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Characterization of *Pseudomonas putida* 1K1 Capable of Growing on Extremely High Concentration of Toluene

Cho, Kyung-Yun, Hyo-Kon Chun, Dong-Cho Han and Yung-Hee Kho*

Genetic Engineering Center, Korea Advanced Institute of Science and Technology, P.O.Box 131, Cheongryang, Seoul 130-650, Korea

고농도 Toluene에서 생육 가능한 *Pseudomonas putida* 1K1의 특성

조경연 · 전효곤 · 한동초 · 고영희*

한국과학기술원 유전공학센터

The isolated bacterial strain 1K1 able to grow on extremely high concentration of toluene was morphologically and physiologically best described as *Pseudomonas putida*. This strain could grow on at least eight aromatic compounds, e.g., benzene, benzoate, phenol, *o*-cresol, *m*-cresol, toluene, *m*-toluate, and xylene, but did not grow on alkanes, such as hexane, octane, decane, and cyclohexane. Strain 1K1 could grow on above 95% toluene, but it could not grow on above 1% of other aromatic compounds. In the point of survival, strain 1K1 was resistant to high concentration of alkanes, appreciably resistant to toluene and xylene, and damaged by to other aromatic compounds. Strain 1K1 which grew on high concentration of toluene had irregular cell shape in comparing with normal cell shape of the genus *Pseudomonas*. Strain 1K1 was shown to have at least two aromatic compound dissimilation pathway, one for benzoate and the other for toluene.

Introduction

Organic solvents are in general very toxic to the microorganisms and do not support for the growth of microorganism above the concentration of 1%. Several bacterial strains capable of growing on organic solvents such as toluene, xylene, benzene, hexane, cyclohexane, and phenol had been isolated (1). But the concentration of organic solvents for their bacterial growth was very low. Thus the microorganism couldn't grow on above the concentration of 2%. A few microorganisms which could growth on high concentration of alkanes had been reported (2). But, no microorganism capable of growing on above 2% of toluene had been described. Horikoshi *et. al.* isolated *P. putida* IH-2000, which was able to thrive in high cocentration of tolvene but unable to utilize touene as a sole carbon source (3).

From an industrial viewpoint, the use of microbial strain capable of growing on high concentration of solvent could offer some advantages in the production of chemical feedstuff from solvent or solvent soluble substrate (4), and in the treatment of industrial waste water or waste gas containing solvent (5).

In this paper we deal with the isolation and characterization of *P. putida* 1K1 capable of growing on extremely high concentration of toluene.

Materials and Methods

Media and cultivation condition

Basal minimal salt medium described previously (6) was used. Toluene was added to the sterilized basal minimal salt medum. To avoid evaporation of toluene, flasks were sealed with silicon rubber caps. For agar plate media, toluene was provided as vapor by placing toluene soaked tissue on inside of peri-

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*Corresponding author

dish cover. L-Broth was used as rich medium. Cultures were incubated at 30°C.

Organism

Strain 1K1 was isolated by successive plating of enriched culture broth on minimal salt agar medum supplemented with toluene as a sole carbon source. Taxonomic characterization tests were performed by the conventional test methods (7) and by the API-20NE kit (API SYSTEM S.A. France).

Enzyme assay

The activity of catechol 1, 2-dioxygenase was assayed in cell-free extracts by the procedure of Hegeman (8). Catechol 2, 3-dioxygenase was assayed in cell-free extracts by the procedures of Nozaki and Hegeman (9). Protocatechuate 3, 4-oxygenase was assayed spectrophotometrically by the method of Stainer and Ingraham (10). Protocatechuate 4, 5-oxygenase was assayed by the method of Dagley *et. al.* (11). Gentisate 1, 2-dioxygenase was assayed by the procedure described by Ronald *et. al* (12).

Sample preparation for scanning electron microscope

Bacterial cell fixed with 3% glutaraldehyde was treated with 1% OsO_4 (W/V) and dehydrated with ethanol. After the bacterial sample was dried by critical point drier, it was attached to a pedestal and coated with gold ion by ion sputtering device (13). Prepared sample was investigated using Scanning Electron Microscope JEOL JSM-35CF.

Determining the solvent resistance

Fully grown culture broth was mixed with each solvent with 1:1 ratio. After shaking at 30°C for 1hr, 0.1ml of sample was transfered to 9.9ml of saline to block further toxic effect of solvent to the bacterial cells. And the number of survived cell was counted.

