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# Application of a Ribosomal DNA Integration Vector in the Construction of a Brewer's Yeast Having α-Acetolactate Decarboxylase Activity

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**An integration plasmid, pIARL28, containing the ribosomal DNA gene as a homologous recombination sequence was constructed for introduction of the α-acetolactate decarboxylase gene into brewer's yeast. The transformation efficiency of pIARL28 was 20- to 50-fold higher than those of the other YIp vectors, as yeast cells had approximately 140 copies of the ribosomal DNA gene. All transformants showed very high α-acetolactate decarboxylase activity due to the multiple integrated copies of the plasmid. The transformants were grown in nonselective conditions, and segregants which had maintained the α-acetolactate decarboxylase expression cassette but no other vector sequences were isolated. Southern analysis showed that these marker-excised segregants contained more than 20 copies of the α-acetolactate decarboxylase gene and were stably maintained under nonselective conditions. Fermentation tests confirmed that the diacetyl concentration was considerably reduced in wort fermented by these marker-excised segregants. The degree of reduction was related to the copy number of the α-acetolactate decarboxylase gene.**

Diacetyl (DA) is a well-known component of yeast-derived off-flavor in beer. During fermentation, an intermediate of the isoleucine-valine pathway in yeasts, α-acetolactate, is leaked into fermented wort and converted to DA nonenzymatically (8). α-Acetolactate decarboxylase (ALDC; EC 4.1.1.5) is an enzyme that converts α-acetolactate directly to acetoin, which has no effect on beer flavor. This enzyme was found in many bacteria but not in yeasts (3).

Recently, we cloned the ALDC gene from *Enterobacter aerogenes* (14) and expressed this gene in brewer's yeast on a YEp plasmid (yeast episomal plasmid containing the origin of 2μm DNA) to reduce the DA content of fermented wort (young beer) (13). Fermentation tests showed that total DA (vicinal diketone and acetohydroxy acid) production of the transformants was considerably lower than that of the parent yeast.

Generally, YEp plasmids are not stably maintained in yeast cells under nonselective conditions; therefore, it was suspected that the transformants might lose the ALDC activity during successive fermentation. To construct a strain with stable ALDC activity, YIp plasmids (yeast integration plasmids) might be the most practical to use. However, YIp plasmids do have some disadvantages for genetic engineering of brewer's yeast. First, transformation efficiency with YIp plasmids is much lower than that with YEp plasmids (15). Since the transformation frequency of brewer's yeasts is low, it is fairly difficult to obtain a sufficient number of transformants with YIp plasmids. Second, since the integrants usually have one or two copies of the YIp plasmid, the expression level of the introduced gene product is fairly low.

To overcome these disadvantages, we constructed a new YIp plasmid, pIARL28, which had a ribosomal DNA (rDNA) sequence as a homologous recombination sequence. Since the rDNA sequence of pIARL28 exists at about 140 copies in a yeast cell, integration at the rDNA genes occurs very efficiently. By using this plasmid, multiple copies of the ALDC gene were introduced into brewer's yeast. Segregants which maintained more than 20 copies of the ALDC gene but no undesirable bacterial sequences were isolated from the transformants.

Fermentation tests showed that the segregants were able to reduce the total DA concentration of the fermented wort without any effect on the other characteristics of the beer. The stability and expression level of the ALDC gene on plasmid pIARL28 are also discussed.

## MATERIALS AND METHODS

**Microorganisms.** Brewer's yeast, *Saccharomyces carlsbergensis* IFO0751, was obtained from the Institute for Fermentation, Osaka, Japan, and was used as the parent brewer's yeast strain. *S. cerevisiae* TD4 (**a** *his4-519 ura3-52 leu2-3 leu2-112 trp1*) was used to check the transformation efficiency of pIARL28. *Escherichia coli* DH1 (F *recA1 endA1 gyrA96 thi-1 hsdR17 supE* [*relA1*?]) was used as a host for plasmid construction.

**Plasmids.** pIARL28 was constructed from yeast integration vector YIp5. The rDNA gene was obtained from a bacteriophage library of *S. cerevisiae* F16C (α *pho5*), and a 3.0-kilobase (kb) *Eco*RI fragment containing a part of the 5.8S and 25S rDNA genes was used as the homologous recombination sequence. The ALDC expression cassette consisting of the yeast *ADC1* (alcohol dehydrogenase) promoter, ALDC coding region, and the yeast *HIP1* (histidine permease) (18) terminator was inserted into the *Bgl*II site of the 3.0-kb rDNA fragment.

The Geneticin G418-resistant gene was cloned from pUC4K (Pharmacia, Uppsala, Sweden) as a *Sal*I fragment. The *E. coli lacZ* fragment, which lacks its promoter and the first eight nonessential N-terminal amino acid codons, was cloned from pMC1871 (Pharmacia) as a *Bam*HI fragment and ligated to the yeast HIP1 promoter and its N-terminal amino acid codons in frame.

