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Isolation and Expansion of the Catabolic Potential of a *Pseudomonas putida* Strain Able To Grow in the Presence of High Concentrations of Aromatic Hydrocarbons

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Pseudomonas putida **DOT-T1 was isolated after enrichment on minimal medium with 1% (vol/vol) toluene as the sole C source. The strain was able to grow in the presence of 90% (vol/vol) toluene and was tolerant to organic solvents whose log P_{ow} (octanol/water partition coefficient) was higher than 2.3. Solvent tolerance was inducible, as bacteria grown in the absence of toluene required an adaptation period before growth restarted. Mg^{2+} ions in the culture medium improved solvent tolerance. Electron micrographs showed that cells growing on high concentrations of toluene exhibited a wider periplasmic space than cells growing in the absence of toluene and preserved the outer membrane integrity. Polarographic studies and the accumulation of pathway intermediates showed that the strain used the toluene-4-monooxygenase pathway to catabolyze toluene. Although the strain also thrived in high concentrations of *m*- and *p*-xylene, these hydrocarbons could not be used as the sole C source for growth. The catabolic potential of the isolate was expanded to include *m*- and *p*-xylene and related hydrocarbons by transfer of the TOL plasmid pWW0-Km.**

Reports of the toxicity of aromatic solvents to microorganisms first appeared early in this century (reviewed in reference 21). Aromatic hydrocarbons become toxic when they are partitioned into lipid bilayer membranes, leading to significant changes in the structure and functioning of membrane components, e.g., disruption of the membrane potential, removal of lipids and proteins, and loss of Mg^{2+} and Ca^{2+} cations as well as other small molecules (21). Although a number of microbes able to grow at the expense of aromatic compounds have been isolated, their addition to the culture medium, in general, prevented growth and bacteria were able to survive only when these compounds were supplied in the vapor phase (7, 27).

There is considerable interest in the isolation of microbes able to thrive in high concentrations of organic solvents, because these microbes can be used as vehicles for the elimination of low-molecular-weight aromatic compounds such as toluene, styrene, benzene, and xylenes, the removal of which is of high priority (14). Furthermore, these aromatic hydrocarbons can be converted into value-added compounds such as *cis*-diols, epoxides, and indigo, among others (6, 7, 15, 26). Their current synthesis by biological means requires large amounts of water, a major cost in the fermentation industry (10). Therefore, synthesis in double-phase fermentors could be more economical. Lastly, understanding of the mechanisms of solvent tolerance can be exploited in the future to generate microbes with enhanced biocatalytic potential.

Inoue et al. (12, 13) reported that certain *Pseudomonas* strains were able to thrive in the presence of more than 50% (vol/vol) toluene, although these strains were not able to grow with the aromatic hydrocarbon alone and other sources of carbon and energy were required. Cruden et al. (3) and Weber et al. (23) reported the isolation of bacterial strains able to grow at the expense of *p*-xylene provided at 5 to 50% (vol/vol) and styrene provided at 1% (vol/vol) in the culture medium.

We now report some physiological properties of *Pseudomonas putida* DOT-T1, which grows at the expense of toluene as the sole carbon source provided at 0.1 to 90% (vol/vol) in the culture medium. The strain uses the toluene-4-monooxygenase pathway to metabolize this aromatic hydrocarbon. The spectrum of aromatic compounds which support growth of the strain was expanded to include *m*- and *p*-xylene and related compounds by transfer of the TOL plasmid pWW0-Km.

MATERIALS AND METHODS

Bacterial strains, plasmids, culture media, and growth conditions. The bacterial strains and plasmids used are listed in Table 1. Bacterial strains were routinely grown on liquid Luria-Bertani (LB) medium or on M9 minimal medium as described before (1). For strains tolerant to high concentrations of toluene, aromatic hydrocarbons supplied as the sole C source were usually added at a concentration of 10% (vol/vol). For strains sensitive to toluene, aromatic hydrocarbons were supplied in the vapor phase. All flasks were sealed, incubated at 30°C, and shaken on an orbital platform operating at 150 to 200 strokes per min. Growth was measured as the increase in turbidity at 660 nm and by determining the most probable number of bacterial cells on solid medium. When water-soluble organic compounds other than aromatic hydrocarbons were used as the sole C source, they were usually supplied at a final concentration of 10 mM.

