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Degradation of Trichloroethylene by a Growth-Arrested *Pseudomonas putida*

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A toluene-oxidizing strain of *Pseudomonas mendocina* KR1 containing toluene-4-monooxygenase (TMO) completely degrades TCE with the addition of toluene as a co-substrate in aerobic condition. In order to construct *in situ* bioremediation system for TCE degradation without any growth-stimulating nutrients or toxic inducers such as toluene, we used the carbon-starvation promoter of *Pseudomonas putida* MK1 (Kim, Y. *et al.*, *J. bacteriol.*, 1995). Upon entry into the stationary phase due to the deprivation of nutrients, this promoter is strongly induced without further cell growth. The TMO gene cluster (4.5 kb) was spliced downstream of the carbon starvation promoter of *Pseudomonas putida* MK1, already cloned in pUC19. TMO under the carbon starvation promoter was not expressed in *E. coli* cells either in stationary phase or exponential phase. For TMO expression in *Pseudomonas* strains, *tmo* and carbon starvation promoter region were recloned into a modified broad-host range vector pMMB67HES which was made from pMMB67HE (8.9 kb) by deletion of *tac* promoter and $lacI^q$ (about 1.5 kb). Indigo was produced by TMO under the carbon starvation promoter in a *Pseudomonas* strain of post-exponential phase on M9 (0.2% glucose and 1mM indole) or LB. 18% of TCE was degraded in 14 hours after entering the stationary phase at the initial concentration of 6.6 μM in liquid phase.

Key words: *trichloroethylene, Pseudomonas putida, carbon starvation promoter, toluene monooxygenase*

INTRODUCTION

Trichloroethylene (TCE) is a major contaminant found in U.S.E.P.A. superfund sites. The large scale contamination by TCE cause a serious environmental problem because of its carcinogenecity. The degradation of TCE by the indigenous microorganism is extremely slow and produces the secondary toxic chemicals such as dichloroethylene or vinylchloride under the anaerobic condition [1]. Recently, more attention has been paid to the aerobic digestion of TCE. TCE is known to be completely degraded through the cometabolism of a certain *Pseudomonas* strain to CO_2, Cl^-, and several nonvolatile products like formic acid and glyoxylic acid which can be readily mineralized by other bacteria in nature [2, 3]. Although little has been known about the degradation mechanism of TCE, it has been reported that several microbial oxygenases which intrinsically catalyse aliphatic or aromatic hydrocarbons e.g. toluene mono- (TMO) [1, 4] or dioxygenase (TDO) [3] and phenol hydroxylase [5] can decompose TCE or other chlorinated compounds. However, most of xenobiotic bacteria indigenous to the polluted envirommenent exhibit the catalytic activities only during the growing phase and also require the inducer like toluene or benzene for the maximum activity. The addition of huge amount of nutrients to sustain the cell growth and the toxic inducers are costly and also cause another pollution on site.

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To uncouple the biodegradation phase of TCE from the growing phase, the carbon starvation promoter (CSP) of *P. putida* was applieded to *in situ* TCE bioremediation. The promoter is controlled by σ^{54} and only induced when the cell enter the stationary phase due to the carbon deprivation without any inducer. It was previously reported that TMO controlled by *E. coli* starvation promoter such as *cstC* and *groEL* can degrade TCE and phenol during the stationary phase [4]. TCE degradation by tac promoter-controlled gene fragment from *P. mendocina* has been also tried in *E. coli* [1]. However, these systems cannot be applied to the real fields because *E. coli* is not indigenous to the polluted sites and IPTG is necessarily required for the activity in latter case. In general, *Pseudomonas* is a well-known microgranism to have an intrinsic activity of being able to degrade toxic chemicals like aromatic or aliphatic compounds as well as to show the strong resistancy against various kinds of toxic solvents. Therefore, we used *Pseudomonas* strain as a source of TCE degrading gene and an expression host as well. From the basis of these literatural backgrounds, we devised a *Pseudomoas* promoter-driven *in situ* bioremediation system.