**Media and Materials.** *E. coli* strains were grown in LB

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medium at 37°C. Plasmid-containing strains were cultivated in medium containing ampicillin at a final concentration of 50 μg/ml.

Restriction endonucleases, T4 DNA ligase, the Klenow fragment of *E. coli* polymerase I, and the *Bam*HI linker were purchased from Takara Shuzo, Kyoto, Japan, and Boehringer Mannheim, Pentzberg, Federal Republic of Germany.

The antibiotic Geneticin G418 was purchased from GIBCO, Chagrin Falls, Ohio, and X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside was from Boehringer Mannheim.

**Transformations and marker excisions.** *E. coli* transformations were carried out by the method of Hanahan (5). Yeast transformations were performed by the method of Ito et al. (7). When transformants were selected on the basis of G418 resistance, the following modifications were carried out. After a cell suspension containing polyethylene glycol 4000 and DNA was incubated for 1 h at 30°C, the suspension was heat shocked and suspended in a mixture of 0.9 ml of YPD medium (2% Bacto-Peptone, 1% yeast extract, 2% glucose) and 0.1 ml of 10% filtered yeast extract. After incubation at 30°C for 18 to 20 h with shaking, a sample of the cell suspension was plated on a selective YPD agar plate containing Geneticin G418 (200 to 700 μg/ml).

To obtain marker-excised clones, the *lacZ* gene was used as a marker. The transformants were cultivated in YPD. After 30 to 40 generations, a sample of the cell suspension was plated onto an M63 plate (4) containing X-Gal. The agar plates were incubated at 30°C for 5 to 7 days, and the white colonies were isolated as marker-excised clones, that is, clones which had lost undesirable bacterial sequences. These were tested for ALDC activity.

Transformants of the first- and second-round transformations which have plasmid pIARL28 at the rDNA locus are referred to as 1AGL and 2AGL clones. The marker-excised clones of the first- and second-round marker excisions which have lost all undesirable sequences but still maintain the ALDC expression cassettes are referred to as 1A and 2A, respectively.

**Southern hybridization.** Total yeast DNA was prepared by the method of Holm et al. (6). DNA samples were separated on agarose slab gels, and Southern hybridization was carried out as described by Maniatis et al. (9). Plasmids pUC4K and pMC1871 were used as probes to detect the G418 resistance gene and the *lacZ* gene, respectively. To deduce the copy number of the ALDC gene, the *Hin*cII-*Bam*HI fragment of the ALDC gene (13) was used as a probe. The intensity of the bands was measured with a densitometer (SC-930; Shimazu Co., Kyoto, Japan).

**ALDC assays and fermentation tests.** To measure ALDC activity, yeast cells were grown to an optical density at 600 nm of 5.0. ALDC assays and the fermentation tests were carried out as described previously (13, 14).

## RESULTS

**Construction of plasmids.** The construction of integration plasmid pIARL28 is shown in Fig. 1. This plasmid has the following characteristics.

The rDNA gene sequence was used as a homologous recombination sequence to increase transformation frequency and to introduce multiple copies of the plasmid into brewer's yeast. The rDNA genes consist of approximately 140 copies of a tandemly repeated segment in the yeast genome (1, 11). A 3.0-kb *Eco*RI fragment containing part of 5.8S and 25S rDNA genes was used as a target sequence for integration (Fig. 1). This fragment does not have an ARS sequence, which has been reported by Skryabin et al. (12). For efficient selection of transformants, the G418 resistance gene and the *lacZ* gene were used as markers.

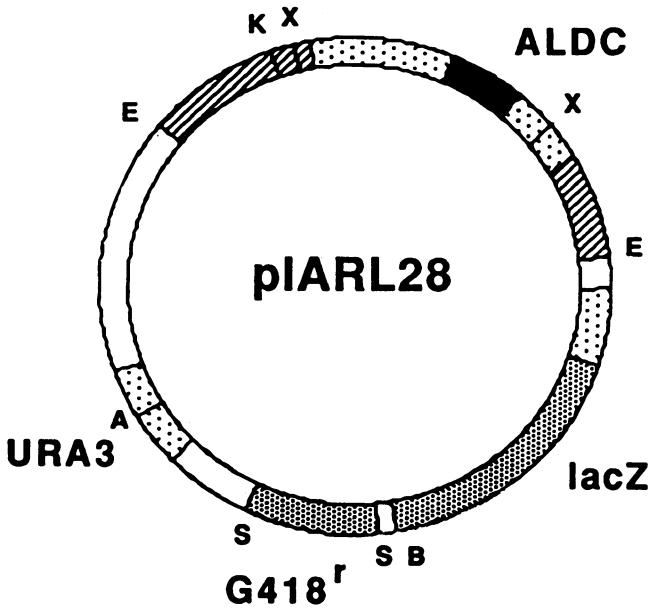


FIG. 1. Construction of pIARL28. The ALDC gene is represented by the filled area; the *S. cerevisiae* rDNA sequences, by hatched areas; the G418-resistant gene and the *E. coli lacZ* gene by heavily stippled area; other yeast DNA sequences, by slightly stippled areas; and pBR322 sequences, by open areas. E, *Eco*RI; K, *Kpn*I; A, *Apa*I; S, *Sal*I; X, *Xba*I.

