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Molecular Cloning of the Gene Coding for 3-Isopropylmalate Dehydrogenase of *Kluyveromyces fragilis*

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Kluyveromyces fragilis 의 LEU gene 의 Cloning

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In order to clone the gene coding for 3-isopropylmalate dehydrogenase of *Kluyveromyces fragilis*, a shuttle plasmid vector pHN114 was used. It can serve as a cloning vector in *Saccharomyces cerevisiae* DBY746 for other Sau3AI-cleaved DNA segment of *Kluyveromyces fragilis*. Two cloned fragments which complement the leu2 mutation of *Saccharomyces cerevisiae* and *E. coli* were obtained. Their length was 4.4 kb an 3.5 kb, and their orientation was opposite each other. From the fact that the two recombinant plasmids were expressed in *Saccharomyces cerevisiae* and *E. coli*, probably the two inserts had the promoter of *Kluyveromyces fragilis* and that of *Kluyveromyces fragilis* was efficiently assosiated with RNA polymerase of *Saccharomyces cerevisiae* and *E. coli*. According to the result of Southern hybridization, we thought that the cloned fragment has low homology with 3-isopropylmalate dehydrogenase coding region of *E. coli* and *Saccharomyces cerevisiae*.

The LEU2 gene of *Saccharomyces cerevisiae* codes for the enzyme 3-isopropylmalate dehydrogenase (1). This enzyme catalyzes the conversion of 3-isopropylmalate into -ketoisocaporate, the third step in the leucine biosynthesis pathway. Unlike other amino acid biosynthesis gene in yeast, the expression of the LEU2 gene is specially repressed by leucine or threonine (2).

Structural features of the 5'end (3) and 3'end of the LEU2 gene, control mechanisms involving mRNA secondary structure, and a leucine-rich leader polypeptide have been proposed (4).

In addition to serving as an interesting model system for the study of gene expression, the LEU2 gene is an essential component of yeast shuttle plasmid vector (5), that is, pYE(LEU2)10 (6), pJDB219 (7), YEp13 (8), YIp26, YIp27 and so on. Because the LEU2 gene can complement *in vivo* both *E. coli* leuB6 mutation (6) and the leu2-3, leu2-12 mutation in yeast (5), it can be used to select transformants of both hosts on leucine deficient medium. The LEU2 gene mentioned above is derived from *Saccharomyces cerevisiae.*

On the other hand, it is well known fact that *Kluyveromyces fragilis* is superior to *Saccharomyces cerevisiae* in secreting proteins into medium (9-11).

In the present paper, cloning of the gene coding for 3-isopropylmalate deydrogenase of *Kluyveromyces fragilis* is described.

Material and Methods

Strains and plasmid

E. coli strains used were JM109 (recA1, △ lacpro, endA1, gyrA96, thi-1, hsdR17, F⁻, traD36) to select inserted plasmids and C600 (F⁻, thi-1, thr-1, leuB6, lacY1, tonA21, supE44, λ⁻) to assay of cloned LEU

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gene product. Yeast strains used were *Kluyveromyces fragilis* Y610 and *Saccharomyces cerevisiae* DBY746 (α, trp1-289, his3-Δ1, leu2-3, leu2-12, ura3-52) as the recipient cell in the transformation. pHN114 (29) was used as a cloning vector plasmid.

Media and culture condition

L-broth (1% polypeptone, 0.5% yeast extract, 0.5% NaCl and 0.1% glucose) and Davis-salt minimal medium (12) were used for *E. coli* strains. The synthetic medium for yeast contained 0.67% yeast nitrogen base, 2% glucose, 20 μg/m*l* of histidine and uracil. YPED medium (1% yeast extracts, 2% glucose and 2% bactopeptone) was also used.

DNA techniques

Kluyveromyces fragilis Y610 chromosomal DNA was isolated by the method of Cryer (13). Plasmid DNA was isolated and purified by routine Alkaline-SDS method (14). Ligation of the partial digested chromosomal DNA and vector plasmid was carried out as follows; *Kluyveromyces fragilis* chromosomal DNA was partially digested by Sau3AI and fractionated on agarose gel (0.8%) by electrophoresis. Then larger than 3 kb fragments were isolated, and used to ligate. Vector plasmid DNA was digested with *Bam*HI and treated with BAP, and also used to ligate. Other DNA techniques were carried out according to the standard methods (12).

