conditions. In addition, the integrants made here are "all yeast" constructs, and contain no bacterial DNA. This feature is a

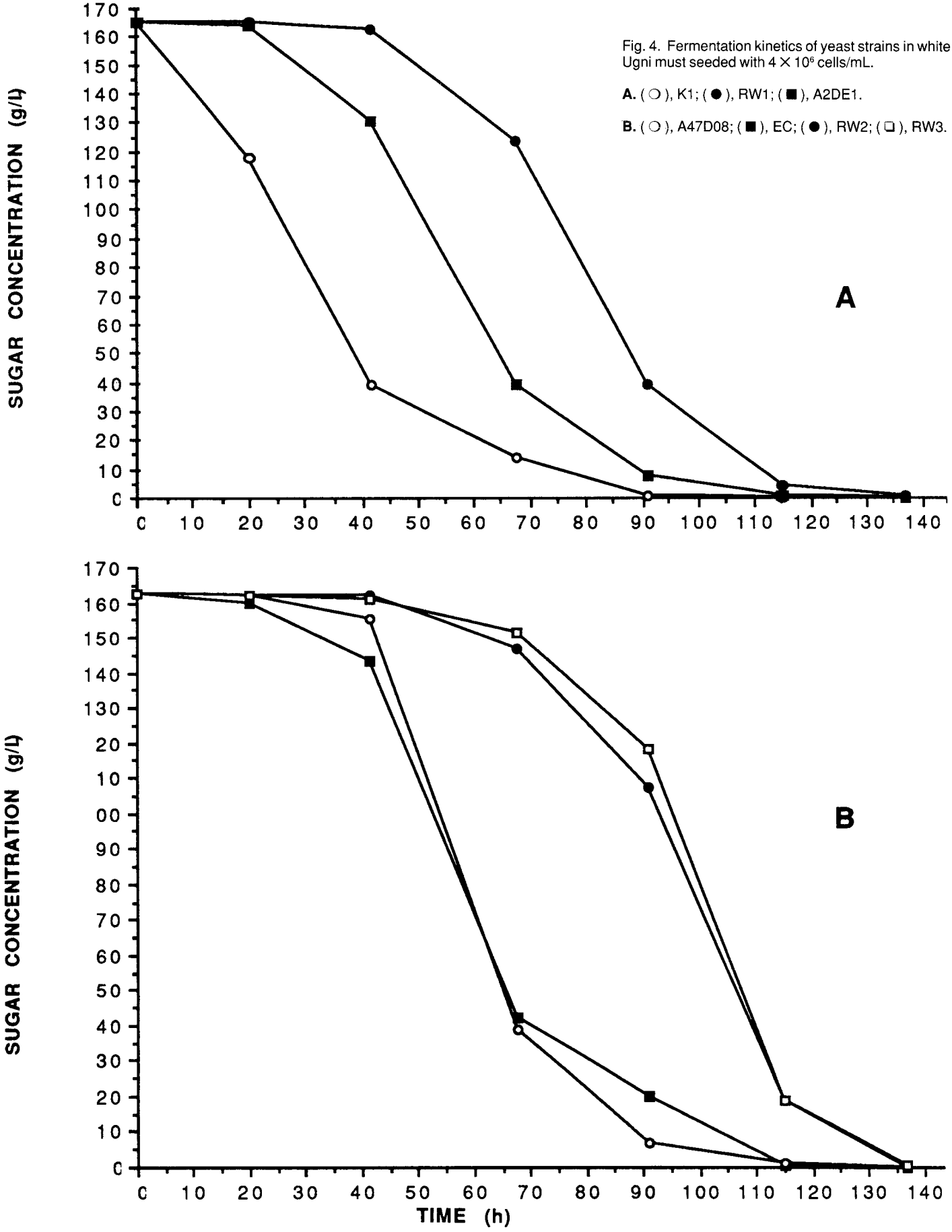

Fig. 4. Fermentation kinetics of yeast strains in white Ugni must seeded with 4×10^6 cells/mL.

A. (○), K1; (●), RW1; (■), A2DE1.

B. (○), A47D08; (■), EC; (●), RW2; (❑), RW3.

desirable one on aesthetic, safety, and regulatory grounds for commercial use of such recombinant industrial strains. We show that the K1 killer can be stably integrated into the genome of two K2 killer marker strains used in wine production to generate new yeasts that can kill both K1 and K2 killers (5, and this work). Such strains would be improved in their anticontaminant properties against sensitive K1, and K2 killer yeasts and would be resistant to indigenous K1 and K2 killer yeasts when used in wine production. Although the data are limited, results from microvinifications using killer integrant strains suggest that fermentations and products can be similar to those from the parental strains. The efficiency of transformation of sugar into ethanol and the fermentation rate during the active phase of fermentation were not affected, although variation in latency was observed. Integrant RW1, a non-LEU2 integrant, was not significantly different from its parent strains in any test. RW3, also a non-LEU2 integrant, had an apparently longer lag phase than its parent, but otherwise it was indistinguishable from its parent strain. RW2, the only killer integrant at the LEU2 locus examined here, had a longer lag phase. Further work would be needed to confirm if this was indeed due to the integration of the killer gene at this locus, but it argues against using this locus as an integration site. Overall, it appears that the killer gene can be integrated into industrial strains without adversely altering their phenotypes. Apart from the intrinsic and specific use of the killer gene as an anticontaminant, the gene also provides a way to introduce other genes into yeast. Integrants such as those made here with some other useful gene included could be made in industrial strains. The use of the yeast killer gene would then be that of a natural dominant selectable marker that would also allow selection for the retention of the integrant by killing of rare cells that lose the integrated genes, usually under normal growth conditions when the killer toxin is produced.

The choice of a site to integrate genes into industrial yeasts is an important consideration. The noncoding 3' region of the LEU2 gene used here, was chosen simply because of availability and convenience of selection in laboratory strains. However, we have some evidence that suggests that in haploids with a functional LEU2 locus, integration at this site is deleterious. In general, there is relatively little information concerning the location of sites in the yeast genome where integration of new material can be safely made. The study of Goebl and Petes (9) suggested that 70% of random integrants into the haploid yeast genome had no apparent affect on the phenotype, despite the fact that some 50% of the yeast genome is thought to be transcribed. One specific successful strategy that has been adopted for polyploid industrial yeast (23) is to target integration to the HO gene, a gene whose expression is restricted to haploids. Certainly the determination of some additional safe harbors for integration of genes into polyploid yeasts would be a useful exercise.

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