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Conjugal Transfer of NAH, TOL, and CAM::TOL* Plasmid into *n*-Alkane Assimilating *Pseudomonas putida*

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방향족 탄화수소 분해 Plasmid의 *n*-Alkane 자화성 *Pseudomonas putida*에로의 전이

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The conjugally transferred TOL plasmid or NAH plasmid was stably maintained and expressed in *n*-alkane assimilating *Pseudomonas putida* KCTC 2405. However, these plasmids were not able to coexist in this strain because of incompatibility. The incompatibility of TOL and NAH plasmid was bypassed using CAM::TOL* plasmid, which was constructed by the transposition of only *tol* gene without incompatibility system in TOL plasmid into CAM plasmid. *P. putida* 3SK capable of growing on *m*-toluate, naphthalene, camphor, and *n*-alkane(C8-C24) was constructed by the conjugal transfer of NAH plasmid into *n*-alkane assimilating *P. putida* SK carrying CAM:: TOL* plasmid. CAM::TOL* plasmid in *P. putida* 3SK was stable on the selective media but unstable on the nonselective media.

Certain microorganisms, especially *Pseudomonas* sp., have the capacity to degrade a variety of hydrocarbons and are thus potentially useful in the control of various environmental pollutants. Such organisms often possess degradative plasmids that encode all or part of enzymes related in hydrocarbon oxidative pathways that feed into central metabolism. The plasmids conferring degradation ability for salicylate(SAL), naphthalene(NAH), toluene and xylene(TOL, XYL), camphor(CAM), and octane(OCT) have been well characterized (1-3).

Substrate range of *Pseudomonas* sp. may be extended by constructing strains that carry a number of degradative plasmids. Mutiplasmid strains capable of degrading various hydrocarbons in crude oil have been obtained by plasmid transfer. Friello *et al.*(4) constructed a strain of *Pseudomonas* (termed Superbug) that harbored a hybrid CAM-OCT, an NAH, and a TOL plasmid.

In this paper, we show transfer of various plasimids conferring degradative potential for *m*-toluate, naphthalene, and camphor into *n*-alkane assimilating *P. putida* KCTC 2405 by conjugation and their maintenance and stability in the constructed strain.

Materials and Methods

Bacterial strain and plasmid

The bacterial strains and their carrying plasmids used in this experiment are shown in Table 1.

Media and cultivation

Basal minimal salt medium(BSM) described earlier(5) was used. Carbon sources were added to the sterilized BSM at final concentration of 5 mM. L-Broth (10g tryptone, 5g yeast extract, and 10g NaCl per 1 liter of distilled water) was used as rich

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Table 1. The bacterial strains and their degradative plasmids.

Strain		Plasmid and Genotype	Sources
P.	KCTC1643	TOL	ATCC33015
putida	KCTC2405	*alk*	NCIB9571
	KCTC2403	NAH	NCIB8859
	CST3A	CAM::TOL*, TOLΔ	KCTC8422P
	TK	TOL, *alk*	this experiment
	3K	NAH, *alk*	this experiment
	3SK	CAM::TOL*, NAH, *alk*	this experiment

medium. Agar at 1.5% was added to solidify the media. For agar plate media, carbon sources were provided as vapor by placing naphthalene and camphor crystal, and octane soaked tissue on inside of petri-dish cover. *m*-Toluate(5mM) was directly added to BSM agar. Cultures were incubated at 27 °C, unless otherwise mentioned. Selective medium of exconjugant strain was containing kanamycin and each of a sole carbon source (naphthalene, camphor, and *m*-toluate).

Transfer and detection of plasmid

Conjugation experiments were carried out with broth cultures containing donor strains mixed with the recipient strain in a 1:10 ratio. Cultures were incubated overnight at 30 °C without shaking and plated on the selective media containing kanamycin (100 ug/m*l*) and a single carbon source. The recombinant strains growing on this medium were isolated (6). Plasmid was detected by the procedure of Kado and Liu(7).

Assay of catechol 2,3-dioxygenase activity

In a cuvette having 1 cm light path, 2.8 m*l* of 50 mM phosphate buffer (pH 7.5) and 0.1 m*l* of cell-free extract was placed. To this 0.1 m*l* of 10 mM catechol solution was added and absorbance was taken at 375 nm every 30 sec. A reference cuvette contained 2.9 m*l* of 50 mM phosphate buffer and 0.1 m*l* of cell-free extract. One unit of enzyme is defined as that amount which oxidizes 1 μmole of catechol per min. A molar extinction coefficient of 33,000 was used for these calculations (8). Protein concentration was measured by the phenol method of Lowry *et al.*(9) using crystalline serum albumin as the reference protein.