Transformation

Transformation of yeast was based upon Li-acetate method (14) and established condition by Hong (15). That of *E. coli* was carried out by the conventional $CaCl_2$ method (16).

Assay of 3-isopropylmalate dehydrogenase

Enzyme solution: *E. coli* C600, containing cloned plasmid was cultured to log phase, and cell pellet was washed with phosphate buffer (0.05 M, pH 6.9) two times, then the washed cell pellet was inoculated into leucine (−) minimal medium. Each 0 hr, 3 hr and 6 hr incubated solutions were collected by centrifugation at 12,000 rpm. for 10 min. The cell pellet was suspended in phosphate buffer and disrupted by sonication. The supernatant obtained was used as enzyme solution. Protein determination was performed by the method of Lowry *et al.* (22) using bovine serum albumin as a standard.

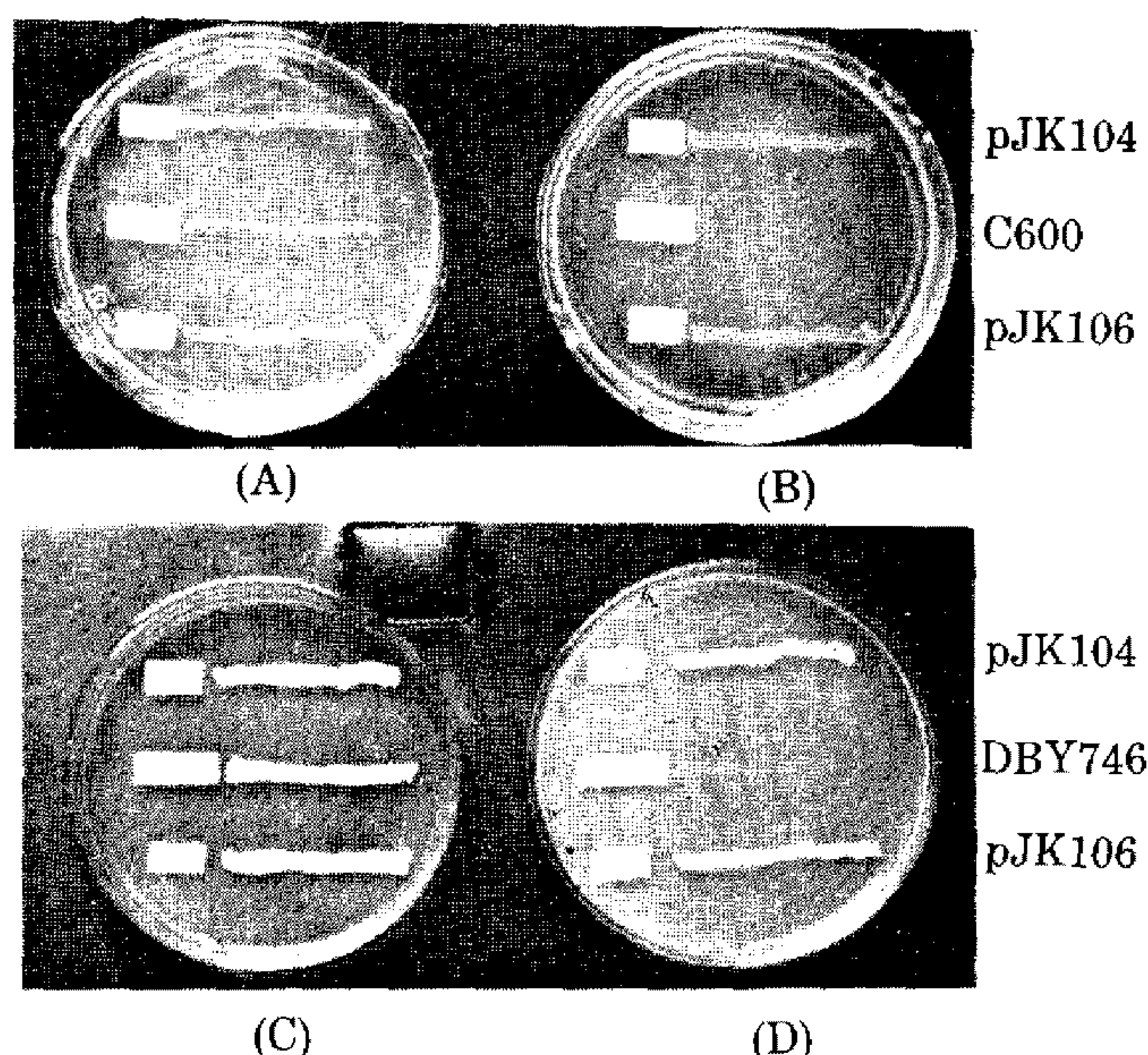

Fig. 1. Expression of pJK104 and pJK106.
(A) LB, (B) Minimal medium for *E. coli* (leu^-), (C) YEPD, (D) Minimal medium for yeast (leu^-).

