21 Genetically Engineered Microorganisms and Bioremediation

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List of Abbreviations

BBIC	bioluminescent bioreporter integrated circuit
BTEX	benzene, toluene, ethylbenzene, and xylene
2,4-D	2,4-dichlorophenoxyacetic acid
DBT	dibenzothiophene
FAV	field application vector
GEM	genetically engineered microorganisms
IC	integrated circuit
IGP	Igepal CO-720®
IPB	isopropylbenzene
IPTG	isopropyl thio-β-galactoside
PAH	phenanthrene, pyrene and benzo[a]pyrene
PCB	polychlorinated biphenyls
PCP	pentachlorophenol
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
sMMO	soluble methane monooxygenase
2,4,5-T	2,4,5-trichloro phenoxy acetic acid
TCE	trichloroethylene
TMO	toluene monooxygenase
TNT	2,4,6-trinitrotoluene
TOL	toluene plasmid pWWO

1 Introduction

During the past 20 years, recombinant DNA techniques have been studied intensively to improve the degradation of hazardous wastes under laboratory conditions. Only one field test has been successfully implemented (SAYLER et al., 1999). Recombinant bacteria can be obtained by genetic engineering techniques or by natural genetic exchange between bacteria. Applications for genetically engineered microorganisms (GEM) in bioremediation have received a great deal of attention, but have largely been confined to the laboratory environment. This has been due to regulatory risk assessment concerns, and to a large extent the uncertainty of their practical impact and delivery under field conditions. There are at least four principal approaches to GEM development for bioremediation application. These include:

(1) modification of enzyme specificity and affinity,
(2) pathway construction and regulation,
(3) bioprocess development, monitoring, and control, and
(4) bioaffinity bioreporter sensor applications for chemical sensing, toxicity reduction, and end point analysis.

There are many reports on the degradation of environmental pollutants by different bacteria. However, only potential bioremediation related to GEM will be reviewed, and the construction and application of GEM will be the focus of this review. Some examples of the relevant use of genetic engineering technology to improve bioremediation are listed in Tab. 1. These genetically engineered microorganisms have higher degradative capacity and have been demonstrated successfully for the degradation of various pollutants under defined conditions. However, ecological and environmental concerns and regulatory constraints are major obstacles for testing GEM in the field. These problems must be overcome before GEM can provide an effective clean-up process at lower cost.

The use of genetically engineered microorganisms has been applied to bioremediation

Tab. 1. Genetic Engineering for Biodegradation of Contaminants

Microorganism	Modification	Contaminants	Reference
Pseudomonas sp. B13	pathway	mono/dichlorobenzoates	REINEKE and KNACKMUSS, 1979, 1980
P. putida	pathway	4-ethylbenzoate	RAMOS et al., 1987
P. putida KT2442	pathway	toluene/benzoate	PANKE et al., 1998
Pseudomonas sp. FR1	pathway	chloro-, methylbenzoates	ROJO et al., 1987
C. testosteroni VP44	substrate specificity	*o*-, *p*-monochlorobiphenyls	HRYWNA et al., 1999
Pseudomonas sp. LB400	substrate specificity	PCB	ERICKSON and MONDELLO, 1993
E. coli JM109(pSHF1003)	substrate specificity	PCB, benzene, toluene	KUMAMMRU et al., 1998
P. pseudoalcaligenes KF707-D2	substrate specificity	TCE, toluene, benzene	SUYAMA et al., 1996
E. coli FM5/pKY287	regulation	TCE, toluene	WINTER et al., 1989

process monitoring, strain monitoring, stress response, end point analysis, and toxicity assessment. Examples of these applications are listed in Tab. 2. The range of tested contaminants includes chlorinated compounds, aromatic hydrocarbons, heavy metals, and nonpolar toxicants, etc.

Development of effective and cost-efficient bioremediation processes is the goal for environmental biotechnology. The combination of microbiological and ecological knowledge, biochemical mechanisms, and field engineering designs are essential elements for successful *in situ* bioremediation using GEM.

2 Degradative (Catabolic) Genes

Extensive review information is available on the biochemical pathway analysis, operon structure, and molecular biology of biodegradative pathways important in bioremediation. Much of this information, critical to the development of appropriate GEM, is confined to aerobic catabolic and cometabolic pathways (SAYLER et al., 1998).

