Breeding of a Brewer's Yeast Possessing Anticontaminant Properties[1]

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ABSTRACT

Antibacterial and anti-wild yeast characters were introduced into a brewer's yeast to achieve a more positive microbial assurance in brewing. An antibacterial yeast that inhibits the growth of 11 beer-spoilage bacteria was screened from our stock cultures and mated with a killer yeast active against many brewery wild yeasts. After sporulation of the hybrid, a haploid clone (AGK27) possessing the two anticontaminant properties was obtained. This clone was protoplast-fused with our brewery yeast, and one fusant, MAK61, was selected out of many hybrid products. MAK61 had both anti-wild yeast and antibacterial characters, and the beer produced was considered acceptable, although the fermentation rate was a little slower than that of our current brewery yeast. This work demonstrates the practical breeding of a brewer's yeast using protoplast fusion to improve brewing characteristics.

Key words: Antibacterial, Breeding, Brewing, Killer yeast, Protoplast fusion, Yeast

Microbial contamination during fermentation may cause off-flavors and biological haze in the product, especially nonpasteurized beer. Microbial control in brewing and effective plant sanitation should be requisite, but an additional positive microbial assurance can be achieved by improving a brewer's yeast itself, that is, by introducing anticontaminant properties that inhibit the growth of wild yeasts and/or beer-spoilage bacteria.

The property of some yeasts to kill a wide range of wild yeasts is well known as the killer phenomenon (2). Sensitive yeasts are killed by what is called a killer factor (KIL) produced by such yeasts (1,17). The factor is a protein (mol wt = 11,000) encoded by dsRNA (a viruslike particle) in the cytoplasm (11,14). Introduction of the killer property into saké (13), wine (6), and brewer's yeast (16) has been reported recently.

Only a few reports, on the other hand, have been on the property of yeasts to inhibit the growth of beer-spoilage bacteria. Dolezil and Kirsop (4) found that five of 31 commercial beers were completely resistant to 16 strains of lactic acid bacteria, perhaps owing to some substance(s) produced by each brewer's yeast. Hudson (7) reported that two strains of 14 brewer's yeasts produced certain small molecules that inhibited the growth of beer-spoilage bacteria. The nature of the inhibiting material has not been defined, however, and no evidence is available of trials to introduce this character into brewer's yeast.

This article describes the breeding—through a combination of mating, sporulation, spore isolation, and protoplast fusion— of a new brewer's yeast possessing the two anticontaminant properties. Our current brewery yeast, a killer yeast, and an antibacterial yeast screened out for this purpose were used.

EXPERIMENTAL

Microorganisms

The killer yeast strain used was *Saccharomyces cerevisiae* G706 (*a,thr*4, *leu*2, *his*4 [ρ^+, killer]). The antibacterial yeast was *S. cerevisiae* A2 (*a*/α [ρ^+, nonkiller]). Both were screened from the Sapporo Breweries culture collection (BSR1 YB4-1). Sapporo yeast (*S. carlsbergensis*) is the lager yeast used in our plants.

The 11 beer-spoilage bacteria (listed with their Brewing Science Research Institute [BSRI] numbers) used as test organisms in the screening process were:

Lactobacillus brevis, B04-2
Pediococcus cerevisiae, B04-12
Corynebacterium sp., B11-2 and B11-3
Enterobacter sp., B07-3 and B07-4
Klebsiella sp., B06-2 and B06-3
Hafnia sp., B07-2
Acinetobacter sp., B11-1
Bacillus sp. (nonspore variant c-3), B05-11.

Growth Media

Hopped wort (11°P) and YEPD medium (yeast extract 1%, peptone 2%, dextrose 2%) were used for yeast growth. Minimum medium contained 0.67% of yeast nitrogen base without amino acids (Difco) and 2% of glucose (minimum glucose medium) or 4% of glycerol (minimum glycerol medium).

For bacterial growth, Heart infusion broth or Schaedler broth (BBL) was used. Actually, residual nutrition of normal beers, including 13 foreign and four domestic brands, was adequate to maintain the growth of all bacteria used in the study, which supports the concept that a yeast factor, and not nutrition, is responsible for inhibition. Precise investigations of the isolation, purification, and identification of the factor(s) produced by yeast A2 are now in progress.

Screening of Antibacterial Yeasts

We screened 150 strains from our culture collection for their antibacterial characters: 74 strains of brewer's yeast, 12 of wine yeast, 11 of saké yeast, 34 of baker's yeast, and 19 of distiller's yeast. Cells were inoculated in 100 ml of wort and incubated for 10 days at 20°C. The fermented wort was membrane-filtered (0.45 μm) and poured into 12 sterilized test tubes (5 ml each). The 11 beer-spoilage bacteria were inoculated (approximately 10^6 cells per tube) into the beer filtrate and incubated for 10 days at 25°C. After incubation, the turbidity of the culture solution in each test tube was measured.