The advantages of our TCE-degrading construct are as follows: first, since it shows little dependence of the biodegradation activity on the active growth, we can minimize the need for nutrients to sustain the activity and at the same time, the competition between the substrates for growth and target polluents which sometimes results in the negative effects on the biodegradation efficiency; second, the optimized gene expression for TCE degradation can be achieved without

Table 1. Strains and plasmids used in this study

Bacterial strain or plasmid	Relevant characteristics	Reference or source
Strains		
E. coli		
DH5α'	*hsdR17*($r_K^- m_K^-$) *supE44 thi-1 RecA1 relA1 gryA (lacIZYA-argF)*	NEBioLab
JM109	JM107 *recA1*	NEBioLab
Pseudomonas putida		
JS388	PpG2 *recA*	11
MK1	Derivative of ATCC12633: Rif^r	10
AMS1000	JS388 with pAM103	This study
Pseudomonas fluorescens		
FAJ2025	OE28.3 *recA*	12
AMS2000	FAJ2025 with pAM103	This study
Pseudomonas mendocina		
KR1	Original strain of *tmoABCDEF*	13
Plasmids		
pRK600	Cm^r *ori* ColE1 RK2-Mob^+ RK2-Tra^+	ATCC#
pGEM7Zf(+)	T7/SP6 cloning and transcription vector	Promega
pMMB67HE	*Tac* expression cloning vector with cloning sites of pUC18; Ap^r	ATCC#
pMMB67HES	pMMB67HE with *PvuII-HindIII* fragment deleted; Ap^r	This study
pMKU101	pUC19 with 0.65kb-CSP; Ap^r	10
pMKY341	pT7-5 with *tmo*ABCEDF	14
pAM101	pGEM7Zf(+) with *tmo*ABCEDF	This study
pAM102	pMKU101 with *tmo*ABCEDF	This study
pAM103	pMMB67HES with CSP and *tmo*ABCEDF	This study

#American Type Culture Collection.

the exogenous inducer. To improve the ability of TCE degradation, the screening of TCE-resistant *Pseudomonas* strain and the chromosomal integradation for the stable expression of the system should be addressed to the future work.

All strains and plasmids used in this study are listed in Table 1. 50 ml of M9 or LB medium with 0.1% glucose and 1000 μg/ml carbenicillin in a 250 ml-flask was used for most of batch cultivation. *Pseudomonas* Isolation Agar for the isolation of *Pseudomonas* transformants from *E. coli* was purchased from Difco Co. The cell growth was determined by the absorbance at 660 nm and the residual glucose concentration in culture broth was determined by using Glucose [HK] 10 enzyme kit from Sigma Co. The total cell protein (in milligram per milliliter) was determined by the Bio-Rad DC protein assay kit (Bio-Rad, CA) using bovine serum albumin as a protein standard. Indole and indigo dye for an assay of TMO were purchased from Sigma Co. All plasmid manipulations were performed in *E. coli* DH5α' or JM109. For the transformation of a plasmid from *E. coli* to *Pseudomonas* strains, the electroporation or triparental mating in the presence of pRK600 (helper plasmid). The donor, recipient and helper cells were withdrawn respectively at the ratio of 1:2:1 and after filtering each broth by 0.2 μm syringe filter (Nalgene Co.), the filter was placed on LB agar plate. After 8 hours incubation at 30°C, the mating mixture grown on the filter were suspended in 0.1% phosphate buffer and then streaked on the appropriate selective agar. Because the ampicillin is heat labile and *Pseudomonas* strains are generally known to resist ampicillin, we used carbenicillin upto 1000 μg/ml to select the correct *Pseudomonas* transformants.

