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Degradation Properties of *n*-Alkane Assimilating *Pseudomonas putida* 3SK Carrying CAM::TOL* Plasmid and NAH Plasmid

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Pseudomonas putida 3SK, which was constructed by the conjugal transfer of the CAM::TOL* plasmid of *Pseudomonas putida* CST3A and the NAH plasmid of *Pseudomonas putida* KCTC 2403 into *n*-alkane assimilating *Pseudomonas putida* KCTC 2405, showed a broad degradation spectrum and floc-forming ability. This strain degraded *m*-toluic acid, naphthalene, camphor and decane simultaneously. Hg^{2+} at the concentration of 1 ppm in the minimal medium could not inhibit the growth of this strain. The degradation of *m*-toluic acid by *Pseudomonas putida* 3SK was not repressed by the easily utilizable compounds, such as glucose and succinate. But, the addition of formalin inhibited the growth of *Pseudomonas putida* 3SK. After the cultivation of this strain on the artificial wastewater containing *m*-toluic acid, naphthalene, camphor and decane for 24 hr, the initial COD value (1500) of the artificial wastewater was declined to 300.

The multi-plasmid strain constructed by the conjugation experiment encounters many environmental factors in industrial wastewater treatment system. These factors may be stimlulative or inhibitory to the degradation of pollutants by the constructed strain, or may not give any effect. In the industrial wastewater treatment system, the strain capable of forming floc is preferred since the cells of floc forming strain are not lost with effluent in the wastewater treatment system. A promising bacterial strain for the treatment of pollutant must possess the following properties; broad degradation spectrum, high degradation rate, floc forming ability and adaptability to adverse conditions. *Pseudomonas putida* 3SK was previously constructed by conjugal transfer of the CAM:: TOL* plasmid of *P. putida* CST3A and the NAH plasmid of *P. putida* KCTC 2403 into *n*-alkane assimilating *P. putida* KCTC 2405 (5, 7).

In this paper, the practical values of *P. putida* 3SK for the treatment of pollutants in wastewater were evaluated in the aspects of degradation spectrum, substrate competition, effect of other compounds, degradation rate and floc formation.

MATERIALS AND METHODS

Bacterial Strain and Media

Pseudomonas putida 3SK was previously constructed by conjugation (5, 7). This strain carries the CAM::TOL* plasmid and NAH plasmid. The media used in this experiment were described previously (4).

Quantitative Assay of Compound

The amount of a known aromatic hydrocarbon solely present in culture broth was measured by spectrophotometer. In the case of the mixture of several compounds, each compound was quantitatively assayed by high performance liquid chromatography. Sample was prepared by mixing 9 volumes of methanol with 1 volume of culture broth and filtering the mixture through membrane filter (0.2 μm). Camphor, naphthalene and *m*-toluic acid were detected by UV detecter, and decane by RI detector.

COD Measurement

The chemical oxygen demand of the sample was measured by A.O.A.C method (2).

Sample Preparation for Scanning Electron Microscope

Bacterial cells fixed with 3% glutaraldehyde was treated with 1% OsO_4 and dehydrated with ethanol. After drying bacterial cell by critical point drier, the dried cells were attached to a pedestal and coated with gold ion

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by ion sputtering device. The prepared sample was observed using JEOL JSM-35CF.

RESULTS AND DISCUSSION

Degradation Spectrum

P. putida 3SK was constructed by the conjugal transfer of the CAM::TOL* plasmid of *P. putida* CST3A and the NAH plasmid of *P. putida* KCTC 2403 into *n*-alkane assimilating *P. putida* KCTC2405. *P. putida* 3SK was capable of degrading the compounds shown in Table 1. This strain had the degradation potentials for camphor, naphthalene, salicylic acid and *m*-touic acid which were acquired by obtaining plasmids (1, 6, 9) and also degraded *n*-alkane (C8-C22) whose degradative genes were encoded on chromosomes. The strain degraded all of the compound efficiently. The metabolic intermediates of the plasmid-encoded degradation pathway could also be degraded by *P. putida* 3SK. However, *P. putida* 3SK carrying CAM::TOL* plasmid and NAH plasmid degraded toluene and *p*-xylene poorly, while *P. putida* mt-2 carrying TOL plasmid degraded toluene very well. This result indicates that the degradation of toluene and xylene by bacterial strain requires a resistant mechanism to these solvents as well as the total dissimilatory pathway.