Another characteristic of plasmid pIARL28 was that, following integration of the plasmid sequence into the rDNA genes loci, all plasmid sequences except the ALDC expression cassette could be excised by spontaneous homologous recombination.

**Transformation efficiency of pIARL28.** Plasmid pIARL28 was used to transform the laboratory yeast strain TD4 after being linearized with *Apa*I (to integrate the plasmid into the *URA3* locus) or *Kpn*I (to integrate the plasmid into the rDNA locus). Integrants were selected on the basis of uracil prototrophy or resistance to G418, respectively. The number of transformants obtained with the *Kpn*I-linearized plasmid was more than 20- to 50-fold higher than that obtained with the *Apa*I-linearized plasmid (Table 1). This result indicated that the rDNA genes were useful target sequences because they enhanced integration efficiency due to their high copy number in the genome.

**Transformation of brewer's yeast.** Brewer's yeast strain IFO0751 was transformed with plasmid pIARL28 linearized

TABLE 1. Transformation efficiency of pIARL28[a]

| Agar | Transformation efficiency of pIARL28 (colonies per μg of DNA) | |
|---|---|---|
| | *Apa*I cut | *Kpn*I cut |
| Uracil | 9 | 463 |
| YPD | | |
| 200 μg of G418 per ml | 3 | 63 |
| 400 μg of G418 per ml | 2 | 40 |

[a] With no DNA, transformation efficiency was 0.

TABLE 2. ALDC activity and relative ALDC gene copy number of transformants and marker-excised segregants

| Clone no. | ALDC activity (U/mg) | Relative ALDC gene copy no. |
|---|---|---|
| 2-2(1AGL) | 2.00 | 27 |
| 2-3(1AGL) | 1.70 | NT[a] |
| 9(*URA3*) | 0.06 | NT |
| 10(*URA3*) | 0.04 | NT |
| 2166(1A) | 0.23 | 10 |
| 21613(1A) | 0.24 | 12 |
| 21(1A) | 0.10 | 8 |
| 22(1A) | 0.05 | 4 |
| 131(1A) | 0.06 | 2 |
| 134(1A) | 0.04 | 2 |
| 137(1A) | 0.02 | 1 |
| 133(2AGL) | 2.40 | NT |
| 140(2AGL) | 2.00 | NT |
| 13313(2A) | 0.74 | 23 |
| 13314(2A) | 0.79 | 23 |
| 14015(2A) | 0.75 | NT |

[a] NT, Not tested.

with either *Apa*I or *Kpn*I. The frequency of transformation of *Kpn*I-linearized pIARL28 was also about 20-fold higher than that of *Apa*I-linearized pIARL28 (data not shown). To confirm the sites of integration, 10 clones from each were analyzed by Southern hybridization. In all clones transformed with *Kpn*I-linearized plasmids, pIARL28 integrated only into the rDNA locus. In all clones transformed with *Apa*I-linearized plasmids, pIARL28 integrated into the *URA3* locus, but in eight of these clones pIARL28 also integrated into the rDNA locus (six clones) or another unknown locus (two clones).

All transformants with plasmid pIARL28 integrated at the rDNA locus (Table 2, clones 2-2 and 2-3) showed about 30- to 50-fold-higher ALDC activity than transformants with plasmid pIARL28 integrated at the *URA3* locus (Table 2, clones 9 and 10). It was assumed that this was caused by multiple copies of the plasmid integrated at the rDNA locus.

**Excision of undesirable sequences.** To isolate clones free of undesirable sequences (G418 resistance gene, *lacZ* gene, and bacterial vector sequences), the 1AGL clones (see Materials and Methods) were grown in nonselective medium to obtain clones which were sensitive to G418 and appeared as white colonies on the X-Gal plate. Integration of pIARL28 by homologous recombination generated a nontandem direct duplication (Fig. 2B). Possible structures after excision are indicated in Fig. 2C. When recombination occurred at the 5′ side of the ALDC expression cassette, the yeasts lost the entire plasmid, but when it occurred at the 3′ side, the ALDC expression cassette was retained in the genome without undesirable vector sequences.

To confirm this hypothesis, several 1A clones were obtained and subjected to Southern analysis. Plasmids pUC4K and pMC1871 were used as probes to detect the G418 resistance and the *lacZ* genes, respectively, and this confirmed that the 1A clones had completely lost their vector sequences (Fig. 3B). The size of the ALDC expression cassette was also checked, using the ALDC gene as a probe. No unexpected gene rearrangement was detected in this region (Fig. 3C).

