3

Genetic Transfer Applied to Traditional Sake Brewing

KATSUHIKO KITAMOTO, KATSUYA GOMI, KUNIYASU GOTO AND SHODO HARA

National Research Institute of Brewing, Tokyo, Japan

Introduction

In Japan there are many kinds of traditional fermented foods and beverages, which are made by use of *koji*-mould and yeast. For instance, sake brewing is done with *koji*-mould (*Aspergillus oryzae*) and sake yeast (*Saccharomyces cerevisiae*). In addition, *shoyu* (soy sauce) and *miso* (soybean paste) are made with the aid of *A. oryzae* or *A. sojae* and salt-tolerant yeast (*Zygosaccharomyces rouxii*) (Hesseltine, 1983). These micro-organisms have been selected and cultivated from ancient times like domestic animals. Since these micro-organisms are specialized for their production and, in general, have no sexual life cycle, breeding by classical genetics is relatively difficult (Hara, Kitamoto and Gomi, 1991).

Accordingly, breeding of sake yeasts and *koji*-moulds has long been investigated in attempts to obtain further favourable characteristics, using mutation, protoplast fusion and selection methods.

Recent astounding progress of techniques for gene isolation and gene analysis of yeast and fungi has allowed the development of DNA-mediated transformation systems, and genetic engineering of these micro-organisms has been started.

In this chapter, we review the new techniques which transfer genes to yeasts and *koji*-moulds used in sake brewing.

Outline of sake brewing

Sake brewing is complicated compared with the brewing of beer and wine, since two kinds of micro-organisms, *koji*-mould and sake yeast, are used for

Abbreviations: dsRNA, double-stranded RNA; ECA, ethyl carbamate; EtdBr, ethidium bromide; FOA, 5-fluoro-orotic acid; OCTase, ornithine carbamoyltransferase; OFAGE, orthogonal-field-alternation gel electrophoresis; PEG, polyethylene glycol; SD, synthetic minimum medium; SM, sulphometuron methyl; TAA, Taka-amylase A; UV, ultraviolet; YPD, complex medium.

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saccharification of starch and the ethanol-producing fermentation, respectively (*Figure 1*). Briefly, the raw materials and brewing process (Nunokawa, 1972) are as follows.

The raw materials used in sake brewing are water and rice. Since water accounts for about 80% (v/v) of the end-product, clean water for sake brewing is very important. The peripheral layers of brown rice contain large amounts of ash, vitamins, fats, and proteins that are undesirable for sake brewing. Thus brown rice is polished by milling to remove these materials, with a polishing rate of 50–70%. The polished rice is washed to remove the bran on the surface of the grains, and then steeped in water for several hours to absorb 28–30% (w/w) water. The drained rice is steamed for about 1 hour. The steaming procedure increases the moisture of the starch granules within the kernel by about 10%. For *koji* preparation, about 20% of the total rice is used, while the rest is used directly for sake brewing. *Table 1* shows a typical preparation of raw materials for sake brewing. The steamed rice is cooled down to about 36°C and transferred to the *koji*-making room (*koji-muro*)

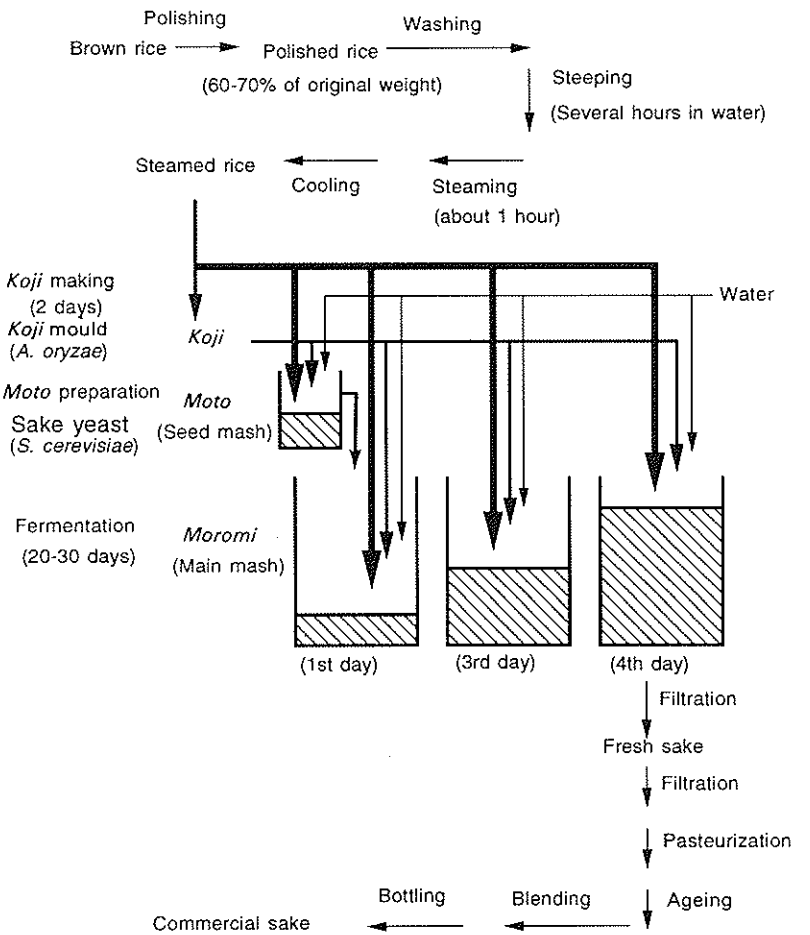


Figure 1. Process flow chart of sake brewing.

Table 1. Raw materials for sake brewing

	Amount of rice (kg) for			
	steaming	*koji*	Water (l)	Mash temperature (°C)
Moto	140	70	230	20
Soe (1st)	320	130	440	15
Naka (2nd)	700	200	1050	9
Tome (3rd)	1140	330	2000	7
Total	2500	700	4000	

where temperature and humidity can be controlled. *Tane-koji* (spores of *Aspergillus oryzae*) is scattered over the surface of the rice (containing approximately 35% moisture) at an inoculation rate of 1 g of *tane-koji* preparation per kg of raw rice, at about 30°C, then mixed thoroughly and covered with cloths. After 40–45 hours since spore scattering, the *koji*-rice in the trays is moved out of the warm *koji-muro* so that the lower temperature (about 5°C) outside stops the growth. *Koji* so prepared is white in color, since the growth stage is halted prior to sporulation of the mould.

About 7% of the total rice is used for *moto* preparation (*Table 1*), and in 2–4 weeks it is ready for use as a starter for the main mash. *Moto* plays an important role as a starter of the yeast culture in carrying out the fermentation of *moromi* (main mash). *Koji* is mixed with water, steamed rice, and sake yeasts (*Saccharomyces cerevisiae*) in the prefermentation tanks. This seed mash is a concentrated culture of pure and healthy living cells of sake yeast.

On the first day in the *moromi* mash preparation, a mixture of steamed rice, water, *koji* and *moto* is put into the main fermentation tank (*moromi* preparation temperature, 15°C). On the third (9°C) and fourth (7°C) days, additional volumes of steamed rice, water and *koji* are added to the tank in order to hold the yeast cell count at about 10^7–10^8/ml in the mash. During the fermentation of *moromi*, which is maintained at about 10–15°C, the starch in the rice is liquefied and saccharified by α-amylase and glucoamylase produced by the *koji*, and this converted mixture is fermented into ethanol by the action of sake yeast. Both processes, saccharification and alcohol fermentation, proceed simultaneously in a well-balanced manner. This unique, complex method, called 'multiple parallel fermentation', contributes an alcohol content of about 20% (v/v), higher than any other naturally fermented beverage. When the fermentation is finished, after 20–30 days, the mash is press-filtered to separate the sake from the solids. The mash residue remaining on the filtering cloth is called sake cake.

The fresh sake is heated to 60–65°C by passage through a heat exchanger. This pasteurization inactivates the enzymes, amylases, proteases, and so on, and kills lactic acid bacteria, the so-called *hiochi* bacteria, undesirable micro-organisms that spoil sake during storage. Immediately after pasteurization, the hot sake is transferred to a sealed vessel for storage.

KOJI-MOULD

Tane-koji (spores of *A. oryzae*) is available from about 10 *tane-koji* manufacturers in Japan, who provide the product to sake Companies. *Aspergillus oryzae* is also used for *shoyu* (soy sauce) and *miso* (soybean paste) manufacture. However, the properties of strains used for sake brewing are different from the others with respect to enzymatic activity. In sake brewing, the following mycological characteristics have been found empirically to be of major importance:

1. Rapid growth on and into the kernel of the steamed rice.
2. Production of abundant amylases (α-amylase and glucoamylase), a little acid carboxypeptidase and less tyrosinase.
3. Low production of coloured substances such as deferriferrichrome, flavines, etc.

SAKE YEAST

The Brewing Society of Japan (*Nihon Jozo Kyokai*) provides sake yeast strains (*Saccharomyces cerevisiae*) used for sake brewing, called *Kyokai* No.6, No.7, No.9, No.10, and so on. *Kyokai* No.6 and No.7 strains are used for brewing of standard sake. *Kyokai* No.9 and No.10 are used for *ginjo-shu* (high-quality sake) because of their ability to cause higher aroma formation. The society also provides *Kyokai* No.601, No.701, No.901 and No.1001, which are non-foaming mutants (Ouchi and Nunokawa, 1973) from numbers 6, 7, 9 and 10, respectively. Breweries are increasingly using their own sake yeast strains for a part of the brewing. The characteristics needed for sake yeast are both high fermentation activity at low temperature (5–15°C) and high resistance to high alcohol content (about 20%).

Breeding of killer sake yeasts

The first successful transfer of genes to sake yeast was in the construction of killer sake yeasts (Ouchi and Akiyama, 1976). Since the killer toxin is encoded by a double-stranded RNA plasmid, introduction of the killer characteristic to sake yeast was carried out by protoplast fusion or crossing, and not by recombinant DNA techniques.

Raw materials for sake brewing are not perfectly sterile and the fermentation is done in open conditions. Therefore, there is a danger of contamination with wild yeasts during fermentation. In *Saccharomyces* killer yeasts, which were discovered by Bevan and Makower (1963), there might be strains tolerant of a high concentration of ethanol or carbohydrate fermentation starters or mash. Thus, if sake *moromi* mash were to be contaminated with killer wild yeasts, it would cause death of the cultured sake yeasts and displacement, resulting in the production of inferior quality sake (Imamura, Kawamoto and Takaoka, 1974, Akiyama, Ouchi and Ueda, 1976).

To protect sake mash from the killer yeasts, studies on killer phenomena and the breeding of industrial killer yeasts have started. In *Saccharomyces*

yeasts, several types of killer have been reported (Berry and Bevan, 1972; Young and Yagiu, 1978; Wickner, 1981; *Table 2*), and the genes coding for the killer toxins known as K1, K2 and KT28 (Schimitt and Radler, 1987; Schimitt *et al.*, 1989) have been located on double-stranded RNA (dsRNA) plasmids. In addition, weak killers (Kitano *et al.*, 1984) have been reported. The gene encoding the weak killer, whose activity is much weaker than that of the K1 or K2 killer, is located on the chromosome.

KILLER IN SAKE YEASTS

Killer wine and beer yeasts were discovered by Naumova and Naumova (1973) and Maule and Thomas (1973), respectively. In 1974, Imamura, Kawamoto and Takaoka found killer yeasts for the first time in a sake brewery, where only an inferior quality of sake was produced every year, in spite of the addition of cultured sake yeasts. Then the 410 strains stocked in the National Research Institute of Brewing (NRIB) sake yeast collection, (which had been isolated from sake breweries in the whole of Japan during 1906 to 1967) were examined to see whether they were killer yeasts or not, and 72 strains were found to be killers (Ouchi and Kawashima, 1974). However, in the investigation of *moromi* mash in 1975 killer yeasts were found in only nine out of 211 sake breweries (Akiyama, Ouchi and Ueda, 1976). These results suggested that wide distribution of killer yeasts in sake breweries was reduced because pure culture sake yeasts have been added as starters since the 1930s. Almost all the killer strains isolated from sake mash were K1 type. In contrast, K2 type killer yeasts were isolated from wine and beer production facilities in Japan (Ouchi and Akiyama, 1981).

