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Nucleotide Sequencing Analysis of a Gene Coding for 3-Isopropylmalate Dehydrogenase of *Kluyveromyces fragilis*

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A 3-isopropylmalate dehydrogenase (3-IPMD) gene was cloned from *Kluyveromyces fragilis*. pJK104 could complement *Escherichia coli* leuB and *Saccharomyces cerevisiae leu2* auxotrophs. The coding region was subcloned and the nucleotide sequence was determined. A 1.8 Kb *Eco*RI/*Sph*I fragment of pJK104 subcloned in pUC18 could still complement the *leuB* mutation. An open reading frame of 1164 bp that corresponds to a polypeptide of 387 amino acids was found in the cloned fragment. The homology between the 3-isopropylmalate dehydrogenase of *S. cerevisiae* and that of *K. fragilis* was 68.13% in nucleotides.

Lower eukaryotes, especially *S. cerevisiae*, have long been used as model organisms in studying the cellular biology of higher eukaryotes. Researches on the expression and secretion of heterologous proteins have resulted in considerable advances in the techniques of producing proteins and peptides in yeast. Other than *S. cerevisiae*, *K. fragilis* have been investigated as possible host organisms for the expression and secretion of heterologous proteins (13, 17).

The yeast *LEU2* gene from chromosome III coding for β-isopropylmalate dehydrogenase has been cloned on several hybrid yeast-*E. coli* plasmids. This enzyme catalyzes the conversion of 3-isopropylmalate to α-ketoisocaporate in the leucine biosynthesis pathway. Unlike other amino acid biosynthesis genes in yeast, the expression of *LEU2* gene is repressed specifically by leucine and threonine (4). The structural features of the 5′ end and 3′ end of *LEU2* gene, the control mechanism involving mRNA secondry structure, and a leucine-rich leader polypeptide have been proposed (16). *LEU2* gene is an essential component of yeast shuttle plasmid vectors. Because the *LEU2* gene can complement *in vivo* both the *E. coli leuB6* mutation (10) and the *leu2-3, leu2-12* mutation in yeast, it can be used to select the transformants of both hosts from a leucine deficient medium. The *LEU2* gene mentioned above is derived from *S. cerevisiae*. It is a well known fact that *K. fragilis* is superior to *S. cerevisiae* in secreting proteins into the medium. So we determined the base sequence of 3-isopropylmalate dehydrogenase gene to obtain information about the primary structure. And we are scheduled to use this gene as a part of new plasmid vector for development of a new host-vector system. In this paper, the sequencing analysis of the gene coding for 3-isopropylmalate dehydrogenase and the comparison analysis with other LEU2 gene are described.

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MATERIALS AND METHODS

Strains, Media and Plasmids

E. coli MC1061 (F^-, *araD139*, Δ*ara*ABO1 (leu^-) 7679, Δ*lac*X74, *gal*U, *gal*K, *rps*L, *hsd*R), JM109 (*rec*A1, *lac*Δ*pro*, *end*A1, *gir*A96, *thi*-1, *hsd*R17, F^- *tra*D36) and C600 (*leu*B, *thr*, *thi*, rk^-, mk^-, Nal^r) which were grown in either L-broth or M9 medium with appropriate supplements were used as bacterial strains. *K. fragilis* Y610 and *S. cerevisiae* DBY746 were used as yeast strains. pUC18 was used as plasmid for the subcloning the fragment which contains enzyme activity, while M13mp18 and M13mp19 were used as plasmids for the nucleotide sequencing.

Transformation of *E. coli* and Yeast

The transformation of yeast was done by the method

described by Ito *et al.* (5), and the transformation of *E. coli* was carried out by the conventional $CaCl_2$ method described by Hanahan *et al.* (2) with slight modifications. Other genetic techniques were carried out according to the methods described by Maniatis *et al.* (7).

DNA Technique and Subcloning

E. coli plasmid was isolated by the Birnboim and Doly's Alkaline-SDS method (1).

Yeast chromosomal DNA was isolated from *K. fragilis* digested partially by *Sau*3AI and fractionated by agarose gel electrophoresis. Fractions larger than 3.0 Kb were pooled, ligated to pHN114 (8) digested by *Bam*HI, and transformed into *E. coli*. About 5,000 colonies were obtained from this transformation step. And DNA isolated from the *E. coli* was used as a gene library. pJK104, which complemented *leu2* mutation in *S. cerevisiae* in the previous paper (9), was isolated from the *Leu*$^+$ transformant with gene library of *K. fragilis*. A 1.8 kb *Eco*RI/*Sph*I fragment (pJS101~pJS 108) of pJK104 was subcloned in pUC18 and activity of *leu* gene was checked.

