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Isolation and transposon mutagenesis of a *Pseudomonas putida* KT2442 toluene-resistant variant: involvement of an efflux system in solvent resistance

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Abstract A toluene-resistant variant of *Pseudomonas putida* KT2442, strain TOL, was isolated after liquid cultivation under xylene followed by toluene for 1 month in each condition. Almost all the populations of the variant strain formed small but readily visible colonies under toluene within 24 h at 30°C. The toluene-resistant strain also showed an increase in resistance to some unrelated antibiotics. Several toluene-sensitive *Tn5* mutants have been isolated from the toluene-resistant strain and showed various levels of sensitivity. Most of these mutations did not cause significant changes in antibiotic resistance; however, one of the mutants (TOL-4) was highly susceptible to both organic solvents and various antibiotics, especially β-lactams. Sequencing analysis revealed that the mutation in TOL-4 had been introduced into a gene that may encode a transporter protein of an efflux system. This efflux system is very similar to one of the multidrug efflux systems of *Pseudomonas aeruginosa*. These observations indicate that a multidrug efflux system plays a major role in the organic solvent resistance of *P. putida* TOL. However, several other genes may also be involved.

Key words *Pseudomonas putida* · Solvent resistance · Organic solvent · Efflux pump · *Tn5* transposon mutagenesis

Introduction

After Inoue and Horikoshi (1989) reported that Pseudomonas putida strain IH-2000 could grow under toluene and introduced log P_{ow} values [the common logarithm of a partition coefficient (P) of a given solvent between equimolar *n*-octanol and water] (Harnisch et al. 1983) as a toxicity index of solvents, several organic solvent-tolerant microorganisms have been isolated and prepared (Aono et al. 1992; Cruden et al. 1992; Komatsu et al. 1994; Moriya and Horikoshi 1993; Ramos et al. 1995; Shima et al. 1991; Weber et al. 1993). Organic solvents with lower log P_{ow} values are more toxic to cells than those with relatively higher log P_{ow} values (Inoue and Horikoshi 1989). Aromatic chemicals such as *p*-xylene (log P_{ow} = 3.1), toluene (log P_{ow} = 2.8), and benzene (log P_{ow} = 2.1) are highly toxic to microorganisms, even at low concentrations. Solvents are preferentially partitioned in the cell membranes, causing expansion of the membrane, an increase in fluidity, and impairment of its functions (Heipieper and de Bont 1994; Sikkema et al. 1995). This is probably the reason why studies on physicochemical changes in the cells, which respond to organic solvent stresses, have mainly focused on the membrane components (Heipieper et al. 1992; Pinkart et al. 1996; Weber et al. 1994).

The *cis* configuration in unsaturated fatty acids causes an increase in membrane fluidity, and the *trans* configuration has a steric structure similar to that of the saturated acids (MacDonald et al. 1985). Increases in the degree of saturation and the *cis* to *trans* isomerization of unsaturated fatty acids may compensate for the effect of organic solvents. Ramos and colleagues (1997) recently reported a *Tn5* mutagenesis of *P. putida* DOT-T1 and summarized mechanisms that increase its tolerance to toluene: change in membrane lipid fatty acid composition was also included. They also proposed two additional cellular mechanisms that decrease the concentration of toluene. One is a solvent exclusion system, which followed the report showing the involvement of an active efflux of toluene in a solvent-resistant *P. putida* S12 (Isken and de Bont 1996). The other system involves metabolic removal of solvent via oxidation, although the mechanism was not fully characterized. Three genes have been reported to contribute to organic solvent resistance in *Escherichia coli* (Aono et al. 1994; Ferrante et al. 1995; Nakajima et al. 1995). Very recently, Levy and coworkers reported that the *acrAB* locus which encodes the acriflavine resistance proteins AcrAB is linked to intrinsic organic solvent tolerance and also to solvent tolerance me-

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diated by the transcriptional activators MarA, SoxS, or RobA in *E. coli* (White et al. 1997). However, these genes or gene products have not been employed to confer solvent tolerance on microorganisms other than *E. coli*.