Tab. 2. Genetic Engineering for Biodegradation Process Efficacy

Microorganism	Application	Contaminants	Reference
A. eutrophus H850Lr	process monitoring	PCB	VAN DYKE et al., 1996
P. putida TVA8	process monitoring	TCE, BTEX	APPLEGATE et al., 1998
P. fluorescens HK44	process monitoring	naphthalene, anthracene, phenanthrene	SAYLER et al., 1999
B. cepacia BRI6001L	strain monitoring	2,4-D	MASSON et al., 1993
P. fluorescens 10586s/pUCD607	stress response	BTEX	SOUSA et al., 1998
P. fluorescens 10586s/pUCD607	toxicity assessment	chlorobenzenes, chlorophenols, BTEX	BOYD et al., 1998; SINCLAIR et al., 1999; GLOVER et al., 1999
Pseudomonas strain Shk1	toxicity assessment	Cd, 2,4-dinitrophenol, hydroquinone	KELLY et al., 1999
A. eutrophus 2050	end point analysis	nonpolar narcotics	LAYTON et al., 1999

2.1 Branched Aromatic Hydrocarbons

2.1.1 *Pseudomonas* spp.

The TOL plasmid pathway of *Pseudomonas putida* has been manipulated extensively to expand its catabolic capability on different branched compounds. RAMOS et al. (1987) modified the TOL metabolic pathway to use 4-ethylbenzoate as a substrate. These restructured strains were mutants isolated by either altering a pathway regulator XylS (RAMOS et al., 1986) or by modifying substrate specificity of enzymes (catechol-2,3-dioxygenase).

The complete upper TOL operon of plasmid pWW0 with its regulator gene, *xylR*, was reconstructed as a single gene cassette and yielded a hybrid mini-Tn5 [*upp* TOL] transposon. The transposon was further inserted into the chromosome of *P. puida* KT2442. The hybrid strain, KT2442::mini-Tn5 [*upp* TOL]Δ[*Km*^r/*xylE*], was stable and grew on toluene as the sole carbon source. Toluene was degraded to benzoate by the TOL upper pathway and benzoate was further metabolized through an *o*-cleavage pathway (PANKE et al., 1988).

2.2 Chlorinated Compounds

2.2.1 Chlorobenzoates

2.2.1.1 *Pseudomonas* sp. B13 – Hybrid Pathways

The plasmid pWR1 encoding 3-chlorobenzoate and 4-chlorophenol degradation genes in strain B 13 have been modified extensively to generate hybrid pathways that extend vertical degradation of haloaromatic compounds. REINEKE and KNACKMUSS (1979, 1980) first reported the introduction of TOL plasmid pWW0, of *P. putida* mt-2 into strain B13 via conjugation. The transconjugants demonstrated the capability to utilize mono- and dichlorobenzoates as growth substrates. The inactivation of the TOL plasmid encoded *m*-pathway (by loss of catechol-2,3-dioxygenase activity)

was an obligatory requirement for the hybrid strain, *Pseudomonas* sp. WR211, to utilize 4-chlorobenzoate as the sole carbon source (REINEKE et al., 1982).

SCHWIEN and SCHMIDT (1982) reported that a benzoate and phenol degrader, *Alcaligenes* sp. A7, acquired chlorocatechol degrading capacity from strain B13 through conjugation. The recombinant strain, *Alcaligenes* sp. A7-2, exhibited the ability to utilize all three isomeric chlorophenols as the sole source of carbon and energy.

The *xylXYZ* (toluate-1,2-dioxygenase) and *xylL* (carboxylate dehydrogenase) genes from TOL plasmid (pWW0-161) of *P. putida* and *nahG* (salicylate hydroxylase) from NAH7 plasmid were cloned into strain B13 to extend its substrate range (LEHRBACH et al., 1984). A hybrid plasmid, including genes (*xylXYZL*), positive regulator (*xylS*), and their native promoter (Pm), was introduced into strain B13 by conjugation. The resulting *Pseudomonas* sp. B13(TOL) was capable of utilizing 3-chloro-, 4-chloro-, and 3,5-dichlorobenzoate. The transconjugants containing the *nahG* gene were able to degrade salicylate, 3-, 4-, and 5-chlorosalicylate.

