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Construction of Genetically Engineered Microorganisms for Overexpression of *xylE* Gene Encoding Catechol 2,3-dioxygenase and the Functional Stability of the Recombinant Plasmid pSW3a Containing *xylE* in Aquatic Environment.

Hyo-Yung Han, Chi-Kyung Kim[1], Yong-Keun Park[2], Jong-Ok Ka[2], Byeong-Jae Lee[3], and Kyung-Hee Min*

Department of Biology, Sookmyung Women's University, Seoul 140-742,
[1]Department of Microbiology, Chungbuk National University, Cheongju 361-763, and
[2]Research Center for Molecular Microbiology and
[3]Institute for Molecular Biology & Genetics, Seoul National University, Seoul 151-742, Korea

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The regulation of *xylE* gene expression was examined by using vector promoter and construction of genetically engineered microorganisms (GEMs) for application in microcosm. When the *xylE* gene was subcloned into pBluscript SK(+) under the control of *lac* promoter(pTY1) in *E. coli*, and the expression was induced by IPTG, the enzyme activity of catechol 2,3-dioxygenase was increased 4.7 times more than that of the crude extracts from transformanants harboring pTY1. We suggest that the *xylE* gene has its own promoter at the upstream portion, because it was able to be expressed even in the absence of IPTG. A recombinant plasmid, pSW3a harboring the *xylE* gene under the T7 promoter, showed the activity of 14.5 units/mg protein, higher than that of parental strain, *E. coli* PYT1. The *xylE* gene in recombinant plasmid pSW3a was used as reporter gene for the application in microcosm ecosystem, since it was used for detection of *xylE*-positive clones by catechol spray on the agar plates. The pSW3a in *E. coli* was introduced into *Pseudomonas putida* to construct GEM strain, and examined for the expression and functional stability in microcosms.

Key words: Construction of GEMs, overexpression of *xylE* gene, activities of C230, stability of GEMs in microcosm

The *Pseudomonas putida* TOL plasmid contains two *xyl* operons that contain thse genetic information required for the degradation of toluene and related aromatic compounds (13, 14, 15). The *xylE* gene product of *Pseudomonas putida*, catechol-2,3-dioxygenase (C230), catalyzes the extradiol *meta*-cleavage of catechol to a yellow compound, 2-hydroxymuconic semialdehyde.

Removal of xenobiotic compounds has become a major issue of wastewater treatment in recent years. In order to achieve an effective and improved removal of xenobiotics, it is necessary to maintain the metabolic activities of specific degrading bacteria at sufficiently high levels. However, as degrading bacteria generally grow slowly and cannot exhibit their degrading activities fully in the mixed substrates or aquatic ecosystem, it is difficult to utilize their activities efficiently in an actual wastewater treatment process. Some strains have been constructed to solve these problems. As a countermeasure to this problems, it was necessary to construct GEMs carrying catabolic genes to wastewater treatment and discussed the feasibility from the view point of both genetic and ecological stabilities (6, 8). It has been suggested that if properly degrading GEMs could be created, the biodegradation rates of phenol compounds in the wastewater treatment process would be enhanced. For this purpose it is essential and important to construct such GEMs which not only can degrade xenobiotic compounds but at the same time can grow rapidly in the absence or under the low concentration of the target compounds.

Recent interest in the possibility of introducing genetically engineered microorganisms into the environment (16, 21, 34) had led us and others to try to develop sen-

* To whom correspondence should be addressed

sitive methods for detecting specific genetically modified microorganisms. Several reports have addressed the difficulty and limited efficiency of recovering bacteria from natural environments by methods that depend on culturing those microbes (1, 28). Some recent studies have focused on detecting methods of microorganism in environmental samples that do not require direct culturing of the organism.

Reporter genes, such as the *lacZ* gene or *xylE* gene, which encode an easily assayed protein and allow a quantitative assessment of gene expression in colonies on agar plates (6, 12), have been applied to the analysis of gene regulation in bacterial cells and used for detection of GEMs in aquatic environment (12).