BREEDING OF KILLER YEASTS FOR SAKE BREWING

One of the most important considerations in breeding industrial yeasts is to ensure that the fermentability and flavour formation characteristics are not impaired during the breeding process. Bred strains, which were produced by an ordinary cross or protoplast fusion between a sake yeast strain and a wild killer strain, would give favourable properties to the hybrid sake yeast.

From 1976 to 1983, Ouchi and co-workers developed several breeding methods for industrial killer yeasts, for example repeated backcrossing between haploids of a sake yeast, *Kyokai* No.7 (K-7) strain, and a wild killer yeast. After the sixth generation, hybrids were backcrossed with haploids of K-7. Several strains of killer hybrids were comparable to K-7 with regard to abilities of fermentation, flavour formation and acid production in laboratory-scale sake brewing. The constructed killer sake yeast protected against contamination by wild yeasts in sake brewing, and produced sake of as good quality as that of K-7 in industrial-scale fermentation (Ouchi and Akiyama, 1976). The wine and sherry killer yeasts were also bred with this method (Hara, Iimura and Otsuka, 1979).

In the cytoduction method (Ouchi *et al.*, 1979), a haploid clone of K-7 was mated with a karyogamy-defective mutant (*kar1*) carrying killer plasmids, to

Table 2. Some characteristics of killer strains of *Saccharomyces cerevisiae*

Type	Toxin (kDa)	Optimum pH	Stability		Mechanism	Reference
			pH	Temp. (°C)		
K1	9·5 (α) 9·0 (β)	4·6–4·8	3·5–6·0	25	Ionophore formation	Bostian *et al.* (1984) Martinac *et al.* (1990)
K2	16·0	4·2–4·4	3·0–5·0	30	K1 like	Pfeiffer and Radler (1984)
KT28	16	ND	ND	ND	DNA synthesis inhibition	Schimitt and Radler (1987), Schimitt *et al.* (1989)
KHR	20	5·8	5·0–7·0	35	ND	Goto *et al.* (1990a)
KHS	75	4·0–4·5	2·0–5·5	30	K1 like	Goto *et al.* (1991)

ND, not determined.

yield segregant clones which had the nucleus of sake yeast and the cytoplasmic factors of both parents. Seki, Choi and Ryu (1985) also bred a killer wine yeast with this method.

In the UV-killed protoplast fusion method (Ouchi, Nishiya and Akiyama, 1983), the killer plasmid from UV-killed donor cells, was directly transferred to a sake yeast through protoplast fusion. A killer sake yeast bred by this method was able to produce sake of a satisfactory quality and also prevented the growth of wild yeasts. This method is applicable to breeding of a killer strain of any industrial yeast irrespective of its ploidy and mating type. Other breeding methods were reported to be a rare mating method (Young, 1981) and a miniprotoplast method (Fukuda and Kimura, 1980; Shimoda, Mizuguchi and Fujita, 1984).

CHROMOSOME-DEPENDENT KILLER

Kitano *et al.* (1984) found new types of killer strains in wine must in Japan. The killer activities of these strains were weaker than those of K1 and K2 killer strains and were not cured by treating with cycloheximide or by incubating at 38°C, under which conditions both K1 and K2 killer plasmids were cured (Kitano *et al.*, 1984). By measuring the optimum pH and thermostability, these killer strains were classified into two groups. The thermostable and unstable killers were designated KHR (*k*iller of *h*eat *r*esistant) and KHS (*k*iller of *h*eat *s*ensitive), respectively. The genes encoding KHR and KHS were mapped on the left arm of chromosome IX and the right arm of chromosome V, respectively (Goto *et al.*, 1990a).

The properties of these killers, the characteristics of these killer toxins and the structure of these killer genes were reported by Goto *et al.* (1990a,b, 1991; *Table 2*). These killer strains are mainly distributed in wine and brewing yeasts. Application of these killers to sake brewing is in progress.

RED SAKE BREWING USING ADENINE AUXOTROPHIC KILLER SAKE YEAST

Red sake has been produced by adenine auxotrophic sake yeast (Ouchi *et al.*, 1983). The *ade*$^-$ mutants accumulate red pigments in their cells and the fermented mash becomes reddish pink. However, since the mutant ferments slowly because of its auxotrophy, it is easily contaminated with wild yeasts.

The red killer yeast was bred with the protoplast cytoduction method (Nishiya, Ishikawa and Iimura, 1984). The red sake yeast No.3038 (a/α *ade*$^-$/*ade*$^-$) was used as a recipient, and the K1 type killer yeast No.5038 (α *his4 kar1-1* [KIL-K1]) was used as a donor of K1 killer plasmid. The 178 fusants between No.3038 and No.5038 strains were selected as ade$^-$ (accumulate a red pigment), His$^+$ and [KIL-K1] strains, and the selection rate was 23·6%. The strain named PK-2 was finally selected with its good fermentability. This strain made the red sake mash without allowing the outgrowth of wild yeast contaminants (Nishiya *et al.*, 1984) (*Figure 2*).

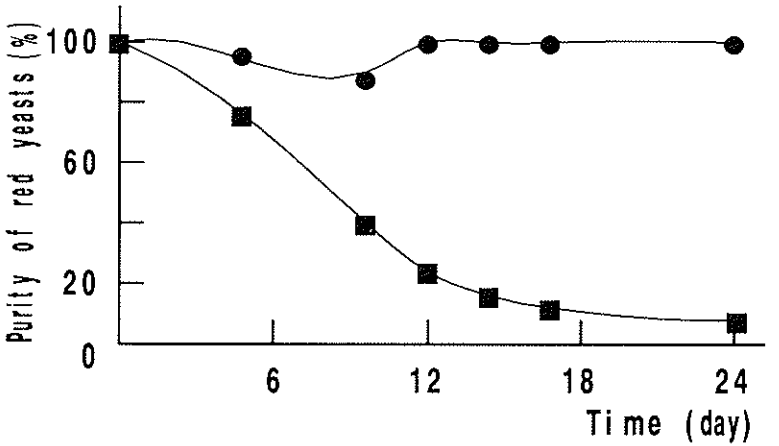


Figure 2. Distribution of the killer and non-killer red yeasts during sake brewing (Nishiya, Ishikawa and Iimura, 1984). The *moromi* mash started with about $5{\cdot}5 \times 10^6$ cells ml^{-1} of the adenine auxotrophic sake yeast (non-killer red yeast) or the red killer yeast and about $1{\cdot}8 \times 10^4$ cells ml^{-1} of wild-type sake yeast. ■, Non-killer red yeast; ●, killer red yeast.

Breeding of useful sake yeasts by gene disruption

CONSTRUCTION OF URACIL AND TRYPTOPHAN AUXOTROPHIC SAKE YEASTS

In 1978, recombinant DNA techniques for yeast *S. cerevisiae* were developed by Hinnen, Hicks and Fink (1978). Since then the molecular genetics of *S. cerevisiae* has developed vigorously. However, since industrial yeast strains have two or more ploidies, genetic engineering of industrial yeasts has not achieved so much. One of the problems was that there was no convenient host strain like *ura3, trp1* or *leu2* mutants. Several dominant selection markers are reported to be available for use in *S. cerevisiae*, including resistance to G418, hygromycin B, cycloheximide and sulphometuron methyl (SM). There are, in general, some difficulties in their use with industrial yeasts, especially sake yeasts. Therefore, at first, uracil and tryptophan auxotropic mutants were constructed from sake yeast (Kitamoto *et al.*, 1990) by a gene disruption method (Rothstein, 1983; Winston, Chumley and Fink, 1983). In transformation experiments with *Saccharomyces cerevisiae* the isolation of transformed cells largely depends upon the complementation of recessive auxotrophic mutations. In this system *ura3, trp1* and *leu2* mutants are commonly used as recipient strains in genetically defined haploid strains. With respect to the possibility that the mutagenesis treatment also damaged some of the many useful genes for sake brewing and that such a mutant reverts to wild type with a certain frequency, construction of the mutant by gene disruption is thought to be the most suitable method for the auxotrophic mutants of recipient strains for molecular breeding. Since a sake yeast is diploid and has two *URA3* genes, two kinds of plasmids, pURA36 and pURA38 (*Figure 3*) were constructed (Kitamoto *et al.*, 1990). *Figure 4a* shows a strategy for isolation of uracil auxotrophic mutants from sake yeasts. First, sake yeasts, *Kyokai*

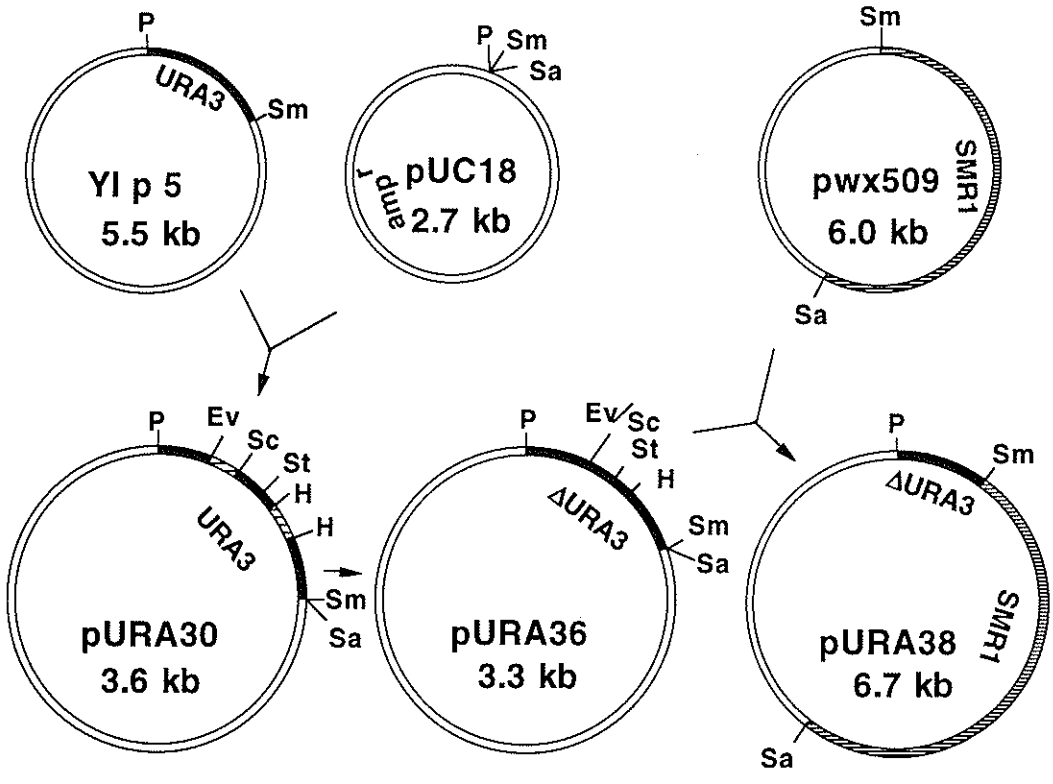


Figure 3. Construction of the plasmids for *URA3* gene disruption (Kitamoto *et al.*, 1990). The 0·9 kb *Pst*I–*Sma*I fragment containing the *URA3* gene was isolated from YIp5 and inserted into the *Pst*I–*Sma*I site of pUC18 to form pURA30. This was digested with *Hin*cII and ligated to form pURA34. Then pURA34 was digested with *Eco*RV and *Sca*I and ligated to construct pURA36. pURA38 was constructed by insertion of a *Sma*I–*Sca*I fragment containing the *SMR1* gene of pWX509 into the *Sma*I–*Sac*I site of pURA36. P, *Pst*I; Sm, *Sma*I; Sa, *Sac*I; Ev, *Eco*RV; Sc, *Sca*I; St, *Stu*I; H, *Hin*cII.