Enzyme assay: 3-Isopropylmalate dehydrogenase had been assayed as follows (17-19); The standard reaction mixture contained 0.1 m*l* of 0.01 M $MgCl_2$, 1.0 M KCl, 0.2 m*l* NAD^+ (0.01 M Nicotinamide dinucleotide), 0.3 m*l* of potassium phosphate (1.0 M, pH 8.0), and 0.3 m*l* of 0.01 M citraconate in 1 m*l*. One unit of enzyme activity is defined as the amount of enzyme which gives one micromole of the product per min. at 37°C. This method is based on titrating product of -ketobutyrate converted from citraconate as substrate instead of 3-isopropylmalate (20, 21).

Southern hybridization

The hybridization was performed according to Southern (23) and detected by Biotinyl-11-deoxy UTP labelling method (24, 25, 26).

Results

Cloning of 3-isopropylmalate dehydrogenase gene

E. coli JM109 was transformed with the ligation mixture, and plated onto selective plates consisting of X-gal medium. Then *Saccharomyces cerevisiae* DBY746 was transformed wit selected plasmid. As a result, two recombinant plasmids which complements the leu2 mutation in *Saccharomyces cerevisiae* and leuB mutation in *E. coli* (Fig. 1) were obtained.

Restriction mapping of cloned fragment

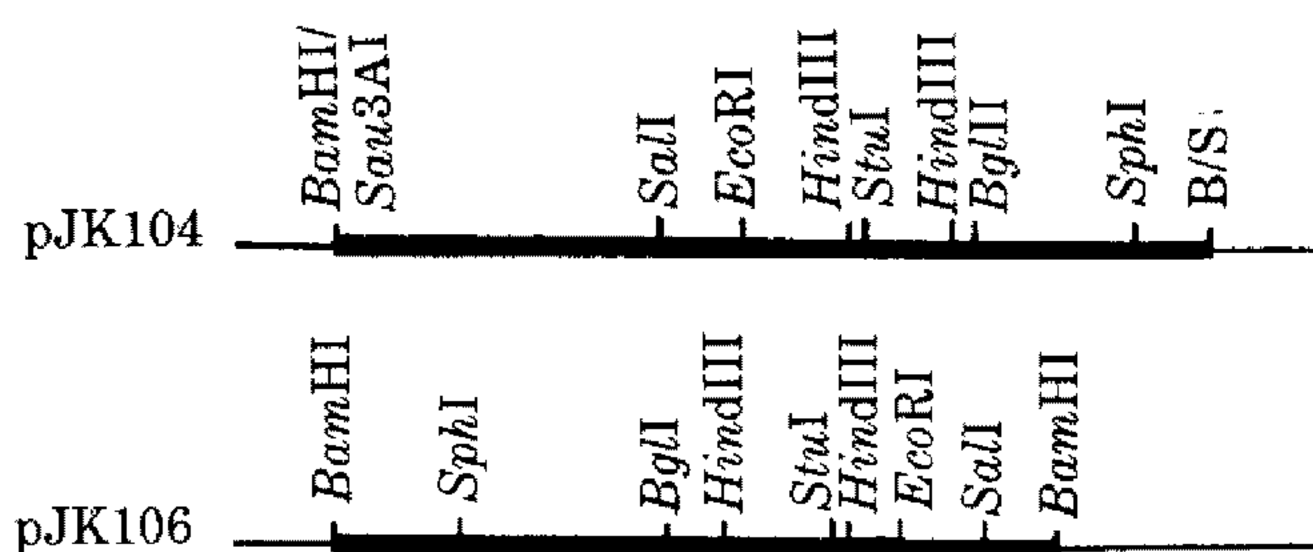

Fig. 2. Restriction maps of recombinant plasmid.
—: PHN114 plasmid vector. ▬: insert DNA