Here we report the isolation and subsequent *Tn5* transposon mutagenesis of a toluene-resistant variant of *P. putida* KT2442, strain TOL. Six toluene-sensitive *Tn5* mutants isolated from the toluene-resistant strain have been classified into three distinct groups, suggesting several genes are involved in solvent resistance in *P. putida* TOL.

Materials and methods

Bacterial strains and growth conditions

Pseudomonas putida KT2442 (Franklin et al. 1981) and its derivatives were grown aerobically at 30°C in PB-I medium [0.5% each of Polypepton and Polypepton-S (Nihon Pharmaceutical, Tokyo, Japan), yeast extract (Difco, Detroit, MI, USA), and NaCl, 0.1% glucose in 10 mM $MgSO_4$] supplemented with 100 mg/l rifampicin and 50 mg/l kanamycin for the *Tn5* mutants. Doubling time was determined by measuring the increase of OD_{660} while grown in logarithmic phase. Solid media contained 1.5% agar and for solvent-resistance studies were prepared in 90-mm glass petri dishes. Organic solvents (10–15 ml) were overlaid 2 h after plating. *Escherichia coli* strain DH5α (Gibco BRL, Rockville, MD, USA) was grown at 37°C in Terrific broth (Sambrook et al. 1989) supplemented with 100 mg/l ampicillin to prepare pUC18 or pUC19 derivatives.

Tn5 transposon mutagenesis

Transposon *Tn5* was used to obtain toluene-sensitive (Tol^-) mutants because *P. putida* TOL was sensitive to kanamycin at a concentration of 50 mg/l. *E. coli* S17-1(pSUP2021) (Simon et al. 1983) cells grown at 37°C in LB [1% Tryptone (Difco), 0.5% yeast extract, 0.5% NaCl] were used to generate transconjugants. About 10^9 cells of both *P. putida* TOL and *E. coli* S17-1(pSUP2021) were mixed in 200 µl PB-I and incubated on a nylon membrane at 30°C for 6 h. Cells were collected and then spread on PB-I plates supplemented with rifampicin (200 mg/l) and kanamycin (50 mg/l), and colonies thus formed were replicated after 2 days. As described in the Results section, Tol^- mutants showed varied sensitivity toward toluene. In this study, the Tol^- phenotype was defined as a strain in which not more than 10% of the population could form colonies on PB-I plates under toluene after 40 h incubation at 30°C.

Determination of minimum inhibitory concentration of antibiotics

Minimum inhibitory concentrations (MICs) were determined by twofold serial broth dilution in LB medium containing 0.1% glucose and 10 mM $MgCl_2$ in microtiter plates. The inoculum was 1×10^6 cells/ml, and results were read after 40 h incubation at 30°C. Cell growth was measured at OD_{660}; an OD_{660} less than 0.2 was considered to be negative.

DNA sequencing and nucleotide sequence accession number

General DNA manipulations were performed as described in published protocols (Ausbel et al. 1990; Sambrook et al. 1989). An alkaline lysis method was used to prepare double-stranded plasmids followed by polyethylene glycol-NaCl precipitation. DNA sequencing reactions were carried out by using a PRIZM DyeDeoxy Terminator Cycle Sequencing Kit (Perkin-Elmer, Norwalk, CT, USA) with the aid of a thermal cycler. The DNA sequence was analyzed using a 373A DNA sequencing system (Perkin-Elmer). The nucleotide sequence of the 7192-bp *Not*I-*Hin*dIII fragment reported in this article will appear in the DDBJ, EMBL, and GenBank nucleotide sequence databases with the accession number AB008909.