In addition, a major reservation for the release of genetically engineered microorganisms (GEMs) has emphasized the need to monitor the dispersal and persistence of such organisms in the environment. GEMs may be introduced into the environment to detect or to degrade toxic substance (31). The proliferation of GEMs in the natural environment depends in part on the stability of the engineered genetic materials.

In our previous paper, we reported cloning of xylE gene from TOL plasmid and determining its nucleotide sequences(18). In this study, we introduced a novel way for genetic manipulation of metabolic pathway to enhance the bacterial degradation activities. The *xylE* gene, encoding catechol-2,3-oxygenase (C230), isolated from the chromosome of toluene-degrading *Pseudomonas putida*, was used to detect IPTG-driven expression under *lac* promoter and reintroduced in the strain as a recombinant plasmid pET-3a for overexpression as a reporter gene.

For the production of GEMs, we constructed a recombinant DNA having *xylE* gene as an insert into an expression vector pET-3a in an attempt to overproduce catechol-2,3-dioxygenase. The enzymatic activity of the cell extract was measured to identify the possibility of applying the GEMs in ecosystem. The potential for ecological usage is enhanced if, under conditions existing outside the laboratory, the recombinant DNA can be mobilized into organisms capable of growth in the natural environment. In order to apply the GEMs in environment we transferred the recombinant DNA into a *Pseudomonas putida* SM25 by conjugation in laboratory scale. The growth and catechol degradation of *Pseudomonas putida* SM25 were investigated and the effect of this amplification of the expression of genes was evaluated in refer to stability in microcosm environment.

Materials and Methods

Bacterial strains and plasmids

Table 1. Bacterial strains and plasmids used in this study

Bacteria and plasmids	Characteristics	Source or reference
Escherichia coli		
BL21	pLysS	Novagen*
PTY	DH5α harboring pTY1	(18)
PSW	BL21 harboring pSW3a	this study
Pseudomonas putida		
SM25		this study
41a	SM25 harboring pSW3a	this study
51a	SM25 harboring pSW3a	this study
Plasmid		
pBluecript SK(+)	Amp	Stratagene**
pET3a	Amp. T7 promoter	Novagen
pTY1	*xylE* in pBluescript SK(+)	(18)
pSW3a	*xylE* in pET3a	this study

* Novagen Inc., Madison, WI53711
** Stratagene, La Jolla CA92037

The bacterial strain used was *E. coli* DH5α, BL21, and PTY. The bacterial strains, *E. coli* PTY, is a derivative of *E. coli* DH5α harboring recombinant plasmid pTY1, and PSW is a derivative of BL21 harboriing pSW3a. The plasmid vectors used were pBluescript SK(+), pET3a, pTY1, and pSW3a as shown in Table 1.

Medium and culture condition

The medium used throughout the experiment was Luria-Bertani (LB) broth (1% tryptone, 0.5% yeast extract, 0.5% NaCl). Agar was added to 1.5% in the LB broth medium to prepare LB agar plate. Incubation were usually carried out at 37°C. For enzyme induction and conjugation experiment, the cells were grown at various temperatures as indicated below.

Purification of plasmid DNA

Recombinant plasmid DNA was isolated by the alkaline extraction method (30).

Restriction enzyme analysis and gel electrophoresis

Restriction endonucleases *Bam*HI and *Sau*3AI were products of Bethesda Research Laboratories, Bethesda, Md. Other restriction endonucleases were purchased from Takara Shuzo Co. Ltd., Kyoto, Japan. Digestion with restriction endonuclease was carried out according to the instructions of the manufactures. Analysis of restriction fragments and purification of digested DNA fragments by electrophoretic elution have been described previously (30).