Results and Discussion

Identification of isolate

Strain 1K1 was isolated from the Han River by enrichment cultivation. As shown in table 1, strain 1K1 was motile, gram negative, aerobic, rod bacteria. It could assimilate glucose, arabinose, mannose, gluconate, caprate, malate, citrate, and phenylacate, but did not assimilate mannitol, N-acetyl-glucosamine, maltose, and adipate. This strain showed oxidase positive, nitrate reduction negative, lipase negative, and arginine dihydrolase negative. It hydrolyzed gelatin and starch, produced fluorescent pigment and accumulated poly-β-hydroxybutyrate. White reference to Bergey's Mannual of Systematic Bacteriolgy, this strain was identified as *Pseudomonas putida* (14). This result was also supported by API-20NE kit test.

Table 1. Characteristics of the isolated strain 1K1.

Characteristics	Isolate 1K1
Cell diameter, μm	0.5-0.6
Cell length, μm	1.0-1.6
Flagellar number	>1
Fluorescent pigments	+
Pyocyanin production	-
Phenazine-1-carboxylate	-
Yellow-orange cellular pigments	-
Oxidase	+
PHB accumulation	+
Levan formation from sucrose	-
Gelatin liquefaction	-
Starch hydrolysis	-
Lipase	-
Growth at 4°C	-
Growth at 41°C	-
Denitrification	-
Arginine dihydrolase	+
Glucose, Arabinose, Mannose, Gluconate, Caprate, Malate, Citrate, Phenylacetate	+
Mannitol, N-Acetyl-glucosamine, Maltose, Adipate	-

Growth on extremely high concentration of toluene

P. putida 1K1 could grow on at least eight aromatic compounds, e. g., benzene, benzoate, phenol, *o*-cresol, *m*-cresol, xylene, and *m*-toluate below the concentration of 1% except toluene (Table 2). As shown in Fig. 1, strain 1K1 grew very well on 95% of toluene, and it was appeared that the growth of strain 1K1 should not be affected by the concentration of toluene if there existed water and minerals that could support the cell growth. In the case of the growth on toluene, almost all of cells were located in water layer or on the boundary of water and

Table 2. Effect of substrate concentration on growth of *P. putida* 1K1.

Substrate	Concentration (%)					
	0.05	0.1	0.3	0.5	1.0	20
Benzene	0.69	0.84	0.48	-		
Benzoate	0.41	0.59	0.97	1.01		
Phenol	0.44	-				
o-Cresol	0.44	-				
m-Cresol	0.46	0.22	-			
p-Cresol	-					
Toluene	0.79	0.71	0.67	0.61	0.63	1.37
o-Toluate	-					
m-Toluate	0.36	0.65	1.00	0.59	0.14	-
p-Toluate	-					
Xylene	0.09	0.16	0.17	-		

cf. Each value represents absorbance of culture broth at 660nm after 48hr incubation at 30°C which was compensated by absorbance of control.

toluene, and no living cell was found in toluene layer. In the mean time, strain 1K1 did not grow at all on alkanes, such as hexane, octane, decane, and cyclohexane.

The resistance test of *P. putida* 1K1 to six solvents showed that the strain was strongly resistant to nonpolar aliphatic compounds, such as hexan, decane, cyclohexan, appreciably resistant to two aromatic compounds, toluene and xylene, and damaged by benzene as shown in Table 3. HB grown cells of *P. putida* 1K1 showed also same resistance property. This result reperesented that resistance of the 1K1 to alkanes and two aromatic compounds, toluene and xylene, should be constitutive property.

Though *P. putida* 1K1 was weakly resistant to toluene, it grew with high growth rate on toluene and growth rate of this strain might overcome the death rate, therefore it could survive and might grow on high concentration of toluene. But, *P. putida* 1K1 grew with very low growth rate on xylene (Table 2) and death rate might be overwhelming the growth rate so it could not grow on high concentration of xylene.

Variation of cell shape by growing on high concentration of toluene.

