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Isolation and characterization of solvent-tolerant *Pseudomonas putida* strain T-57, and its application to biotransformation of toluene to cresol in a two-phase (organic-aqueous) system

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Abstract *Pseudomonas putida* T-57 was isolated from an activated sludge sample after enrichment on mineral salts basal medium with toluene as a sole source of carbon. *P. putida* T-57 utilizes *n*-butanol, toluene, styrene, *m*-xylene, ethylbenzene, *n*-hexane, and propylbenzene as growth substrates. The strain was able to grow on toluene when liquid toluene was added to mineral salts basal medium at 10–90% (v/v), and was tolerant to organic solvents whose log P_{ow} (1-octanol/water partition coefficient) was higher than 2.5. Enzymatic and genetic analysis revealed that *P. putida* T-57 used the toluene dioxygenase pathway to catabolize toluene. A cis-toluene dihydrodiol dehydrogenase gene (*todD*) mutant of T-57 was constructed using a gene replacement technique. The *todD* mutant accumulated *o*-cresol (maximum 1.7 g/L in the aqueous phase) when cultivated in minimal salts basal medium supplemented with 3% (v/v) toluene and 7% (v/v) 1-octanol. Thus, T-57 is thought to be a good candidate host strain for bioconversion of hydrophobic substrates in two-phase (organic-aqueous) systems.

Keywords Two-phase system · Solvent tolerant · Toluene dioxygenase pathway · *Pseudomonas putida* · Monooxygenation

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Introduction

There is considerable interest in the development of technology for the production of value-added chemicals from hydrophobic substrates such as petroleum hydrocarbons by using biocatalysts. Enantiospecific oxidation and reduction of aromatic and aliphatic hydrocarbons have especially attracted much interest, because many important chemicals can be produced by these reactions. Since cofactors and their regeneration are required for oxidation and reduction reactions, whole cells are favored as biocatalysts. Therefore, solvent-tolerant bacteria as hosts, and genes encoding enzymes that catalyze reactions of interest are necessary for the development of bioconversion processes for hydrophobic substrates. Some microorganisms found in soil and water are known to have the capacity to metabolize a wide range of aromatic hydrocarbons as sole source of carbon and energy. Most of these bacteria have been demonstrated to belong to *Pseudomonas* [13].

We are investigating bioconversion of toluene to *o*-cresol, because *o*-cresol is one of the most important chemical products in the petrochemical industry. Toluene with a low log P_{ow} (1-octanol/water partition coefficient) value (2.5) is extremely toxic to living organisms, and there are only a few reports of organisms that can grow with toluene in two-phase (organic-aqueous) systems [4, 7, 12]. We recently isolated a *Pseudomonas* strain able to grow in the presence of 90% (v/v) toluene and identified it as *Pseudomonas putida*. This toluene-tolerant *P. putida* strain could be used as a bacterial host for the production of cresol from toluene. Although this strain utilized toluene as sole source of carbon and energy, not much cresol accumulated in the culture broth. In this study, we performed a genetic investigation of the pathway for toluene oxidation in the toluene-tolerant *P. putida* strain, and modified the toluene dioxygenase pathway for overproduction of cresol from toluene in the two-phase system.

Materials and methods

Strains and plasmids

The bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* MV1184 was used for plasmid construction and DNA manipulation. *P. putida* strains were grown at 28°C with shaking in mineral salts basal (MSB) medium supplemented with appropriate carbon sources. MSB medium consisted of 4.5 g K_2HPO_4, 3.4 g KH_2PO_4, 2.0 g $(NH_4)_2SO_4$, 0.16 g $MgCl_2{\cdot}6H_2O$, 0.001 g $MnCl_2{\cdot}4H_2O$, 0.006 g $FeSO_4{\cdot}7H_2O$, 0.026 g $CaCl_2{\cdot}2H_2O$, 0.02 mg $Na_2MoO_4{\cdot}2H_2O$, 0.01 mg $ZnCl_2{\cdot}7H_2O$, 0.01 mg $CoCl_2{\cdot}6H_2O$, 0.01 mg $CuSO_4$, 0.001 mg $NiSO_4{\cdot}6H_2O$, and 0.001 mg Na_2SeO_4 per liter of deionized water. Luria-Bertani (LB) medium [9] was used as complete medium. For solid media, 2% agar was added to MSB medium and LB medium. *E. coli* was grown at 37°C with shaking in 2×YT medium [9] supplemented with appropriate antibiotics.