Ligation and transformation

Ligation of DNA fragments with T4 DNA ligase (Takara Shuzo Co. Ltd.) and transformation of *E. coli*

cells were carried out as described previously (30). Antibiotic concentrations used for selection of transformants on LB agar plate were 50 μg/ml for ampicillin, and 150 μg/ml for streptomycin. The transformants containing *xylE* gene were selected directly by the spray of 0.1 M catechol to form the yellow 2-hrdroxymuconic semialdehyde on LB agar plates.

Recombinant pTY1 plasmid

The recombinant plasmid pTY1 used in this study was constructed by subcloning the *xylE* gene responsible for *meta*-cleavage in degradation of toluene into pBluescript SK(+) vector as previously reported by Kim *et al* (18). The size and restriction map of recombinant pTY1 are shown in Fig. 1. The pTY1 was isolated from the cloned cells of *E. coli* PTY by the alkaline lysis methods described by Sambrook *et al.* (30).

Determination of catechol 2,3-dioxygenase activity

The catechol-2,3-dioxygenase activity in cells was directly detected by color production of the colonies on LB agar plates. When the cells had the induced catechol-2,3-dioxygenase the colonies turned to yellow due to 2-hydroxymuconic semialdehyde produced from catechol (0.1M) sprayed on the colonies(14).

Cultures were inoculated from precultures grown in LB and subsequently grown in LB broth containing ampicillin (50 μg/ml) at 37℃ for 16 hr with shaking (180 rpm). The crude extract was prepared in 50nM phosphate buffer (pH 7.0) by sonic oscillation followed by centrifugation to remove cell debris precipitants. The supernatant solution was fractionated and the crude extract was used for enzyme activity measurement.

Catechol-2,3-dioxygenase activity was assayed with crude extracts according to the methods of Inouye *et al* (14). Cell extract was incubated at 55℃ for 10 minutes to diminish catechol-1,2-dioxygenase activity. Subsequent to adding 0.1 ml of 0.01 mM catechol in 0.8 ml of 50 mM phosphate buffer (pH 7.0) contained 0.1 ml of cell-extract to a cuvette of spectrophotometer, and optical density at 375 nm was measured by spectrophotometer. One unit corresponds to the increase of O.D. at 375 nm by 0.98 per minute at 37℃ as a result of formation of 2-hydroxymuconic semialdehyde (HMS) from 0.1 μM catechol degradation (25, 33).

Measurement of protein

The amount of protein was measured by the method of Lowry *et al.* (22).

IPTG induction

The cloned *xylE* gene in DH5α under the control of *lac* promoter on the pBluescript SK(+) vector was induced by adding IPTG in the M9 media containing 0.4% glucose. In order to analyze the effect of an inducer on promoter, the expression of *xylE* gene was examined, and the induction of enzymes was investigated as follows.

After growing cells to mid-log phase at 37℃, target gene expression was induced by adding 1 mM IPTG to M9 minimal medium containing 0.4% glucose. The prepared culture broth was incubated overnight at 37℃ with shaking. In a typical assaying conditions of catechol 2,3-dioxygenase, 0.1 ml of 10 mM catechol was mixed with 0.1 ml of cell extract from IPTG-induced culture plus 0.8 ml of 50 mM Tris-Cl buffer (pH 7.5). The measurement of the enzyme activities was described as above.

Conjugation experiment

For the environmental application, recombinant plasmids were transferred by conjugation in microcosm. In a dialysis sac(Spectrapor membrane tubing, MWCO 10,000, USA), GEM strain, helper strain and recipient strains were added to perform conjugation (4, 32). Approximately 6×10^7 cells of a donor strain (*E. coli* PSW), a recipient strain (*P. putida* SM25), and a helper strain, *E. coli* C600 harboring pRK2013, from exponentially growing bacterial cultures in LB broth were washed with filtered Han river water, which was filtered through 0.2 μm Nucleopore membrane filter. The cells pellets were suspended in 1 ml of filtered Han river water in microcosm. The mixtures were incubated for 16 hours at various temperatures.

