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Identification and Expression of the *cym*, *cmt,* and *tod* Catabolic Genes from *Pseudomonas putida* KL47: Expression of the Regulatory *todST* Genes as a Factor for Catabolic Adaptation

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***Pseudomonas putida* KL47 is a natural isolate that assimilates benzene, 1-alkylbenzene (C_1-C_4), biphenyl, *p*-cumate, and *p*-cymene. The genetic background of strain KL47 underlying the broad range of growth substrates was examined. It was found that the *cym* and *cmt* operons are constitutively expressed due to a lack of the *cymR* gene, and the *tod* operon is still inducible by toluene and biphenyl. The entire array of gene clusters responsible for the catabolism of toluene and *p*-cymene/*p*-cumate has been cloned in a cosmid vector, pLAFR3, and were named pEK6 and pEK27, respectively. The two inserts overlap one another and the nucleotide sequence (42,505 bp) comprising the *cym, cmt,* and *tod* operons and its flanking genes in KL47 are almost identical (>99%) to those of *P. putida* F1. In the cloned DNA fragment, two genes with unknown functions, labeled *cymZ* and *cmtR,* were newly identified and show high sequence homology to dienelactone hydrolase and CymR proteins, respectively. The *cmtR* gene was identified in the place of the *cmtI* gene of previous annotation. Western blot analysis showed that, in strains F1 and KL47, the *todT* gene is not expressed during growth on Luria Bertani medium. In minimal basal salt medium, expression of the *todT* gene is inducible by toluene, but not by biphenyl in strain F1; however, it is constantly expressed in strain KL47, indicating that high levels of expression of the *todST* genes with one amino acid substitution in TodS might provide strain KL47 with a means of adaptation of the *tod* catabolic operon to various aromatic hydrocarbons.**

***Keywords*:** *tod* operon, TetR-type regulator, toluene, catabolic adaptation, *Pseudomonas*

Benzene, toluene and xylenes (BTX) are used as bulk industrial chemicals for solvents and as starting/ intermediate compounds for synthesis of numerous chemicals, including plastics and polymers (Swoboda-colberg, 1995). The total production of BTX in Western European was estimated at around 14 million tons in 2004 (http://www.aromaticsonline.net). These compounds are relatively soluble in water and are toxic. Benzene is also known as a carcinogen. Substantial amounts of BTX are often accidentally introduced into the environment. The most recent disaster involved a mass benzene spill in China's Songhua River in 2005 as a result of an explosion at a chemical plant; this spill seriously threatened drinking water sources. Microbial degradation is one key attribute of the recycling process of those spilled chemicals (van Hamme *et al.*, 2003). Biochemical study of BTX degradation by aerobic bacteria began late in the 1960s, and was undertaken by the Gibson group using *Pseudomonas putida* F1 (Gibson *et al.*, 1968; Gibson *et al.*, 1970).

P. putida F1 utilizes benzene, toluene, and ethylbenzene for growth via a *meta*-cleavage Tod pathway (Gibson *et al.*, 1968). The Tod pathway enzymes are encoded with the *tod* catabolic operon and degrade the aromatic hydrocarbons to TCA cycle intermediates (Finette *et al.*, 1984; Zylstra *et al.*, 1988; Zylstra and Gibson, 1989). The pathway is

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positively regulated by a two-component phosphorelay system, a hybrid sensor kinase (TodS), and a response regulator (TodT) (Lau *et al.*, 1997). However, bulky aromatic hydrocarbons such as biphenyl and propyl-, isopropyl-, butyl- and *sec*-butylbenzene cannot be utilized by this pathway because they cannot induce the *tod* catabolic operon (Choi *et al.*, 2003). Even the induced Tod pathway enzymes cannot completely hydrolyze 2-hydroxy-6-oxo-hexa-2,4-dienoate (HOHD) derivatives due to the limited substrate specificity of TodF (refer to Fig. 1 in Choi *et al.*, 2003) (Cho *et al.*, 2000; Furukawa *et al.*, 1993; Gibson *et al.*, 1968). *P. putida* F1 can also utilize another aromatic hydrocarbon, *p*-cymene, for growth by means of a separate *meta*-cleavage pathway with *p*-cumate as a central intermediate. The pathway enzymes are encoded on two separate *cym* and *cmt* operons, which both are regulated by a TetR-type repressor, CymR (Eaton, 1996; Eaton, 1997). Recently, a *sep* gene cluster, *ttgDEF* genes, encoding a solvent efflux or multi-drug pump was identified downstream of the *todT* gene (Mosqueda and Ramos, 2000; Phoenix *et al.*, 2003). In the chromosome, the *cym*, *cmt*, *tod,* and *sep* gene clusters are positioned in a row within about 45 kilobases.