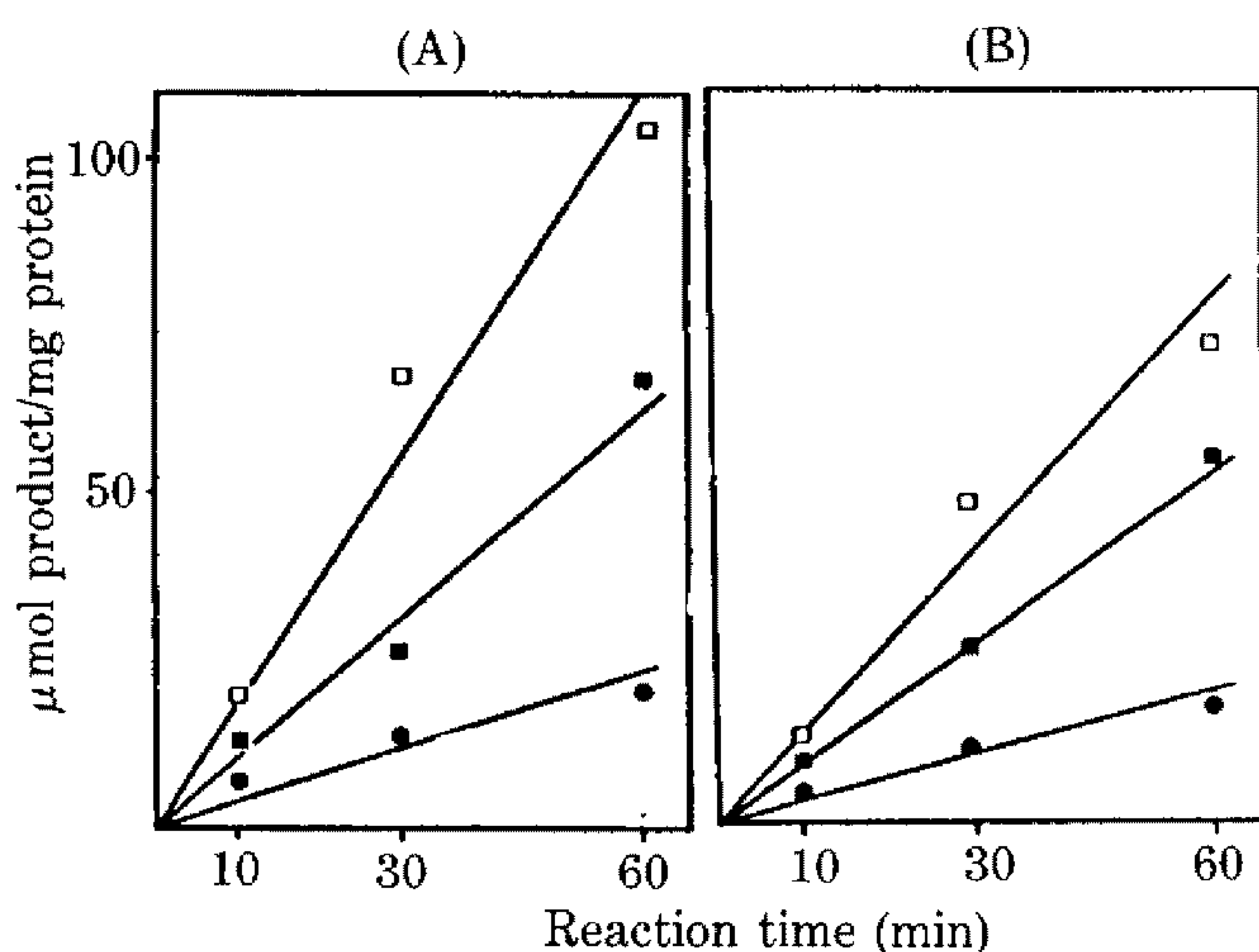

Fig. 3. Activity of 3-isopropylmalate dehydrogenase in the cell extract of *E. coli* C600 carrying pJK104 (A), pJK106 (B).
●—● : 0 hr., □—□ : 3 hr., ■—■ : 6 hr. inducted time in (leu$^-$) medium. See the text.