The mixture solution (100 μl) was transferred to LB plate containing ampicillin and streptomycine. When *E. coli* PTY was used as a donor strain in conjugation procedure, conjugants were screened on LB plate including 100 μg/ml of ampicillin and 150 μg/ml of streptomycine. Screening the conjugants was performed by spraying 0.5 ml of 0.1M catechol solution on LB agar plates. Subsequent conjugants harboring *xylE* were identified by the production of yellow colonies on LB agar plate.

Water microcosm

The water microcosm was provided as described by Kim *et al.* (19). A dialysis sac was filled with 10 ml of Han river water, which was filtered through 0.2 μm Nucleopore membrane filter. The microcosm was suspended with *Pseudomonas putida* 41a, 51a, at a final concentration of 100×10^2 cells/ml, respectively. Water temperature of the microcosms in Han river water was controlled to 15℃ during the experiment for the determination of the stability of GEMs. After several days of GEMs release, the conjugants in the microcosms were counted on the LB agar plates by the method of catechol

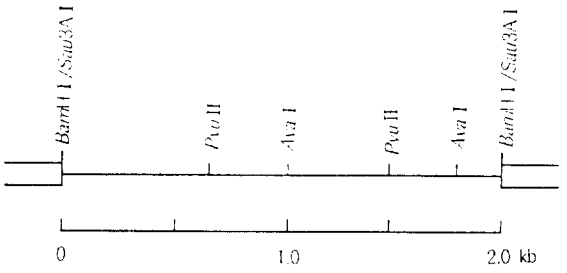

Fig. 1. Restriction map of recombinant pTY1 carrying *xylE* gene.

Table 2. Induction of C230 from the *E. coli* PTY containing pTY1 in the presence or absence of IPTG

Temp (℃)	Presence of IPTG	Cell concn. (OD at 600 nm)	Protein (μg/ml)	C230 (10^2 units*)	Specific activities (units/mg)
30℃	-IPTG	0.16	1.50	0.71	4.73
	+IPTG	0.20	2.04	4.59	22.50
37℃	-IPTG	0.10	1.30	1.94	14.92
	+IPTG	0.13	1.41	2.50	17.73

*On unit of C230 represents the increase of O.D. at 375 nm by 0.98 per minute at 37℃ as a result of formation of 2-hydroxymuconic semialdehyde from 0.1 μM catechol degradation (25, 33).

spray.

Results and Discussion

Regulation of *xylE* gene expression

Partially digested TOL plasmids with *Sau*3AI were ligated to the *Bam*HI site of pBluescript SK(+) plasmids and resulting recombinant plasmids were transformed into *E. coli* DH5α. A clone of pTY1 carrying 2 kb fragments containing *xylE* gene has obtained(18). Figure. 1. summarizes the results of previous and present studies on the physical map of recombinant pTY1.

In order to examine whether *xylE* gene is expressed using vector promoter, *xylE* gene was cloned into pBluescript SK(+) to construct recombinant plasmid pTY1 and was placed under the control of *lac* promoter (Fig. 1). The specific activities of catechol 2,3-dioxygenase (C230) were determined in the crude extracts of transformants harboring pTY1 grown in the absence or presence of the inducer IPTG.

The *xylE* gene of *P. putida* encodes a catechol-2,3-dioxygenase that converts catechol, a colorless compound, to 2-hydroxymuconic semialdehyde, which is yellow in color. To determine whether this chromogenic reation could be used to detect IPTG-driven expression of *xylE*, *E. coli* transformant containing pTY1 were tested onto LB agar plates, which were sprayed with a 0.5 M catechol solution after 2 days of growth. Colonies without pTY1-containing cells grown on LB agar plates remained white after exposure to catechol, while colonies of pTY1-containing cells grown on LB agar plates were intensively yellow.

In addition, when the cells grown in the presence of IPTG in the M9 minimal medium, the activities of C230 was increased 4.7 times higher than that without IPTG as shown in Table 2. At 37℃, in the presence of IPTG in M9 plus glucose media, protein production increased to 1.3 μg/ml and specific activity of catechol-2,3-dioxygenase increased somewhat higher than that without IPTG. These results indicate that the *xylE* gene is expressed well in *E. coli*, and that this expression is regulated appropriately by the *lac* promoter at 30℃.