The indigo assay for TMO was carried out as decribed previously [6]. 1 mM of indole was initially added for the assay. Indigo produced was measured by removing 1.0 ml of the culture broth at various time intervals and extracting with an equal volume of ethyl acetate. After the centrifugation to separate both phase, the absorbance of ethyl acetate solution at 600 nm was measured. Because the indole considerably inhibited the initial growth of *Pseudomonas* sp. even at low concentrations, it was sprayed on the colonies of agar plates after substantial initial growth had occurred.

The degradation of TCE was determined by injecting 500 μl of gas samples from the headspace into a gas chromatograph (HP5890) equipped with 30 m DB624 capillary column from J&W Scientific Co. and a photo ionization detector (PID). 160 ml of serum cap vials containing 50 ml of M9 (0.1% glucose) medium were used for batch cultivation at 30°C. A calculated amount of TCE was injected to each serum bottle by a gas-tight syringe right after the inoculation. The amount of TCE in the culture broth was calculated from TCE concentration in the headspace [7]. At each time, 0.5 ml of culture broth and headspace were sampled for measuring the cell growth and TCE degradation, respectively.

ApaI and EcoRI were created at both ends of *tmo* gene by PCR because of absense of the appropriate restriction sites to be used for the cloning. The TMO in pMKY341 was used as a template of PCR. Before the direct cloning into pMMB67HE, a broad host range vector, a commercial vector, pGEM7Zf(+), was used to facilitate the accomodation of PCR products of *tmoABCDEF* gene (4.5 kb) and to verify *tmo* expression at this step. After the sequential digestions of PCR products of *tmo* with *ApaI* and *EcoRI*, it was spliced downstream of T7 promoter of pGEM7Zf(+) digested with the same restriction enzymes.

Since TMO oxidizes indole to indigo, a deep blue dye, TMO expression in *E. coli* cells is indicated by the presense of indigo color of the colonies grown on LB

agar plates (the tryptophan in LB is first converted to indole by the tryptophanase of *E. coli*) [6]. With this construct, we confirmed the expression of *tmo* gene of pAM101 in *E. coli* DE3 by the addition of IPTG.

A broad host range vector, pMMB67HE (8.9 kb) (ATCC No. 37623), was used for the expression of *tmo* gene in *Pseudomonas* sp. The plasmid can be replicated and highly express the cloned gene by *tac* promoter in both *P. putida* and *E. coli* [8]. In order to restrict TMO expression only under the control of CSP of *P. putida*, the plasmid pMMB67HES was constructed from pMMB67HE by deleting the DNA region of *PvuII-EcoRI* (about 1.5 kb) including the region of tac promoter and *lacI*^{q}. (Fig. 1)

To begin with, *tmo* was excised from pAM101 with *ApaI* and *EcoRI* and cloned into the same sites of pMKU101, a commercial plasmid pUC18 carrying CSP of *P. putida* MK1. The construct was designated by pAM102. At this step, we tried to express *tmo* of pAM102 in *E. coli*. M9 agar plate with 0.05% glucose and 1 mM indole were used to isolate a transformant producing deep blue color. However, *tmo* under CSP of *P. putida* MK1 was not expressed in *E. coli* on either LB or low glucose-M9 agar plate containing indole. Although *E. coli* genes can be easily expressed in *Pseudomonas* sp., it is difficult to express *Pseudomonas* genes in *E. coli* because some *Pseudomonas* promoters do not follow a typical -10/-35 concensus sequences [9]. Since the both CSP and TMO were originated from *P. putida*, it is most likely that TMO is expressed better in *Pseudomonas* than *E. coli*.

In the second step, we subcloned the whole DNA fragment of CSP:*tmo* from pAM102 to pMMB67HES for the TMO expression in *Pseudomonas*. At first, the whole region of CSP and *tmo* from pAM102 was excised with *SphI* and *EcoRI* and then ligated to pMMB 67HES which was also digested with same restriction enzymes (Fig. 1). The final construct was named pAM 103.

