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Genetic Engineering of a Sake Yeast Producing No Urea by Successive Disruption of Arginase Gene

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Urea is reported to be a main precursor of ethyl carbamate (ECA), which is suspected to be a carcinogen, in wine and sake. In order to minimize production of urea, arginase-deficient mutants (Δ*car1*/Δ*car1*) were constructed from a diploid sake yeast, Kyokai no. 9, by successive disruption of the two copies of the *CAR1* gene. First, the yeast strain was transformed with plasmid pCAT2 (Δ*car1 SMR1*), and strains heterozygous for *CAR1* gene were isolated on sulfometuron methyl plates. Successively, the other *CAR1* gene was disrupted by transformation with plasmid pCAT1 (Δ*car1 G418*^{r}) and the resulting *car1* mutants were isolated on a G418 plate. Arginase assay of the total cell lysate of the mutants showed that 70% of transformants isolated on G418 plates had no detectable enzyme activity, possibly as a result of the disruption of the two copies of the *CAR1* gene. Further genomic Southern analysis confirmed this result. We could brew sake containing no urea with the Δ*car1*/Δ*car1* homozygous mutant. It is of additional interest that no ECA was detected in the resulting sake, even after storage for 5 months at 30°C. This molecular biological study suggests that ECA in sake originates mainly from urea that is produced by the arginase.

Ethyl carbamate (ECA) is a suspected carcinogen (8) found in a variety of fermented beverages and foods (12). Several years ago, trace levels of ECA in some types of wine, sherry, whisky, brandy, and sake were detected in Canada (17). Since this problem is worldwide and involves all fermented beverages, many studies on a mechanism of ECA formation and methods for lowering of ECA content have been done (2, 9, 16, 17). It was reported that this compound can be formed by spontaneous chemical reaction of urea and ethanol in wine (9, 13, 14) and sake (2). Removal of the major precursor, urea, by an acid urease from wine (15, 22) and sake (25) has been examined.

It is known that in the yeast *Saccharomyces cerevisiae*, urea is formed by arginase (7), which is encoded by the *CAR1* gene (21). Recently, we have reported that an arginase-deficient mutant was constructed by *CAR1* gene disruption from a laboratory yeast strain, YNN27 (*MAT***a** *trp1 ura3*) and that this *car1* mutant produced no urea (20). But, since this mutant does not originate from sake yeast, normal sake could not be obtained by a fermentation test. However, these results suggested that there is a possibility to brew sake containing no urea. Therefore, in order to elucidate a role of the *CAR1* gene product, arginase, in sake brewing, we tried to construct an arginase-deficient sake yeast mutant by double disruption of the *CAR1* gene. Since sake yeast is a diploid, it is necessary to disrupt two copies of the *CAR1* gene. Two kinds of dominant selection markers are required for this purpose, because there is no sake yeast having recessive markers such as *ura3*, *trp1*, and *leu2*, which are commonly used markers in DNA recombinant experiments.

In this report, we describe the construction of the *car1* mutants by gene disruption (Δ*car1*/Δ*car1*) from the sake yeast Kyokai no. 9 (K-9) by using two kinds of plasmids with *G418*^{r} or *SMR1* genes as dominant selection markers. The resulting mutant has been used to brew sake which contains no urea and no ECA.

