Molecular Cloning of β -Isopropylmalate Dehydrogenase Gene from *Clostridium butyricum* M588

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The gene for β -isopropylmalate dehydrogenase of *Clostridium butyricum* M588 has been cloned in *E. coli*. The genome of *Clostridium butyricum* M588 was digested with restriction endonuclease *Eco*RI and joined to plasmid pBR322. Competent *E. coli* HB101 cells were transformed with the hybrid plasmids and the leucine⁺ transformants were selected. The plasmid, pCE4, containing four *Eco*RI fragments, was isolated from the transformants. It was found that the 2.2 kb *Eco*RI fragment on a reconstructed plasmid pCE1 contained the β -isopropylmalate dehydrogenase gene. Restriction analysis showed that the β -isopropylmalate dehydrogenase gene was located in the 1.4 kb *Bgl*II-*Eco*RI fragment and the *Bam*HI site was inside the β -isopropylmalate dehydrogenase gene.

Clostridium butyricum M588 is a gram-positive anaerobe which is commercially available as a gastrointestinal tonic.^{1~4)} It was found that this microbe produced H₂ gas and accumulated anthranilate, a precursor of tryptophan, in the medium (unpublished data). Genetic information on *Clostridium butyricum* is quite limited. There is no report on the cloning of the genes from *Clostridium butyricum*. Therefore, it is interesting to clone the genes of *Clostridium butyricum* and to investigate the expression of the genes in *E. coli*.

In this paper, we will describe the cloning of the β -isopropylmalate dehydrogenase gene from *Clostridium butyricum* M588 and analysis of the cloned fragment.

MATERIALS AND METHODS

Bacterial strains and plasmid used. The bacteria and plasmid used are listed in Table I.

Preparation of DNA. *Clostridium butyricum* M588 was anaerobically grown to the early log phase at 37°C in medium containing 10 g of polypeptone, 5 g of beef extract, 3 g of NaCl and 10 g of glucose (pH 7.0). The chromosomal DNA of *Clostridium butyricum* M588 was purified by the method of Saito and Miura.⁵⁾ The *E. coli*

plasmid was detected by the miniscreening method.⁶⁾ Plasmid DNAs were purified according to the method of Bolivar *et al.*⁷⁾

Construction of recombinant plasmids. DNAs were digested with *Eco*RI at 37°C for 1 hr (for plasmid DNA) or 4 hr (for chromosomal DNA). After the digestion, 1 μ g of pBR322 and 3 μ g of the chromosomal DNA were mixed and ligated with T4 ligase for 24 hr at room temperature. This ligated DNA fraction was used for the following transformation process.

Transformation. Transformation of *E. coli* with the plasmid was carried out in the presence of calcium chloride as described.⁸⁾

Gel electrophoresis. Electrophoresis of the plasmids was performed on 1% agarose slab gel or 5% acrylamide slab gel with 0.09 M Tris-borate buffer, pH 8.3, at 14.5 V/cm for 1.5~5 hr. After the electrophoresis, the gel was stained with ethidium bromide solution (50 μ g/ml). The DNA separated was detected with a short wavelength UV transilluminator (Ultra-Violet Products, Inc.) and the molecular weight was estimated. As molecular size references, DNA fragments of lambda phage digested with *Hind*III were used.

Nick-translation and hybridization of DNA digests. DNA was labeled by nick-translation using *E. coli* DNA polymerase I (BRL) in the presence of ³²P-dATP as described.⁹⁾ The hybridization technique used was essentially the same as that described by Southern.¹⁰⁾