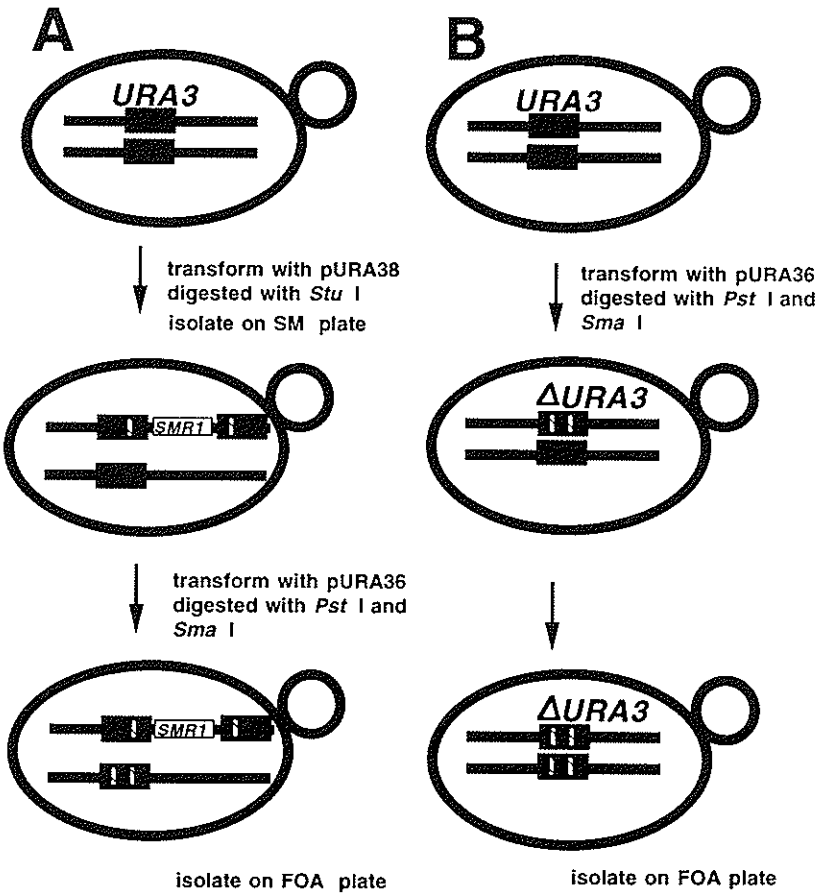


Figure 4. Procedures for *URA3* gene disruption (Kitamoto *et al.*, 1990).

No.701 (K-701) and No.901 (K-901) were transformed using pURA38 (△*URA3 SMR1*) linearized at the *Stu*I site between the two deletions, and the resulting transformants, which are expected to have two disrupted *URA3* genes on one chromosome and one normal *URA3* gene on the other one, were selected on the SM plate containing sulphometuron methyl (Casey, Xiao and Rank, 1988). Each of the four strains of K-701 and K-901 transformants were successively transformed using pURA36 (△*URA3*) digested with *Pst*I and *Sma*I. As the result of two transformations, the uracil auxotrophic mutants, of which all *URA3* genes were disrupted, were isolated on a 5-fluoro-orotic acid (FOA) plate (Boek, LaCroute and Fink, 1984) on which only *ura3* mutants can grow. Kitamoto *et al.* (1990) also tried a direct gene disruption using only one plasmid without a selection marker (pURA36) (*Figure 4b*) and isolated nine colonies from K-901 on the FOA plate.

Four kinds of mutants were isolated from Southern blot analysis, as shown in *Figure 5*. It is noted that mutant types C and D have no bacterial sequences; this is desirable with respect to the practical use of sake brewing.

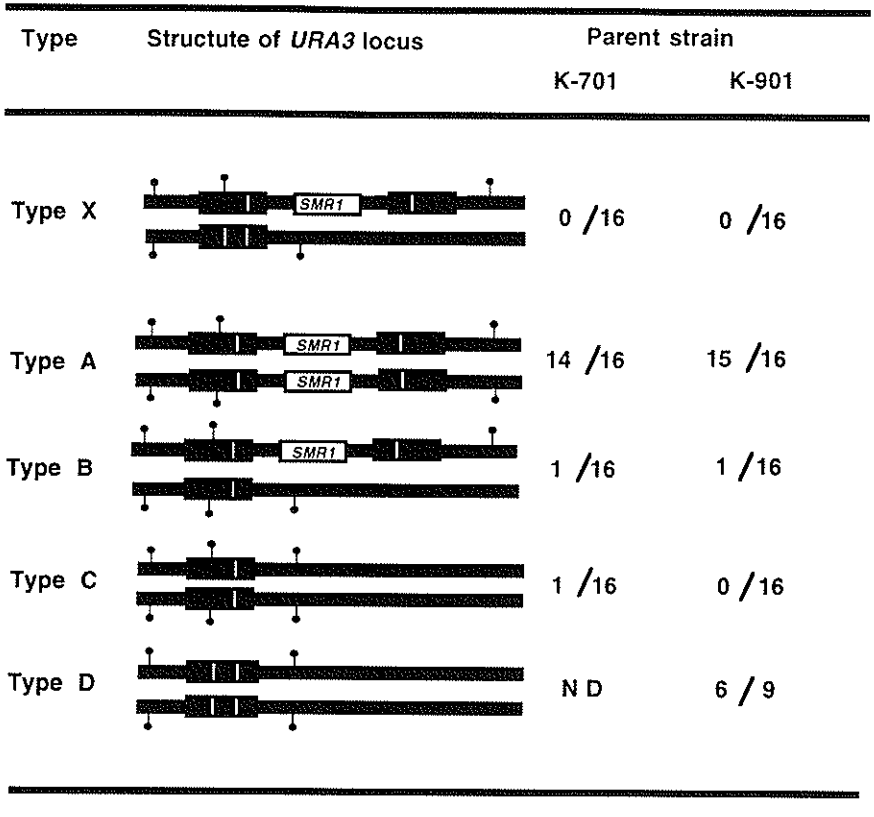


Figure 5. *ura3* mutants obtained from sake yeast K-701 and K-901 (Kitamoto *et al.*, 1990). By the procedure given in *Figure 4a*, types A, B and C were obtained. The expected type X was not isolated from the 16 strains examined by Southern blot analyses (↑ *Eco*RV site). Type D was isolated by the procedure given in *Figure 4b*. ND, not done.

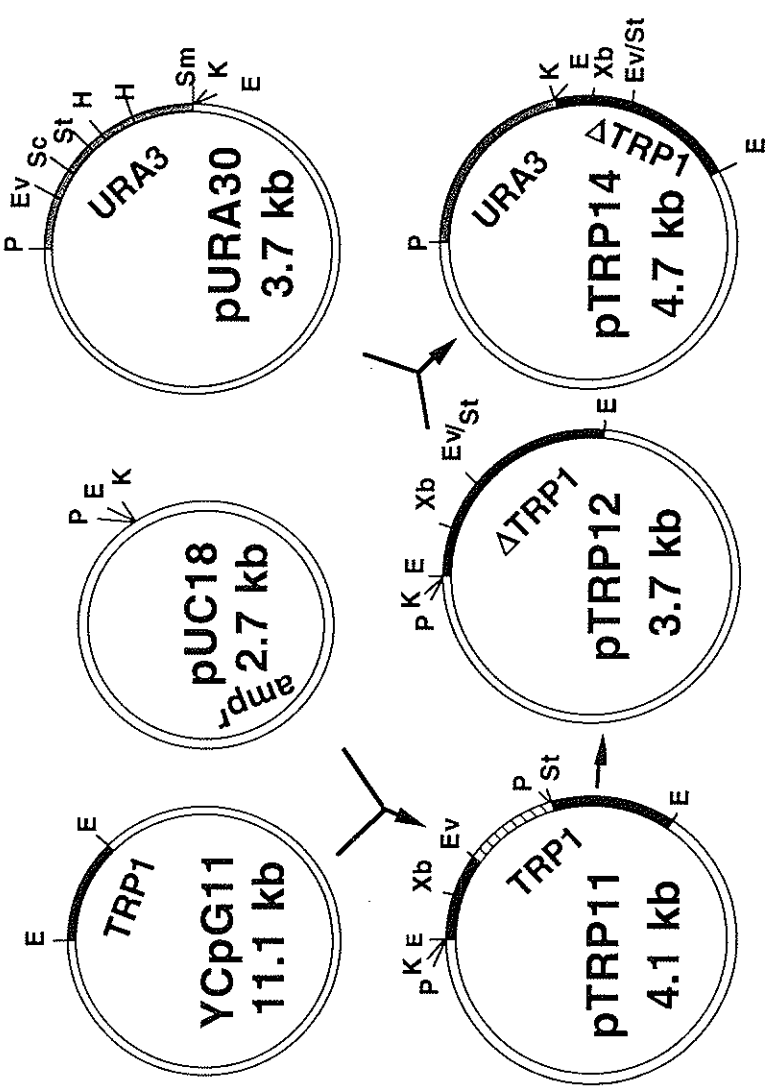


Figure 6. Construction of the plasmids for *TRP1* gene disruption (Kitamoto *et al.*, 1990). The 1·4 kb *Eco*RI fragment containing the *TRP1* gene was cloned into the *Eco*RI site of pUC18. The resulting pTRP11 was digested with *Eco*RV and *Stu*I and ligated to form pTRP12, the 1·0 kb *Eco*RI fragment of pTRP14. For integration into the *TRP1* locus, the *Xba*I site within the deleted *TRP1* gene is unique. E, *Eco*RI; K, *Kpn*I; Xb, *Xba*I; Ev, *Eco*RV; St, *Stu*I; P, *Pst*I; Sm, *Sma*I; Sc, *Sca*I; H, *Hin*dII.

Kitamoto *et al.* (1990) also constructed tryptophan auxotrophic mutants using a plasmid, pTRP14 (*Figure 6*) from the uracil auxotrophic mutant, U-4-12. *Figure 7* shows the strategy for isolation of tryptophan auxotrophic mutants from uracil auxotrophic mutants. First, a uracil auxotrophic mutant from U-4-12 was transformed with pTRP14 (△*TRP1 URA3*) digested with *Xba*I and the transformants were isolated on the SD plate (synthetic minimal medium: 0·67% Bact-yeast nitrogen base and 2% glucose). Next, after the

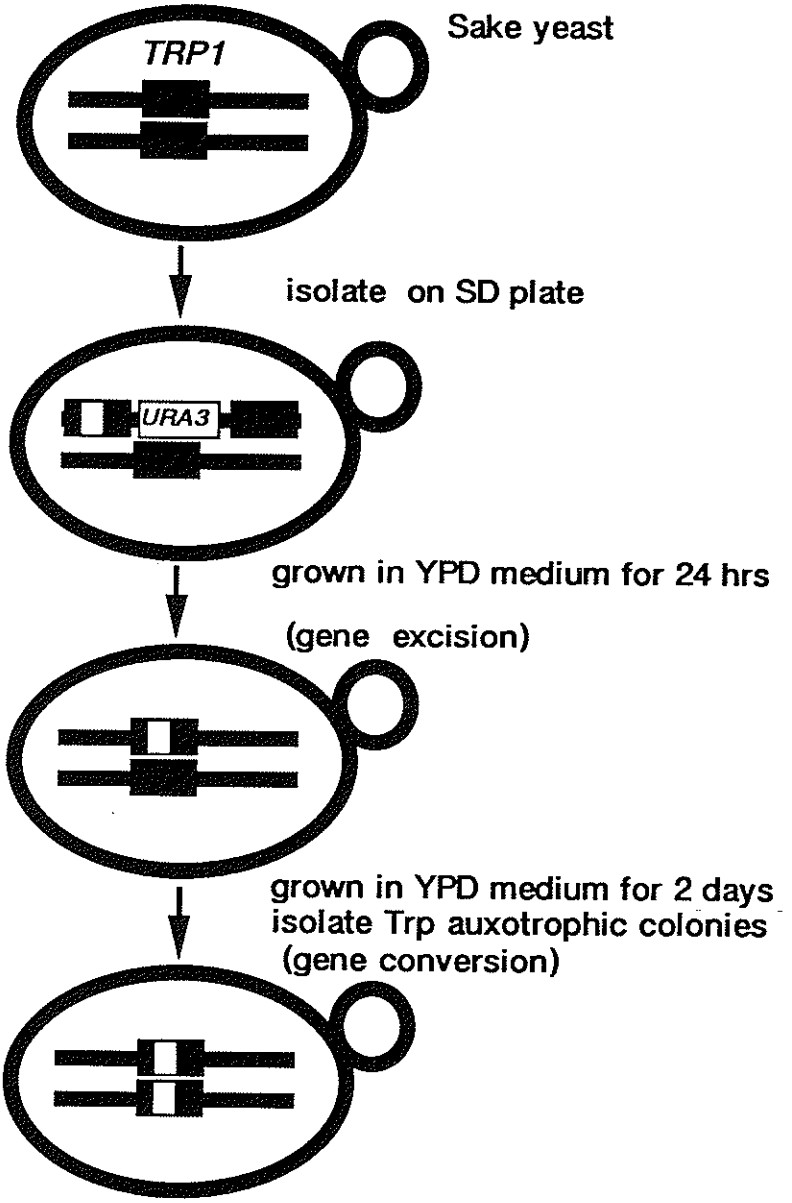


Figure 7. Procedure for *TRP1* gene disruption (Kitamoto *et al.*, 1990).

transformants were grown in YPD medium (1% yeast extract, 2% peptone and 2% glucose) for 24 h at 30°C, an aliquot of the culture was spread onto the FOA plate. Revertants to uracil auxotrophic mutants can grow on FOA plates by gene excision. The revertants were picked up independently and the expected heterozygous *TRP1* disruptants (*TRP1/trp1*) were screened by Southern blot analysis. Two strains were obtained and were grown in YPD medium at 30°C with shaking for 2 days, long enough for a gene conversion event to occur. Then an appropriate amount of cultures were spread on Trp-deficient plates so that 100–200 colonies appeared on one plate. From about 10 000 colonies, about 20 strains forming very small colonies were replica-plated onto uracil-supplemented SD plates with or without tryptophan. Finally, two strains of tryptophan auxotrophic mutants, of which both *TRP1* genes were disrupted, were isolated. They were designated UT-1 and UT-2.