Results

Adaptation of *P. putida* KT2442 to toluene

Pseudomonas putida KT2442 was not able to form colonies in toluene under any conditions so far tested, and cells were killed by the organic solvent within 1 min. However, a small portion of the population formed colonies on LB plates under *p*-xylene, which has a higher log P_{ow} value than toluene. About 10^{-8} of the population were *p*-xylene tolerant when exposed to the organic solvent immediately after plating on LB, and a xylene-tolerant variant was used as a starter strain. The variant strain was initially cultured in PB-I containing 5% (v/v) xylene, because it has been reported that precultivation in less toxic solvents supports cell adaptation to more toxic solvents (Komatsu et al. 1994). It was then constantly subcultured into PB-I medium every 3–4 days with a weekly 5% stepwise increase (5%–20%) of *p*-xylene. After 1 month, the strain was subcultured under toluene (5%–20%) for an additional month. A toluene-resistant variant of *P. putida* KT2442, strain TOL, which formed small colonies under toluene, was isolated from the final culture. The strain did not lose its resistance after several subcultures, and grew slightly slower than the parental strain without toluene (Table 1). We have noticed that a short time (1–2 h) of preincubation between cell plating and solvent pouring stabilized the colony formation in resistant strains under the most toxic solvent. If the strain TOL cells were exposed to toluene immediately after plating, about 90% of the population were not able to form colonies. However, such preincubation was not necessary for *p*-xylene.

Isolation, solvent sensitivity, and MIC of antibiotics of toluene-sensitive mutants

Tn5 transposon mutations were introduced into *P. putida* TOL by conjugation with *E. coli* S17-1(pSUP2021), and six

Table 1. Organic solvent and antibiotic resistance of *Pseudomonas putida* strains

Strain	Resistant to:[b]	Colony formation under toluene	Td[c] (min)	MIC (mg/l)[a]				
				AMP	PENG	ERY	NOV	TC
KT2442	Xyl	$<10^{-7}$	40	256	512	2048	256	64
TOL	Tol	>0.8	55	>1024	>4096	>2048	>1024	128
TOL-1	Xyl	$1–3 \times 10^{-2}$	55	>1024	>4096	2048	1024	64
TOL-2	Xyl	$<10^{-7}$	60	>1024	>4096	2048	>1024	128
TOL-3	Xyl	$2–5 \times 10^{-2}$	55	>1024	>4096	2048	1024	128
TOL-4	Hex	$<10^{-7}$	55	32	64	512	64	16
TOL-5	Xyl	$0.5–1 \times 10^{-4}$	55	>1024	>4096	>2048	1024	128
TOL-6	Xyl	$<10^{-7}$	70	>1024	>4096	>2048	>1024	128

[a] Abbreviations: MIC, minimum inhibitory concentration; AMP, ampicillin; PENG, penicillin G; ERY, erythromycin; NOV, novobiocin; TC, tetracycline. All strains were resistant to more than 32 mg/l of acryflavin, 128 mg/l of chloramphenicol, and 2048 mg/l of vancomycin

[b] Abbreviations: Xyl, xylene (log P_{ow} = 3.1); Tol, toluene (log P_{ow} = 2.8); Hex, hexane (log P_{ow} = 3.9)

[c] Td, doubling time

Table 2. *Pseudomonas putida* TOL and other solvent efflux system proteins

Organism	Linker Size (identity)[a]	Transporter	Outer membrane channel
Pseudomonas putida	MepA 384 (100%)	MepB 1050 (100%)	MepC 484 (100%)
Pseudomonas aeruginosa	MexA 383 (66%/383)	MexB 1046 (78%/1043)	OprM 477 (70%/466)
	MexC 387 (42%/381)	MexD 1043 (49%/1038)	OprJ 479 (42%/470)
	MexE 414 (31%/360)	MexF 1062 (42%/1048)	OprN 472 (29%/459)
Escherichia coli	AcrA 397 (53%/394)	AcrB 1049 (66%/1039)	TolC 495 (23%/422) [YlcB 457 (42%/465)]
Neisseria gonorrhoeae	MtrC 412 (41%/375)	MtrD 1067 (47%/1059)	MtrE 467 (40%/464)

