Characteristics of Transposon Insertion Mutants of Mandelic Acid-utilizing *Pseudomonas putida* Strain A10L

Masayuki SHIMAO,¹ Tomoyuki NAKAMURA,* Akira OKUDA,* Shigeaki HARAYAMA†

Department of Medical Biochemistry, University of Geneva, 1211 Geneva 4, Switzerland
*Department of Biotechnology, Tottori University, Tottori 680, Japan

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A soil isolate, *Pseudomonas putida* strain A10L, that utilizes mandelate via the mandelate pathway was mutagenized by transposon Tn5-Mob insertion and a mutant 168 lacking mandelate racemase (MR) and a mutant 254 lacking benzoylformate decarboxylase (BFDC) were obtained. Expression of (S)-mandelate dehydrogenase (MDH), BFDC, NAD⁺-dependent benzaldehyde dehydrogenase (BDH) and NADP⁺-dependent BDH in the MR-lacking mutant was not affected by the insertion, and it was inducible similarly to the wild type strain. On the other hand, expression of MR and MDH in the BFDC-lacking mutant was low and constitutive, and NAD⁺- and NADP⁺-dependent BDHs were produced at a rather high level under non-induced conditions by the mutant. Genes for MR (mdlA), MDH (mdlB), and BFDC (mdlC) were indicated to be organized in an operon in the order of mdlC-BCA. Optical resolution to obtain (R)-mandelate, a useful synthon for pharmaceuticals, was shown to be performed with the MR-lacking mutant.

Key words: mandelate pathway; *Pseudomonas putida*; transposon mutant; optical resolution; gene organization

Mandelic acid is a naturally occurring chiral aromatic compound which is found in plant tissues.¹¹ So far, many microorganisms metabolizing the compound and its derivatives have been found.¹³⁻¹⁵ The optically active mandellic acid is useful in synthesis of pharmaceuticals, as a chiral synthon for semi-synthesized antibiotics and in optical resolving reagents⁹; various methods utilizing microorganisms and enzymes have been proposed to prepare the optically active compound.⁶⁻⁸⁻¹³

In the course of studies on microbial degradation of aromatic compounds, we isolated *Pseudomonas putida* strain A10L, which utilizes mandelate as a sole source of carbon and energy. Our study showed that the strain metabolizes mandelic acid via what is called the mandelate pathway as shown in Fig. 1, the same pathway reported for *P. putida* ATCC 12633.¹⁴⁻¹⁷ The pathway involves five enzymes, mandelate racemase (MR), (S)-mandelate dehydrogenase (MDH), benzoylformate decarboxylase (BFDC), NAD⁺-dependent benzaldehyde (BDH), and NADP⁺-dependent BDH, and mandelate is converted to benzoate via the pathway. The genes for the enzymes of the pathway, mdlA, B, C, D, E have been cloned from *P. putida* ATCC 12633.¹⁸⁻¹⁹

On the basis of the metabolic flow of the pathway, MR-lacking mutants may metabolize (S)-mandelate specifically and could be used to prepare optically active (R)-mandelate from the racemate. BFDC-lacking mutants may also be useful as a biocatalyst to convert the racemate to benzoylformate, which is used as a substrate for asymmetric reduction to optically active mandelate.⁶,¹¹,¹² However, there has been no report concerning metabolic mutants of mandelate-utilizing *P. putida* as such microbial catalysts.

In this study, we obtained a MR-lacking mutant and a BFDC-lacking mutant from *P. putida* strain A10L by transposon mutagenesis, and cloned genes for some enzymes of the pathway from the transposon insertion mutants. This report describes some properties of the mutants and organization of the genes in the strain A10L.

**Materials and Methods**

*Materials.* (R)-, (S)-, and (RS)-mandelate, and benzoylformate were purchased from Fluka Chemie AG (Buchs, Switzerland). Restriction endonucleases and T4 DNA ligase were from New England Biolabs (Beverly, Mass). Calf intestinal alkaline phosphatase (CIP) was from Boehringer Mannheim GmbH (Mannheim, Germany).