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MATERIALS AND METHODS

Strains, plasmids, and media. Sake yeast strain K-9 (*S. cerevisiae*), which is one of the most commonly used strains in sake brewing in Japan, was used. This strain is a prototroph and has a diploidy. Yeast cells were grown at 30°C in YPD medium (1% yeast extract [Difco Laboratories, Inc., Detroit, Mich.], 2% polypeptone [Wako Junyaku Co., Osaka, Japan], 2% glucose). G418 plates (11) (1% yeast extract, 2% polypeptone, 2% glucose, 2% agar, 600 μg of geneticin per ml [Sigma Chemical Co., St. Lous, Mo.]) and sulfometuron methyl (SM) plates (1) (0.67% yeast nitrogen base without amino acids, 2% glucose, 2% agar, 100 μg of SM per ml [DuPont Japan, Tokyo, Japan]) were used for detection of transformants. Geneticin, which was dissolved in 20% of ethanol sterilized by filtration, was added to autoclaved YPD medium just before pouring the medium into petri dishes. SM plates were incubated in the dark, as SM is light sensitive. Arg plates (0.17% yeast nitrogen base without amino acids and ammonium sulfate [Difco], 2% glucose, 20 mM arginine, 2% agar) and Orn plates (Arg plates, but with 20 mM ornithine instead of arginine) were used to examine the phenotypes of the transformants. Arginase noninducible medium (0.17% yeast nitrogen base without amino acids and ammonium sulfate, 5 mM ammonium sulfate, 2% glucose) and arginase inducible medium (10 mM arginine was added to the noninducible medium) were used for arginase assays. *Escherichia coli* HB101 was used for the propagation of plasmids and for subcloning. Bacterial methods were as described elsewhere (5). The plasmids used for construction of the disruption plasmids were YCpG11 (11), pWX509 (1), pCAR112 (20), and pUC18 (6).

Yeast cell transformation and gene disruption. Yeast cells were transformed by the lithium acetate method (3). Gene disruption methods used were based on a one-step gene disruption by a double recombination event (18) and an integration by one recombination event at the homologous site (24). Arginase activities of transformants were assayed according to the method of Whitney and Magasanik (23).

DNA isolation and Southern blot analysis. Yeast DNA was isolated in a manner essentially the same as that described

TABLE 1. Raw materials for sake brewing[a]

Addition no.	Amt of: Rice (g) for: Steaming	Koji	Water (ml)	Mash temp (°C)
1	25	10	55	15
2	55	10	75	9
3	80	20	130	7

[a] Precultured yeast cells (about 2.5×10^9 cells) and 1.2 ml of 7.5% lactic acid were added into moromi mash of the first addition. Two days after steamed rice was added, rice koji and water were added into the moromi mash (second addition), and the next day the indicated materials were added (third addition). The sake brewing started at 7°C after the third addition, and the temperature was raised 1°C a day until it reached a maximum of 15°C, where it was kept to the finish of the fermentation. Fermentation profiles were monitored by weighing a loss of CO_2. When the loss was about 60 g, the moromi mash was centrifuged and the supernatant was obtained as sake.

by Sherman et al. (19). Southern hybridization was carried out by using Hybond-N nylon membrane (Amersham) according to the supplier's instructions. Radioactive DNA probe was prepared by nick translation (Boeringer Mannheim) with [α-^{32}P]dCTP (Amersham). Plasmid pCAR112 (20) was used as a probe for analyzing integration of *G418*^{r} or *SMR1* markers in the chromosomal *CAR1* gene.

Sake brewing with *car1* mutants by gene disruption. Laboratory-scale sake brewing was carried out with the *car1* mutants (Table 1). Fermentation profiles were monitored by weighing a loss of CO_2 evolution. When about 60 g (total) was lost (after about 20 days of fermentation), the moromi mash was centrifuged and the supernatant was obtained as sake. General components of the obtained sake were analyzed by the standard method established by the National Tax Administration Agency (10). The composition of amino acids was analyzed in a Hitachi 835 amino acid analyzer. The urea content of the sake was measured by the enzymatic method (2), and the ECA content was measured with a Hewlett-Packard 5890A gas chromatograph (4).