In a previous study, we isolated F1 mutants spontaneously generated in the presence of biphenyl-loaded vapor and found that they were able to grow on bulky aromatic hydrocarbons such as *n*-propylbenzene, *n*-butylbenzene, cumene, and biphenyl, as well as on original aromatic hydrocarbon growth substrates (Choi *et al.*, 2003). Molecular dissection showed that these mutants displayed a reconstituted hybrid pathway with mutation that was not limited to the *cymR* gene; this allowed the recruitment of CmtE, which is encoded on the *cmt* operon to hydrolyze the derivatives of HOHD formed from new growth substrates, but also in the *todS* gene to allow the *tod* operon to be induced by new chemicals. In this study, we have isolated a *P. putida* strain from soil, the KL47 strain, which utilizes the growth substrate range employed by a hybrid pathway present in the F1 mutants. We then investigated how the catabolic pathway genes present in *P. putida* F1 have evolved in strain KL47 to degrade new aromatic hydrocarbons.

Materials and Methods

Plasmids, bacterial strains and culture conditions

The plasmids used in this study are listed in Table 1. Strain KL47 was isolated from soils in an industrial complex in Changwon, Republic of Korea, using toluene as a carbon and energy source in minimal salt basal (MSB) medium (Stanier *et al.*, 1966). Strain KL47 has routinely been grown in Luria-Bertani (LB) medium (Bertani and Bertani, 1970). Toluene was supplied in the vapor phase as the sole source of carbon and energy in MSB medium. Biphenyl was supplemented on the lids of the plates or added at 0.04% (w/v) in the liquid culture. Other volatile chemicals were also supplied in the vapor phase. *Escherichia coli* DH5α (Ausubel *et al.*, 1990) was used as a host strain for the maintenance of plasmids. To culture transformed *E. coli* or *Pseudomonas*, ampicillin (Ap), gentamycin (Gm), kanamycin (Km), or tetracycline (Tc) was incorporated into the culture

Table 1. Plasmids used in this study

Plasmid	Description[a]	Reference or source
pET-22b(+)	Carries C-terminal His·Tag sequence, Ap^r	Novagen
pBluescript SK(-)	Routine cloning vector, Ap^r	Stratagene
pLAFR3	Broad-host-range cosmid vector, Tc^r	(Staskawicz *et al.*, 1987)
pEN12	Reporter vector for the *tod* catabolic operon, carries P_{todX}-*gfp*, Gm^r	(Choi *et al.*, 2003)
pEN19	Reporter vector for the *cmt* catabolic operon, carries P_{cmtAa}-*gfp*, Gm^r	(Choi *et al.*, 2003)
pEN56	*Nco*I/*Xho*I digested 0.63 kb PCR product, made using primers CTC CGG CCC ATG GGT GAT GCA TC and CGA CTA CTC GAG GCT ATC CTT GAG with chromosomal DNA from strain KL47 as template, inserted into pET-22B(+), TodT-6xHis expression vector, Ap^r	This study
pEK6	Partial *Sau*3AI digested 24,838bp fragment, inserted at *Bam*HI in pLAFR3, contains *tod* and partial *sep* operons, Tc^r	This study
pEK27	Partial *Sau*3AI digested 21,625bp fragment, inserted at *Bam*HI in pLAFR3, contains *cym/cmt* operons, Tc^r	This study

[a]Ap^r, ampicillin-resistant; Gm^r, gentamycin-resistant; Tc^r, tetracycline-resistant

media at the previously indicated amounts (Choi *et al.*, 2003). *E. coli* and *Pseudomonas* cells were grown at 37°C and 28°C, respectively. In liquid culture, cells were grown in a 50-ml culture volume in 250 ml Erlenmeyer flasks with shaking in an incubator at 180 rpm. *P. putida* G7.C-1 is an NAH-7 plasmid-cured derivative of strain G7 (Dunn and Gunsalus, 1973).