Enzyme Assay

α-Ketobutylate formed from citraconate was assayed as the activity of the 3-isopropylmalate dehydrogenase in the following manner. The reaction mixture contained in total volume of 2 m*l*; 0.3 m*l* citraconate (0.01 M) as a substrate, 0.1 m*l* $MgCl_2$ (0.01 M), 0.1 m*l* KCl (1.0 M), 0.2 m*l* NAD^+ (nicotinamide adenine dinucleotide), 0.3 m*l* potassium phosphate buffer (1.0 M pH 8.0), and cell-free extracts. Incubation was carried out at 37℃ for 10 minutes and the reaction was stopped by the addition of 3 m*l* 2,4-dinitrophenyl hydrazine (0.25 mg/m*l* in 0.5 M HCl). Finally 1 m*l* of KOH (40%) was added for colorization. The enzyme activity was measured with the absorbance at 540 nm.

Determination and Analysis of Nucleotide Sequence of *LEU* Gene

Each fragment of within the 1.8 kb *Eco*RI/*Sph*I fragment of pJK104 was subcloned in M13mp18 and M13 mp19, and the DNA sequencing was performed according to the method described by Sanger (12) using the Sequenase kit from United States Biochemical Corp. (Cleveland, OH, U.S.A) following the supplier's protocol. Comparison of sequence between *K. fragilis LEU2* gene and *S. cerevisiae LEU2* gene was done by using DNASIS.

RESULTS AND DISCUSSION

Determination of Coding Region

Localization of 3-isopropylmalmalate dehydrogenase coding region in pJK104 was examined in the following manner. Several parts as shown in Fig. 1, were subcloned in pUC18, and the 3-isopropylmalate dehydrogenase activity of the deletion plasmids was examined by doing complementation tests of *E. coli leuB* auxotroph. It was concluded that the 3-isopropylmalate dehydrogenase coding region was located in a *Eco*RI/*Sph*I fragment of 1.8 kb.

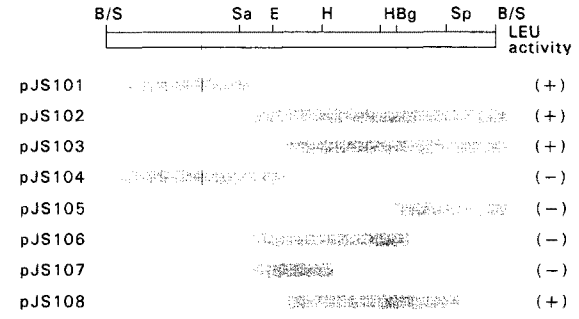

Fig. 1. Identification of *Kluyveromyces fragilis* LEU gene by subcloning.

The open bars represent the fragments (4.4 kb) of pJK104 with *K. fragilis* LEU2 gene.

These fragments were tested for complementation of an *E. coli leu2* mutation and the results were indicated on the right. End restriction site of each fragment is following to

pJS101 : E-Sa pJS102 : Sa-Sa
pJS103 : E-E pJS104 : E-E
pJS105 : H-H pJS106 : Sa-Bg
pJS107 : Sa-H pJS108 : E-Sp
pJS106 was ligated to Sa-B of pUC18

Abbreviations; B, *Bam*HI; S, *Sau*3AI; E, *Eco*RI; H, *Hind*III; Bg, *Bgl*II; Sp, *Sph*I; Sa, *Sal*I.

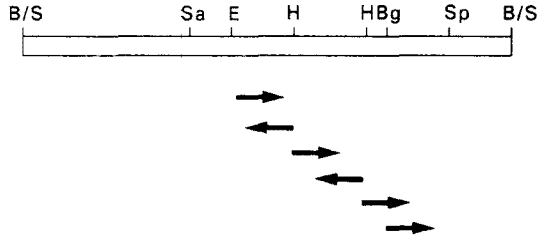

Fig. 2. DNA sequence strategy for the fragments containing the LEU gene.