RESULTS

Plasmid construction for *CAR1* gene disruption. Since a sake yeast cell is diploid, two kinds of plasmids were constructed to disrupt both copies of the *CAR1* gene. In the first plasmid, pCAT1, a 1.7-kb fragment from the *G418*^{r} gene was inserted into the *Bgl*II-*Bgl*II gap within the coding region of the *CAR1* gene (Fig. 1A). A 6.5-kb *Bam*HI-*Bam*HI fragment of this plasmid contains the truncated *CAR1* gene and *G418*^{r} marker gene. The second plasmid, pCAT2, was constructed from an *SMR1* marker gene and a 0.9-kb *Hin*dIII-*Pst*I fragment containing only the middle part of the *CAR1* gene (Fig. 1B). The linearization with homologous ends, which arise from digestion of pCAT1 with *Bam*HI and digestion of pCAT2 with *Ava*I, enhanced integration into the recipient *CAR1* locus.

Successive disruption of the *CAR1* gene in sake yeast cells. For *CAR1* gene disruption, first, sake yeast K-9 was transformed with pCAT2 (Δ*car1 SMR1*) linearized by digestion with *Ava*I to integrate at the *CAR1* locus. The resulting integrants, which were expected to have a disrupted *CAR1* gene on one chromosome and a normal *CAR1* gene on the other, were isolated on SM plates. Successively, the three independent transformants were retransformed with pCAT1 (Δ*car1 G418*^{r}) digested with *Bam*HI to disrupt the remaining copy of the *CAR1* gene. As the result of two transformations, the transformants, in which two copies of *CAR1* gene were expected to be disrupted, were isolated on G418 plates.

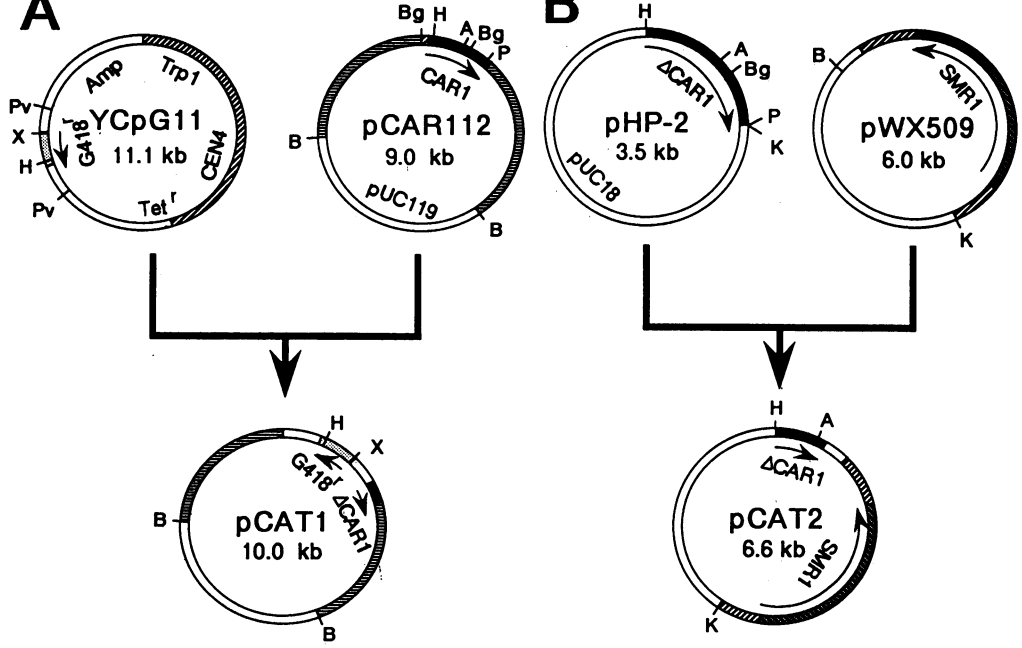

FIG. 1. Plasmid construction for *CAR1* gene disruption. (A) A 1.7-kb *Pvu*II-*Pvu*II fragment containing the *G418*^{r} gene was isolated from YCpG11 (11) and inserted into the *Bgl*II-*Bgl*II gap of pCAR112 (20) to form pCAT1. (B) A 3.2-kb *Bam*HI-*Kpn*I fragment containing the *SMR1* gene from pWX509 (1) was inserted into the *Bgl*II-*Kpn*I gap of pHP-2 to form pCAT2. *G418*^{r} and *SMR1* are genes encoding G418 (geneticin) and SM resistance and were used for dominant selection markers. A, *Ava*I; B, *Bam*HI; G, *Bgl*II; H, *Hin*dIII; K, *Kpn*I; P, *Pst*I; Pv, *Pvu*II; X, *Xho*I.