Molecular cloning and analyses of DNA and deduced amino acid sequences

A *P. putida* KL47 genomic library was constructed with the pLAFR3 cosmid in *E. coli* VCS257 (Stratagene) and mobilized to *P. putida* G7.C-1 as previously described (Jeong *et al.*, 2003). Recombinant *P. putida* G7.C-1 cells containing toluene and *p*-cymene catabolic genes were positively selected in a Tc-containing MSB medium with a chemical supplied in the vapor phase. Other molecular genetic techniques were performed using standard procedures and as recommended by the supplier of the reagent. The insert in pEK27 and pEK6 was digested with various restriction enzymes and subcloned in pBluescript SK(-) (Stratagene). Nucleotide sequences from the subclones or cosmid vectors were determined by Genotech Co. (Taejeon, Korea) with an automated sequencing unit (ABI PRISM 377, PE Biosystems Inc. USA) using M13 and sequence-based primers. The primers used to sequence the ends of the insert were oligo L3H (5'-GGATGTGCTGCAAGGCG-3') and oligo L3E (5'-GCTTCCGGCTCGTATGTT-3') for the *Hind*III and *Eco*RI ends, respectively. Searches for specific nucleotide or amino acid sequences were carried out using the BLAST program (Altschul *et al.*, 1997) provided by DDBJ/GenBank/EMBL and available on the Internet. The amino acid sequence homology was calculated using the BioEdit program, which can be accessed at http:// www.mbio.ncsu.edu/BioEdit/page2.html. The nucleotide sequence of the partial 16S rRNA gene of strain KL47 was determined by direct sequencing of the PCR product amplified using 27F and 1522R primers (Johnson, 1994) with Ex-Taq DNA polymerase (TaKaRa, Japan). The presence of the structural *cymR* gene in strain KL47 was verified by PCR analysis with primers 5'-ATGAGTCCAAA GAGAAGAACACAGG-3' and 5'-CTAGCGCTTGAA TTTCGCGTACCGC-3'. PCR was carried out for 1 min under previously described conditions (Cho *et al.*, 2000).

Fluorescence and oxygen uptake measurements

Specific fluorescence from cells containing a *gfp* reporter vector was measured as previously described (Kim *et al.*, 2004). Oxygen uptake was measured with a Clark-type oxygen electrode (Rank Brothers, England) with washed cells grown on glucose as previously described (Cho *et al.*, 2000).

Preparation of antigen and polyclonal antibody, and Western blot analysis

The expression of the C-terminal hexahistidine-tagged form of TodT (TodT-6xHis) from *E. coli* BL21(DE3) (pEN56) was carried out as previously described (Cho *et al.*, 2003). The TodT-6xHis was purified by nickel affinity chromatography on Ni^{2+}-NTA columns (Peptron, Korea) as recommended by the manufacturers. The protein concentrations were measured using the BCA protein assay (Pierce) with bovine serum albumin as the standard. The procedure for constructing a polyclonal antibody against TodT- 6xHis follows. A 5-week-old female *Balb/c* mouse was immunized with 30 μg of purified TodT-6xHis antigen emulsified in Freund's complete adjuvant (Sigma, USA). On days 14 and 21, the mouse was given an intravenous, intraperitoneal injection with the antigen emulsified in Freund's incomplete adjuvant (Sigma, USA). After three injections, serum was obtained by sacrificing the mouse. For Western blot analysis of expressed TodT, each lane in SDS-PAGE (15% polyacrylamide) contained 75 μg of cell extracts and 0.2 μg of purified control TodT-6xHis. The gels were blotted onto the Immobilin-P transfer membrane (Millipore) for 1 h at 300 mV. The membrane was then incubated with a 1:1,500 dilution of polyclonal antibody raised against TodT-6xHis for 1 h and then washed twice with a TBST buffer (0.2M Tris, pH 7.6; 1.37M NaCl, 0.1% Tween 20) for 10 min each. The reacting signal was detected by a 1:1,000 dilution of anti-mouse IgG HRP-conjugated antibody (Sigma) using a Western ECL-detection system (Pierce).