[a] Size, the number of amino acid residues of a protein. Identity (percent identity/aligned residues including gaps) was calculated by using amino acid sequences of MepA, MepB, and MepC (DDBJ accession no. AB008909), and those deduced from nucleotide sequence data obtained from GenBank: MexA and MexB (L11616); OprM (L23839); MexC, MexD, and OprJ (U57969); MexE, MexF, and OprN (X99514); AcrA and AcrB (U00734); TolC (X00016); YlcB (P77211); MtrC (U14993); MtrD (U60099); MtrE (X95635)

toluene-sensitive strains designated as TOL-1 to TOL-6 were obtained. These mutations, apart from TOL-6, caused a minimal effect on growth rate (see Table 1). These mutants showed various levels of toluene sensitivity. Three mutants (TOL-2, TOL-4, and TOL-6) were unable to form colonies under toluene. The mutation in TOL-4 made the strain hypersensitive to organic solvents: the strain was resistant to hexane (log P_{ow} = 3.9) but was sensitive to *p*-xylene and cyclohexane (log P_{ow} = 3.4). Another three strains formed limited numbers of colonies under toluene. The colony-forming ability of these strains under toluene showed little fluctuation between experiments and was not dependent on the growth phase (data not shown).

Strain TOL was several times more resistant to β-lactams (ampicillin, penicillin G) and novobiocin than its parental strain KT2442 (see Table 1). Mutations that caused impairment of solvent resistance, except for TOL-4, resulted in minimum changes in MICs. TOL-4 was hypersusceptible to several antibiotics tested, especially β-lactams. Its MICs were not only far lower than those of the parental strain TOL, but also several times less than those of KT2442.

TOL-4 is an efflux pump-deficient mutant

As described, a mutation in TOL-4 affected solvent tolerance as well as antibiotic resistance. A 9-kb TOL-4 *Eco*RI fragment that contained the *Tn5*-derived Km^r gene was cloned into pUC19, and the nucleotide sequence from the TOL-4 chromosome was determined. A database search revealed that the nucleotide sequence illustrated considerable similarity with *Pseudomonas aeruginosa mexB*, the product of which (MexB) is a transporter protein of a multidrug efflux system (Li et al. 1995). It has been considered that the efflux system is transcribed as a *mexA-mexB-oprM* operon. Following a gene walking study, the nucleotide sequence of the 7.3-kb fragment that encoded four possible *mep* (multidrug efflux pump) genes was determined. Gene products of three open reading frames (ORFs) designated *mepA*, *mepB*, and *mepC* are most similar to one of the *P. aeruginosa* multidrug efflux proteins MexA, MexB, and OprM, respectively (Table 2). The MexA-MexB-OprM efflux system belongs to the RND (resistance-nodulation-division) family efflux system (reviewed by Nikaido 1996). We have also identified an ORF (*mepR*) that is transcribed in the opposite direction to that of *mepABC*. The *mepR* gene product (MepR) was most similar to a possible repressor protein (AcrR) that regulates a multidrug efflux system, the *E. coli acrAB* operon (Okusu et al. 1996). Genes *mepR*, *mepA*, *mepB*, and *mepC* encode proteins of 210, 384, 1050, and 484 amino acid residues, respectively, with calculated molecular masses of 23723, 41258, 112834, and 52863 Da, respectively. These