TABLE 2. Phenotypes of the transformants and the parent strain, K-9

Strain	Genotype	Growth[a] on:			
		Orn plate	Arg plate	SM plate	G418 plate
Type A	Δ*car1*::*SMR1*/Δ*car1*::*G418*^{r}	+	−	+	+
Type B	Δ*car1*::*G418*^{r}/*CAR1*	+	+	−	+
Type C	Δ*car1*::*SMR1*/*CAR1*	+	+	+	−
K-9	*CAR1*/*CAR1*	+	+	−	−

[a] Growth was checked after incubation at 30°C for 3 days. +, Growth; −, no growth.

The frequency of the first transformation with pCAT2 and that of the second with pCAT1 were about nine and seven transformants per 1 μg of plasmid DNA, respectively. Table 2 shows growth phenotypes of the transformants arisen from the course of this study on Orn, Arg, G418, and SM plates. The transformant (type C) with pCAT2 (Δ*car1 SMR1*) was able to grow on the Orn, Arg, and SM plates but not on the G418 plate. On the other hand, the second transformants with pCAT2 (Δ*car1 SMR1*) and pCAT1 (Δ*car1 G418*^{r}) were divided into two groups with different phenotypes. One of them (type A) showed resistance to both SM and G418, but the other (type B) showed resistance only to G418. Type A transformants, which were expected products with both copies of the disrupted *CAR1* gene, were not able to grow on Arg plates, whereas type B transformants grew on both Arg and Orn plates. Analysis of the growth phenotypes showed that 70% of the transformants selected on G418 plates were type A. These results suggested that a replacement of the normal *CAR1* gene in the type C strain with the 6.5-kb *Bam*HI-*Bam*HI fragment of pCAT1 caused type A and that a replacement of the disrupted CAR1 gene caused type B (Table 2).

Arginase activities of the *car1* mutants by gene disruption. Arginase activities in the cell extracts from the parent strain, K-9, and three types of transformants were assayed (Table 3). Arginase activities of type B and C strains were almost the same as that of the parent strain, K-9, while no activity was detected in the type A strain. This result supports the possibility that both copies of the *CAR1* gene are disrupted in type A strains and that one copy of the *CAR1* gene is left intact in types B and C.

Southern blot analysis of *car1* mutants by gene disruption. Genomic DNAs isolated from K-9 and type A, B, and C transformants were digested with *Bam*HI, and then Southern blot analysis was carried out with a pCAR112 DNA fragment as a probe (Fig. 2). In the parent strain, K-9, a single 6-kb fragment from the intact *CAR1* gene was detected. In type B and type C transformants, 7- and 13-kb fragments, respectively, in addition to the 6-kb fragment were observed. Type A transformant showed two bands (13 and 7 kb), which were expected from *Bam*HI digestion of integrants with pCAT1 and pCAT2 at the *CAR1* locus. Some of the transformants showing the same phenotype as type A strain showed an abnormal fragment pattern (Fig. 2, lane 5), suggesting that a rearrangement occurs upon integration with a certain frequency.

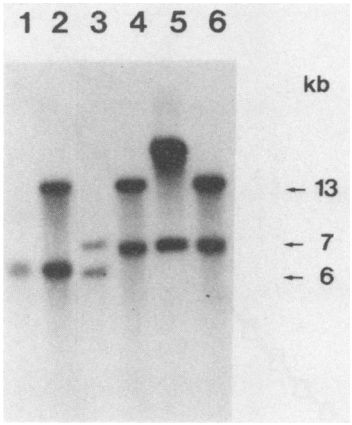

FIG. 2. Southern blot analysis of the *car1* mutants by gene disruption. Total yeast genomic DNA was digested with *Bam*HI, electrophoresed on 0.8% agarose gels, transferred to a nylon membrane, and hybridized with a ^{32}P-labeled pCAR112 probe. Lane 1, K-9 (wild-type parent); lane 2, type C transformant; lane 3, type B transformant; lanes 4 to 6, type A transformants. The transformant in lane 5, which shows the same growth phenotype as type A, may be caused by a rearrangement of the *CAR1* locus.