Chemicals and enzymes

The aromatic chemicals used in this study were obtained from Aldrich in the U.S.A. Reagents and enzymes used for DNA manipulation were obtained from Takara, Bio-Rad, Promega, or Pharmacia. The commercial phenotype identification API 20NE kit was obtained from bioMérieux (France).

GenBank accession numbers

The GenBank accession numbers for the sequences reported in this study are DQ157469 (*cym*, *cmt, tod,* and partial *sep* gene clusters of strain KL47) and DQ157470 (the partial sequence of 16S rDNA of strain KL47).

Results and Discussion

Identification of strain KL47 and its growth on aromatic hydrocarbons

In a previous study in our laboratory, *P. putida* F1

mutants containing a hybrid pathway consisting of *cym/cmt* and *tod* pathways were found to broaden the spectrum of aromatic hydrocarbons for growth (Choi *et al.*, 2003). In order to study natural adaptation of the pathways, *Pseudomona*s strains capable of growing on toluene and biphenyl were isolated from soil. One of the strains, KL47, showed a very similar range of growth substrates as the laboratory-evolved *P. putida* F1 strains (Fig. 1). The almost complete nucleotide sequence of 16S rDNA in strain KL47 showed 99% sequence identity to *Pseudomonas putida*. Additionally, biochemical testing with API 20NE showed 99.6% identity, which indicates that strain KL47 belongs to *P. putida*. In comparison with strain F1 (GenBank accession number D87108), the four nucleotides are different from the 1367 bases of 16S rDNA, and the two strains have very similar electrophoretic patterns of cellular proteins in SDS-PAGE, indicating that both strains are very similar, but are not identical.

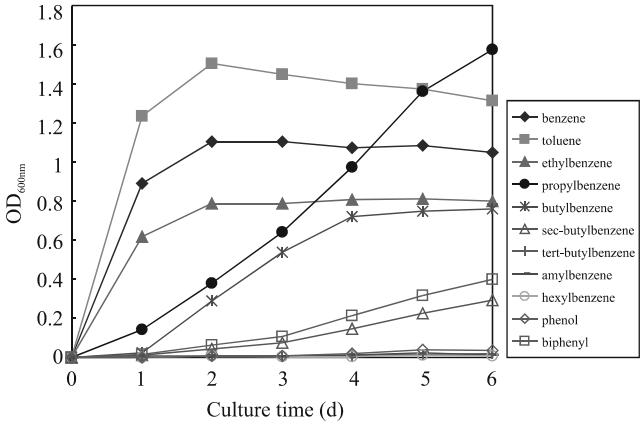

Fig. 1. Growth of *P. putida* KL47 using aromatic hydrocarbon as the sole source of carbon and energy.

Expression of cmt and tod operons in strain KL47

The above results indicate that strain KL47 may possess *tod* and *cym/cmt* operons that are present in strain F1. In order to test the presence of those operons in strain KL47, the previously constructed green fluorescent protein (GFP)-reporter vectors for the *cmt* promoter (pEN19) and the *tod* promoter (pEN12) (Choi *et al.*, 2003) based on strain F1 were introduced into strain KL47. GFP induction by various chemicals was determined using the resulting recombinant KL47 strains. The results are shown in Table 2 and indicated that, in strain KL47, the *cmt* operon is constitutively expressed and the *tod* operon is induced by toluene and biphenyl, the latter of which does not elicit the expression of *tod* operon in strain F1, but does in the adapted F1 strains (Choi *et al.*, 2003). Additionally, due to the constitutive expression of *cym/cmt* operons, washed cells of strain KL47 that were grown on glucose showed high oxygen consumption (greater than 10 O_2 μM/min/OD_{600}) in the presence of 2,3-dihydroxybiphenyl, catechol, 3-, 4-methylcatechol, *p*-xylene, and *p*-cymene. The catechols are substrates for 2,3-dihydroxy-*p*-cumate dioxygenase and *p*-xylene for *p*-cymene monooxygenase. These results indicate that strain KL47 has expression patterns of *tod* and *cym/cmt* operons similar to those present in biphenyl-adapted F1 mutants with broad growth-substrate specificity, as characterized previously (Choi *et al.*, 2003).

