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Isolation and characterization of toluene-sensitive mutants from *Pseudomonas putida* IH-2000

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Abstract

Two toluene-sensitive mutants were generated from *Pseudomonas putida* IH-2000, the first known toluene-tolerant isolate, by Tn*5* transposon mutagenesis. These mutants were unable to grow in the presence of toluene (log P_{ow} 2.8) but they could grow in medium overlaid with organic solvents having a log P_{ow} value higher than that of toluene such as *p*-xylene (log P_{ow} 3.1), cyclohexane (log P_{ow} 3.4) and *n*-hexane (log P_{ow} 3.9). The Tn*5* transposable element knocked out a *cyoB*-like gene in one mutant and a *cyoC*-like gene in the other mutant. Seven open reading frames were found in a 5.5-kb region containing the *cyoB*- and *cyoC*-like genes of strain IH-2000. ORFs 3–7 showed significant identity to the *cyoABCDE* gene products of *Escherichia coli*, but ORFs 1 and 2 showed no significant homology to any protein reported so far. The growth patterns of the Tn*5* mutants with the inactivated *cyo*-like gene were similar to that of the wild-type strain in the absence of organic solvents, although the doubling times were slightly longer than that of the wild-type strain. Our findings indicate that *cyo* is an important gene for toluene tolerance, although its role is still unclear.

Keywords: *Pseudomonas putida* IH-2000; Tn*5* transposon mutagenesis; *cyoABCDE*; Cytochrome *o*; Toluene tolerance

1. Introduction

Pseudomonas putida strain IH-2000 was the first isolate found to be capable of growth in a culture medium containing more than 50% (v/v) toluene [1]. Inoue and Horikoshi introduced the concept of using the log P_{ow} value, which is the common logarithm of the partition coefficient (*P*) of a given solvent between equimolar amounts of *n*-octanol and water [2], as an index of the toxicity of solvents. Toluene (log P_{ow} 2.8) is one of the most toxic organic solvents as it can readily permeate into the phospholipid membranes of cells and disrupt essential membrane functions [3].

Although organic solvents with low log P_{ow} values are highly toxic to most living organisms, independent laboratories have isolated *P. putida* strains tolerant to various aromatic hydrocarbons, such as toluene, styrene and *p*-xylene [4,5] since the first report by Inoue and Horikoshi [1]. Tolerance to organic solvents in these *P. putida* strains is achieved by a

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series of biochemical mechanisms that actively remove the organic solvent from cell membranes and by physical barriers that help the cell to become impermeable to the solvent [6–11]. One of mechanisms for changing membrane fluidity is the isomerization of *cis* to *trans* configuration in unsaturated fatty acids [6,7,9]. This conversion is catalyzed by an energy-independent isomerase [12]. A transposon mutant of the solvent-tolerant *P. putida* DOT-T1 is both solvent-sensitive and unable to perform this isomerization [9]. However, the *cis*/*trans*-isomerization is unlikely to be the only necessary mechanism of adaptation to organic solvents because strains are known that can perform the isomerization and that are still solvent sensitive [7,9]. This indicates that changes in the *cis*/*trans* ratio may occur as part of a general stress response in microbes.

Degradation or detoxification of organic solvents partly contributes to organic solvent tolerance in several microorganisms [6,7,9]. However, *P. putida* IH-2000, isolated in our laboratory, is not able to metabolize toluene. An alternative means of removal of organic solvents from the cells is through active efflux of organic solvents. Such efflux systems are well known for lipophilic cytotoxic agents such as antibiotics [13,14]. Many energy-dependent export systems play an important role in drug resistance and toxic organic solvent tolerance as they are able to pump out a wide range of compounds. Recent studies have suggested that toluene-tolerant *P. putida* strains have an efficient active efflux system which expels toluene [8,10,11].

We were interested in elucidating the mechanisms of bacterial adaptation to toxic solvents and on identifying which genes are involved in toluene tolerance in *P. putida* IH-2000. In this study, we generated toluene-sensitive mutants by Tn*5* transposon mutagenesis. Here we report the analysis of the knocked out genes in two Tn*5* mutants and discuss the relationship between the gene products and toluene tolerance.