Isolation of toluene-metabolizing bacteria

Activated sludge, soil, and sediment samples were taken from chemical plants and roadsides in Hiroshima and Kumamoto, Japan. Samples were acclimated to toluene by incubating in 50 mL screw-capped vials containing a small tube of toluene for 1 week at 28°C. After acclimation, 1 g of sample was dispersed in 4 mL sterile water and allowed to stand for 30 min; 1 mL of this suspension was added to 9 mL MSB medium, and 0.5 mL toluene was added to the small tube inside the screw-capped vial. The vial was incubated with shaking (120 rpm) at 28°C for 5 days. Subsequently, 1 mL of the culture was transferred to 9 mL fresh MSB medium for another subsequent 2-day incubation. Serial dilutions of the second culture were plated on MSB agar plates and incubated in a desiccator with a beaker containing liquid toluene. Colonies obtained were reinoculated into liquid medium to confirm utilization of toluene. Pure cultures utilizing toluene as a sole carbon source were maintained on LB agar plates.

Hydrocarbons used for growth substrate analysis were provided in the vapor phase or directly added to MSB medium at 10% (v/v). Growth in the presence of organic solvents was measured in LB medium with 10% (v/v) solvent. The total volume of medium plus solvent was 20% of the volume of the screw-capped vial. Each vial was incubated at 28°C with shaking (120 rpm). Growth was measured by the increase in turbidity at 600 nm. When water-soluble organic compounds were used as the sole carbon source, they were usually supplied at a final concentration of 10 g/L.

Taxonomic analysis of strain T-57

Strain T-57 was characterized by phenotypic and morphological analyses. Phenotypic analysis was carried out using a BactoLab non-fergram S-1 kit (Wako, Osaka, Japan).

Amplification and sequencing of the 16S ribosomal RNA gene

Genomic DNA was prepared as described by Goldberg and Ohman [3]. The following primers were used for PCR amplification of the 16S ribosomal DNA (rDNA): 63f (5′-CAGGCCTAACACATGCAAGTC-3′) and 1387r (5′-GGGCGGWGTGTACAAGGC-3′). The PCR mixtures (50 μL) contained 25 pmol of each primer, 200 μM each deoxynucleoside triphosphate, TaKaRa EX-*Taq* PCR buffer (TaKaRa Bio, Ohtsu, Japan), 0.5 U TaKaRa EX-*Taq* (TaKaRa Bio), and 10 ng DNA per microliter. The thermocycling conditions consisted of a denaturation step at 96°C for 3 min, 25 amplification cycles of 96°C for 30 s, 50°C for 1 min, and 72°C for 2 min, and final

Table 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Source or reference
Escherichia coli		
MV1184	*ara*Δ (*lac*-it proAB) *rpsL thi* (*ϕ*80 *lacZ*Δ M15) Δ (*srl*-it recA) *306*::Tn *10*(Tc^R) F'[*traD36 proAB*$^+$ *lacI*^{q}*lacZ*Δ M15]	[11]
Pseudomonas putida		
T-57	Solvent-tolerant toluene-oxidizing bacterium	This study
TODD1	T-57 derivative, *todD*::Km^R	This study
Plasmids		
pUC118	Cloning vector, Ap^R	[11]
pHSG298	Cloning vector, Km^R	TaKaRa Bio, Ohtsu, Japan
SuperCosI	Cosmid vector, Ap^R, Km^R, *cos*	Stratagene, La Jolla, CA
pKT240	Broad-host-range plasmid vector, IncQ, Km^R, Cb^R	[1]
pT57COS045	SuperCosI containing approximately 30-kb fragment from *P. putida* T-57, Ap^R, Km^R	This study
pT57Tod01	pUC118 containing 6.2-kb *Eco*RI fragment from pT57COS045, *todC1C2BADE*, Ap^R	This study
pHSG298todD	pHSG298 containing the 0.5-kb internal region of *todD* open reading frame, Km^R	This study