Degradation of Hydrocarbon in the Mixed State

If a bacterial strain is grown in the media composed of many utilizable compounds as the carbon sources, simultaneous or preferential degradation will occur. In the case of wastewater treatment, a strain capable of degrading pollutants simultaneously is preferred because preferential degradation requires lag time for the induction of enzyme systems for each compound, thus does not produce an efficiency for total degradation of these compounds. To investigate whether *P. putida* 3SK show simultaneous or preferential degradation in the mixed state, *P. putida* 3SK was cultured in the media containing naphtalene, *m*-toluic acid, camphor and decane. The amount of each compoud was monitored by HPLC. As shown in Fig. 1, *P. putida* 3SK was assumed to degrade these compound simultaneously.

Table 1. Degradation spectrum of *Pseudomonas putida* 3SK

Compounds	Gene	*P. putida* 3SK
Toluene, *m*-toluic acid, p-toluic acid, benzene, p-xylene, benzoate benzyl alcohol	TOL	+*
Salicylic acid, naphthalene anthracene	NAH	+
Alkane (C8-C22) fatty acid (C12-C48)	*alk*	+
Camphor	CAM	+

*: Degradation activity.

Effect of Some Compounds

There may be two types of coumpounds which can unfavorably affect the degradation of hydrocarbon by *P. putida* 3SK. One is easily utilizable compounds which may retard the degradation of hydrocarbon by the carbon catabolite repression. The other is the toxic compounds which are bacteriocidal or bacteriostatic. The effects of these two types of compounds on the degradation of hydrocarbon by *P. putida* 3SK were investigated. As shown in Fig. 2, the addition of easily utilizable compound, such as glucose, to the media did not repress the degradation of *m*-toluic acid, but increased the degradation rate of *m*-toluic acid by increasing the number of bacterial cells. The addition of succinate resulted in the same trend. Among the tested compounds (formalin, phenol, aniline and o-cresol) which could not be utilized by *P. putida* 3SK, formalin was very toxic to the growth of *P. putida* 3SK on *m*-toluic acid. Phenol, aniline and *o*-cresol at the concentration of 5 mM showed moderate toxic effects on the growth of *P. putida* 3SK on *m*-toluic acid (Table 2). The problem of toxic compounds in wastewater treatment using *P. putida* 3SK could be solved by the mixed culture with the strains capable of degrading the toxic compounds (1, 3). As shown in Table 2, *P. putida* 3SK was resistant to Hg^{2+} at the concentration of 1 ppm in the minimal medium.

COD Decrease by *P. putida* 3SK

After adjusting the initial COD of liquid media containing minimal salts, *m*-toluic acid, naphthalene, camphor and decane to 1500, the bacterial cells precultured for

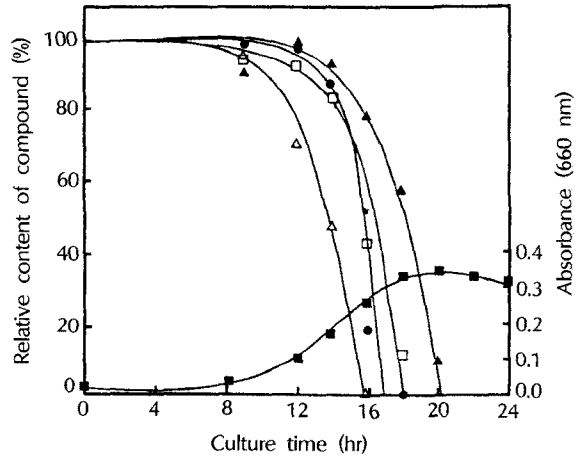

Fig. 1. Degradation of each compound by *P. putida* 3SK in synthetic wastewater containing camphor (●), naphthalene (▲), *m*-toluic acid (△) and decane (□).
Synthetic wastewater was the basal minimal salts media containing each compound at the concentration of 1.25 mM, ■; Cell growth.

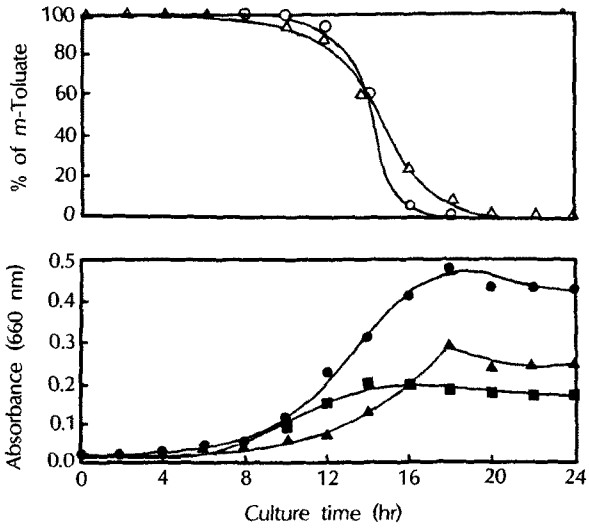

Fig. 2. Effects of dual substrate media containing *m*-touic acid and glucose on the growth of *P. putida* 3SK.
▲▲; growth on *m*-toluic acid, ■■; growth on glucose, ●●; growth on *m*-toluic acid and glucose, △—△; residual *m*-toluic acid (%) in the media containing *m*-toluic acid, ○—○; residual *m*-toluic acid (%) in the media containing *m*-toluic acid and glucose.