To investigate the gene dosage effect of several independent clones (1AGL, 1A, and 2A), their relative ALDC gene copy numbers and ALDC activities were checked. To check the relative copy number, Southern analysis was carried out and the signal of the *LEU2* gene was used to normalize the DNA for loading. The copy number of the ALDC gene varied considerably among independent 1A clones. Clone 21613(1A) had >10 times more copies of the ALDC gene than clone 137(1A) (Table 2). That is, more than 10 copies of the ALDC gene were integrated into the chromosome of clone 21613(1A). It was also observed that most 1A clones had ALDC activity fairly proportional to ALDC gene copy number (Table 2). However, the ALDC activity of clone 2-2(1AGL) was about 10 times higher than that of clone 21613(1A), although the ALDC gene copy number of clone 2-2(1AGL) was only about twice that of clone 21613(1A) (Table 2). These results indicated that there might be some difference in the expression level of the ALDC gene in 1AGL and 1A clones.

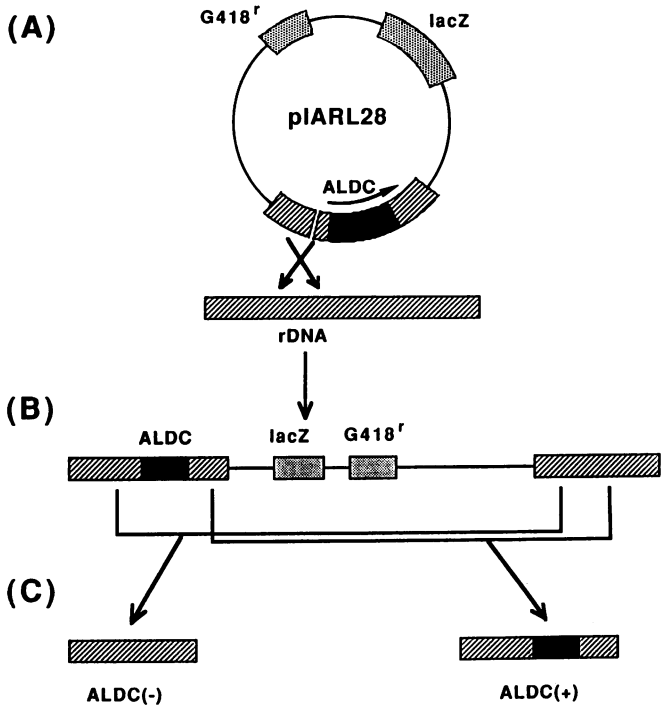


FIG. 2. Excision of undesirable sequences. (A) Homologous recombination at rDNA locus between pIARL28 and yeast chromosomal DNA. (B) Structure of rDNA gene with integrated plasmid. (C) Possible structures after excision of transforming DNA.

**Construction of clones with increased ALDC activity.** As the rDNA genes consist of 140 copies of a tandemly repeated segment and the 1A clones had only 10 copies of the ALDC expression cassettes, it was expected that, by repeating transformation and marker excision, further copies of the ALDC gene would be introduced into the 1A clones. Clone 21613(1A) was chosen as the host for secondary transformation and marker excision. All 2AGL clones showed ALDC activity as high as that of 1AGL (Table 2). The 2A clones had 0.1 to 0.9 U/mg of protein. Several 2A clones with higher ALDC activity than clone 21613(1A) were isolated and analyzed further. Southern analysis indicated that such 2A clones contained more copies of the ALDC expression cassette than clone 21613(1A) (Table 2). It was also noted that these clones had completely lost the undesirable vector sequences (Fig. 3B).