TABLE I. PROPERTIES AND ORIGIN OF STRAINS AND VECTOR USED

Bacterial strains and plasmid	Genotypes and properties	Origin and Reference
<i>Clostridium butyricum</i> Miyairi 588 (M588)	Used as gastro-intestinal tonic	C. Miyairi (1)
<i>E. coli</i> C600	<i>leuB</i> ⁺ , <i>thr</i> ⁺ , <i>trpB</i> ⁺ , <i>thi</i> ⁻ , <i>r</i> ⁻ , <i>m</i> ⁻	K. Nagahari
<i>E. coli</i> HB101	<i>pro</i> ⁺ , <i>leuB</i> ⁺ , <i>B1</i> ⁺ , <i>lacY</i> ⁺ , <i>hsdR</i> ⁺ , <i>hsdM</i> ⁺ , <i>ara</i> ⁺ , <i>gal</i> ⁺ , <i>xyl</i> ⁺ , <i>mtl</i> ⁺ , <i>sup44</i> ⁺ , <i>F</i> ⁻ , <i>endoI</i> ⁻ , <i>recA</i> ⁻	R. H. Doi
pBR322	<i>tet</i> ^r , <i>amp</i> ^r	(7)

Enzymes. Restriction endonucleases and T4 ligase were purchased from Bethesda Research Laboratories and used according to the methods suggested by the manufacturer.

RESULTS

Isolation of recombinant plasmids carrying the β -isopropylmalate dehydrogenase gene of *Clostridium butyricum* M588

As shown in Figs. 1 and 2, ligated DNA fragments (pBR322 and chromosomal DNA) mentioned above were introduced into *E. coli* C600 (*leuB*: β -isopropylmalate dehydrogenase less mutation) and plated onto selection plates consisting of M-9 minimal medium supplemented with tryptophan (50 μ g/ml), threonine (50 μ g/ml) and ampicillin (50 μ g/ml). Seven colonies of leucine⁺ amp^r transformants were picked up after 2 days of incubation at 37°C. Plasmids were isolated from the strains by the rapid boiling method and analyzed by agarose gel electrophoresis. The recombinant plasmid, pCE4, which contained pBR322 and four *EcoRI* fragments was purified and could transform *E. coli* C600 at a frequency of about 10⁶ leucine⁺ amp^r transformants/ μ g of DNA. This fact indicates that the genes determining amp^r and Leu⁺ are coded on the same recombinant plasmid. To reduce the size of DNA fragments, pCE4 was digested with *EcoRI* and ligated with T4 ligase. Ligated DNA fragments were introduced into *E. coli* HB101 and leucine⁺ amp^r transformants were obtained.

FIG. 1. Construction of Plasmids Containing the β -Isopropylmalate Dehydrogenase Gene of *Clostridium butyricum* M588.

The thickened line indicates *Clostridium butyricum* M588 DNA.

FIG. 2. Electrophoretic Patterns of Plasmid DNAs Digested with *EcoRI* in 1% Agarose Gel.

Lanes 1 and 6, λ DNA digested with *HindIII* as size markers; lane 2, pCL1; lane 3, pCE1; lane 4, pCE4; lane 5, pBR322.

The plasmid, pCE1, which contained pBR322 and the *EcoRI* fragment (2.2 kb) is shown in Fig. 1.

Restriction mapping

Figure 3 shows the electrophoretic patterns of pCE1 after single or double digestions with several restriction endonucleases. The recognition map of pCE1 obtained on this analysis is shown in Fig. 1.

Homology between plasmid pCE1 and the chromosomal DNA

To analyze the origin of DNA inserted in the pBR322 plasmid, we hybridized ^{32}P -labeled pCE1 prepared by nick-translation to the restriction enzyme-digested chromosomal DNAs of *Clostridium butyricum* M588 and *E. coli* which had been immobilized on nitrocellulose filters. Radioactively labeled pCE1 hybridized to the digested fragments of unlabeled pCE1 (Fig. 4b, lanes

FIG. 3. Electrophoretic Patterns of pCE1 Plasmid DNA Digested with Restriction Endonucleases in 1% Agarose Gel.