2. Materials and methods

2.1. Bacterial strains and growth conditions

P. putida IH-2000 (*Tol*$^+$, *Km*$^-$, *Nx*$^+$) and its derivatives were grown aerobically at 30°C in LB liquid medium or on LB agar plates containing 10 mM $MgSO_4 \cdot 7H_2O$. For Tn*5* mutants, 50 mg $^{-1}$ nalidixic acid (Nx) and 50 mg $^{-1}$ kanamycin (Km) were added to the medium. *E. coli* S17-1 (pSUP2021) [15] was used as a Tn*5*-donor strain to generate transconjugants. *E. coli* VCS257 was used as a host strain to prepare Charomid 9-36 (Nippon Gene, Japan) derivatives.

2.2. Gene manipulations

Isolation of chromosomal DNA from *P. putida* IH-2000 and its derivatives was as described previously [16]. The pattern of digestion of chromosomal DNA with each restriction enzyme was analyzed by 0.9% agarose gel electrophoresis and the digested fragments were vacuum-blotted onto a Hybond N$^+$ nylon membrane (Amersham, USA). A digoxigenin-labeled Km resistance gene (3.4 kb) was used as a DNA probe to detect fragments containing the Tn*5* element. Southern hybridization [17] was performed using a DIG labeling and detection kit (Boehringer, Mannheim, Germany).

2.3. Transposon mutagenesis

Transposon Tn*5* was used to prepare toluene-sensitive (*Tol*$^-$) mutants. *E. coli* S17-1 cells grown at 37°C on an LB agar plate were mixed with *P. putida* IH-2000 cells grown at 30°C in LB liquid medium. Four hundred microliters of the cell mixture was spread onto a sterilized cellulose filter on an LB agar plate. After a 6-h mating period at 30°C, the cells were resuspended in LB liquid medium and spread onto an LB agar plate supplemented with nalidixic acid (50 μg ml^{-1}) and kanamycin (50 μg ml^{-1}) and the colonies that had formed after a 2-day incubation period at 30°C were replicated onto an LB agar plate. To detect toluene-sensitive mutants, each replica plate was overlaid with pure toluene and incubated for 2 days at 30°C.

2.4. DNA sequencing and ORF analysis

Sequencing was performed using a DNA sequencer ABI PRISM 373 and a Dye Terminator Cycle Sequencing Kit (Perkin Elmer, CT, USA).

The sequence of the 5.5-kb DNA fragment reported in this paper has been deposited in DDBJ with the accession number AB016787. The sequences were analyzed for the locations of putative ORFs using the GeneWorks program (version 2.5. 1N) from IntelliGenetics (CA, USA). The deduced amino acid sequences of the identified ORFs were compared with sequences reported previously in a search of the non-redundant protein databank using the FASTA and BLAST network service (GenomeNet WWW server, http://www.genome.ad.jp).

3. Results and discussion

3.1. *Tn5 transposon mutagenesis and isolation of Tn5 mutants*

Two toluene-sensitive mutants, nos. 30 and 31, were selected from among approximately 5000 transconjugants, which were unable to grow in the presence of toluene even after repeated subculture. Southern hybridization analysis revealed that Tn*5* was inserted at only one site in the chromosomal DNA of each mutant. Although neither of these mutants could grow in the presence of toluene, they flourished on LB agar plates overlaid with *p*-xylene (log P_{ow} 3.1), cyclohexane (log P_{ow} 3.4) or *n*-hexane (log P_{ow} 3.9), all with log P_{ow} values higher than that of toluene (log P_{ow} 2.8) (Table 1). These mutants showed similar growth behavior in liquid cultures in the presence of each of these organic solvents at 10% (v/v) final concentration. These results suggest that each of these knocked out genes plays an important role in toluene-tolerance in *P. putida* IH-2000.

3.2. *Cloning and partial sequencing of EcoRI and KpnI fragments*

A 3.0 kb *Kpn*I fragment derived from mutant 30 and a 1.9-kb *Eco*RI fragment derived from mutant 31 were cloned in Charomid 9-36 (data not shown). These fragments, each containing the Tn*5* transposon, were partially sequenced (ca. 1 kb) around the Tn*5* insertion site using charomid primers (5′-AAAATAGGCGTATCACGAGG-3′ and 5′-TGACAGCTTGTATGTTTCTG-3′), and a Tn*5* primer (5′-GGAGGTCACATGGAAGTCAGAT-3′), the sequence of which begins at a point 50 bp within the IS*50* sequence. The partially sequenced fragments showed significant homology to *E. coli* genes encoding cytochrome *o*, which is one of two terminal ubiquinol oxidases in the aerobic respiratory chain, belonging to the heme-copper terminal oxidase superfamily [18,19]. Tn*5* insertion was accompanied by a 9-bp duplication which commonly occurs when Tn*5* is transposed into various locations in bacterial or bacteriophage genomes and at many sites in a single gene [20]. Tn*5* was inserted into ORF4 at 4055–4063 bp in mutant 31 and into ORF5 at 2844–2852 bp in mutant 30 as shown in Fig. 1.

