Intraspecific Hybridization of *Aspergillus awamori* var. *kawachi* by Protoplast Fusion

Kihachiro OGAWA, Hitoshi OHARA and Nobuo TOYAMA

Applied Microbiology Laboratory, Department of Agricultural Chemistry,
Faculty of Agriculture, Miyazaki University,
Miyazaki 889-21, Japan

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Protoplast fusion was studied to breed a koji-mold, *Aspergillus awamori* var. *kawachi*, used for making a Japanese spirit, shochu. Protoplasts were obtained from mycelia of two biochemical mutants using an enzyme system from *Trichoderma viride* consisting of mainly β -1,3-glucanase and chitinase. Approximately 10~20% of these protoplasts regenerated on a solid culture medium with a soft agar overlay. When the two types of protoplasts were treated with a fusogen including 35% polyethylene glycol, they fused effectively and formed a heterokaryon. A strain obtained from the heterokaryon using *d*-camphor showed high stability (98.8%) and numerical sporulation, and produced the same levels of amylase and citric acid as those by the original strain. From these results and the formation of recombinants, the new strain was assumed to be a heterozygous diploid. The appearance frequency of the diploid was 2.9×10^{-2} . The existence of a parasexual cycle in this fungus was also discovered, using the protoplast fusion technique.

Shochu is a traditional alcoholic drink in Japan. *Aspergillus awamori* var. *kawachi*, *Aspergillus kawachii* Kitahara (a mutant of *A. awamori* var. *kawachi*) and *Aspergillus oryzae* have been commonly used for making shochu, but there have not reports of studies on genetic and active breeding of koji-molds used for making shochu.

In recent years, fungal breeding using protoplast fusion techniques has been performed throughout the world. Many studies concerned the intra- and inter-specific protoplast fusion of filamentous fungi, including strains of *Aspergillus*,^{1~4)} *Penicillium*,⁵⁾ *Mucor*⁶⁾ and *Trichoderma*.^{7,8)} Although heterokaryon formation through protoplast fusion seems to be an effective method for analyzing a parasexual cycle, no studies concerning koji-molds used for making shochu have been reported as yet.

The present study was undertaken to determine the feasibility of koji-mold breeding through protoplast fusion and to obtain a useful strain for making shochu. We attempted to clarify the conditions for the intraspecific

fusion and regeneration of *A. awamori* var. *kawachi* and to establish a method for obtaining heterokaryons from among different biochemical mutants derived from the fungus. The heterozygous diploid derived from the heterokaryons obtained was shown to be stable and useful as an original strain.

MATERIALS AND METHODS

Organisms. The citric acid hyper producer, *A. awamori* var. *kawachi* KGS (Kawachi Genichiro Shoten), used for making shochu, was employed as the original strain. Conidia of this strain were treated with UV rays in a routine manner. The auxotrophic single mutants obtained were checked as to their stability over several vegetative generation, and as to their characteristic citric acid and amylase production. Strain KGS-1 (*met*) and KGS-2 (*arg*) are mutants of *A. awamori* var. *kawachi* KGS, respectively. These parental strains also produced single nucleus conidia on a complete medium.

Mycolytic enzyme sources. Commercial enzyme preparations, Zymolyase 5,000 (Seikagaku Kogyo Co., Ltd.) and Halicase (Reactif de Laboratoire), and a *Trichoderma viride* BIA preparation⁹⁾ were used in this work.

Cultivation of microorganisms. Czapek Dox medium

containing 2% glucose was used as a minimal medium (MM). The minimal medium supplemented with 0.5% yeast extract and casamino acid was used as a complete medium (CM).