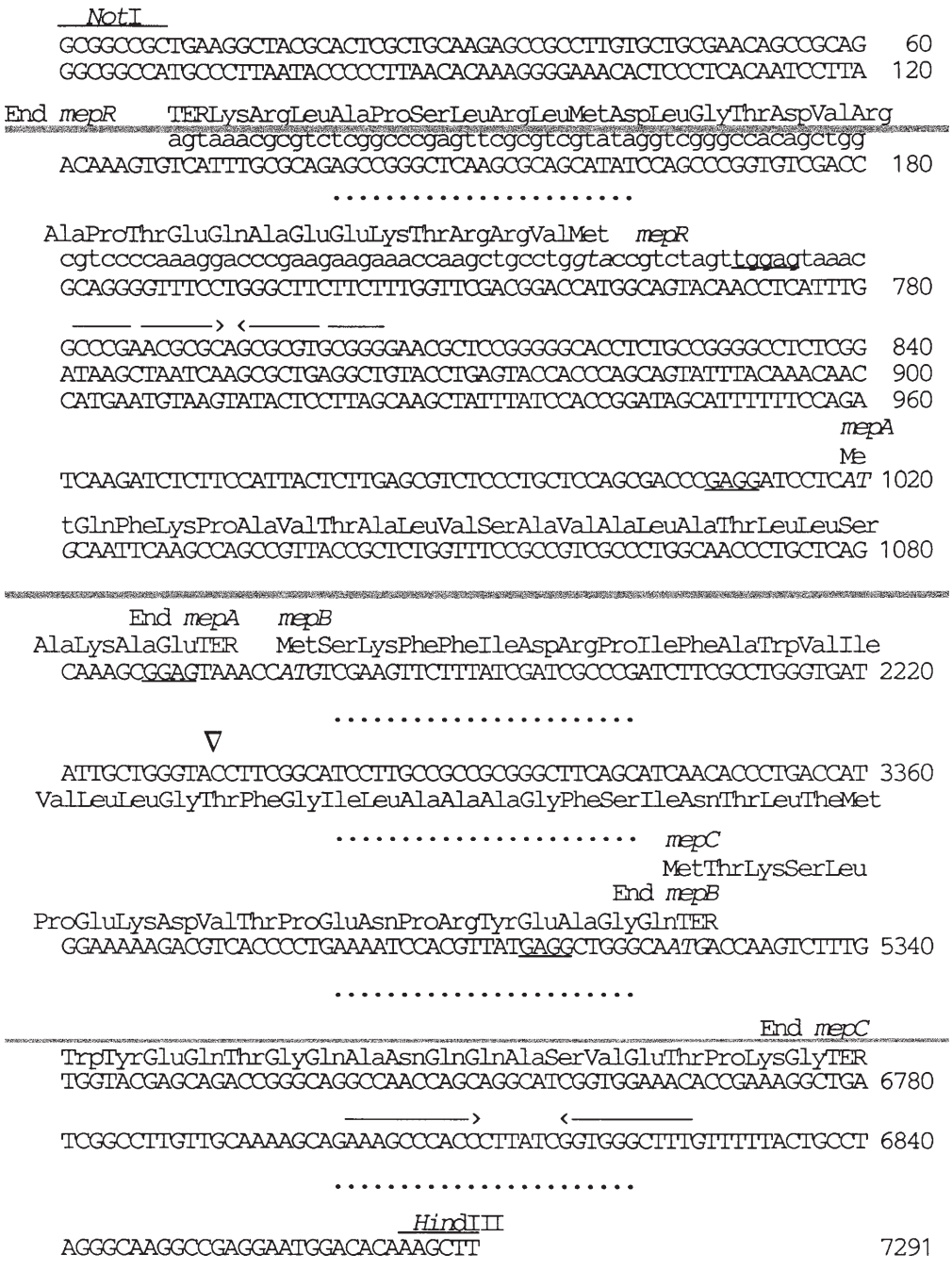

Fig. 1. Relevant portions of the *Not*I-*Hin*dIII fragment of *P. putida* TOL. The first G of the *Not*I site is numbered as *1*, and *dotted lines* indicate gaps in the nucleotide sequence. The amino acid sequence of the predicted proteins are shown *above* the nucleotide sequence. Possible translational start codons are *italicized*, and stop codons are marked as *TERs*. Potential ribosome-binding sites are *underlined*. Inverted repeat sequences between *mepR* and *mepA* and downstream of *mepC* are represented as *inverted pairs of arrows*. A *reverse open triangle* indicates the insertion site of *Tn5*

genes are preceded by typical ribosome-binding sites (Fig. 1). Stable stem and loop structures that may function as transcriptional terminators are found between *mepR* and *mepA*, and downstream of *mepC* (Fig. 1).