3.3. *Sequencing and analysis of ORFs in the 5.5 kb fragment*

The 5.5 kb fragment containing the *cyo* gene cluster, in the case of both mutants 30 and 31, was

Table 1
Growth properties of toluene-sensitive Tn*5* mutants 30 and 31 under various hydrophobic organic solvents

Solvents	Low P_{ow}	Strains		
		IH-2000	No. 30	No. 31
Toluene	2.8	+	−	−
p-Xylene	3.1	+	+	+
Cyclohexane	3.4	+	+	+
n-Hexane	3.9	+	+	+

Growth patterns of bacteria were examined in LB liquid medium and on LB agar medium each containing 10 mM $MgSO_4$. In the case of liquid medium, each of the cultures was incubated with shaking at 30°C for 10 h in the presence of 10% (v/v) organic solvent. The cells were streaked onto agar plates and the plates were overlaid with the organic solvent to be tested. The plates overlaid with organic solvent were incubated at 30°C for 24 h and then colony formation was assessed.

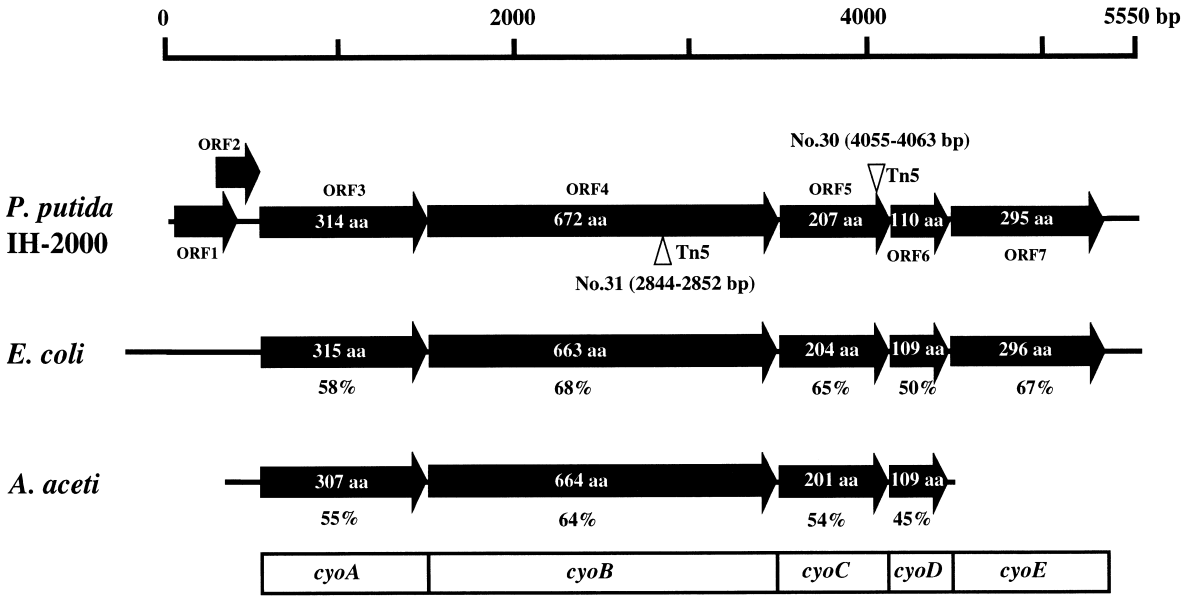

Fig. 1. Cytochrome *o* gene (*cyo*) homolog in *P. putida* IH-2000. Arrows show possible open reading frames (ORFs) and a solid line indicates the sequenced region. A small arrowhead indicates the position of Tn*5* transposon insertion. The amino acid sequence of the Cyo homologue in *P. putida* IH-2000 was compared with other quinol oxidases from Gram-negative bacteria, *E. coli* cytochrome *o* and *A. aceti* cytochrome a_1. The % identity between each ORF and the other two quinol oxidases, from *E. coli* and *A. aceti*, is shown below each arrow.