Subcloned plasmids were analyzed by agaroge gel electrophoresis with several restriction enzymes, the small one was designated as pJK106, cosisted of pHN114 and *Bam*HI, *Sal*I, *Sau*3AI, *Eco*RI, *Hin*dIII, *Stu*I, *Bgl*II, *Sph*I. According to the result of restriction mapping, the inserted fragment had inserted different directions to each other.

Expression of cloned LEU gene

Activity of 3-isopropylmalate dehydrogenase from *E. coli* C600 carring pJK104 and pJK106 was shown in Fig. 3. In the case of pJK104, when the cell-free extracts of *E. coli* C600 were incubated for 0, 3 and 6 hours, the activities were 25.4, 102.7 and 64.7 μmole product/mg protein, respectively. On the other hand, the activity of the enzyme encoded from pJK106 was 10.9, 68.1, 49.5 mol product/mg protein, respectively. It show that the activity of cloned 3-isopropylmalate dehydrogenase is inhibited by accumulation of leucine, product of biosynthesis. The reason that the activity

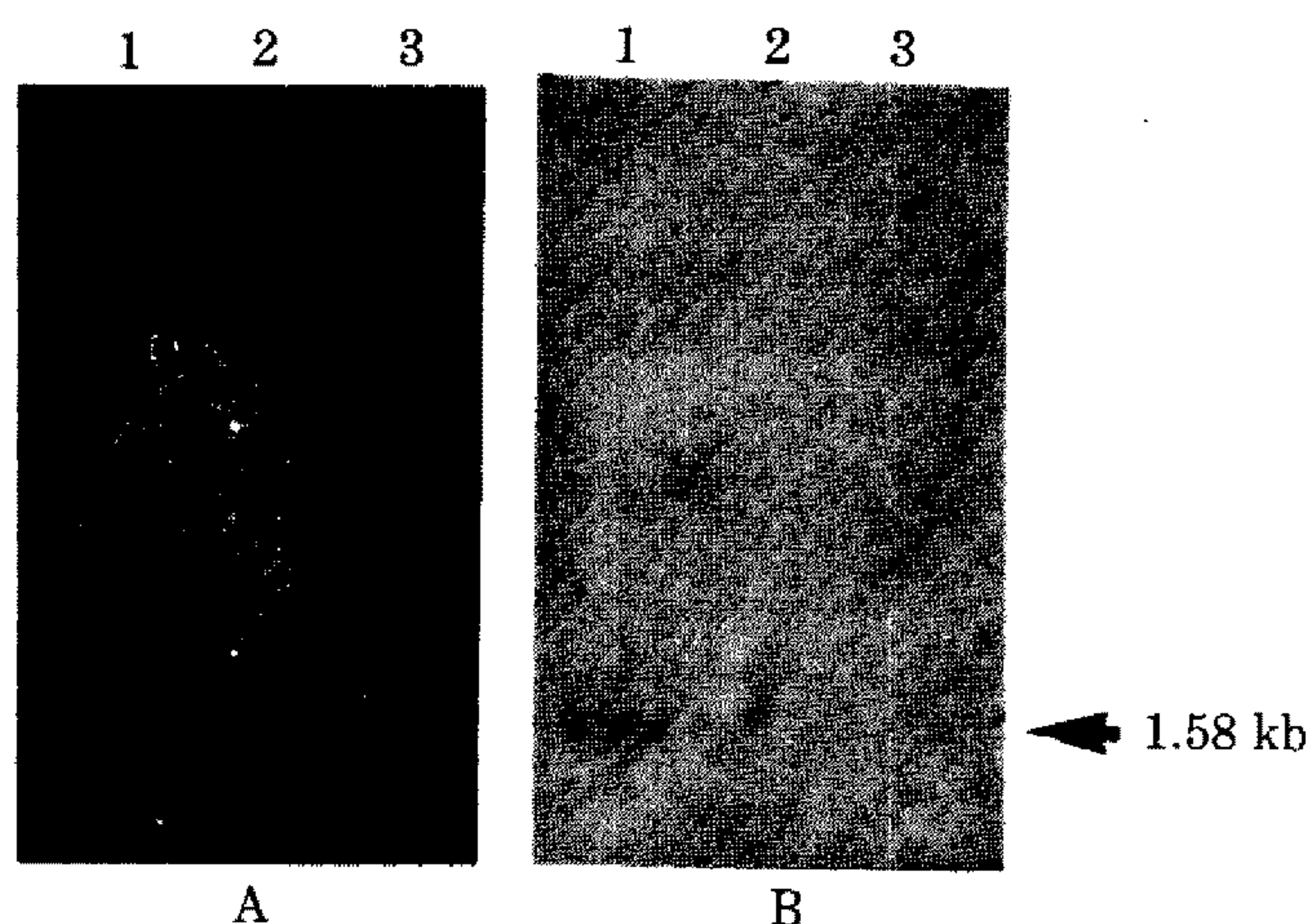

Fig. 4. Southern hybridization.
Chromosomal DNAs were digested with *Bgl*II. Probe DNA was prepared from pJK104 digested with *Bgl*II and *Sal*I (1.58 kb).
A : Gel electrophoresis (0.8% agarose)
B : Nitrocellulose filter
1 : *Kluyveromyces fragilis* DNA
2 : *Saccharomyces cerevisiae* DNA
3 : *E. coli* DNA

of the enzyme encoded from pJK104 is superior to pJK106 is, perhaps, due to addition the enzyme of the activity of lac promoter expression.

Hybridization

To confirm that the inserted fragment is originally come from *Kluyveromyces fragilis* DNA, Southern hybridization was carried out. Total DNA of *Kluyveromyces fragilis, E. coli* and *Saccharomyces cerevisiae* was digested completely with *Bgl*II and *Sal*I, and was carried out on agarose gel; then the DNA fragments were transferred to a nitrocellulose filter. The 1.58 kb *Bgl*II and *Sal*I fragment of pJK104, labelled with biotinyl-dUTP was hybridized as a probe to the nitrocellulose filter. Fig. 4 shows that the inserted fragment is hybridized to DNA of only *Kluyveromyces fragilis*. It was concluded that the cloned fragment was originated from *Kluyveromyces fragilis* DNA, which would complement te leuB mutation of *E. coli* and the leu2 mutation of *Saccharomyces cerevisiae.*