ROJO et al. (1987) reported a modified *o*-cleavage pathway, in strain B13, for simultaneous degradation of chloro- and methylaromatic compounds. This hybrid pathway included:

(1) toluate 1,2-dioxygenase and carboxylate dehydrogenase from the TOL pathway to degrade 4-chlorobenzoate and transform methylbenzoates into methyl-2-enelactones (strain FR1),
(2) 4-methyl-2-enelactone isomerase from *Ralstonia eutropha* (formerly *Alcaligenes eutrophus*) JMP134 to transform 4-methyl-2-enelactones to 3-methyl-2-enelactones to complete the *o*-pathway for 4-methylbenzoate [strain FR1(pRFC20P)],
(3) phenol hydroxylase from B13 to mineralize chloro- and methylphenols (cresols) to corresponding catechols [strain FR1(pFRC20P)-1].

A hybrid plasmid, pFRC4P (Tn5::*xylXYZLS*), containing Pm promoter was introduced into strain B13 through triparental mating using *E.*

coli HB101(pFRC4P) as a donor, and *E. coli* HB101(pRK2013) as a helper. The hybrid Tn5 transposon that carried TOL genes was transposed into the chromosome of B13 and yielded strain FR1. Gene encoded 4-methyl-2-ene-lactone isomerase from *R. eutropha* JMP134 was cloned and transferred by conjugation into FR1. The resulting strain containing a hybrid plasmid, pFRC20P, was designated as B13FR1(pFRC20P) and was capable of growing on 4-methylbenzoate. Spontaneous mutants of FR1(pFRC20P), such as FR1-(pFRC20P)-1 and -2, were able to use 4-methyl-phenol as a sole source of carbon and energy. The engineered bacterium *Pseudomonas* sp. B13FR1(pFRC20P)-1 was capable of growing on and mineralizing mixtures of 3-chloro-, 4-chloro-, and 4-methylbenzoate and 4-chloro- and 4-methylphenol via the modified *o*-pathway. Studies on the survival rate of the engineered strain, the stability and expression of the recombinant pathways, and gene transfer in the environment were conducted in an activated sludge microcosm (NüßLEIN et al., 1992). Strain FR1(pFRC20P) was also examined in two different aquatic sediments and the results showed enhancement of the degradation rate of contaminants (PIPKE et al., 1993).

2.2.1.2 *Pseudomonas aeruginosa* AC869(pAC31)

Strain *P. putida*, containing plasmid pAC25 that encodes genes for the degradation of 3-chlorobenzoate, has been shown to be structurally homologous to pWR1 (CHATTERJEE and CHAKRABARTY, 1983). Genetic rearrangements occurred between plasmids pAC25 and TOL under chemostat maintenance and yielded pAC31. The new plasmid contained *xylDGEF* genes located on the chromosome. The resulting strain AC869(pAC31) showed the capability to degrade 3,5-dichlorobenzoate and 3- and 4-chlorobenzoate (CHATTERJEE and CHAKRABARTY, 1982).

2.2.1.3 *Pseudomonas* sp. US1 ex.

A monochlorobenzoate and 2,4-D degrader, strain US1 ex. containing pJP4 was obtained through conjugation. The *E. coli* JMP397, harboring plasmid pJP4 (no expression), was used as a donor and the 4-chloro-benzoate degrader, US1, was the recipient. The new strain released stoichiometric amounts of chloride when grown on respective chloroaromatics as carbon source (SAHASRABUDHE and MODI, 1991).

There are reports on the horizontal transfer of 3-chlorobenzoate catabolic plasmid (pBRC60) in a freshwater ecosystem (FULTH-THROPE and WYNDHAM, 1991) and in an activated sludge unit (pD10; MCCLURE et al., 1989).

2.2.2 Polychlorinated Biphenyls (PCB) and Chlorobiphenyls

2.2.2.1 *Ralstonia eutropha* (formerly *Alcaligenes eutrophus*) AE707/AE1216

Chromosomally located PCB catabolic genes of *R. eutropha* A5 (SHIELDS et al., 1985), *Achromobacter* sp. LBS1C1 (PETTIGREW et al., 1990), and *Alcaligenes dentrificans* sp. JB1 (PARSONS et al., 1988) were transferred into a heavy metal resistant strain *R. eutropha* CH34 through natural conjugation. All donor strains degraded biphenyl and monochlorobiphenyls to corresponding benzoate and chlorobenzoates. Benzoate was further metabolized via the *o*-cleavage pathway in strains A5 and LBS1C1, and via the *m*-cleavage pathway in strain JB1. Strain A5 harbored a catabolic transposon, Tn4317, which carried biphenyl and 4-chlorobiphenyl degradation genes (SPRINGEAL et al., 1993a). The dehalogenase activity was mediated by plasmid pSS50 in strain A5 (LAYTON et al., 1992).