Moon *et al.*(24) has reported that a *tet*A gene encoding tyrosine-phenol lyase from *Erwinia herbicola* was fused to *lac* promoter of pUC18 to construct pTPL51 and introduced into *E. coli*. The enzyme was expressed at high level in the resulting strain in the presence of IPTG or lactose as an inducer. Fugita *et al.*(8) cloned DNA fragments containing phenol or cresol catabolic genes from *Pseudomonas putida* into the plasmid pSO_4-92 as vector harboring *lac* promoter and introduced into *E. coli* JM103 or HB101 and *P. putida* KT2440. *E. coli* with the recombinant plasmids did not require phenol as a cosubstrate for trichloroethylene degradation, the rate of which was comparable to that of fully-induced *P. putida*. However, it did require IPTG for induction of the *lac* promoter on the vector. The transcription of the C230 gene in pCNU 401 contained *xylE* gene in pUC 18 with *lac* promoter could be regulated by P1 promoter(24). Moon *et al.*(24) also indicated that the C230 gene of *Alcaligenes* sp. KF711 was overexpressed in *E. coli* HB101 by using the *lac* promoter of pUC18.

In our experiment, it was also found that *xylE* gene was regulated and induced fully in the presence of IPTG using *lac* promoter as shown in Table 2. However, the activities of C230 at 30℃ was 4.73 units/mg protein in the absence of IPTG. This result implies that the activities of C230 in pTY1 may be dependent on the own promoter in pTY1.

Inoue *et al.*(14, 15) reported that the *xylE* genes were organized into two regulatory uints; the *xylABC* operon and the *xylDEFG* operon, which were regulated by *xylR* and *xylS*. They determined the nucleotide sequences of the operator-promoter region of the *xylDEFG* operon, showing the promoter was located at upstream of *xylDEFG* operon. However, Keil *et al.*(17) found that there is internal promoter sequence at upstream of *xylE* gene in pWW53-4.

From this experimental result we suggest that *xylE* has internal promoter at upstream the *xylE* in pTY1 as

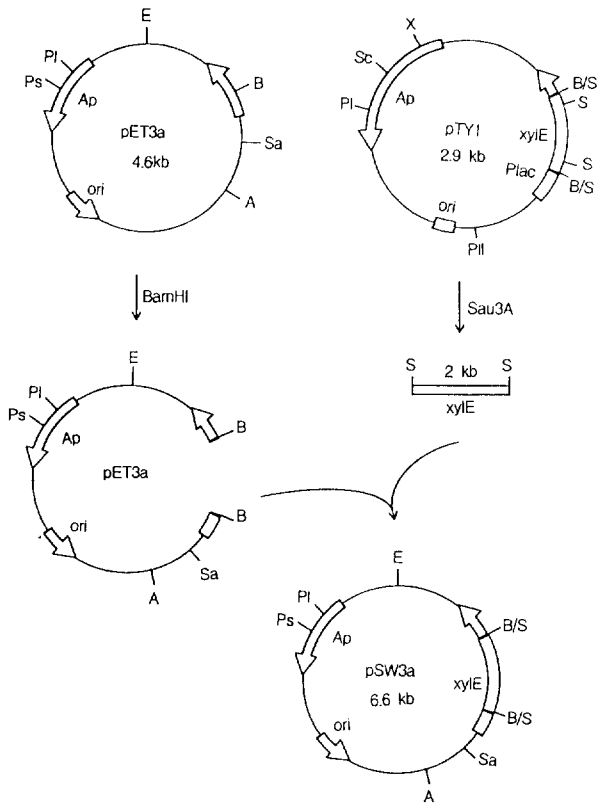

Fig. 2. Construction of recombinant plasmid pSW3a carrying *xylE* gene. A *Sau*3AI digestion fragment carrying *xylE* from pTY1 was inserted into *Bam*HI site of pET3a expression vector to from the recombinant plasmid, pSW3a vector, for its overexpression. A; *Ava*I, B; *Bam*HI, E; *Eco*RI, PI; *Pvu*I, PII; *Pvu*II, Ps; *Pst*I, S; *Sau*3AI, Sa; *Sal*I, Sc; *Sca*I, X; *Xmn*I.

reported by Keil *et al.*(17).

