Transformation of *Aspergillus oryzae* through Plasmid-mediated Complementation of the Methionine-auxotrophic Mutation

Yuzuru IMURA, Katsuya GOMI, Hiroshi UZU
and Shodo HARA

National Research Institute of Brewing, 2-6-30,
Takinogawa, Kita-ku, Tokyo 114, Japan

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A methionine-auxotrophic mutant (Met^-) of *Aspergillus oryzae* was transformed to prototrophy (Met^+) with plasmid pKA5-1 carrying a 3.5 Kbp genomic DNA fragment of *A. oryzae* through treatment of spheroplasts with polyethylene glycol 4000 and CaCl_2 . The highest transformation frequency obtained was 32 transformants per μg DNA. We recovered the transforming plasmid from the Met^+ transformant cell, showing that this plasmid was derived from the original plasmid, pKA5-1. The mitotic stability of the Met^+ transformant was about 70% after growth without selective pressure; the transforming DNA was lost in the Met^- clone. These findings suggest that the transforming plasmid, pKA5-1, exists in the transformant cell as a self-replicating circular DNA.

The gene in the 3.5 Kbp DNA fragment, which complements the methionine-auxotrophic mutation, can be used as a selectable marker to construct efficient plasmid vectors for *A. oryzae*.

A DNA-mediated transformation system is essentially required for molecular genetics and molecular breeding of *Aspergillus*. Efficient transformation systems have been developed in *Aspergillus nidulans*^{1~6)} and *Aspergillus niger*.^{7,8)} Therefore, genetic studies have been made possible at the molecular level for these fungi.^{7,9~11)}

Aspergillus oryzae is extensively used for the production of industrial enzymes and for *saké* making, etc. Therefore, useful strains of *A. oryzae* have been constructed by mutagenesis,^{12,13)} or spheroplast fusion.¹⁴⁾ However, because of the lack of vectors carrying selectable genetic markers, studies on DNA recombination have never been performed in the case of *A. oryzae*.

In this paper, we report the successful complementation of the methionine-auxotrophic mutation in *A. oryzae* with a vector DNA containing a genomic DNA fragment of *A. oryzae* and the subsequent recovery of this transforming hybrid vector from *E. coli*. These findings will serve as a basis for the con-

struction of efficient transformation systems and plasmid vectors carrying genetic marker for *A. oryzae*.

MATERIALS AND METHODS

Strains. A strain of *E. coli*, JA221 (*hsdM*⁺ *hasdR*⁻ *lacY* *leuB6* *trpE5* *recA1*), was used as a gene library for *A. oryzae* and the recovery experiments on the transforming vector from *A. oryzae*. Two mutants of *A. oryzae*, M-28 and M-28W, having the phenotypes of Met^- and Met^- white conidia, respectively, were used as recipient strains in the transformation experiments. Mutant M-28 was derived from the wild strain, *A. oryzae* RIB203, and M-28W from mutant M-28. Another wild strain, *A. oryzae* RIB40, was also used as a DNA donor for a gene library.

Media and growth conditions. Strains M-28, M-28W, RIB203 and RIB40 were grown in DP medium, which was composed of 2.0% dextrin, 1.0% polypeptone, 0.5% KH_2PO_4 , 0.1% NaNO_3 and 0.05% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, with vigorous shaking at 30°C for 48 hr. We also used Czapek-Dox medium, which was composed of 0.2% NaNO_3 , 0.1% K_2HPO_4 , 0.05% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.05% KCl, 0.001% FeSO_4 , 3.0% sucrose and pH 5.5, as a minimal medium. If necessary, 30 mg/liter methionine was added to the Czapek-Dox medium. For conidia formation, the trans-

formant was incubated on KE medium, which was composed of rice-koji extract (ca. 8% in sugar concentration) containing 2% agar, at 30°C for 5 days.

Plasmids. The plasmid used as a gene library was the yeast-bacterial shuttle vector, YEp13,¹⁵⁾ which is composed of bacterial plasmid pBR322, a yeast DNA fragment carrying the *LEU2* gene and the yeast 2 μ m DNA. Plasmid pBR322 was also used in control experiments.

Preparation of mycelial spheroplasts. Mycelia after washing with chilled water were suspended in the cell wall lytic solution, which was composed of 50 mM phosphate buffer (pH 6.0), 5.0% NaCl, as an osmotic stabilizer, and 0.5 mg/ml *Oerskovia*'s enzyme.¹⁶⁾ After incubation with gentle shaking at 30°C for 2 hr, the residual mycelia were removed by filtration on a glass filter. The mycelial spheroplasts in the filtrate were collected by centrifugation.

