Cloning and Expression of the *Pseudomonas* Gene for Utilization of D-Leucine in *Escherichia coli*

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Two genes of *Pseudomonas putida* (IFO 12996) which code for enzymes participating in amino acid metabolism, were cloned in *Escherichia coli* C600 using pBR322 as a vector. pST7549 is a 7.9 kb hybrid plasmid DNA which is composed of four *Sal*I fragments (0.3, 1.4, 1.9 and 4.3 kb), and codes for β -isopropylmalate dehydrogenase (EC 1.1.1.85) in L-leucine biosynthesis. The enzyme activity in the crude extract from *E. coli* C600 bearing pST7549 was 80~90% lower than that of *E. coli* K12 or *P. putida*. When the foreign *Sal*I fragments derived from *P. putida* were subcloned, a 1.9 kb *Sal*I fragment was found to encode β -isopropylmalate dehydrogenase and it did not contain the promoter of *P. putida* DNA. Plasmid pST6961 has a 1.8 kb insert derived from the *P. putida* DNA in the *Sal*I site of pBR322. *E. coli* cells carrying this recombinant plasmid show no leucine racemase activity and no D-leucine transaminase activity, but five-times higher D-leucine oxidation activity than the host strain, *E. coli*. Enzymological studies have suggested that plasmid pST6961 codes for D-amino acid dehydrogenase, a key enzyme in D-amino acid metabolism.

The genetic recombination technique has enabled us to clarify the mechanism of gene expression and gene regulation, and to amplify specific enzymes producing useful materials abundantly. We are interested in the role of enzymes involved in bacterial D-amino acid metabolism. *Pseudomonas putida* (IFO 12996) has high activities of amino acid racemase with low substrate specificity,^{1,2)} D-hydantoinase^{3,4)} and other enzymes participating in D-amino acid metabolism. The present study was undertaken to clone the genes of *P. putida* for phenotypic alteration of L-leucine requirement, and for the utilization of D-leucine instead of the L-enantiomer in *Escherichia coli* C600 $r_k^- m_k^- leuB$. Each *E. coli* transformant has a hybrid plasmid encoded β -isopropylmalate dehydrogenase or D-amino acid dehydrogenase.

MATERIALS AND METHODS

Strains and media. The following bacterial strains were

used: *E. coli* C600 $r_k^- m_k^- thi thr leu$, *P. putida* (= *P. striata* IFO12996) and *E. coli* K12 (IFO3208) as a representative of the wild strain of *E. coli* C600. Transformed *E. coli* cells were grown in L broth containing 1% polypeptone, 0.5% yeast extract, 0.5% NaCl and 0.1% glucose, supplemented with 2% agar and appropriate antibiotics. Antibiotic concentrations used for the selection of transformants were 25 μ g/ml of ampicillin and 15 μ g/ml of tetracycline. For the assay of D-amino acid dehydrogenase activity, bacteria were grown at 37°C in a minimum medium (pH 7.2) containing 0.1% $(NH_4)_2SO_4$, 0.15% K_2HPO_4 , 0.05% KH_2PO_4 , 0.02% $MgSO_4 \cdot 7H_2O$, 0.01% yeast extract, 0.05 μ g/ml thiamine, 20 μ g/ml L-threonine and 10 μ g/ml D- or L-leucine. DL-Alanine, DL-leucine or glucose (0.3%) was added as a sole source of carbon and energy.

Purification of DNA. *P. putida* chromosomal DNA was isolated according to the method of Saito and Miura.⁵⁾ Plasmid DNA was prepared by a modification of the method of Oka.⁶⁾ After removal of RNA by gel filtration on Bio-Gel A-5m (Bio-Rad laboratories), covalently closed circular plasmids were purified by cesium chloride-ethidium bromide density gradient centrifugation. Ethidium bromide was removed by extraction with *n*-butanol followed by dialysis against 10 mM Tris-HCl buffer (pH 7.5) containing 0.2 mM EDTA.

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Restriction endonuclease analyses. All the restriction endonucleases (*Eco*RI, *Bam*HI, *Hind*III, *Sal*I and *Pst*I) used were obtained from Takara Shuzo Co. (Kyoto, Japan). Restriction endonuclease assays were performed under the conditions recommended by the supplier. Restriction endonuclease digestion products were separated by electrophoresis on a 0.7% agarose slab gel in 89 mM Tris-borate buffer (pH 8.3) containing 2.5 mM EDTA and 0.5 μ g/ml ethidium bromide.