Overexpression of *xylE* gene.

In order to detect directly GEMs in microcosms or aquatic environment, it is required to overexpress the enzyme activities of C230 using expression vector. We constructed recombinant plasmid pSW3a carrying transcriptional fusions of a *xylE* to pET3a vector. The resulting recombinant plasmid pSW3a was introduced into *E. coli* strain BL21 for the overproduction of C230 (Fig. 2).

In this experiment, the cells harboring pSW3a plasmid was turned to yellow-colored clone strongly by catechol spray for the direct detection of presence of C230 on the plate.

Catechol 2,3-dioxygenase activity was expressed in *E. coli* PTY, carrying the recombinant plasmid pTY1, showing 14.51 units/mg protein higher than that of the parental strain, DH5α. Activities of C230 in the crude extract from *E. coli* cells carrying recombinant plasmids, pSW3a were also determined. Specific activities of PSW strain was the highest, 73.06 units/mg protein, more than those of *E. coli* PTY, and BL21, as host strains (Table 3).

Table 3. Activities of C230 in *E. coli* strain carrying recombinant plasmids

E. coli strains	Catechol 2,3-dioxygenase* (units/mg protein)
DH5α	0
BL21	0
PTY	14.51
PSW	73.06

*Specific activities represent the units of enzyme per mg of protein.

This results suggest that the overexpression of the cloned *xylE* gene in *E. coli* PSW carrying pSW3a is a result of the read through higher transcription initiated at T_7 promoter of pET3a than at *lac* promoter of PTY 1(Table 3).

Kobayashi *et al.*(20) has also reported overexpression of *Pseudomonas putida* C230 in plasmid pHT3, which contained *xylE* gene at the downstream of the P_L promoters as well as the cI_{ts} gene encoding the temperature-sensitive repressor of bacteriophage λ on pBR322 vector. The cloned *xylE* gene encoding C230 from TOL plasmid in *P. putida* mt-2 has been expressed in *E. coli* W3110 to a level of 15% of the total soluble protein.

xylE as a reporter gene

Several studies has been reported on *xylE* as a reporter gene. The *xylE* gene encoding C230 has been used for reporter gene as recombinant vaccine development (29), the study of *gal* P1, a catechol-controlled promoter (12), and the catabolite control of *gal* operon (12).

Ingram *et al.*(12) described the development of a convenient and sensitive reporter gene system for *Streptomyces* spp. based on the use of a promoterless copy of the *xylE* gene of *Pseudomonas putida*. A promoterless copy of *xylE* gene was placed under the transcriptional control of *gal* P1, a glucose-repressed and galactose- induced promoter from *Streptomyces lividans*, and its expression was examined in bacterial colonies on agar plates or in liguid cultures grown in the presence of glucose or galactose as the sole carbon source. On agar plates, colonies of bacteria grown on galactose turned to bright yellow within a few minutes after sprayed with catechol solution, whereas colonies on glucose-containing plates remained white colored, even after extensive incubation. The properties of *xylE* gene as a reporter gene thus make it suitable not only for monitoring expression of regulated promoters, but also for recovering colonies that show the expression levels of promoters of interest.

In our experiment, the *xylE* gene has also been used for detection of colonies by catechol spray on agar plates

because the *xylE* gene is an efficient reporter gene.