polymerization for 4 min with a GeneAmp PCR System 9600 (Perkin–Elmer, Norwalk, CT). The PCR products were visualized on 1.0% agarose gels, and the products were excised and purified with glassmilk following the manufacturer's instructions (Gene Clean; Bio 101, Vista, CA), prior to sequencing. The PCR product for strain T-57, a unique fragment of 1.5 kb, was cloned in a pGEM-Teasy vector (Promega, Madison, WI) by following the manufacturer's instructions. Several clones containing a 1.5-kb insert were isolated, and one was selected for sequencing by the dideoxynucleotide method [10]. Nucleotide sequence similarities were determined using BLAST (National Center for Biotechnology Information databases). The 609-bp partial rDNA sequence is registered with the GSDB, DDBJ, EMBL, and NCBI nucleotide sequence databases under the accession number AB188094.

DNA manipulation and sequencing

Standard procedures were used for plasmid DNA preparations, restriction enzyme digestions, ligation, transformations, and agarose gel electrophoresis [9]. The nucleotide sequence was determined with an ABI model 310 automated DNA sequencer with dye primer or dye terminating sequencing protocols (DYEnamic ET Terminator Cycle Sequencing, Amersham, Piscataway, NJ). DNA sequence similarity searches were carried out using the program BLAST with the GenBank.

Electroporation

P. putida strain T-57 was transformed by electroporation using an Electro Cell Manipulator 620 (BTX, San Diego, CA). The following optimized protocol was devised from different experiments. Bacterial cells, grown overnight in LB medium, were inoculated into fresh LB medium (a 1% inoculum) and, after 4 h of incubation, cells were harvested by centrifugation (12,000 *g*, 5 min, 4°C). Pelleted cells were resuspended in ice-cold HS buffer [7 mM *N*-2-hydroxyethylpiperazine-it N′-2-ethanesulfonic acid (HEPES) plus 252 mM sucrose, pH 7.0], washed three times with the same buffer, and resuspended in 0.1 of the original volume of HS buffer. The electroporation was performed in electrocuvettes (BTX) with gaps of 2 mm and the following settings: 6.5 kV/cm, 725 Ω, and 50 μF. Pulsed cells were immediately diluted with 4 mL LB medium and regenerated at 28°C for 10 h before being plated on appropriate selective media.

Cloning of toluene dioxygenase operon of strain T-57

A cosmid genomic library of strain T-57 was constructed using a SuperCos I cosmid vector kit (Stratagene, La Jolla, CA) following the manufacturer's instructions. Southern hybridization was carried out using the *P. putida* F1 *todC1C2BA* genes [15] as a probe to select cosmid clones containing the toluene dioxygenase operon.

Biotransformation of toluene in a two-phase system

Bacterial cells were inoculated into 10 mL MSB medium in the screw-capped vial, with 1-octanol as a growth substrate and toluene as an inducer for toluene dioxygenase being provided in the vapor phase. The vial was incubated with shaking (120 rpm) at 28°C for 2 days. Subsequently, cells were harvested by centrifugation (10,000 *g*, 10 min, room temperature). Pelleted cells were resuspended in 9 mL fresh MSB medium. About 9 mL cell suspension, 0.7 mL 1-octanol, and 0.3 mL toluene were added to the screw-capped vial, which was then incubated with shaking (120 rpm) at 28°C. Samples were periodically taken and the aqueous supernatant was subjected to gas-liquid chromatography (GC) to measure cresol concentrations.