Fermentation profiles and analytical data of the sake resulting from use of these mutants are shown in *Figure 8* and *Table 3*. All uracil auxotrophic mutants fermented more slowly than the parent sake yeasts. However, by introduction of one *URA3* gene into the mutant (U-4-12) transformed with the fragment containing *URA3* gene, the fermentation profile became like the parent strain, K-701 Since UT-1 originated from the uracil auxotrophic mutant, U-4-12, it is a tryptophan and uracil auxotrophic mutant, having the genotype (a/α *trp1/trp1 ura3/ura3*). A tryptophan auxotrophic mutant was constructed from UT-1 by gene replacement of the fragment containing the *URA3* gene and a resulting mutant was designated UT-1U (a/α *trp1/trp1 ura3/URA3*). The fermentation profile of UT-1U is almost the same as that of the parent strain, K-701.

BREEDING OF SAKE YEAST PRODUCING α-AMYLASE OF *KOJI*-MOULD

As described above, sake is brewed using *koji*-mould and sake yeast. One of the most important roles of *koji*-mould is to produce amylases, which digest starch of steamed rice into glucose. If yeast producing the amylases could be constructed, it might be possible to brew sake without *koji*-mould. Therefore,

Table 3. Sake brewing by use of the constructed uracil and tryptophan auxotrophic mutants (Kitamoto *et al.*, 1990)

Strain	Sake meter*	Alcohol concn (%)	Acidity†	Amino acidity‡	Sensory score§
K-701	− 2·0	17·7	2·6	2·2	1·2
U-4-12	−22·0	13·1	6·1	2·1	2·7
U-4-12-U	− 1·0	17·2	2·3	2·5	1·8
UT-1	−27·0	14·3	6·0	2·1	3·0

K-701, Wild-type sake yeast; U-4-12, uracil auxotrophic mutants (*ura3/ura3*) from K-701; U-4-12U, *URA3* integrant (*URA3/ura3*) from U-4-12; UT-1, uracil and tryptophan auxotrophic mutant (*ura3/ura3 trp1/trp1*) from U-4-12.

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

† Acidity is expressed as the volume (ml) of 0·1 N NaOH which titrates 10 ml of sake to neutrality.

‡ Amino acidity is expressed as the volume (ml) of 0·1 N NaOH which titrates formol nitrogen in 10 ml of sake.

§ The sensory test was performed by six judges, using three scores: 1, excellent; 2, average; 3, bad.

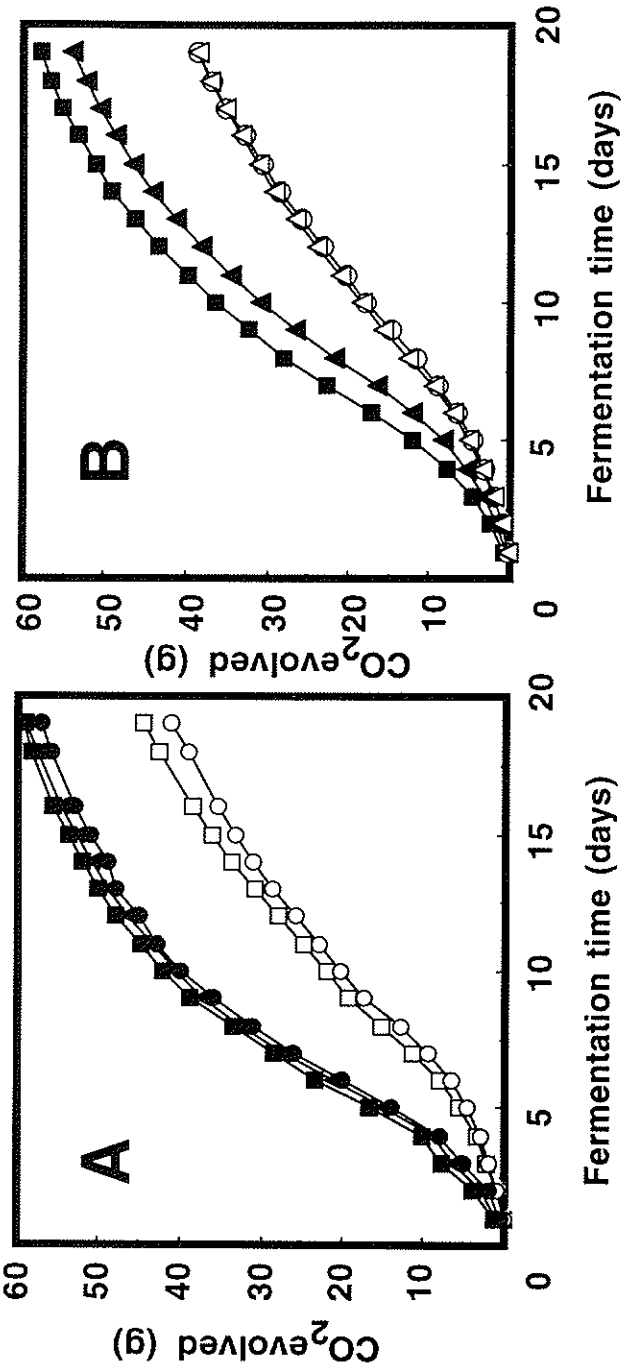


Figure 8. Sake brewing using *URA3* and *TRP1* disruptants (Kitamoto *et al.*, 1990). (A) Laboratory-scale sake brewing using *URA3* disruptants (○, U-4-12; □, U-9-11). Fermentation by *URA3* disruptants was weaker than fermentation by wild-type parent strains (■, K-701; ▲, K-901). *URA3* integrant from U-4-12 (●, U-4-12-U) showed a similar fermentation profile to that of the parent strain. (B) The fermentation profile of the *TRP1* disruptant (▲, UT-1U) was almost the same as that of the parent strain (■, K-701). Since UT-1 (○) and UT-1T (△) have uracil auxotrophy, the fermentations were weaker than fermentation of the parent strain.

as the first step of the genetic transfer, the introduction of α-amylase-encoding cDNA of *A. oryzae* into sake yeast has been tried (Kitamoto, 1991). First, the cDNA was isolated from a cDNA library from *A. oryzae*, using genomic α-amylase-encoding DNA (*amyB*) as a probe. The cloned cDNA was inserted into the *Eco*RI site of the yeast expression vector, pYcDE, (which has the *TRP1* gene for a selection marker) and was introduced into laboratory strain YPH250 (a, *ura3, ade2, lys2, trp1, his3, leu2*), producing the α-amylase-secreting transformant. Next, the constructed tryptophan auxotrophic sake yeast having the homozygous *trp1* mutation, UT-1U, was used for the transfer of the cDNA of *A. oryzae* to sake yeast. The resulting sake yeast formed a large halo on the SD plate containing starch, indicating that it produces α-amylase (*Figure 9*; Kitamoto, 1991). Even if the genetically engineered sake yeast was used for sake brewing, it would be impossible to brew sake without *koji*, because the sake yeast can only produce α-amylase. For digestion of starch without *koji*-mould, in addition to the α-amylase, glucoamylase-encoding cDNA would have to be introduced into the sake yeast. The glucoamylase-encoding cDNA from *A. oryzae* has been cloned by Hata *et al.* (1991), and sake yeast producing both the α-amylase and the glucoamylase from *A. oryzae* has been constructed (Kitamoto *et al.*, unpublished results).

GENETICALLY ENGINEERED SAKE YEAST PRODUCING NO UREA BY ARGINASE GENE DISRUPTION

Ethyl carbamate (ECA) is a suspected carcinogen (Mirvish, 1968) found in a variety of fermented beverages and foods (Ough, 1976). In 1986 trace levels of ECA in some types of wine, sherry, whisky and brandy, as well as in sake, were detected in Canada. It was reported that this compound can be formed by the spontaneous chemical reaction of urea and ethanol in wine (Ough, Cromwell and Gutlove, 1988; Monteiro, Trouosdale and Bisson, 1989; Ough

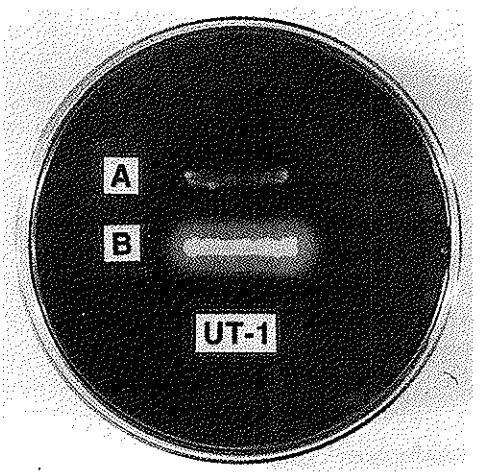


Figure 9. Sake yeast producing α-amylase from *koji*-mould, *A. oryzae*. A, UT-1; B, UT-1 introduced with the cDNA encoding α-amylase. The white area shows digestion of starch.

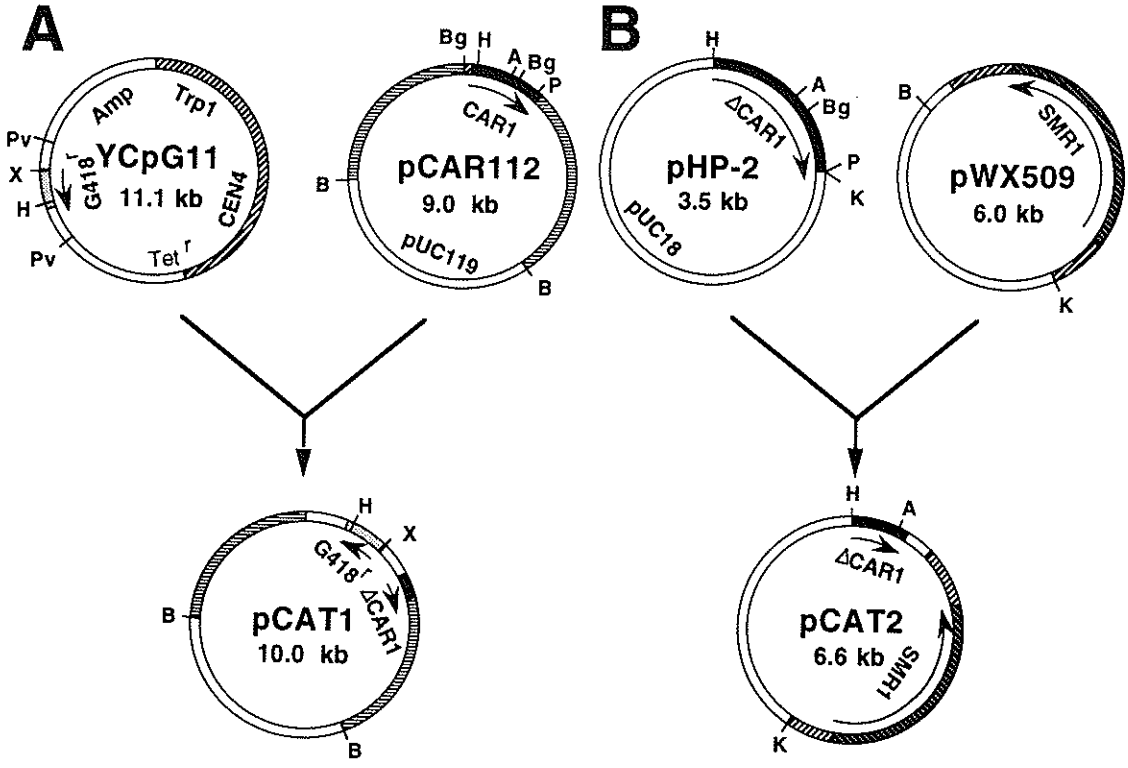


Figure 10. Plasmid construction for *CAR1* gene disruption (Kitamoto *et al.*, 1991). A 1·7 kb *Pvu*II–*Pvu*II fragment containing *G418*^{r} gene was isolated from YCpG11 and inserted into the *BGl*II–*Bgl*II gap of pCAR112 (Suizu *et al.*, 1990) to form pCAT1. A 3·2 kb *Bam*HI–*Kpn*I fragment containing the *SMR1* gene from pWX509 was inserted into the *Bgl*II–*Kpn*I gap of pHP-2 to form pCAT2. *G418*^{r} and *SMR1* are genes conferring G418 (Geneticin) and SM (sulphometuron methyl) resistance, respectively, 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.