Construction of GEMs by conjugation

In this study, GEMs made by the transformation did not survive after transferring to the microcosm environment. The *xylE* portion of pTY1 was ligated to pKT 230 DNA and transformed into *E. coli* BL21 and DH5α cells, the recombinant microorganisms were screened in the test of catechol spray assay on LB agar plates contained kanamycine (50 μg/ml). When the transformants were transferred to a new agar plate, that did not show *xylE* gene activity. It appears that the transformants are unstable in the microcosm environment.

The plasmid can be mobilized among different bacterial strains by conjugation if fertility (*tra*) functions are provided by a mobilizing plasmid. An *E. coli* strain containing a helper plasmid, such as pRK2013, is used to allow a recombinant plasmid be transferred from a donor to a recipient. Since helper plasmids were designed not to be replicated in recipient cells, only the target recombinant plasmid can be mobilized to a recipient on a selective plate after conjugation (9).

Based on our experiment conjugation procedure seems to be a better method than transformation procedure for the DNA transfer in *Pseudomonas putida* SM25. In this experiment, in order to examine the stability of GEMs in microcosm, we constructed GEM strains, *P. putida* 41a, 51a. At 15℃ in the presence of helper, conjugation of pSW3a from *E. coli* donor strain to *Pseudomonas putida* SM25 occurred almost same level as at 30℃. At 37℃ there was low frequency of conjugation, showing the optimum temperature for conjugation is about 30℃ (Table 4). Recombinant DNA pSW3a was mobilized to *Pseudomonas putida* SM25 by conjugation in microcosm in the presence of a helper strain *E. coli* C600 harboring pRK 2013 at the frequencies of $1\sim3\times10^{-7}$.

Stability of GEMs in artificial aquatic environment

The stability of the genetically engineered microorganisms released in microcosm has been regraded as one of the molecular ecological topics. Genetically engineered microorganisms (GEMs) may be introduced into the environment to detect (7, 31) or to degrade toxic substances (4, 31) in aquatic environment. Sometimes, a major reservation for the release of GEMs has centered around the need to monitor the dispersal and persistence of such organisms in the environment.

We have examined the use of *xylE* gene for detection of GEMs released into the artificial microcosm before ap-

Table 4. Conjugation frequencies in the presence or absence of helper strain

Temp(℃)	Helper	
	Without	With
15	0	3×10^{7}
30	0	1×10^{7}
37	0	4×10^{8}

Conjugation was carried out in microcosm. The conjugation frequencies were measured by counting yellow colored colonies on LB agar plate containing antibiotics after spraying 0.1M catechol.