Antibiotics and growth inhibitors were used at the following concentrations (in micrograms per milliliter): kanamycin, 50; rifampin, 20; phosphinothricin, 1,000.

Plasmid pWW0-Km is a derivative of the archetypal TOL plasmid pWW0 bearing a mini-Tn*5*, which encodes resistance to kanamycin and allows the host *Pseudomonas* sp. strain to grow on toluene, *m*- and *p*-xylene, and related hydrocarbons (18).

Transfer of the TOL plasmid pWW0-Km to *P. putida* DOT-T1-1. Matings between the rifampin-resistant, solvent-tolerant *P. putida* DOT-T1-1 and the kanamycin-resistant, rifampin-sensitive, and solvent-sensitive *P. putida* EEZ15 (pWW0-Km) were carried out for 16 h at 30°C on Millipore filters placed on the surface of an LB plate as described previously (18). Then, the bacteria were resuspended in 50 mM phosphate buffer (pH 7.0), and serial dilutions were spread on glass petri dishes with solid M9 minimal medium supplemented with 1% (vol/vol) *m*-xylene as the sole C source plus rifampin and kanamycin. Transconjugants appeared at a rate of about 10^{-2} per recipient cell.

Electron microscopy. *P. putida* DOT-T1 was grown on LB medium in the absence of toluene or in the same medium with toluene supplied in the vapor phase or added to the liquid medium to a final concentration of 10% (vol/vol). Cells were harvested by centrifugation, washed once in 50 mM phosphate buffer

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TABLE 1. *P. putida* strains and plasmids used in this study

Strain or plasmid	Relevant characteristics[a]	Source or reference
Strains		
DOT-T1	Utilizes toluene in a two-phase system in which the hydrocarbon is present at 0.01 to 90%[b]	This study
DOT-T1-1	Rif^r derivative of DOT-T1	This study
DOT-T1-5	DOT-T1-1(pWW0-Km)	This study
EEZ15	Ben^+ Pt^r	18
Plasmid		
pWW0-Km	Tol^+ *m*-Xyl^+ Km^r	17

[a] Rif^r, Pt^r, and Km^r, resistance to rifampin, phosphinothricin, and kanamycin, respectively. Tol^+, *m*-Xyl^+, and Ben^+, ability to grow with toluene, *m*-xylene, or benzoate as the sole C and energy source, respectively.

[b] By volume.

(pH 7.0), immediately fixed with 2.5% (vol/vol) glutaraldehyde plus 1% (vol/vol) formaldehyde, postfixed with osmium tetroxide in the presence of 0.15% (wt/vol) ruthenium red, and embedded in Eponate 12. Thin sections were poststained with uranyl acetate and lead citrate, and examined in a Zeiss transmission electron microscope at an accelerating voltage of 75 kV.

Analysis of metabolites and analytical methods. *P. putida* DOT-T1 was grown on M9 minimal medium with toluene supplied in the vapor phase. Cells were removed by centrifugation at 5,000 × *g* for 10 min. The culture fluid was analyzed directly by high-performance liquid chromatography (HPLC). HPLC analyses were done on a Hypersil C_{18} column with acetonitrile-water (60:40, vol/vol) as the mobile phase, at a flow rate of 0.7 ml/min. Metabolites were identified by coelution in HPLC with pure compounds and UV-visible spectra.

Substrate oxidation. Oxygen uptake was determined polarographically with a Clark electrode. Cells in the exponential growth phase were harvested by centrifugation, washed twice in M9 minimal medium without a C source, and suspended in the same medium to an optical density (OD_{660}) at 660 nm of 1. One milliliter of the cell suspension was placed in the electrode chamber, which was equilibrated at 30°C for 5 min. The rate of endogenous oxygen consumption was determined for 5 min; then, 20 μl of the substrate dissolved in dimethylformamide was added to a final concentration of 0.5 mM, and oxygen consumption was recorded for at least 5 min. The rate of substrate-dependent oxygen consumption is given as the difference between the oxygen uptake rate in the presence of the substrate and the endogenous respiration rate. Oxygen consumption was expressed as micromoles of O_2 per unit of OD_{660} per minute.

Enzymatic assays. Catechol 2,3-dioxygenase was assayed in cell extracts as described previously (27).

Microbial deposit. The nucleotide sequence of *P. putida* DOT-T1-5 has been deposited with the Colección Española de Cultivos Tipo in Valencia, Spain, under accession number 4501.