The direction and length of sequence determinations are shown with arrows. The putative coding region for the LEU gene is shaded.

Sequencing Strategy and DNA Sequencing of the 3-Isopropylmalate Dehydrogenase Gene of *K. fragilis*

To analyze the DNA sequence of the 3-isopropylmalate dehydrogenase gene of *K. fragilis*, we determined

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1                                                                   48
ATG TTT GGC TCT GGT CAT GAT TAC GGC ATT GAT ATC GTC CAA CTG CAT
Met Phe Gly Ser Gly His Asp Tyr Gly Ile Asp Ile Val Gln Leu His
49                                                                  96
GGA GTG AGT CGT GGC AAG AAT ACC AAG AGT TCC TCG GTT TGC CAG TTA
Gly Val Ser Arg Gly Lys Asn Thr Lys Ser Ser Ser Val Cys Gln Leu
97                                                                 144
TTA AAA GAC TCG TAT TTC CAA AAG ACT GCA ACA TAC TAC TCA GTG CAG
Leu Lys Asp Ser Tyr Phe Gln Lys Thr Ala Thr Tyr Tyr Ser Val Gln
145                                                                192
CTT CAC AGA AAC CTC ATT CGT TTA TTC CCT TGT TTG ATT CAG AAG CAG
Leu His Arg Asn Leu Ile Arg Leu Phe Pro Cys Leu Ile Gln Lys Gln
193                                                                240
GTG GGA CAG GTG AAC TTT TGG ATT GGA ACT CGA TTT CTG ACT GGG TTG
Val Gly Gln Val Asn Phe Trp Ile Gly Thr Arg Phe Leu Thr Gly Leu
241                    Hind III                                    288
GAA GGC AAG AGA GCC CCG AAA GCT TCC AAG AAA GCT GAT GCA GTA TTA
Glu Gly Lys Arg Ala Pro Lys Ala Ser Lys Lys Ala Asp Ala Val Leu
289                                                                336
TTG GGT GCA GTG GGT GGT CCA AAG TGG GGT ACA GGT TGC GTT AGA CCT
Leu Gly Ala Val Gly Gly Pro Lys Trp Gly Thr Gly Cys Val Arg Pro
337                                                                384
GAA CAA GGT TTA TTG AAG ATA AGA AAG GAA TTG GGA TTG TAT GCT AAC
Glu Gln Gly Leu Leu Lys Ile Arg Lys Glu Leu Gly Leu Tyr Ala Asn
385                                                                432
TTG TGC CCT TGT AAT TTG CCT CAA GAC TCT TTG CTC GAT CTA TCC CCA
Leu Cys Pro Cys Asn Leu Pro Gln Asp Ser Leu Leu Asp Leu Ser Pro
433                                                                480
TTG AAA CCT GAG TAC GCC AAG GGA ACT GAC TTT GTT GTC GTT AGA GAA
Leu Lys Pro Glu Tyr Ala Lys Gly Thr Asp Phe Val Val Val Arg Glu
481                                                                528
TTG GTG GGT GGT ATC TAC TTT GAT GAG AGG AAG GAA GAT GAA GGT GAT
Leu Val Gly Gly Ile Tyr Phe Asp Glu Arg Lys Glu Asp Glu Gly Asp
529                                                                576
GTT GTT GCT TGG GAT TCT GAA AAG TAC AGT GTA CCA GAA GTT CAA AGA
Val Val Ala Trp Asp Ser Glu Lys Tyr Ser Val Pro Glu Val Gln Arg
577                                                                624
ATC ACC AGA ATG GCT GCA TTT TTA GCT TTA CAA CAC AAC CCA CCT TTA
Ile Thr Arg Met Ala Ala Phe Leu Ala Leu Gln His Asn Pro Pro Leu
625                                                                672
CCA ATC TGG TCA TTG GAT AAA GCA AAT GTC TTG GCA TCA TCT AGA TTG
Pro Ile Trp Ser Leu Asp Lys Ala Asn Val Leu Ala Ser Ser Arg Leu
673                                                                720
TGG AGA AAA ACA GTA GAA GAA ACT ATC AAG AAT GAG TTC CCT CAA TTG
Trp Arg Lys Thr Val Glu Glu Thr Ile Lys Asn Glu Phe Pro Gln Leu
721                                                                768
ACT GTC CAA CAT CAA TTG ATC GAT TCT GCT GCT ATG ATC CTC GTC AAG
Thr Val Gln His Gln Leu Ile Asp Ser Ala Ala Met Ile Leu Val Lys
769                                                                816
TCT CCA ACC AAG TTA AAT GGT ATA GTT ATT ACG AAT AAC ATG TTT GGT
Ser Pro Thr Lys Leu Asn Gly Ile Val Ile Thr Asn Asn Met Phe Gly
817                    Hind III                                    864
GAT ATC ATT TCT GAT GAA GCT TCC GTC ATT CCA GGT TCA TTG GGC TTA
Asp Ile Ile Ser Asp Glu Ala Ser Val Ile Pro Gly Ser Leu Gly Leu
865                                                                912
CTA CCA TCC GCT TCA TTG GCC TCG CTA CCA GAC ACC AAC AAA GCA TTC
Leu Pro Ser Ala Ser Leu Ala Ser Leu Pro Asp Thr Asn Lys Ala Phe
913                                                                960
GGT TTG TAC GAA CCA TGT CAC GGT TCT GCG CCC GAC TTG CCA GTA AAT
Gly Leu Tyr Glu Pro Cys His Gly Ser Ala Pro Asp Leu Pro Val Asn
961                                                               1008
AAG GTC AAC CCA ATT GCA ACA ATT CTA TCT GCC GCT TAG ATG TTG AAA
Lys Val Asn Pro Ile Ala Thr Ile Leu Ser Ala Ala Met Met Leu Lys
1009        Bgl II                                                1056
CTT TCC CTA GAT CTA GTG GAA GAA GGT AGA GCC GTT GAG GAA GCC GTT
Leu Ser Leu Asp Leu Val Glu Glu Gly Arg Ala Val Glu Glu Ala Val
1057                                                              1104
AGA AAA GTT TTG GAT TCT GGA ATC AGA ACT GGC GAT CTA GGC GGT TCC
Arg Lys Val Leu Asp Ser Gly Ile Arg Thr Gly Asp Leu Gly Gly Ser
1105                                                              1152
AAC TCT ACC ACG GAG GTT GGT GAC GCT GTT GCA AAG GCT GTA AAG GAG
Asn Ser Thr Thr Glu Val Gly Asp Ala Val Ala Lys Ala Val Lys Glu
1153       1164
ATT TTG GCA TAA
Ile Leu Ala ***
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Fig. 3. Nucleotide and amino acid sequence of the 3-isopropylmalate dehydrogenase gene of *K. fragilis*. ***represents the termination codon. The underlined region represents a possible substrate binding site proposed by Anderadis *et al.* (1984).

