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Cloning and Expression of Leu 2 Gene (β-isopropylmalate dehydrogenase) from the Basidiomycete *Flammulina velutipes* in *E. coli*

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팽나무버섯 균사체에서 β-isopropylmalate dehydrogenase(Leu 2) gene 의 cloning 및 *E. coli* 에서 발현

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ABSTRACT: Gene libraries of DNA from *Flammulina velutipes* were constructed using *Escherichia coli* plasmid pBR 322. Leu 2 gene coding β -isopropylmalate dehydrogenase from *F. velutipes* was cloned by complementation of leucine requiring mutant of *E. coli*. The size of inserted DNA fragment of this clone is about 1 Kbp. The fragment has Bam H1 and Ava 1 restriction sites.

KEYWORDS: *Flammulina velutipes*, β-isopropylmalate dehydrogenase, Cloning.

Flammulina velutipes is white rot fungi and having been cultivated using sawdust medium in far-east Asia such as Japan, Taiwan and South Korea.

Gene transfer and its expression in mushroom are interested. A major problem in developing of transformation for the mushroom has been the deficiency of appropriate selectable markers.

Recent interest on molecular biology led to development of vector with auxotrophic gene and transformation system for two basidiomycetes *Coprinus cinereus* and *Schizophyllum commune.*

The Leu 2 locus of *F. velutipes* encodes beta-isopropyl malate dehydrogenase E.C.1.1.1.85, an enzyme which catalyses the conversion of beta-isopropyl malate to alpha-ketoisocaprate in the leucine biosynthesis pathway.

The gene was cloned by direct selection for complementation in an *Escherichia coli* leu B mutant. Subsequent analysis showed that DNA fragment contains the structural gene.

Materials and Methods

Strains

Bacterial strains used were *E. coli* DB 6507 (ATCC 35673) (Leu B6, Pyr F74::Tn5) which was the host for the plasmid-gene library. *E. coli* JA300 (ATCC 33588) (F-thr leuB6B1 TrpC 117rpsL hsdR hsdM-) was used for complementation of leu B gene. A wild type strain of *Flammulina velutipes* ASI 4003 from our collection was the source of DNA for gene libraries.

Media

Fammulina velutipes mycelia, for the isolation of DNA, were grown in liquid mushroom complete medium as previously described (Raper *et al.*, 1972). *E. coli* cells for transformation and for the isolation of plasmid DNA were grown in LB broth. Media for selection of hybrid plasmid containing Leu B gene was the glucose minimal media.

DNA isolation of *F. velutipes*

DNA of *F. velutipes* was isolated using modifi-

cation of gentle extraction method described by Specht (1982). Mycelium of *F. velutipes* (60-80 gram wet weight) was harvested, washed with TE-buffer (50 mM Tris, 20 mM EDTA, pH 8.0) and ground in a mortar under liquid nitrogen. It was suspended in 100 m*l* 50 mM Tris. HCl pH 8, 100 mM EDTA pH 8, 150 mM NaCl, 2% SDS, 20% toluene and shaken at 60 rpm for three days at room temperature on a rotary shaker.

The suspension was filtered through 2 layers of nylon cloth to remove mycelial debris. The filtrate was shaken with 1 volume of phenol:chloroform:isoamylalcohol(25:24:1) solution for 10 min. (Phenol was saturated with 750 mM NaCl, 75 mM Na citrate pH 7.0 and 0.1% hydroxy quinoline). The phases were separated by centrifugation for 30 min at 12000 rpm in Hitachi RPR 20-2 rotor. The aqueous phase was taken off carefully, and saturated with chloroform:isoamyl alcohol (24:1) again. After centrifugation, upper phase was mixed with 0.1 volume 3 M sodium acetate and 2.5 volumes cold ethanol, and centrifuged for 30 min at 12000 rpm. The pellet was dried and resuspended in TE. DNA was further purified by CsCl density gradient centrifugation (1.1g of CsCl/m*l* and Ethidium Bromide). The solution was centrifuged for 6 hours at 65000 rpm in Bechman VTi 80 rotor at 20°C. Flourescing band was taken off the gradient and ethidium bromide was extracted several times with saturated n-butanol. The solution was dialyzed against TE.

Construction of *F. velutipes* partial gene library

Total DNA of *F. velutipes* was partially digested with Sau3A to give a mixture of fragments with an average size of 1-4 Kilo base pairs. This DNA fragments was ligated with Bam H1 digested, dephosphorylated PBR 322. The ligation mixture was used for transformation into *E. coli* DB 6507 by the method of Hannahan (1983). Transformants were obtained from ampicillin resistant (Ap) colonies. Of the colonies 50% contained recombinant plasmids.

Complementation of *E. coli* Leu mutants

Transformants with recombinant plasmids were incubated aerobically at 37°C overnight. To select the Leu^+ cells, overnight culture was centrifuged at 5000g for 10 min at 4°C, the cells were resuspended in 1 m*l* of distilled water and 0.1 m*l* samples were spread on the surface of glucose minimal agar medium supplemented with all the appropriate growth factors without leucine (Munoz Rivas *et al.*, 1986). Plates were incubated at 37°C and Leu^+ colonies were visible after 3-7 days.

Plasmid DNA restriction analyses and Southern hybridization

Plasmid DNA was isolated by a method involving alkaline treatment (Birnboim and Doly, 1979).

Restriction enzymes were used according to the manufacturer's instructions and DNA fragments generated by digestion were separated on a 0.7% agarose gel. After denaturation, DNA fragments were transferred from the agarose gel onto a sheet of Gene screen plus (Dupont) as described by Southern (1975) and the use of 10×SSPE as the transfer buffer.