TABLE 3. Arginase activities of the wild-type and *car1* mutant strains

Strain	Arginase activity[a] (μmol of urea/h/mg of protein) in:	
	Induced medium	Noninduced medium
Type B-1	85.2	30.3
Type B-2	85.6	25.2
Type C-1	83.7	34.4
Type C-2	100.4	31.8
K-9	111.8	41.9

[a] No activity was detected in type A-1 and A-2 strains. The limit of detection in the assays for urea was 0.5 μg/ml.

Sake brewing with *car1* mutants by gene disruption. To investigate the effect of the *CAR1* gene disruption on sake brewing, laboratory-scale sake brewing was carried out with the parent strain and the three types of transformants (Table 1 and Fig. 3). The fermentation profile of each transformant was the same as that of the parent strain, K-9, suggesting that the disruption of the *CAR1* gene of sake yeasts does not inhibit normal sake brewing.

Analyses of the general components of the brewed sake showed that there is no difference between the wild-type and the *car1* mutant strains except the concentration of urea and the amounts of arginine and ornithine (Table 4 and Fig. 4). The sensory test also showed that the sake made with the mutant had quality similar to that of sake made with the parent strain. No urea was detected in sake made with the type A transformant, whereas the urea concentrations of sake made with the type B and type C transformants were a little lower than that of sake made with the parent strain, K-9 (Table 3). The amount of arginine in sake made with the type A strain, which was not able to cleave arginine to urea and ornithine, was apparently higher than in those made with K-9, type B, and type C (Fig. 4). On the contrary, the amount of ornithine in sake made with type A strain was lower. There was little difference in the other amino acid amounts. The stability of the disrupted *CAR1* locus was checked by determining the growth phenotypes of isolated yeast cells just before centrifugation of sake moromi mash. Type A cells carried nearly 100% double disruption, judging from the test of *G418*^{r} and *SMR1* markers.

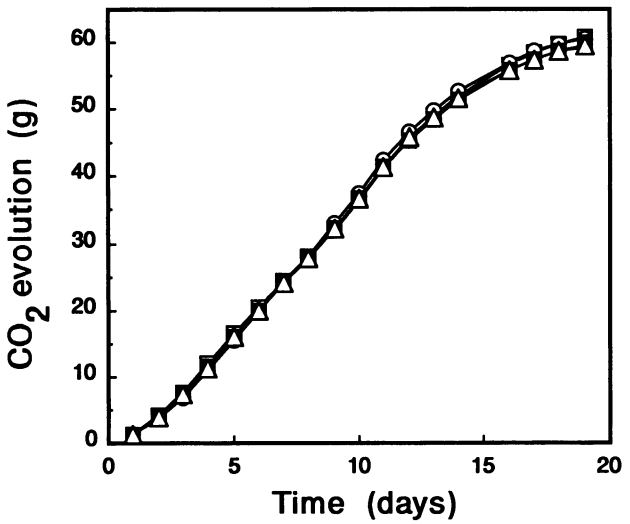

FIG. 3. Sake brewing with the *car1* mutants. Laboratory-scale sake brewing was carried out with the *car1* mutants (△, type A; ■, type B; ○, type C) and parent strain K-9 (□).

Formation of ECA after storing the resulting sake. The brewed sake was pasteurized at 65°C for 30 min and then stored at 30°C for 45 days or 5 months. After storage, the ECA concentration was analyzed (Table 5). Even after 5 months, ECA was not detected in the sake made with type A strain, whereas more than 100 ng of ECA per ml was formed in sake made with K-9, type B, and type C strains. This result strongly suggests that ECA is formed only from urea and ethanol in sake.