**Fig. 1.** Mandelate Pathway.

[Diagram showing metabolic pathway involving MR, MDH, BFDC, and BDH enzymes, with key metabolites labeled: (R)-Mandelate, (S)-Mandelate, Benzoylformate, Benzaldehyde, and Benzoate.]
Table 1. Bacterial Strains and Plasmids Used in This Study

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Genotype or phenotype</th>
<th>Reference or source</th>
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</thead>
<tbody>
<tr>
<td><strong>Strains</strong></td>
<td></td>
<td></td>
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<tr>
<td>E. coli</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S17-1</td>
<td>thi pro hisB recA (chr.: RP4-2 (Tc:: Mu)</td>
<td>Kms::Tn7)</td>
</tr>
<tr>
<td>LE392</td>
<td>supF44 supF48 hisD14 gagK2 galT2</td>
<td>mcvB1 (pRP5 lac Y1)</td>
</tr>
<tr>
<td><em>P. putida</em></td>
<td></td>
<td></td>
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<tr>
<td>A10L</td>
<td>Soil isolate which assimilates mandelate</td>
<td>This study</td>
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<tr>
<td>A10L80a</td>
<td>Nal</td>
<td>This study</td>
</tr>
<tr>
<td>168</td>
<td>mdA:: Tn5-Mob</td>
<td>This study</td>
</tr>
<tr>
<td>254</td>
<td>mdC:: Tn5-Mob</td>
<td>This study</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
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<tr>
<td>pSUP5011</td>
<td>pBR325 derivative:: Tn5-Mob, Amp'</td>
<td>22</td>
</tr>
<tr>
<td></td>
<td>Cm' Km'</td>
<td></td>
</tr>
<tr>
<td>pACYC184</td>
<td>Cm' Te'</td>
<td>23</td>
</tr>
<tr>
<td>pMS401</td>
<td>pACYC184 derivative carrying</td>
<td>EcoRII fragment (mdA:: Tn5-Mob)</td>
</tr>
<tr>
<td>pMS411</td>
<td>pACYC184 derivative carrying</td>
<td>EcoRII fragment (mdC:: Tn5-Mob)</td>
</tr>
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</table>

Bacterial strains and plasmids. The source of bacterial strains and plasmids and their relevant characteristics are shown in Table 1. Mandelate-utilizing strain A10L was isolated from a soil sample obtained in Tottori, Japan, by selective enrichment culture with a medium containing (RS)-mandelate as a sole source of carbon and energy. A naldixic acid (Nal)-resistant mutant, A10L80a, was obtained spontaneously from strain A10L. Escherichia coli strain S17-1 was used as a donor strain in bacterial mating. pSUP5011 is a mobilizable suicide vector containing Tn5-Mob.

**Growth conditions.** Media used in this study were the basal medium, antibiotic medium 3 (Difco Laboratories, Detroit, Mich.), and LB medium.25 The basal medium contained (per liter) 7 g of Na₂HPO₄, 3 g of KH₂PO₄, 1 g of NH₄Cl, 0.5 g of NaCl, 2.5 ml of metal solution, and 1 ml of vitamin mixture in H₂O (pH 7.0). The metal solution contained (per liter) 5.375 g of MgO, 61.62 g of MgSO₄·7H₂O, 1 g of CaCO₃, 4.75 g of FeSO₄·7H₂O, 0.72 g of ZnSO₄·7H₂O, 0.56 g of MnSO₄·H₂O, 0.125 g of CuSO₄·5H₂O, 0.14 g of CoSO₄·7H₂O, 0.03 g of K₂BO₃·4H₂O, and 290 mmol of HCl in H₂O. The vitamin mixture contained (per liter) 400 mg of Ca pantothenate, 200 mg of nicotinic acid, 200 mg of p-amino benzoic acid, 100 mg of pyridoxine hydrochloride, 400 mg of thiamine hydrochloride, 2 mg of biotin, and 0.5 mg of vitamin B₁₂ in H₂O. For agar media, agar was added at 15 g/liter. Unless otherwise specified, tetracycline (Tc) was used at 10 μg/ml, kanamycin (Km) at 50 μg/ml, naldixic acid (Nal) at 100 μg/ml, chloramphenicol (Cm) at 50 μg/ml, and ampicillin (Amp) at 100 μg/ml.

**Tn5-Mob mutations.** The suicide plasmid pSUP5011 was used to generate Tn5-Mob insertion strain A10L80a. After conjugation with strain S17-1 (pSUP5011) as the donor, cells were plated on antibiotic medium 3 agar containing Km and Nal. Survivors on the plate were replica plated onto basal agar media containing (RS)-mandelate (1 g/liter) and benzoate (1 g/liter) as a sole source of carbon and transconjugants that grow on benzocate but not on (R)-mandelate were selected as metabolic mutants for the mandelate pathway.