et al., 1990) and sake (Hara, Yoshizawa and Nakamura, 1988). Removal of the major precursor, urea, by an acid urease from wine (Ough and Trioli, 1988; Trioli and Ough, 1989) and sake (Yoshizawa and Takahashi, 1988) has been examined. It is known that in yeast (*Saccharomyces cerevisiae*) urea is formed by arginase (Middelhoven, 1964), which is encoded by the *CAR1* gene (Sumrada and Cooper, 1982). An arginase-deficient mutant was constructed by *CAR1* gene disruption from a laboratory yeast strain, YNN27, and this *car1* mutant produced no urea (Suizu *et al.*, 1990). However, since this mutant did not originate from sake yeast, normal sake could not be obtained by a fermentation test. These results suggested that there is a possibility of brewing sake containing no urea. Therefore, in order to elucidate the role of the *CAR1* gene product, arginase, on sake brewing, a deficient sake yeast was constructed by double disruption of the *CAR1* gene (Kitamoto *et al.*, 1991). Two kinds of plasmids, pCAT1 and pCAT2 (*Figure 10*), were constructed to disrupt both copies of the *CAR1* gene. The strategy for *CAR1* gene disruption of sake yeast is shown in *Figure 11*. First, sake yeast, *Kyokai* No.9 (K-9), was transformed with pCAT2 (△*car1 SMR1*) linearized by digestion with *Ava*I to integrate at the *CAR1* locus. The resulting integrants, which are expected to have a disrupted *CAR1* gene on one chromosome and a normal *CAR1* gene on the other, were isolated on SM plates (Casey, Xiao and Rank, 1988). Successively, the transformants were

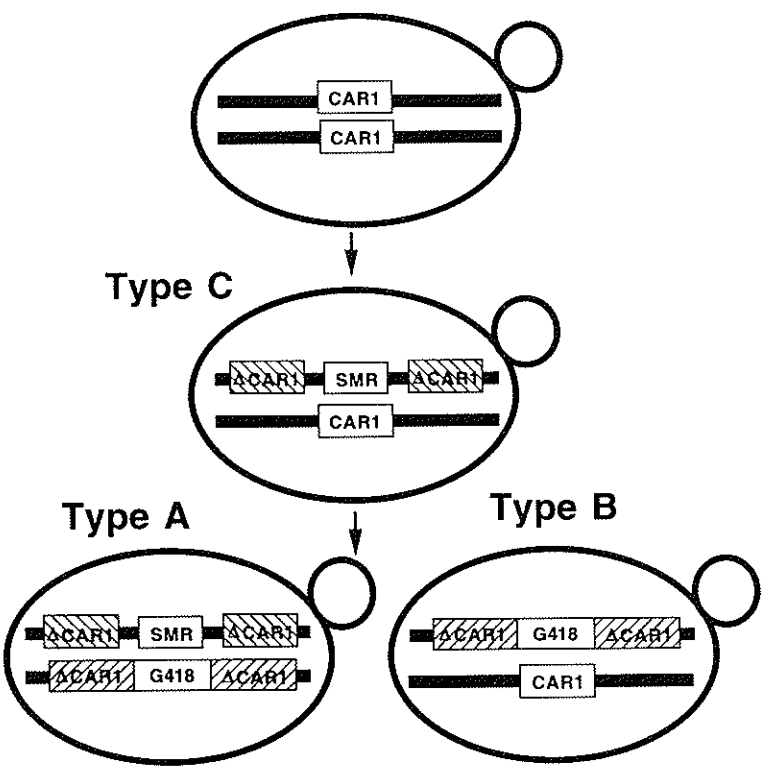


Figure 11. Procedure for *CAR1* gene disruption (Kitamoto *et al.*, 1991).

re-transformed with pCAT1 (△*car1 G418*^{r}) digested with *Bam*HI to disrupt the remaining copy of the *CAR1* gene. Transformants with *SMR1* and *G418*^{r} genes show resistance to sulphometuron methyl and G418 (Geneticin), respectively. As the result of two transformations, the transformants, in which two copies of the *CAR1* gene were expected to be disrupted, were isolated on G418 plates. The transformant (type C) with pCAT2 (△*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 (△*car1 SMR1*) and pCAT1 (△*car1 G418*^{r}) were divided into two groups with different phenotypes. One group (type A) showed resistance to both SM and G418, but the other (type B) showed resistance only to G418. Type A transformants, which are the expected ones 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.

Arginase activity of type B and C strains was almost the same as that of the parent strain, K-9, while it was not detected in the type A strain (*Table 4*). It was confirmed by Southern blotting that the two copies of the *CAR1* gene were disrupted in the type A strain. To investigate the effect of the *CAR1* gene disruption on sake brewing, laboratory-scale sake brewing was carried out by using the parent strain and the three types of transformants. 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 for the concentration of urea and the amounts of arginine and ornithine (*Table 5*). The sensory test also showed that the sake produced by the mutant had a similar quality to that produced by the parent strain. No urea was detected in sake when the type A transformant was used (*Table 6*). The brewed sake was pasteurized at 65°C for 30 min, then stored at 30°C for 45 days or 5 months. After storing, the ECA concentration was determined (*Table 6*). ECA was not detected in the sake produced by type A strain even after 5 months, whereas more than 100 ng ml^{-1} of ECA was formed in sake when K-9, type B and type C strains were used.

Molecular approaches for the breeding of *koji*-mould, *Aspergillus oryzae*

As mentioned above, *koji* is used as the saccharifying and diastatic agent (which is comparable to malt in brewing) in the manufacture of sake. *Koji*

Table 4. Arginase activities of wild type and *car1* mutant strains (Kitamoto *et al.*, 1991)

Strain	Induced	Non-induced
Type A-1	ND	ND
Type B-1	85·2	30·3
Type C-1	83·7	34·4
K-9	111·8	41·9

Arginase activities are expressed as μmole urea h^{-1} mg^{-1} protein.
ND, not detected.

Table 5. Analysis of sake brewed using the *car1* mutants (Kitamoto *et al.*, 1991)

Strain	Sake meter*	Alcohol content (%)	Acidity†	Amino acidity‡	Sensory score§
Type A	−2·0	18·2	2·7	1·9	1·7
Type B	−2·5	18·1	2·7	2·1	2·0
Type C	−2·0	18·3	2·8	2·1	2·0
K-9	−1·5	18·2	2·8	1·9	1·7

*, †, ‡ and § are the same as those in *Table 3*.

Table 6. Urea concentration in the brewed sake and ECA concentration after storing (Kitamoto *et al.*, 1991)

Strain	Urea ($\mu g\ ml^{-1}$)	ECA ($ng\ ml^{-1}$) after storing at 30°C	
		for 45 days	for 150 days
Type A	ND	ND	ND
Type B	32·3	36·0	146·5
Type C	33·3	37·1	127·4
K-9	39·4	45·7	180·5

ND, not detected.
The limits of detection in the assays for urea and ECA are 0·5 $\mu g\ ml^{-1}$ and 5 $ng\ ml^{-1}$, respectively.

also contributes to the colour, flavour and aroma which are important for the overall attributes of the resulting sake. The quality of the *koji* greatly depends upon the properties of the fungal strain used. Therefore, the *koji*-mould is the most important factor in determining the distinctive quality of sake. Accordingly, the breeding of *koji*-moulds has long been attempted, mainly by the mutation method.

Recently, the application of recombinant DNA technology to *Aspergillus* has allowed strain improvement by increasing the number of genes encoding enzymes of industrial importance. Furthermore, as *koji*-moulds have no sexual life cycle, little is known about their genetics. Recombinant DNA technology also contributes to our understanding of the organization and regulation of the genes concerning the fermentation process.

HOST-VECTOR SYSTEMS IN *ASPERGILLUS ORYZAE*

Among the filamentous fungi, *Aspergillus nidulans* and *Neurospora crassa* have been most extensively investigated in molecular genetics, owing to a great store of knowledge on their physiology, biochemistry and genetics. Initially, DNA-mediated transformation of *N. crassa* was accomplished (Case *et al.*, 1979) and then the successful transformation of *A. nidulans* was reported (Ballance, Buxton and Turner, 1983; Tilburn *et al.*, 1983). Based on the molecular genetic systems of these fungi, systems for several industrially important species, including *A. niger* (Buxton, Gwynne and Davies, 1985; Kelly and Hynes, 1985) and *A. oryzae* (see below), have also been developed. *Table 7* and *Figure 12* show the selectable markers and the plasmid vectors used for transformation of the *koji*-mould, *A. oryzae*, respectively.

Generally, to introduce the exogenously added DNA into the *koji*-mould, the use of protoplasts is required. Protoplasts are prepared by treating the mycelia with fungal cell wall lytic enzymes. Nowadays, commercial enzyme preparations, produced mainly from *Trichoderma* sp., are available. When

Table 7. Selectable markers used for the transformation of *koji*-mould, *A. oryzae*

Marker	Encoded enzyme	Source	Reference
argB	Ornithine carbamoyltransferase	*A. nidulans*	Gomi, Iimura and Hara (1987) Hahm and Batt (1988)
pyrG	Orotidine-5′-phosphate decarboxylase	*A. niger*	Mattern *et al.* (1987)
		A. oryzae	de Ruiter-Jacobs *et al.* (1989)
niaD	Nitrate reductase	*A. oryzae*	Unkles *et al.* (1989)
amdS	Acetamidase	*A. nidulans*	Christensen *et al.* (1988)
		A. oryzae	Gomi, Kitamoto and Kumagai (1991)
met	Undetermined	*A. oryzae*	Iimura *et al.* (1987)

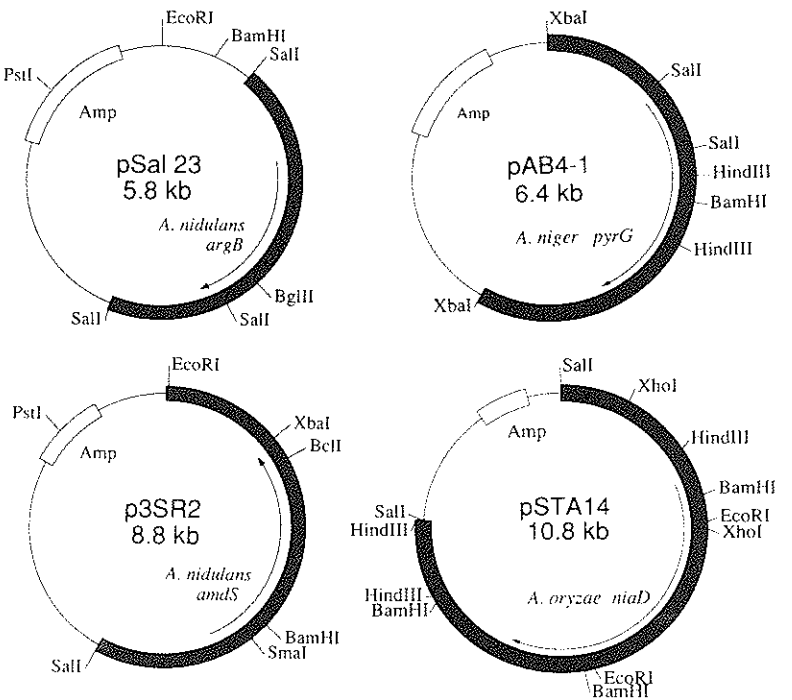


Figure 12. The structures of the plasmid vectors used for transformation of the *koji*-mould, *A. oryzae*.

protoplasting, 0·8 M NaCl or KCl is used as an osmotic stabilizer. Then protoplasts are mixed with transforming DNA and fused in the presence of 40% polyethylene glycol (PEG) and 10–50 mM $CaCl_2$. Fused protoplasts are embedded in the regeneration agar medium. Recently, *A. niger* was transformed by electroporation (Ward, Kodama and Wilson, 1989), but *A. oryzae* has not been transformed by this method.