Lanes 1 and 16, λ DNA digested with *HindIII*; lanes 2~15, pCE1 digested with endonucleases as follows (lane 2, *SalI*; lane 3, *BamHI*; lane 4, *EcoRI*; lane 5, *HindIII*; lane 6, *BglII*; lane 7, *PstI*; lane 8, *SalI* + *BglII*; lane 9, *BamHI* + *BglII*; lane 10, *EcoRI* + *BglII*; lane 11, *EcoRI* + *HindIII*; lane 12, *BamHI* + *EcoRI*; lane 13, *PstI* + *EcoRI*; lane 14, *PstI* + *BglII*; lane 15, *PstI* + *HindIII*).

FIG. 4. Confirmation That pCE1 DNA Contains a Fragment Derived from the Genome of *Clostridium butyricum* M588.

a) Agarose gel electrophoresis of endonucleases digests of various DNAs. The sources of DNA were: Lane 1, λ DNA digested with *Hind*III (0.2 μ g); lane 2, pCE1 digested with *Sal*I (0.2 μ g); lane 3, pCE1 digested with *Eco*RI (0.2 μ g); lane 4, *Clostridium butyricum* M588 DNA digested with *Eco*RI (5 μ g); lane 5, *E. coli* DNA digested with *Eco*RI (5 μ g).

b) Hybridization analysis of Southern transfer of the DNAs from gel a), using a 32 P-labelled pCE1 DNA probe.

2 and 3), and also hybridized to a 2.2 kb DNA fragment from *Clostridium butyricum* (Fig. 4b, lane 4). No complementary sequences to pCE1 were detected in *E. coli* (Fig. 4b, lane 5).

Localization of the β -isopropylmalate dehydrogenase gene in the cloned 2.2 kb fragment of *Clostridium butyricum* M588

To determine the localization of the β -isopropylmalate dehydrogenase gene, plasmid pCE1 was restricted with *Bgl*II, ligated with T4 ligase, and transformed into *E. coli* HB101. The transformants were checked as to whether the reduced size fragment substituted for the *leuB* phenotype of *E. coli* HB101 or not. When the 0.8 kb *Bgl*II fragment was eliminated from pCE1, plasmid pCL1 containing the 1.4 kb fragment still substituted for the *leuB* pheno-

type. Then, plasmid pCE1 was digested with *Bam*HI plus *Eco*RI, ligated with T4 ligase, and introduced into *E. coli* HB101. But no leucine⁺ transformants were obtained, although the plasmids contained the 0.9 kb fragment or 1.3 kb fragment. It was concluded that the 1.4 kb *Bgl*II-*Eco*RI fragment contained the β -isopropylmalate dehydrogenase gene and the *Bam*HI site was inside the β -isopropylmalate dehydrogenase gene (Fig. 1).

DISCUSSION

We have cloned the β -isopropylmalate dehydrogenase gene from *Clostridium butyricum* M588. It was found that β -isopropylmalate dehydrogenase of *Clostridium butyricum* M588 was expressed in *E. coli*, the 2.2 kb *Eco*RI fragment contained the β -isopropylmalate

dehydrogenase gene and the fragment cloned on pCE1 was hybridized to a *Eco*RI fragment (2.2 kb) of *Clostridium butyricum* M588 chromosomal DNA. Leucine genes are clustered on the chromosome in *E. coli* and *B. subtilis*.^{11,12)} Plasmid pCE1 may have other leucine genes beside that for β -isopropylmalate dehydrogenase.

The molecular weight of β -isopropylmalate dehydrogenase of *Salmonella typhimurium* consisting of two subunits is about $35,000 \times 2$.¹³⁾ It is reasonable that the β -isopropylmalate dehydrogenase gene of *Clostridium butyricum* M588 is included in the 1.4 kb of *Bgl*II-*Eco*RI fragment. But the 0.9 kb *Bam*HI-*Eco*RI fragment did not substitute for the *leuB* phenotype. These results indicated that the *Bam*HI site was inside the β -isopropylmalate dehydrogenase gene. These results suggest that the β -isopropylmalate dehydrogenase gene is a useful negative selection marker of a plasmid vector.

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