RESULTS

Isolation and characterization of *P. putida* DOT-T1. Strain DOT-T1 was isolated from water collected at a wastewater treatment plant in the city of Granada. Equal volumes of wastewater and M9 minimal medium were mixed, and toluene was supplied in the vapor phase. After 48 h of growth at 30°C, the suspension was diluted 100-fold in M9 minimal medium with 1% (vol/vol) toluene. This culture was kept for several days at 30°C with agitation until turbidity increased. Then, serial dilutions were spread on glass petri plates with solid M9 minimal medium and 1% (vol/vol) toluene as the sole C source. Plates were sealed and incubated at 30°C for 72 h. A single type of colony was found, and a random clone, called DOT-T1, was chosen. The strain is a motile, oxidase-positive and catalase-positive gram-negative rod. It grows at 25 and 30°C but not at 37°C. It uses as the sole C source toluene, ethylbenzene, *p*-cresol, *p*-hydroxybenzoate, protocatechuate, glucose, acetate, and succinate but is unable to use benzyl alcohol, xylenes, *o*- or *m*-cresol, *m*- or *p*-methylbenzoate, fucose, arabinose, mannose, rhamnose, sucrose, or melibiose.

The strain did not denitrify and did not produce hydrolases for Tween 80 and gelatin. Phospholipid analyses (Microbial Identification Systems Inc., Essex Junction, Vt.) identified this strain as a bacterium of the *P. putida* biovar A group.

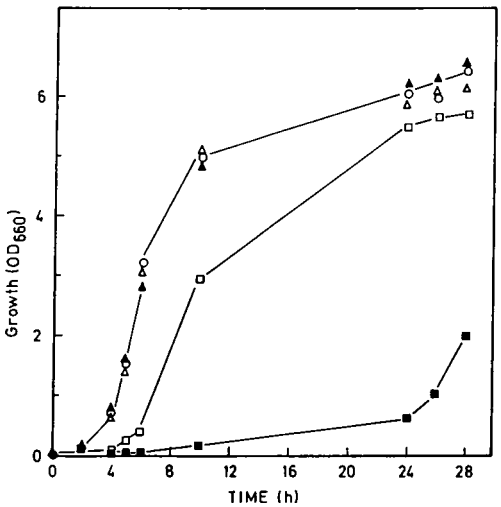

FIG. 1. Growth of *P. putida* DOT-T1 on LB medium in the absence and in the presence of toluene. Cells pregrown overnight on LB medium were harvested, washed in LB medium, and resuspended in LB medium without toluene (○) or with 10% (vol/vol) toluene in the absence of magnesium ions (■) or in the presence of 10 mM $MgSO_4$ (□). Cells pregrown overnight on LB medium plus 10% (vol/vol) toluene were treated as above and resuspended in LB medium without toluene (△) or LB medium with 10% (vol/vol) toluene (▲). Turbidity was determined at 660 nm.

Solvent tolerance. The viability and tolerance of *P. putida* DOT-T1 to different concentrations of toluene were tested. Bacteria were grown on LB medium with and without 10% (vol/vol) toluene, and each culture was harvested by centrifugation, washed twice, and resuspended in LB medium. Four aliquots of each culture were made, and 0, 1, 10, or 90% (vol/vol) toluene was added. After 5 min, serial dilutions were spread on LB plates and the number of viable cells was determined. We found that cells grown on LB medium were sensitive to toluene and that after 5 min the viability decreased by 5 orders of magnitude in 1% (vol/vol) toluene and by more than 7 orders of magnitude at higher concentrations of toluene. In contrast, cells initially grown on 10% (vol/vol) toluene were fully viable after a 5-min exposure to 90% (vol/vol) toluene.

The maintenance or loss of viability of the strain at different concentrations of solvent was reflected in the growth curve of the strain. In fact, *P. putida* DOT-T1 cells pregrown on LB medium with 10% (vol/vol) toluene immediately grew on LB medium supplemented or not with 10% (vol/vol) toluene, whereas a prolonged lag was observed for bacteria pregrown in the absence of the aromatic hydrocarbon (Fig. 1). The doubling times of the strain grown on LB medium with and without toluene when cells were in the exponential phase were similar (about 50 to 60 min), reaching in both media about 2×10^9 CFU/ml.

We also determined growth rates on minimal medium with toluene supplied in the vapor phase or at 1 and 10% (vol/vol). In all cases bacteria showed doubling times of about 75 min, and the highest cell density was 8×10^8 CFU/ml.