Construction of hybrid plasmids. *P. putida* chromosomal DNA (50 μ g) was partially digested with *Sal*I restriction endonuclease, and the resulting fragments were ligated into the *Sal*I site of pBR322 (10 μ g) with T4 DNA ligase (Takara Shuzo Co.) in 66 mM Tris-HCl buffer (pH 7.6), 6.6 mM MgCl₂, 10 mM dithiothreitol and 66 μ M ATP. The ligation mixture was chromatographed on a Sephadex G-100 column (Pharmacia Fine Chemicals). The DNA fractions were collected and used for transformation.⁷⁾ Transformants containing the hybrid plasmids obtained are resistant to ampicillin, but sensitive to tetracycline.

Hybridization analysis of cloned *P. putida* genes. Hybridization was carried out essentially by the procedure of Southern.⁸⁾ Foreign fragments of hybrid plasmids were labeled with ³²P-dCTP using a Nick Translation kit (Amersham International Ltd.). After the hybridization, the nitrocellulose filter was washed according to the method of Jaffreys and Flavell^{9,10)} to avoid a high background. The filter was autoradiographed by exposure to Fuji RX film with an intensifying screen at -70°C.

Preparation of a cell-free extract. Cells harvested by centrifugation were washed twice with 0.85% NaCl. The washed cells were suspended in 10 mM potassium phosphate buffer (pH 7.2) containing 0.01% 2-mercaptoethanol, and subjected to sonication in a 19 kc Kaijo Denki oscillator (Tokyo, Japan) at 0°C for 5 min. After centrifugation at 40,000 $\times$ g for 30 min, the supernatant solution was dialyzed against the same buffer for 7 hr and used as the cell-free extract.

Enzyme assays. β -Isopropylmalate dehydrogenase activity was assayed by measuring the formation of α -ketobutyrate from citraconate according to the procedure of Kisumi *et al.*¹¹⁾ The reaction mixture (1.0 ml) contained 20 mM citraconate, 50 mM KCl, 10 mM MnCl₂, 5 mM NAD⁺, 100 mM potassium phosphate buffer (pH 8.0) and enzyme. Spectrophotometric measurement was carried out with a Carl Zeiss PMQ-II spectrophotometer.

D-Amino acid transaminase activity was measured by following the formation of D-glutamate with a Hitachi model 835 amino acid analyzer. The reaction mixture (1.0 ml) contained 30 mM D-leucine, 5 mM α -ketoglutarate, 0.8 mM pyridoxal 5'-phosphate (PLP), 30 mM potassium phosphate buffer (pH 8.0) and enzyme. The reaction was performed at 37°C under anaerobic conditions.

Amino acid racemase activity was measured by determination of D- or L-enantiomers of amino acids. The reaction mixture (1.0 ml) consisted of 120 mM D- or L-leucine, 0.5 mM PLP, 120 mM potassium phosphate buffer (pH 7.5) and enzyme. After incubation at 37°C under anaerobic conditions, 10% trichloroacetic acid was added to the mixture. An aliquot of the supernatant solution was subjected to high performance liquid chromatography according to the procedure of Gil-Av *et al.*¹²⁾ The conditions were as follows: Column, 30 $\times$ 0.46 cm octadecyl silica gel (JASCO PACK SC-02, Japan Spectroscopic Co., Ltd., Tokyo, Japan); mobile phase, aqueous solution of 0.008 M copper acetate and 0.017 M L-proline (pH 5.0); flow rate, 0.5 ml/min; pump pressure, 20 ~ 30 atm; solution for detection, 6 mM *o*-phthalaldehyde, 0.2% 2-mercaptoethanol and 0.4 M H₂BO₄ (pH 9.7).

The oxidation of D-leucine was followed by measuring oxygen consumption with a Hansatech oxygen electrode. The reaction mixture (1.0 ml) contained 50 mM D-leucine, 0.01 mM FAD, 0.1 mM 2,4-dichlorophenolindophenol (DCPIP), 50 mM potassium phosphate buffer (pH 8.0) and enzyme.

D-Amino acid dehydrogenase activity was measured at 600 nm by following the reduced DCPIP with a Union Giken SM-401 spectrophotometer (Kyoto, Japan). The reaction mixture contained 50 mM D-leucine, 0.1 mM DCPIP, 10 mM KCN, 50 mM potassium phosphate buffer (pH 7.8) and enzyme.

RESULTS AND DISCUSSION

Cloning in *E. coli* of *P. putida* genes

When the ligation mixture was transformed into *E. coli* C600 *r*_k⁻ *m*_k⁻ *thi* *thr* *lenB* cells, two Am^rTc^s transformants grew at 37°C in a minimum medium containing 0.3% glucose as a carbon source and 10 μ g/ml D-leucine. One transformant, no. 7549, was L-leucine

FIG. 1. Circular Restriction Maps of Plasmids pST7549 and pST6961.