Analytical methods

Metabolites in culture supernatants were determined by GC with a DW-WAX column (30 m ×0.32 mm; Shimadzu, Kyoto, Japan). The following conditions were used for GC: He 6.25 mL min^{-1}, on-column injection mode; oven temperature, 40°C for 5 min; thermal gradient, 5°C min^{-1} to 250°C, and then held at 250°C for 30 min.

Results and discussion

Identification of strain T-57

Strain T-57 was isolated from activated sludge collected at a wastewater treatment plant in Kumamoto, Japan. T-57 was isolated by serial batch enrichments in mineral salts medium (MSB medium) containing toluene as a sole source of carbon. T-57 could grow on toluene when liquid toluene was added to MSB medium at 10–90% (v/v) (data not shown). T-57 is a Gram-negative, motile, strictly aerobic rod, which can grow at 33°C, but not at 42°C. Analysis of rDNA sequence revealed that T-57 is closely related to *P. putida*, with more than 99.8% similarity to other *P. putida* strains. To verify identification of T-57 as *P. putida*, T-57 was characterized by phenotypic analysis, and was found to be negative for lysine and ornithine decarboxylation, nitrate reduction, acidic amidase, urease, and indole production, and to hydrolyze arginine, but not starch, DNA, or escrine. T-57 can utilize citrate as sole sources of carbon and energy, and can produce acids from glucose and xylose, but not from mannitol, lactose, maltose, or phenylpyruvate. These data support classification of T-57 as *P. putida*.

Hydrocarbon utilization and tolerance

P. putida T-57 uses organic solvents, including *n*-butanol, toluene, *p*-xylene, ethylbenzene, and propylbenzene, as sole sources of carbon and energy. It grew well on these organic solvents when they were supplied in the vapor. *P. putida* T-57 was unable to grow on benzene, styrene, *o*-xylene, *m*-xylene, cyclohexane, diethylphtalate, *n*-hexane, *n*-octane, or *n*-decane.

P. putida T-57 could grow in LB medium when toluene was supplied in a two-phase system where toluene was present at 10% (v/v). A lag phase of about 18 h was followed by a period of exponential growth with a cell doubling time of 1.5 h (Fig. 1). Maximum growth was obtained after 22 h. A role for Mg^{2+} has been implicated in solvent tolerance for *P. putida* strains IH-2000 and DOT-T1, which are tolerant to high concentrations of toluene [4, 5, 7]. To test the role of Mg^{2+} in solvent tolerance of T-57, T-57 was grown on LB medium supplemented or not with 10 mM $MgSO_4$ in the presence or absence of 10% (v/v) toluene (Fig. 1). As was the case with *P. putida* IH-2000 and DOT-T1, the lag period was significantly shorter in the presence of 10 mM $MgSO_4$.

The ability of *P. putida* T-57 to grow on LB medium in the presence of a wide range of organic solvents at 10% (v/v) was also tested. The solvents used are listed in Table 2 in decreasing order of hydrophobicity, expressed as log P_{ow}. Strain T-57 grew in the presence of organic solvents whose log P_{ow} was equal to or greater than 2.5. Similar resistance to solvents has been observed in *P. putida* IH-2000, S12, and DOT-T1 [4, 7, 12].