As in *A. nidulans*, transformation systems in *A. oryzae* have been developed by complementation of nutritional requirements. The successful transformation of *A. oryzae* through complementation of a methionine-auxotrophic mutation was first reported by Iimura *et al.* (1987). A genomic library of *A. oryzae* was screened for a DNA fragment complementing the methionine-auxotrophic mutation. As a result, a plasmid carrying a 3·5 kb

*Bam*HI fragment of *A. oryzae* was isolated, and this plasmid could be recovered in *E. coli*, suggesting that it might exist, in part, as a free plasmid in the transformants. The enzyme in the methionine biosynthetic pathway encoded by the isolated DNA fragment has not been determined. However, these results have opened the way to investigate the *koji*-moulds at the molecular level.

Shortly thereafter, Gomi, Iimura and Hara (1987) and Mattern *et al.* (1987) further developed the transformation systems using an *argB* gene encoding ornithine carbamoyltransferase (OCTase) of *A. nidulans* and a *pyrG* gene encoding orotidine-5′-phosphate decarboxylase of *A. niger*, respectively, as selectable markers. The recipient *argB* mutants can be easily distinguished among many arginine-auxotrophic mutants by their growth phenotype on a medium containing citrulline, although there are no available positive selection procedures for isolating the *argB* mutants. In contrast, the *pyrG* mutants having uracil or uridine auxotrophy can be isolated by positive selection for 5-fluoro-orotic acid resistance.

In both cases, the transformation occurred by integration of the transforming plasmid into the chromosome, as in other filamentous fungi. The two genes used as selectable markers were derived from *A. nidulans* and *A. niger*, species related to *A. oryzae*, and could function in *A. oryzae*. However, Southern blot analysis revealed that the genes did not have a high degree of homology to the corresponding genes of *A. oryzae*. Therefore, it is likely that the transformation occurred by integration at non-homologous regions. This might account for the low frequency of transformation (less than 10 transformants per μg of plasmid DNA). The transformants generally contained many copies of the foreign gene in tandem arrays on the chromosome. Gomi, Iimura and Hara (1987) estimated the copy number of the integrated *argB* gene in the transformants, one of which (transformant-2) contained at least 60 copies and the others also had more than 15 copies (*Figure 13*). These phenotypes were extremely stable for many generations under non-selective conditions. The activity of OCTase encoded by the *argB* gene in the transformants increased in proportion to the copy number of the integrated gene (*Table 8*), suggesting that the transformation system would allow improvement in the production of industrially important enzymes, in addition to the possibility of the production of valuable heterologous proteins.

Table 8. Specific activity of ornithine carbamoyltransferase in the transformants (Gomi, Iimura and Hara, 1987)

Strain	Specific activity*	Activity relative to FN-16†
A. oryzae		
FN-16‡	17·1	1·0
Transformant-1	469·9	27·5
Transformant-2	904·6	52·9
Transformant-3	514·3	30·1
A. nidulans		
IAM 2006	22·2	1·3

* Specific activity; μg citrulline min^{-1} mg^{-1} protein.
† Specific activity of FN-16 is expressed as 1·0.
‡ Parent strain of the recipient, M-2-3.

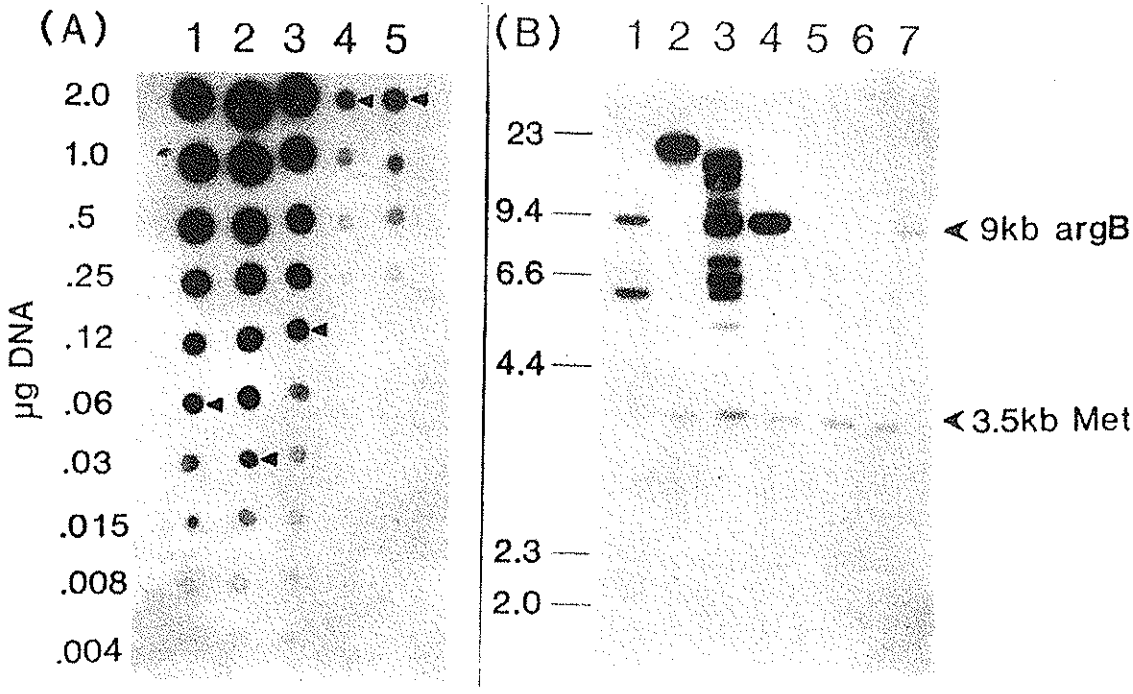


Figure 13. Southern blot analysis and determination of the copy number of the plasmid in the transformants (Gomi, Iimura and Hara, 1987). (A) Genomic DNA was serially diluted twofold and then spotted on a nylon membrane, followed by hybridization with the plasmid carrying the *A. nidulans argB* gene and *A. oryzae met* gene used as an internal standard for the estimation. Lanes: 1, transformant-1; 2, transformant-2; 3, transformant-3; 4, recipient strain, *A. oryzae* M-2-3; 5, *A. nidulans* IAM 2006. The spots showing the same intensities are indicated by arrows. (B) Genomic DNA was digested with *Bam*HI and electrophoresed, followed by hybridization with the plasmid used in (A). Lanes: 1, pSal43 digested with *Bam*HI and *Eco*RI; 2, transformant-1; 3, transformant-2; 4, transformant-3; 5, recipient strain, *A. oryzae* M-2-3; 6, parent strain, FN-16; 7, *A. nidulans* IAM 2006. The 3·5 kb band in lanes 2–6 and the 9 kb band in lane 7 correspond to the genomic *A. oryzae met* gene and *A. nidulans argB* gene, respectively.

Recently, de Ruiter-Jacobs *et al.* (1989) isolated a *pyrG* gene from *A. oryzae*, and showed that the transformation using this gene occurred by homologous recombination, which leads to an increase of the transformation frequency.

In general, transformation systems using complementation of the auxotrophic mutation are available for fundamental investigations. For instance, the regulational analysis of the α-amylase-encoding gene has been performed (Tada *et al.*, 1991a) using this system. However, this system may be unsuitable for the breeding of the useful strain for an industrial use, because it is possible to cause damage to genes important in the fermentation process when auxotrophic mutants are isolated. To avoid this disadvantage, positive transformation systems using dominant selectable markers are obligatory. *Aspergillus oryzae* is resistant to most antibiotics used for positive selection in yeast (*Saccharomyces cerevisiae*) and most filamentous fungi, but is sensitive to benomyl (1–2 μg ml^{-1}) and phleomycin (50 μg ml^{-1}). A transformation system with the genes responsible for resistance against these compounds is not yet available (Mattern *et al.*, 1987; Gomi, unpublished results). Another dominant selectable marker, the acetamidase-encoding gene (*amdS*) from *A. nidulans*, has been widely utilized for the transformation of many filamentous fungi, which are unable to assimilate acetamide as a nitrogen source. Industrial species, including *A. niger* (Kelly and Hynes, 1985), *Penicillium chrysogenum* (Beri and Turner, 1987) and *Trichoderma reesei* (Penttila *et al.*, 1987), have been transformed with the *amdS* gene as a selectable marker. Unlike these fungi, *A. oryzae* can grow on a medium containing acetamide as a sole nitrogen source. However, *A. nidulans* strains with an ability of acetamide assimilation show more vigorous growth on the medium containing acetamide as the sole nitrogen source by the introduction of multiple copies of the *amdS* gene (Kelly and Hynes, 1987). A similar selection system could be developed for the transformation of *A. oryzae*. In fact, Christensen *et al.* (1988) reported a successful transformation of *A. oryzae* using the *A. nidulans amdS* gene. More recently, an *amdS* gene from *A. oryzae* has been cloned (Gomi, Kitamoto and Kumagai, 1991) and has proved to be applicable for the homologous transformation of the wild type of *A. oryzae*. Additionally, the promoter of the *amdS* gene was replaced with the strong expression promoter of the α-amylase-encoding gene of *A. oryzae*, thereby enhancing selection of transformants (Gomi *et al.*, unpublished results).

Unkles *et al.* (1989) developed an efficient transformation system for *A. oryzae* involving a gene of the nitrate assimilation pathway. They used a *niaD* gene encoding nitrate reductase as a selectable marker for the transformation of an *A. oryzae niaD* mutant unable to utilize nitrate as the sole nitrogen source. In particular, the transformation using a plasmid carrying the *niaD* gene of *A. oryzae* occurred only by homologous integration at a resident locus on the host genome, and the event of gene replacement arose to some extent. Transformation frequency was much higher than that using heterologous genes as selectable markers. Furthermore, this transformation system has some advantages which make it extremely favourable for the improvement of industrially important *koji*-moulds. First, since *niaD* mutants can be easily isolated as strains resistant to chlorate without any induction procedures of

mutation, there is little deleterious effect on the genes important for industrial use. Secondly, the pathway for nitrate utilization is entirely dispensable, so mutation within the pathway should not cause any secondary growth defects. Finally, since transformation occurs only by homologous integration, the regulational control of the expression of the genes of interest can be analysed.