It has been suggested that hydrocarbons induce the loss of Mg^{2+} ions (4, 13). To test the role of magnesium ions in tolerance to the solvent, bacteria were grown on LB medium, harvested, and resuspended on LB medium supplemented or not with 10 mM $MgSO_4$ in the presence and in the absence of 10% (vol/vol) toluene. In the presence of 10 mM $MgSO_4$, the

TABLE 2. Growth of *P. putida* DOT-T1 in the presence of organic solvents[a]

Solvent	log P_{ow}[b]	OD_{660}[c]
n-Decane	5.6	>2.0
n-Octane	4.5	>2.0
n-Heptane	4.1	>2.0
Propylbenzene	3.6	>2.0
Diethylphthalate	3.3	>2.0
Cyclohexane	3.2	>2.0
Ethylbenzene	3.1	>2.0
p-Xylene	3.1	>2.0
Styrene	3.0	>2.0
Toluene	2.5	>2.0
l-Heptanol	2.4	>1.0
Dimethylphthalate	2.3	>1.0
Benzene	2.0	<0.1
Chloroform	2.0	<0.1
Butanol	0.8	<0.1

[a] Cells were grown on LB medium in the presence of 10% (vol/vol) solvent as described in the text.
[b] From references 12 and 13.
[c] After 24 h.

lag period was significantly shorter (Fig. 1). Other divalent cations such as Ca^{2+}, Cu^{2+}, Zn^{2+}, and Mn^{2+} did not exert any beneficial effect as determined by a shorter lag phase or higher cell yield (data not shown).

The ability of *P. putida* DOT-T1 to grow on LB medium in the presence of a wide range of organic solvents at 10% (vol/vol) was also tested. The solvents used are listed in Table 2 in decreasing order of hydrophobicity, expressed as log P_{ow}. Strain DOT-T1 was grown on LB medium with 10% (vol/vol) toluene, and, after removal of the hydrocarbon by washing the cells twice with LB medium, cells were suspended in fresh LB medium supplemented with the organic solvent being tested, at 10% (vol/vol), to an initial turbidity at 660 nm of 0.05. Growth was determined 24 h later as culture turbidity. We found that the strain grew in the presence of organic solvents whose log P_{ow} (octanol/water partition coefficient) was equal to or greater than 2.3 (Table 2).

Ultrastructure of *P. putida* DOT-T1 grown in the absence and in the presence of different concentrations of toluene. Bacteria were grown on LB medium in the absence of toluene or with toluene supplied through the gas phase or added to the culture medium at 10% (vol/vol). Thin sections were prepared from exponentially growing cells. In *P. putida* DOT-T1 growing on LB medium with or without toluene in the gas phase, the outer and the cytoplasmic membranes were close together and ribosomes were well defined (Fig. 2A). Cells growing with 10% (vol/vol) toluene exhibited altered structure, the periplasmic space was usually larger (Fig. 2B and C) than in cells growing in the absence of toluene, and occasionally membrane evaginations were observed (Fig. 2D).

Metabolic studies. Five different metabolic pathways for the metabolism of toluene in aerobic microorganisms have been defined (7, 16, 19, 24, 25, 27, 29). When the aromatic ring is oxidized by toluene dioxygenase, *cis*,*cis*-diol is produced, which is further oxidized to 3-methylcatechol. This product undergoes *meta*-cleavage fission (7, 8). Different toluene monooxygenases are involved in the other four pathways. The product of toluene oxidation is benzyl alcohol, which is further oxidized to catechol (27) or *o*-, *m*-, or *p*-cresol according to the specificity of each enzyme (16, 19, 29). *P. putida* DOT-T1 was not able to grow on *m*-xylene, benzyl alcohol, *m*-methylbenzyl alcohol, or *m*-methylbenzoate, and, because the strain grown on toluene did not exhibit catechol 2,3-dioxygenase activity against catechol, this was taken as evidence that metabolism of toluene did not occur through the oxidation of the lateral chain. *P. putida* DOT-T1 grown on toluene did not exhibit catechol 2,3-dioxygenase activity against 3-methylcatechol either, suggesting that the aromatic ring did not undergo a dioxygenase attack. *P. putida* DOT-T1 grew on *p*-cresol but not on *o*- or *m*-cresol. This was taken as evidence that toluene could be metabolized via *p*-cresol by the toluene-4-monooxygenase pathway (Fig. 3) (24, 29). To further confirm this possibility, bacteria were grown on minimal medium with toluene as the sole C source, and, after removal of the aromatic hydrocarbon by washing in minimal medium, we determined the rate of oxidation of the substrate. Cells grown with toluene oxidized toluene, *p*-cresol, *p*-hydroxybenzyl alcohol, *p*-hydroxybenzaldehyde, *p*-hydroxybenzoic acid, and protocatechuate at similar rates (2.5 to 4.5 μmol of O_2 per unit of OD_{600} per min). In contrast, cells grown on protocatechuate did not oxidize toluene or the other aromatic compounds.