Plasmid stability

The strain was subcultured at the interval of 12 hrs in the selective media and the nonselective media(LB). Each culture was plated on LB agar media and the colonies formed were toothpicked on to BSM agar media supplimented with naphthalene, camphor, and *m*-toluate, and also on to LB agar media. Plasmid stability was expressed as the percentage of the number of colonies formed on the selective agar media per the number on the LB agar.

Results and Discussion

Transfer of NAH and TOL plasmid

To transfer the TOL plasmid of *Pseudomanas putida* KCTC1643 into *n*-alkane assimilating *P. putida* KCTC2405, plate mating was performed using strain KCTC1643 as donor and strain KCTC2405 as recipient cell. An exconjugant TK was selected on the minimal salt agar media containing *m*-toluate and kanamycin. Exconjugant TK was able to utilize *m*-toluate and octane as a sole carbon source. As shown in Fig. 1, exconjugant TK harboured the TOL plasmid of strain KCTC1643. To determine the transmissibility of the NAH plasmid in *P. putida* KCTA2403 into *P. putida* KCTC2405, plate mating was performed using the kanamycin resistant strain KCTC2405 as recipient cell and the naphthalene utilizing strain KCTC 2403 as donor cell. A kanamycin resistant exconjugant capable of utilizing naphthalene and octane as a sole carbon source was selected and named as exconjugant 3K. This exconjugant harboured the NAH plamid of

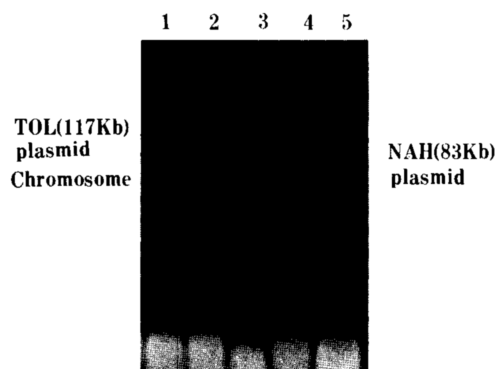

Fig. 1. Agarose gel electrophoretic pattern of plasmids in *P. putida*
lane 1: KCTC1643, lane 2: KCTC2405, lane 3: TK, lane 4: KCTC2403, and lane 5: TK3.

strain KCTC2403 (data not shown). These results indicated that separate transfer of TOL plasmid or NAH plasmid into *n*-alkane assimilating strain KCTC2405 can be successfully achieved.

Incompatibility of NAH and TOL plasmid in *P. putida* KCTC2405

It was reasonable to assume that an exconjugant capable of degrading naphthalene, *m*-toluate and *n*-alkane might be obtained if the NAH plasmid was transferred to the exconjugant TK carrying TOL plasmid. Thus, plate mating was performed using kanamycin resistant exconjugant TK as recipient cell and naphthalene utilizing *P. putida* KCTC2403 as donor cell. Many exconjugants were formed on the selective media containing kanamycin and naphthalene but none of these utilized *m*-toluate as a sole carbon source. An exconjugant TK3, which lost the degradative potential for *m*-toluate but obtained naphthalene catabolism, was selected. Plasmid in exconjugant TK3 was detected on the agarose gel. As shown in Fig. 1, exconjugant TK3 lost the TOL plasmid of exconjugant TK but obtained the NAH plasmid of *P. putida* KCTC 2403. When the TOL plasmid was transferred to exconjugant 3K carrying NAH plasmid all selected exconjugants obtained *m*-toluate catabolism but lost the degradative potential for naphthalene. These results indicate that the NAH plasmid of *P. putida* KCTC2403 and the TOL plasmid of *P. putida* KCTC1643 were not able to coexist in *P. putida* KCTC2405. It was therefore concluded that the NAH and TOL plasmids are incompatible. Austen *et al.*(10) also reported that selection for transfer of the TOL plasmid into and maintenance in a strain whih harboured the NAH plasmid coused segregation of the NAH plasmid and that a similar selection involving transfer and maintenance of the NAH plasmid caused segregation of the TOL plasmid.