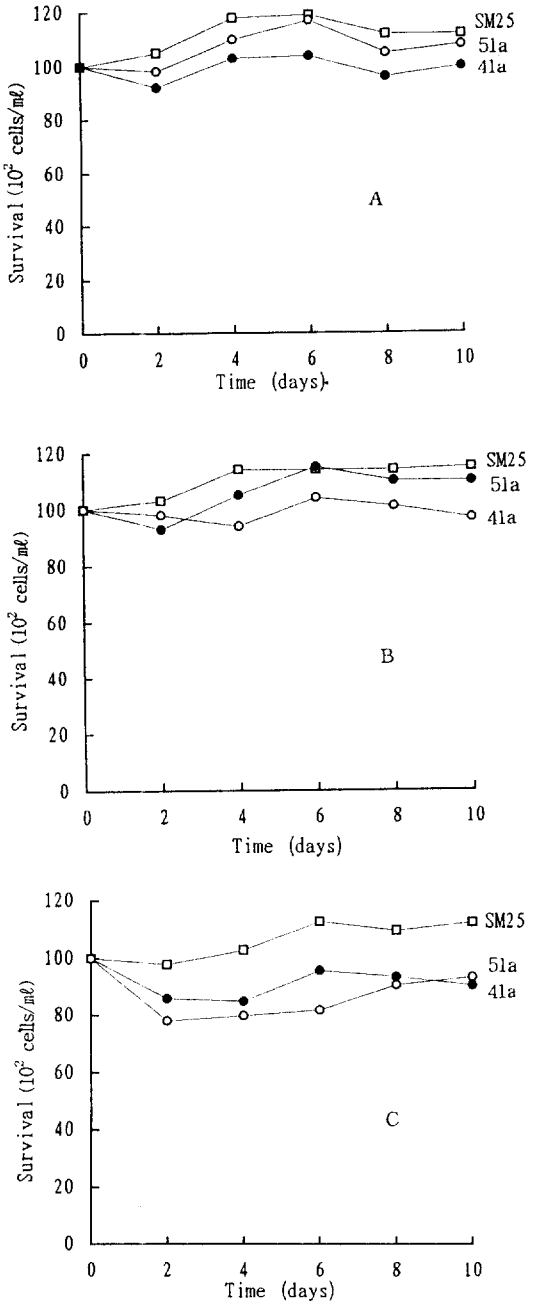

Fig. 3. Survival of conjugants, 41a, 51a, and recipient strain, *Pseudomonas putida* SM25, The numbers of bacteria in the microcosms were encountered on LB agar plates. A; 37℃, B; 30℃, C; 16℃.

plication to natural environment. For the examination of the stability of the recombinants, the selected clones were subcultured by transferring continually to new microcosm. The conjugants were examined the presence of the enzyme activities on LB plates by catechol spray and counted the numbers of yellow colonies in microcosms on LB plates at various time points several days after release of GEMs.

The conjugants with recombinant DNAs were maintained in the recipient cells after 4th transfer to microcosm that gained the ability to convert catechol into 2-hydroxymuconic semialdehyde. Subsequent to conjugation of *E. coli* cells, conjugants appeared to direct the synthesis of C230. The selected conjugants still exhibited enzymatic activities after 4th transfer to microcosm plate, showing yellow color on LB plates. It clearly showed that the conjugants were quite stable in kepping the recombinant vectors in a microcosm (data not shown).

We examined the survival of GEMs, *P. putida* 41a, 51a, and donor strain SM25, in microcosms at 15℃, 30℃, and 37℃. The conjugants produced in a microcosm showed over 90% of survival in microcosms after 10 days of release as shown in Fig. 3. Generally survival of *P. putida* 41a and 51a was decreased more or less at the initial stage of release in microcosms. The conjugants, GEMs, may need the time to be adapted for survival in the microcosms. Morever, almost 100% of bacterial colonies were able to show yellow color production by catechol spray.

As shown in Table 4, when the conjugantion was carried out with only donor strains (PTY, PSW) and recipient strains (SM25), conjugants were not formed at any temperatures. The conjugation occurred only in the presence of a helper strain, suggesting that helper strain is needed for conjugation in microcosm. Since the conjugation will occur quite rarely in the natural aquatic situation, artificial transferring of a specific gene with helper strain is needed for the environmental usage.

Shaw *et al.*(31) reported the utilization of bioluminescence for detection of GEMs released sensitives as plating methods for detecting the GEMs in environmental samples. Gealt *et al.*(10) described the transfer of plasmids pBR322 and pBR325 in waste water from laboratory *E. coli* strains to bacteria indigenous to the waste disposal system. They demonstrated the general increase in the number of wastewater bacteria resistant to tetracycline, chloramphenicol, and carbenicillin when the indigenous bacteria were coincubated with both on *E. coli* harboring pBR322 and on *E. coli* mobilizer strain (X178) containing the R100-1 conjugative plasmid. The transfer of pBR325 or pBR322 plasmid does occur from laboratory *E. coli* strains into sterilized wastewater (10).

Survival and catabolic activity of natural and genetically engineered bacteria in a laboratory-scale activated-sludge unit were examined(23). The GEMs were adapted for survival and enhanced breakdown of 3-chlorobenzoate in the environment.

Pseudomonas sp. B13 FRI was a GEM which is able to degrade chloro- and methylaromatics through a constructed *ortho*-cleavage pathway(27). The GEM survived in lake and river sediments at high density of bacterial cells throughout a 4-week period of investigation. According to several criteria, the microcosm system was stable and healthy during the experiment.

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