Enzyme assays. β -1,3-Glucanase activity: A reaction mixture consisting of 0.5 ml of a 1% laminarin (Tokyo Chem. Ind. Co., Ltd.) solution (0.1 M acetate buffer, pH 5.0) and 0.5 ml of an adequately diluted enzyme solution was incubated at 45°C for 30 min. The reducing sugar was determined by the 3,5-dinitrosalicylic acid method.¹⁰⁾ One unit of β -1,3-glucanase activity was defined as the amount of enzyme which produced 1 μ mol of reducing sugar as glucose per min. Chitinase activity: in a test tube, 1 ml of a 1% colloidal chitin suspension (prepared from chitin, shrimp; Wako Pure Chem. Ind. Ltd.) and 1 ml of an appropriately diluted enzyme solution were incubated in a water bath at pH 5.0 and 40°C for 60 min. The amount of NAG (N-acetyl-D-glucosamine) was determined by the modified method of Reissig.¹¹⁾ One unit of chitinase activity was defined as the amount of enzyme which liberated 1 μ mol of NAG.

Amylase activity: This activity was assayed according to our β -1,3-glucanase activity assay method using a 2% soluble starch (Wako Pure Chem. Ind.) solution. The enzyme unit was also defined in the same manner as before.

Preparation of protoplasts. A loopful of a conidia-mycelial mixture was taken from a slope, added to 50 ml of the liquid complete medium in a 100 ml Erlenmeyer flask, and then incubated at 30°C and 18 hr on a rotary shaker (150 rpm). After incubation, the mycelia were collected on filter paper (Toyo No. 2) and then washed with distilled water under aseptic conditions. About 200 mg (wet weight) of mycelia was then added to 5 ml of a 3% (w/v) *T. viride* B1A mycolytic enzyme solution, made to 0.6 M with respect to NaCl (or NH_4Cl), followed by incubation in a Monod shaking apparatus at pH 5.0 and 35°C for 1 to 2 hr. The reaction mixture was filtered through G2 and G3 porosity sintered glass filter, and the filtrate was centrifuged at $700\times g$ for 5 min. The sedimented protoplasts were suspended in a 1 ml solution (0.6 M NaCl), which was osmotically balanced.

Protoplast fusion. Fusion was carried out according to a modification of the method of Anne and *et al.*¹²⁾ Protoplasts were prepared from both biochemical mutants. Protoplasts of each mutant were mixed and then centrifuged at $700\times g$ for 5 min. This procedure was repeated several times to purify the protoplasts. The sedimented protoplasts were resuspended in 1 ml of a solution of 35% (w/v) polyethylene glycol 4,000 (PEG; av. mol. wt., 3,000; Wako Pure Chem. Ind. Ltd.) and 0.05 M $\text{CaCl}_2\cdot 2\text{H}_2\text{O}$ in 0.05 M glycine-NaOH buffer (pH 7.5). After standing at 20°C for 15 min, the suspension was

centrifuged at $700\times g$ for 5 min. The sedimented protoplasts were resuspended in 1 ml of an osmotically balanced medium and then plated onto hypertonic MM and CM containing 2% agar and 0.6 M NaCl in Petri dishes, and then overlaid with the same MM containing 0.5% agar. After incubation at 25°C for 4 days, the colonies showing successful fusion were counted and the fusion frequency was estimated. The fusion frequency was defined as the percentage ratio of the numbers of colonies developing on MM and CM after the fusion treatment.

Diploidization of heterokaryons. A solution of 0.1 g of *d*-camphor dissolved in 1 ml of 99% ethanol was added to 99 ml of a MM solution containing 2 g of powdered agar. This mixture was autoclaved at 120°C for 15 min. The autoclaved MM agar solution was poured into a Petri dish and allowed to solidify. Heterokaryon strain KGS-1-2-H was inoculated onto the MM agar and kept at 25°C for 7 days.

Solid culture for enzyme preparation. Twenty grams of rice grains moistened with tap water in a 300 ml Erlenmeyer flask was autoclaved at 25°C for 20 min. The flask was inoculated with a loopful of mycelia and then incubated at 25°C for 5 days. Enzyme activity was assayed in the filtrate prepared by extracting the solid culture with 10 volumes of distilled water for 1 hr.

Determination of DNA. Determination of DNA was carried out according to the method of Burton¹³⁾ after extraction by the method of Schneider.¹⁴⁾ The DNA content per conidium was calculated as follows: $100 \times (\text{the amount of DNA in the conidia}) / (\text{the number of conidia tested}) (10^{-6} \mu\text{g})$.