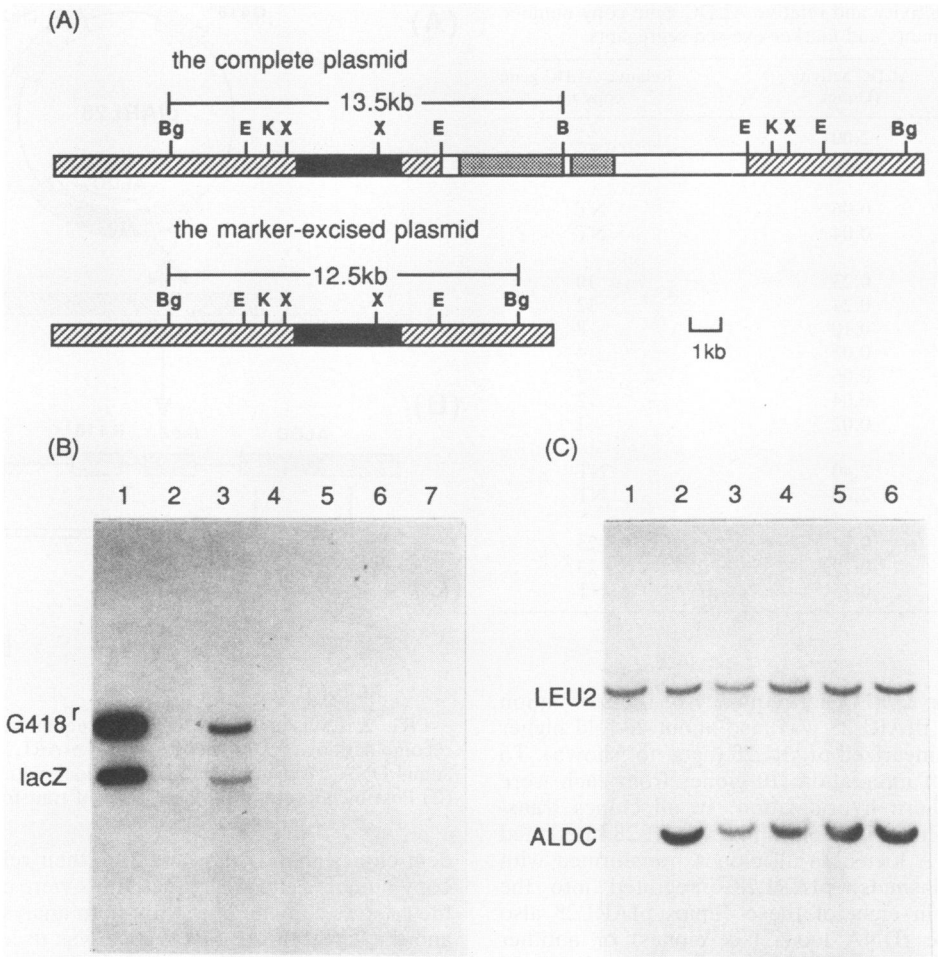


FIG. 3. Southern analysis of the 1AGL, 1A, and 2A clones. All DNA samples were cleaved with *Bam*HI and *Xba*I and electrophoresed in 0.8% agarose gels. (A) Restriction map of pIARL28 integrated at rDNA genes (the complete plasmid, before marker excision; the marker-excised plasmid, after marker excision). The ALDC expression cassette is represented by the filled area; the G418 resistance gene and the *lacZ* gene, by stippled areas; the yeast rDNA gene, by the hatched areas; and YIp5 sequences, by open areas. B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; K, *Kpn*I; X, *Xba*I. (B) DNA was hybridized with G418 (pUC4K) and *lacZ* (pMC1871) probes. Lane 1, pIARL28 was cleaved with *Bam*HI and *Xba*I; lane 2. IFO0751; lane 3, 2-2(1AGL); lane 4, 2166(1A); lane 5, 21613(1A); lane 6, 13313(2A); lane 7, 13314(2A). (C) DNA was hybridized with ALDC and *LEU2* (internal marker) probes. Lane 1, IFO0751; lane 2, 2-2(1AGL); lane 3, 21613(1A); lane 4, 2166(1A); lane 5, 13313(2A); lane 6, 13314(2A).

**Stability of ALDC activity and the ALDC gene.** By culturing the 1AGL, 1A, 2AGL, and 2A clones in nonselective conditions, the stability of the ALDC activity was investigated (Fig. 4A to D). The 1AGL and 2AGL clones initially showed high ALDC activity; however, their activity decreased by 50% after 40 generations. In contrast, initial ALDC activity of the 1A and 2A clones was lower than that of the 1AGL and 2AGL clones, but both clones 1A and 2A stably maintained ALDC activity for more than 80 generations.

To investigate the cause of the different ALDC stability between the 1AGL and 2AGL clones and the 1A and 2A clones, chromosomal DNA was prepared from a 1AGL clone after nonselective cultivation and was digested with *Bam*HI and *Bgl*II. Southern analysis with the ALDC gene as a probe was carried out. The 1AGL and 2AGL clones were expected to contain only the complete plasmid, and the 1A and 2A clones were expected to contain only the marker-excised plasmid (the ALDC expression cassette without vector sequences). If this was the case, a single 13.5-kb band would be detected in the 1AGL and 2AGL clones and a single 12.5-kb band would be detected in the 1A and 2A clones (Fig. 3A).

In fact, an additional 12.5-kb band corresponding to the marker-excised plasmid was detected along with the 13.5-kb band in the 1AGL clones (Fig. 5A). This result suggested that excision by spontaneous recombination had already occurred even under selective conditions. The same experiment was carried out with the 1A, 2A, and 2AGL clones and the same results were observed for the 2AGL clones (data not shown). During nonselective cultivation, the complete plasmid was lost rapidly, while the marker-excised plasmid was stably maintained (Fig. 5A). To determine the loss of the complete plasmid (13.5 kb) more accurately, we also carried out Southern hybridization with the *lacZ* gene as a probe to detect only the complete plasmid. The copy numbers of the two types of plasmid were calculated from the intensity of the bands. It appeared that the decrease in ALDC activity was closely related to that in the copy number of the complete plasmid (Fig. 5B).