The specific resistance of *P. putida* 1K1 to high

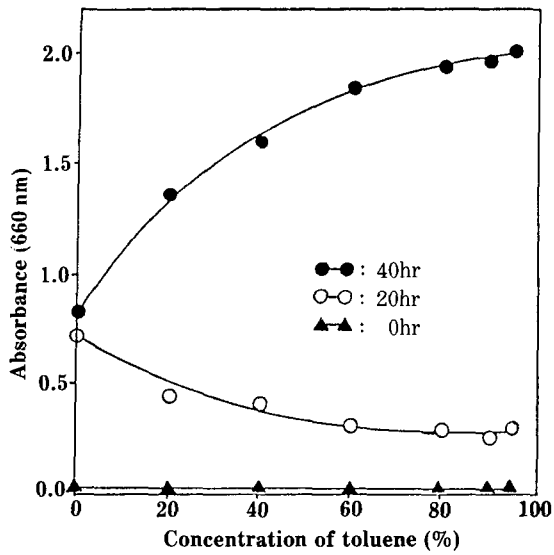

Fig. 1. Growth of *P. putida* 1K1 on the various concentration of toluene.

concentration of toluene revealed that this strain might have special resistance mechanism for toluene. The fact that resistance to toluene was constitutive property and some cells of *P. putida* 1K1 were also damaged by toluene indicated that resistance mechanism was not related to metabolim but related to composition and structure of the cell envelope. To study the resistance mechanism, we inspected the effect of toluene on the cell surface of *P. putida* 1K1. The microorganisms of the genus *Pseudomonas* are all rod and have smooth outer cell surface. Strain 1K1 which grew on L-broth was also rod and had smooth cell surface. But, the bacterial cells, which grew on minimal salt media containing 50% of toluene, had

Table 3. Effect of solvent at the concentrration of 50% on survival of *P. putida* 1K1.

Solvent	LB grown cell	Toluene grown cell
Benzene	L	L
Toluene	S	S
Xylene	S	S
CCl_4	S	S
Cyclohexane	R	R
Decane	R	R
Hexane	R	R

cf. R represent resistant (100 to 90% survial), S: sensitive (89 to 1% survival), and L: lethal (100% death).

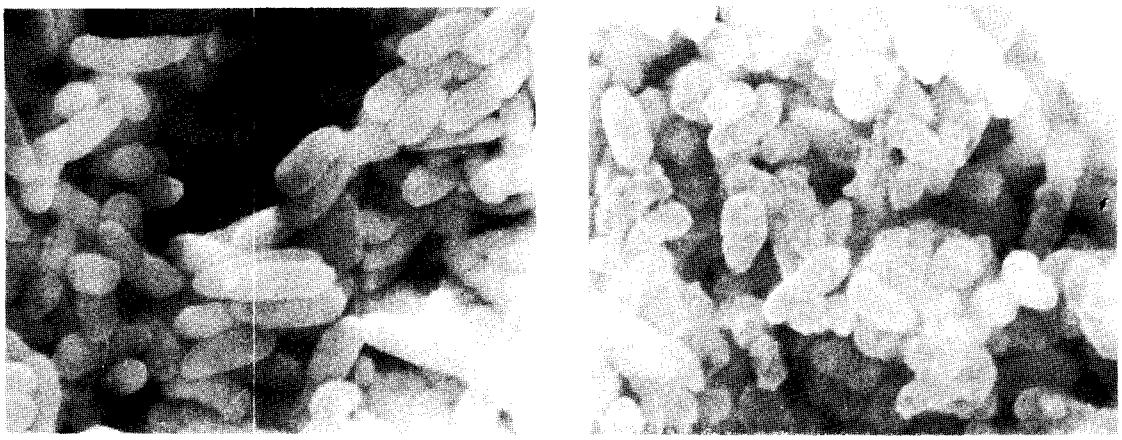

Fig. 2. Scanning electron micrographs of *P. putida* 1K1.
Cells were grown on following media; a) LB b) minimal media containing 50% of toluene.

irregular cell shape and rough cell surface as shown in Fig. 2. And, many ruptured cells appeared among the bacterial cells grown on toluene. These results indicated that the cells of strain 1K1 were severely affected by toluene, so the cell shape was changed and some cells were destroyed.