RESULTS

Protoplast formation and regeneration

The mycolytic enzyme activities of *T. viride* B1A and commercial enzyme preparations were assayed (Table I). Mycelial pellets of *A. awamori* var. *kawachi* prepared from rotary shaken cultures were used in this study. Numerous protoplasts appeared within 1 to 2 hr from mycelia degraded with the *T. viride* enzyme preparation. Although the enzyme from *T. viride* gave the highest yield of protoplasts, the other enzyme preparations did not act effectively in case of *A. awamori* var. *kawachi*. The protoplasts purified by washing were plated on CM agar. In the early stage, the protoplasts swelled somewhat, and later showed two types of regeneration: the for-

mation of chains of yeast-like cells and the direct formation of hyphae.¹⁵⁾

Heterokaryon formation

Protoplasts from the biochemical mutants, KGS-1 and KGS-2 were fused as described under MATERIALS AND METHODS. Protoplast fusion usually occurred between two cells or among three cells. The regeneration ratio for the protoplasts was defined as the percentage ratio of the number of colonies appearing on the osmotically balanced CM and the total number of protoplasts of the original mutant. The regeneration ratios of the biochemical mutants used in this test was 10 to 20%, respectively. The fusion frequency was 9×10^{-3} (Table II). After 4 days incubation, 10 to 25 colonies developed on the MM agar in the Petri dish.

Properties of the fusants

The colonies of fusants that appeared on the

TABLE I. MYCOLYTIC ENZYME ACTIVITIES OF THE ENZYME PREPARATIONS

Preparation	β -1,3-Glucanase (units/ml)	Chitinase (units/ml)
<i>T. viride</i> lytic enzyme ^a preparation	17.20	0.392
Zymolyase-5000	0.69	0.006
Helicase	1.80	0.096

^a This preparation was prepared by the tannic acid polyethylene glycol method developed in our laboratory.

MM agar were transferred to MM agar slants, respectively. Most of the colonies showed fine growth on the slants. Most fusants also segregated exactly into two types of parental auxotrophs, and produced sectors. These results evidently showed that the fusants were heterokaryons.

Formation of heterozygous diploids

It has been shown that the frequency of diploidization from heterokaryons can be enhanced by treatment with *d*-camphor.¹⁶⁾ A loopful of heterokaryon mycelia from the slant was incubated on MM agar containing 0.1% *d*-camphor, followed by further incubation at 25°C for 7 days. After incubation, some sectors were also seen for the colonies grown on MM agar. It was suggested that these sectors were due to the formation of diploids in the colonies. When appropriately diluted conidia in a sector were plated on fresh MM agar, the conidia were almost able to germinate on the MM agar, as evident in Fig. 1. If these conidia are heterokaryotic, they ought to segregate into both types of parental auxotrophic conidia on the MM agar. These conidia could not consequently grow on the MM agar. Therefore, the resulting strains that could grow on the MM agar were regarded as heterozygous diploids. The frequency of diploidization was 2.9×10^{-2} (Table III). As shown in Fig. 2, it was suggested that a diploid strain KGS-1-2-D, grew more vigorously than a heterokaryotic strain, KGS-1-2-H,

TABLE II. FUSION FREQUENCY BETWEEN PROTOPLASTS OF AUXOTROPHIC MUTANTS

Protoplast pair	Protoplasts regenerated on hypertonic CM		Fusant formed on hypertonic MM	Fusion frequency
	Before fusion treatment	After fusion treatment	After fusion treatment	
KGS-1 (<i>met</i>)	2.51×10^6	2.65×10^5		
+				
KGS-2 (<i>arg</i>)	2.50×10^6	2.90×10^5	5.0×10^3	9×10^{-3}

Czapek-Dox medium containing 2% glucose was used as the minimal medium (MM). MM supplemented with 0.5% yeast extract and casamino acid was used as the complete medium (CM). Fusion was accomplished by suspending the protoplasts in 35% PEG 4,000, 0.05 M CaCl₂, 0.05 M glycine-NaOH buffer, pH 7.5 (25°C for 15 min). After washing with 0.6 M NaCl, a series of appropriately diluted protoplast suspensions were plated onto hypertonic MM and CM agar.