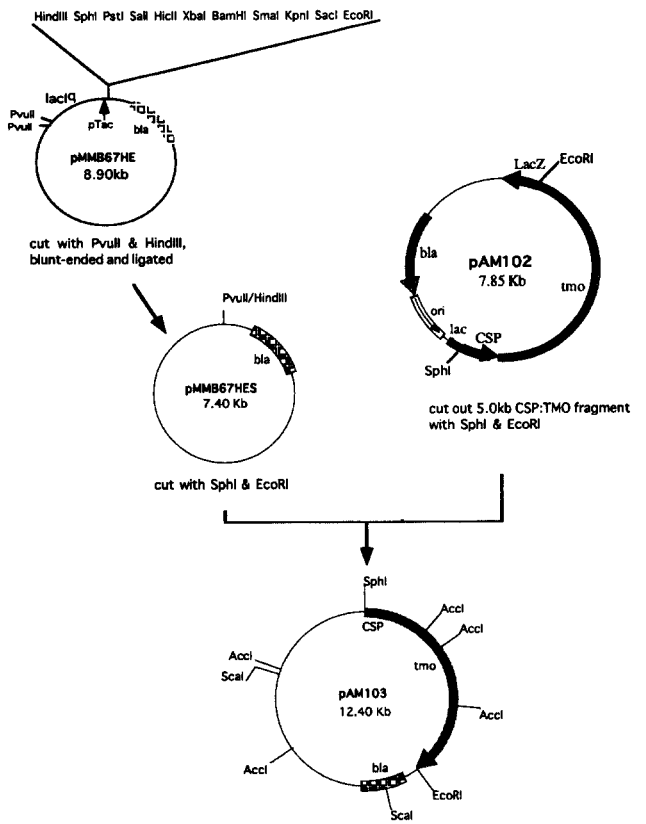

Fig. 1. Construction of TMO expression vector pAM103.

By triparental mating in the presence of a helper plasmid, pRK600, pAM103 was transformed into several *Pseudomonas* strains. In order to enhance the genetic stability of the plasmid in host cells, two *recA* mutants of *P. putida* JS388 and FAJ2025, were also used as expression hosts. However, we could not observe any significant difference with respect to the amount of indigo produced from indole between *recA* mutants and their parental strains, PpG2 and OE28.3 (data not shown). It was found that *P. putida* JS388 and its parental strain PpG2 were the best strains for TMO expression by indigo assay. After overnight incubation at 30°C, the recombinant *P. putida* carrying the plasmid pAM103 (designated by *P. putida* AMS 1000) showed deep blue color both on M9 agar plate and in M9 liquid culture with 0.05% glucose and 1mM indole (Fig. 2).

In M9 liquid culture, the indigo started to accumulate in the culture broth of *P. putida* AMS1000 and *P. fluorescens* AMS2000 when the cells entered the stationary phase whereas same strains without the plasmid did not show indigo production during all the experimental periods. We finally could get 0.0088 and 0.0058 mmoles of indigo from 0.2 and 0.1 mmoles of indole added in *P. putida* AMS1000 and *P. fluorescens* AMS2000, respectively. Because one molecule of indigo is synthesized from two molecules of indole, there was ca. 8.8 and 11.5% conversions of indole to indigo in *P. putida* AMS1000 and *P. fluorescens* AMS 2000, respectively. As a control, we performed the TMO expression of *P. mendocina* KR1, from which TMO was originated, in M9 minimal medium with 0.15% glucose (data not shown). The pattern of indigo production was totally growth-associated in *P. mendocina* KR1.