Discussion

To construct host systems for *Kluyveromyces cerevisiae,* we cloned a LEU gene of *Kluyveromyces fragilis.* As a result, we obtained two cloned fragments which had inserted in pHN114 in different direction to each other.

There are several reports about orientation-dependent expression by Loison *et al.* (27), using the URA1 gene of *Saccharomyces cerevisiae* in *E. coli* (pyrD$^-$) and by Kawamura *et al.* (28), using a LEU gene of *Candida maltosa* in *E. coli* (leuB$^-$). According to the reports, LEU gene expression in *E. coli* was found to depend on the orientation of the insertion. If the *Kluyveromyces fragilis* DNA segment is inserted in the same diraction onto *E. coli* promoter, the expression of the pJK104 would be superior to that of pJK106. It also should be taken into consideration that 5' and 3' flanking region of pJK104 is longer than that of pJK106.

Nevertheless pJK104 was still expressed in *E. coli* and *Saccharomyces cerevisiae.* It means that the inserted fragment has its promoter sequences of *Kluyveromyces fragilis*, which are funtional in *E. coli* as well as yeast.

In addition, we are investigating about homology of cloned gene with other LEU gene through the nucleotide sequencing analysis.

요 약

Shuttle plasmid vector인 pHN114를 이용하여 *Kluyveromyces fragilis*의 3-isopropylmalate dehydrogenase 유전자를 cloning 하였다. 그 결과 *Saccharomyces cerevisiae*의 leu2 변이와 *E. coli*의 leuB 변이를 상보하는 두 가지의 clone체 pJK104와 pJK106을 얻었다. Restriction mapping 결과 이들은 서로 반대방향으로 삽입되어 있었으며 expression activity는 pJK104가 높았다. pJK104에 삽입된 유전자를 BglII와 SalI으로 끊은 1.6 kb fragment를 probe로 하여 Southern Hybridization한 결과 유전자의 유래가 *Kluyveromyces fragilis* 임을 확인하였다.

Acknowledgement

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