Transfer of PCB degradation genes from A5 to the heavy metal resistant strain CH34 was carried out by conjugation (SPRINGAEL et al., 1993b). The constructed strain, AE707, exhibited phenotype of Bph$^+$Cbp$^+$, and degraded 4-chlorobiphenyl to 4-chlorobenzoate in the presence of heavy metals. In resting cell assays (grown on biphenyl), strain AE707 cometabolized di- and trichlorinated congeners of Aroclor 1242® in the presence of heavy metals. The

PCB catabolic chromosomal genes of strain JB1 were transferred into CH34 through RP4::Mu3A mediated R-prime plasmid formation (SPRINGAEL et al., 1994). A transconjugant, strain AE1216, utilized 2-, 3- and 4-chlorobiphenyl and exhibited properties of metal resistance.

2.2.2.2 *Pseudomonas* sp. – Hybrid Strains

Pseudomonas putida BN10 grew on biphenyl and accumulated metabolites of 2-, 4-chlorobenzoate and 3-chlorocatechol from corresponding monochlorobiphenyls. 3-Chlorobiphenyl degraders were obtained from conjugation between *Pseudomonas* sp. B13 and strain BN10. The resulting *Pseudomonas* strain BN210 (gained chlorocatechol degradation genes) and B131 (acquired biphenyl degradation genes) were able to grow on 3-chlorobiphenyl. Both strains exhibited the capability to degrade monochlorobiphenyls and 2 of the dichlorobiphenyls found in Aroclor 1221® (MOKROSS et al., 1990).

Another hybrid *Pseudomonas* sp. strain UCR2 was isolated from multi-chemostat mating between a chlorobenzoate degrader, *P. aeruginosa* JB2, and a 2-chlorobiphenyl utilizer, *Arthrobacter* sp. strain B1Barc (HICKEY et al., 1992). Strain UCR2 exhibited ability to mineralize 2-chloro- and 2,5-dichlorobiphenyl. The UCR2 showed higher phenotypic similarity and higher genomic DNA homology to strain JB2. No hybridization was observed when the parental strains were probed against each other.

Recombinant *Pseudomonas* sp. strain CB15 was obtained by multi-chemostat mating by mixing *Pseudomonas* sp. strain HF1 (3-chlorobenzoate utilizer) and *Acinetobacter* sp. strain P6 (biphenyl utilizer) on ceramic beads (ADAMS et al., 1992). 3-chlorobiphenyl, 3-chlorobenzoate, and biphenyl could be utilized as growth substrates by strain CB15. Results of DNA hybridization suggested strain CB15 was closely related to parent strain HF1.

An *in vivo* recombinant plasmid, pDD530, containing the *bphABCD* genes from *Burkholderia* sp. strain LB400 was isolated from *P. putida* KT2442. The *bph* genes on pDD530 were further cloned into a pUT transposon vector to yield plasmid pDDPCB. Plasmid pDDPCB was mobilized into *Pseudomonas* sp. strain B13, and its genetically engineered derivative B13FR1 via conjugation. A transconjugant, *Pseudomonas* sp. strain B13FR1::*bph*, with a chromosomally integrated *bph* gene removed approximately 90% of added 4-chlorobiphenyl after 5 d in lake sediment microcosms (DOWLING et al., 1993). The plasmid, pDDPCB, was later transferred into a rhizosphere pseudomonad, *Pseudomonas fluorescens* F113, by conjugation to generate a genetically modified strain F113pcb (BRAZIL et al., 1995). The *bph* operon was chromosomally located and was stable in non-sterile soil microcosms for 25 d after inoculated onto sugar beet seeds. Strain F113pcb gained the ability to utilize biphenyl as a sole carbon source.