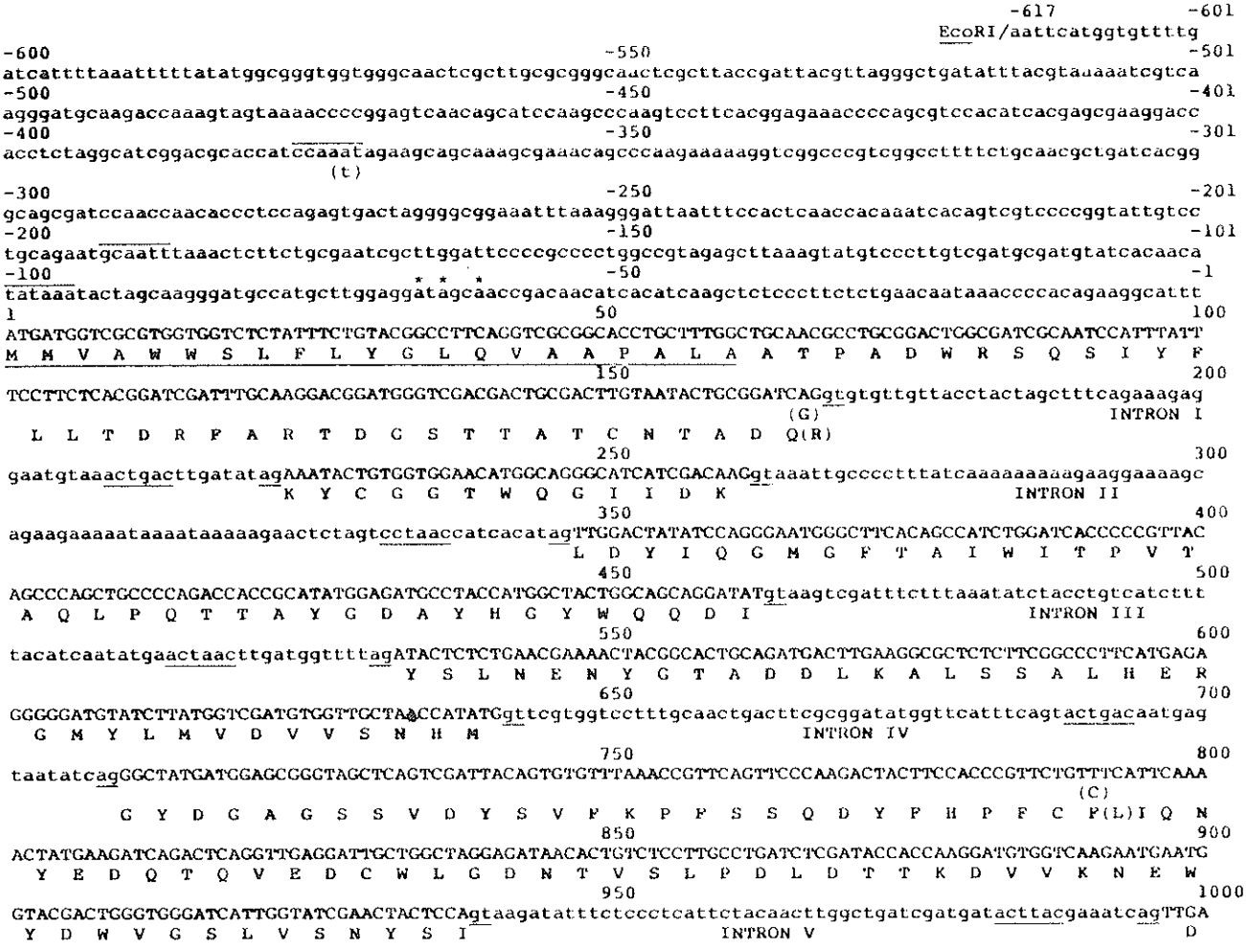
In contrast to yeasts, many attempts have been made to develop autonomously replicating plasmids in filamentous fungi, but so far they have failed, except for a few cases, because of a lack of extrachromosomal DNA. Autonomously replicating plasmids will allow the increase of the transformation frequency and the re-isolation of plasmids carrying the genes complementing the mutations. In the zygomycete fungus *Mucor circinelloides*, a chromosomal sequence which confers autonomous replication of plasmids was found (van Heeswijck, 1986). Recently, a sequence conferring autonomous replication in *Aspergillus* was also isolated from *A. nidulans* (Gems, Johnstone and Clutterbuck, 1991). The sequence designated *AMA1* could enhance the transformation frequency over 200-fold and the plasmid containing the sequence was maintained extrachromosomally in the *A. nidulans* transformants. Moreover, the sequence could also function in a similar fashion in *A. oryzae*, and thus it will be a prominent tool for the isolation of genes from *A. oryzae* by complementation.

CLONING AND ANALYSIS OF THE GENES OF IMPORTANT ENZYMES IN SAKE BREWING

In sake brewing, *koji* is used for saccharification of starch of steamed rice, and produces many kinds of hydrolases, of which the most important for sake brewing are α-amylase and glucoamylase. The α-amylase of *A. oryzae* is known as Taka-amylase A (TAA) and has been investigated extensively. It is a glycoprotein consisting of a single polypeptide chain of 478 amino acid residues (Toda, Kondo and Narita, 1982). The carbohydrate chain structure and X-ray crystallographic analysis of the enzyme have been reported (Yamaguchi, Ikenaka and Matsushima, 1971; Matsuura *et al.*, 1984). In contrast to many biochemical analyses, little work has been done by means of genetical analysis. The reason is that *A. oryzae* has no sexual life cycle and the multinucleate conidia and hyphae make mutant isolation very difficult. Recently, in order to elucidate the function of TAA at the molecular level, the TAA gene was cloned and the nucleotide sequence was determined by Tada *et al.* (1989).

TAA genes were isolated from a λEMBL3 genomic library of *koji*-mould, *A. oryzae* RIB 40, by plaque hybridization using two kinds of 26mer oligonucleotide probes, which were synthesized according to the amino acid sequence (from 54 to 62, and from 448 to 456) reported by Toda, Kondo and Narita (1982). The probes contained deoxyinosine at positions where the nucleotide could not be assigned due to codon degeneracy.

Genomic Southern blot analysis with the cloned TAA gene as a probe suggested the existence of at least three TAA genes on the chromosomes in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-100 * * * -50 -1
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1 50 100
ATGATGGTCGCGTGGTGGTCTCTATTTCTGTACGGCCTTCACGTCGCGGCACCTGCTTTGGCTGCAACGCCTGCGGACTGGCGATCGCAATCCATTTATT
M M V A W W S L F L Y G L Q V A A P A L A A T P A D W R S Q S I Y F
150 200
TCCTTCTCACGGATCGATTTGCAAGGACGGATGGGTCGACGACTGCGACTTGTAATACTGCGGATCAGgtgtgttgttacctactagctttcagaaagag
(G) INTRON I
L L T D R F A R T D G S T T A T C N T A D Q(R)
250 300
gaatgtaaactgacttgatatagAAATACTGTGGTGGAACATGGCAGGGCATCATCGACAACGgtaaattgccccttttatcaaaaaaaaaagaaggaaaagc
K Y C G G T W Q G I I D K INTRON II
350 400
agaagaaaaatataaaataaaaagaactctagtcctaaccatcacatagTTGGACTATATCCAGGGAATGGGCTTCACAGCCATCTGGATCACCCCCGTTAC
L D Y I Q G M G F T A I W I T P V T
450 500
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A Q L P Q T T A Y G D A Y H G Y W Q Q D I INTRON III
550 600
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Y S L N E N Y G T A D D L K A L S S A L H E R
650 700
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G M Y L M V D V V S N H M INTRON IV
750 800
taatatcagGGCTATGATGGAGCGGGTAGCTCAGTCGATTACAGTGTGTTTAAACCGTTCAGTTCCCAAGACTACTTCCACCCGTTCTGTTTCATTCAAA
(C)
G Y D G A G S S V D Y S V F K P F S S Q D Y F H P F C F(L)I Q N
850 900
ACTATGAAGATCAGACTCAGGTTGAGGATTGCTGGCTAGGAGATAACACTGTCTCCTTGCCTGATCTCGATACCACCAAGGATGTGGTCAAGAATGAATG
Y E D Q T Q V E D C W L G D N T V S L P D L D T T K D V V K N E W
950 1000
GTACGACTGGGTGGGATCATTGGTATCGAACTACTCCAgtaagatatttctccctcattctacaacttggctgatcgatgatacttacgaaatcagTTGA
Y D W V G S L V S N Y S I INTRON V D

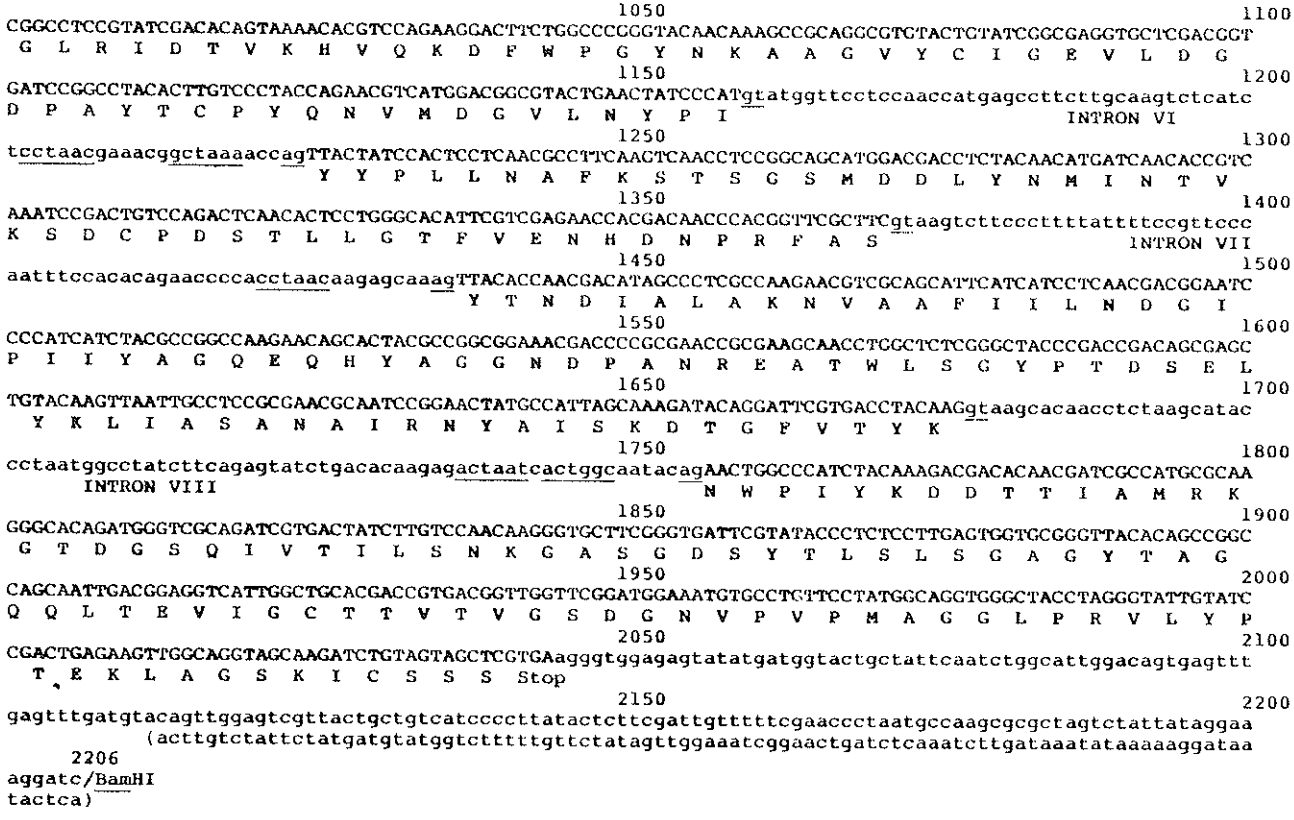


Figure 14. Nucleotide and deduced amino acid sequence of α-amylase-encoding gene of *A. oryzae* (Tada *et al.*, 1989). A putative TATA box and CAAT boxes are overlined. GT/AG and the sequence related to an internal consensus sequence, PuCTPuAC, in introns are underlined. The signal peptide sequence is also underlined. Major transcription start points are indicated by asterisks. Different nucleotides and amino acids of *amyA* compared to those of *amyB* and *amyC* are shown in parentheses.

A. oryzae. The nucleotide sequences of the two genes designated as *amyB* and *amyC* were completely identical throughout, including the coding region, and the 5′ and 3′ non-coding regions. Another gene (*amyA*) had almost the same sequence as the others, differing by only two nucleotides in the coding region, but differing significantly around the 3′ non-coding region. The nucleotide sequence of the genes showed that the TAA-coding regions consisted of eight introns and nine exons, and coded for 499 amino acids (*Figure 14*). All eight introns started with GT and terminated with an AG sequence, general features of introns (Gurr, Unkles and Kinghorn, 1987). In addition, a sequence homologous to the internal consensus sequence found in fungi, PuCTPuAC, was observed (Gurr, Unkles and Kinghorn, 1987). An interesting finding was that the region of the ninth exon seems to correspond to the C-terminal domain found by Matsuura *et al.* (1984) on X-ray analysis, of which the function is unclear. Compared with the amino acid sequence of the mature TAA reported by Toda, Kondo and Narita (1982), an additional 21 amino acids, which are thought to be a signal sequence, were observed at the N-terminus of the cloned TAA gene. Furthermore, one insertion and one deletion, as well as 10 substitutions, of amino acid residues were found throughout the whole sequence in the two identical genes (*amyB*, *amyC*). It is likely that these changes are due to a strain variation. Additionally, the transformation experiment, where each of the three genes was introduced into *A. nidulans* or introduced back to *A. oryzae*, revealed that all of the genes are functional in the fungi.