To further confirm the catabolic pathway, we isolated pathway intermediates from culture supernatants according to the following strategy: *P. putida* DOT-T1 cells were grown on glucose until they reached mid-log phase, washed twice on M9 minimal medium without a C source, and transferred to fresh medium with toluene supplied in the vapor phase. We found that, before growth resumed, several aromatic compounds accumulated in the culture medium (Fig. 4). These compounds were coeluted with *p*-cresol (2.31 min), *p*-hydroxybenzaldehyde (1.69 min), and *p*-hydroxybenzoate (1.30 min). Toluene was also identified in the culture medium (4.02 min). Two products whose retention times were found to be 2.69 and 6.04 min were also found, but their nature remains to be determined.

Toluene-4-monooxygenase genes (*tmoABCDE*) from *Pseudomonas mendocina* KR1 hybridized total DNA prepared from *P. putida* DOT-T1, confirming the presence of genes homologous to those already cloned (29).

Expansion of the catabolic potential of *P. putida* DOT-T1. The TOL plasmid pWW0 encodes a catabolic pathway that allows, in addition to toluene mineralization, the metabolism of *m*- and *p*-xylene, *m*-ethyltoluene, and 1,2,4-trimethylbenzene. A TOL plasmid containing a mini-Tn*5* that confers kanamycin resistance and inserted outside the catabolic operons was transferred to DOT-T1-1 as described in Materials and Methods, and a derivative clone called DOT-T1-5 was isolated; this clone grew, as expected, on *m*- and *p*-xylene. Cell extracts of *P. putida* DOT-T1-5, grown on *m*-xylene exhibited catechol 2,3-dioxygenase as expected (about 1,000 mU/mg of protein). *P. putida* DOT-T1-5 also grew on *m*-ethyltoluene, 1,2,4-trimethylbenzene, *m*- and *p*-methylbenzyl alcohol, *m*- and *p*-methylbenzoate, and 3,4-dimethylbenzoate, as expected, after acquisition of the TOL plasmid. This clone conserved the solvent tolerance of the parental strain, as well as its ability to grow on *p*-cresol, *p*-hydroxybenzyl alcohol, *p*-hydroxybenzaldehyde, *p*-hydroxybenzoate, and protocatechuate.

DISCUSSION

Microbes able to grow on low concentrations of aromatic hydrocarbons as the sole source of carbon and energy were first identified early this century (reviewed by Gibson and Subramanian [8]). However, bacteria able to thrive and grow in the presence of high concentrations of aromatic solvents have been reported only recently (2, 3, 12, 13, 20, 22, 23). Most strains isolated were identified as *P. putida*. Three of the *P. putida* strains able to thrive on toluene did not use this compound as the sole C source; however, a previous isolate, *P. putida* Idaho