Preparation of *A. oryzae* whole DNA. The spheroplasts were suspended in NTE buffer, which was composed of 100 mM NaCl, 50 mM Tris-HCl (pH 8.0) and 5% SDS. The debris was removed by centrifugation at 10,000 rpm for 10 min. The whole DNA in the resultant supernatant was precipitated with ethanol and treated with RNase A.

Construction of a gene library. Plasmid YEp13 was digested with *Bam*HI and then dephosphorylated with bacterial alkaline phosphatase to prevent recircularization, whereas the genomic DNA of *A. oryzae* RIB40 (Met⁺) was partially digested with *Bam*HI or *Sau*3A and then fractionated by sucrose density gradient centrifugation. Then, the DNA fragments of 3~5 K bp in length were collected and mixed with the *Bam*HI-digested YEp13. After ligation with T4 DNA ligase, the mixture was used to transform *E. coli* JA221. About 18,000 colonies of ampicillin-resistant and tetracycline-sensitive transformants were collected as a gene library for *A. oryzae* DNA.

Transformation of *A. oryzae*. The transformation procedure was based on the method of Hinnen *et al.*¹⁷⁾ with some modifications, as follows. The mycelial spheroplasts were prepared as described above. The resultant spheroplasts (1.0×10^8 cells) were suspended in 100 μ l of 50 mM Tris-HCl (pH 7.5) containing 5.0% NaCl and 10 mM CaCl₂, and then incubated with 1~10 μ g of the transforming DNA at room temperature for 5 min. Then, the suspension was added to an equal volume of 70% (w/v) polyethylene glycol (PEG) 4000-10 mM Tris-HCl (pH 7.5)-10 mM CaCl₂ solution, followed by incubation at room temperature for 10 min. After washing with water twice, the spheroplasts were spread on Czapek-Dox medium containing 5.0% NaCl and 2% agar, and then overlaid with 0.5% agar containing 5.0% NaCl. The plate was incubated at 30°C.

Transformation of *E. coli*. The conventional method was used.¹⁸⁾

Southern blotting and hybridization. The DNA fragments obtained on *Bam*HI-digestion were electrophoretically separated in 1% agarose gel, and then transferred onto a nitrocellulose filter by Southern's method.¹⁹⁾ The probe DNAs were labeled with α -³²P-dCTP by means of nick-translation according to Rigby *et al.*²⁰⁾ DNA immobilized on the nitrocellulose filter was hybridized to ³²P-labeled probe DNA at 68°C according to Maniatis.¹⁹⁾

RESULTS

*Transformation of *A. oryzae* with a recombinant plasmid from the gene library*

Two methionine-auxotrophic mutants of *A. oryzae*, M-28 and M-28W, were used as recipients in the transformation experiments. The spontaneous reversion frequencies of these mutants to prototrophy (Met⁺) were low (below 2×10^{-8}). The mycelia of these mutants were efficiently spheroplastized by *Oerskovia*'s enzyme.¹⁶⁾ As compared with the number of the spheroplasts determined by microscopically, the regeneration frequency of the spheroplasts on DP medium containing 5.0% NaCl was 80~86% for both M-28 and M-28W.

The hybrid plasmids from the gene library were amplified in *E. coli*, and then introduced into spheroplasts of M-28 or M-28W in the presence of CaCl₂ and PEG4000. Out of about 8,000 hybrid plasmids tested, we found that one, designated as pKA5-1, was able to transform both M-28 and M-28W to prototrophy (Met⁺).

Structure of recombinant plasmid pKA5-1

To characterize the inserted DNA in plasmid pKA5-1, we digested the pKA5-1 DNA with various restriction endonucleases. The inserted DNA fragment (3.5 Kbp) and linearized YEp13 DNA (10.7 Kbp) were observed in the *Bam*HI-digested pKA5-1 (Fig. 1). Several restriction sites were determined in the 3.5 Kbp inserted fragment (Fig. 2).

Construction of pKA5-2

In control experiments, we constructed a new hybrid plasmid, designated as pKA5-2, by

FIG. 1. The Structures of Plasmids pKA5-1 and pKA5-2.

Agarose gel electrophoresis of *Bam*HI-digested pKA5-1 (2), pKA5-2 (3) and marker DNAs (*Hind*III-digested λ phage) (1). The band corresponding to 3.5 Kbp is the inserted fragment from the genomic DNA of *A. oryzae*.

FIG. 2. Restriction Sites of Plasmid pKA5-1.

integrating the 3.5 Kbp fragment into the *Bam*HI-site of pBR322 (Fig. 1).