the nucleotide sequence of the 1.8kb fragment of pJK 104 by using the dideoxy chain termination method according to the sequencing strategy as shown in Fig. 2. Several fragments, which are indicated with arrows in Fig. 2, were subcloned in the multiple cloning site of M13mp18 and M13mp19. The DNA sequence of the 3-isopropylmalate dehydrogenase gene of *K. fragilis* is shown in Fig. 3.

Sequence Analysis

The nucleotide sequence, the codon usage, the amino-acid composition and the amino acid sequence of the enzyme were compared with those of *S. cerevisiae* and *B. subtilis*. When the base sequence of 3-isopropylmalate dehydrogenase gene of *K. fragilis* was compared with that of *S. cerevisiae* by using DNASIS, a considerable homology (68.13%) was found. And in many cases, only the 3rd letter of corresponding codon differed; in contrast to the 72% homology between the base pairs and the 76% homology between the deduced amino acids of *Candida maltosa* and the *S. cerevisiae* (15). The homologies of two amino acid sequences are 45.7% for *S. cerevisiae-Bacillus coagulans*, 42.0% for *B. coagulans-Thermus thermophilus* and 38.6% for *S. cerevisiae-T. thermophilus* (14). The sequence of 14 amino acids in *Saccharomyces cerevisiae* underlined in Fig. 3. was considered by Andreadis *et al.* (1984) to be the substrate binding site of the *LEU2* gene products. The homology in this region was 84.6%.

In many cases, cloned *leu* fragment also contained an ARS(autonomously replicating sequence) site of *Candida maltosa, Mucor circinelloides* and *Rhodotorula glutins* (3, 6, 11). So a putative 'core' consensus sequence of the ARS (A/T)TTTAT(A/G)TTT(A/T) has been found in the cloned fragment.