Characterization of toluene pathway in *P. putida* T-57

To determine the metabolic pathway of toluene in *P. putida* T-57, we first tested catechol 2,3-dioxygenase (C23O) activity. The rapid spot test described by Pankhurst [6] was carried out to assess C23O activity. Catechol or 3-methylcatechol dissolved in acetone was sprayed onto 1-day-old T-57 colonies grown on toluene vapor plates. The spot test revealed bright yellow colonies, indicative of C23O activity against catechol and 3-methylcatechol. To further investigate the metabolic pathway of toluene, Southern blot analysis was conducted using toluene dioxygenase genes (*todC1C2BA*) and catechol 2,3-dioxygenase gene (*todE*) from *P. putida* F1 [15], and toluene monooxygenase genes (*xylAM*) from the TOL plasmid pWW0 [2], as probes. The *todC1C2BA* and *todE* probe hybridized with a 10-kb *Eco*RI fragment of T-57 genomic DNA, while the *xylAM* probe did not hybridize with this fragment (data not shown). These results suggest that T-57 possesses sequences homologous to the *todC1C2BA* genes and *todE*, but not sequences homologous to the *xylAM* genes. To confirm that T-57 possesses toluene dioxygenase genes, we constructed a cosmid genomic library of T-57 and screened it with the *todC1C2BA* probe. One positive clone, designated pT57COS045, was obtained. The 6.2-kb *Eco*RI fragment of pT57COS045 that hybridized with the *todC1C2BA* probe was then subcloned into pUC118 to construct pT57Tod01. The 6.2-kb insert of pT57Tod01 was digested with various restriction enzymes and the fragments were subcloned into pUC118. About 500 bases of both ends of the fragments were sequenced using the resulting plasmids as templates. Sequence analysis found sequences highly homologous (more than 99% similarities) to the *todC1*, *todC2*, *todB*, *todA*, *todD*, and *todE* genes, and the gene order was same as that of the *P. putida* F1 *tod* operon (Fig. 2c). These results confirmed the presence of the toluene dioxygenase pathway in *P. putida* T-57.

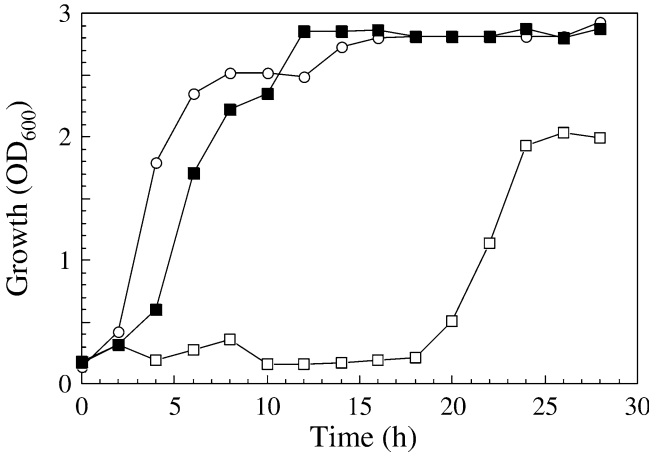

Fig. 1 Growth of *Pseudomonas putida* T-57 on Luria-Bertani (LB) medium in the presence and absence of toluene. Cells, grown overnight in LB medium, were inoculated into LB medium without toluene (*open circles*), or with 10% (v/v) toluene in the absence (*open squares*) or presence (*closed squares*) of 10 mM $MgSO_4$

Construction of *todD* mutant of T-57

Enzyme assays and genetic analysis suggested that strain T-57 utilizes toluene via the toluene dioxygenase pathway. In the initial step of this pathway, toluene is oxidized to cis-1,2-dihydroxy-3-methylcyclohexa-3,5-diene (cis-toluene dihydrodiol), which is further oxidized to 3-methylcatechol (Fig. 2). This product undergoes meta-cleavage fission catalyzed by C23O [15]. It is known that the first intermediate, cis-toluene dihyrodiol, is nonenzymatically dehydrated to cresol [8]. cis-Toluene dihydrodiol dehydrogenase (the gene product of *todD*) further oxidizes cis-toluene dihydrodiol to 3-methylcatechol. Therefore, a *todD* mutant strain of T-57, which is unable to further metabolize cis-toluene dihydrodiol, is predicted to accumulate cresol when cultivated in the presence of toluene. To assess this possibility, we constructed a *todD* knockout mutant of T-57 using a gene replacement technique.