further sequenced by the primer walking method, and the whole sequence of this fragment was determined. Seven open reading frames (ORFs) were identified in the 5.5-kb fragment containing the *cyoB*- and *C*-like genes. Although five ORFs, ORFs 3–7, were similar to *E. coli cyoABCDE* gene products which constitute cytochrome *o*, showing 58, 68, 65, 50 and 67% identity, respectively, the another two (ORFs 1 and 2) showed no significant homology to any other protein reported so far. In addition, ORFs 3–6 showed extensive identity to *Acetobacter aceti cyaABCD* gene products which have been identified as components of a terminal oxidase for ethanol oxidation (Fig. 1) [21].

Cytochrome *o* has been purified and subunits, I, II, III and IV of the oxidase complex have been named CyoB, CyoA, CyoC and CyoD, respectively [22,23]. Subunit I binds all the redox metal centers, low-spin heme *b*, high-spin heme *o*, and Cu_B, and the last two centers form the heme-copper binuclear center where molecular oxygen is reduced to water. Also, it has been suggested that subunits II, III, and IV of the oxidase complex are required for assembly of the metal centers in subunit I. The deduced amino acid sequences of ORFs 4 and 5, knocked out in mutants 31 and 30, respectively, were aligned and compared with CyoB and CyoC in *E. coli* and CyaB and CyaC in *A. aceti*, respectively (Fig. 2). The amino acid sequence of ORF4 showed overall homology with the two terminal oxidases (CyoB and CyaB) of the Gram-negative bacteria, *E. coli* and *A. aceti*, although the C-terminal region showed variation. The amino acid sequence of ORF5 also showed overall homology with *E. coli* and *A. aceti* proteins although the N-terminal and central regions are somewhat more variant (Fig. 2).

3.4. Growth patterns of Tn5 mutants with a defective *cyo* gene

The growth patterns of the cytochrome *o* deficient mutants (30 and 31) were investigated to determine whether proliferation of these mutants is affected under normal laboratory growth conditions. The growth patterns (not shown) of the mutants were quite similar to that of the wild-type strain in the