2.2.2.3 *Pseudomonas cepacia* JHR22

HAVEL and REINEKE (1991) reported a hybrid bacterium, *Pseudomonas cepacia* JHR2, was constructed by filter mating with a biphenyl-grown donor and a chlorobenzoate-grown recipient (*P. cepacia* JH230). Strain 230 is a hybrid strain that originated from the transfer of chlorocatechol degradative genes from strain B13 into *Pseudomonas* sp. WR401 (HARTMANN et al., 1989). Strain JHR2 was able to grow on 3- and 4-chlorobiphenyl. A 2-chlorobiphenyl degrader, JHR22, was obtained by growing JHR2 with 4-chlorobenzoate (1 mM) in the presence of 2-chlorobiphenyl (3 mM). The new hybrid strain, *P. cepacia* JHR22, showed capability to utilize 2-chloro-, 3-chloro-, 4-chloro-, 2,4-dichloro-, and 3,5-dichlorobiphenyl as sole source of carbon and energy. The strain JHR22 also exhibited ability to degrade all monochlorobiphenyls in Aroclor 1221® when tested with soils (HAVEL and REINEKE, 1993).

2.2.2.4 *Pseudomonas acidovorans* M3GY

Recombinant bacterium, strain M3GY, was produced within a multi-chemostat culture by

mixing *P. acidovorans* CC1 (a chloroacetate and biphenyl degrader) and *Pseudomonas* sp. strain CB15 (a hybrid strain) on ceramic beads that were coated with 3,3′-dichlorobiphenyl (MCCULLAR et al., 1994). Strain M3GY expressed catabolic ability to utilize 3,4′-dichlorobiphenyl as a sole carbon and energy source. The recipient strain was determined by phenotypic similarity and genetic homology between strains M3GY and CC1.

In a recent study, 2,3′-dichloro- and 2,4′-dichlorobiphenyl were mineralized by a two-member consortium, *Burkholderia* (formerly *Pseudomonas*) sp. strain LB400 and *P. putida* mt-2a (POTRAWFKE et al., 1998). The strain mt-2a was obtained by intergeneric mating with the chlorocatechol genes transferred from LB400 to mt-2 (TOL). Strain mt-2a exhibited the ability to grow on 3-chloro- and 4-chlorobenzoate.

2.2.2.5 *Pseudomonas putida* (pDA261)

Different biphenyl degradative genes (*bphABCD*) from *Comamonas testosteroni* B-356 were subcloned into *P. putida* and *E. coli* separately, and their degradative capabilities were examined (AHMAD et al., 1992). The *hphC* and *bphD* genes were expressed well in both cells, however, *bphA* and *bphB* in *E. coli* were poorly expressed even though located downstream of the *tac* promoter. A review of the strain B-356 was published earlier (SYLVESTRE, 1995).

2.2.2.6 *Comamonas testosteroni* VP44(pE43)/VP44(pPC3)

Introduction of dehalogenase genes into the biphenyl degrading strain *C. testosteroni* VP44 resulted in complete mineralization of *o*- and *p*-substituted monochlorobiphenyls (HRYWNA et al., 1999). Plasmid pE43, containing the *ohbAB* gene, encodes iron–sulfur protein (ISPOHB) of the *o*-halobenzoate-1,2-dioxygenase in *P. aerugrinosa* 142. This plasmid, pE43, was transformed into strain VP44. The resulting recombinant strain VP44(pE43) mineralized 2-chlorobiphenyl and 2-chlorobenzoate. The other recombinant strain VP44-(pPC3) contained the *fcbABC* genes (from *Arthrobacter globiformis* KZT1) that catalyze hydrolytic *p*-dechlorination of 4-chlorobenzoate. This strain demonstrated the capability to grow on 4-chlorobiphenyl and 4-chlorobenzoate.

2.2.2.7 *Escherichia coli* JM109 (pSHF1003)/(pSHF1007) – Hybrid Biphenyl Dioxygenase

The *bphA1* genes of biphenyl dioxygenase in *Pseudomonas pseudoalcaligenes* KF707 and *Burkholderia cepacia* LB400 were recombined randomly by DNA shuffling (KUMAMARU et al., 1998). The shuffled *bphA1* genes were cloned into pJHF18 (MulI), upstream of *bphA2A3A4BC*, to replace the disrupted *bphA1* genes in the pJHF18 (HIROSE et al., 1994). The resulting plasmids containing *bphA1* (shuffled) and *bphA2A3A4BC* (KF707) were transformed into *E. coli* JM109 by electroporation. Some chimeric biphenyl dioxygenases in *E. coli* cells exhibited enhanced degradation of different biphenyl compounds. Biphenyl dioxygenase (pSHF 1003) showed 3 amino acid substitutions in KF707 *bphA1* (H255Q, V258I, and D303E), derived from strain LB400, and acquired degradation capabilities not only for PCB, but also for benzene and toluene. Plasmid pSHF1007 showed 4 amino acid substitutions (H255Q, V258I, G268A, and T376N) in KF707 *bphA1* gene and increased substrate affinities for some PCB congeners. The above results suggested the substitutions of His with Gln at 255 and of Val with Ile at 258 leading to the differences in substrate specificity and mode of oxygenation between the two enzymes.