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REFERENCES

1. **Birnboim, H.C. and J. Doly.** 1979. A Rapid Alkaline Extraction for Screening Recombinant Plasmid DNA. *Nucl. Acid. Res.* **7**: 1513-1523.
2. **Hanahan, D.** 1983. Studies on Transformation of *E. coli* with Plasmid. *J. Mol. Biol.* **166**: 557-570.
3. **Ho, Y.R. and M.C. Chang.** 1988. Cloning of a LEU gene and an ARS site of *Rhodotorula glutinis*. *Chung-Hua-Min-Kuo-Wei-Sheng-Wu-Chi-Mien-I-Hsueh-Tsa-Chih.* **21**(1): 1-8.
4. **Hus, Y. and G. Kohlaw.** 1982. Overproduction and control of the LEU2 gene product, β-isopropylmalate dehydro-

genase, in transformed yeast strains. *J. Biol. Chem.* **25**: 39.

5. **Ito, H., K. Fukuda, A. Murata and A. Kimura.** 1983. Transformation of intact yeast cell treated with alkali cations. *J. Bacteriol.* **153**: 163-168.
6. **Kawamura, M., M. Takagi and K. Yano.** 1983. Cloning of a LEU gene and an ARS site of *Candida maltosa*. *Gene.* **24**: 157-162.
7. **Maniatis, T., E.F. Fritsch and J. Sambrook.** 1982. Molecular cloning. A Laboratory Manual. Cold Spring Harbor Laboratory, *Cold Spring Harbor*, NY, 76.
8. **Park, Sung-Hee, Taeg-Kyu Kwon, Jong-Guk Kim and Soon-Duck Hong.** 1990. Construction of a novel shuttle plasmid vector pHN114. *Kor. J. Appl. Microbiol. Biotech.* **18**: 199-202.
9. **Park, Sung-Hee, Dong-Sun Lee, Ju-Hyung Woo, Jong-Guk Kim and Soon-Duck Hong.** 1990. Molecular cloning of the gene coding for 3-Isopropylmalate dehydrogenase of *Kluyveromyces fragilis*. *Kor. J. Appl. Microbiol. Biotech.* **18**: 305-308.
10. **Ratzkin, B. and J. Carbon.** 1977. Functional expression of cloned yeast DNA in *Escherichia coli*. *Proc. Natl. Acad. Sci.* **74**: 487-491.
11. **Roncero, M.I.G., L.P. Jepsen, P. Stroman, R. Van Heeswijck.** 1989. Characterization of a leuA gene and an ARS element from *Mucor circinelloides*. *Gene.* **84**: 335-343.
12. **Sanger, F., S. Nicken and A.R. Carson.** 1977. DNA Sequencing with chain Terminating Inhibitors. *Proc. Natl. Acad. Sci.* **74**: 5463-5467.
13. **Schultz, K., D. Moyer, M. Richman and J.R. Shuster.** 1990. *Kluyveromyces* as a host for heterologous gene expression: expression and secretion of prochymsin. *Bio /Technology.* **8**: 135.
14. **Sekiguchi, T., J. Ortega-Cesena, Yoshiaki. Nosoh, S. Ohashi, K. Tsuda and S. Kanaya.** 1986. DNA and amino acid sequences of of 3-isopropylmalate dehydrogenase of *Bacillus coagulans*. Comparison with the enzymes of *Saccharomyces cerevisiae* and *Thermus thermophilus*. *BBA.* **867**: 36-44.
15. **Takagi, M., N. Kobayashi, M. Sugimoto, T. Fujii, J. Watari and K. Yano.** 1987. Nucleotide sequencing analysis of a LEU gene of *Candida maltosa* which complements leuB mutation of *Escherichia coli* and leu2 mutation of *Saccharomyces cerevisiae*. *Curr. Genet.* **11**: 6-7. 451-457.
16. **Yanofasky, C.** 1983. Prokaryotic mechanisms in eukaryotes. *Nature.* **302**: 751-752.
17. **Yu, J.H., I.D. Lew, C.K. Park and I.S. Kong.** 1987. Lactic acid fermentation of soymilk by mixed cultures of *Lactobacillus acidophilus* and *Kluyveromyces fragilis*. *Kor. J. Appl. MIcrobiol. Bioeng.* **15**: 162-169.

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