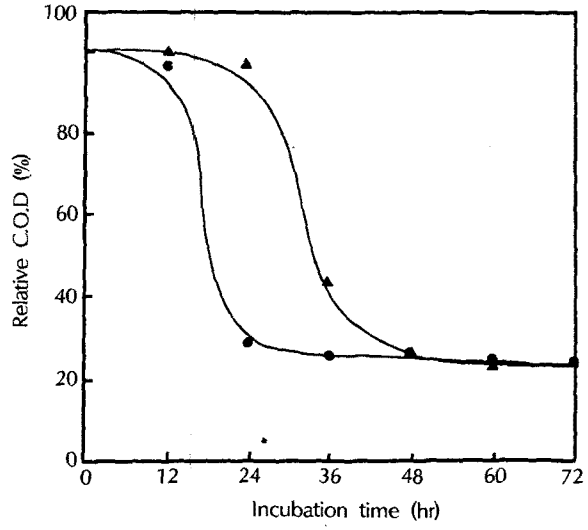

Fig. 3. Decrease of COD in synthetic wastewater by *P. putida* 3SK.
Initial COD: 1450. ●●: *P. putida* 3SK (inoculated by the cells precultured for 12 hr) ▲▲: *P. putida* 3SK (inoculated by the cells precultured for 24 hr).

Table 2. Effect of some compound on the growth[a] of *P. putida* 3SK on *m*-toluic acid

Compound	Culture time / Concn (mM)	24 hr		36 hr	
		1	5	1	5
No addition		0.39		0.37	
Phenol		0.37	0.06	0.34	0.20
Aniline		0.38	0.20	0.35	0.15
Formalin		0.02	0.01	0.03	0.02
Cresol (-o)		0.32	0.03	0.32	0.13
$HgCl_2$*		0.36	0.02	0.34	0.03

* ppm [a] Absorbance at 660 nm.

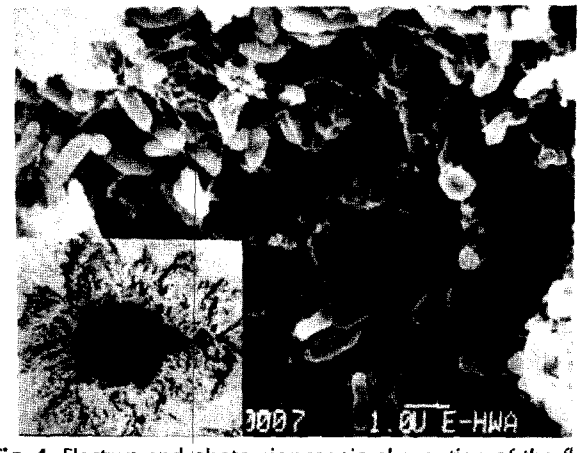

Fig. 4. Electron and photomicroscopic observation of the floc formed by *P. putida* 3SK.
Insert: photomicroscopic appearance, actual size: 1 mm.

12 hr were inoculated. The COD of culture supernatant was measured at the interval of 12 hr. As shown in Fig. 3, about 300 (mgCOD/l) remained in culture supernatant after 24 hr cultivation. The remaining COD may be due to the metabolic products, which could be easily degraded by other bacteria.

Floc Formation by *P. putida* 3SK

The floc forming property of the microorganisms is very important in the wastewater treatment. If a microbe cannot form floc, this strain is easily lost through the overflow water in wastewater treatment system. Thus we constructed *P. putida* 3SK by using a strain, *P. putida* KCTC2405, which is capable of forming floc, as a recipient strain in the conjugation experiment. *P. putida* 3SK formed the floc of cells (0.5~1.5 mm), when grown in the minimal media containing *m*-toluic acid, naphthalene or camphor as a sole carbon source, as shown in Fig. 4. Through the electron microscopic inspection, we found that the floc of *P. putida* was composed of thread-like material and cells of bacteria (Fig. 4). Such a floc-forming phenomena occurred very frequently in nature. It was reported that flocculent was generally composed of high molecular weight material, such as polysaccharide, protein, lipid, or their complex, but their structure was not studied in detail.

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