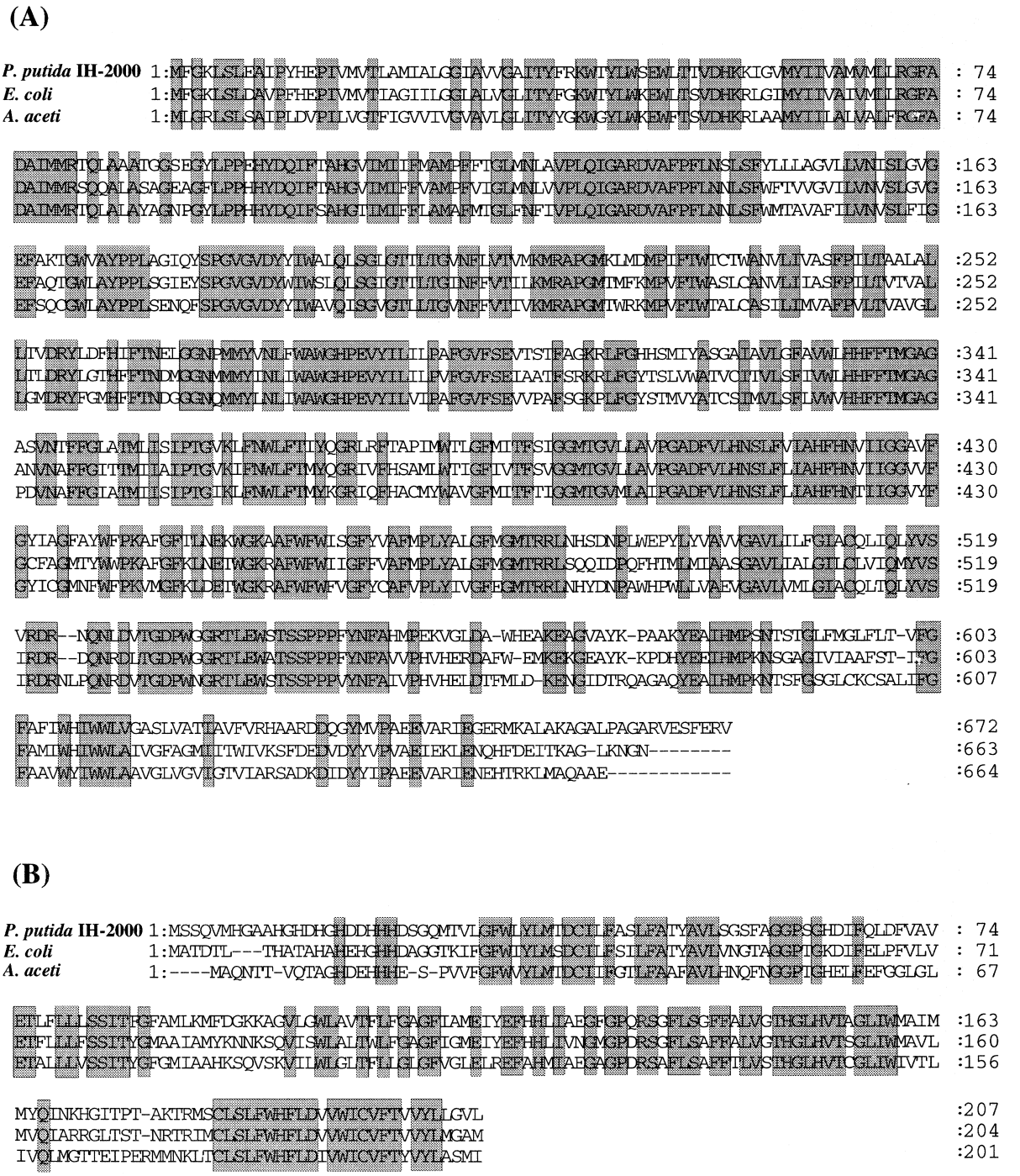

Fig. 2. Alignment of amino acid sequences of IH-2000 CyoBC homologs, *E. coli* CyoBC and *A. aceti* CyaBC. (A) CyoB homologs. (B) CyoC homologs. Common amino acid residues among the three proteins are boxed.

absence of organic solvents, although a slight difference in doubling time was evident among the three strains as follows: *P. putida* IH-2000, 48.1 min; mutant 30, 59.5 min; mutant mutant 31, 52.9 min. Our findings seem to be consistent with the report by Au et al. indicating that there was no difference in oxygen utilization in *E. coli* membrane preparations comparing *cyo*$^-$ and *cyo*$^+$ strains [24]. It was indicated in this report that the cytochrome *o* branch of the respiratory chain is dispensable under normal laboratory growth conditions [24]. Actually, it is known that cytochrome *d* (*cyd*) is expressed mainly in the stationary phase of growth in Gram-negative bacteria, such as *E. coli* and *P. putida* [25,26] and *cyd* is used instead of *cyo* in *cyo*-deficient mutants of *E. coli*. Tn*5* mutants may also use *cyd*, as in *E. coli*, although *cyd* has not been identified yet in *P. putida* IH-2000.

Studies by Isken and de Bont [8], and Ramos et al. [11] have suggested that toluene-tolerant *P. putida* S-12 and DOT-TIE have efficient active efflux systems which expels toluene and that this system is energy-dependent. Their recent research findings identified efflux pumps as the previously unidentified factor which makes a major contribution to toluene tolerance in *P. putida* S-12 and DOT-TIE. On the other hand, Kieboom et al. reported that the gene responsible for the export of toluene in *P. putida* S12 shows homology to other well-known export systems responsible for the active efflux of antibiotics from the cell [10]. The homology between the *srpABC* genes and genes encoding proton-dependent efflux pumps, such as those in the *acrAB* operon in *E. coli*, suggests that solvent efflux is dependent on the proton motive force [27]. Cytochrome *o* in *E. coli* functions as a redox-driven proton pump and the respiratory chain is an important system serving to provide energy for bacterial growth [28]. We speculate that cytochrome *o* in the respiratory chain might affect an intrinsic proton-dependent efflux system which contributes to toluene tolerance in *P. putida* IH-2000, although no substantial change in antibiotic resistance was evident comparing the wild-type strain IH-2000 and its *cyo*-deficient mutants.

We intend to focus on the efflux system in *P. putida* IH-2000 and are investigating the relationship between the product of the *cyo*-like gene and toluene tolerance. We are also interested in evaluating whether ORFs of unknown function (ORFs 1 and 2) located upstream of the *cyoA*-like gene are involved in the intrinsic function of cytochrome *o* in toluene-tolerant *P. putida* IH-2000.

Acknowledgments

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