DISCUSSION

An arginase-deficient mutant was constructed from sake yeast strain K-9 by successive disruption of the *CAR1* gene. One copy of the *CAR1* gene was disrupted with pCAT2 (Δ*car1 SMR1*), followed by disruption of the other copy with pCAT1 (Δ*car1 G418*^{r}). Judging from the growth phenotypes, about 70% of the second transformants have two copies of disrupted *CAR1* gene (type A). In the rest of the transformants, the *CAR1* gene disrupted by the first transformation

TABLE 4. Analysis of sake brewed with the *car1* mutants

Strain	Sake meter[a]	Alcohol content (%)	Acidity[b]	Amino acidity[c]	Sensory score[d]
Type A-1	−2.0	18.2	2.7	1.9	1.7
Type A-2	−4.0	18.1	2.8	2.0	1.8
Type B-1	−2.5	18.1	2.7	2.1	2.0
Type B-2	−2.0	18.2	2.7	2.1	1.5
Type C-1	−2.0	18.3	2.8	2.1	2.0
Type C-2	−3.0	18.3	2.8	2.1	1.7
K-9	−1.5	18.2	2.8	1.9	1.7

[a] The sake meter indicates the apparent specific gravity of sake and is basically a Baumé meter, which works on the principle that alcohol is lighter than water while glucose is heavier. Water is given a value of 0 at 15°C, and Baumé value 1 corresponds to sake meter value −10.

[b] The volume (in milliliters) of 0.1 N NaOH which titrates 10 ml of sake.

[c] The volume (in milliliters) of 0.1 N NaOH which titrates formol nitrogen in 10 ml of sake.

[d] The sensory test was done by six judges, and the sake was scored as follows: 1, excellent; 2, average; 3, bad.

TABLE 5. Concentrations[a] of urea in the brewed sake and of ECA after storing

Strain	Urea concn (μg/ml)	ECA concn (ng/ml) after storing at 30°C for:	
		45 days	150 days
Type B-1	32.3	36.0	146.5
Type B-2	32.3	35.7	147.0
Type C-1	33.3	37.1	127.4
Type C-2	33.6	43.0	124.3
K-9	39.4	45.7	180.5

[a] No urea or ECA was detected in type A-1 and A-2 strains. The limits of detection in the assays for urea and ECA were 0.5 μg/ml and 5 ng/ml, respectively.

was replaced with a 7.0-kb *Bam*HI fragment of pCAT1 and the other copy of the normal *CAR1* gene was kept intact. Southern blot analysis confirmed that the two copies of the *CAR1* gene were disrupted as expected in most type A transformants, but rearrangements at the *CAR1* locus were observed in some cases (Fig. 2).

Since the stability of the disrupted *CAR1* locus is very important for a strain used in sake brewing, losses of selection markers of *SMR1* and *G418*^{r} were checked after about 100 generations of culture in nonselective medium, YPD. G418 resistance was perfectly maintained, but about 2% of the mutants lost SM resistance. A little less stability of SM resistance may result from the strategy of disruption. These results showed that this mutant has full stability for sake brewing. Sake-brewing tests using the mutants and the parent strain showed that the disruption of both *CAR1* genes of the sake yeast strain had no effect on its fermentation profiles and that the resulting sake had almost the same sensory scores and components, except for urea, as did sake made with the parent strain. No urea was detected in the sake made with type A transformant (Δ*car1*::*G418*^{r}/Δ*car1*::*SMR1*), and no ECA was formed in it, even after storage for 5 months at 30°C. Ough et al. (13) reported that possible precursors for ECA formation in fermented beverages are most N-carbamyl compounds, such as urea, citrulline, allantoin, carbamyl phosphate, and so on. And it is known that urea is carried over into moromi mash from steamed rice and that rice koji is used for raw materials in processes other than urea production by yeast arginase (25). However, the present results strongly suggest that urea contained in sake is produced only by arginase of sake yeast and that ECA formed in long storage of sake originates from the urea. Urea existing at the first stage of sake brewing must be completely consumed by the sake yeast, since urea is a favorite nitrogen source for the yeast *S. cerevisiae*.