The main barrier in the construction of multiplasmid strain by conjugal transfer of degradative plasmid is the incompatibility of the plasmids. Gunsalus *et al.*(11) bypassed the incompatibility of CAM and OCT by fusing the two plasmids with U.V irradiation and forming the recombinant plasmid CAM-OCT. But we bypassed the problem of incompatibility in constructing *n*-octane and camphor utilizing strain using *P. putida* KCTC2405, whose genes for the *n*-alkane assimilatory enzymes were encoded on chromosome as recipient cell. Unlike the report of Friello *et al.* (4), we were not able to construct the strain carrying both TOL and NAH plasmids. This may be due to the incompatibility of NAH plasmid. We used the NAH plasmid of *P. putida* KCTC2403 whose properties were not well characterized. These results indicate that the NAH plasmid of *P. putida* KCTC2403 belongs to the same incompatibility group as the TOL plasmid, namely IncP-9(12).

Transfer of CAM::TOL* plasmid

The recombinant plasmid CAM::TOL* plasmid was previously constructed by transposition of the *tol* gene in TOL plasmid into CAM plasmid (Results being published elsewhere). Using the strain *Pseudomona putida* CST3A containing CAM::TOL* as donor cell and *P. putida* KCTC2405 as recipient cell, plate mating was performed. The resulting mixture of two strains was plated on the selective medium containing camphor as a sole carbon source. The colonies formed on this medium were toothpicked on to the media containing octane and kanamycin, and the media containing *m*-toluate and kanamycin. All kanamycin resistant and camphor utilizing strains could grow on *m*-toluate and octane as a sole carbon source. One of this strain was selected and named as *P. putida* SK.

Furthermore, to transfer NAH plasmid into *P. putida* SK, we performed the plate mating which

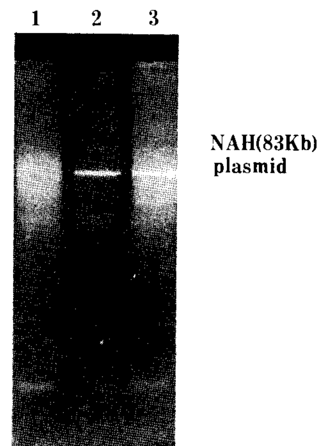

Fig. 2. Agarose gel electrophoretic pattern of plasmids in *P. putida*
lane 1: SK, lane 2: KCTC2403, and lane 3: 3SK.

used kanamycin resistant *P. putida* SK as recipient cell and naphthalene utilizing *P. putida* KCTC2403 as donor cell. An exconjugant 3SK capable of growing on the selective media containing naphthalene as a sole carbon source was able to utilize naphthalene, *m*-toluate, camphor, and octane. As shown in Fig. 2, exconjugant 3SK harboured the NAH plasmid of KCTC2403. The presence of NAH in *P. putida* SK containing CAM::TOL* did not cause the segregation of the latter plasmid.

In our initial attempts, we failed to construct the *m*-toluate, naphthalene and *n*-alkane utilizing strain by the conjugal transfer of NAH and TOL plasmid into *n*-alkane assimilating *P. putida* KCTC 2405 because of incompatibility of two plasmids. However, we have shown here that the incompatibility of NAH and TOL plasmid can be bypassed using CAM::TOL* plasmid.

The detection of CAM::TOL* plasmid in *P. putida* 3SK on the agarose gel was not successful probably because of larger size of the plasmid(>250 Kb) and sticky character of the cells. But we could indirectly manifest the existence of CAM::TOL* plasmid in *P. putida* 3SK by its instability on the nonselective media such as LB media. When the colonies of *P. putida* 3SK formed on the LB media were toothpicked on the minimal salt agar media containing *m*-toluate, naphthalene, camphor, and octane, respectively, several colonies lost *m*-toluate and camphor catabolism simultaneously and irreversibly (Fig. 3). This simultaneous and irreversible loss of *m*-toluate and camphor catabolism in *P. putida* 3SK on the nonselective media provided the indirect evidence for the presence of CAM::TOL* plasmid.