First, we optimized an electroporation procedure for construction of the *todD* knockout mutant using the broad-host-range plasmid pKT240 (12.9 kb, Km^R),

Table 2 Growth of *P. putida* T-57 in the presence of organic solvents. Cells were grown on LB medium in the presence of 10% (v/v) solvents. Log P_{ow} 1-octanol/water partition coefficient

Solvents	Log P^{a}_{ow}	OD^{b}_{600}
n-Decane	5.6	> 2.0
n-Octane	4.5	> 2.0
n-Hexane	3.9	> 2.0
Propylbenzene	3.6	> 2.0
Diethylphthalate	3.3	> 2.0
Cyclohexane	3.2	> 2.0
m-Xylene	3.2	> 2.0
p-Xylene	3.1	> 2.0
o-Xylene	3.1	> 2.0
Ethylbenzene	3.1	> 2.0
Styrene	3.0	> 2.0
Toluene	2.5	> 2.0
Benzene	2.0	< 0.2
Chloroform	2.0	< 0.2
n-Butanol	0.8	< 0.2

[a]From Ref. [7]
[b]After 24 h

which can replicate in *Pseudomonas* strains including *P. putida* [1, 14]. Different growth phase, electroporation media, field strength, external resistance, and cultivation times before plating were tested. Based on the results, an optimized electroporation procedure for *P. putida* T-57 was devised (described in detail in Materials and methods). The optimized procedure gave a transformation efficiency of 1×10^{7} transformants/μg DNA.

We then constructed a plasmid for the disruption of the chromosomal *todD* gene. A 0.5-kb *Stu*I– *Nru*I fragment containing part of the *todD* gene, but lacking its 5′ and 3′ ends, was cloned into pHSG298 (TaKaRa Bio) to construct pHSG298todD (3.2 kb, Km^{R}). pHSG298todD contains the ColE1 replication origin and cannot replicate in *P. putida*. pHSG298todD was introduced into T-57 using the optimized electroporation procedure, and kanamycin-resistant transformants were selected on LB plates supplemented with kanamycin. The results of Southern analysis confirmed that the *todD* gene was properly disrupted by single-cross-over recombination (data not shown). The chromosomal mutant was designated TODD1. TODD1 was unable to grow in MSB medium containing toluene as a sole source of carbon.

Biotransformation of toluene to *o*-cresol by the *todD* mutant

P. putida wild-type T-57 and *todD* mutant TODD1 were assessed for bioconversion of toluene. Strains T-57 and TODD1 were cultivated in MSB medium containing 3% (v/v) toluene and 7% (v/v) 1-octanol (sources of carbon and reducing power) and aqueous supernatants were analyzed by GC. Both strains grew very well in this two-phase system. Less than 0.2 g/L cresols were detected in aqueous supernatants of T-57. TODD1 accumulated a