This result suggested that the high ALDC activity of the 1AGL clones might be related to high-level expression of the

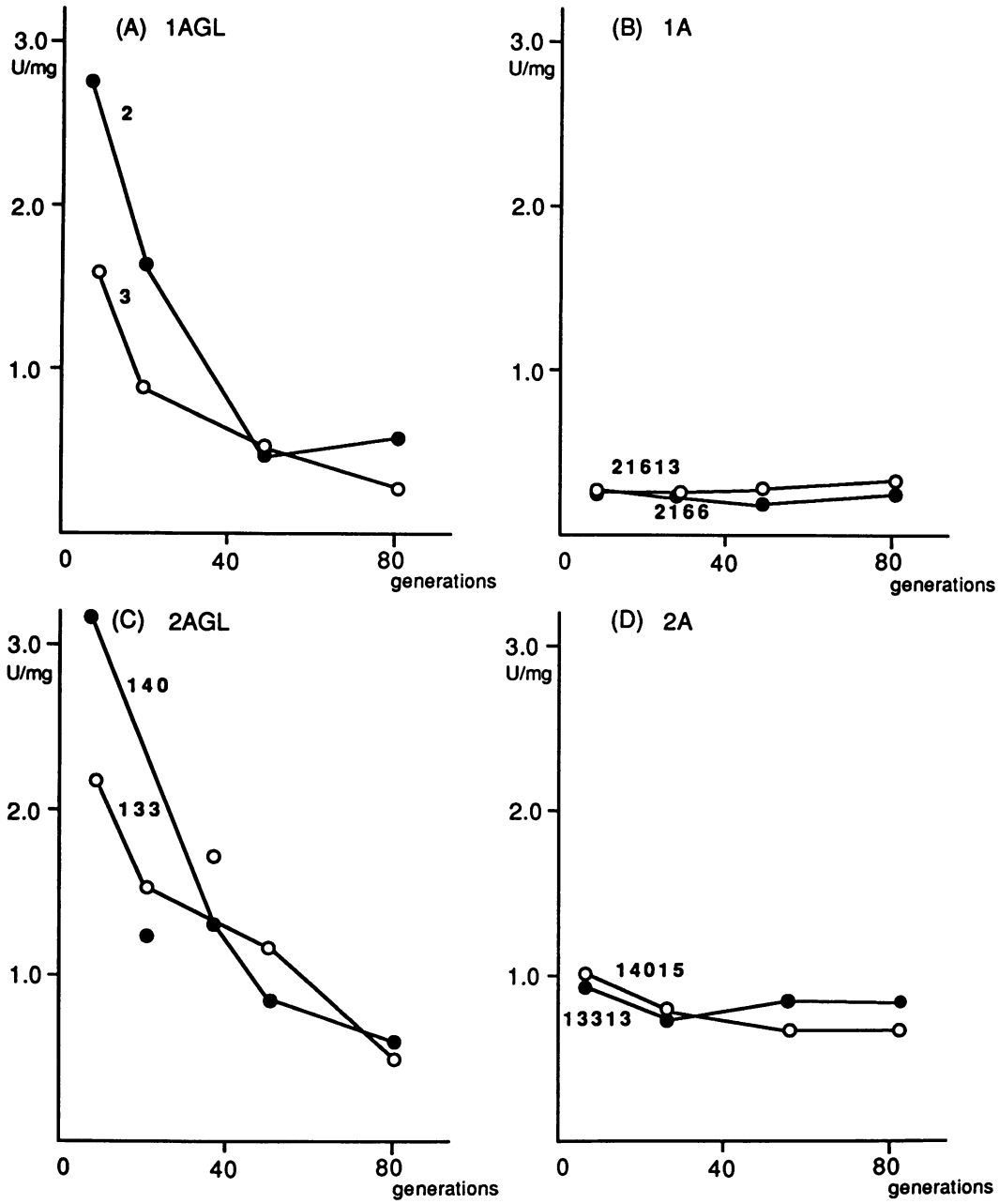


FIG. 4. Stability of ALDC activity under nonselective conditions.

ALDC genes of the complete plasmid. The complete plasmid was gradually lost from the 1AGL clones under nonselective conditions and the level of ALDC activity also decreased, but the marker-excised plasmid was stably maintained and expressed a low level of ALDC activity.

**Fermentation tests.** We carried out laboratory-scale fermentation tests, using clones 21613(1A) and 13313(2A). Compared with the parent strain, total DA concentrations in the fermented wort were reduced by one-half and two-thirds, respectively (Table 3). This result suggested that both segregants had sufficient activity to reduce the total DA of fermented wort and that the reduction ratio of total DA was related to the ALDC activities of the clones. It was also shown that the growth rate of yeasts and its alcohol production ability were not affected by the multicopy integration of pIARL28 into rDNA genes.