Table 2. Expression of *cmt* and *tod* catabolic operons in *P. putida* KL47 and F1 strains[a]

Strain	Carbon source	OD_{600nm}	Specific GFP expression[b]
KL47(pEN-19)	Succinate	0.9 ± 0.2	106 ± 3
KL47(pEN-19)	Succinate + *p*-cymene	1.5 ± 0.4	122 ± 16
F1(pEN-19)	Succinate	0.9 ± 0.0	3.0 ± 0
F1(pEN-19)	Succinate + *p*-cymene	2.0 ± 0.7	57 ± 6
KL47(pEN-12)	Succinate	0.8 ± 0.0	5.4 ± 0.3
KL47(pEN-12)	Succinate + toluene	2.7 ± 0.3	469 ± 73
KL47(pEN-12)	Succinate + biphenyl	1.6 ± 0.0	49 ± 2
F1(pEN-12)	Succinate	0.8 ± 0.0	2.9 ± 0.4
F1(pEN-12)	Succinate + toluene	2.8 ± 0.3	692 ± 16
F1(pEN-12)	Succinate + biphenyl	0.8 ± 0.0	3.6 ± 0.2

[a]Cells were grown for four days on the carbon source(s) indicated in MSB medium with shaking as described in Materials and Methods.
[b]Units are arbitrary.

Cloning and analysis of the deduced amino acid sequences of cym/cmt and tod operon genes from strain KL47

In order to identify the genes responsible for the degradation of those aromatic hydrocarbons in strain KL47 and to compare differences in genes between strains F1 and KL47, a genomic library was made using pLARF3, as described in Materials and Methods. pLAFR3 is a cosmid vector and it is capable of replicating in *Pseudomonas*. The genomic library in *E. coli* cells was transferred via conjugation to *P. putida* G7.C-1, which cannot grow on those chemicals. The recombinant G7.C-1 strains obtained were screened for their ability to grow on toluene or *p*-cymene as a sole source of carbon and energy. The plasmids that have been obtained from the recombinant *P. putida* G7.C-1 were named pEK6 and pEK27, respectively. *E. coli* DH5α (pEK6) formed indigo in LB agar, but *E. coli* DH5α (pEK27) did not. The recombinant strains that can grow on biphenyl have not been obtained, which indicated that the size of gene cluster for biphenyl catabolism in strain KL47 is beyond the DNA size for the cosmid package. The inserts in pEK6 and pEK27 overlapped and enclosed 42,505 base pairs. The genetic organization obtained from the nucleotide sequence analysis of the insert in pEK6 and pEK27 is shown in Fig. 2.

The genetic organization obtained from the recombinant plasmids was almost identical to that found in strain F1, and the nucleotide sequence of the cloned genes showed more than 99% identity to that of strain F1 (GenBank accession numbers J04996, M64080, U09250, U24215, and U72354) and of *cmt* genes from *P. putida* CE2010 (GenBank accession number AB042508), and the *tod* genes in *P. putida* DOT-T1 (GenBank accession number Y18245) and *P. putida* T-57 (Faizal *et al.*, 2005). The deduced amino acid sequence of the cloned genes identified two new genes encoding dienelactone hydrolase (named *cymZ*) and a TetR-type transcriptional regulator (named *cmtR*). Their locations in the gene cluster are indicated in Fig. 2. Dienelactone hydrolase is found in the bacterial intradiol dioxygenase-dependent degradation of chloroaromatics (Ngai *et al.*, 1987). With 55% identity and 66% similarity, the deduced amino acid sequence of the *cymZ* gene is most homologous to the gene tag, Bcep02004989, in the *Burkholderia fungorum* LB400 genome. The *cmtR* gene was identified in the place of the *cmtI* gene of previous annotation (Eaton, 1996). The deduced amino acid sequence of the *cmtR* gene showed 37% identity and 53% similarity to CymR (Fig. 3). It has been previously shown that the *cmtE* gene, which is located downstream of the *cmtR* gene, is constitutively expressed by the mutation of the regulator *cymR* gene (Choi *et al.*, 2003). Thus, it is concluded that the *cmtR* gene is also a component of the *cmt* operon and is not expressed independently. Because CymR controls the *cmt* operon (Eaton,