In the first screening, hopped wort (O.G. 1.040) was fermented by each strain of the yeasts. Membrane filtrate (0.45 μm) was tested for inhibition of two bacteria, *Lactobacillus* and *Enterobacter*. Of the 150 strains, 113 did not inhibit either bacteria, 26 inhibited one or the other, and 11 inhibited both.

In the second screening, the 11 bacteria listed were used. Four of the 11 strains that inhibited both bacteria in the first screening showed antibacterial activity against all of the tested bacteria, with yeast A2 YB2-2 showing the highest overall activity (Table I). Yeast A2 YB2-2 was also strongly inhibitory to *all* of the 11 diverse bacterial cultures. It should be noted that the 11 bacteria tested were all resistant to beer pH, alcohol level, and to five times the normal hop rate for lager beer, with yeast A2 still showing an antibacterial property after an adjustment to pH 5.2.

Sporulation and Separation of Spores

We used the procedures described by Gjermansen and Sigsgaard (5), except that Zymolyase-5000™ (2 mg/ml) (Seikagaku Kogyo Co., Ltd., Tokyo) was substituted for β-glucuronidase.

Assay of Killing Activity

Killing activity by killer yeasts was assayed by a cylinder plate method modified by Ouchi et al (12), with *S. pastorianus* as a sensitive yeast.

Preparation of Protoplasts

To obtain protoplasts at a high regeneration rate, yeast cells were incubated in YEPD medium (100 ml) with shaking for 24 hr at

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30°C, then centrifuged, resuspended in fresh YEPD medium (100 ml), and incubated for 3–4 hr at 30°C with shaking. After centrifugation, the cells were washed once with 50 ml of a hypertonic buffer containing 1 *M* of KCl and 20 m*M* of tris-HCl (pH 7.5).

The washed cells were incubated in 50 ml of the buffer containing 0.1 ml of concentrated β-mercaptoethanol for 20 min at 30°C, then centrifuged and suspended in 20 ml of the hypertonic buffer solution containing 4–8 mg of Zymolyase-5000. After 30–60 min of incubation at 30°C, the cells were nearly completely converted to protoplasts (>99.9%). They were washed three times with the hypertonic buffer.

Fusion of Protoplasts

We used the conditions described by van Solingen and van der Plaat (15).

RESULTS AND DISCUSSION

Screening of Antibacterial Yeasts

There have been several reports on antibacterial yeasts (3,9,10), but none has been well elucidated. We screened 150 strains from our yeast culture collection for antibacterial properties. Only four strains completely inhibited the growth of all the bacteria, and we selected *S. cerevisiae* A2 (a baker's yeast) for further studies. This strain is a diploid; it sporulates and germinates well but has no killer activity. It is prototrophic and respiratory-sufficient.

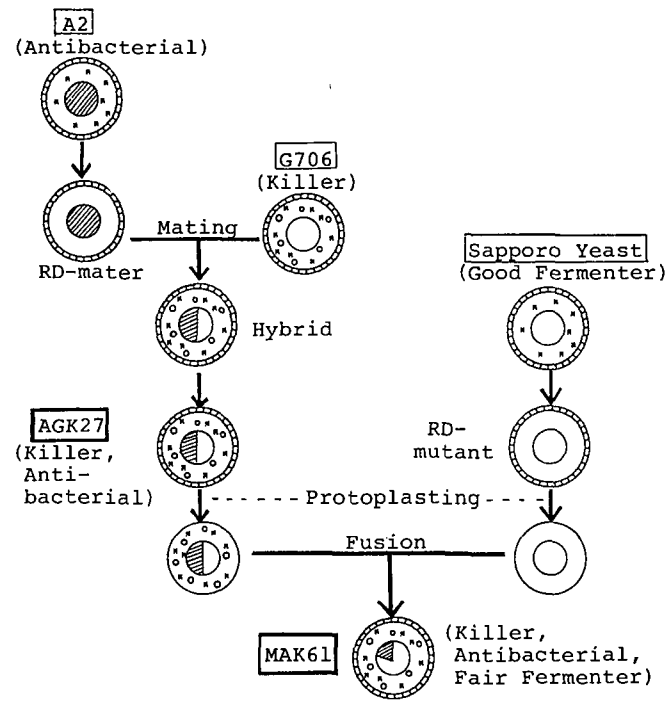

Fig. 1. Genetic manipulation for the production of AGK27 and MAK61. Outmost circle = cell wall, central circle = nucleus, M = mitochondria, small closed circle = killer plasmid (dsRNA), oblique lines = antibacterial gene(s).

The A2 cells produced an antibacterial substance in the culture broth that was heat-stable (100°C for 20 min), effective at acidic pH, and sufficiently small to be dialyzable. Work on purification of the substance is in progress.