Site-directed mutagenesis on the LB400 *bphA* gene was reported previously (ERICKSON and MONDELLO, 1993). 4 amino acids in LB400 were converted to the corresponding KF707 sequence. The modified biphenyl dioxygenase exhibited broader substrate specificity with PCB.

The *bphB* gene, encoding the *cis*-biphenyl dihydrodiol dehydrogenase from *P. putida* OU83 was cloned into the pQE31 vector and

expressed in IPTG-induced *E. coli* recombinant cells (KHAN et al., 1997).

2.2.2.8 *Pseudomonas putida* IPL5 (Field Application Vectors)

Field application vectors (FAV) are a combination of a selective substrate that can be used easily by host (not indigenous) microorganisms, and a cloning vector to provide a temporary niche for the host bacterium in harsh environments (LAJOIE et al., 1992). FAV can stabilize and enhance the expression of foreign genes in contaminated sites. The chromosomally encoded PCB catabolic genes (*bphABC*) from *Pseudomonas* sp. strain ENV307 were cloned into broad host range plasmid pRK293. The resulting plasmid was transferred to the host *Sphingomonas paucimobilis* 1IGP that utilizes non-ionic surfactant Igepal CO-720® (IGP) as selective substrate. The recombinant strain 1IGP4(pCL3) exhibited ability to degrade individual PCB congeners in Aroclor 1242® without biphenyl as an inducer (LAJOIE et al., 1993). The transposon encoded PCB degradative genes (*bphABC*) were more stable than plasmid encoded after insertion into the surfactant utilizing strain, *Pseudomonas putida* IPL5 (LAJOIE et al., 1994).

2.2.3 Trichloroethylene (TCE)

2.2.3.1 *Escherichia coli* HB101/pMY402 and FM5/pKY287

A XhoI fragment (4.7 kb), containing toluene monooxygenase (TMO) genes from *Pseudomonas mendocina* KR-1, was subcloned into a broad host range vector, pMMB66EH, which contains an *E. coli tac* promoter to yield plasmid pMY402. The same fragment was also inserted into another *E. coli* expression vector, pCFM1146, containing the temperature inducible *E. coli* phage λP_{L} promoter, to yield plasmid pKY287. Recombinant *E. coli* strains, *E. coli* HB101/pMY402 and FM5/pKY287, were able to oxidize both toluene and TCE (WINTER et al., 1989).

Recently, TMO genes in pMMB503EH, a broad host range vector, were introduced into *P. putida* KT2440 by electroporation (WARD et al., 1998).

2.2.3.2 *Pseudomonas pseudoalcaligenes* KF707-D2 and *Pseudomonas putida* KF715-D5

HIROSE et al. (1994) reported a hybrid dioxygenase gene cluster between the *tod* and the *bph* operons in *E. coli* JM109. Plasmid pJHF101, containing *todC1::bphA2orf3A3A4*, was constructed by deleting a 1.3 kb *Ppu*MI fragment from *bphB* and *bphC* genes from pJHF10 (FURUKAWA et al., 1993). The vector pUC118 was used for the construction of pJHF10. This recombinant strain, *E. coli* (pJHF101), degraded TCE at an initial rate of 1.8 µg mL^{-1} h^{-1} which was much faster than *E. coli* cells carrying the toluene dioxygenase genes (*todC1C2BA*) or the biphenyl dioxygenase genes (*bphA1A2A3A4*, FURUKAWA et al., 1994). The hybrid gene cluster, *todC1::phA2orf3A3A4*, was further inserted into the chromosomal *bph* operons by SUYAMA et al. (1996). The plasmid pJHF101 was subcloned into suicide vector, pSUPB30, to yield plasmid pASF101. The pASF101 was inserted into *E. coli* S17-1 (chromosomally integrated RP4-2-Tc::Mu-Km::Tn7) cells via transformation, and the resulting strain was used as a donor in the mating with the recipient biphenyl utilizing *P. pseudoalcaligenes* KF707 and *P. putida* KF715. The resulting double crossover strains, KF707-D2 and KF715-D5, maintained *todC1* in LB broth under no selective pressure. Both single and double crossover strains carrying *todC1* on chromosomes of KF707 and KF715 degraded TCE efficiently, and grew on toluene and benzene.