The probe used was derived from a recombinant plasmid. Single stranded probe DNA was obtained by oligo labelling procedure (Pharmacia) using ^{32}P dCTP.

Hybridization and washing were performed at 65°C by dupont's gene screen plus methods. Autoradiography using Fuzi film was carried.

Results and Discussion

F. velutipes DNA was isolated and purified. Purified DNA was partial digested with Sau 3A.

E. coli strain DB 6507 (Leu B) was used as a host for construction of genomic library in the Bam H1 site of pBR 322 and selection of the Lue 2 gene of *F. velutipes* by complementation.

Transformed cells were plated on selective minimal medium lacking leucine and incubated at 37°C. After 3-7 days of incubation, 10 colonies with correct phenotype (Ap^r, Leu^+) appearred. Of those colonies, tetracycline sensitive colonies were two and the other 8 colonies tetracycline resistant.

After several transfer to new media, one ampicillin resistant, tetracycline sensitive, leucine independent colonies and 3 ampicillin resistant, tetracycline resistant, leucine independent colonies were obtained.

Plasmid was prepared from ampicillin resistant, tetracycline sensitive, leucine independent clone for complementation anaylsis with Leu B mutants, restriction mapping and Southern hybridization to *Flammulina velutipes* DNA.

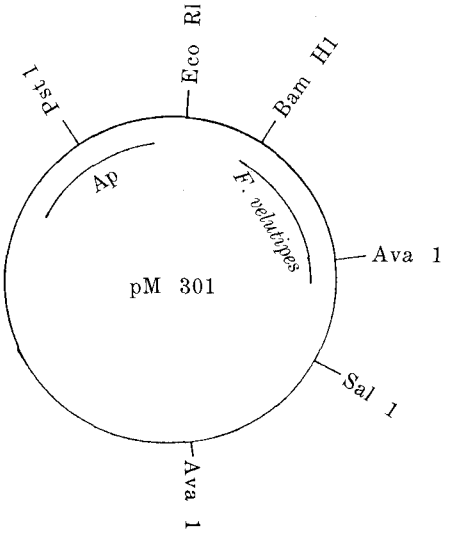

Fig.1. Restriction map of pM 301 vector.

The presence of the Leu B gene was confirmed by transforming the other Leu B mutant strain JA 300. An efficiency of Leu$^+$ transformation was showed to 10^5 to 10^6 Leu$^+$ transformants per μg DNA.

These results indicated that the transformed lencine independent colony contained a recombinant plasmid with suppressed the Leu$^-$ phenotye of the Leu B strain. Single and double digestions of the plasmid by different restriction enzymes allowed us to construct a physical map of the recombinant plasmid pM 301. The insert is about 1 kilo base pairs and has Aval and Bam H1 restriction sites (Fig.1). No Bam H1 site was regenerated by the ligation of Sau 3A and Bam H1 cohesive ends.

The insert contains no restriction site for Eco R1, Pst 1, Hind 3 and Sal 1.

The recombinant plasmid was nick-translated and used to probe genomic DNA of wild type *F. velutipes* DNA partial-digested with Sau 3A. One Sau 3A fragment hybridized with the probe DNA of recombinant plasmid. Therefore the insert DNA was confirmed to be derived from *F. velutipes* DNA.

To investigate the homology with yeast Leu 2 gene, middle size Pst 1 fragment in YEP 13 vector was extracted from agarose gel. The Pst 1 fragment of yeast Leu 2 gene was hybridized with pM 301 probe. It was considered that *F. velutipes* Leu 2 gene has some homology with yeast Leu 2 gene.

Many yeast genes have been cloned using complementation of *E. coli* mutants (Gurr, 1987). In contrast, only a few genes filamentous fungi were found to complement bacterial mutants perhaps due to the presence of more introns in the coding sequences of these organisms than in yeast genes. Intron-less fungal genes in filamentous fungi were pyr 4, qa, trp 1 of *Neurospora crassa* and argB, aromA, trpC of *Aspergillus nidulans*. (Gurr, 1987). 3-isopropyl malate dehydrogenase gene of *Candida utilis* was open reading frame (ORF) of 1089 bp encoding 363 amino acids. *F. velutipes* fragment of Leu 2 gene was about 1 kb.

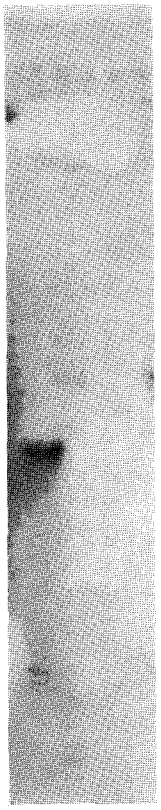

Fig.2. Autoradiograph showing hybridization of ^{32}P-labelled pM 301 to *F. velutipes* chromosomal DNA.

We feel that this strategy to isolate gene is adaptable to other basidiomycete organisms.

摘 要

팽나무버섯 균사체 DNA를 대장균 프라스미드인 PBR322를 이용하여 gene library를 작성하였다. 팽나무버섯에서 β-isopropyl malate dehydrogenase 유전자 클론을 얻었으며 이 클론은 대장

균 Leucine 요구성 균주를 complementation 시켰다. 이 클론의 팽나무버섯 DNA 크기는 약 1Kb였으며 Bam H1과 Ava 1 제한효소 절단부위를 지니고 있었다.

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