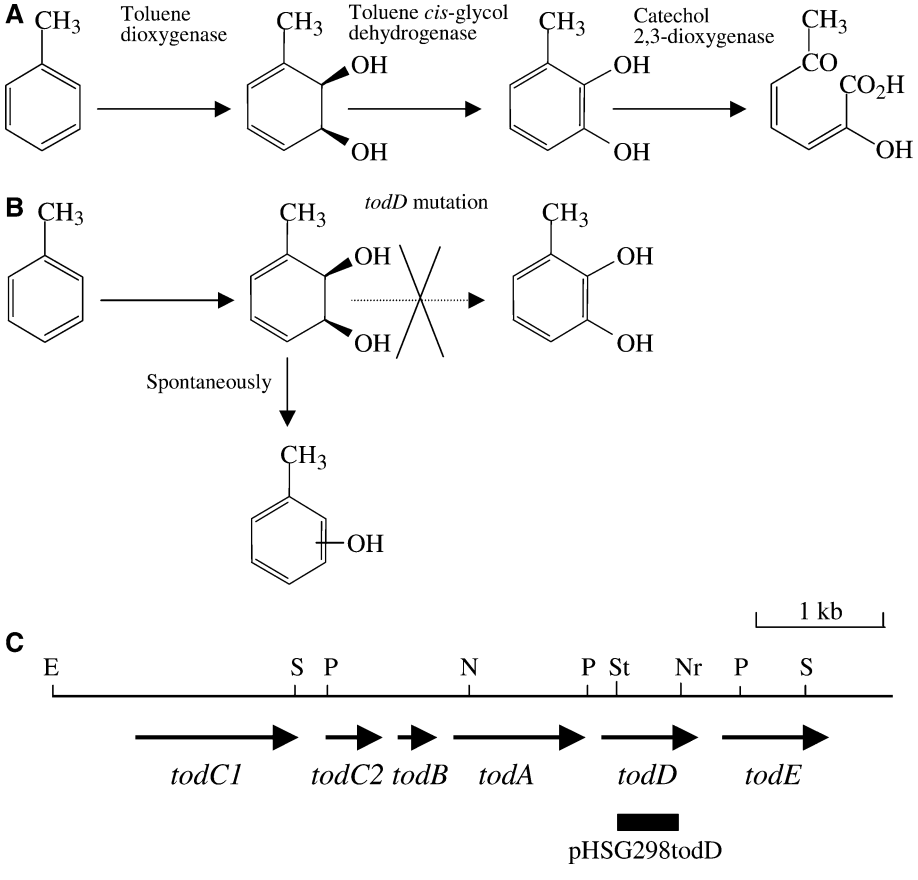

Fig. 2 Proposed catabolic pathways for toluene metabolism by *P. putida* T-57 (**a**) and TODD1 (**b**). **c** Restriction map of plasmid pT57Tod01 containing the *P. putida* T-57 *tod* genes and the subclone pHSG298todD. The locations and orientations of *todC1*, *todC2*, *todB*, *todA*, *todD*, and *todE* are indicated by *horizontal arrows* below the restriction map. Restriction sites: *EEco*RI, *NNot*I, *NrNru*I, *PPst*I, *SSal*I, *StStu*I

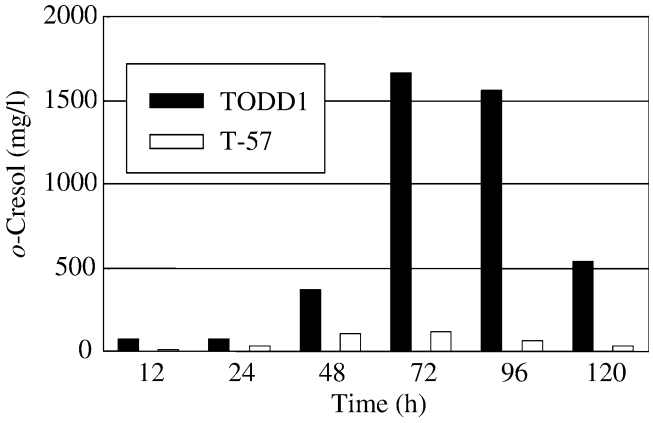

Fig. 3 Accumulation of *o*-cresol in aqueous supernatants. Wild-type T-57 (*open bars*) and *todD* knockout mutant TODD1 (*solid bars*) were cultivated in mineral salts basal (MSB) medium supplemented with 7% 1-octanol (v/v) and 3% toluene (v/v)

maximum of 1.7 g/L *o*-cresol at 72 h after the start of cultivation (Fig. 3). Interestingly, *m*-cresols and *p*-cresols were rarely detected in the culture supernatants of TODD1.

In summary, we have isolated a toluene-oxidizing bacterium, *P. putida* T-57, which is highly tolerant to organic solvents. This strain utilizes toluene via the toluene dioxygenase pathway. We modified the toluene dioxygenase pathway of T-57 by inactivating the chromosomal *todD* gene encoding toluene cis-dihydrodiol dehydrogenase, and demonstrated that toluene could be converted to *o*-cresol by the *todD* mutant in a two-phase (organic-aqueous) system. Thus, T-57 is thought to be a good candidate host strain for bioconversion of hydrocarbons in two-phase systems.

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