Cloning of Tn5-Mob inserted DNA fragments. Total DNA was isolated by the method of Marmur.24 Plasmid DNA isolation, transformation, restriction endonuclease digestion, ligation, and agarose gel electrophoresis were done by the procedures described by Maniatis et al.25 Total DNA from a Tn5-Mob insertion mutant was completely digested with EcoRI, dephosphorylated with CIP, and ligated to EcoRI-digested pACYC184. E. coli strain LE392 was transformed by the ligation mixture, and transformants were selected on antibiotic medium 3 agar plates containing Km. A hybrid plasmid that carries the Tn5-Mob inserted DNA fragment was isolated from a Km-resistant transformant.

**Preparation of cell extract.** Cells were grown in 100 ml of a medium in a flask at 30 °C for 16 h with reciprocal shaking and then harvested by centrifugation (10,000 x g, 10 min) at 4 °C. The cells were washed twice with 0.1 mol potassium phosphate buffer (KPB, pH 7.0) and then suspended in 20 ml of the same buffer. The washed cells were disrupted with an ultrasonic oscillator (19 kHz) for 10 min at 0 to 10 °C. The cell debris was removed by centrifugation (14,000 x g, 20 min) at 4 °C and the supernatant was used as the cell extract.

**Preparation of membrane fraction.** Cells of the mutant strain 168 lacking MR was grown in 500 ml of basal medium containing (RS)-mandelate at 5 g/liter in a flask at 30 °C for 16 h, and cell extract was prepared as described above except that the sonication was for 20 min and the centrifugation was followed by an ultracentrifugation at 100,000 x g for 60 min at 4 °C. The precipitate was suspended in 1 ml of 50 mm KPB (pH 7.0) and used as the membrane fraction.

**Enzyme assay.** All assays for activities of the enzymes were carried out at 30 °C with 1.2 ml reaction mixtures. One unit of the enzyme was defined as the amount which catalyzes conversion of 1 μmol of the substrate indicated below per min under the assay conditions.

MR was assayed by a modification of the method described by Hegeman.26 Commercially available (R)-mandelate contained a trace of (S)-mandelate. The trace of (S)-mandelate in commercial (R)-mandelate was removed with the membrane fraction of the MR-lacking mutant 168 as a MR-free MDH preparation. The reaction mixture contained 100 mm (R)-mandelate, 0.4 mm 2,6-dichlorophenol-indophenol (DCPIP), 1.2 mm potassium cyanide, 66.7 mm KPB (pH 7.5) and the membrane fraction at 1.3 μl MDH activity per ml. The reaction was carried out at 30 °C for 1 h and then the reaction mixture was used as the substrate solution for the MR assay. The assay mixture for MR contained 80 μmol of KPB (pH 7.5), 100 μl of the (R)-mandelate solution, 4 μmol of magnesium chloride, 1.2 μmol of phenazin ethosulfate (PES), 0.12 μmol of DCPIP, 1.2 μmol of potassium cyanide, the membrane fraction of the MR-lacking mutant 168, and an enzyme preparation. The membrane fraction having 2 μl MDH activity was used for each reaction. The rate of reduction of DCPIP was measured from the decrease in absorbance at 600 nm with 19.1 as the millimolar absorption coefficient of the compound.

The assay mixture for MDH contained 80 μmol of KPB (pH 7.5), 10 μmol of (S)-mandelate, 1.2 μmol of PES, 0.12 μmol of DCPIP, 1.2 μmol of potassium cyanide, and an enzyme preparation. The rate of reduction of DCPIP was determined by the same way as the MR assay.

BFDc was assayed by a modification of the method described by Hegeman.26 The assay mixture for BFDc contained 80 μmol of glycine NaOH buffer (pH 9.0), 1.2 mol of Na⁺, 0.12 μmol of benzaldehyde, and an enzyme preparation. The rate of decarboxylation of benzylformate was determined from the decrease in absorbance at 334 nm with 0.081 as the millimolar absorption coefficient of the substrate.

The assay mixture for NAD⁺-dependent BDH contained 80 μmol of glycine NaOH buffer (pH 9.0), 1.2 mol of Na⁺, 0.12 μmol of benzaldehyde, and an enzyme preparation. The assay mixture for NAD⁺-, dependent BDH was the same except for containing 1.2 μmol of NAD⁺ as the substitute for NAD⁺. The rate of reduction of NAD⁺P⁺ was measured from the increase in absorbance at 340 nm with 6.3 as the millimolar absorption coefficient of NAD⁺P⁺.