Because the final goal of this study is to construct a recombinant *Pseudomonas* strain that can completely mineralize TCE on site in a growth-arrested stage, we studied the TCE degradation pattern by *P. putida* AMS1000 by using 160 ml of serum cap vials as a culture vessel. Three different concentrations of TCE (6.6, 56.0 and 139.1 μM in liquid phase) were applied for the

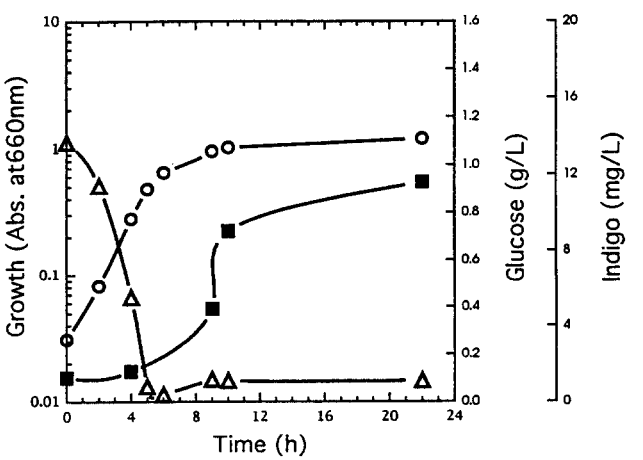

Fig. 2. TMO expression during exponential and stationary phase of *P. putida* AMS1000 in 0.1% glucose M9-medium. ○, Cell growth; △, Residual glucose; ■, Indigo production.

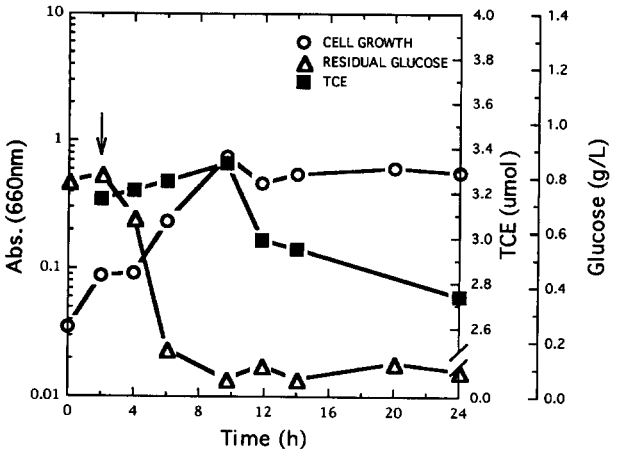

Fig. 3. Degradation of TCE by *P. putida* AMS1000 in 0.1% glucose M9-medium. About 3.3 μmol of TCE (which is equal to 6.6 μM in liquid phase at 30°C) was initially injected into the serum cap vial as indicated by arrow. ○, Cell growth; △, Residual glucose; ■, TCE concentration.

experiment. There was little degradation observed at 56.5 and 139.1 μM of TCE either in the exponential phase or stationary phase. Although the culture aggregated right after TCE was injected to the bottle because of the growth inhibition by TCE, it was confirmed by indigo assay that *tmo* was still active in those cells. However, in case of 6.6 μM, about 18% of TCE was degraded for 14 hours after the stationary phase when compared with that of the control strain without the plasmid (Fig. 3). TMO in AMS1000 was expressed in LB with indole as well as M9 minimal medium. By the sampling with narrow intervals, it was found that TMO expression was initiated from the late-exponential phase (data not shown).

Until now, only two articles regarding TCE degradation by a genetically engineered microorganism have been reported [1, 4]. In case of the former, they utilized the intrinsic promoter of TMO gene and *E. coli* cell as an expression host. Therefore, a substrate, toluene should be added to induce TCE-degrading activity, which can cause another pollution on site. Recently, TCE-degrading system using *E. coli* starvation promoter such as *cstC* or *groEL* which works in the stationary phase was developed [4]. Although about 65% of the initial TCE after 20 hours later at 10 μM of TCE was degraded, their system could not show TCE degradation in the post-exponential phase which might be due to the low viability of host cells against TCE. Because our system uses *Pseudomonas* which is indigenous in TCE-contaminated sites as a host cell, a long term operation with little decreasing of TCE-degrading activity can be achievable under the nutrient-starved environment such as the real fields. Searching a more solvent-resistant strain of *Pseudomonas* sp. as an expression host and testing the stability of TCE-degrading activity of the construct in chemostat are on progress.

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