Independently, the TAA genes from *A. oryzae* were cloned and sequenced by other groups (Gines, Dove and Seligy, 1989; Wirsel *et al.*, 1989). Wirsel *et al.* also reported that there are three TAA genes with almost the same nucleotide sequences, and that, by detecting the corresponding mRNAs, at least two genes are thought to be expressed.

In order to elucidate the location of the TAA genes on the chromosomes, physical mapping of the genes has been done. The three TAA genes were located on three different chromosomes by orthogonal-field-alternation gel electrophoresis (OFAGE) Southern blot analysis, as shown in *Figure 15* (Gomi *et al.*, unpublished results). In *A. sojae*, the *koji*-mould for *shoyu* making, only one chromosome was hybridized with the TAA gene. It is very interesting that *A. oryzae*, which is used for sake brewing and is needed for higher α-amylase activity, has more TAA genes.

Northern blot analysis of the TAA gene was performed in order to analyse the regulational control of the gene expression. Total RNA was extracted from glucose- or starch-grown mycelia of *A. oryzae* RIB 40 and separated by formaldehyde-gel electrophoresis. The analysis with the TAA gene as a probe revealed a discrete RNA species which was prevalent when starch was used as the sole carbon source (Tada *et al.*, 1991b). Ethidium bromide staining showed that almost the same amounts of RNA were present in each sample. This result confirmed that the production of TAA is controlled at a transcriptional level, consistent with the result from an *in vitro* translation experiment (Erratt *et al.*, 1984).

Two extensively related α-amylase genes have also been cloned from *A.*

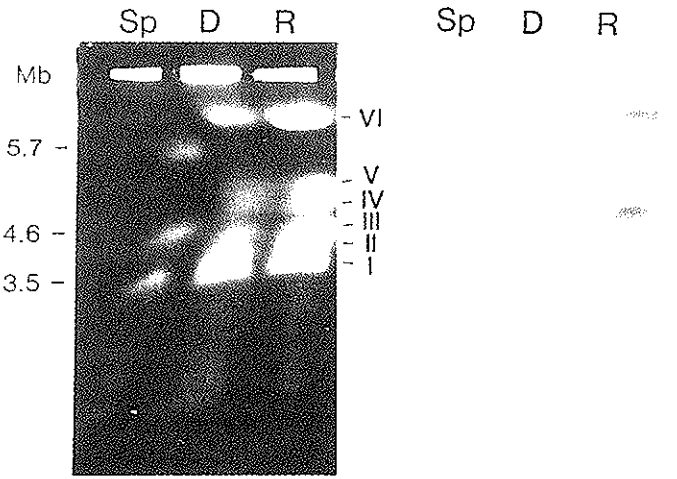


Figure 15. Chromosomal location of the three α-amylase genes in *A. oryzae*. (Left panel) Ethidium bromide (EtdBr) staining of the agarose gel. Lanes: Sp, *Schizosaccharomyces pombe* as molecular size markers, which are given in the panel; D, *A. oryzae* RIB 40 (DNA source of the genes); R, *A. oryzae* M-2-3 (*argB* auxotrophic mutant strain used for transformation). The six chromosome bands resolved are consecutively numbered from the smallest (I) to the largest (VI). (Right panel) Autoradiogram of the blot following hybridization to the 3 kb *Eco*RI–*Bam*HI fragment containing *amyB*.

niger var. *awamori* and their nucleotide sequences have a very high degree of homology (more than 98%) to that of the TAA gene from *A. oryzae* (Korman *et al.*, 1990).

Hata *et al.* (1991a, b) isolated cDNA and genomic DNA encoding glucoamylase from *A. oryzae*, and determined their nucleotide sequences (*Figure 16*). The homology with *A. niger* glucoamylase (Boel *et al.*, 1984) was about 70% at the amino acid level. Particularly, *A. oryzae* glucoamylase was found to lack the threonine and serine-rich region, which is present between the catalytic domain and the raw-starch binding domain of *A. niger* glucoamylase. Northern blot analysis indicated that glucoamylase production was also controlled by starch or maltose at the transcriptional level.

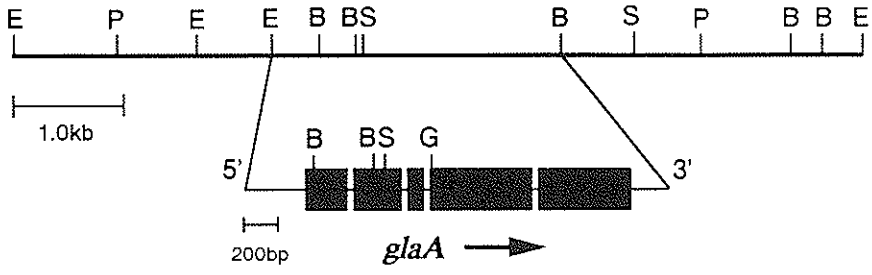
The cDNA encoding alkaline protease, which is important for *shoyu* making, has also been cloned from *A. oryzae* (Tatsumi *et al.*, 1989). The cDNA was expressed and the protease was secreted in *Zygosaccharomyces rouxii* (*shoyu* yeast) with a yield of about 100 mg l^{-1} (Ogawa *et al.*, 1990).

In addition to these genes concerning sake brewing, genes encoding ribonuclease T_2 (Ozeki *et al.*, 1991), acetamidase (Gomi, Kitamoto and Kumagai, 1991) and 3′-phosphoglycerate kinase (Ogawa *et al.*, manuscript in preparation) have been cloned from *A. oryzae* and their nucleotide sequences have also been determined.

BREEDING OF USEFUL *ASPERGILLUS* STRAINS

By introduction of the cloned TAA gene into *A. oryzae* using the transformation system (Gomi, Iimura and Hara, 1987), high-α-amylase-producing

A



B

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                                                                                gaattcacgggcgaaatcaatttgtggctgcatcctcatgtcttgaaacaagcagtgtga  -289
ttggaagtggtggcgtcttggctccagccagctgcatgactatgagaaaatagtccaactagtcttggcttgcagaatgacttccggaaatttaac  -193
ctggtggctgctctcagtattggctgacgctccgcacctctaattgtgcttcaggtcagtcaaagatgttttgaaccaccaacgagccgaaccctt  - 97
ccgccgctacaatactcgggttattataaaacgcatcgtggttcatcgtcccagctataggattattttcacatcagcaaacgaagtcgaagcaag  -   1
ATGGTGTCTTTCTCCTCTTGTCTCCGGGCCTTAGCCCTCGGATCCTCAGTTCTCGCGGTCCAACCTGTCCTTAGACAGGCGACTGGTCTAGATACC    96
 M  V  S  F  S  S  C  L  R  A  L  A  L  G  S  S  V  L  A  V  Q  P  V  L  R  Q  A  T  G  L  D  T     32
TGGCTGAGCACAGAAGCAAATTTTTCCCGCCAGGCAATCTTGAATAATATCGGCGCAGATGGCCAGTCGGCGCAGGGCGCAAGTCCAGGGGTGGTG    192
 W  L  S  T  E  A  N  F  S  R  Q  A  I  L  N  N  I  G  A  D  G  Q  S  A  Q  G  A  S  P  G  V  V     64
ATTGCCAGCCCTAGCAAAAGTGACCCAGATTgtacgtgctctggccttttgcaatccttcatttgacttactggccgtagATTTCTATACCTGGAC    288
 I  A  S  P  S  K  S  D  P  D                    IVS 1                    Y  F  Y  T  W  T     80
CCGTGACTCGGGTCTCGTCATGAAAACCCTGGTCGATCTGTTCAGAGGCGGAGATGCCGATCTTCTCCCTATCATCGAGGAGTTCATTAGCTCCCA    384
  R  D  S  G  L  V  M  K  T  L  V  D  L  F  R  G  G  D  A  D  L  L  P  I  I  E  E  F  I  S  S  Q    112
GGCTCGGATCCAAGGCATCTCAAACCCTTCTGGTGCTCTTTCCAGTGGGGGTCTGGGCGAGCCTAAGTTCAATGTCGACGAGACAGCATTTACCGG    480
  A  R  I  Q  G  I  S  N  P  S  G  A  L  S  S  G  G  L  G  E  P  K  F  N  V  D  E  T  A  F  T  G    144
CGCATGGGGTCGGCCGCAGCGTGACGGACCAGCTTTGCGCGGCGACCGCTATGATCTCGTTTGGAGAATGGCTAGTTgtaagttccatccgtctttc    576
  A  W  G  R  P  Q  R  D  G  P  A  L  R  A  T  A  M  I  S  F  G  E  W  L  V                        169
agaatgggcgtagcttaccttatagGAAAATAGTCATACAAGCATAGCGACGGACCTGGTATGGCCTGTTGTTAGGAATGATCTATCCTATGTAGC    672
 IVS 2                    E  N  S  H  T  S  I  A  T  D  L  V  W  P  V  V  R  N  D  L  S  Y  V  A    193
TCAGTATTGGAGCCAATCCGGGTTCGgtgagcttctagagaaatggcccgcagctatcagtagctaacttccacagATCTCTGGGAGGAAGTCCAA    768
  Q  Y  W  S  Q  S  G  F                          IVS 3                    D  L  W  E  E  V  Q    208
```

Figure 16. Nucleotide and deduced amino acid sequence of the glucoamylase-encoding gene of *A. oryzae* (Hata *et al.*, 1991b). (A) Restriction endonuclease map of the *A. oryzae* genome surrounding the *glaA* gene. The location and orientation of the *glaA* gene are shown below; protein-encoding regions are indicated as solid boxes. Abbreviations: B, *Bam*HI; E, *Eco*RI; G, *Bgl*II; S, *Sal*I. (B) Nucleotide sequence and deduced amino acid sequence of the *glaA* gene. Putative TATA and CAAT boxes in the 5′ non-coding region, consensus sequence in introns, and a putative polyadenylation signal in the 3′ non-coding region are underlined. The nucleotide sequence of the protein-coding region is given in capital letters and the deduced amino acid sequence is shown below each codon.

koji-moulds were constructed (Tada *et al.*, 1989). The *Eco*RI fragment of the cloned TAA gene was inserted into the *Eco*RI site of pSa123. The transformants appeared to have two- to sixfold higher TAA activity (Table 9).

Similarly, high-glucoamylase-producing *koji*-moulds were constructed by the transformation of *A. oryzae* with the cloned glucoamylase gene (Hata *et al.*, 1991b). The glucoamylase activity of the transformants was about eightfold higher than the parent strain. In this experiment, interestingly, the transformant had about one-tenth lower α-amylase activity. The mechanism of this effect is unclear. Furthermore, when the gene encoding the *A. usamii* mut. *shirousamii* glucoamylase, which has the ability to digest raw starch, was introduced into *A. oryzae*, the transformants showed an increased level of raw-starch digesting activity (Shibuya *et al.*, 1990).

The transformants have not yet been used to make *koji* for sake brewing. However, since it has been made clear that *koji*-moulds constructed through a recombinant DNA technique have high enzyme activity, by increasing the copy number of the corresponding enzyme gene, this method will be a powerful tool for the breeding of *koji*-moulds.

Conclusion

It is only a few years since studies on gene cloning and gene transfer into sake yeasts and *koji*-mould started. There is as yet little work on genetic engineering in sake brewing, but the systems and tools for the genetic transfer have now been established. Since this technique is superior to the mutation, crossing, or protoplast fusion techniques previously used, it will provide powerful tools for the breeding of micro-organisms for sake brewing and for analysis of the complex mechanisms of sake brewing. In addition, since sake yeast and *koji*-mould have been used for sake brewing for more than 1000 years, *S. cerevisiae* and *A. oryzae* are believed to be safe as hosts for the production of heterologous protein, such as prochymosin, interleukin and so on (Saunders *et al.*, 1989). The system for genetic transfer to yeasts and moulds would also contribute to the production of proteins important to human life.

Table 9. α-Amylase activity produced by transformants (Tada *et al.*, 1989)

Strain	Plasmid	α-Amylase activity	
		Submerged culture*	Rice-*koji*†
FN-16‡		8800	785
M-2-3	pSal23	8200	546
M-2-3	pSal23+*amyB*	32 100	2190
	pSal23+*amyB*	21 400	2980
	pSal23+*amyB*	49 050	2110

* Units: per g dry mycelium.
† Units: per g rice-*koji*.
‡ Parent strain of *argB* mutant, M-2-3.

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