## DISCUSSION

**Efficient transformation and multicopy integration of pIARL28.** We constructed a new YIp plasmid, pIARL28, with the rDNA sequence as a homologous sequence and were successful in transforming the laboratory (TD4) and brewer's (IFO0751) strains efficiently. Skryabin et al. (12) previously reported the presence of an ARS sequence in the yeast genomic rDNA genes. However, the 3.0-kb *Eco*RI fragment used as a recombination target sequence contains no ARS sequence, so all of the plasmids should be inte-

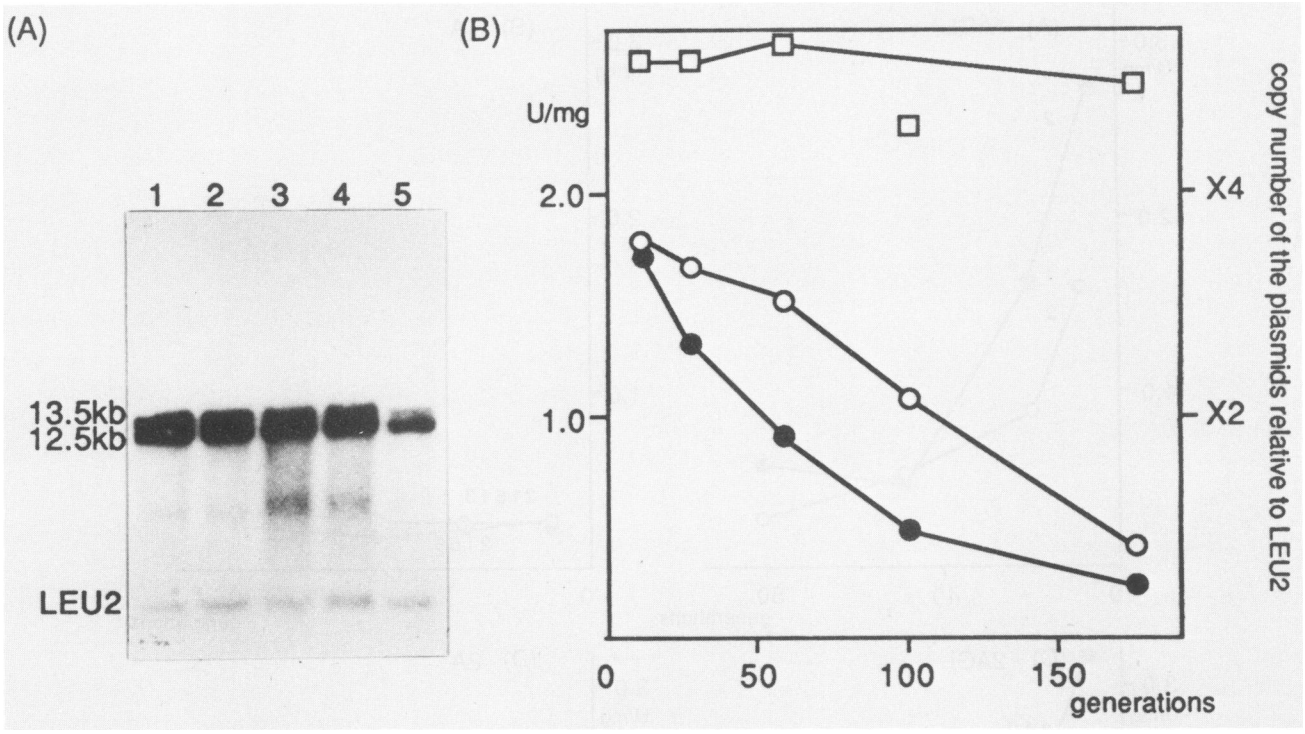


FIG. 5. Stability of two types of plasmids and ALDC activity. (A) 2-2(1AGL) was cultured under nonselective conditions and total DNA was prepared at the respective generation as described by Holm et al. (6). All samples were cleaved with *Bam*HI and *Bgl*II and electrophoresed in 0.4% agarose gel. The ALDC coding region was used as a probe. Lane 1, 13th generation; lane 2, 28th generation; lane 3, 58th generation; lane 4, 102nd generation; lane 5, 178th generation. (B) Stability of the two types of plasmid and ALDC activity. Symbols: ○, copy number of complete plasmids (measured by using the *lacZ* gene as a probe); □, copy number of marker-excised plasmids (measured by using the ALDC gene as a probe); ●, ALDC activity. The *LEU2* gene was used to normalize the DNA for loading.

grated. Southern analysis was carried out, and this confirmed that no episomal plasmid was present (data not shown).

It was also shown by Southern analysis that, in most of the transformants, each copy of pIARL28 had been integrated into separate loci of the tandemly repeated 140 copies of the rDNA gene, and a tandemly integrated structure which was reported by Szostak and Wu (16) was detected in a few clones.