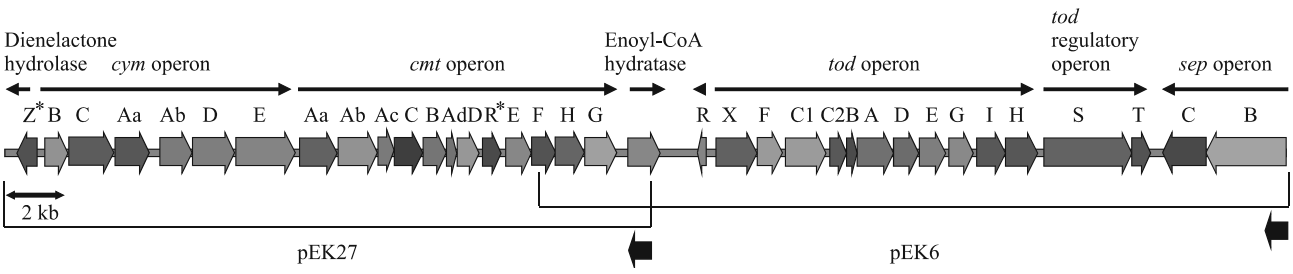

Fig. 2. Physical and genetic map of *cym, cmt, tod,* and *sep* operons from strain KL47. The insert in pK27 and pEK6 is presented in the map. The small and large arrowheads represent the directions of transcription and the position of the *lac* promoter in pLAFR3, respectively. The function of each gene has been described in previous reports (NCBI GenBank accession number DQ157469). The newly identified genes are marked with an asterisk.

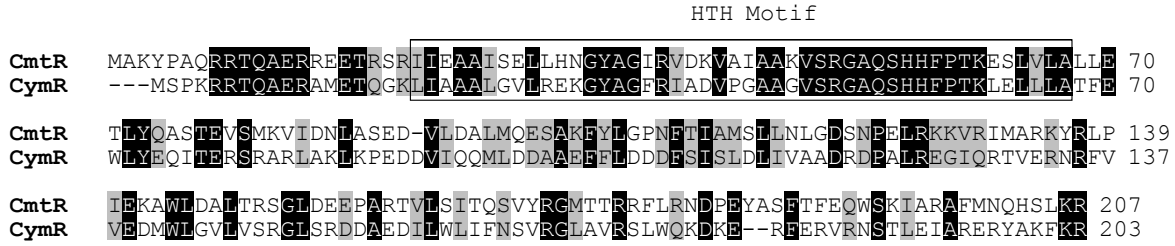

Fig. 3. Alignment of amino acid sequences of CymR and CmtR. The DNA-binding domain is boxed.

1997), CmtR may be in charge of controlling genes other than the *cmt* operon, such as extra genes for surviving with the toxic aromatics. In the *p*-cumate catabolic *psb* genes from a photosynthetic bacterium, *Rhodopseudomonas palustris*, the *psbI* gene, which shows high amino acid sequence homology with CymR, is also found in the middle of the catabolic genes (Puskas *et al.*, 2000), although the genetic organization of the *psb* gene clusters is different from that found in the KL47 or F1 strain.

Lack of cymR and amino acid substitutive mutation in todS from strain KL47

In order to investigate the molecular mechanisms underlying the metabolic versatility present in strain KL47, the nucleotide sequence encoding the regulators CymR, TodS, and TodT and the promoters controlling the expression of *cymR* and *todS* genes were further compared to those in strain F1. The *cym/cmt* and *tod* catabolic promoters and the *todT* gene in strain KL47 have the same nucleotide sequences as those in strain F1. Because the *cymR* gene was not cloned in pEK27, the presence of *cymR* in strain KL47 was determined by PCR analysis. PCR with primers based on the *cymR* gene, as described in Materials and Methods, yielded an expected size of 0.6 kb from strain F1, but not from strain KL47 (data not shown), indicating that a functional *cymR* gene was not present in strain KL47. Furthermore, the deduced amino acid sequence of the $todS_{KL47}$ gene has Arg at position 153, which is located at the boundary of the first PAS domain (Choi *et al.*, 2003), where Lys is located in strain F1. Because TodS is known as an effector sensor, it is predicted that the amino acid substitution may play a role in the recognition of new growth substrates, as previously described (Lau *et al.*, 1997; Ramos-Gonzalez *et al.*, 2002; Choi *et al.*, 2003).