TABLE III. SEGREGATION OF HETEROKARYONS AFTER FUSION AND THE APPEARANCE FREQUENCY OF DIPLOIDS

Protoplast pair	Reversion rate (less than)	Segregation into auxotrophs (%)	Appearance frequency of diploids
KGS-1 (<i>met</i>) +	2.1×10^{-6}	70	2.9×10^{-2}
KGS-2 (<i>arg</i>)	3.5×10^{-6}	30	

The reversion rate was defined as the ratio of the numbers of protoplasts regenerating on CM and MM agar.

Appearance frequency of diploids was defined as the ratio of the numbers of conidia regenerating on CM and MM agar.

FIG. 1. Germination of Diploid Conidia on Minimal Medium.

The photomicrograph was taken after 18 hr incubation at 25°C ($\times 150$). The bar marker represents 30 μ m.

on the MM agar. The strain showed an about 1.4 fold DNA content per conidium (Fig. 3 and Table IV). Moreover, the conidial diameters of the diploids were about two fold those of the parental or original strains. Although 98.8% of the total conidia were stable, some recombinants were spontaneously produced from the conidia (Table V). In the solid rice grain culture of the diploid strain, KGS-1-2-D, the citric acid and amylase productivities were the same as those

FIG. 2. Difference in Growth between the Heterokaryon and Diploid on Minimal Medium.

The photomicrograph was taken after 4 days incubation on 25°C.

TABLE IV. SIZE AND DNA CONTENT OF CONIDIA OF THE HAPLOID AND DIPLOID

Strain	Size (dia.) (μ m)	DNA content (10^{-6} μ g per conidium)
Strain KGS (haploid) ^a	2.5~4.5 (Ave. 4.0)	10.69
Strain KGS-1-2-D (diploid)	5.0~7.5 (Ave. 5.2)	21.17

^a The conidial sizes of the original strain and the biochemical mutants, KGS-1 (*met*) and KGS-1 (*arg*), were much smaller than that of the diploid, respectively. In this table, the conidial size of the original strain is shown.

of the original strain, KGS. Those of KGS-1, KGS-2 and KGS-1-2-H, however, were very low (Table VI). Moreover, strains KGS-1, KGS-2 and KGS-1-2-H showed poor growth

and poor conidiation on the rice grain culture. On the other hand, strain KGS-1-2-D grew well on the rice grain culture and showed

healthy sporulation on it.

DISCUSSION

TABLE V. SEGREGATION OF BIOCHEMICAL MUTANTS FROM THE DIPLOID

Prototroph	Auxotrophic mutant		Total
	<i>met</i>	<i>arg</i>	
2191	3	1	2195
Black conidia	2174		
No conidia and color changed conidia	17		

A conidial suspension of the diploid strain, KGS-1-2-D, was plated on CM agar and then incubated at 25°C for 4 days. The colonies appearing were transferred onto MM agar. The colonies that did not grow were further tested as to their nutritional requirements.

A useful strain for making shochu needs to exhibit high citric acid and amylase productivities. Protoplast fusion techniques have not yet been applied to the field of shochu koji-mold breeding for making shochu. We obtained new diploid strains through protoplast fusion, and for the first time, found the presence of a parasexual cycle in *A. awamori* var. *kawachi*.

The protoplast fusants obtained in this study were able to grow on MM agar, and conidia from the fusants completely segregated into two types of parental auxotrophs. These results showed that the protoplast fusants were

FIG. 3. Conidial Sizes of the Haploid and Diploid.

A, haploid from original parent strain ($\times 600$); B, diploid ($\times 600$). The bar marker represents 15 μm .

TABLE VI. PROPERTIES OF THE HETEROKARYON AND DIPLOID

Strain	Citric acid produced in koji (g)	pH of ^a koji extract	Amylase activity ^b (units/ml)
<i>Asp. awamori</i> var.			
<i>kawachi</i> KGS	0.753	2.85	9.25
KGS-1 (<i>met</i>)	0.018	4.30	0.74
KGS-2 (<i>arg</i>)	0.035	4.15	0.59
KGS-1-2-H (heterokaryon)	0.072	3.50	1.00
KGS-1-2-D (diploid)	0.806	2.80	9.25

^a The koji extract was prepared by extracting a solid culture with 10 volumes (for a fresh culture) of 0.1 M acetate buffer, pH 5.0, at room temperature for 1 hr.