Yocum (19) reported that by repeating transformation and marker excision several copies of a gene of interest could be introduced into a polyploid strain, since a polyploid strain has several copies of the homologous recombination sequence. However, repeating so many rounds of transformation and marker excision is time-consuming. The possible copy number of the integrated gene at one time is limited by the copy number of the homologous recombination sequence, so it is impractical to introduce more than 10 copies by using a gene which exists as 2 or 3 copies as a target for integration. Our results showed that the rDNA gene was one of the most useful target sequences for integrating multiple copies of the plasmid into the chromosome of yeasts. Using plasmid pIARL28, clones which had more than 20 copies of the ALDC gene and no undesirable sequences were easily obtained after only two rounds of transformation and marker excision.

TABLE 3. Analysis of young beer brewed by 1A and 2A

| Strain | Total DA (mg/liter) | Apparent extract (Plato)[a] | Apparent attenuation (%) | Ethanol (%, vol/vol) | Yeast multiplication ratio[b] |
|---|---|---|---|---|---|
| Expt 1 | | | | | |
| IFO0751 | 0.98 | 2.3 | 80.0 | 4.8 | 3.8 |
| 21613(1A) | 0.48 | 2.1 | 80.1 | 4.8 | 3.9 |
| Expt 2 | | | | | |
| IFO0751 | 0.63 | 1.9 | 82.6 | 5.0 | 3.3 |
| 13313(2A) | 0.21 | 2.0 | 82.8 | 5.0 | 3.3 |

[a] [(Extract content of wort − apparent extract of beer)/extract content of wort] × 100.

[b] Yeast cell wet weight after fermentation/yeast cell wet weight before fermentation.

Previously, Boeke et al. (2) reported that yeast retrotransposon Ty can be used to insert multiple copies of a gene into a new site in the genome. However, since Ty integration occurs at random throughout the yeast chromosome, it would be necessary to evaluate the effect of Ty integration on the metabolism of each individual transformant before selecting a clone for practical application. The results of our fermentation tests suggested that multicopy integration into the rDNA has no adverse effect on beer fermentation.

**Expression and stability of pIARL28.** The 1A and 1AGL clones differ in the stability of their ALDC activities in nonselective conditions. Southern analysis showed that the 1A clones contained only the marker-excised plasmid but the 1AGL clones contained both the marker-excised and the complete plasmids and the former was stable but the latter was unstable under nonselective conditions. This result suggested that the difference in the stability of ALDC activity in the 1AGL and 1A clones was caused by the

different stability of the two types of plasmid. It is suggested that the spontaneous homologous recombination between the plasmid and chromosomal rDNA sequences occurs frequently so that the 1AGL clones lose the complete plasmid rapidly. Previous reports have also suggested that the plasmid which has a nontandemly duplicated structure (like the complete plasmid) is less stably maintained than the plasmid which has lost one of the duplications (like the marker-excised plasmid) under nonselective conditions (10, 15). The size difference between the two types of plasmid also could be responsible for stability, as the size of the complete plasmid is 18 kb while that of the marker-excised plasmid is 3.5 kb.

Szostak and Wu (17) reported that unequal crossing over occurred at the rDNA locus during mitosis. To confirm that this event does not seriously affect the stability of pIARL28, clone 2(1A) was plated onto the plate containing X-Gal after culturing under nonselective conditions. More than 98% of the colonies were blue even after 100 generations, meaning that the unequal crossing over occurs rarely in our strain.

The initial ALDC activities of the 1A and 1AGL clones were quite different. From the observation that the ALDC activity of clone 2-2(1AGL) was almost 10 times higher than that of clone 21613(1A), although the copy number of clone 2-2(1AGL) was almost twice that of clone 21613(1A), it is suggested that the expression level of the complete plasmid is higher than that of the marker-excised plasmid. This hypothesis is also supported by evidence showing that the decrease in ALDC activity of clone 2-2(1AGL) under nonselective conditions was closely related to loss of the complete plasmid but not to that of the marker-excised plasmid (Fig. 5). The mechanism causing the high expression level of the ALDC gene of the complete plasmid is not clear. The chromatin structure might be involved, but further experiments are needed to clarify this.

The results of our fermentation test have suggested that both 21613(1A) and 13313(2A) strains had sufficient ALDC activity to reduce the total DA concentration in fermented wort. The reduction ratio of total DA in the fermented wort was related to the copy number of the ALDC gene. In our previous report (13), clones with the ALDC gene on a YEp plasmid showed ALDC activity of 2.0 U/mg of protein and the total DA reduced to less than one-fourth of that produced by the parent strain. The expression level of the ALDC gene and the decreased ratio of total DA of 21613(1A) and 13313(2A) is lower than that of the clones with the ALDC gene on a YEp plasmid, but it is expected that the expression level of the ALDC gene on pIARL28 could be further increased by fusion to a stronger promoter.

Plasmid pIARL28 is a useful vector for gene transfer into a variety of industrial *Saccharomyces* strains to meet the requirements of commercial application.

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