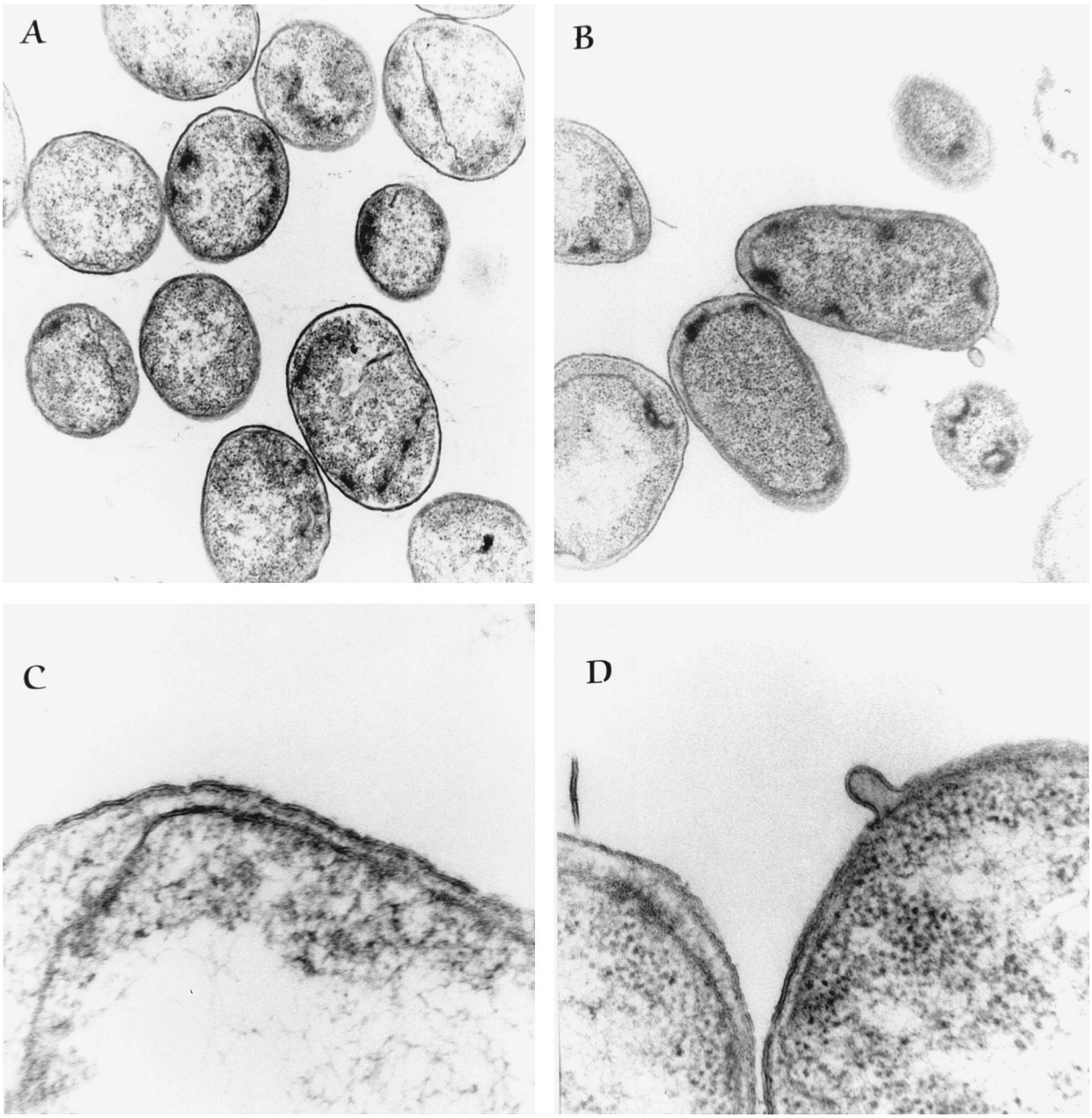

FIG. 2. Ultrastructure of *P. putida* DOT-T1. Cells were grown on LB medium without toluene (A) or LB medium with 10% (vol/vol) toluene (B). (C) Detail of the periplasmic space (D) and an evagination of cells grown on 10% (vol/vol) toluene. Magnification, ×31,500 (A and B), ×125,000 (C), or ×100,000 (D).

(3), and the strain isolated in the present study, *P. putida* DOT-T1, thrive in the presence of high concentrations of toluene and grow at its expense. In contrast with *P. putida* Idaho, which used the benzyl alcohol pathway to grow on toluene, the strain we isolated in this study used the toluene-4-monooxygenase pathway for toluene catabolism. This suggests that the ability to thrive on organic solvents is strain specific rather than pathway dependent. In fact, transfer of the TOL plasmid pWW0-Km to the DOT-T1 strain resulted in bacteria that retained their solvent tolerance but were able to grow with *m*- and *p*-xylene as the sole C source.