Transformation frequency with pKA5-1 and pKA5-2

Using plasmid pKA5-1 or pKA5-2 as a

TABLE I. TRANSFORMATION OF *Aspergillus oryzae* THROUGH COMPLEMENTATION OF THE METHIONINE-AUXOTROPHIC MUTATION

Recipient	Plasmid	Transformation frequency ^a		
		Large colony ^b	Small colony ^c	Total
M-28	pKA5-1	4	10	14
M-28	pKA5-2	5	9	14
M-28W	pKA5-1	6	15	21
M-28W	pKA5-2	5	27	32

^a Number of colonies obtained on transformation with 1 μ g of transforming DNA.

^b Colonies showing vigorous growth.

^c Abortive colonies.

FIG. 3. Growth Rates of the Met⁺ Transformants and the Recipients.

The transformant and recipient cells were grown on the minimal medium and the minimal medium supplemented with 30 mg/liter methionine, respectively. The growth is expressed as the diameter of colonies. ■, transformant M-28 (pKA5-1); □, transformant M-28W (pKA5-1); ○, recipient M-28; ⊙, recipient M-28W.

transforming vector, we examined the transformation frequency of the recipients, M-28 and M-28W (Table I). The frequency was 14~32 colonies per μ g DNA, while no transformant was obtained in the experiments using YEp13 or pBR322. These results show that the Met⁺ phenotype of these transformants was due to plasmid-mediated complementation of the Met⁻ mutation, depending on the 3.5 Kbp inserted DNA, and not on the yeast DNAs in YEp13, or on pBR322.

The frequency in these experiments was similar to that observed for the trans-

formation of *Aspergillus*,^{1~3)} but much less than that reported in other transformation studies.^{4~6,11)}

Two types of transformant colonies were observed on growth on the minimal medium. One showed abundant growth and subsequent sporulation; the other showed poor growth, and was abortive. A similar phenomenon was reported for the transformation of *A. nidulans* by Tilburn *et al.*²⁾

Growth rates of transformants

We isolated two transformants with pKA5-1, which showed vigorous growth on the minimal medium, and estimated their growth rates (Fig. 3). The growth rates of the transformants were lower than those of the auxotrophic recipients (Met^-) grown on the minimal medium supplemented with methionine, although these transformant cells were morphologically similar to the recipient cells.

Stability of transformants

To determine the mitotic stability of the Met^+ transformants, the cells were incubated on KE medium, which contained methionine, at 30°C for 10 days, and then the resultant conidia were transferred onto the minimal medium to assay the reversion frequency to Met^- . As shown in Table II, the reversion frequency was high, showing that all the Met^+ transformants were unstable.

TABLE II. MITOTIC STABILITY OF THE Met^+ PHENOTYPE IN THE TRANSFORMANTS

Transformant ^a	Number of colonies tested ^b	Number of colonies (Met^+) ^c	Reversion rate ^d (%)
M-28 (pKA5-1)	108	69	36
M-28 (pKA5-2)	103	76	26
M-28W (pKA5-1)	116	81	30
M-28W (pKA5-2)	118	86	27

^a Transformation was due to the plasmid in parentheses.

^b Colonies which formed conidia on KE medium.

^c Colonies grown on minimal medium after incubation without selective pressure.

^d Rate of reversion to Met^- ; $b - c/b \times 100$.

Fate of the transforming plasmid in transformant cells

To confirm that the Met^+ transformation of *A. oryzae* was due to plasmid-mediated complementation of the Met^- mutation, we investigated as to whether or not the transforming plasmid was present in the whole DNA prepared from Met^+ transformant cells of M-28W, which were grown in DP medium, by means of Southern blotting analysis using labeled pKA5-1 as a probe. Consequently, for the *Bam*HI-digested whole DNA of the transformant, two distinct bands were observed, which corresponded to the 3.5 Kbp inserted DNA and linearized YEp13 DNA, respectively, while for the *Bam*HI-digested whole DNA of M-28W (Met^-), only one band corresponding to the 3.5 Kbp DNA was observed (Fig. 4-I). In control experiments using labeled YEp13 as a probe, only one band corresponding to the linearized YEp13 was observed for

FIG. 4. Southern Blotting Analysis of Whole DNA of *A. oryzae*.

Whole DNAs were prepared from the M-28W transformant obtained with pKA5-1 and the recipient cells. Ten micrograms of whole DNA was loaded in each lane. ³²P-Labeled probe DNA (ca. 17 $\mu\text{Ci}/\mu\text{g}$ DNA) was hybridized at 68°C for 16 hr; filters were washed at 42°C in 0.1 $\times$ SSC for 60 min. (I) and (II) show hybridization using ³²P-pKA5-1 and ³²P-YEp13, respectively. (1) and (4) *Bam*HI-digested pKA5-1 (control); (2) and (5) *Bam*HI-digested whole DNA of the recipient cells; (3) and (6) *Bam*HI-digested whole DNA of the transformant cells.

the *Bam*HI-digested whole DNA of the transformant, while no band was seen for that of M-28W (Met⁻) (Fig. 4-II).