Construction of an Antibacterial Yeast Donor

To introduce killer and antibacterial characters into a brewer's yeast, an intermediate anticontaminant donor strain was created by manipulating *S. cerevisiae* G706 and A2. The donor thus obtained was protoplast-fused with a Sapporo brewer's yeast (Fig. 1).

Haploid clones from the antibacterial yeast A2 were gained through sporulation and spore isolation. Among 10 α-maters (haploids), only four showed antibacterial character. One was treated with acriflavine to convert it to a respiratory-deficient (RD) mutant.

The RD mutant (*a*,antibacterial [ρ^-]) of A2 was mated with killer yeast G706 (*a*,*thr*4, *leu*2, *his*4 [K1L, ρ^+]). After the two strains were mixed and incubated in YEPD medium at 25°C for 16 hr, the culture was diluted and spread on minimum glycerol agar plates to obtain hybrid colonies.

After incubation, five selected prototrophic colonies showed both killer and antibacterial activities as well as high sporulation and germination rates.

After sporulation and spore isolation of one of the hybrids (*a*/α,*thr*4/+, *leu*2/+, *his*4/+, −/antibacterial [K1L, ρ^+]), haploid clones were selected for killer and antibacterial characters as well as for auxotrophy (one or more markers from G706). This auxotrophy was necessary for the succeeding protoplast fusion. The 18 clones obtained were all killers but only three were antibacterial. A clone designated AGK27 was selected as a good fermenter; AGK27 possessed the two anticontaminant properties and was an α-mater requiring histidine for growth (*his*4). Thus, a fusion donor became available.

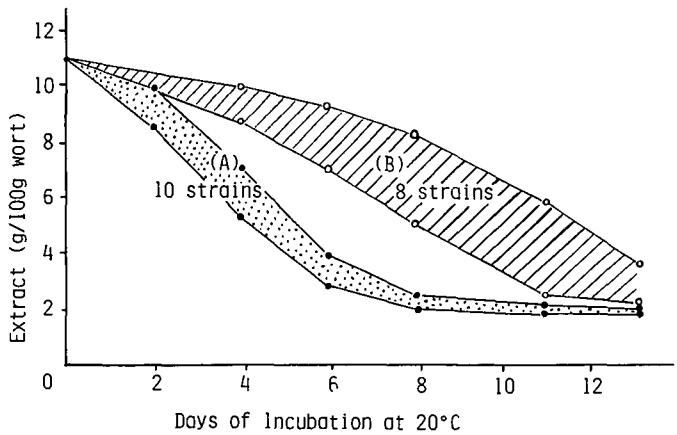

Fig. 2. Relationship between growth on a crystal-violet (CV) plate and wort fermentation rates of fusion products. Ten clones that did not grow (A) and eight that grew (B) on CV plates were randomly selected from among the fusion products. Each clone was incubated in 200 ml of wort, and the apparent extract was measured.

TABLE I
Antibacterial Activities of Four *Saccharomyces* Yeast Strains

Yeast	Bacteria[a] 1	2	3	4	5	6	7	8	9	10	11
S. cerevisiae A2 YB2-2	+++	+++	++	+++	+++	++	+++	+++	+++	++	+++
S. carlsbergensis YB5-1	++	++	+++	+	+++	+	+	+	+++	++	++
S. cerevisiae YB3-1	+	+++	+	+	+++	+++	+	++	+	++	++
S. carlsbergensis YB6-12	+	++	+	+	++	+++	+	++	+	++	+

[a] Bacterial growth in beer inhibited for +++ = 14 days, ++ = 10 days, and + = seven days of incubation at 25°C under a large inoculum.

Protoplast Fusion with a Brewer's Yeast

Protoplast fusion is an advantageous method of simultaneously introducing cytoplasmic (killer) and chromosomal (antibacterial) genetic factors into a lager yeast that generally lacks mating ability.

Fusion Between AGK27 and Sapporo Yeast. To add a selective marker (RD), Sapporo yeast was treated with acriflavine. Cells of the RD mutant of Sapporo yeast (1.3×10^9) and those of AGK27 (*his*4) (3.2×10^9) were protoplasted and fused in 3 ml of PEG4000 (40%) solution containing 50 m*M* of $CaCl_2$. Each 0.1 ml of the fusion solution was embedded in 12 plates of hypertonic minimum glycerol agar. After incubation for two weeks at 30°C, 250 colonies appeared. The apparent frequency of fusion was 2×20^{-6} per regenerated protoplast.

Selection of the Fusants. Fifty-five fusion products were picked randomly from the plates, and properties derived from the parental yeasts were tested. Those from AGK27 were killer, antibacterial, and respiratory-sufficient; those from Sapporo yeast were autotrophic, were unable to grow on crystal-violet (CV) agar (8), and had good fermentation ability. Cell size and giant colony appearance of each fusion product were also recorded.