Pseudomonas oleovorans was reported to thrive in high concentrations of *n*-octane and 1-octene. Growth of this bacterium with high concentrations of *n*-octane resulted in cell damage, as shown by cell membrane disruption (4, 5). Similarly, Cruden et al. (3) showed that *P. putida* Idaho growing on 20% (vol/vol) *p*-xylene has evident membrane damage in electron microscopic images. We found that *P. putida* DOT-T1 cells growing in the presence of high concentrations of toluene exhibited a wider periplasmic space than cells growing in the absence of toluene. In some cases ribosomes appeared in the periplasmic space, suggesting that damage occurred at the level of the cytoplasmic membrane. We sometimes observed cell membrane evaginations in cells growing on 10% (vol/vol) toluene (Fig. 2B and D); the significance of these evaginations is unknown. *P. putida* Idaho was reported to be extremely sensitive to toluene upon reaching stationary phase; in contrast, the viability of the strain, DOT-T1, isolated in the present study

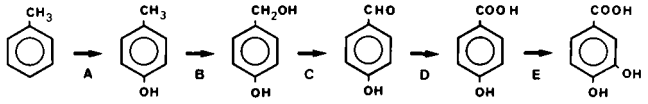

FIG. 3. Proposed catabolic pathway for toluene metabolism by *P. putida* DOT-T1. A, toluene-4-monooxygenase; B, *p*-cresol methylhydroxylase; C, *p*-hydroxybenzyl-alcohol dehydrogenase; D, *p*-hydroxybenzaldehyde dehydrogenase; E, *p*-hydroxybenzoate monooxygenase.

remained high, as the number of CFU per milliliter did not decrease significantly when DOT-T1 reached stationary phase.

The mechanism of toluene tolerance remains to be elucidated, although it has been reported that *P. putida* (9, 22) and *Escherichia coli* (11) strains exposed to aromatics such as phenol and toluene exhibited an increased *trans*-to-*cis* ratio of fatty acids. This resulted in an increase in the transition temperature of the membrane, suggesting decreased fluidity. However, the *trans*/*cis* ratio of fatty acids may not have been the only factor that affected solvent tolerance. In fact, for the first isolate tolerant to high concentrations of toluene, *P. putida* IH-2000 (12, 13), and in the case of *P. putida* DOT-T1 a role of Mg^{2+} has also been implicated in solvent tolerance. The combination of Mg^{2+} plus the increase in the *trans*-to-*cis* ratio of lipids in the cell membrane could be reflected as a decrease in fluidity of the membranes and increased solvent tolerance.

P. putida DOT-T1 seems to grow on toluene via the *p*-cresol pathway described for *P. mendocina* KR1 (24, 29). This is supported by several lines of evidence: (i) cells growing exponentially on toluene oxidized all proposed intermediates of the p-cresol pathway, (ii) several of the pathway intermediates have been identified in culture supernatants, (iii) toluene-4-monooxygenase genes from *P. mendocina* KR1 hybridized total DNA prepared from the DOT-T1 strain, and (iv) enzyme activity of alternative pathways for toluene metabolism was not found. We propose, therefore, that the catabolic pathway used by this strain is the same as that used by *P. mendocina* (Fig. 3).

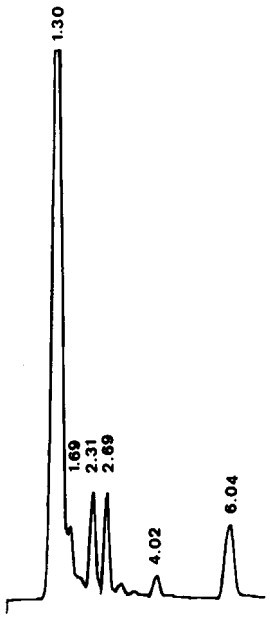

FIG. 4. Accumulation of pathway intermediates after exposure of *P. putida* DOT-T1 to toluene. Bacteria were grown to an OD_{660} of 0.5 on M9 minimal medium with glucose and exposed to toluene for 30 h as described in the text. Culture fluids were analyzed by HPLC. Products identified were toluene (4.02 min), *p*-cresol (2.31 min), *p*-hydroxybenzaldehyde (1.69 min), and *p*-hydroxybenzoic acid (1.30 min).

Genes that encode enzymes for the catabolism of toluene via the *p*-cresol pathway have been located on the chromosome in *P. mendocina* (28), and this may also be the case in *P. putida* DOT-T1, as the ability to grow on toluene was not transferred from this strain to other *P. putida* strains. Further studies are in progress to elucidate the genetic organization of the toluene-4-monooxygenase pathway in *P. putida* DOT-T1 and to identify the genetic components responsible for solvent tolerance in this strain.

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