Biodegradation pathway of *P. putida* 1K1 for toluene

Toluene can be served as a growth substrate for a several *Pseudomonas* species. However, the metabolic pathway for toluene degradation is not the same in all species that have been examined. For example, *Pseudomonas putida* mt-2 oxidizes the methyl group of toluene to form benzyl alcohol (15). Subsequent oxidative reactions lead to the formation of catechol, which is the substrate for *meta*-ring fission of the aromatic nucleus. In contrast, Gibson *et. al.* has revealed two aromatic pathway by which toluene is dissimilated to tricarboxylic acid (TCA) cycle intermediates (16, 17). *Pseudomonas mendocina* oxidizes toluene to protocatechuate, which serve as the substrate for *ortho*-ring fission of the aromatic nucleus. The other strains of *P. putida* initiate the oxidation of toluene by incorporating oxygen in to the aromatic nucleus to form *cis*-toluene dihydrodiol. Further oxidation of *cis*toluene dihydrodiol leads to the formation of 3-methylcatechol, which is then degraded to intermediates of the TCA cycle via *meta*-ring cleavage pathway.

Table 4. Induction of aromatic ring-fission enzymes of *P. putida* 1K1 by several substrates.

Ring-fission enzymes	Succinate	Benzoate	*m*-Toluate	Toluene
Catechol 1, 2-dioxygenase	0.001	0.038	0.004	0.002
Catechol 2, 3-dioxygenase	0.001	0.064	0.006	0.004
Protocatechuate 4, 5-dioxygenase	0.0	0.0	0.0	0.0
Protocatechuate 4, 5-dioxygenase	0.0	0.0	0.0	0.0
Gentisate 1, 2-dioxygenase	0.0	0.0	0.0	0.0

cf. Each value represents specific activity of enzyme.

When *P. putida* 1K1 was incubated with benzoate for 24hr at 30°C, both activities of catechol 2, 3-dioxygenase and catechol 1, 2-dioxygenase were appreciably high to support the growth though these activities were one tenth of those of *P. putida* mt-2 induced by benzoate. But, both of these enzyme activities of *P. putida* 1K1 grown on toluene were as low as those of *P. putida* mt-2 grown on succinate which did not induce the enzymes. In all cases, pro tocatechuate 3, 4-oxygnase, protocatechuate 4, 5-oxygenase, and gentisate 1, 2-dioxygenase activities were not detected. So, it was supposed that *P. putida* 1K1 assimilate toluene and *m*-toluate through another pathway not through catechol or protocatechuate. Though pathway for toluene through 3-methylcatechol existed (17), strain 1K1 was not shown to have this pathway because generally

catechol and methylcatechols were attacked by the same enzymes, catechol dioxygenases (16). Therefore, strain 1K1 might dissimilate toluene by unknown pathway. Anyway, *P. putida* 1K1 was shown to have two metabolic pathways for aromatic compound dissimilation, one is that pathway through catechol for benzoate, and the other is undifined pathway for toluene and *m*-toluate assimilation. This suggestion was also supported by the production of different pigments from *m*-toluate by benzoate grown cell, brown color, and toluene grown cell, pink color. The elucidation of the pathway for toluene assimilation by strain 1K1 requires further studies.

요 약

고농도의 톨루엔에서도 자랄 수 있는 균주 *Pseudomonas putida* 1K1은 benzene, benzoate, phenol, *o*-cresol, *m*-cresol, toluene, *m*-toluate, xylene 등 8종의 방향족 화합물을 분해 이용 성장 가능하였으나 hexane, octane, decane, cyclohexane과 같은 지방족 화합물은 이용하지 못했다. 방향족 화합물의 분해 이용에 있어서 *P. putida* 1K1은 0.1%의 낮은 농도에서는 이들 8종의 화합물을 모두 이용 성장 가능하였지만 1% 이상의 농도에서는 자랄 수 없었고, 다만 toluene의 경우에 있어서만 특이하게도 95%의 고농도에서도 빠르게 성장할 수 있었다. 여러 유기화합물에 대한 이 균주의 저항성을 조사하여 본 결과 1K1은 고농도의 지방족 화합물에 대해 아주 큰 저항성을 갖으나 대부분의 방향족 화합물에 의해서는 쉽게 사멸되었으며, toluene과 xylene에 대해서는 예외적으로 상당한 저항성을 갖고 있었다. 한편, 이 균주는 toluene에서 성장할 경우 toluene의 영향으로 인해 세포의 형태가 정상적인 *Pseudomonas*의 형태와 상당히 다른 모습을 보였다. 또한 이 균주는 benzoate 분해를 위한 대사경로와 함께 toluene 분해를 위한 또다른 경로가 있어 적어도 두개의 서로 다른 방향족 고리분해 대사경로를 갖는 것으로 보여졌다.

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