Catechol 1,2-dioxegonate (Cat1,2-DDO) was assayed by a modification of the method of Nakazawa.27 The assay mixture for Cat1,2-DDO contained 80 μmol of KPB (pH 7.5), 0.4 μmol of catechol, and an enzyme preparation. The rate of formation of cis,cis-muconic acid was determined from the increase in absorbance at 260 nm with 16.0 as the difference between the millimolar absorption coefficients of catechol and cis,cis-muconic acid.

Catechol 2,3-dioxegonate (Cat2,3-DDO) was assayed by a modification of the method of Nozaki.28 The assay mixture for Cat2,3-DDO was the same as Cat1,2-DDO. The rate of formation of γ-hydroxymuconic α-semialdehyde was measured from the increase in absorbance at 375 nm with 44 as the millimolar absorption coefficient of the compound.

**Analyzes.** Growth was measured turbidimetrically at 660 nm. Protein was measured with the Bio-Rad protein assay (Bio-Rad Laboratories, Richmond, Calif.). The amounts of mandelate and its metabolites were determined by high-performance liquid chromatography (HPLC) at room temperature with a Lichrosorb RP-18 column (4 by 250 mm, particle size 7 μm, F. Merck AG, Darmstadt, Germany). The mobile phase was methanol water acetic acid (19:8 : 79.2 : 1.0 and 44.55 : 54.45 : 1 by volume)
and the flow rate was 1.0 ml/min. An appropriate amount of culture supernatant prepared by centrifugation (16,000 x g, 2 min, twice at room temperature) was injected into the system. The enantiomers of mandelate were determined by HPLC at 50°C with a Chiralpak WH column (4.6 by 250 mm, particle size 10 μm, Daicel Chemical Ind., Osaka, Japan). The mobile phase was 0.25 mM CuSO₄ aqueous solution and the flow rate was 1.0 ml/min. The culture supernatant was acidified with 0.1 volume of 2M HCl and extracted with 2 volumes of ethyl ether three times. All the ethyl ether layer was collected, combined and dried in vacuum at room temperature. The residue was dissolved with 1 volume of H₂O and centrifuged (16,000 x g, 10min, twice, at room temperature) and the supernatant was injected into the system. Detection was based upon the absorbance at 254 nm.

**Results**

**Mandelate-utilizing strain A10L**

The taxonomic characteristics of strain A10L were investigated at the National Collection of Industrial and Marine Bacteria Ltd. (Aberdeen, United Kingdom). Strain A10L was a Gram-negative, mobile bacterium that formed short rod cells. The strain was catalase positive, oxidase positive, nitrate reduction negative, indole production negative, arginine dehydrolase positive, urease negative, ascapsulase hydrolysis negative, gelatin hydrolysis negative, and β-galactosidase negative, and did not produce acid from glucose fermentatively. The strain grew at 37°C but not at 41°C. Colonies of the strain on nutrient agar were buff, round, regular, entire, flat, smooth, shiny, and translucent and produced a diffusible yellowish green fluorescent pigment. The strain assimilated glucose, mannose, gluconate, caprate, malate, citrate, phenylacetate, and benzyllamine, but not arabinose, mannnitol, N-acetylglucosamine, maltose, or adonitol. From these results and the 16S rDNA sequence (S. Yamamoto, unpublished result), the strain was identified as *Pseudomonas putida*.

**Enzymes for mandelate metabolism in strain A10L**

Expression of the mandelate pathway enzymes was examined with cells of the wild type strain grown on basal media containing (RS)-mandelate, glutamate, or glutamate plus (RS)-mandelate as a carbon source (Table II). The mandelate pathway enzymes, MR, MDH, BDFC, NAD⁺- and NADP⁺-dependent BDHs were produced at a trace level in the glutamate medium but abundantly in the mandelate medium and glutamate plus mandelate medium. This result showed that all the mandelate pathway enzymes are inducibly produced in the wild type strain in the presence of mandelate. Cat1.2-DO was also induced, but Cat2.3-DO was not detected in cells. This indicated that the mandelate pathway in the strain is followed by the β-ketoacidopate pathway but not a meta-cleavage pathway.