Discussion

Although several microbes have been reported to survive in the presence of high concentrations of organic solvents, little is known about the molecular mechanisms. We propose that several genes or gene products, including a solvent efflux system, are involved in the organic solvent resistance. Six toluene-sensitive *Tn5* mutants have been isolated from a toluene-adapted strain, *P. putida* TOL. It is noteworthy that these mutants showed different levels of solvent sensitivity. They could be classified into three distinct groups: one that was more solvent sensitive than the original strain KT2442 (TOL-4), those which had similar levels to KT2442 (TOL-2 and TOL-6), and those that were less resistant to toluene than strain TOL but more resistant than KT2442 (TOL-1, TOL-3, and TOL-5). This finding would suggest that the genes that had been

disrupted by *Tn5* contribute differently to organic solvent resistance.

We have shown that disruption of a possible efflux pump system greatly influenced solvent resistance. Strain TOL-4 was sensitive not only to toluene but also to *p*-xylene and cyclohexane, to which the original strain KT2442 was tolerant. Also, the mutation in the strain rendered the cell hypersusceptible to some antibiotics. Our results agree with two major findings showing that an energy-dependent efflux pump involved in the export of toluene in a *P. putida* strain (Isken and de Bont 1996) and a multidrug efflux system (MexA-MexB-OprM) contributed to antibiotic resistance in *P. aeruginosa* (reviewed by Nikaido 1996). The latest study indicates that upregulation of *acrAB* could increase the efflux of organic solvents, resulting in an increase of organic solvent tolerance in *E. coli* (White et al. 1997).

Our results strongly support the hypothesis that an efflux system plays a major role in organic solvent resistance. The *P. putida* efflux system that was responsible for solvent resistance is most similar to the *P. aeruginosa* MexA-MexB-OprM system, but is still significantly similar to the *E. coli* AcrAB system except for the proposed outer membrane protein TolC (see Table 2). However, a database search suggested that *E. coli* YlcB was more similar than TolC (Table 2). MepR rather resembled AcrR and MtrR, which would regulate *E. coli* AcrAB and *Neisseria gonorrhoeae* MtrCDE systems, respectively (data not shown). The *P. putida* efflux system may be regulated in different ways than that of *P. aeruginosa*. A database search by the BLAST program (Altschul et al. 1990) failed to illustrate any significant similarity between the possible repressor protein MepR in *P. putida* and the *P. aeruginosa* MexR, which regulates the *mexA-mexB-oprM* operon.

Our research has also focused on a molecular factor that produces toluene resistance in strain TOL. TOL cells must express a molecular factor(s) for resistance in the absence of the solvent as *P. putida* KT2442 was unable to survive under toluene. Upregulation of the efflux system is probably the major factor that increased toluene resistance in KT2442. Another factor could be an alkyl hydroperoxide reductase, of which the small subunit (AhpC) is constitutively overexpressed in the strain TOL (Fukumori and Horikoshi, manuscript in preparation). Genetic analysis of toluene-sensitive mutants as well as a functional study of AhpC would greatly contribute to the understanding of solvent tolerance mechanisms.

Note added in proof. A paper describing involvement of an efflux system in solvent resistance in *P. putida* was published while this study was being reviewed [Kiebroom J, Dennis JJ, de Bont JAM, Zylstra GJ (1998) Identification and molecular characterization of an efflux pump involved in *Pseudomonas putida* S12 solvent tolerance. J Biol Chem 273:85–91].

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