Western blot analysis for the expression of todT from the strains F1 and KL47

Sometimes the expression of the regulatory gene for the catabolic operon is not always constitutive, but is instead controlled by environmental conditions such as the presence of effector molecules (Tropel and van der Meer, 2004; Velazquez *et al.*, 2005). As an alternative approach in our search for the induction of the *tod* catabolic operon in the presence of biphenyl, the level of TodT protein in strains F1 and KL47 was examined by Western blotting with anti-TodT antibodies, as described in Materials and Methods. The results are shown in Fig. 4. Both F1 and KL47 cells grown in LB complex medium did not express TodT. In strain F1, the expression of TodT was very low or negligible in the MSB medium with succinate or biphenyl plus succinate, and was induced in the presence of toluene. In contrast, in strain KL47, the level of TodT was almost the same as that shown in cells grown on succinate, biphenyl, or toluene. Because the *todST* genes most likely form a transcriptional unit, the expression level of TodS and TodT in the two strains may be the same. This result indicates that the constitutive expression of TodS and TodT in strain KL47 with minimal basal salt medium might be another possible factor governing the induction of the *tod* catabolic operon and may lead to versatile catabolism of strain KL47. The constitutive expression of the *todST* genes was also observed in a toluene-tolerance strain, *P. putida* DOT-T1E (Mosqueda *et al.*, 1999). Additionlly, experiments with a chemostat showed that the expression of another two-component regulatory system coded in the *styST* genes controlling the expression of styrene catabolic operons in strain *P. putida* CA-3 was growth condition-dependent (O'Leary *et al.*, 2002). It would be interesting to investigate further how these regulatory operons, including the *todST* genes, are controlled by culture conditions.

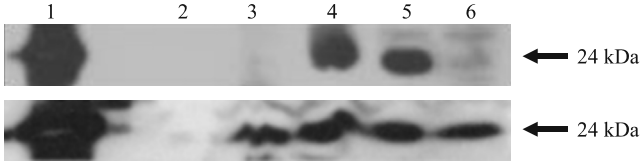

Fig. 4. Expression of TodT in strains F1 and KL47, as detected by Western blot analysis with polyclonal antibody. Lane 1 is purified TodT-6xHis. The upper and lower panels are from cell extracts of *P. putida* F1 and KL47, respectively, grown in LB (lane 2), MSB + succinate (lane 3), MSB + toluene (lane 4), MSB + succinate + toluene (lane 5), and MSB + succinate + biphenyl (lane 6).

Conclusions

Microbial adaptation to man-made chemicals is an important feature in environmental microbiology because it contributes to the recycling of synthetic chemicals (van der Meer *et al.*, 1992; Song *et al.*, 2004; Kim *et al.*, 2005). Because toluene is a ubiquitous pollutant, bacterial strains with common metabolic pathways can be easily isolated all over the world. One aerobic toluene catabolism is carried out by the Tod pathway present in *P. putida* F1. High sequence homology in the nucleotide sequences for the *tod* genes from *P. putida* DOT-T1E (Mosqueda *et al.*, 1999), CE2010 (Ohta *et al.*, 2001), T-57 (Faizal *et al.*, 2005), and KL47, which were isolated in Spain, Japan, and the Republic of Korea, indicates that all of these strains contain the same Tod pathway. Those strains are further adapted to expand the range of growth substrates to (polychlorinated) biphenyl (KL47 and CE2010) or to be solvent-tolerant (CE2010, DOT-T1E and T-57), providing usefulness not only

for biodegradation, but also for biotransformation in the presence of solvent. *P. putida* KL47 is identified in this study as possessing almost the same *cym, cmt,* and *tod* gene clusters as *P. putida* F1, but the strain is defective in the *cymR* gene and shows a higher expression of *todST* genes with one amino acid substitution in TodS, leading to constitutive expression of *cym/cmt* operons and a changed inducer spectrum of TodS. Strain KL47 is supposed to be an environmental version of the laboratory-evolved *P. putida* F1 mutants that have a broad growth-substrate range (Choi *et al.*, 2003). This result indicates that the presence of multiple pathways for similar substrates in one strain can be beneficial for building up a new degradation pathway, such as a hybrid pathway for xenobiotic compounds, even in natural environments. In this type of genetic adaptation, it can be concluded from this and previous studies that mutation in regulatory function is more critical than mutation in structural genes.

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