**Growth of transposon insertion mutants on metabolic intermediates of the mandelate pathway**

Mutant strains 168 and 254 were selected from 14,000 transconjugants obtained by the transposon mutagenesis as metabolic mutants for the mandelate pathway. Growth ability of the Tn5-Mob insertion mutants was examined with basal agar medium containing (R)-mandelate, (S)-mandelate, benzoylformate, or benzaldehyde, the intermediates in the mandelate pathway, as sole carbon source. The compounds were used at 1 g liter except benzaldehyde at 0.5 g liter. The wild type strain A10L grew on all the compounds. Strain 168 did not grow on the (R)-mandelate but grew on the other compounds. Strain 254 grew on benzaldehyde and benzoate but not on (R)-mandelate, (S)-mandelate, or benzoylformate.

**Enzyme activities of transposon insertion mutants**

Expression of the mandelate pathway enzymes in the mutants was examined to clarify their defects in enzyme activities (Table II). MR was not expressed by strain 168 but the strain produced the other mandelate pathway enzymes inducibly as did the wild type strain. Strain 168 is thus a MR-lacking mutant in which other enzymes of the mandelate pathway are not affected by the Tn5-Mob insertion.

The mutant 254 did not express BDFC but produced a trace of MR and MDH constitutively (Table II). Strain 254 is a BDFC-lacking mutant in which the expression of MR and MDH is also changed to low and constitutive by the Tn5-Mob insertion. As well as these characteristics, the mutant showed a difference from the strains A10L and 168 in expression of NAD⁺- and NADP⁺-dependent BDHs. These enzyme activities were expressed rather abundantly even under the non-inducible conditions, and were inducibly increased in the presence of mandelate.

**Tn5-Mob insertion in the mutants**

To physically locate the Tn5-Mob insertion in mutants 168 and 254, we cloned Tn5-Mob inserted DNA fragments from both mutants using pACYC184 as a vector: pMS401 carries a Tn5-Mob-inserted EcoRI fragment from strain.
168 while pMS411 carries a Tn5-Mob-inserted EcoRI fragment from strain 254. Analysis by cleavage with restriction enzymes showed that the cloned EcoRI fragments on the plasmids are the same except for their insertion sites of the Tn5-Mob (Fig. 2). The size of the original fragment without Tn5-Mob insertion was 12.2 kbp.

Expression of the mandelate pathway enzymes by the plasmids was examined in E. coli strain LE392 (Table III). MDH and BFDC but not MR were expressed from pMS401. On the other hand, trace levels of MR and MDH, and no BFDC, were expressed from pMS411. These results showed that pMS401 has the insertion of Tn5-Mob within the MR gene, mldA, and that pMS411 has the insertion within the BFDC gene, mldC.

NADP⁺-dependent BDH was not expressed from the plasmids. It was also concluded that NADP⁺-dependent BDH was not expressed from the plasmids, as low NADP⁺-dependent BDH activities detected in cells carrying the plasmids were similar to the activity levels in cells carrying pACYC184 (Table III) and in plasmid-free cells of E. coli strain LE392 (data not shown).

### Table III. Enzyme Activities of E. coli Strain LE392 Carrying pMS401 and 411

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>MR</th>
<th>MDH</th>
<th>BFDC</th>
<th>NAD⁺-BH</th>
<th>NADP⁺-BDH</th>
</tr>
</thead>
<tbody>
<tr>
<td>pMS401</td>
<td>ND</td>
<td>3.7</td>
<td>1.3</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>pMS411</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>pACYC184</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

a Strain LE392 carrying a plasmid was cultivated at 30°C for 16 h in 100 ml of LB medium containing (RS)-mandelate at 10 μM, Tc at 10 μg/ml, and Km (at 25 μg/ml for pMS401 and 411, and at 0 μg/ml for pACYC184).

b ND: Activity was not detected.

### Optical resolution of (RS)-mandelate by the MR-lacking mutant

Expression of the other enzymes of the mandelate pathway in the MR-lacking mutant 168 was not affected by the insertion. The characteristics of the strain may make it suitable as a biocatalyst for optical resolution of a racemic mixture of mandelate, because only (S)-mandelate in the racemate is expected to be utilized as the carbon and energy source. To examine this possibility, we cultivated the strain on a basal medium containing (RS)-mandelate as a sole carbon source (Fig. 3). In the culture, (S)-mandelate was selectively removed, and (R)-mandelate remained at the initial concentration level. (S)-Mandelate was not detected in the culture at 9 h by the HPLC analysis and the enantiomeric excess of the remaining (R)-mandelate in the culture was estimated to be at least more than 98%. Metabolic intermediates of the mandelate pathway were in trace levels in the culture: at 24 h, only benzoyleformate (0.14 mg/liter), and benzyaldehyde (0.34 mg/liter) were detected, while benzoate and any other metabolites were not detected.