Recovery of the plasmid from the transformant

To examine the possibility that pKA5-1 might exist as a self-replicating plasmid in the Met⁺ transformant cells, we attempted to transfer the plasmid from Met⁺ transformant cells of either M-28 or M-28W, which were grown in DP medium at 30°C for 48 hr, to *E. coli*. The transformation frequency in these experiments was low (10-16 colonies per µg). One colony, designated as ECK-5, was isolated from the ampicillin-resistant and tetracycline-sensitive *E. coli* transformants. On the other hand, no transformant was obtained on transformation of *E. coli* using the whole DNA of the Met⁻ revertant, which was generated on incubation without selective pressure.

Hybridization analysis of the plasmid recovered in *E. coli*

To prove that *E. coli* transformant ECK-5 contained the original plasmid, pKA5-1, we carried out Southern blotting analysis for the whole DNA of ECK-5 (Fig. 5). On the hy-

bridization of the *Bam*HI-digested whole DNA to labeled pKA5-1, two distinct bands were observed, which corresponded to the 3.5 Kbp inserted DNA of pKA5-1 and linearized YEp13 DNA, respectively. Besides, on hybridization to labeled YEp13, only one band corresponding to the linearized YEp13 was detected. These results show that the transforming DNA in ECK-5 is derived from the original plasmid, pKA5-1.

DISCUSSION

In the gene cloning of *Aspergillus*, the complementation of a mutant allele in a heterologous organism has been used for the identification of an isolated gene.^{9,21} Besides, since this method is limited to genes which can be expressed in a heterologous organism, the direct cloning of genes has been achieved through the complementation of a mutation with plasmid DNA carrying a fragment from the genomic DNA of a wild type strain for *A. nidulans*,^{2-4,6, 11} but not for *A. oryzae*.

In this study, we attempted the direct cloning of the gene which complements the methionine-auxotrophic mutation of *A. oryzae* and isolated a hybrid plasmid from the gene library for *A. oryzae*, which was able to change the phenotype from Met⁻ to Met⁺, being the first instance of transformation in *A. oryzae*. The Met⁺ phenotype of this transformant was probably due to the complementation of the mutant allele with the gene carried by the 3.5 Kbp inserted DNA fragment of pKA5-1.

Generally, transforming DNA within a transformant cell is integrated into a homologous region of the chromosome, or present as a self-replicating circular plasmid. In this study, the occurrence of abortive transformants (Table I) and the instability of the transformant on growth without selective pressure (Table II), or loss of the transforming plasmid during mitotic division, suggest that this hybrid plasmid exists as a self-replicating DNA in the transformant. Besides, the plasmid recovered from the Met⁺ transformant was proved by Southern blotting analysis to be

FIG. 5. Southern Blotting Analysis of the *Bam*HI-digested Whole DNA of the *E. coli* Transformant.

Whole DNA was prepared from the *E. coli* transformant cells obtained by introducing the whole DNA of the Met⁺ transformant of *A. oryzae*. ³²P-Labeled probe DNA was hybridized at 68°C; filters were washed at 50°C in 0.1 × SSC for 60 min. (I) and (II) show hybridization using ³²P-pKA5-1 and ³²P-YEp13, respectively. (1) and (3) *Bam*HI-digested whole DNA; (2) and (4) *Bam*HI-digested YEp13, as a control.

derived from the original plasmid (Figs. 4 and 5). The frequency and the stability of the transformation with hybrid plasmid pKA5-2, which was composed of the 3.5 Kbp inserted DNA and pBR322, were similar to those in the case of pKA5-1 (Table I). These results show that the self-replication of these hybrid plasmids was due to the 3.5 Kbp inserted DNA in pBR322, and not to the yeast DNA in YEp13. However, we cannot conclude that the transformation depends only on the self-replicating plasmid, because there is a possibility that the occurrence of free circular plasmid is due to excision of the integrated plasmid on recombination, as reported for the transformation of *A. nidulans* by Johnstone.²²⁾ In this study, the fate of the transforming DNA was not clarified. Further studies are necessary.

In the recovery test of the transforming plasmid from the Met⁺ transformant, the transformation frequency of *E. coli* was found to be low. This shows that the copy-number of the transforming DNA in the transformant cell is small. The delay in the growth of the transformant (Fig. 3) also suggests that the copy-number of the gene complementing the Met⁻ mutation is small.

A vector with a high-transformation frequency and a high copy-number is desired for practical use in *A. oryzae* transformation. To construct such an effective vector, the gene which is involved in the complementation of the Met⁻ mutation can be used as a marker.

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