2.2.3.3 *Pseudomonas* sp. strain JR1A::*ipb* – Hybrid Strains

A multi-component isopropylbenzene (IPB) dioxygenase from strain *Pseudomonas* sp. JR1, growing on IPB exhibited the capability to co-oxidize TCE (PFLUGMACHER et al.,

1996). Organization of *ipb* genes encodes IPB dioxygenase (*ipbA1A2A3A4*), 2,3-dihydro-2,3-dihyroxy-IPB dehydrogenase (*ipbB*), and 3-IPB-2,3-dioxygenase (*ipbC*). Recently, a recombinant, *Pseudomonas* sp. JR1A, exhibited constitutive TCE oxidation activity was reported (BERENDES et al., 1998). A transposon vector pC8 (pUT/miniTn5Km::*ipbABC*) was constructed, then transformed into *E. coli* S17.1(*pir*). The pC8 vector in *E. coli* was conjugatively transferred into different recipients. The *ipb* genes were detected in following transconjugants: strain JR1A (spontaneous IPB negative mutants of strain JR1), *P. putida* 548, and *Pseudomonas* sp. strain CBS-3. Two of these hybrid strains, JR1A::*ipb* and CBS-3::*ipb*, were stable for more than 120 generations in antibiotic free medium and degraded TCE and IPB constitutively. Further studies showed that strain JRlA::*ipb* can oxidize TCE without inducer.

2.2.3.4 *Escherichia coli* JM109(pDTG601)

Toluene dioxygenase from *P. putida* F1 has been reported as an enzyme responsible for degradation of TCE (WACKETT and GIBSON, 1988). The structural genes (*todC1C2BA*) of toluene dioxygenase were cloned from plasmid pKK223-3 then transformed into strain JM109. The resulting strain *E. coli* JM109 (pDTG601) degraded TCE at a slower initial rate when compared to *P. putida* F39/D, a mutant strain of strain F1 that does not contain *cis*-toluene dihydrodiol dehydrogenase (ZYLSTRA et al., 1989).

2.2.3.5 *Pseudomonas putida* G786(pHG-2)

A recombinant *P. putida* G786(pHG-2), containing two multicomponent enzyme systems, cytochrome *P450*$_{cam}$, and toluene dioxygenase, capable of degrading pentachloroethane and TCE was reported by WACKETT et al. (1994, 1995). The toluene dioxygenase genes (*todC1C2BA*) and *tac* promoter (*Ptac*) were cloned into plasmid pKT230 to form plasmid pHG-1. The *lac*I^{Q} gene cassette from

plasmid pMMB24 was cloned into plasmid pHG-1 to yield pHG-2, then transformed into *E. coli* DH5. Plasmid pRK2073 was used as a helper to transconjugate plasmid pHG-2 into *P. putida* G786 containing cytochrome *P450*$_{cam}$ genes on the CAM plasmid. The new hybrid strain, G786(pHG-2), transformed pentachloroethane to TCE by the cytochrome *P450*$_{cam}$, then TCE was further mineralized by the toluene dioxygenase.

2.2.3.6 *Pseudomonas putida* F1/pSMMO20

Slow growth rate, copper repression of the *smmo* locus, and strong competition between TCE and methane for soluble methane monooxygenase (sMMO) are restrictions to the use of strain OB3b to degrade TCE. To overcome these impediments, the complete *smmo* cluster of *Methylosinus trichosporium* OB3b was cloned into a wide host range vector, pMMB277, to form pSMMO20 (JAHNG and WOOD, 1994). pSMMO20 is a 14.7 kb plasmid and contains the IPTG-inducible *tac* promoter upstream of the *smmo* cluster. Plasmid pSMMO20 was transformed into *P. putida* F1, P. putida KT2440, *P. mendocina* KR1, *B. cepacia* G4, and *B. cepacia* G4 PR1 through electroporation. *P. putida* F1/pSMMO20 was the only bacterium that was able to degrade TCE. However, inconsistent sMMO activity was a major drawback in the recombinant strain. Furthermore, the sMMO protein bands were detected on SDS-PAGE gels only when TPTG was present to induce the *tac* promoter. The constructed strain, *P. putida* F1/pSMMO20, showed a lower TCE degradation rate and a much higher growth rate than strain OB3b. This recombinant strain also demonstrated the capability to mineralize chloroform.