For lowering urea content in sake, an acid urease is generally used. It is relatively easy to lower the level to less than half of the original urea content in sake by this method, but it is very difficult to remove urea perfectly, and it is labor-intensive and expensive. We think that the use of the *car1* mutant described here is the best method for sake brewing without a fear of ECA formation during storage.

We are constructing the *car1* mutants by gene disruption from wine yeasts. Similar wine-making tests will reveal whether only urea contributes to ECA formation in wine also.

The *CAR1* locus of the *car1* mutant constructed in this study has bacterial DNA sequences, since the *G418*^{r} gene and pUC18 for construction of the disruption plasmid originate from *E. coli*. It is not desirable for industrial yeast

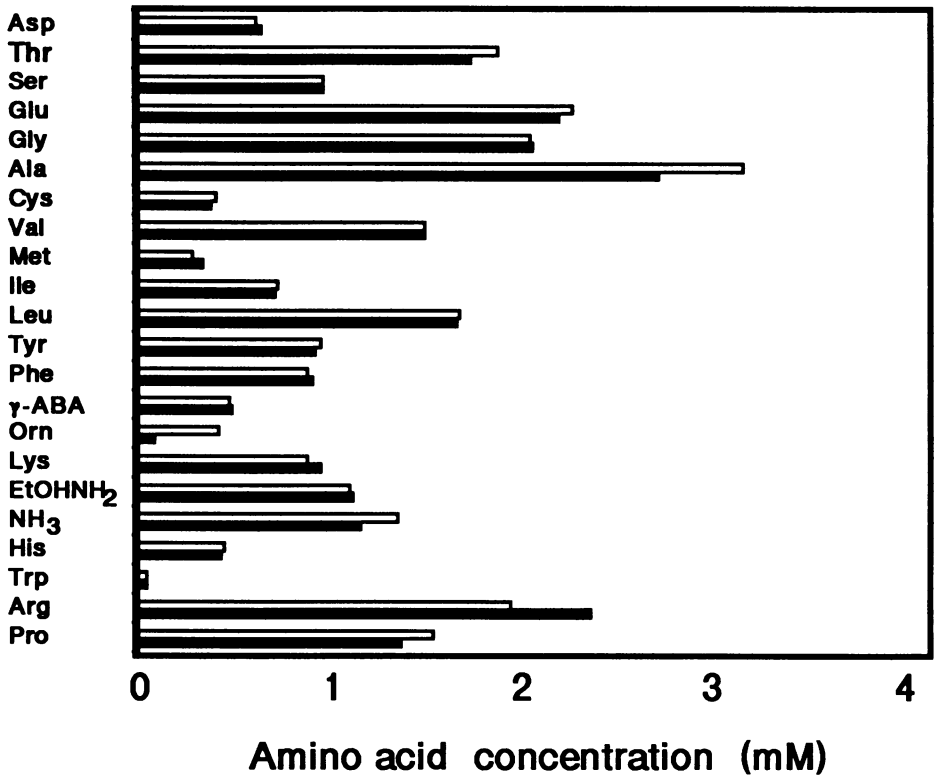

FIG. 4. Amino acid composition of sake made with a transformant and the parent strain. The sake obtained with the type A transformant (■) had higher arginine and lower ornithine contents compared with that made with the parent strain, K-9 (□). There is little difference between the other amino acid contents. γ-ABA, γ-Aminobutyric acid; $EtOHNH_2$, ethanolamine.

strains to have bacterial sequences. Now we have tried to construct by gene disruption *car1* mutants without bacterial sequences from sake and wine yeasts. These strains will be more useful for fermented-beverage production.

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