The restriction maps are based on the relative sizes of the restriction fragments. The heavy line represents *P. putida* chromosomal DNA, and the light line represents DNA from the pBR322 cloning vector. The restriction sites for *Eco*RI, *Hind*III, *Bam*HI, *Sal*I and *Pst*I are marked. pST6961 has no site for these endonucleases on the 1.8 kb *Sal*I fragment.

independent, and the other, no. 6961, utilized D-leucine instead of the L-enantiomer. The Leu⁺ transformant, no. 7549, contained a 7.9-kb hybrid plasmid (pST7549) which was composed of four *Sal*I fragments (0.3, 1.4, 1.9 and 4.3 kb). Transformant no. 6961 contained a 6.1-kb plasmid (pST6961) with a 1.8-kb insert in the *Sal*I site of pBR322. From the digestion patterns of plasmid DNAs with several restriction endonucleases on agarose gel electrophoresis, the circular restriction maps of plasmids pST7549 and pST6961 can be depicted as shown in Fig. 1.

Hybridization of plasmid DNAs and chromosomal DNA

Southern blot analysis⁸⁾ of plasmids and chromosomal DNA was carried out to establish that the cloned insert is derived from *P. putida*. *P. putida* chromosomal DNA, and plasmids pST7549 and pST6961 DNAs were digested with *Sal*I. Samples were electrophoresed on a 0.7% agarose gel, transferred to nitrocellulose, and probed with nick translated foreign DNA fragments of pST7549 or pST6961. *Sal*I fragments of plasmid DNAs labeled with ³²P-dCTP were hybridized to the same size of DNA fragments from *P. putida*. The results indicate that the foreign fragments of hybrid plasmids are derived from *P. putida*.

β -Isopropylmalate dehydrogenase activity in *E. coli* carrying pST7549

Plasmid pST7549 was found to encode β -isopropylmalate dehydrogenase (EC 1.1.1.85) in L-leucine biosynthesis. Table I shows that the enzyme activity in the cell-free extract from *E. coli* C600 bearing plasmid pST7549 is 80~90% lower than that of *E. coli* K12 or *P. putida*, when assayed by measuring the formation of α -ketobutyrate from citraconate. Several workers reported reduced expression in *E. coli* of certain *Pseudomonas* genes,^{13~16)} and the dehydrogenase gene of *P. putida* is not fully expressed in *E. coli* either. Several possible explanations can be considered for these low activities; 1) the promoter region or an appropriate terminator is lacking in the DNA

TABLE I. β -ISOPROPYLMALATE DEHYDROGENASE ACTIVITY IN CELL-FREE EXTRACTS

Strain	Specific activity ^a
<i>E. coli</i> K 12	0.25
<i>E. coli</i> C600-pBR322	0.00
<i>E. coli</i> C600-pST7549	0.04
<i>P. putida</i> IFO 12996	0.48

^a Specific activity is expressed as nmol of product formed per mg of protein per min.

fragments derived from *P. putida* DNA. 2) These enzymes cannot be produced effectively in *E. coli* cells, because of the non-fitting of the *E. coli* ribosome, RNA polymerase and initiation factors to the mRNA produced by the *P. putida* genes.

Subcloning of pST7549

In an attempt to localize the gene and reduce the size of the insert, the 3.6-kb *Sal*I fragments of pST7549 were subcloned. The foreign fragments of pST7549 digested with *Sal*I were ligated into the *Sal*I site of pBR322. Figure 2 shows various derivatives of pST7549, their phenotypes and the enzyme activities of *E. coli* cells carrying these plasmids. The results indicate that the 1.9kb-A fragment encodes the *leuB* gene. The 2.2 kb-AC fragment and 1.9kb-A fragment with the same orientation as pST7549 confer the Leu⁺ phenotype, and express the enzyme activity. These results suggest that plasmid pST7549 does not contain the promoter region of *P. putida* DNA.

Clone bearing plasmid pST6961.

E. coli C600 bearing plasmid pST6961 could utilize D-leucine instead of the L-enantiomer with a low concentration of D-leucine (10 μ g/ml). This result indicates that plasmid pST6961 has a gene encoding the specific enzyme participating in D-leucine metabolism. From the results obtained by amino acid analysis and high performance liquid chromatography, it was found that *E. coli* bearing this recombinant plasmid showed no leucine racemase and no leucine transaminase activity (Table II). However, enhancement of D-leucine

FIG. 2. Construction of Various Derivatives of Plasmid pST7549.