2.2.3.7 *Burkholderia cepacia* G4 5223-PR1

An aerobic bacterium, *Burkholderia cepacia* G4, cometabolically degrades TCE to CO_2 and non-volatile products by toluene *o*-monooxygenase (SHIELDS et al., 1989). Tn5 mutants of strain G4 were constructed via triparental

mating among G4, *E. coli* C600(pRZ102), and *E. coli* HB101(pRK2013). The resulting Tn5 mutants could not express toluene *o*-monooxygenase activity, and were unable to degrade TCE, toluene, and phenol. A mutant spontaneously reverted to express toluene *o*-monooxygenase constitutively and was designated as G4 5223-PR1. This strain metabolized TCE and *m*-trifluoromethyl phenol without induction (SHIELDS and REAGIN, 1992).

2.2.3.8 *Ralstonia eutropha* AEK301/pYK3021

Strain AEK301, a Tn5 mutant that lost phenol hydroxylase activity, was derived from strain JMP134. Plasmid pYK3021 encoding phenol hydroxylase was subcloned into pMMB67EH (vector from pJP4) and exhibited TCE degradation capability without phenol induction. Triparental mating was used to transfer plasmid pYK3021 from *E. coli* to *R. eutropha* AEK301 with the helper plasmid pRK2013. The recombinant strain AEK301/pYK3021 expressed phenol hydroxylase activity constitutively and degraded TCE efficiently (KIM et al., 1996). The removal rate of TCE by the strain could be influenced by growth substrates (AYOUBI and HARKER, 1998).

2.2.4 2,4-Dichlorophenoxyacetic Acid (2,4-D)

2.2.4.1 *Pseudomonas putida* PPO300(pRO101) and PPO301(pRO103)

Plasmid pJP4 encodes the degradation genes of 2,4-D and 3-chlorobenzoate in *R. eutropha* JMP134 (DON and PEMBERTON, 1985). GEM containing catabolic genes for the degradation of 2,4-D to 2-chloromaleylacetate were reported by HARKER et al. (1989). Plasmid pRO101 (pJP4::Tn1721) was constructed by insertion of transposon Tn1721 into pJP4, then further transferred by conjugation to different *Pseudomonas* strains. One of the resulting strains, *P. putida* PPO300(pRO101),

with chromosomally encoded phenol hydroxylase, also degrades phenoxyacetate in the presence of an inducer (2,4-D or 3-chlorobenzoate) of the 2,4-D pathway. A mutant plasmid, pRO103, derived from pRO101 by the spontaneous deletion of negative regulatory gene (*tfdR*) was isolated from strain PPO300-(pRO101). The deletion of *tfdR* resulted in the constitutive expression of the *tfdA* gene (encodes 2,4-D monooxygenase), and enabled mutant strain PPO300(pRO103) to grow on phenoxyacetate as the sole carbon source. Strain PPO301, derived from PPO300 (ATCC 17514) and resistant to nalidixic acid, was used to harbor plasmid pRO103 to yield a constitutive 2,4-D degrader, PPO301(pRO103), that was used in agricultural soil studies (SHORT et al., 1990). A dual substrate (2,4-D and succinate) chemostat study with *P. cepacia* DBO1-(pRO101) indicated that succinate can act as repressor of the 2,4-D catabolic pathway. But, this repression can be relieved with appropriate adjustments, such as lower 2,4-dichlorophenol accumulation or reduced succinate concentration ($<$2,4-D) in the media (DAUGHERTY and KAREL, 1994).

2.2.4.2 *Pseudomonas cepacia* RHJ1

Burkholderia cepacia (formerly *P. cepacia*) RH1 is a recombinant strain created by performing conjugation between *R. eutropha* JMP134 (2,4-D degrader) and *B. cepacia* AC1100 (2,4,5-trichlorophenoxyacetate degrader). The self-transmissible 2,4-D degradative plasmid, pJP4, in *A. eutrophus* JMP134 was transferred into a 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) degrader, *B. cepacia* AC1100. This new strain, designated RHJ1, was capable of degrading mixtures of 2,4-D and 2,4,5-T simultaneously (HAUGLAND et al., 1990).