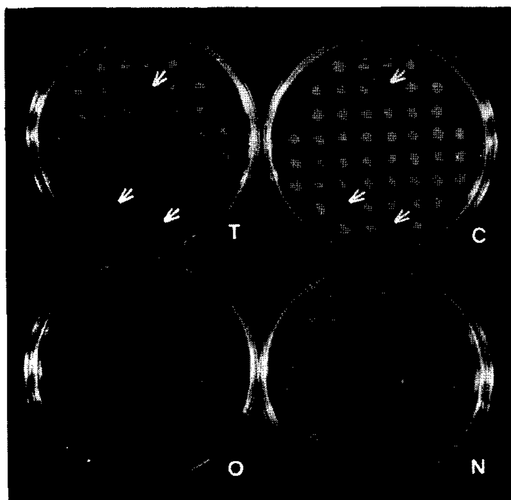

Fig. 3. Simultaneous loss of *m*-toluate and camphor catabolism by the cultivation of *P. putida* 3SK on the LB media.

T: minimal salts agar media with *m*-toluate as sole carbon source
C: minimal salts agar media with camphor as sole carbon source
O: minimal salts agar media with octane as sole carbon source
N: minimal salts agar media with naphthalene as sole carbon source

Table 2. Induction level of catechol 2,3-dioxygenase on various carbon sources

Strain / Carbon source	*Pseudomonas putida*		
	KCTC1643	KCTC2403	3SK
Benzoate	0.80	0.03	0.80
Naphthalene	—	0.03	0.13
Salicylate	—	0.11	0.22
m-Toluate	0.41	—	0.22
Camphor	—	—	0.00
Octane	—	—	0.00

Unit: μmoles/min/mg protein
—: Not determined because of absence of cell growth

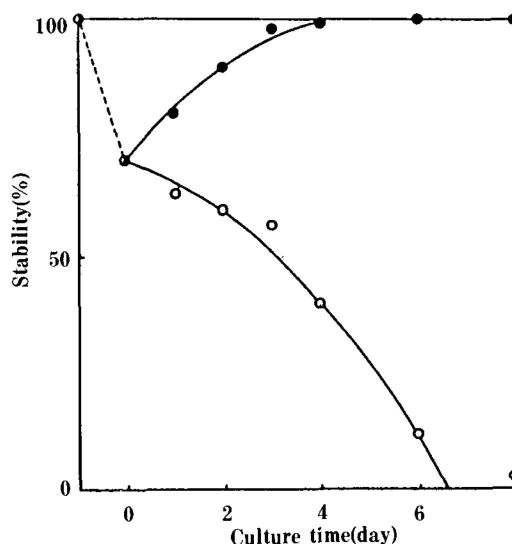

Fig. 4. Stability and instability of CAM::TOL* plasmid in *P. putida* 3SK on the selective media(●) and nonselective media(○), respectively.

Expression and stability of degradative plasmid

Catechol 2,3-dioxygenase catalizes *meta*-cleavage of catechol, the common intermediate of plasmid encoded catabolic pathway for *m*-toluate and naphthalene degradation. *P. putida* KCTC 2405, the recipient cell of plate mating in this experiment, didn't show catechol 2,3-dioxygenase activity. However, when strain 3SK carrying NAH and CAM::TOL* was grown on benzoate which was known as an inducer of catechol 2,3-dioxygenase, the enzyme activity was present. Salicylate, naphthalene and *m*-toluate also induced this enzyme in the strain 3SK (Table 2).

CAM::TOL* plasmid in *P. putida* SK was unstable on the nonselective media but stable on the selective media. As shown in Fig. 4, *P. putida* 3SK simultaneously lost the capacity to utilize *m*-toluate and camphor on the LB media. But, when this strain was grown on the minimal media containing camphor, the ability of strains to grow on camphor and *m*-toluate was stable. NAH plasmid in *P. putida* 3SK was stable on both selective and nonselective media.

요 약

TOL 플라스미드와 NAH 플라스미드는 *n*-알칸을 자화하는 *P. putida* KCTC 2405에 접합에 의해 각각의 이동은 가능하나 두 플라스미드는 불화합성에 기인하여 본 균주내에 공존할 수 없었다. TOL plasmid에서 불화합성 체계는 남겨두고 *tol* 유전자만이 CAM plasmid내로 transposition 되어 형성된 CAM : TOL* 플라스미드는 NAH 플라스미드와 *P. putida* KCTC 2405에서 공존할 수 있어 *m*-toluate, naphthalene, camphor 및 *n*-alkane(C8-C24)를 분해할 수 있는 *P. putida* 3SK 균주를 육종하였다. CAM : TOL* 플라스미드는 선택성 배지에서 안정하였으나 비선택성 배지에서는 불안정하였다.

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