The heavy line represents *P. putida* chromosomal DNA: A; 1.9 kb, B; 1.4 kb, C; 0.3 kb fragment. The restriction sites for *Eco*RI and *Sal*I are marked: E, *Eco*RI; S, *Sal*I.

TABLE II. THE ENZYME ACTIVITIES PARTICIPATING IN D-LEUCINE METABOLISM IN CELL-FREE EXTRACTS

Activity ^a	Strain		
	<i>P. putida</i>	<i>E. coli</i> C600	<i>E. coli</i> pST6961
Transamination	+ ^b	—	—
Racemization	+	—	—
Oxidation	+	+	+

^a Substrate, D-leucine.

^b +, detected; —, not detected.

TABLE III. D-LEUCINE OXIDATION ACTIVITY IN CELL-FREE EXTRACTS

Strain	Leucine added in minimum medium ^a	Activity ^b
<i>E. coli</i> C600-pBR322	100 µg/ml D-leucine	16
<i>E. coli</i> C600-pBR322	10 µg/ml L-leucine	22
<i>E. coli</i> C600-pST6961	10 µg/ml D-leucine	100
<i>P. putida</i> (IFO12996)	complete medium ^c	105

^a The minimum medium contained 0.3% glucose, 0.1% (NH₄)₂SO₄, 0.15% K₂HPO₄, 0.05% KH₂PO₄, 0.02% MgSO₄·7H₂O, 0.01% yeast extract, 0.05 µg/ml thiamine and 20 µg/ml L-threonine (pH 7.2).

^b Activity is expressed as nmol of oxygen consumed per mg of protein per 15 min.

^c L broth was used as the complete medium.

oxidation activity was observed. The host strain, *E. coli*, could grow in the minimum medium supplemented with a high concentration of D-leucine (100 µg/ml), but no appreciable increase in D-leucine oxidation activity was observed. *E. coli* bearing plasmid pST6961 showed five-times higher oxidation activity than did the host strain, *E. coli* (Table III). The oxygen consumption was inhibited by addition of 1 mM KCN, which is an uncoupler of the respiratory chain. This suggests that D-amino acid dehydrogenase, which cannot use molecular oxygen as a direct electron acceptor, participates in the oxidation of the D-leucine. D-Amino acid dehydrogenase is a bacterial membrane-bound enzyme that catalyzes the oxidation of a variety of D-amino acids to

produce the corresponding imino acids and ammonia, but the oxidation is coupled with an energy requiring process such as the active transport of various solutes.¹⁷⁻¹⁹⁾ The growth of *P. putida* in a medium containing 3 g/liter of DL-alanine or DL-leucine as a sole carbon source resulted in the induction of D-amino dehydrogenase (Table IV). In *E. coli* bearing plasmid pST6961, this activity was found in the membrane fraction of the crude extract and was also induced by DL-alanine or DL-leucine. These results indicate that plasmid pST6961 encodes D-amino acid dehydrogen-

TABLE IV. ENZYME ACTIVITY OF D-AMINO ACID DEHYDROGENASE

Strain	Carbon source for growth ^a		
	Specific activity ^b		
	Glucose	DL-Alanine	DL-Leucine
<i>P. putida</i> IFO 12996	0.5	2.9	2.9
<i>E. coli</i> C600-pST6961	0.7	1.5	1.1
<i>E. coli</i> C600-pBR322	0.5	0.7	ND ^c

^a The medium contained 0.3% of the carbon source indicated, 0.1% (NH₄)₂SO₄, 0.15% K₂HPO₄, 0.05% KH₂PO₄, 0.02% MgSO₄·H₂O, 0.01% yeast extract, 0.05 µg/ml thiamine, 20 µg/ml L-threonine and 10 µg/ml L-leucine (pH 7.2).

^b Specific activity is expressed as nmol of DCPIP reduced per min per mg of protein.

^c ND, not determined.

ase.

The host strain, *E. coli*, cannot grow on 3 g/liter of DL-leucine as a sole carbon source, suggesting that the oxidation activity of D-leucine is too low to fulfill the nutritional requirement for L-leucine through the corresponding α -keto analogue. Massey *et al.*⁽²⁰⁾ reported that a mutant of *P. putida* which had lost the ability to grow on L-leucine, had lowered levels of a few enzymes participating in L-leucine metabolism. Thus, the host strain, *E. coli*, may have a similar property to the *P. putida* mutant. *E. coli* bearing plasmid pST6961 can grow on DL-leucine under the same conditions. These results suggest that the oxidation of D-leucine is performed more effectively by increasing the level of D-amino acid dehydrogenase activity. Further investigation is in progress to obtain information on the gene regulation and the enzyme structure.

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