All 55 fusion products were prototrophic and killer, but only 32 were antibacterial and only 15 grew on CV agar. Their fermentation rates at 20°C varied over a wide range. Of the 17 fusants studied for behavior in wort fermentation at 10°C and for flavor of the beer produced, one, MAK61, was selected and studied in detail.

Wort fermentation ability was correlated with yeast growth response on CV agar. AGK27 grew on CV agar but Sapporo yeast did not. Accordingly, the fusants were divided into group A, those that did not grow on CV plates and fermented wort rapidly, and group B, those that grew on CV plates and did not ferment wort rapidly (Fig. 2). This technique was useful for easy, rapid selection of good fermenters from among many candidates after a mating or protoplast fusion between a brewer's yeast and a wild yeast.

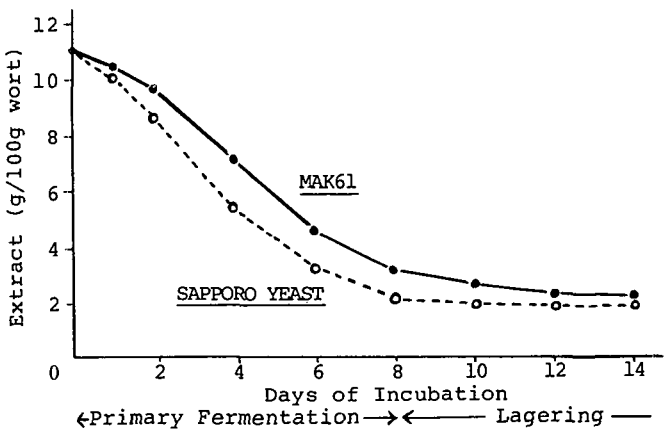

Fig. 3. Wort fermentation rates. MAK61 (closed circle) and Sapporo yeast (open circle) were pitched (0.4%, w/v, pressed yeast) separately into 20 L of wort. Fermentation and lagering were done for eight days at 10°C and for 32 days at 0°C, respectively.

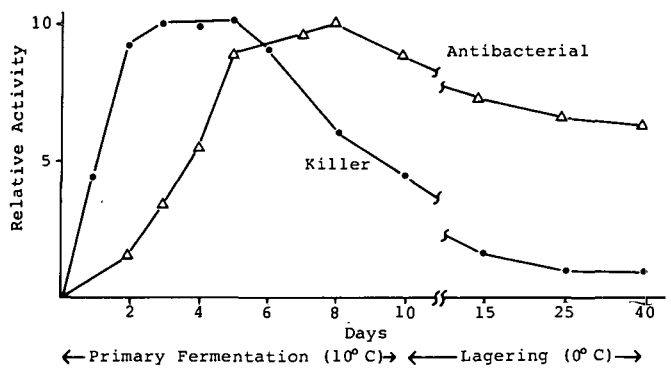

Fig. 4. Formation and fate of killer and antibacterial activities during fermentation. Killer factor was formed at an early stage of primary fermentation, and activity was maintained at a high level for several days, then gradually decreased. Antibacterial activity was highest toward the end of primary fermentation and was maintained at a relatively high level throughout the lagering stage.

Properties of MAK61. In a pilot-scale fermentation using MAK61, 80 g of pressed yeast cells was pitched into 20 L of wort. Throughout fermentation at 10°C and lagering at 0°C, the apparent extract, killer, and antibacterial activities of the beer were measured.

MAK61 fermented wort more slowly than the parent Sapporo yeast (Fig. 3), perhaps because of undesirable genes introduced from AGK27 through the fusion process. The killer activity of the fusant increased greatly during the early stage of primary fermentation, was maximum for three days, then decreased rapidly, with little significant activity retained in finished beer (Fig. 4). This might reflect instability of the killer factor (12). Contamination by wild yeasts, however, can be avoided by using such "engineered" killer brewer's yeast.

The antibacterial activity of the fusant was lower under ordinary conditions than in small-scale fermentations at 20°C. The maximum activity in the beer failed to inhibit the growth of three of the 11 beer-spoilage bacteria at a dosage of 2×10^5 cells per milliliter after incubation at 25°C for 10 days. This could be due to complex regulation or expression of the antibacterial gene(s).

Although our taste panel judged the beer obtained in this trial brew acceptable with respect to flavor and foam, the fusant had a weaker fermentation ability than Sapporo yeast and lower antibacterial activity than A2. Further improvement is desirable.

Manipulation of Brewing Yeast

In general, when a useful genetic character is introduced into a brewer's yeast from other yeasts through direct mating or protoplast fusion, unfavorable characters derived from the yeast partners are also introduced. Our proposal to overcome such a disadvantage is to improve the brewing characteristics of a yeast partner before mating or protoplast fusion with a current brewery yeast.

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