^b Amylase activity is expressed as saccharifying activity.

heterokaryons. When the heterokaryotic strains were incubated on MM agar containing 0.1% *d*-camphor, black sectors developed, and then germ tubes from a part of the conidia in the sectors were observed.

In this study, more than 50 strains were isolated, 20 of which were subjected to conidial analysis. One of the conidia that germinated on MM agar, strain KGS-1-2-D, was also able to grow on MM agar, and segregated spontaneously into same recombinants. Moreover, the conidium from strain KGS-1-2-D, showed a 2 fold DNA content and an about 1.4 fold conidial diameter compared with those of the original strain, KGS. On the basis of these facts, it seemed reasonable to assume that strain KGS-1-2-D was a heterozygous diploid. The diploid strain, KGS-1-2-D, was regarded as a useful strain exhibiting high strong citric acid and amylase productivities, corresponding to those of the original strain, KGS.

Furuya *et al.*⁴⁾ have successfully obtained stable diploids from *A. oryzae* through protoplast fusion of a protease-improved mutant and its healthy parent. It is known that a diploid strain obtained through fusion of single nucleic protoplasts shows an about 1.3 fold conidial diameter compared to the haploid and a 2 fold DNA content per conidium. The DNA content per conidium or nucleus of *A. awamori* var. *kawachi* KGS was similar to those *Aspergillus nidulans*¹⁷⁾ and *Aspergillus sojae*.¹⁸⁾

From the results presented it would appear

that protoplast fusion will be a useful tool as to the genetics and mechanisms of breeding of koji-mold.

REFERENCES

- 1) L. Ferenzy, F. Kevei and M. Szegei, *Experimentia*, **31**, 1028 (1975).
- 2) L. Ferenzy, M. Szegei and F. Kevei, *Experimentia*, **33**, 184 (1976).
- 3) J. Anne, H. Essen and P. De Somer, *Nature*, **262**, 719 (1976).
- 4) T. Furuya, M. Ishige and K. Uchida, *Nippon Nogeikagaku Kaishi*, **57**, 1 (1983).
- 5) B. Lasker and T. P. Borgia, Abstracts of the Meeting, National Meeting of the American Microbial Society, Hawaii, 1979, p. 129.
- 6) T. Ohnuki, Y. Etoh and T. Beppu, *Agric. Biol. Chem.*, **46**, 451 (1982).
- 7) N. Toyama, H. Ohara and K. Ogawa, *Bull. Fac. Agric. Miyazaki Univ.*, **31**, 91 (1984).
- 8) K. Ogawa, J. A. Brown and T. M. Wood, *Enzyme Microb. Technol.*, **9**, 229 (1987).
- 9) A. J. Brown, K. Ogawa and T. M. Wood, *Enzyme Microb. Technol.*, **9**, 527 (1986).
- 10) J. B. Sumner, *J. Biol. Chem.*, **65**, 393 (1925).
- 11) K. Ogawa, H. Toyama and N. Toyama, *Bull. Fac. Agric., Miyazaki Univ.*, **26**, 387 (1981).
- 12) J. Anne and J. F. Peberdy, *J. Gen. Microbiol.*, **92**, 413 (1976).
- 13) K. Burton, *Biochem. J.*, **62**, 315 (1956).
- 14) W. C. Schneider, *J. Biol. Chem.*, **164**, 747 (1946).
- 15) I. Garcia Acha, F. Lopez-Belmonte and J. R. Villanueva, *J. Gen. Microbiol.*, **45**, 515 (1966).
- 16) G. Pontecorvo, *Adv. in Genetics*, **5**, 141 (1953).
- 17) G. C. Heagy and J. A. Roper, *Nature*, **170**, 713 (1952).
- 18) S. Ushijima and T. Nakadai, *Agric. Biol. Chem.*, **51**, 1051 (1987).