### Discussion

In this study, the mandelate-utilizing P. putida strain A10L was isolated from a soil sample in Tottori, Japan and transposon insertion mutants for the mandelate pathway, the MR-lacking mutant strain 168 and the BFDC-lacking mutant strain 254, were obtained for the first time. Such transposon insertion mutants for the mandelate pathway have never been reported before.

In the MR-lacking mutant 168, the mandelate pathway enzymes except MR were expressed inducibly at a similar level to the wild type strain and it was confirmed that (S)-mandelate was selectively assimilated from the racemic mixture by the strain.

On the other hand, in the BFDC-lacking mutant 254, MR and MDH were repressed at a low constitutive level. The transposon insertion causes what is called a polar effect, that is, transposon insertion in a gene inhibits transcription of the downstream genes from a natural promoter in an operon. The repressed MR and MDH activities in strain 254 can be concluded to result from the polar effect of the transposon, as described above.

Restriction map analysis and expression study with plasmids pMS401 and 411 showed that genes for MR (mldA), MDH (mldB), and BFDC (mldC) were located in the 12.2-kbp EcoRI fragment. The locus of mldA is in the region around the Tn5-Mob insertion site on pMS401 and the
locus of mldC is in the region around the Tn5-Mob insertion site on pMS411. The expression of mldA and mldB was affected in the case of the pMS411, which has a transposon insertion in mldC and the expression of mldB and mldC was not affected in the case of pMS401 which has a transposon insertion in mldA. These observations indicated that the expression of the mldA and B from pMS411 and in the mutant 254 is affected by a polar effect of the transposon and that mldA, B, and C are organized in an operon in the order of mldICBA. The gene order is the same as that reported for the P. putida ATCC 12633.

NAD⁺- and NADP⁺-dependent BDHs were not expressed from pMS401 and pMS411, indicating that the genes for benzaldehyde dehydrogenases, mldID and E may not be present on the plasmid. The genes may exist outside the operon of mldICBA, as reported for the P. putida strain. On the other hand, interestingly, NAD⁺- and NADP⁺-dependent BDHs in the mutant 254 were found to be expressed in a different manner than from the wild type strain. The activities of NAD⁺- and NADP⁺-dependent BDHs were at a rather high level under the non-induced conditions and further increased in the presence of mandelate. This phenomenon is clearly distinguishable from the polar effect, although the unusual expression of the BDHs is also thought to be caused by the transposon insertion. Its mechanism, however, remains to be clarified in future work.

The mutant 168 was confirmed to perform efficient optical resolution of mandelate in a short time. Although various studies concerning biological preparation of optically active mandelate have been reported, as to the biological optical resolution of mandelate, only one study with Alcaligenes bronchisepticus has been reported. This strain had (S)-mandelate specific mandelate dehydrogenase but did not assimilate the compound. Our work is then the first example of optical resolution by a mutant of mandelate-utilizing P. putida, and such efficient optical resolution of mandelate by microorganisms has not been reported before. In addition to its high efficiency, optical resolution with the mutant 168 has other advantageous features: the optical resolution by the strain based on selective utilization of (S)-mandelate for growth, and therefore could be carried out in a rather simple process. With microorganisms, like the A. bronchisepticus strain, that do not assimilate (S)-mandelate, optical resolution must be done either in a resting cell system, which needs a cultivation process with a growth substrate and harvesting process to obtain cells before reaction or in a growing cell system, which must contain an additional carbon source to support growth during the optical resolution. Optical resolution with strain 168 can be performed without such processes and additional carbon sources. Furthermore, metabolism of (S)-mandelate in the mutant does not generate any dead end metabolite, indicating that the remaining (R)-mandelate in a culture of the strain may be isolated by a rather simple procedure. Optical resolution with A. bronchisepticus needed 4 days of cultivation with a growth substrate before reaction and rather long 3 days reaction time, and accumulated benzoylformate and benzoate in the reaction.

Furthermore, the deficiency in MR activity in strain 168 was stably maintained during successive transfers on an agar medium for more than one year. Stability of the mutant may be suitable for use as a biocatalyst for the optical resolution of mandelate. On the other hand, as shown in the mutant 254, transposon insertion mutagenesis is unsuitable to obtain BFDC-deficient mutants that can be used as biocatalysts for conversion of mandelate to benzoylformate, since the polar effect causes significant repression of MR and MDH.

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References